The Dissertation Committee for Jingya Xia Certifies that this is the approved version of the following dissertation:

THE ROLE OF TLR3- AND MYD88-DEPENDENT SIGNALING IN THE DEVELOPMENT OF ANTI-WEST NILE VIRUS ADAPTIVE IMMUNE RESPONSES INDUCED BY A VACCINE CANDIDATE, REPLIVAX WN.

Committee:

Gregg N. Milligan, Ph.D. Supervisor or Mentor

Alan D. Barrett, Ph.D.

Nigel Bourne, Ph.D.

Joan E. Nichols, Ph.D.

Frank Scholle, Ph.D.

Dean, Graduate School

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by

Jingya Xia, B.S.

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Dedication

To my family and friends who always offer help and spiritual support no matter what situation I am in.

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I appreciate the effort by Dr. Peter Mason's group for developing the successful RepliVAX WN. It allows us to establish the mouse model to study the immunomodulatory mechanisms that are responsible for anti-WNV memory responses and contribute to WNV vaccine development.

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Recognition of conserved pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs) results in the activation of innate signaling pathways that drive the innate immune response and ultimately shape the adaptive immune response. RepliVAX WN, a single-cycle flavivirus (SCFV) vaccine candidate derived from West Nile virus (WNV), is intrinsically adjuvanted with multiple PAMPs and induces vigorous anti-WNV T and B cell responses. However, the innate mechanisms that link pattern recognition and development of vigorous antigen-specific T and B cell responses are not completely understood. Moreover, the roles of individual PRR signaling pathways in shaping T and B cell responses to this live attenuated SCFV vaccine have not been established. We examined and compared the role of TLR3- and MyD88-dependent signaling in the development of anti-WNV-specific T cell responses by examining the kinetics of WNV-specific IFN-γ secreting $CD4^+$ and $CD8^+$ T cell responses, the functional pattern of primary and secondary effector WNV-specific IFN-γ secreting $CD8^+$ T cells, the development of anti-WNV $CD4^+$ and $CD8^+$ memory T cells and the role of TLR3- and MyD88-dependent signaling in modulating antigen presenting function of myeloid dendritic cells. We examined and compared the role of TLR3- and MyD88-dependent signaling in the development of anti-WNV-specific B cell responses by detecting the kinetics of antibody-secreting cell responses, anti-WNV IgG subclass switch, anti-WNV IgG affinity and neutralizing capacity and memory B cell responses induced by RepliVAX WN. We found that MyD88 deficiency significantly diminished the antigen presenting function of myeloid DCs, T cell proliferation, memory T cell generation, effector cytokine expression by $CD8^+$ T cells, B cell activation, cellularity of germinal centers, and the generation of long-lived plasma cells (LLPCs) and memory B cells (MBCs). In contrast, TLR3 deficiency had more impact on the functional pattern of T cell responses, memory T cell generation and reactivation, the development of LLPCs, whereas differentiation of MBCs was unaffected. Our data suggest that both TLR3- and MyD88-dependent signaling are involved in the intrinsic adjuvanting of RepliVAX WN

and differentially contribute to the development of vigorous WNV-specific primary and memory T and B cell responses following immunization with this novel SCFV vaccine.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
AICD	activation induced cell death
AID	Activation-Induced (Cytidine) Deaminase
AP-1	activator protein 1
APC	antigen presenting cell
APE1/2	apyrimidic/apurinic (AP)-endonuclease
ASC	apoptosis-associated speck-like protein containing a C-terminal CARD
ASC	antibody secreting cell
BAFF	B cell-activating factor of the TNF family
BCL-1	B-cell lymphoma line
BCR	B cell receptor
BHK	baby kidney hamster
BIR	baculovirus inhibitor repeat
C	capsid
C	cytosine
CARD	caspase activation and recruitment domain
CCL	chemokine (C-C motif) ligand
CDC	Centers for Disease Control and Prevention
CDR	complementary determinant region
CH	constant domain on the heavy chain
CL	constant domain on the light chain
CLEC5A	C-type lectin domain family 5 member A
CLR	C-type lectin receptor
CpG ODN	CpG oligodeoxynucleotide
CR	complement receptor
CRD	carbohydrate-recognition domain
CSR	class switch recombination
CTD	C-terminal domain
CTL	cytotoxic T lymphocyte
CTLD	CLRs containing C-type lectin-like domain
CXCL	(C-X-C motif) chemokine ligand
CXCR	(C-X-C motif) chemokine receptor
DI	Domain I
DII	Domain II
DIII	Domain III
dpi	day post immunization
DRBD	dsRNA binding domain
DSB	DNA strand break
dsRNA	double stranded RNA
E	envelope
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay

ELISPOT	Enzyme-linked immunospot assay
ERK	extracellular signal-regulated kinase
FDC	follicular dendritic cell
FcRN	neonatal Fc receptor
FITC	fluorescein isothiocyanate
FLUC-SCFV	Firefly luciferase-expressing SCFV particle
FP	food pad
FO	follicular
FTH cell	follicular T helper cell
GC	germinal center
HEL	hen egg lysozyme
hpi	hour post immunization
HTLV-1	human T cell leukemia virus type
IFN	interferon
IKK	I kappa B kinase
IKKε	I kappa B kinase ε
Ig	immunoglobulin
IL	interleukin
iLN	Inguinal lymph node
i.p.	intraperitoneal
IPS-1	interferon promoter stimulator-1
IRF-3	interferon regulatory factor 3
ITAM	immunoreceptor tyrosine-based activation motif
ivag	intravaginal
IVIS	*in vivo* imaging system
JAK1	Janus Kinase 1
JEV	Japanese encephalitis virus
LBP	laminin-binding protein
LGP2	Laboratory of Genetics and Physiology 2
LLPC	long-lived plasma cell
LPS	Lipopolysaccharides
LRR	leucine-rich repeat
Lyn	Lck/Yes novel tyrosine kinase
M	membrane
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral-signaling protein
MBC	memory B cell
miRNA	microRNA
MDA5	Melanoma Differentiation-Associated Gene 5
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MHV-68	murine gammaherpesvirus 68
MIP-1	macrophage inflammatory protein 1
MLC	memory lymphocyte cluster
MVEV	Murray Valley encephalitis virus
MyD88	Myeloid differentiation primary response gene (88)

MZ	marginal zone
NCR	Noncoding region
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NiNLP	nanolipoprotein particle
NK cells	natural killer cells
NLR	nucleotide oligomerization domain (NOD)-like receptor
NOD	nucleotide oligomerization and binding domain
NS	nonstructural protein
NTPase	RNA triphosphatase
HSV-2	herpes simplex virus type 2
NVU	neurovascular unit
OD	optical density
ORF	open reading frame
PAMP	pathogen associated molecular pattern
PE	phycoerythrin
Percp-Cy5.5	peridinin-chlorophyll proteins-Cy5.5
PKR	protein kinase R
pLN	Popliteal lymph node
PNS	peripheral nervous system
PRR	pattern recognition receptor
RdRp	RNA-dependent RNA polymerase
RGD	Arg-Gly-Asp
rgD2	recombinant glycoprotein D2
rgG2	recombinant glycoprotein G2
RGE	Arg-Gly-Glu-Ser
RIG-I	Retinoic acid-Inducible Gene I
RIP1	Receptor-Interacting Protein 1
RLR	retinoic acid-inducible gene I (RIG-I)-like receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
SAP	signaling lymphocytic activation molecule-associated protein
s.c.	subcutaneous
SC	secreting cell
SCFV	single cycle flavivirus virus
SHM	somatic hypermutation
siRNA	short interfering RNA
sfRNA	subgenomic flaviviral RNA
S region	switch region
ssRNA	single stranded RNA
SVP	subviral particle
Syk	spleen tyrosin kinase
TAK1	TGF-β Activated Kinase 1
TAP	transporter association protein
TBK1	TANK-binding kinase-1
TCM	central memory T cell
TCR	T cell receptor

TEM	effector memory T cell
Th1	type 1 T helper cells
TI-2	thymus independent type 2
TIR	Toll/interleukin-1 receptor
TNF	tumor necrosis factor
TLR	toll-like receptor
TReg	regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRIM21	tripartite motif-containing 21
TRM	resident memory T cell
Tyk2	tyrosine kinase 2
U	uracil
UTR	untranslated region
VCAM	vascular cell adhesion molecule
VH	variable domain on the heavy chain
VL	variable domain on the light chain
VLP	virus-like particle
WNV	West Nile virus
YFV	yellow fever virus

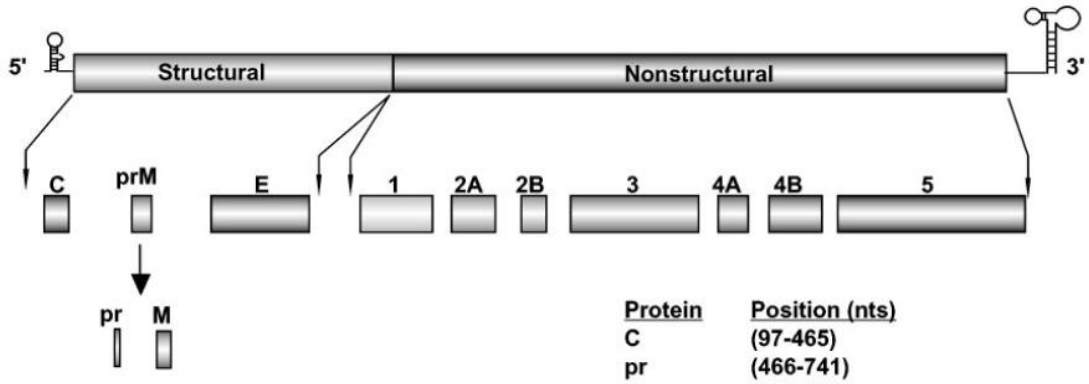
Chapter 1: Introduction

West Nile virus (WNV) is a member of the Japanese encephalitis virus (JEV) serogroup which is classified in the Flavivirus genus in the Flaviviridae family. WNV is divided into two genetic lineages based on signature amino acid substitutions or deletions in the envelope protein genes. WNV isolates that were identified from human WNV encephalitis and meningitis cases are included in lineages 1 and 2. Lineage 1 is subdivided into clade 1a and clade 1b. Clade 1a includes strains isolated from Europe, the Middle East, Asia and North America while the strains from Australia belong to clade 1b. WNV lineage 2 strains are isolated mainly in South Africa and Madagascar and are generally less pathogenic in humans. However, the repeated WNV outbreaks in Europe caused by lineage 2 suggesting the neuropathogenicity of this lineage in human. (Hernández-Triana, et al. 2014) A few strains that were isolated from Austria, Russia and India are identified as lineage 3, lineage 4 and lineage 5 respectively (Donadieu, et al. 2013). These are less well studied because they are rarely isolated. WNV was first identified in the West Nile region of Uganda in 1937. In the enzootic cycle, WNV circulates between the prime vector, mosquitoes and the prime reservoir, birds. Horses and humans are only incidental hosts with low viremic levels and are not involved in the transmission cycle. WNV is transmitted by a number of different genera of mosquitoes, e.g. *Culex*, *Aedes*, *Anopheles*, *Minomyia*, and *Mansonia*. Through these transmission vectors, WNV rapidly spread over regions including Africa, the Middle East and western Asia. It was first introduced into New York City in 1999 and isolated as the strain NY99. In the following decade, it spread over the entirety of North America and into the Caribbean and Latin America causing disease that ranges from inapparent infection to encephalitis. Although humans are generally infected through the bite of an infected mosquito, human-to-human transmission occasionally occurs from blood transfusion,

organ transplantation, intrauterine exposure, and breast feeding. (Kramer, Styer and Ebel 2008) Although WNV infection is asymptomatic in about 80% of the infected population (Suthar, Diamond and Gale 2013), it has been more and more considered as a severe public health issue in the U.S. because of the increasing prevalence and severity of disease. In the U.S., WNV caused 34,113 human infection cases and 1,487 deaths between 1999 and 2012. (CDC. 1999-2012) Recently, the WNV outbreak of 2012 in the US led to 5,387 human disease cases including 243 deaths. (CDC. 1999-2012) The aged and immunocompromised populations are most susceptible to symptomatic WNV infection. Symptomatic infection commonly causes a self-limiting West Nile fever with symptoms like headache, muscle weakness and disorientation. A minority of WNV infections cause severe neurological manifestations (encephalitis or meningitis), long-term sequelae, hepatitis or even death in the population aged 60 and older (Suthar, Diamond and Gale 2013).

Genome structure and viral proteins of WNV.

Figure 1.1: The genome structure and viral protein of WNV.



Protein	Position (nts)
C	(97-465)
pr	(466-741)
M	(742-966)
E	(967-2469)
NS1	(2470-3525)
NS2A	(3526-4218)
NS2B	(4219-4611)
NS3	(4612-6468)
NS4A	(6469-6915)
NS4B	(6916-7680)
NS5	(7681-10395)

Representation of WNV genome structure and viral proteins that are expressed from the viral genome. (Reprinted with permission from Brinton MA. 2002. The molecular biology of West Nile Virus: a new invader of the western hemisphere. Annu Rev Microbiol. 56:371-402.).

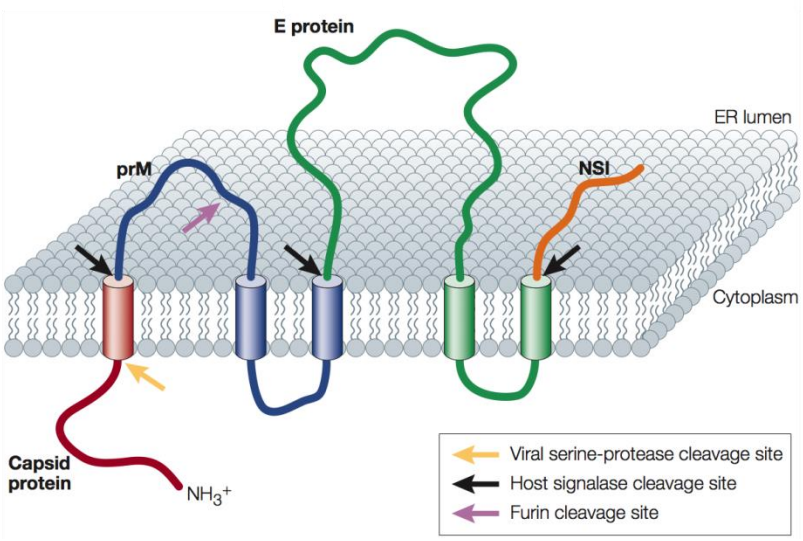
The genome of WNV is a positive-sense, single-stranded ribonucleic acid (RNA) molecule (11,029 nt) with a 5' cap and a 3' terminus.(**Fig.1.1**) Generally, in nature, there are 3 types of mRNA cap structure, N7-methyl guanine (m7G), N7-methyl guanosine diphosphate (m7GDP), and N7-methyl guanosine triphosphate (m7GTP). WNV RNA carries the type 1 cap structure (m7GpppAmp). Unlike most viruses, the 3' end of flavivirus RNA terminates with CUOH as opposed to a 3' poly (A) tail (polyadenylic acid tail). It is still poorly understood how this special 3' end functions in the flavivirus. However, studies have shown that the untranslated regions (UTRs) at the 5' and 3' ends which contain numerous secondary structures (stem loops and pseudoknots) are important for viral gene replication, translation and pathogenesis. The 3' UTR after the

stop codon is degraded by the cellular 5'-3' exoribonuclease XRN1 and processed into a series of unique subgenomic flaviviral RNAs (sfRNAs). (Roby, et al. 2014) The sfRNA generated from 3' UTR was shown to suppress antiviral RNA interference activity in insect and mammalian cells (Schnettler, et al. 2012). One study showed that deletion of the 3' UTR impaired the generation of full length sfRNA species and attenuated viral cytopathicity and virulence. (Pierson 2008)

There is a single ORF (open reading frame) of 10,301 nt in the WNV genome. The ORF encodes a single polyprotein (more than 3,000 amino acids) which is processed into structural and nonstructural proteins. The structural proteins are encoded in the N terminal portion followed by the nonstructural proteins. The polyprotein is cleaved into three structural proteins (capsid (C), membrane (prM/M), and envelope (E)) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The cleavage is attributed to the viral protease (NS2B-NS3 which will be discussed below) and cellular proteases (furin and signal peptidase).(**Fig.1.2**) Signal peptidases, usually identified as serine proteases, cleave the signal peptides from the N terminals of the proteins at the extracytoplasmic site of the ER membrane. Although there are no studies reporting how host signal peptidase processes WNV polyproteins, studies with dengue virus, yellow fever virus and hepatitis C virus show that the signal peptidase is responsible for cleaving hydrophobic regions at the N termini of structural and nonstructural proteins. (Ruiz-Linares, et al. 1989) An *in vitro* processing system showed that the signal peptidase is responsible for the cleavage at the capsid-prM and prM-E junctions of dengue virus. (Markoff 1989) One study used recombinant vaccinia viruses which encoded various lengths of sequences up- and down-stream of NS1 to identify the cleavage site and found that the host signal peptidase cleaved the N-terminal signal sequence of NS1 protein. (Falgout, Chanock and Lai 1989) The signalase cleavage site at 2K/4B on yellow fever virus (YFV) was also confirmed by using cell-free translation of *in vitro* transcribed YF RNAs. (Lin, et al. 1993)

The host signalase and viral protease proteolytically process the polyprotein precursor into structural proteins for virion assembly and nonstructural proteins for modulating viral genome replication, post-translational processing and suppress antiviral responses.

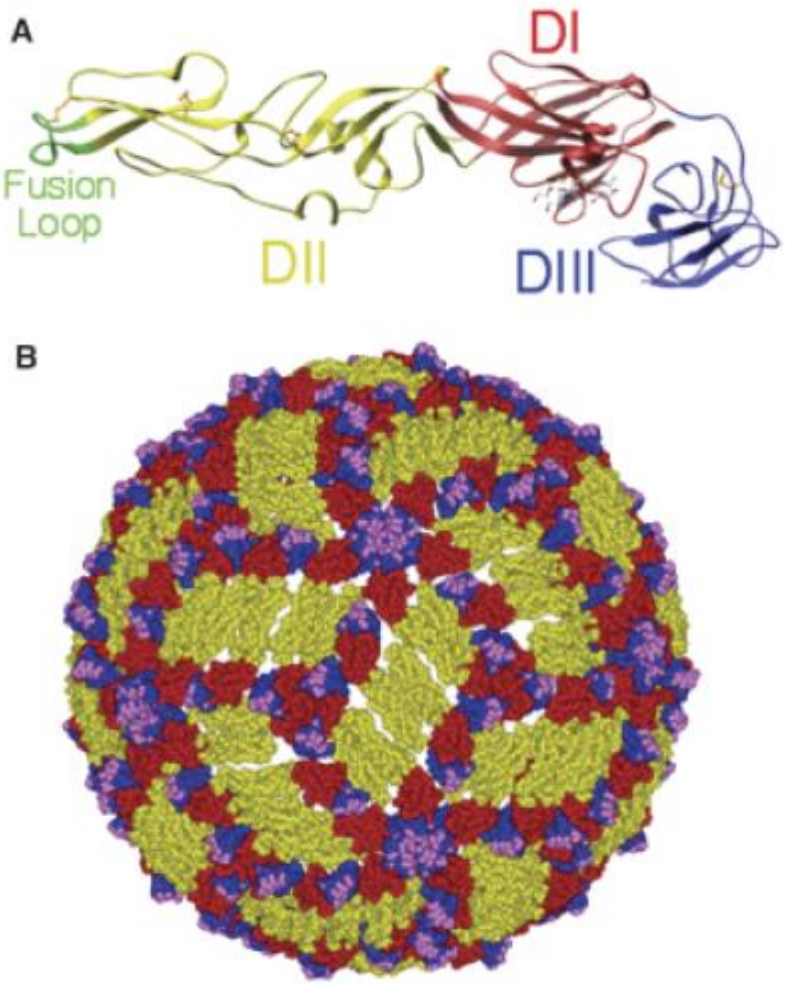
Figure 1.2: Membrane topology of the flavivirus structural proteins.



Representation of the protein processing of flavivirus across ER membrane. (Reprinted with permission from Mukhopadhyay S, Kuhn RJ, Rossmann MG. A structural perspective of the flavivirus life cycle. Nat Rev Microbiol. 2005. 3(1):13-22).

STRUCTURAL PROTEINS.

Figure 1.3: E protein and mature WNV virion structure.



Representation of WNV E protein crystal structure (A) and the pseudoatomic model of mature WNV viron based on cryo-electron microscopy studies (B). (Reprinted with permission from Diamond MS, Pierson TC, Fremont DH. 2008. The structural immunology of antibody protection against West Nile virus. Immunol Rev. 225:212-25.).

C protein.

The WNV C protein is about 120 amino acids in length and is cleaved from the polyprotein by viral protease at the N-terminal and host signalase at the C-terminal. The C proteins contain α helixes which stabilize dimerization. Multiple copies of the capsid protein dimers and a copy of the viral genome are assembled by electrostatic interaction and form the nucleocapsid. The hydrophobic region of the α helixes is exposed on the surface of the nuclear capsid and facilitates its binding to the lipid membrane. (Ma , Jones, et al. 2004); (Kofler, Heinz and Mandl 2002) The WNV capsid protein is also a pathogenic determinant. The capsid protein of WNV was shown to act as a pro-apoptotic factor which induces apoptotic cell death through the mitochondrial-activated apoptotic pathway or through the local inflammation in the mouse brain. (Yang, et al. 2003) The capsid protein may also suppress the transcription factor activator protein 1 (AP-1), thereby downregulating type I interferon (IFN) transcription and thus compromising innate immune responses. (Urbanowski, Ilkow and Hobman 2008)

prM protein.

Following C protein on the polyprotein, prM (~165 amino acids) is cleaved from the polypeptide by signalase and the calcium-dependent serine endoprotease furin. prM is a seven β stranded glycoprotein containing two transmembrane domains. It plays an important role in viral maturation. prMs heterodimerize with E proteins and stablize the oligometric state of E proteins to prevent irreversible conformational change during the release of virus particles. (Lorenz, et al. 2002) In the acidic environment of the trans-Golgi network, prMs are cleaved by furin and produce M proteins (~75 amino acids). Following this cleavage, prM-E heterodimers are rearranged into E homodimers followed by the maturation and release of virions. (Pierson and Diamond 2012) A study showed that these rearranged mature WNV virions had a decreased sensitivity to human neutralizating antibodies developed from WNV infection. (Nelson, et al. 2008) This

suggested the important role of prM impacting the accessibility of viral epitopes to the neutralizing antibodies.

E protein.

E protein is a 53kDa glycoprotein composed of about 495 amino acids. A pair of E protein monomers each of which has three β-barrel domains are dimerized. Ninety of the E protein dimers are assembled into a virion. There are three domains in each E protein monomer. Domain I (DI, central domain, in red) acts as the flexible loop linking domain II (DII, in yellow) and Domain III (DIII in blue) (shown as Fig 1.3 A). DII is the region where a pair of E proteins interact to form an anti-parallel dimer. A highly conserved 13 amino acid hydrophobic fusion loop resides at the distal end of DII and was shown to be responsible for viral fusion with the endosomal membrane (Allison, et al. 2001). The immunoglobulin-like DIII forms small protrusions or exposed loops on the particle surface. On DIII, the RGD (Arg-Gly-Asp)/RGE (Arg-Gly-Glu-Ser) sequence could be recognized by integrins and is thought to act as the putative receptor-binding site. (Mukhopadhyay, Kuhn and Rossmann 2005) Additionally, monoclonal antibodies derived from immunization with purified soluble E proteins were used to detect and map neutralizing antibody epitopes in DIII. (Oliphant, Engle, et al. 2005) Therefore, DIII segments are being considered as an antigenic target for WNV vaccine candidates.

NONSTRUCTURAL PROTEINS.

NS1.

NS1 (~48kDa) is a nonstructural glycoprotein that is cleaved from the polyprotein by host signalase. Following WNV infection, NS1 can be secreted from or attached on the surface of infected mammalian cells. The membrane-associated NS1 is a hydrophobic homodimer while the secreted NS1 is a hexamer composed of three homodimers. (Muller

and Young 2013) NS1 in infected cells plays an important role in modulating viral replication and in immune evasion. Introduction of mutations in NS1 resulted in impaired viral replication, especially the initial synthesis of negative strand RNA. (Lindenbach and Rice 1997) Another study also showed that NS1 modulated WNV replication by interacting with NS4B. (Youn, Li, et al. 2012) With regards to immune evasive function, the secreted and intracellular NS1 have been shown to inhibit TLR3 (toll-like receptor 3) signal transduction. (Wilson, et al. 2008); (Crook, et al. 2014) Secreted extracellular NS1 has been shown to act as an antagonist and inhibited the activation of the complement pathway that facilitates the phagocytosis of antibody-coated virus. (Chung, Liszewski, et al. 2006); (Avirutnan, Fuchs, et al. 2010); (Avirutnan, Hauhart, et al. 2011) Additionally, the surface-associated and secreted NS1, which could be detected between day 3 and 8 post-infection (Macdonald, et al. 2005), stimulated the production of serum anti-NS1 immunoglobulins that were shown to trigger the clearance of WNV via antibody-dependent cell-mediated cytotoxicity (ADCC) (which will be further discussed in the section on B cell responses). (Chung, Thompson, et al. 2007) As an early diagnostic approach to identify WNV infection, recombinant NS1 ELISA test system was developed to detect anti-NS1 IgM in patient serum. (Saxena, et al. 2013)

NS2A.

NS2A (~25kDa) is a small hydrophobic protein and cleaved from the WNV polyprotein by viral protease NS2B/NS3. One study showed that single amino acid substitutions in NS2A attenuated WNV replication. (Rossi, Fayzulin, et al. 2007) WNV containing the NS2A/A30P mutation was shown to lose this ability to suppress type I IFN production. (Audsley, et al. 2011) Additionally, the NS2A/A30P mutant has also been shown to rescue WNV infected cells from IFN response-independent apoptosis. (Melian, et al. 2013) These studies showed the capacities of NS2A to facilitate viral replication

(Rossi, Fayzulin, et al. 2007), virus-induced apoptosis (Melian, et al. 2013), and inhibit the production of type I IFN. (Liu, Wang and Clark, et al. 2006) Therefore, it suggests that NS2A is associated with WNV induced cytopathicity and virulence. (Audsley, et al. 2011)

NS2B.

NS2B is a small protein cofactor with a hydrophobic domain of 40 amino acids. The hydrophobic region is essential for the protease activity and the membrane-association of NS3 that cleaves at the NS2A-NS2B and NS2B-NS3 junctions. (Falgout, Miller and Lai 1993)

NS3.

NS3 (~70 KDa) is a multifunctional protein with several enzymatic functions. The N terminus of NS3 is highly conserved and has a trypsin-like serine protease activity. The proteolytic activity depends on interaction between the N terminus and NS2B. (Chappell, et al. 2005) The central region of NS3 acts as a helicase while the C terminal region has an RNA triphosphatase (NTPase) activity. (Wengler G 1993); (Feito, et al. 2008) The NTPase/helicase domain is essential for 5' cap synthesis and unwinding of double-stranded RNA during viral replication. Additionally, the protease and helicase domains of NS3 have been shown to contribute to NS2B-NS3 induced apoptosis in WNV infected mammalian cells. (Ramanathan, et al. 2006)

NS4A.

NS4A (~16kDa) is a small hydrophobic protein. In the infection of Australian WNV strain, Kunjin virus, NS4A was shown to be essential for viral replication by contributing to cellular membrane biogenesis and targeting the replication complex

(discussed below) to the Golgi membrane. (Roosendaal, et al. 2006) This protein was shown to be involved in inhibiting IFN-α/β signaling by inducing the ER stress response and thus helping the viruses evade from antiviral immune responses. (Liu, Wang and Mokhonov, et al. 2005); (Ambrose and Mackenzie 2011) NS4A also acts as a cofactor to sustain the activity of NTPase/helicase, NS3. (Shiryaev, et al. 2009)

NS4B.

NS4B (~27 kDa) is a small hydrophobic protein that is cleaved by host signalase and viral protease. The complete cleavage between NS4A and NS4B generates the 2K peptide which plays a direct role in viral RNA synthesis. (Zou, et al. 2009) NS4B was shown to be important in viral replication by modulating viral RNA synthesis. (Youn, Li, et al. 2012); (Puig-Basagoiti, et al. 2007) Additionally, NS4B inhibits interferon signaling by preventing the accumulation of phosphorylated Janus Kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2) which phosphorylate downstream proteins for signaling transduction. (Ambrose and Mackenzie 2011); (Evans and Seeger 2007)

NS5.

NS5 is the largest nonstructural protein and contains 900 amino acids. The C terminal region of NS5 has a RNA-dependent RNA polymerase (RdRp) activity while the N terminus has a methyltransferase domain. (Brinton 2002) The polymerase and methyltransferase activities are essential for the synthesis of the type I cap and viral RNA. (Malet, et al. 2008); (Tan, et al. 2013) NS5 is also involved in inhibiting type I IFN-mediated JAK-STAT signaling (Laurent-Rolle, et al. 2010) and thus facilitates the evasion of the antiviral responses.

Life cycle of WNV.

The ecological cycle of WNV transmission requires WNV infection of competent mosquito vectors. The dominant vectors, female *Culex* mosquitoes, feed on WNV infected viremic birds and ingest WNV which passes through the antimicrobial peritrophic matrix in the mosquito midgut (Villalon, Ghosh and Jacobs-Lorena 2003) and replicates in the midgut epithelial cells. After WNV spreads to the salivary glands through the haemolymph, the infected mosquitoes become competent vectors for viral transmission. Each blood meal by the viremic female mosquitoes can deliver up to 10^6 plaque-forming units of WNV into the new host. Meanwhile, the mosquito saliva facilitates the initial WNV infection by suppressing inflammation and altering the host's immunity. Studies have shown that mosquito saliva inhibited B and T cell activation and proliferation. (Wassenrman, Singh and Champagne 2004) The saliva was also shown to suppress the production of type I cytokines (IFN-γ) but not type II cytokines (IL-4) and therefore to inhibit the polarization to type 1 T helper cells (Th1) cells which are responsible for antiviral activity. (Cross, Cupp and Enriquez 1994) Suppression of the production of proinflammatory and inflammatory cytokines by saliva was also observed. (Bissonnette, Rossignol and Befus 1993) The modulatory function of saliva on the host immune response therefore facilitated viral replication and spread in hosts. (Titus, Bishop and Mejia 2006)

WNV is thought to initially infect and replicate in keratinocytes, Langerhans cells and skin-resident dermal dendritic cells. (Suthar, Diamond and Gale 2013) Langerhans cells, the resident dendritic cells in skin, were considered as the initial target for WNV infection. B cells, T cells, and macrophages could also be infected by WNV (Samuel and Diamond 2005). Additionally, some studies showed that the absence of Type I IFN or RIG-I like receptors (RLR), which are critical for antiviral responses, expanded the spectrum of WNV infection tropism. (Samuel and Diamond 2005); (Suthar, Brassil, et al. 2013)

The cellular receptors on mammalian cells for WNV entry are not well identified. One study suggested that the ligation of WNV E protein with the receptors LBP (laminin-binding protein) and αvβ3-integrin was the putative mechanism for viral attachment. (Bogachek, et al. 2010) However, contrary to this hypothesis, another study concluded that WNV entry was αvβ3 independent by showing that WNV could normally infect β3-/- cells. (Medigeshi, et al. 2008) Additionally, a separate study supported this conclusion by showing that the permissiveness for WNV infection was not impacted by the blockage or deficiency of either integrin subunit. This study showed that the viral entry into the β3-/- cells was enhanced by the transfection of a plasmid encoding β3 integrin indicating that αvβ3 integrin was not required for but modulated WNV entry. (Schmidt, et al. 2013) Three identified different types of viral fusion proteins have been identified based on their common post-fusion structural motif. Class II fusion, utilized by flavivirus, is characterized by the trimerization of fusion proteins which fold into hairpin structures during insertion into the target membrane. (Kielian 2006) After the internalization of WNV, E proteins fuse with the endosomal membrane through Class II fusion in the endosomal acidic environment (Weissenhorn, Hinz and Gaudin 2007). Nucleocapsids are released and uncoated in the cytoplasm where viral positive sense (+) ssRNAs are transcribed, replicated and translated. For viral genome replication, viral (+) ssRNAs utilize the RdRp replication complex to synthesize the full-length negative-sense (-) ssRNAs. Noncoding regions (NCRs) on (-) ssRNAs act as the initiation site for the synthesis of the positive strand. Viral RNA synthesis is asymmetric with the generation of (+) ssRNA more than 10 times greater than that of (-) ssRNA. These non-self RNA replicates can be recognized by the host via cytoplasmic or membrane bound pattern recognition receptors (PRRs) (e.g. TLR3, TLR7/8, RIG-I) and initiate innate immune responses, especially the secretion of type I IFN. Meanwhile, the ORF on the (+) ssRNA encodes the WNV polyprotein and generates the nonstructural and structural proteins. (+) ssRNAs are targeted to the ER membrane and translated into polyproteins by ribosomes

on the ER. The viral genome in the ER is encapsidated by C proteins. The capsids, E protein dimers and prM proteins are assembled into immature virions. In the acid environment of the trans Golgi network and secretory vesicles, E proteins are glycosylated and the pr portion of prMs is cleaved by furins. E proteins lie flat against the viral surface and the heterotrimeric prM-E is presented as spikes on the immature virion. Following the release of immature virions into the extracellular neutral milieu, the 60 prM-E heteromeric spikes on each virion are rearranged into 90 anti-parallel E protein dimers. (Pierson and Diamond 2012) Progeny virions are exocytosed 10 to 12 hours after infection while mature viral particles are detectable at 24 hours post-infection. The progeny viral particles spread to the peripheral organs (e.g. spleen, liver, kidney and brain) and cause viremia.

Pathogenesis of WNV.

Following the report of WNV infection of horses in Egypt in 1959 (Schmidt and Elmansoury 1963) and birds in the Sindbis region of Egypt in 1946 (Work, Hurlbut and Taylor 1955), studies were undertaken to investigate the pathogenesis of WNV in these animals. In natural infection through mosquito vectors, equids can develop a subclinical infection or neurological disease. WNV spreads to tissues including the CNS, spinal cord, lower brain stem, ventral horns, basal nuclei, thalamus, and midbrain. Polioencephalomyelitis and petechiae are commonly observed. (Angenvoort, et al. 2013) Although viruses are not detected early in the blood stream of mammals, WNV can be detected in avian blood samples very early after infection. This suggests that WNV infection in birds is different from WNV infection in mammals where Langerhans cells transport viruses to the amplification sites (draining lymph nodes) and cause viral dissemination to different organs and blood. On the contrary, in birds, the early primary WNV viremia may not be caused by local viral replication. (Reisen, Fang and Martinez

2007); (Gray and Webb 2014) However, studies have shown that cellular targets of WNV in birds includes neurons, glial cells, myocardial fibers, macrophages, blood monocytes, renal tubular epithelium, adrenal cortical cells, pancreatic acinar cells, islet cells, intestinal crypt epithelium, oocytes, fibroblasts and smooth muscles. (Steele, et al. 2000) Mononuclear cells were identified as important cell targets for viral replication and dissemination in birds. (Weingarti, et al. 2004) In the natural infection, WNV is detected as early as 30-45 min post-infection. The viremia peaks on 3 dpi followed by the viral dissemination to numerous organs, e.g. CNS, eyes, peripheral nervous system (sciatic nerve, myenteric, proventricular, ventricular ganglia), heart, spleen and other lymphoid organs (thymus (Himsworth, et al. 2009)), liver, kidney, lung, gastrointestinal tract, endocrine system, gonads, skeletal muscle, skin, and bone marrow. (Gamino and Hofle 2013) Viremic birds develop clinical signs, e.g. depression, anorexia, dehydration, ruffled feathers, convulsions, ataxia, abnormal head posture and movements, tremors, uncoordinated flight, paresis and disorientation by 5 dpi. (Gray and Webb 2014)

In human beings, 26% of viremic patients developed West Nile Fever with symptoms such as headache, generalized weakness, fever, severe muscle pain, joint pain, chills, painful eyes, or rash. (Gray and Webb 2014) WNV infected patients may develop uncommon symptoms, e.g. fulminant hepatitis, pancreatitis, rhabdomyolysis, myocarditis, myositis, and orchitis due to the recruitment of inflammatory cells to different organs (Reusken, et al. 2011). Less than 1% of WNV infection causes injuries to brain stem, hippocampus, cortex, cerebellum and spinal cord and neuroinvasive diseases. The neuropathogenicity of WNV is dependent on the viral genetic variation. The ability of virulent WNV stains to cause menigoencephalitis is attributed to their neuroinvasive property and efficient replication in astrocytes, endothelial cells and neurons which comprise the neurovascular unit (NVU). (Beasley, Li, et al. 2002); (Hussmann, et al. 2013) WNV can invade into CNS via peripheral blood since higher level of viremia was shown to correlate with the earlier viral entry into CNS. (Diamond, Mehlhop, et al. 2009)

WNV viremia stimulates TLR signaling and results in the production of TNF-α, a cytokine that causes the loss of tight junction in the BBB. Meanwhile, WNV infection of brain microvascular endothelial cells results in the leakage of the BBB (Chai, et al. 2014); (Verma, et al. 2009). The neuronal invasion by WNV resulted in the infection of micoroglia and astrocytes, which secrete matrix metalloproteinase 1, 3, and 9 and further disrupt the BBB. (Rossini, et al. 2013) The increased permeability of BBB facilitates the invasion into CNS by peripheral WNV or infected leukocytes acting as a "Trojan horse" where WNV replicate, reside or adhere (Garcia-Tapia, Loiacono and Kleiboeker 2005); (Rios, et al. 2007) .Additionally, WNV enters into the CNS from the peripheral nervous system (PNS) via retrograde and anterograde axonal transportation (Hunsperger and Roehrig 2006); (Samuel, Wang, et al. 2007).

Immune responses to WNV and RepliVAX WN.

The initial infection by WNV triggers host innate immune responses and antiviral activity by type I IFN signaling, and activation of neutrophils, natural killer (NK) cells and γδ T cells. These innate responses are responsible for viral clearance in the early phase of infection. Type I IFN has been shown to play an important role in control of WNV viremia and neuronal invasion in the early phase (Samuel and Diamond 2005). *In vitro* expanded human primary NK cells also showed antiviral activity against WNV infection. (Zhang, et al. 2010) This antiviral activity is dependent on the NK cell intrinsic Mavs (a protein involved in Rig-I like receptor signaling) and type I IFN signaling. (Suthar, Brassil, et al. 2013) However, WNV has evolved strategies to evade the innate antiviral immune mechanisms. As previously discussed, WNV can suppress type I IFN production or signaling by viral nonstructural proteins, NS2A, NS4A, NS4B, and NS5. (Liu, Wang and Clark, et al. 2006); (Roosendaal, et al. 2006); (Puig-Basagoiti, et al. 2007); (Ambrose and Mackenzie 2011); (Tan, et al. 2013); (Diamond 2009) Considering

these evasion strategies combating the antiviral innate immunity, adequate anti-WNV adaptive immune responses are also desirable for clearing viruses and limiting the tissue damage in the late infection phase. Studies showed that $CD8^+$ T cells, $CD4^+$ T cells and B cells were important in protecting hosts from WNV neuronal invasion and lethal WNV infection. (Shrestha and Diamond 2004); (Sitati and Diamond 2006) The antiviral activity of $CD8^+$ T cells and WNV specific $CD4^+$ T cells was shown to be dependent on perforin, granzyme and effector cytokines. (Shrestha, Samuel and Diamond 2006); (Brien, Uhrlaub and Nikolich-Zugich 2008) Additionally, the development of efficient adaptive immune responses also results in the generation of WNV specific memory T and B cells. The optimal memory immunity which protects the hosts from WNV re-exposure is desirable for vaccination. Therefore, to develop an efficient WNV vaccine, it is important to study how the adaptive immune responses develop.

PRRs and WNV infection.

Innate immune responses to viral infection are initiated by the recognition of pathogen associated molecular patterns (PAMPs) via PRRs. PRRs are expressed on almost all kinds of host cells, e.g. epithelial cells, DCs, macrophages, B cells and T cells. PAMPs expressed by viruses include DNAs, RNAs and glycoproteins. Many studies have investigated the involvement of PRRs, including TLRs, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs) and C-type lectin receptors (CLRs), in recognizing the viral PAMPs. With regard to WNV infection, it is commonly accepted that PRR signaling initiates the innate immune response and modulates the adaptive immune response to protect against WNV infection.

TLRs. TLRs are evolutionarily conserved receptors expressed on the cellular membrane or the inner endosomal surface. There are 13 TLRs. Each TLR monomer

consists of 3 portions, the extracellular leucine-rich repeat (LRR) domain for recognizing ligands, the transmembrane helix and the intracellular Toll/interleukin-1 receptor (TIR) domain for the transduction of downstream signaling. Dimerization of TLR monomers is required for TLR activation. (Song and Lee 2012) TLR3 and TLR7/8 are mainly involved in the recognition of flavivirus infection. (Kong, et al. 2008); (Welte, Reagan, et al. 2009) TLR3 recognizes endocytosed extracellular dsRNA (longer than 100 bp) which might be released during viral replication or from damaged tissues. (Chattopadhyay and Sen 2014) Functional TLR3 is localized in the endosomal lumen since its ligation with dsRNA and dimerization is acidic environment dependent. (Leonard, et al. 2008) There is not a well-defined conserved dsRNA binding domain (DRBD) on TLR3 for recognizing dsRNA in a sequence-independent manner. However, the LRRs on TLR3 interact with dsRNA and thus facilitate the homodimerization which is required for signaling transduction. (Choe, Kelker and Wilson 2005) TLR3-dependent signaling is transmitted through the adapter molecule, TIR-domain-containing adapter-inducing interferon-β (TRIF), and activates two branches of signaling pathways. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) branch is activated via the recruitment of Receptor-Interacting Protein 1 (RIP1), TGF-β Activated Kinase 1 (TAK1) and I kappa B kinase (IKK) and results in the transcription of proinflammatory cytokines. The other signaling branch occurs through interferon regulatory factor 3 (IRF-3) which is activated via phosphorylation by TANK-binding kinase-1 (TBK1) or I kappa B kinase ε (IKKε) (Fitzgerald, et al. 2003); (Sharma, et al. 2003) and triggers antiviral responses through the transcription of inflammatory cytokines and type I IFN. Recently, a new branch of TLR3 signaling has been proposed in which epidermal growth factor receptor (EGFR) associates with the activated TLR3 dimer and recruits Src (Yamashita, Chattopadhyay, et al. 2012); (Yamashita, Chattopadhyay, et al. 2012), a family of kinases that are autophosphorylated. The phosphorylated Src kinases initiate signaling associated with cell migration, adhesion and proliferation (Je, et al. 2014); (Yamashita, Chattopadhyay, et

al. 2012); (Suzuki and Ishikawa 2014), suggesting the involvement of TLR3 signaling in these processes. TLR7 recognizes uridine- and guanosine-rich ssRNA from viruses (Diebold, et al. 2004), short interfering RNA (siRNA) (Hornung, Guenthner-Biller, et al. 2005), microRNA (miRNA) (Chen, et al. 2013) and self RNA (Barrat, et al. 2005) via LRRs. (Tanji, et al. 2013) The ligation of TLR7 triggers signaling through another adaptor molecule, Myeloid differentiation primary response gene (88) (MyD88). The activated signaling also directs the transcription of type I IFN and (pro- or) inflammatory cytokines via the IRF-7 and NF-κB branches. In addition to TLR3 and TLR7, TLR8 that recognizes ssRNA was also shown to be able to sense the yellow fever vaccine YF-17D. (Querec, et al. 2006) With regard to WNV infection, TLR signaling is thought to play an important role in protection against WNV. While TLR7 signaling has been shown to mitigate the susceptibility of infected mice to lethal WNV encephalitis (Town, et al. 2009), the role of TLR3 signaling in WNV infection is controversial. One study showed the presence of TLR3 signaling was responsible for severe neuroinvasion and lethality (Wang, Town, et al. 2004) while another study showed that TLR3 signaling protected mice against WNV neuroinvasion. (Daffis, et al. 2008) The failure of protection by TLR3 signaling in the former publication may be due to severe TLR3-induced inflammation induced by a more virulent WNV NY99 isolate since TLR3 signaling was shown to be responsible for the liver injury by upregulating the expression of inflammatory cytokines and recruiting immune cells in the Con A induced hepatitis. (Xiao, et al. 2009)

RLR. RLRs are localized in the cytosol and recognize cytosolic dsRNA derived from viral replication. There are 3 members of the RLR family, Retinoic acid-Inducible Gene I (RIG-I), Melanoma Differentiation-Associated Gene 5 (MDA5) and Laboratory of Genetics and Physiology 2 (LGP2). Each RLR contains a C-terminal domain (CTD) and a DExD/H-box helicase domain that recognize duplex RNA. (Silverman, Edwalds-Gilbert and Lin 2003) Additionally, RIG-I and MDA5 have a caspase activation and recruitment domain (CARD) on the N terminus for signaling transduction. (Leung and

Amarasinghe 2012) LPG2, lacking CARD, was shown to be an upstream regulator of RIG-I and MDA5 signaling. (Satoh, et al. 2010) RIG-I recognizes ssRNA and dsRNA (longer than 23 bp) (Marques, et al. 2006) containing a 5'tri-phosphate motif (5'ppp) and secondary structures at the 5' end which is a specific non-self viral genome structure and distinguished from self RNA. (Hornung, Ellegast, et al. 2006) MDA5 is thought to recognize ssRNA and dsRNA (longer than 1000 nts) of a higher molecular weight with 5'tri-phosphate motif (5'ppp). (Dixit and Kagan 2013) Although the mechanisms for MDA5 signaling activation are still poorly understood, studies have shown that the ligation with viral RNA triggers the dimerization of CARDs on RIG-I or MDA5 which recruit the adaptor molecules, mitochondrial antiviral-signaling protein (MAVS)/interferon promoter stimulator-1 (IPS-1). These adaptor molecules on the mitochondrial outer membrane form a signalosome complex with TRAF3, TBK1 and IKKε. The signalosome phosphorylates IRF-3 and IRF-7 to initiate the antiviral activities and the transcription of type I IFN. Meanwhile, NF-κB signaling is also activated through FADD and caspase-8/10 dependent pathway and initiates the transcription of proinflammatory cytokines. (Takahashi, et al. 2006) RIG-I and MDA5 have been shown to sense WNV infection (Suthar, Brassil, et al. 2013); (Fredericksen, et al. 2008) and initiate antiviral activity. RIG-I and MDA5 signaling pathways were shown to play an important and nonredundant role in manipulating the production of type I IFN and inflammatory cytokines and limiting viral replication and spread. (Errett, et al. 2013) The role of MDA5 signaling in clearing WNV from CNS has been attributed to its modulation on the antiviral function by $CD8^+$ T cells. (Lazear, Pinto and Ramos, et al. 2013); (Fredericksen, et al. 2008) Although there is no study showing the role of LGP2 in recognizing WNV, the absence of LGP2 was shown to impair the WNV specific CD8+ T cell survival and fitness. (Suthar, Ramos, et al. 2012)

NLRs. There are 23 family members in the NLR family in humans. The NLRs are characterized by a central conserved nucleotide oligomerization and binding domain

(NOD) motif. Similar to TLRs, NLRs have LRRs on the C terminus to recognize PAMPs. The effector regions for signal transduction are localized on the N terminus of NLRs and identified as CARD, pyrin domain (PYD), acidic domain, or baculovirus inhibitor repeats (BIRs). (Fanchi, et al. 2009) The central NOD domain is responsible for ATP hydrolysis which is essential for NLR oligomerization and activation. (Tanabe, et al. 2004) Activated NLRs induce NF-κB and MAPK signaling and the assembly of inflammasomes. The inflammasome is a large protein complex composed of NLR proteins, the adapter apoptosis-associated speck-like protein containing a C-terminal CARD (ASC) and pro-caspase-1 through the CARD-CARD and PYD-PYD interactions. (Fanchi, et al. 2009) The inflammasome activates caspase-1 which cleaves pro-IL-1β and pro-IL-18 into their active forms, IL-1β and IL-18. (Ghayur, et al. 1997) Although there is no report directly showing that WNV is recognized by NLRs, ASC was shown to be critical for IL-1β production and protecting mice from WNV encephalitis suggesting the involvement of inflammasomes in WNV infection. (Kumar, et al. 2013) Studies have shown that IL-1β played an important role in controlling WNV acute infection and neuronal invasion. (Durrant, Robinette and Klein 2013); (Ramos, et al. 2012) These studies suggest that NLRP3, a NLR sensing viral RNA (Lawrence, et al. 2013), may contribute to the formation of NLRP3 inflammasome and the production of IL-1β and IL-18 during WNV infection.

CLRs. There are 2 groups types of family members in the CLR family. One group are CLRs containing C-type lectin-like domains (CTLDs) that do not recognize carbohydrate ligands. The other group involved in recognizing CLR ligands is characterized by carbohydrate-recognition domains (CRDs). Activated CLRs trigger the Syk kinase/CARD9 pathway and activate NF-κB which directs the transcription of proinflammatory or inflammatory cytokines, IL-6, pro-IL-1β and TNF-α. (Drummond and Brown 2013) Additionally, the activation of a CLR, C-type lectin domain family 5 member A (CLEC5A), is critical in the activation of NLRP3 inflammasome in dengue

virus infection suggesting that CLRs are also involved in the activation of inflammasomes. (Wu, et al. 2013) However, at present there is no study investigating the role of CLRs in defending against WNV infection.

Summary. As discussed above, PRR signaling is responsible for the production of type I IFN and proinflammatory and inflammatory cytokines. These cytokines play an essential role in protecting hosts from WNV infection. The proinflammatory cytokine, IL-1β, was shown to play a protective role in WNV infection (shown in the NLR section). The signaling induced by TNF-α, another proinflammatory cytokine, was also shown to protect WNV infected mice by limiting WNV replication in CNS. This limitation is thought to be attributed to the recruitment of inflammatory cells into CNS. (Shrestha, Zhang, et al. 2008)

T cell responses.

The recognition of virus PAMPs by PRRs facilitates pathogen internalization and the maturation of phagosomes for virus degradation. The CLRs, the mannose receptor and DC-SIGN, were shown to be involved in DENV attachment and internalization (Miller, et al. 2008) The ligation of TLRs has also been shown to facilitate viral entry and internalization (Blander and Medzhitov 2004) by promoting the expression of receptors for pathogens (Bieback, et al. 2002.). TLR/MyD88 signaling was shown to facilitate phagosome maturation by regulating the fusion of phagosomes with phagolysosomes (Blander and Medzhitov 2004). The phagosome is a cellular compartment which is formed following the phagocytosis of pathogens. During maturation, phagosomes fuse with the more acidic lysosomes and mature into phagolysosomes where pathogens are digested. The proteasome is another important protein complex that is responsible for degrading pathogens or pathogen-derived proteins. The proteasomes process proteins into peptides by proteolysis. The degradation of pathogens and antigens in phagolysosomes

and proteasomes is essential for APCs to process and present antigens to $CD8^+$ and $CD4^+$ T cells and initiate T cell responses.

$CD8^+$ T cells are primed by recognizing the major histocompatibility complex (MHC) I-peptide complex presented on APCs while $CD4^+$ T cells are primed by recognizing the MHC II-peptide complexes. The two MHC complexes are generated in different pathways. (Blum, Wearsch and Cresswell 2013) With regard to MHC I-peptide complexes, the exogenous and endogenous proteins are proteolytically processed into short peptides in the proteasomes and transported into the ER by the transporter association protein (TAP). In the ER, the short peptides are loaded onto MHC I molecules and become MHC I-peptide complexes. For the biosynthesis of the MHC II-peptide complexes, MHC II is expressed on the inner surface of ER and transit to the mature endosomes via the trans-Golgi network. Meanwhile, antigens or pathogens are internalized, processed into short peptides and delivered to the mature endosomes. In the mature endosome, these short peptides are loaded onto the MHC II and form MHC II-peptide complexes. The MHC I-peptide complex and the MHC II-peptide complex are ultimately transported to the cellular surface and recognized by T cell receptors (TCRs) on $CD4^+$ and $CD8^+$ T cells. The stimulation of TCRs activates naïve T cells and acts as signal 1 to prime T cell responses.

Besides the interaction of TCRs and MHC-peptide (signal 1), the ligation of costimulatory molecules (signal 2) and the cytokine environment (signal 3) are important cooperative signals (discussed below) which influence the type and magnitude of T cell responses. The dynamic of T cell responses includes several processes, T cell activation, T cell expansion and differentiation, T cell contraction and memory T cell persistence.

T cell response development.

T cell response development includes T cell activation, expansion and the ultimate differentiation into memory T cells. T cell activation is modulated by 3 signals,

TCR signaling, costimulatory signaling and cytokine signaling. These signals are also important for shaping the subsequent T cell expansion and differentiation.

Signals to prime T cell activation.

Signal 1. The interaction between MHC-peptide complex and TCR acts as signal 1 for T cell activation. TCR signaling regulates T cell expansion, differentiation and cytokine production. In the presence of the co-receptors, CD4 and CD8, TCR activation triggers the downstream Src-family kinases, p56lck (Lck) and p59fyn (Fyn). (Salmond, et al. 2009) The activation of Lck and Fyn induces the extracellular signal-regulated kinases (ERK)/ mitogen-activated protein kinases (MAPK) pathway which regulates T cell proliferation. (Cuy, et al. 2013); (D'Souza, Chang, et al. 2008) The absence of Lck or Fyn was shown to impair the activation and expansion of $CD4^+$ and $CD8^+$ T cells. (Sugie, Jeon and Grey 2004); (Tewari, et al. 2006) Additionally, studies showed that the intensity and duration of TCR signaling determined the cytokine production and differentiation of T cells. (van Panhuys, Klauschen and Germain 2014); (Miskov-Zivanov, et al. 2013) The affinity of the TCR for peptide/MHC plays an important role in T cell differentiation into memory T cells since a study showed that TCR mutation in transgenic mice impairs the quantity of memory T cells. (Knudson, et al. 2013)

Signal 2. During the maturation of APCs, the expression of co-stimulatory molecules is upregulated. The co-stimulatory molecules expressed on APCs include the Immunoglobulin superfamily (B7.1/2 (CD80/CD86) and ICOSL) and TNF receptor superfamily (CD70, OX40L, and 4-1BBL). (Welten, Melief and Arens 2013) These molecules pair with the related co-stimulatory molecules on T cells including CD28, ICOS, CD27, OX40, and 4-1BB. The ligation of costimulatory molecules between APCs and T cells acts as a second signaling 2 for modulating T cell responses. (Welten, Melief and Arens 2013) The absence of CD80/CD86 or CD28-CD80/CD86 ligation was shown to cause an impaired $CD8^+$ T cell or $CD4^+$ T cells expansion, IFN-γ secretion and viral clearance following murine gammaherpesvirus 68 (MHV-68) (Fuse, Obar, et al. 2006),

influenza A virus (Dolfi, et al. 2011) or vaccinia virus (Fuse, Tsai, et al. 2011) infection. The B7-CD28 pathway was shown to be associated with $CD4^+$ and $CD8^+$ T cell responses against Epstein-Barr virus (EBV) and influenza A virus infection *in vitro*. (LeBlanc, et al. 2004) The ICOSL-ICOS interaction is also essential for T cell activation, differentiation and function. The production of IFN-γ by $CD8^+$ and $CD4^+$ T cells and the expression of transcriptional factors associated with T cell differentiation were shown to be impaired in ICOS deficient patients. (Takahashi, et al. 2009) The depletion of ICOS from $CD4^+$ T cells diminished their capacity to proliferate and produce effector cytokines (e.g. IL-2, IL-4, IL-10, IFN-γ). (Dong, et al. 2001) The costimulatory molecules in TNF receptor superfamily have a more complex cytoplasmic tail. OX40-OX40L interaction was thought to be essential in modulating primary $CD4^+$ T cell responses compared with $CD8^+$ T cell responses. Studies showed that the absence of OX40-OX40L interaction abrogated expansion, IFN-γ secretion and specific cytotoxicity of primary $CD4^+$ T cells against LCMV and influenza virus infection. (Kopf, et al. 1999); (Chen, et al. 1999) Fewer studies described the important role of OX40-OX40L interaction in manipulating primary $CD8^+$ T cell responses shown as $CD8^+$ T cells differentiation and effector cytokine production (Salek-Ardakani, Moutaftsi and Crotty, et al. 2008). However, there is sufficient evidence showing the essential role of OX40-OX40L interaction in memory CD8+ T cell development by facilitating $CD4^+$ T cell help. (Salek-Ardakani, Moutaftsi and Sette, et al. 2011); (Humphreys, et al. 2007); (Welten, Melief and Arens 2013) For 4-1BB-4-1BBL interaction, the activation of 4-1BB by anti-4-1BB antibodies promoted primary and secondary $CD8^+$ T cell responses against vaccinia or LCMV infection. (Zhao, Tahiliani, et al. 2012); (Vezys, et al. 2011) The absence of 4-1BBL was shown to impair primary $CD4^+$ and $CD8^+$ T cell responses and the survival of $CD8^+$ memory T cells in bone marrow. (Lin, et al. 2012); (Zhao and Croft 2012); (Hirao, et al. 2011) Because of the critical role of OX40 and 4-1BB in the development of memory T cells

(Dawicki, et al. 2004), the stimulation of OX40 and 4-1BB is considered a viable strategy for promoting vaccine efficiency.

Signal 3. Inflammatory cytokines are involved in promoting antiviral T cell responses. The mature APCs and activated T cells secrete various cytokines and create a unique cytokine milieu. These cytokines act as the third signal to shape the development of T cell responses. The addition of IFN-α during antigen stimulation has been shown to promote $CD8^+$ T cell activation, expansion and effector function following vaccination (Sikora, et al. 2009) or cross-priming in which APCs are not directly infected by virus (Le Bon, Durand, et al. 2006). IL-12 has been shown to facilitate the expansion and lytic effector function of Th1 $CD4^+$ and $CD8^+$ T cell following antigen stimulation. (Knutson and Disis 2004); (Curtsinger, et al. 1999) Although there is no direct evidence showing the influence of type I IFN on CD4+ T cell cytotoxicity, the differentiation of $CD4^+$ T cells into Th1 but not Th2 is determined by type I IFN and IL-12. (Sinigaglia, D'Ambrosio and Rogge 1999) IL-21 has also been shown to be important for $CD8^+$ T cells expansion and effector function *in vitro*. (Casey and Mescher 2007) Treatment with IL-1 was shown to facilitate antigen-induced T cell expansion, differentiation and IFN-γ production. (Pape, Khoruts, et al. 1997) This regulatory function of IL-1 was shown to be attributed to its direct interaction with APCs and $CD4^+$ T cells. (Khoruts, Osness and Jenkins 2004); (Ben-Sasson, et al. 2009) Another inflammatory cytokine, TNF-α, has been shown to facilitate the expansion and cytotoxic function of specific $CD8^+$ T cells *ex vivo*. However, this promoting function is not as efficient as other TNF family members (e.g. CD40 ligand). (Yu, et al. 2003) Anti-inflammatory cytokines, e.g. IL-10 and IL-4, in the milieu are responsible for dampening antiviral T cell responses. The absence of IL-10 production by APCs has been shown to promote the production of IFN-γ by Th1 cells against *Chlamydia trachomatis*. (Igietseme, et al. 2000) Treatment with IL-4 has been shown to inhibit the responsiveness of APCs to type I IFN and result in the decreased expression of MHC I and costimulatory molecules which are essential in activating $CD8^+$

T cells. (Sriram, et al. 2007) IL-2, a cytokine secreted by APCs (Granucci, et al. 2003) and activated T cells (Malek and Castro 2010), is essential for T cell expansion and differentiation into memory cells. Studies showed that the administration of IL-2 augmented and sustained $CD8^+$ T cell expansion in the primary immune response. (Yu, et al. 2003); (D'Souza and Lefrancois 2003) IL-2 therapy has been shown to abrogate the adenosine A receptor agonist-mediated inhibition on Th1 expansion suggesting the essential role of IL-2 on $CD4^+$ T cell proliferation. (Erdmann, et al. 2005) IL-2 also directs the differentiation of $CD4^+$ and $CD8^+$ T cells into memory cells and supports T cell memory. The differentiation of memory T cells from adoptively transferred activated $CD4^+$ and $CD8^+$ T cells was promoted by prior IL-2 treatment on the transferred cells *in vitro*. (Carrio, Bathe and Malek 2004); (Dooms, et al. 2007)

T cell expansion and differentiation.

T cells proliferate and become effector $CD4^+$ and $CD8^+$ T cells via modulation by the 3 signals discussed above. $CD4^+$ T cells differentiate into different T cell subsets, Th1, Th2, Th17, and regulatory T cells (T_{Reg}). IFN-γ secreting Th1 cells are the predominant antiviral $CD4^+$ T cells to be studied in this work. During proliferation, both $CD4^+$ and $CD8^+$ T cells also acquire multifunctionality as they differentiate. T cell multifunctionality is defined as the capacity of an individual effector T cell to simultaneously secret different effector cytokines, e.g. IFN-γ, TNF-α and IL-2. (Klatt, et al. 2011) Although it is still not well understood how this functional pattern is associated with T cell proliferation and differentiation, dual IL-2/IFN-γ T cells or single IL-2 T cells versus single IFN-γ T cells are more likely to obtain proliferation capacity. (Zimmerli, et al. 2005) During the differentiation into effector and memory T cells, IL-2 secreting or multifunctional T cells ($TNF\text{-}\alpha^+IL\text{-}2^+$, $IFN\text{-}\gamma^+TNF\text{-}\alpha^+$, $IFN\text{-}\gamma^+IL\text{-}2^+$) are more likely to develop into memory cells (central memory T cells and effector memory T cells which will be introduced below) while single IFN-γ secreting T cells represented terminally differentiated effector T cells. (Harari, et al. 2006) The secretion of TNF-α by T cells, on

the one hand, contributes to their effector function; on the other hand, has been shown to correlate with the expression of memory cell marker CCR7, indicating TNF-α secreting T cells also have memory cell potential. (Darrah, et al. 2007) Therefore, IL-2 and TNF-α secretion endows T cells a longer life span and memory potential while IFN-γ and TNF-α contribute to the effector function. Meanwhile, the function pattern of T cells (multifunctionality and monofunctionality) also influences the potential of the cells to differentiate into memory cells.

The functionality of effector T cells.

Effector T cells express strong effector function (e.g. secreting effector cytokines and CTL activities) but experience a short life span. The effector T cells are responsible for primary cellular immunity and viral clearance. Effector T cells clear virus infected cells through several mechanisms, such as Fas/Fas-L ligation, perforin and effector cytokines. Effector T cells are the major producers of IFN-γ, TNF-α and granzymes which are responsible for limiting viral replication and induce death of infected cells.

The effector cytokine, IFN-γ. IFN-γ, a type II IFN, plays an important role in the antiviral activity. IFN-γ triggers the phosphorylation of JAK1 and JAK2 portion of IFN-γ receptors. The phosphorylated JAK1 phosphorylates Stat molecules which form homodimers (Stat1:Stat1) or heterodimers (Stat1:Stat2) and induce downstream signaling. These signaling pathways direct the transcription of IRF-1, Stat1 and IFN-regulated genes which enhance antiviral activity. Viral clearance was shown to be attributed to direct and indirect IFN-γ-mediated mechanisms. (Schroder, et al. 2004) For the direct mechanisms, IFN-γ was shown to inhibit viral replication by upregulating protein kinase R (PKR). (Ramana, et al. 2000); (Garcia, Meurs and Esteban 2007) PKR was also shown to be involved in the production of inflammatory cytokines and the induction of apoptosis which contribute to the antiviral activity. (Kang and Tang 2012); (Balachandran, et al. 1998) IFN-γ itself has also been shown to upregulate the expression of Fas/FasL and TNF-α receptor which are involved in target cell apoptosis. (Xu, et al.

1998); (Tsujimoto, Yip and Vilcek 1986) With regard to the indirect mechanisms, IFN-γ facilitates antigen presentation and antiviral T cell responses. IFN-γ was shown to upregulate the expression of TAP and MHC molecules which are essential in the generation of MHC-peptide complexes. (Epperson, et al. 1992); (Shirayoshi, et al. 1988) IFN-γ is also involved in shaping T cell responses by upregulating expression of immunomodulatory molecules. Treatment with IFN-γ was shown to upregulate the expression of CD80 and CD86 which are costimulatory molecules for T cell activation (will be introduced below) (Menendez Iglesias, et al. 1997) and chemokines (e.g. MIP-1α/CCL3, MIP-1β/CCL4) which are essential in the recruitment of activated $CD4^+$ and $CD8^+$ T cells to the infection loci (Taub, et al. 1993); (Kasama, et al. 1995).

The effector cytokine, TNF-α. TNF-α secreted by effector T cells displays its antiviral capacity via cytotoxic and non-lytic mechanisms. The cytotoxic activity is attributed to TNF-α-mediated cell death. There are two forms of TNF-α, secreted and membrane-associated. Secreted TNF-α induces the apoptosis of infected cells by binding to TNF-α receptors of which the death domain activates the downstream caspase proteins. (Smyth and Johnstone 2000) The membrane-associated TNF-α triggers cell death in a cell-to-cell contact manner. With regard to the effector function, TNF-α contributes to T cell activation, proliferation and differentiation. TNF-α has been shown to enhance the maturation of dendritic cells which are essential APCs to activate T cells. (Brunner, et al. 2000) Blockage of the TNF receptor was shown to inhibit T cell proliferation. (Harrop, et al. 1998) Additionally, TNF-α has been shown to cooperate with IFN-γ and promote the expansion and differentiation of $CD4^+$ and $CD8^+$ T cells into CTLs. (Roth, et al. 1991) Like IFN-γ, TNF-α is also able to suppress viral gene expression. (Guidotti, et al. 1994)

Perforin-dependent mechanisms. Effector T cells secrete granzymes and perforins which cooperate to kill the virus infected cells. This activity is dependent on the pore forming protein, perforin, which facilitates the delivery of granzymes into infected cells. Perforins insert themselves into the plasma membrane and form a tubular polymer

through which the granzymes can be delivered into the cell cytoplasm. (Tschopp and Nabholz 1990) The granzymes are proteases which degrade intracellular substrates and promote target cell death. Granzymes include granzyme A, B, K, and H. Granzyme B, the most abundantly secreted granzyme, proteolytically processes pre-caspase 3 and 7. Activated caspase 3 and caspase 7 induce apoptosis of infected cells. (Talanian, et al. 1997) Additionally, granzyme B also activates apoptosis indirectly by cleaving and activating BID, a BH3-containing pro-apoptotic protein. BID targets to mitochondria followed by the formation of apoptosomes which activate caspases and induce apoptosis. (Waterhouse, et al. 2005); (Cullen, Brunet and Martin 2010) The other granzymes (A, K and H) may play a non-cytolytic role because of low physiological concentration. (Cullen, Brunet and Martin 2010) Granzyme A has been shown to act as the enzyme to convert the precursor IL-1β to the mature form (Irmler, et al. 1995) which induces cell death. Granzyme K has also been shown to augment the LPS-induced inflammatory response (e.g. TNF-α production) suggesting the antiviral activity of granzyme K. (Wensink, et al. 2014) Granzyme H exhibited suppression of viral replication by cleaving the viral proteins that are required for replication. (Tang, et al. 2012)

Fas/Fas-L mechanism. Effector T cells express FasL and induce Fas/FasL-mediated cytotoxicity. Following T cell activation, the expression of FasL, especially on Th1 cells, was shown to be upregulated. (Norian, et al. 2000) FasL interacts with Fas on the target cells and induces caspase-mediated apoptosis of the target cells. The absence of FasL from granzyme deficient CTL has been shown to further impair cytotoxic function suggesting that Fas-FasL pathway is another important mechanism for CTL function besides perforin-mediated mechanism. (Shresta, Russell and Ley 1997) A study also showed preferential utilization of pre Fas-FasL mechanisms by $CD4^+$, but not $CD8^+$ T cells for cytotoxic function (Traidl, et al. 2000) which is consistent with the preferential expression of FasL by Th1 $CD4^+$ T cells.

T cell contraction.

Following T cell activation and differentiation to become effector T cells, 90%~95% of T cells undergo apoptosis leaving the remaining cells as memory T cells for persistent protection. This process is called T cell contraction. T cell contraction is important in rescuing the host from tissue damage or autoimmunity resulting from uncontrolled T cell cytokine production and cytotoxicity and for maintaining homeostasis. T cell contraction is triggered via different mechanisms including activation induced cell death (AICD), cytokine or growth factor mediated mechanisms and antigen withdrawal.

Studies showed that T cells undergo apoptosis following antigen stimulation. (Shi, et al. 1990) Apoptosis was shown to be induced by the ligation of FAS on T cells with FASL which is secreted by the activated T cells themselves. (Fulda, et al. 2000) Other factors were also shown to be responsible for T cell death. Granzyme B inhibitor, 2-AAD-CMK, has been shown to rescue effector $CD8^+$ but not $CD4^+$ T cells from AICD suggesting the involvement of Granzyme B in $CD8^+$ T cell contraction. (Gorak-Stolinska, et al. 2001) The inhibition of reactive oxygen species (ROS) which are produced during AICD (Zamzami, et al. 1995) has been shown to prevent the apoptosis of cytotoxic T lymphocytes (CTLs) (Norell, et al. 2009) suggesting that ROS also mediate T cell contraction. The apoptosis of activated $CD4^+$ T cells after treatment with chemokines, CCL19 and CCL21, was shown to be promoted indicating chemokines may also be involved in AICD. (Yasuda, et al. 2007) In addition to AICD, T cell contraction is also attributed to antigen withdrawal. Viral clearance and reduction in antigen availability cause T cell apoptosis as shown by the dramatic apoptosis of antiviral $CD4^+$ and $CD8^+$ T cells after HSV-1 clearance. Apoptosis resulting from antigen withdrawal could be attributed to the lack of antigen-induced TCR signaling which is essential for T cell survival. (Marrack and Kappler 2004) On the other hand, the termination of immune responses also causes the shutdown of cytokine production which leads to T cell

contraction. IL-2, IL-15 and IL-7 are important to protect T cell from contraction. The ligation of IL-2 receptor γ chain and IL-7 receptor was shown to sustain or upregulate the expression of anti-apoptotic protein Bcl-2 and protect memory T cells from cell death. (Li, et al. 2001); (Kondrack, et al. 2003) Apoptosis results from antigen withdrawal and immune response termination was shown to be due to Bcl-2-mediated apoptosis pathway which was independent of Fas-FasL mediated cell death. (Pellegrini, et al. 2003); (Davey, et al. 2002)

The development of memory T cells.

Following T cell contraction, only memory T cells survive and are responsible for conferring long term protection. It is still controversial as to how memory T cells are developed. There are several models for memory T cell differentiation: the obligatory linear differentiation model, the optional linear model, the decreasing potential model, the progressive differentiation model and so on. The obligatory linear differentiation model postulates that memory T cells are the direct descendants of effector T cells. The optional linear differentiation model postulates that memory T cells are developed either in a dependent or independent manner from effector T cells. (Gerlach, van Heijst and Schumacher 2011) Memory T cells have a prolonged life-span and are maintained in a quiescent state. Following the re-exposure of memory T cells to pathogen-antigens, memory T cells, compared to naïve T cells, respond more rapidly and differentiate into a larger number of effector T cells for more efficient pathogen clearance. Therefore, developing a high quality memory T cell response is essential for successful vaccination. Memory T cells can be divided into several subsets, central memory T cells (T_{CM}), effector memory T cells (T_{EM}) and resident memory T cells (T_{RM}). T_{CM} are predominantly localized in secondary lymphoid organs while T_{EM} are localized in peripheral compartments, e.g. blood and lung. T_{RM} represent a recently discovered subset and localize initially in inflamed peripheral tissues, e.g. skin, gut, and lung, where they are maintained long after resolution of the infection. Compared with T_{EM} and T_{RM}, T_{CM}

have longer life span and are more proliferative because of the autocrine IL-2. T_{CM}, compared with T_{EM}, were shown to mount a more vigorous recall response to antigens (Bouneaud, et al. 2005) but are less responsive to cytokines, IL-15 and IL-7. (Geginat, Sallusto and Lanzavecchia 2001) However, the responsiveness of T_{CM} and T_{EM} to re-exposure of pathogens might also depend on the infection location since T_{EM} were shown to be prominent in the secondary response against Sendai virus infection in lung. (Roberts and Woodland 2004)

T_{RM} have been localized in different peripheral tissues, e.g. brain, lung, small intestine, vaginal mucosa, salivary glands and thymus. (Mueller, Zaid and Carbone 2014) HSV infection has been shown to cause the generation of T_{RM} in genital tract tissues (Gebhardt, et al. 2011) which I study in Chapter 4 of this dissertation work. T_{RM} display a dendritic morphology which distinguishes them from T_{EM} in peripheral tissues. (Zaid, et al. 2014) A very recent study also showed that T_{RM} were retained as memory lymphocyte clusters (MLCs) independently of circulating T_{EM} in a network of macrophages. (Iijima and Iwasaki 2014) During the infection occurring in the genital tract, antigen bearing tissue-associated DCs migrate to the adjacent draining LNs and activate T cells. (von Andrian and Mempel 2003) Activated T cells have been shown to be attracted to infected tissues (skin) by CXCL9 and CXCL10 secreted by keratinocytes. (Mackay, Rahimpour, et al. 2013) The migrating T cells are characterized by the surface marker, CD103 and CD69. (Casey, Fraser, et al. 2012) CD103 is the α chain of αEβ7 integrin which facilitates T cell migration within the peripheral tissues at a lower velocity. It is still not clear how T_{RM} protect against re-exposure of pathogens. However, a study showed that following antigen re-exposure at the genital tract, T_{RM} produced IFN-γ and recruited more memory T cells to the peripheral tissues. (Schenkel, et al. 2013) Although T_{RM} were shown to respond to antigens and proliferate (Wakim, et al. 2008), whether T_{RM} are involved in killing infected target cells is still poorly understood.

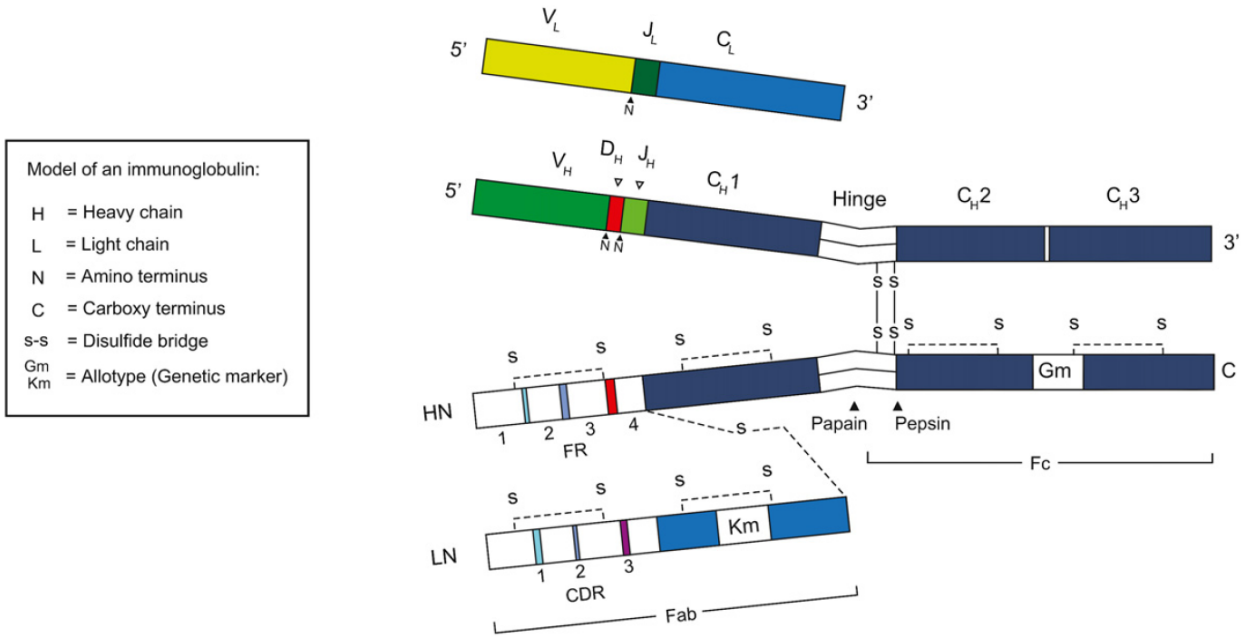
B cell (humoral) responses.

Immunoglobulins and B cells.

The B cell response (humoral response) acts as another important branch of adaptive immunity against viral infection.

Immunoglobulin. The major and well-known function of B cells is their capacity to secrete antibodies (at 2,000 to 20,000 molecules per second) that can neutralize viruses and contribute to viral clearance. Immunoglobulins (shown in Fig.1.4) are divided into different isotypes, IgM, IgA, IgE, IgD, and IgG. IgM is an immunoglobulin pentamer and is secreted by newly activated B cells. IgA is secreted as a monomer, dimer, or tetramer by plasma cells residing in mucosal tissues. IgE is involved in immunity against parasitic worms and developing allergic diseases. IgD is an immunoglobulin monomer that is constitutively expressed on mature naïve B cells which migrate from the bone marrow and reside in the secondary lymphoid organs. (Chen and Cerutti 2010) IgD has been shown to help the survival of naïve B cells (Peckham, et al. 2001) and modulate immunity and inflammation (Chen and Cerutti 2010). IgG is an immunoglobulin monomer secreted by fully activated B cells and plays a major role in mature humoral responses and host defense (discussed below).

Figure 1.4: The structure of an immunoglobulin molecule.



Representation of immunoglobulin structure. (Reprinted with permission from Schroeder HW Jr, Cavacini L. Structure and function of immunoglobulins. 2010. J Allergy Clin Immunol. 125(2 Suppl 2):S41-52.).

Immunoglobulin monomers are composed of two heavy chains and two light chains. The light chain has a variable domain (V_L) and a constant domain (C_L) while the heavy chain has a variable domain (V_H) and three constant domains (C_H1, C_H2 and C_H3). Each light chain pairs with each heavy chain by V_L-V_H interaction and C_L-C_H1 interaction. The two heavy chains are paired by disulfide bonds in the hinge region between C_H1 and C_H2, C_H2 and C_H3 portion on heavy chains. The portion comprising C_H1 through C_H3 is called the F_C region. The F_C region can bind the C1q subunit of the C1 complement complex (will be introduced below) or F_C receptors on the cell membrane of several types of immunocytes. There are 3 hypervariable regions, CDRs (complementary determinant regions), on the V_L and V_H portion. CDRs endow immunoglobulins with the capacity to specifically recognize and bind an individual

epitope. The high diversity of CDRs allows immunoglobulins to recognize and neutralize viruses or antigens and facilitate viral clearance.

Immunoglobulins are involved in viral clearance by opsonizing viruses, activation of the complement pathway, or ADCC mechanisms. For viral opsonization, viruses are bound by the antigen-binding regions of antibodies and phagocytosed though complement-mediated-, F_C receptor-mediated- or tripartite motif-containing 21 (TRIM21) (Keeble, et al. 2008)-mediated mechanisms. The complement system consists of a group of small serum proteins which are involved in the cascade to neutralize pathogens or kill infected cells. During complement-mediated opsonization, C1 complement complex is recruited to the F_C portion of immunoglobulins (IgM and IgG) which coat the viruses via their CDRs. Following the recruitment and cleavage of a series of complement components, the complement-antibody-pathogen complex is formed. C3 on the complex is recognized by complement receptors (CRs) on macrophages. Binding of the C3 by CRs induces phagocytosis and opsonization. (Aderem and Unerhill 1999) During F_C receptor- or TRIM21-mediated phagocytosis, the Fc portion on the virus-immunoglobulin complex is recognized by F_C receptors or TRIM21 on macrophages and granulocytes. The phagocytosed antibody-virus complex is digested in the low pH environment in lysosomes. Besides opsonization, immunoglubulins are involved in viral clearance by ADCC. In ADCC, IgGs recognize and are recruited at the target cell surface which express viral antigens. Effector cells (e.g. NK cells, monocytes, macrophages) that bear Fc receptors on their surface recognize the IgG bound target cells and release IFN-γ (Jegaskanda, et al. 2013), perforin and granzymes (Sanapala, et al. 2012) to kill the infected cells. (Ahmad and Menezes 1996)

B cell subsets.

There are several B cell subsets, B-1 ($CD5^+$ B-1a and $CD5^-$ B-1b) B cells, marginal zone (MZ) B cells, and B-2 B cells. In spleens (spleen structure is shown as Fig. 1.6), ~3% of the B cells are B-1 B cells and 15% are MZ B cells while more than 70%

are B-2 B cells. (Baumgarth 2011) B-1 B cells are described as innate-like B cells because of their constitutive secretion of natural IgM which has minimal rearrangement of V regions in the absence of antigenic challenge. B-1 cells respond to self- or non-self-antigens and are localized in spleens, peritoneal, and pleural cavities. (Berland and Wortis 2002) Foreign antigens which crosslink the BCRs trigger B-1 B cell expansion and polyreactive IgM production. MZ B cells ($CD21^{hi}CD23^{lo}$), another B cell lineage, are also important IgM producers and localize in the marginal zone of spleens. MZ B cells exhibit a similar cellular function to B-1 B cells in responding to thymus independent type 2 (TI-2) antigens (Marin, Oliver and Kearney 2001) which induce humoral responses in a T cell independent manner. The antibody production by both B-1 and MZ B cells contributes to the very early humoral response. B-1 B cells in the peritoneal cavity have been shown to be activated within 1 hour of antigen exposure and are recruited to lymph organs to produce IgM within 1 or 2 days after skin immunization. (Itakura, et al. 2005) MZ B cells have been shown to differentiate into plasma cells by *in vitro* stimulation with LPS within 24 hrs while *in vivo* immunization with T-independent antigens induced the generation of plasmablasts devided from MZ B cells within 3 days. (Marin, Oliver and Kearney 2001) The last lineage of B cells, B-2 B cells, is the conventional B cell population that differentiates into plasma cells and undergoes maturation of the antibody response by class switch recombination (CSR) and somatic hypermutation of Ig genes during the adaptive immune response. B-2 B cells (sometimes referred to as follicular (FO) B cells) become activated, proliferate and differentiate in a T cell dependent manner. B-2 B cells may differentiate into short-lived plasma cells in the extrafollicular area in lymphoid tissues, or into the long-lived plasma cells and memory B cells in the germinal center (GC) reaction in the follicles. The T cell dependent response contributes to the second wave of primary humoral responses. Therefore, B-2 B cells are critical in the late phase and preventing secondary infection.

The developmental process of this conventional humoral response will be further discussed below.

B cells as APCs.

B cells play an important role not only in the production of immunoglobulin but also the modulation of adaptive immune responses by acting as APCs and producing immunomodulatory cytokines. In the section below, these B cell functions will be fully discussed.

In addition tothe secreted form, membrane-associated immunoglobulins are also expressed by B cells. Mature naïve B cells express low affinity membrane-associated mIgM and mIgD. Membrane-associated immunoglobulins are co-expressed with Igα/Igβ heterodimer on the B cell surface which together constitute B cell receptors (BCRs). Antigen capture by BCRs initiates antigen presentation by B cells. BCRs have been shown to facilitate the internalization and processing of multivalent or monovalent antigens. (Siemasko and Clark 2001) Following internalization of the antigen-BCR complex, antigens are degraded into small peptides in late endosomes. Meanwhile, BCR-mediated B cell activation causes the accumulation of MHC II-containing compartments in lysosomes where the processed peptides are loaded onto MHC II. (Lankar, Vincent-Schneider, et al. 2002) Many factors are involved in determining the efficiency of BCR-mediated antigen presentation. The nature of BCR-ligation to antigens (e.g. ligation affinity, antigen concentrations, and BCR signaling transduction) was shown to determine the antigen presentation efficiency by MHC II. A study showed that the affinity of BCR for hen egg lysozymes (HELs) and the concentration of HEL correlated with the different capacity of the B cells to present antigens to T cells. (Batista and Neuberger 1998) This suggests that the efficiency of B cell antigen presentation was dependent on the BCR affinity threshold and the concentration of Ag-BCR complex. Additionally, studies using different B cell lymphomas which were transfected with

normal or mutated Igα, Igβ or Syk-expressing plasmids also showed the important role of BCR signaling in determining the antigen presenting efficiency. The results showed that the Igα/Igβ heterodimer of BCRs accelerated antigen endocytosis and the trafficking of internalized antigen-BCR complexes. Meanwhile, the recruitment of Syk kinases to the accumulated antigen-BCR microclusters facilitated antigen presentation. (Lankar, Briken, et al. 1998); (Siemasko, Eisfelder, et al. 1999) Like other APCs, activated B cells express co-stimulatory molecules (e.g. CD80 and CD86) necessary for activating naive $CD4^+$ T cells. (Clatza, et al. 2003) As will be discussed below, antigen presentation by B cells to $CD4^+$ T cells also provides an additional level of assistance for the GC reaction and facilitates the development of humoral memory immunity. (Barnett, et al. 2014) Among these B cell subsets, MZ and B-1 B cells are the major B cell lineages acting as APCs. MZ B cells infected with parasites *in vitro* or primed with antigens *in vivo* are able to present antigens and activate $CD4^+$ T cells. (Attanavanich and Kearney 2004) Furthermore, the antigen presentation by MZ B cells that were primed *in vivo* was shown to be more efficient than presentation by B-2 (FO) B cells. (Attanavanich and Kearney 2004) Peptide-pulsed peritoneal B-1 B cells were also shown to prime $CD4^+$ T cells more efficiently than splenic B cells which are primarily B-2 B cells. (Margry, et al. 2013) The efficient antigen presentation by B-1 B cells was shown to be attributed to their superior phagocytic capacity compared with B-2 B cells. (Parra, et al. 2012)

The regulatory function of B cells.

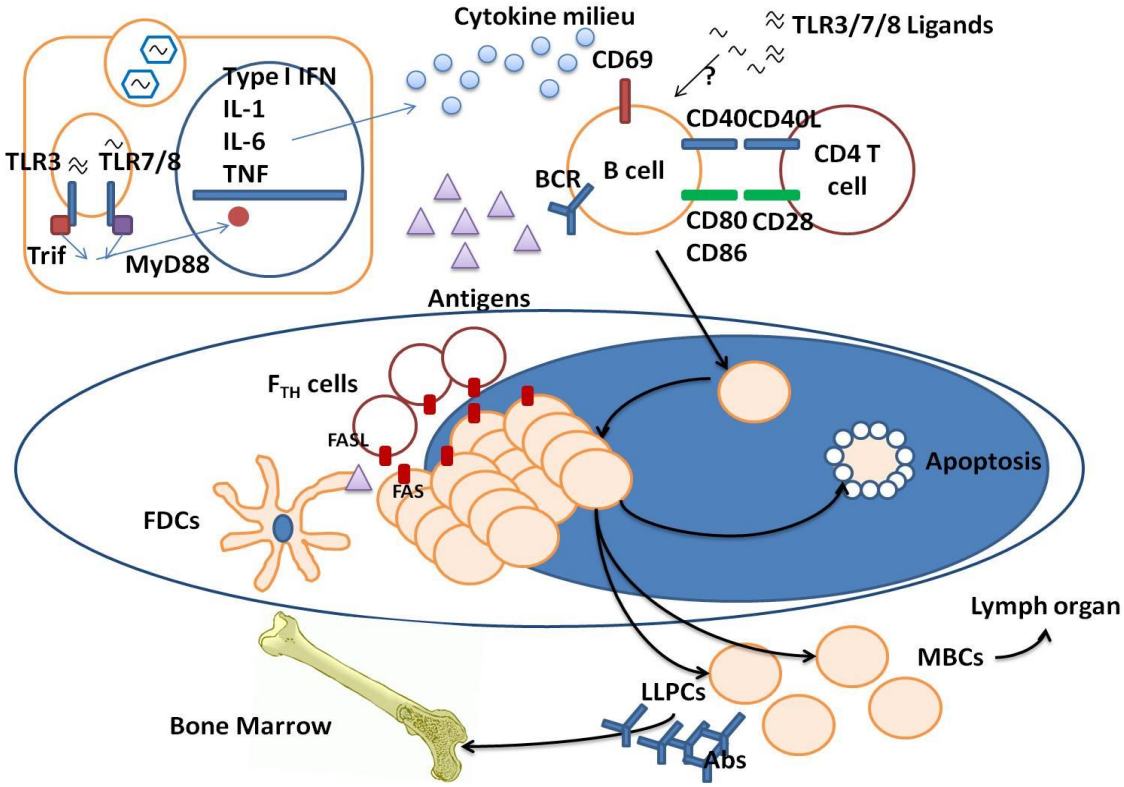
Recently, B cells that are capable of secreting anti-inflammatory cytokines (e.g. IL-10, TGF-β) were identified as regulatory B cells. Regulatory B cells that secrete IL-10 are the most frequently studied and are referred to as B10 B cells. In mice, IL-10-secreting B cells mainly have the $CD1d^+CD5^+$ (Matsushita, et al. 2008) phenotype and may include the $CD5^+$ B-1a B cells and $CD1d^{hi}CD23^-IgM^{hi}CD21^{hi}$ MZ B cell subsets. IL-10 expression at a high level has been described in the peritoneal B-1 B cells but not the splenic B-2 B cells (Fischer, et al. 2001). In *Leishmania donovani* infection or in contact

hypersensitivity reactions, IL-10 secreting B cells have been shown to display a MZ-like phenotype, expressing $CD23^{-}IgM^{hi}CD21^{hi}$ (Yanaba, et al. 2008); (Bankoti, et al. 2012) IL-10 secreting regulatory B cells play an important role in suppressing inflammatory responses induced by infections.

The development of B cell responses.

B2 B cell responses represent the B cell subset most relevant in response to vaccines. The development of B2 B cell responses (Fig.1.5) is precisely programmed and divided into several steps, B cell activation, B cell proliferation, maturation in the GC and the generation and persistence of memory cells. These processes will be further discussed in the section below.

Figure 1.5: B cell response development.



Representation of the developmental process of T cell-dependent B cell responses. After innate immune cells (orange rounded rectangle) take up virus, viral genome or genome replicates (curve) activate TLRs and triggered proinflammatory cytokine or inflammatory cytokine production. B cells (orange circle) are activated following the ligation of BCRs by antigens (purple triangle) and TLR ligation. In the presence of cytokine milieu (blue bubbles) and activated $CD4^+$ T helper cells (brown circle), B cells further mature in GCs (blue oval). In the dark zone (filled blue oval), B cells proliferate, undergo SHM and express FAS on their surface. In the light zone (blank part of blue oval), high affinity B cells are selected to survive with the help of FDCs (orange dendritic cells) which present antigens and F_{TH} cells (red circle) which induce the death of low affinity B cells by FAS-FASL mediated mechanisms. Surviving cells become MBCs (which stay in lymph organs or blood) and LLPCs which migrate to and go into long-term survival in bone marrow. (Details will be discussed in the content below.)

B cell activation. B cells are activated via antigens binding to and crosslinking BCRs. (Kurosaki 1997) Once crosslinked by antigens, BCRs are oligomerized and form lipid-raft-associated microclusters. The cytoplasmic region of the oligomerized BCRs, called the immunoreceptor tyrosine-based activation motif (ITAM), is phosphorylated by

Lck/Yes novel tyrosine kinase (Lyn). The phosphorylated ITAM recruits spleen tyrosine kinase (Syk) to signal the downstream cascades and direct the transcription of proliferation-associated genes (cyclin D and cdk4). Thus, B cells expand and differentiate into antibody secreting plasma cells.

Antigens are captured by DCs or macrophages which migrate to LNs and transfer antigens to BCRs (shown in Fig1.6, Fig1.7). Free antigens in lymphatic vessels spread to lymph nodes and may be presented to B cells by $CD11b^{+}CD169^{+}MHCII^{+}$ subcapsular sinus macrophages. (Junt, et al. 2007) Blood-borne antigens spread to the spleen and may be presented to B cells by marginal zone macrophages (Aichele, et al. 2003) or DCs (Balazs, et al. 2002). The capacity of splenic and LN DCs in transferring antigens and activating naïve B cells has been tested both *in vivo* and *in vitro*. (Wykes, et al. 1998); (Qi, Egen, et al. 2006) Additionally, these DCs were shown to promote the development of subsequent extrafollicular B cell responses. (Qi, Egen, et al. 2006) Following activation, B cells initially secrete IgM. Then the mIgM and mIgD expressed on naïve B cells switch to the more mature Ig isotypes, IgA, IgG and IgE (Pone, Zan, et al. 2010) in response to cytokine signals through the process called CSR. In CSR, the CSR protein complex machinery binds switch (S) regions on the heavy chain gene and forms an R-loop which exposes the non-template DNA region for cleavage. Activation-Induced (Cytidine) Deaminase (AID) is recruited to the R loop and deaminates cytosine (C) to uracil (U) residues which forms U:G mismatches. (Maul, et al. 2011) Uracil residues of U:G mismatches are then deglycosylated by uracil DNA glycosylase and processed into DNA strand breaks (DSBs) by apyrimidic/apurinic (AP)-endonucleases (APE1/2). (Guikema, et al. 2007) DSBs induce the DNA damage response and the formation of an S-S synapse which results in the deletion of the R loop DNA by a loop-out/deletion mechanism (Jack, et al. 1988). CSR is completed by the joining of DNA ends at the S-S synapse. The CSR mechanism also directs IgG switch to different subclasses (e.g. IgG1,

IgG2a, and IgG2c in mice) (Kracker and Durandy 2011) by the same mechanism in responses to different switch factors which will be discussed below.

In addition to BCR ligation (Munthe, et al. 2004), TLR signaling and cytokine signaling are also important in activating B cells. TLR ligands (e.g. LPS, CpG) (Ogata, et al. 2000), cytokines (e.g. type I IFN (Purtha, Chachu, et al. 2008), and BAFF (Lindh, et al. 2008)) directly signal B cells and fine-tune the T-cell-independent B cell response. (Pihlgren, et al. 2013); (Herlands, et al. 2008); (Zhu, Huang and Yang 2007); (Harada, et al. 1983) Cytokines and TLR ligands (Phan, Amesbury, et al. 2003) binding by specific TLR (Bombardieri, et al. 2011) have also been shown to synergize with BCRs in CSR.

Like other lymphocytes, activated B cells upregulate expression of the early activation marker, CD69. (Sacco, et al. 1994) As discussed above, activated B cells also express co-stimulatory molecules (e.g. CD80 and CD86) necessary for priming naïve $CD4^+$ T cells.

B cell proliferation and differentiation. Following activation, B cells proliferate and differentiate into plasmablasts/plasma cells, long lived plasma cells or memory B cells. The expansion and differentiation of activated B-2 B cells takes place in two anatomically separated compartments in secondary lymphoid tissues, the extrafollicular area and the B cell follicles (as shown in Fig1.6 and Fig1.7 and discussed respectively in the content below). B cell proliferation and differentiation in the extrafollicular area generate IgG secreting short-lived plasma cells. This occurs rapidly (as early as day 3 post-infection) and contributes to the early primary antibody response. The proliferation and differentiation in B cell follicles is referred to as the GC reaction which contributes to the development of mature humoral memory immunity. The location where B cell expansion and differentiation occur ultimately determines the development of short-lived primary or long-lived memory humoral responses.

B cell proliferation and differentiation in the extrafollicular region. Naïve B cells are usually localized in follicles in the spleens and LNs. The majority of these B cells stay only briefly before migrating to the blood. (Nieuwenhuis and Ford 1976) B cells in both recirculation and lymphoid tissues can encounter antigens and become activated. Recirculating B cells are activated by antigens in blood or intranodal lymphatics of adjacent LNs where free antigens are available (MacLennan, et al. 2003) and ultimately migrate to the splenic T cell zone (Liu, Zhang, et al. 1991). Free antigens are transferred and presented to B cells as previously discussed. Then antigen-primed B cells migrate to the T cell zone and interact with $CD4^+$ T cells on day 1-2 post-immunization with antigens or viral infection (Baumgarth 2013); (Luther, et al. 1997). During the early phase of the interaction with $CD4^+$ T cells, CSR to different IgG subclasses is induced. For extrafollicular B cells, CSR has been shown to take place early after activation and end after B cell differentiate into plasmablasts. (Marshall, et al. 2011) T helper cells secrete various cytokines (e.g. IL-4, IFN-γ and IL-27) which guide the recombination of μ constant (C) region on the heavy chain genes. The presence of IL-4, a Th2 cytokine, with or without CD40-mediated T cell help has been shown to induce CSR from Cμ to Cε or Cγ1 which results in the switch to IgE or IgG1. (Litinskiy, et al. 2002); (Ma, Wortis and Kenter 2002) IFN-γ (Collins and Dunnick 1993) and IL-27 (Yoshimoto, et al. 2004) have been shown to induce CSR from Cμ to Cγ2a and complete the switch to IgG2a which is the dominant subclass of Ig elicited by viral infection and responsible for ADCC. (Coutelier, et al. 1987)

Following T cell dependent B cell activation, B cells commit to two differentiation lineages, extrafollicular B cells and germinal center B cells. The differentiation choice between extrafollicular plasma cells and GC B cells has been shown to be regulated by the antigen recognition strength shown as the density and affinity of antigen-BCR interaction. (Paus, et al. 2006) The differentiation to extrafollicular plasmablast was associated with the upregulation of Blimp-1 (B

lymphocyte-induced maturation protein-1) (Shaffer, Lin, et al. 2002) and the expression of the surface marker syndecan-1 (CD138). Cells with these markers migrate to and colonize in the extrafollicular area between the T cell zone and the red pulp zone (Jacob, Kassir and Kelsoe 1991) where plasmablasts continue to expand, differentiate into plasma cells and produce short-lived immunoglobulins. The expansion, but not migration or differentiation, of plasmablasts has been shown to be controlled by antigen affinity. (Chan, et al. 2009) The migration of plasmablasts to the red pulp is attributed to the chemokine attraction of CXCR4 expressing plasmablasts to sites of CXCL12 producers. A study showed the expression of CXCL12 was mostly detected in the red pulp by *in situ* hybridization and that the absence of CXCR4 failed to form plasma cell clusters in the red pulp. (Hargreaves, Hyman, et al. 2001) With regard to the plasmablasts growth, only a portion of cells were reported to survive with the assistance by $CD11c^{high}$ DCs in the red pulp zone (Garcia De Vinuesa, et al. 1999) while others die of apoptosis. However, how the DCs protect the plasmablast from apoptosis is poorly understood. The surviving plasmablasts eventually differentiate into short-lived plasma cells. The terminal differentiation depends on the exit of the plasmablasts from the cell cycle. A study showed that the terminal differentiation of B-cell lymphoma line (BCL-1) as defined by the increased expression of CD138 and the secretion of IgM is dependent on the upregulation of Blimp-1 which repressed the promoter activity of *c-myc* which is critical for cell proliferation. (Lin, Wong and Calame 1997) However, another study also showed that the inducible inhibition on c-Myc is not sufficient to drive the BCL-1 to the terminal differentiation suggesting additional mechanisms to terminate the plasmablast differentiation. (Lin, Lin and Calame 2000) The extrafollicular short-lived plasma cells have been shown to start responding on day 3-4 post-immunization and die of apoptosis on day 7 post-immunization followed by death of over 20% of these cells daily. (Smith, Hewitson, et al. 1996)

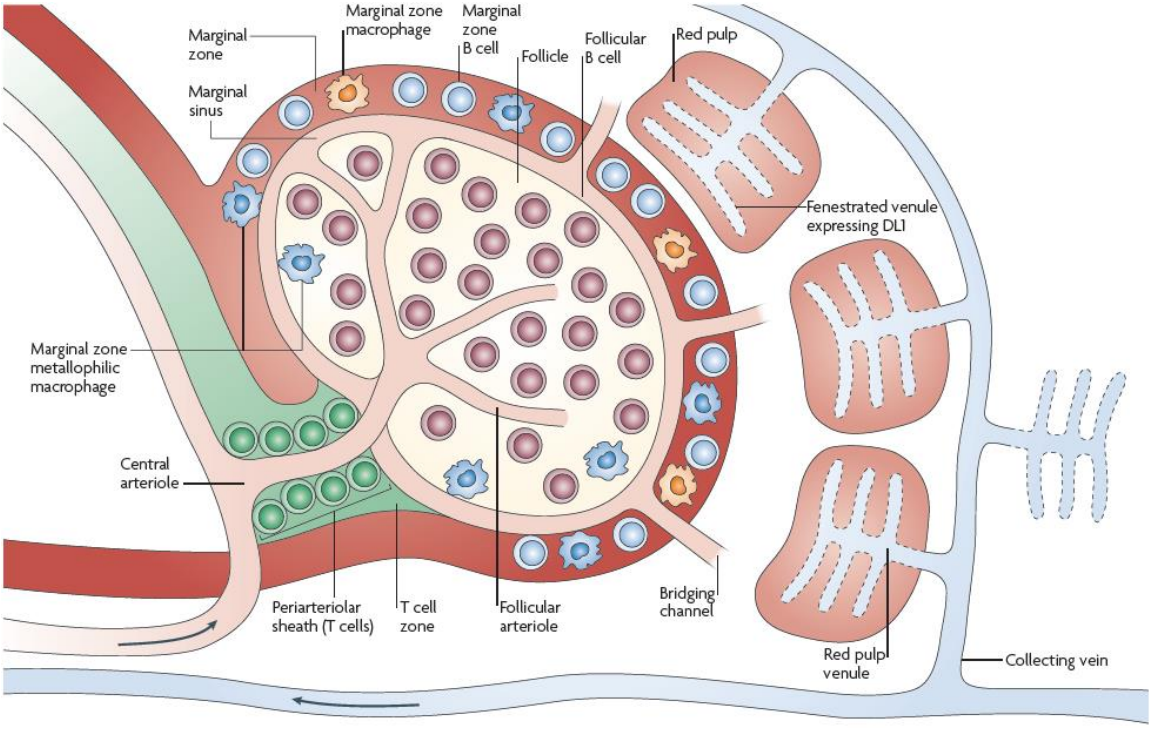
B cell proliferation and maturation in GC reaction. While the extrafollicular plasma cells are responsible for the initial wave of the T cell dependent antibody response, the second antibody response wave is attributed to the plasma cells derived from the GC. Following B-T interaction at the T cell zone-follicle junction, a portion of activated B cells upregulate the expression of CXCR5 and are chemotactically attracted to the follicle region by CXCR13 which is secreted by follicular DCs. (Ansel, et al. 2000) The fate of the progeny B cells derived from the activated B cells has been shown to be determined after the first cell division. (Hawkins, et al. 2009); (Turner, Hawkins and Hodgkin 2008) The activated B cells at the T-B border start to express the Epstein-Barr virus induced molecule 2 (EBI-2) which results in their migration to the perifollicular regions between the central region of follicles and the extrafollicular area. (Pereira, et al. 2009) Some of these cells start to downregulate EBI-2 expression due to the high expression of Bcl-6 and become residents in the GCs. (Shaffer, Yu, et al. 2000) These cells retain CXCR5 and CXCR4 expression and are attracted back to the central follicles for GC maturation.

In the specialized GC area, according to the classic model, B cells undergo two processes, somatic hypermutation (SHM) and affinity selection in two separate anatomical areas, the dark zone and the light zone. The CXCL12 producing stromal cells in the dark zone have been shown to position the CXCR4 expressing B cells in the dark zone (Allen, et al. 2004). In the classic model, GC B cells proliferate and undergo SHM in the dark zone and are referred to as centroblasts. (Liu, de Bouteiller, et al. 1996) SHM is the mechanism inducing frequent somatic mutation (point mutation) occurring in the Ig CDRs of proliferating GC B cells. A study showed that SHM occurred in the CDRs on either the heavy or light chain and the single amino acid is substituted during GC (PNA^{hi}) B cell proliferation phase. (Berek, Berger and Apel 1991) This process provides a large B cell repertoire for affinity selection.

CXCR4 expression on centroblasts is downregulated by an incompletely understood mechanism. Using CXCL13-CXCR5 chemotaxis, these B cells are recruited to the light zone where a network of follicular dendritic cells (FDCs) express CXCL13 and present antigens to B cells. (Vissers, et al. 2001); (Tew, Phipps and Mandel 1980) The abundant CXCL13 also recruits follicular T helper cells (FTH cells) which facilitates the affinity selection. In the light zone, B cells stop proliferating and are referred to as centrocytes. The hypermutated B cells undergo affinity selection during which B cells survive or die depending on their BCR affinity for the original antigen. In this process, free antigens are captured by FDCs and presented to BCRs of the B cells. B cells that express low-affinity BCRs fail to be retained by FDCs and go into apoptosis because of the weakened BCR signaling (Cortez, Kadlec and Pendergast 1995). B cells expressing intermediate affinity BCRs are capable of interaction with FDCs but fail to fully interact with F_{TH} cells. The inadequate help by F_{TH} cells is most likely due to a weakened CD40-CD40L signaling and the lower production of IL-21 and IL-4. These signals have been shown to be essential in GC reaction. The X-linked hyper-IgM syndrome patients that have CD40L defect develop an abortive GC reaction suggesting the important role of CD40-CD40L interaction in GC reaction. (Facchetti, et al. 1995) IL-21 is also involved in GC reaction since the absence of IL-21 was shown to cause incorrect B cell positioning, affinity maturation and memory cell generation from GC reaction. (Linterman, et al. 2010); (Zotos, Coquet, et al. 2010) IL-4 was shown to be produced by FTH cells (Yusuf, et al. 2010) and stimulate the proliferation of follicular lymphoma in the presence of CD40-CD40L interaction (Schmitter, et al. 1997). Additionally, other molecules on F_{TH} cells which decide the duration of T-B conjugates are also important for determining the fate of GC B cells. The deficiency of SAP (signaling lymphocytic activation molecule-associated protein), a binding protein of the glycoprotein CD150 on T/B/dendritic cell, has been shown to disrupt T-B interaction and limit germinal center formation. (Qi, Cannons, et al. 2008); (Howie, et al. 2002) B cells have been shown to go into apoptosis

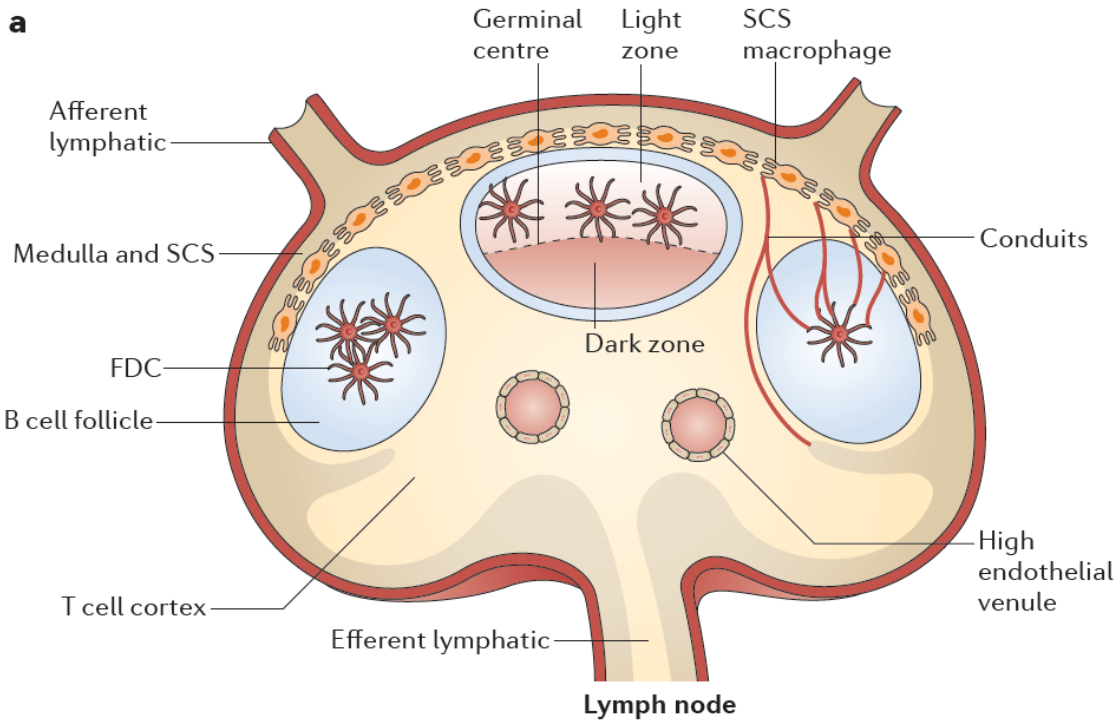
via the Fas-FasL mediated mechanism. (Hao, et al. 2008) Therefore, anti-FAS antibody is commonly used to identify GC B cells via flow cytometry.

Figure 1.6: A schematic view of anatomy of the spleen.



Representation of a schematic view of anatomy of the spleen. (Reprinted with permission from Pillai S, Cariappa A. The follicular versus marginal zone B lymphocyte cell fate decision. 2009. Nat Rev Immunol. 9(11):767-77.).

Figure 1.7: The architecture of lymph node.



Representation of the schematic overview of lymph node anatomy showing the location of B cell follicles and T cell cortex. (Reprinted with permission from Heesters BA, Myers RC, Carroll MC. Follicular dendritic cells: dynamic antigen libraries. 2014. Nat Rev Immunol. 14(7):495-504.).

B cell memory development and persistence. B cell memory includes two cell types, memory B cells (MBCs) and long-lived plasma cells (LLPCs). MBCs are quiescent, residing in the lymphoid organs or recirculating in peripheral blood. MBCs have a low activation threshold and respond to the same antigen which they previously encountered in the GC by proliferation and differentiation into antibody-secreting plasma cells. LLPCs constitutively secrete antibodies and are mainly localized in survival niches in the bone marrow. The surface expression of immunoglobulins (IgM and IgG) and TLRs distinguish MBCs from LLPCs. (Yoshida, et al. 2010) Therefore, the stimulation

with antigens and TLR ligands triggers the expansion and differentiation of MBCs into antibody-secreting plasma cells.

A small portion of MBCs and LLPCs develop as a result of non-specific B cell activation by polyclonal stimulation or during T cell independent responses. Some of these MBCs express unmutated IgM and have been shown to be mainly developed outside of GCs and also give vigorous secondary responses following Ag re-exposure. (Pape, Taylor, et al. 2011); (Dogan, et al. 2009) The quantity of IgG-expressing MBCs that develop independently of T cells and GCs was still considerable. (Kaji, et al. 2012) A small portion of LLPCs have also been found to be generated from immunization with vaccines triggering T cell independent responses. IgM secreting LLPCs (partially developed from B-1 B cells) were mainly localized in bone marrow and produce IgM at a considerable level following the T cell independent B cell response. (Taillardet, et al. 2009) Some IgG secreting LLPCs have been shown to be derived from the extra-GC region in a T cell independent manner and have a long life-span in the bone marrow. (Bortnick, et al. 2012) However, the amount of LLPCs derived from T cell independent responses is much lower than the one from T cell dependent responses. (Taillardet, et al. 2009)

The majority of MBCs and LLPCs are developed from GC reactions in a T cell dependent manner. The antibodies secreted by cells that are generated from GC reaction are more efficient in protecting because they possess a higher affinity for antigen following SHM. But the mechanisms controlling the differentiation of GC B cells into either MBCs or LLPCs are still unknown. However, there is evidence that BCR affinity and help by F_{TH} cells may be involved in determining the differentiation fate. The high affinity BCR-expressing B cells that are derived from the GC reaction are more likely to differentiate into plasma cells than be retained in GCs. (Phan, Paus, et al. 2006) Compared with GC-derived MBCs, GC-derived LLPCs have been shown to have a higher BCR affinity. (Smith, Light, et al. 2000) These results suggest that the

differentiation into PCs but not MBCs is more dependent on BCR affinity. It is still unclear how F_{TH} cells are involved in determining the differentiation into either LLPCs or MBCs in the absence of testing models. However, a proposed model suggests the potential role of CD40 and IL-21 expressed by F_{TH} cells in the fate decision of GC B cells. (Zotos and Tarlinton 2012)

The majority of high-affinity plasma cells leave the GCs and are recruited to the bone marrow for long term survival and contribute to humoral defense by constitutive antibody secretion. Although some of them have been shown to reside in the red pulp blood vessels and collagen bundles (Sze, et al. 2000), the bone marrow is the dominant survival niche with a high number of supporting cells and survival factors. The origin of LLPCs is still controversial. One scenario proposes that LLPCs are derived from self-renewing MBCs following the stimuli of TLR ligands or retaining antigens in the host. (Bernasconi, Traggiai and Lanzavecchia 2002) Another scenario, commonly accepted, suggests that persistent serum antibodies are secreted by LLPCs in the bone marrow survival niche. (Chu and Berek 2013) Although still poorly understood, the longevity of LLPCs is thought to be attributed to the programming status in the GC reaction and the microenvironment in bone marrow survival niches. The supportive microenvironment includes help by the stromal cells, surrounding cytokines and chemokines, and the interaction with hematopoietic cells. The supportive role of bone marrow stromal cells includes recruiting and sustaining LLPCs. The constitutive expression of CXCL12 by bone marrow stromal cells is responsible for the recruitment of plasma cells as a study showed that the CXCR4 (the receptor for CXCL12) deficient plasma cells failed to migrate to the bone marrow. (Tokoyada, et al. 2004) After arrival in the bone marrow, plasma cells are sustained by interacting with vascular cell adhesion molecule (VCAM) on stromal cells. Studies showed that the constitutive expression of α4 integrin by plasma cells mediated their binding and co-localization with VCAM-1 expressing cells (Underhill, et al. 2002) ($CXCL12^{+}VCAM^{+}$ bone marrow reticular stromal cells)

(Belnoue, Tougne, et al. 2012). The viability and IgG production of plasma cells were shown to be maintained by interacting with VCAM using VLA. (Minges Wols, et al. 2002) Additionally, cytokines and other leukocytes play an important role in composing the microenvironment of bone marrow survival niches. With regard to cytokines, APRIL and IL-6 are critical in supporting LLPC survival. The absence of APRIL in APRIL deficient mice has been shown to impair LLPC survival. (Benson, et al. 2008) Another TNF family member, BAFF (B cell-activating factor of the TNF family) was also shown to be essential in the persistence of LLPCs in bone marrow since blocking BAFF receptors significantly shrank the population of BM LLPCs. (Benson, et al. 2008) IL-6 produced by BM stromal cells has been shown to protect plasma cells from apoptosis and promote their adherence on stromal cells. (Lawano, et al. 1995); (Nakayama, et al. 2003) While BM stromal cells were considered as the IL-6 source, various types of leukocytes including the stromal cells from adult BM were shown to produce BAFF and APRIL (Belnoue, Pihlgren, et al. 2008). In adult bone marrow, BAFF and APRIL mRNA were mainly detected in neutrophils, eosinophils and monocytes while the expression of BAFF was mainly detected in neutrophils and the expression of APRIL was mainly detected in monocytes. (Belnoue, Tougne, et al. 2012) Additionally, iNKT (invariant natural killer) cells in bone marrow have been shown to be another contributor for BAFF production in supporting LLPCs survival. (Shah, et al. 2013) MBCs prefer to remain quiescent near the contracted GCs in lymph organs (Aiba, et al. 2010) or patrol in the peripheral blood. MBCs can be stimulated either polyclonally by TLR ligands (e.g. LPS or CpG) or specifically by antigens. MBCs proliferate following the stimulation with TLR9 ligand (CpG ODN) and TLR4 ligand (LPS) *in vitro* but not *in vivo*. (Richard, Pierce and Song 2008) *In vivo*, MBCs were observed to proliferate with the necessary help of adjacent $CD4^+$ T cells on day 2 post-rechallenge. (Aiba, et al. 2010) Therefore, the reactivation of MBCs may occur by non-specific stimuli (e.g. TLR ligands) as well as antigen-specific stimuli with $CD4^+$ T cell help.

Adaptive immune responses and modulatory mechanisms to WNV infection.

Adaptive immune responses, both T and B cell responses, are important in protecting hosts from WNV dissemination (Diamond, Shrestha and Mehlhop, et al. 2003) and are involved in the process of viral clearance.

T cell immunity against WNV.

T cell responses are essential for killing WNV-infected cells and preventing viral dissemination to the CNS and thus protect the host from encephalitis or death. In the aged mouse model, the presence of $CD8^+$ and $CD4^+$ T cells was shown to be the key factor for host survival. (Brien, Uhrlaub and Hirsch, et al. 2009) $CD8^+$ T cells that are specific to WNV epitopes were shown to be cytotoxic to target cells *in vitro* and protect mice from death from a WNV infection. (Purtha, Myers, et al. 2007); (Kim, et al. 2010) In the absence of $CD8^+$ T cells, although humoral responses are still normally developed, more severe WNV infection in CNS and increased mortality rate were detected. (Shrestha and Diamond 2004) The protective role of $CD8^+$ T cells might be attributed to their infiltration into the CNS to clear WNV. (Wang, Lobigs, et al. 2003) The cytotoxic activity of $CD8^+$ T cells which clear WNV infection from CNS is due to secreted cytokines and perforin (Shrestha, Samuel and Diamond 2006) or Fas-FasL mediated cytotoxicity (discussed above) (Shrestha and Diamond 2007). However, for some WNV strains (Sarafend strain), the protection by $CD8^+$ T cells in the CNS was shown to be independent of the cytokine, IFN-γ. (Wang, Lobigs, et al. 2006) The infiltration of $CD8^+$ T cells into the CNS is attributed to the chemotaxis of CXCL10/CXCR3 (the ligation of the chemokine receptor CXCR3 to the chemokine CXCL10). (Klein, et al. 2005); (Zhang, et al. 2008) Reduced $CD8^+$ T cell infiltration and more viral infection in CNS were observed in the absence of CD40 on T cells (Sitati, et al. 2007) suggesting CD40-CD40L interaction is also required for the recruitment of antiviral $CD8^+$ T cells into CNS.

Interestingly, by blocking CXCR4 using antagonism, the infiltration of $CD8^+$ T cells (McCandless, et al. 2008) into CNS is also promoted. WNV-specific $CD4^+$ T cells also produce antiviral cytokines (IFN-γ and IL-2) and are essential for viral clearance in the CNS (Sitati and Diamond 2006) and protecting hosts from WNV infection (Brien, Uhrlaub and Nikolich-Zugich 2008). Additionally, IFN-γ-dependent effector function but not either perforin- or Fas-FasL-dependent mechanisms contributes to protection by $CD4^+$ T cells. Besides antiviral $CD4^+$ T cells, regulatory T cells may also play an important role in controlling WNV infection. Both clinical studies and animal experiment showed that a lower frequency of regulatory T cells (Tregs) was associated with the development of symptomatic WNV infection (Lanteri, et al. 2009). The mechanism by which regulatory T cells facilitate the asymptomaticity is still unknown. However, a mouse study showed that the absence of regulatory T cells during WNV infection resulted in an impaired development of tissue-resident antiviral memory $CD8^+$ T cells in the brain (Graham, Da Costa and Lund 2014) which may abrogate the host defense against WNV re-infection and result in the symptomatic infection. Addionally, innate-like T cells, γδ T cells, are also involved in the protection against WNV infection. Depletion of $V\gamma1^+$ γδ T cells has been shown to cause an enhanced viremia and mortality while the absence of $V\gamma4^+$ γδ T cells showed the opposite phenomenon suggesting differential roles of each γδ T cells subset in WNV infection. (Welte, Lamb, et al. 2008)

B cell immunity against WNV.

B cells, acting as APCs, were shown to present WNV antigens to T cells. (Kulkarni, Mullbacher and Blanden 1991) The major role of B cells is to develop a virus-specific humoral response which is critical in protecting against WNV. Passive administration of immune serum within 2 days after WNV infection was shown to protect infected mice from death. (Engle and Diamond 2003) The presence of antibodies and B cells was shown to prevent WNV dissemination to different organs, e.g. spleen, liver and CNS, and host survival. (Diamond, Shrestha and Marri, et al. 2003) Furthermore, WNV-

specific human monoclonal antibodies were shown to neutralize and inhibit viral infection *in vitro* at the post-attachment step. (Vogt, Moesker, et al. 2009) However, this passive antibody treatment is not sufficient enough to protect immunocompromised mice. This suggests that the presence of anti-WNV MBCs and LLPCs which provide a persistent humoral defense rather than a transient presence of passive antibodies is strongly required for preventing or clearing WNV infection. Therefore, an efficient vaccine that elicits anti-WNV B cell responses is desirable for protection against WNV.

Modulatory mechanisms on adaptive immune responses against WNV.

Previous studies have been performed to demonstrate the important role of innate signaling in modulating T and B cell responses against WNV. The regulatory role of PRRs has been discussed above. In addition to PRR signaling, type I IFN and its relative signaling have been shown to be involved in shaping the immune responses. The depletion of type I IFN by neutralizing antibodies following WNV infection restricts the effector function of $CD8^+$ T cells by suppressing the costimulatory molecule expression on APCs and exhausting $CD8^+$ T cells. (Pinto, et al. 2011) IFN-β deficiency was also shown to increase the population of Tregs. (Lazear, Pinto and Vogt, et al. 2011) Additionally, B cell early activation, shown as the expression of early activation markers and costimulatory molecules, also requires type I IFN. (Purtha, Chachu, et al. 2008) The depletion of transcription factors (e.g. IRF-1, IRF-5) that control type I IFN expression was shown to impair lymphocyte activation, the effector function of WNV-specific $CD8^+$ T cells and antibody responses. (Brien, Daffis, et al. 2011); (Thackray, et al. 2014) in addition, IL-1 signaling also was shown to play an important role in WNV-specific $CD8^+$ T cell in CNS responses by modulating T cell infiltration into the CNS and $CD8^+$ T cell effector function. (Durrant, Daniels and Klein 2014); (Ramos, et al. 2012). IL-1 was thought to modulate $CD8^+$ T cell responses in a DC-intrinsic manner. (Durrant, Robinette and Klein 2013)

WNV Vaccines.

At present, numerous vaccine candidates have been tested in mice, hamsters, birds, horses and non-human primates. Some have been licensed for veterinary use. Several efforts have also been made to develop candidate WNV vaccines for use in humans in the last decade. However, while some vaccine candidates have been tested in phase I and II clinical trials, none of them have been approved for use in humans.

WNV vaccines licensed for veterinary use and vaccine candidates in clinical trials can be characterized as inactivated vaccines, DNA vaccines or recombinant vaccines. The formalin-inactivated vaccines (e.g. the one that was developed by Fort Dodge Animal Health and licensed in 2003) were shown to be protective in horses. (Gould and Fikrig 2004) With regard to nucleic acid or DNA vaccines, the recombinant plasmid (pCBWN) expressing WNV prM and E proteins was shown to elicit vigorous neutralizing antibodies and protect horses against WNV. This vaccine was licensed but not used clinically as the first veterinary DNA vaccine by the US Department of Agriculture in 2005. (Dauphin and Zientara 2007) Another DNA vaccine encodes the WNV prM and E proteins under the control of CMV/R promoter/enhancer where a 5' long terminal repeat of human T cell leukemia virus type (HTLV-1) is linked to the CMV promoter and acts as a transcriptional and post-transcriptional enhancer. Using the CMV/R promoter to improve immunogen expression, this DNA vaccine has been shown to be safe and immunogenic in humans in a phase I clinical trial. (Ledgerwood, et al. 2011) A WNV recombinant Recombitek® vaccine was licensed for veterinary use in 2004 and commercially available in US. (Iyer and Kousoulas 2013) This vaccine is a canarypox/ALVAC vector backbone (a poxvirus immunization vehicle) expressing WNV prM and E proteins and was shown to induce an efficient immune protection against mosquito-borne WNV infection in horses. (Grosenbaugh, et al. 2004) ChimeriVAX-WN02, a human recombinant vaccine candidate, is a yellow fever 17D vaccine clone

expressing NY99 prM gene and E gene with 3 mutations in the E gene to reduce neurovirulence. It was tested in a phase II trial and found to be immunogenic and safe in the young population and elderly adults. (Biedenbender, et al. 2011) But the moderate reactogenicity observed in the clinical trial might prevent ChimeriVAX-WN02 from being licensed for clinical use in human. However, ChimeriVAX-WN02 was used as live equine WN vaccine till 2010 and ultimately remarketed as a killed vaccine.

No subunit WNV vaccines have been approved for either veterinary or human use. Many studies have shown that the DIII domain of E protein is the cellular receptor binding site for viral entry and the target of neutralizing antibodies. (Lee, Chu and Ng 2006); (Beasley and Barrett 2002); (Oliphant, Engle, et al. 2005) Therefore, many studies have focused on utilizing DIII as the lead antigen in subunit WNV vaccines. Several different vaccine platforms have been tested to promote the immunogenicity of the DIII domain. Bacteriophage-derived virus-like particles (VLPs) conjugated to DIII were shown to protect mice from lethal WNV infection. (Spohn, et al. 2010) The linkage of truncated E proteins or DIII immunogens to nanolipoprotein particles (NiNLPs), heat-shock protein (p458) or GPI-0100 increased immunogenicity and efficacy in protecting animals against WNV infection. (Fischer, et al. 2010); (Gershoni-Yahalom, et al. 2010); (Lieberman, et al. 2009) The studies on how to adjuvant the subunit proteins were only performed in animal models (e.g. mice and non-human primates) and still require more investigation. Therefore, how the adjuvant-related signaling modulates the immunogenicity of E proteins remains to be determined. One long-term goal of this dissertation study is to identify the role of TLR3- and MyD88-dependent signaling in the development of anti-WNV adaptive immune responses and thus understand their contribution to adjuvanticity to WNV vaccines.

WNV vaccine candidate, RepliVAX WN.

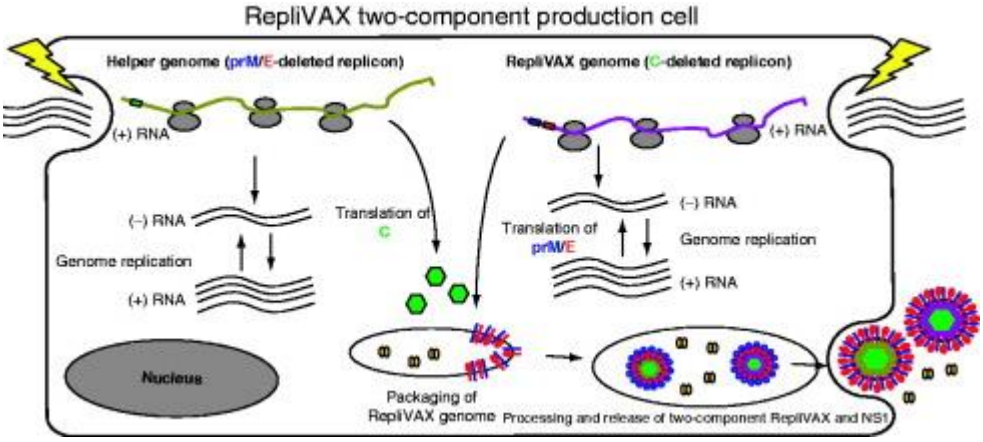
An ideal WNV vaccine should include the following elements: 1. Immunogenic peptides or proteins that include protective WNV B and T cell epitopes; 2. PAMPs that trigger appropriate innate immune responses to optimize the development of strong adaptive memory cells; 3. Safety including a well-controlled innate immune response, the absence of self-reactive adaptive immune response development following vaccination and the irreversibility of the vaccine back to wild type virulence phenotype; and 4. High efficacy in humans. Studies have shown that immunization of mice with subviral particles (SVPs) composed of JEV or Murray Valley encephalitis virus (MVEV) E and prM/M proteins induced protective humoral responses against lethal flavivirus infection suggesting a similar WNV platform would induce vigorous antibody responses. (Konishi, Pincus, et al. 1992); (Kroeger and McMinn 2002) In addition to antibody, $CD4^+$ and $CD8^+$ T cell responses are also required for anti-WNV protection. (Brien, Uhrlaub and Nikolich-Zugich 2008); (Shrestha and Diamond 2004) In this regard, a subset of WNV specific epitopes for mice and humans have also been mapped on C, E and nonstructural proteins (Purtha, Myers, et al. 2007); (Kaabinejadian, et al. 2013). These results suggest that a successful WNV vaccine needs to infect host cells to trigger innate immune responses, express structural and nonstructural proteins for inducing T cell responses against all potential epitopes and produce immunogenic SVPs to induce anti-WNV humoral responses. To induce appropriate innate immunity to ultimately drive adaptive immune response development, the vaccine needs to express PAMPs that trigger PRRs in the vaccine target cells. Replicating genomes and intermediate stages of genome replication and viral mRNA trigger endosomal or cytosolic PRRs and act as intrinsic adjuvants. Live vaccines, including live attenuated vaccines, recombinant vectored vaccines and single cycle vaccines, include all the elements (T and B cell epitopes, PAMPs) discussed above. Compared to live attenuated vaccines which are potentially

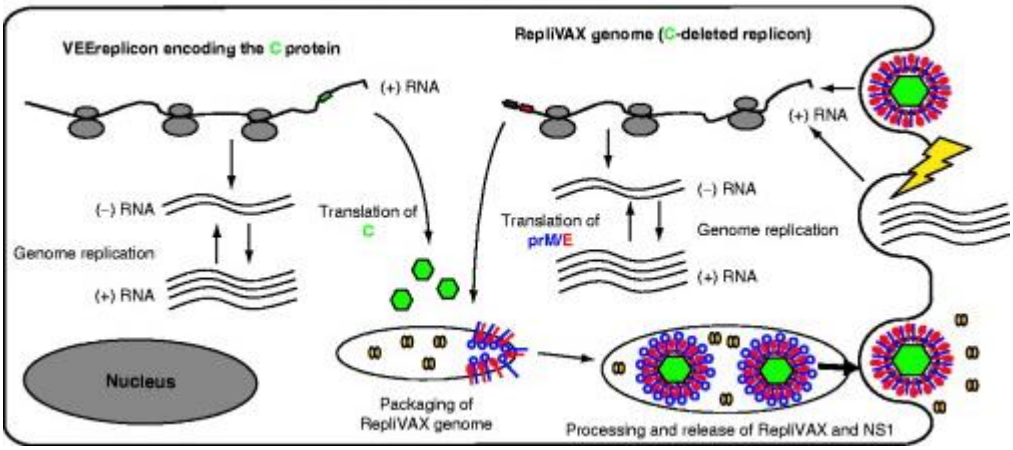
revertible to wild type in hosts and recombinant vaccine vector platforms that also elicit vector specific immune responses, single cycle vaccines that are infectious, irreversible (Widman, Frolov and Mason 2008) and only express WNV specific epitopes represent an excellent options for developing successful WNV vaccines. A successful vaccine should also be able to deliver the replicable genome into the host cells, express nonstructural proteins which facilitate genome replication and express viral proteins eliciting immune responses. In this regard, the deletion of the C gene from flaviviruses prevented infection but still results in expression of the nonstructural proteins required for genome replication and the structural proteins E and prM/M proteins essential for SVP assembly (Mason, Pincus, et al. 1991); (Mason, Shustov and Frolov 2006). Therefore, both structural and nonstructural proteins are available for recognition by B and T cells. Based on this reasoning, RepliVAX WN, a WNV vaccine candidate, was developed by the group of Dr. Peter W. Mason and Dr. Ilya Frolov in 2006. (Mason PW 2006)

RepliVAX WN is derived from the Texas 2002 WNV strain that was isolated originally from an immunocompromised patient (Rossi, Zhao, et al. 2005). It is artificially mutated by deletion of 70 codons from the capsid gene (Widman, Ishikawa and Fayzulin, et al. 2008). The genome of RepliVAX WN replicates normally in target cells but is unable to be encapsidated into infectious particles in the absence of C proteins. Host cells infected with RepliVAX WN release the noninfectious SVPs composed of prM and E proteins that fail to infect neighboring cells. Thus, RepliVAX WN initiates only one infection cycle and is referred to as a single cycle flavivirus virus (SCFV). To generate RepliVAX WN, a novel two-component genome system is utilized. (Shustov, Mason and Frolov 2007) In this system, a helper genome and RepliVAX WN genome are electroporated into the BHK cells. The helper genome complimentarily expresses C proteins to encapsidate the genome replicates of RepliVAX WN and produce infectious RepliVAX WN particles. To amplify RepliVAX WN progeny production, the complementary BHK cells that constructively express C proteins are infected with

RepliVAX WN. The genome replicates are encapsidated, enveloped and released as RepliVAX WN particles. The production (Fig 1.8) and the life cycle (Fig 1.9) of RepliVAX WN is shown as the figure below.

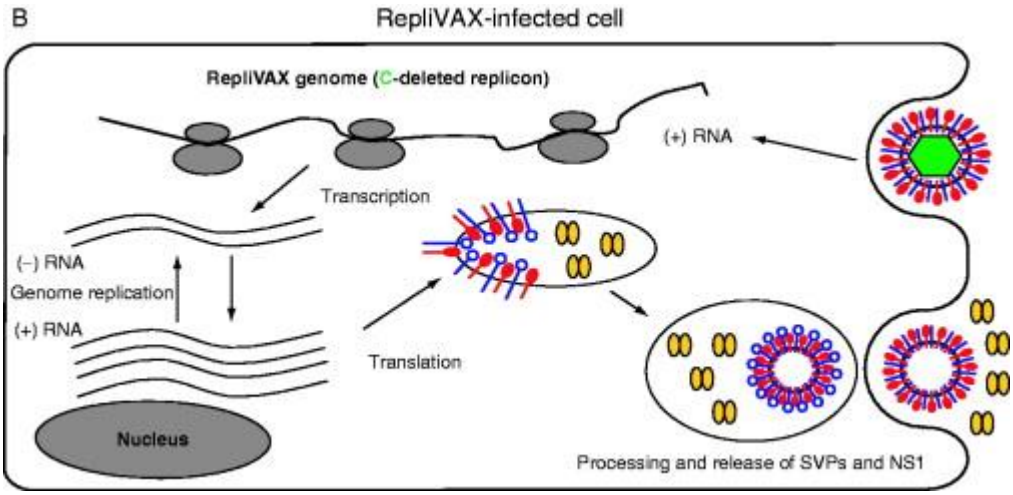
Figure 1.8: The production process of RepliVAX WN progeny.





Representation of the cell system for RepliVAX WN production. The upper figure shows the two component genome system. Following the electroporation of the helper genome and RepliVAX WN genome into BHK cells, nucleocapsids (green particles) are enveloped by M (blue) and E (red) proteins and form RepliVAX WN particles. NS1 proteins (yellow dimers) are also released. The bottom figure shows the generation of RepliVAX WN using C-expressing cell line. (Reprinted with permission from Widman DG, Frolov I, Mason PW. Third-generation flavivirus vaccines based on single-cycle, encapsidation-defective viruses. 2008. Adv Virus Res. 72:77-126.).

Figure 1.9: The life cycle of RepliVAX WN.



Representation of the life cycle of RepliVAX WN in the absence of C gene expression in host cells. RepliVAX WN particles infect and release C-depleted genome into host cells. M proteins (blue) and E proteins (red) form empty envelop particles (SVPs) are formed and released. Meanwhile, NS1 proteins (yellow dimers) are also released. (Reprinted with permission from Widman DG, Frolov I, Mason PW. Third-generation flavivirus vaccines based on single-cycle, encapsidation-defective viruses. 2008. Adv Virus Res. 72:77-126.).

RepliVAX WN particles have been shown to be very immunogenic. The released SVPs contain the same epitopes as WNV and induce vigorous anti-WNV humoral responses. Like virulent WNV, RepliVAX WN infection also causes the release of NS1 which triggers the production of anti-NS1 antibodies (Widman, Ishikawa and Fayzulin, et al. 2008) which facilitate WNV clearance (Chung, Thompson, et al. 2007). For the induction of T cell immune responses, in host cells the RepliVAX WN genome encodes E proteins and nonstructural proteins which contain T cell epitopes and act as excellent immunogens (Konishi, Fujii and Mason 2001); (Qiao, et al. 2004). Numerous animal studies showed that RepliVAX WN immunization induced immune protection against WNV in the mouse, hamster and primate models (Nelson, et al. 2010); (Widman, Ishikawa and Winkelmann, et al. 2009); (Widman, Ishikawa and Giavedoni, et al. 2010); (Winkelmann, et al. 2012).

Although there is evidence demonstrating the immunogenicity of RepliVAX WN, no study has examined the mechanisms which endow RepliVAX WN with such an optimal efficiency. Unlike inactivated vaccines, RepliVAX WN inoculation results in intracellular genome replication which stimulate PRR signaling. Additionally, type I IFN signaling, the downstream signaling of PRR signaling, has been shown to play an important role in modulating anti-WNV adaptive immunity. (Winkelmann, et al. 2012) Therefore, in this study, I aimed to investigate whether the intrinsic adjuvanticity of RepliVAX WN with viral RNA is responsible for the optimized immunogenicity.

Outline of dissertation.

RepliVAX WN has been shown to induce vigorous T and B cell responses as well as quality memory responses against WNV infection. However, the mechanisms responsible for the immunogenicity of single cycle particles such as RepliVAX WN are

still poorly understood. RepliVAX WN carries the viral genome which initiates intracellular RNA replication and thus it is considered as a live virus intrinsically adjuvanted with TLR3 and TLR7 ligands. Therefore, immunization with RepliVAX WN is linked with the stimulation of TLR3 and TLR7 signaling. As previously discussed, PRR signaling is important for optimizing the development of T and B cell responses to viral infection or in this case, immunization. Unlike TLR3 which is mediated through TRIF adaptor molecule, TLR7 signaling is mediated through the MyD88 adaptor molecule. Therefore, in this study, I compared the differential role of TLR3- and MyD88-dependent signaling in the development of adaptive immune responses to RepliVAX WN immunization. **I hypothesize that the absence of either TLR3- or MyD88-dependent signaling will lead to impaired T and B cell responses against the immunization with the intrinsically adjuvanted WNV vaccines candidate, RepliVAX WN.** Since MyD88 (compared to TLR3) is an important adaptor molecule which directs multiple innate immune signaling pathways, **I also hypothesize that the deficiency of MyD88 would compromise T and B cell responses more and by a different mechanism than TLR3 deficiency.** In this project, I quantified and characterized adaptive immune response development in wild type and transgenic mouse strains (TLR3-/- and MyD88-/-) on C57BL/6 background and compared the responses in each strains following RepliVAX WN immunization.

The first specific aim of this project (Chapter 2) was to identify and compare the role of TLR3- and MyD88-dependent signaling in the development of T cell responses. In this chapter, $CD8^+$ T cell activation, the kinetics of WNV specific IFN-γ secreting $CD4^+$ and $CD8^+$ T cells, the polyfunctionality of WNV specific effector $CD8^+$ T cells, secondary responses of $CD8^+$ T cells and the role of the intrinsic TLR3- and MyD88-dependent signaling of $CD11c^+$ myeloid DCs in manipulating these responses in the three mouse strains were studied.

The second specific aim of the project (Chapter 3) was to identify and compare the role of TLR3- and MyD88-dependent signaling in the development of B cell responses. In this chapter, B cell activation, B cell compartmentalization, GC reaction, serological titer of WNV specific IgG, kinetics of anti-WNV B cell development, anti-WNV IgG subclass switch, the affinity and neutralizing capacity of serum IgG, and the generation of memory B cells and long lived plasma cells in the three mouse strains were studied.

In Chapter 2 and 3, RepliVAX WN immunization through either subcutaneous foot pad injection or intraperitoneal injection elicits immune responses in secondary lymphoid organs (peritoneal LNs and spleens). This immune response mainly results in the development of central memory T cell immunity in lymphoid tissues. The immune response in peripheral tissues which generates non-lymphoid tissue resident memory immunity will be discussed in Chapter 4 (or appendix). The peripheral tissue resident immune response is elicited by herpes simplex virus type 2 (HSV-2) infection at the genital tract in the guinea pig model. During the chronic infection phase of HSV-2 infection, humoral and cellular immunity at the infection tissues (genital tracts) and the reactivation tissues (neuronal tissues, e.g. spinal cord and sensory ganglia) are detected. These effector immune cells (antibody secreting B cells and IFN-γ secreting T cells) are mainly localized at the tissue portions where viruses infect or stay latent. The nature of the peripheral tissue resident T and B cell immunity (the compartmentalization of $CD4^+$ and $CD8^+$ T cell and the presence of memory B cells and long lived plasma cells) is compared with the adaptive immunity in lymphoid tissues (spleens and bone marrow).

Chapter 2. The role of TLR3- and MyD88-dependent signaling in the development of T cell responses induced by RepliVAX WN.

INTRODUCTION.

Previous studies have suggested an important role for TLR3- and MyD88-dependent signaling in modulating the development of antiviral T cell responses. For example, application of poly I:C adjuvant (a TLR3 ligand) was shown to facilitate anti-HIV $CD4^+$ and $CD8^+$ T cell responses elicited by vaccination with Gag (HIV group associated antigen) ISCOM (immunostimulatory complexes, a cage structure consists of cholesterol, saponins and phospholipids) by promoting the differentiation of multifunctional memory T cells in mice. (Quinn, et al. 2013) Vaccination with poly I:C adjuvanted Gag in non-human primates also showed induction of a high quantity of anti-HIV $CD4^+$ and $CD8^+$ memory T cells. (Park, et al. 2013)

With regards to MyD88-dependent signaling, MyD88-deficiency caused a significantly diminished vaccinia virus-specific IFN-γ secreting $CD4^+$ T cell response. (Davies, et al. 2014) Additionally, the quantity and effector function of $CD8^+$ memory T cells ultimately residing in vaginal mucosa were shown to be enhanced by stimulating MyD88-dependent signaling using a recombinant adenovirus expressing HSV CTL recognition peptide, gB498-505. (Zhang, et al. 2012) In addition to T cell responses to DNA viruses, an RNA virus, the WNV NS4B-P38G mutant, has been shown to trigger a vigorous $CD4^+$ and $CD8^+$ T cell response via TLR7/MyD88-dependent signaling. (Xie, et al. 2013) These results all suggest that both TLR3- and MyD88-dependent signaling are important in the development of efficient antiviral $CD4^+$ and $CD8^+$ T cell responses.

A previous studies showed that RepliVAX WN is very immunogenic and elicits vigorous T cell responses specific for WNV $CD4^+$ T cell epitopes ($NS3_{1616-1630}$ and $E_{641-655}$) and $CD8^+$ T cell epitopes ($NS4B_{2488-2496}$ and $E_{347-354}$). (Nelson, et al. 2010) RepliVAX WN-induced T cell responses are shaped by type I IFN signaling as shown in a recent report (Winkelmann, et al. 2012). The role of type I IFN in influencing the magnitude of WNV-specific T cell responses was complicated due to the different antigen loads in RepliVAX WN-inoculated B6 and IFNAR-/- mice. However, after equalizing viral loads

between mouse strains, type I IFN signaling was shown to modulate the effector function of developing $CD8^+$ T cells by facilitating the development of multifunctional $CD8^+$ T cells. As previously demonstrated, TLR3/Trif- and TLR7/8/MyD88- signaling pathways direct the transcription of type I IFN. (Sharma, et al. 2003); (Hornung, Guenthner-Biller, et al. 2005) Therefore, in this chapter, the hypothesis tested was that TLR3- and MyD88-dependent signaling pathways differentially influence T cell response development following RepliVAX WN immunization of mice.

SIGNIFICANCE.

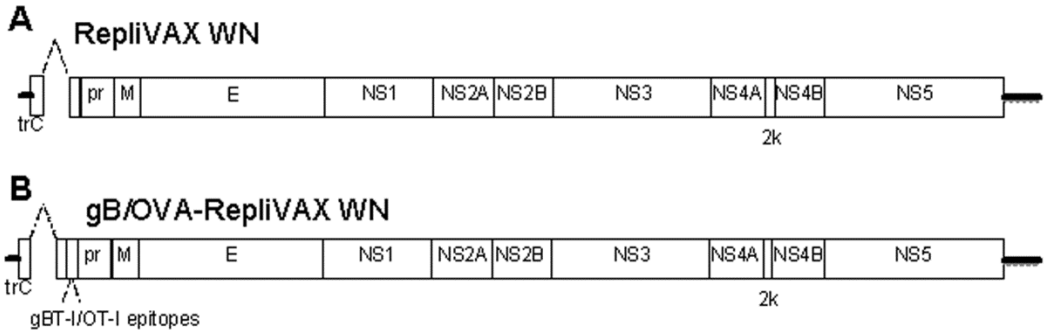
Many vaccine candidates, especially subunit vaccines, fail to elicit vigorous memory T cell responses that will protect against re-exposure to pathogens. But RepliVAX WN is intrinsically adjuvanted by the expression of TLR ligands and causes a sustained stimulation of PRRs during RepliVAX WN genome replication. Therefore, it is important to understand the role of these intrinsic adjuvants in enhancing the immunogenicity of RepliVAX WN and promoting memory T cell responses. Understanding and discriminating the role of TLR3- and MyD88-dependent signaling in modulating anti-WNV T cell immunity and memory will help to understand the role of intrinsic adjuvants in responses to single cycle vaccines to optimize the use of RepliVAX WN as a potential vaccine vector (Rumyantsev, et al. 2013) and may provide insight into the use of TLR ligands to shape the immune responses to recombinant WNV vaccines. Additionally, from the perspective of basic science research, this study will demonstrate the utility of the RepliVAX WN immunized mouse model in investigating of the mechanisms by which PRR signaling pathways drive the development of T cell responses and thus memory T cells.

MATERIAL AND METHOD.

Mice. C57BL/6J (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TLR3 deficient (TLR3-/-) and MyD88 deficient (MyD88-/-) mice on a B6 background were obtained from Dr. Michael Diamond (Washington University, St. Louis) and maintained as a breeding colony in the Association for Assessment and Accreditation of Laboratory Animal Care- approved animal research center of the University of Texas Medical Branch. Animals were age and sex matched for all experiments. All animal research was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch with oversight of staff veterinarians.

Virus and WNV T cell epitope peptides. RepliVAX WN was generated in BHK (VEErep/Pac-Ubi-C*) cells as previously described (Widman, Ishikawa and Fayzulin, et al. 2008). OT-I peptide-expressing SCFV particles (OT-I-SCFV) consisting of a WNV replicon genome expressing an OT-I peptide ($OVA_{257-264}$) gene were generated in BHK (VEErep/C*-prM-E-Pac) cells (VEErep/C*-prM-E-Pac is shown in Figure 2.1). RepliVAX WN and OT-1-SCFV were titrated using Vero cells as previously described (Gilfoy, Fayzulin and Mason 2008). WNV peptides representing $CD4^+$ and $CD8^+$ T cell epitopes were synthesized by New England Peptide, Gardner, MA. WNV $CD4^+$ T cell epitopes ($NS3_{1616-1630}$ and $E_{641-655}$) and $CD8^+$ T cell epitopes ($NS4B_{2488-2496}$ and $E_{347-354}$) were loaded on the stimulator cells as previously described. (Nelson, et al. 2010) Single cell suspensions prepared from spleens or lymph nodes of immunized mice were stimulated by culture with the epitope peptide presenting stimulator cells *ex vivo* to detect anti-WNV IFN-γ secreting $CD8^+$ and $CD4^+$ T cells via ELISPOT. (Nelson, et al. 2010)

Figure 2.1: Schematic representation of the single-cycle flavivirus (SCFV) genomes.



RepliVAX WN genome (A) was used to construct OT-I-SCFV (B) which expresses ovalbumin peptides ($OVA_{257-264}$). (Reprinted with permission from Winkelmann ER, Widman DG, Xia J, Johnson AJ, van Rooijen N, Mason PW, Bourne N, Milligan GN. Subcapsular sinus macrophages limit dissemination of West Nile virus particles after inoculation but are not essential for the development of West Nile virus-specific T cell responses. 2014. 450-451:278-89.).

Immunization with RepliVAX WN. For quantifying WNV-specific serum IgM and IgG antibody titers and WNV-specific antibody secreting cells (ASC), mice were immunized by the intraperitoneal (i.p.) route with 10^6 IU RepliVAX WN. To assess the level of viral gene replication and evaluate early B cell activation in secondary lymphoid tissues, mice were immunized by subcutaneous (s.c.) food pad (FP) injection with 10^6 IU RepliVAX WN or FLUC-SCFV. Inocula were delivered in L-15 medium containing 10mM HEPES and 0.5% FBS.

Enzyme-linked immunospot assay (ELISPOT). ELISPOT assays for IFN-γ secreting $CD4^+$ and $CD8^+$ T cells were performed as described previously (Nelson, et al. 2010) using microtiter filter plates (Millipore corporation, Billerica, MA). Anti-WNV IFN-γ secreting T cells were quantified using an ImmunoSpot reader and data were analyzed with ImmunoSpot software (Cellular Technology Ltd, Cleveland, OH).

Generation and infection of bone marrow-derived dendritic cells (BMDCs). BMDCs were generated according to the protocol published by Inaba et al. (1992) and Lutz et al. (1999). Femur bone marrow cells were harvested and red blood cells (RBCs) were lysed with Red blood cell lysing buffer (Sigma-Aldrich Inc., St. Louis, MO). Bone marrow cells were cultured at 10^7 cells per 100 mm^2 tissue culture petridish (BD Bioscience, San Jose, CA) at 37 °C, 5%CO_2 for 10 days. BMDC medium was made of T cell medium with 20 ng/mL GM-CSF (R&D Sustems, Inc. Minneapolis, MN) and 20 ng/mL IL-4 (BD PharmingenTM, San Diego, CA). Medium was renewed on day 3, 6 and 8 during culture. BMDCs were washed and infected with RepliVAX WN at 100 IU/cell for 2 hrs at 37 °C, 5%CO_2. Infected BMDCs were washed and incubated at 37 °C, 5%CO_2 for T cell assay.

Isolation of $CD8^+$ T cells and T cell assay. Single cell suspensions of splenocytes were prepared from spleens of $Thy1.1^+$ $OT\text{-}I^+$ mice on a B6 background. $CD8^+$ T cells were isolated via $CD8\alpha^+$ T cell isolation kit II, mouse (Macs, Miltenyi Biotec Inc, Auburn, CA). Isolated $CD8^+$ T cells were labeled with CFSE and plated into a 96-well plate at $5x10^4$ cells/well. $CD8^+$ T cells were co-cultured with serially diluted OT-I-SCFV infected BMDCs starting at 10^5 cells/well. Uninfected BMDCs were added to equalize the total cell number among assay wells (to $2x10^5$ cells/well). On day 3, cells were harvested for detecting $CD8^+$ T cell proliferation and cytokine secretion by $CD8^+$ T cells.

Surface staining, Intracellular cytokine staining (ICS), and flow cytometry analysis. Single cell suspensions from immunized and naive spleens were blocked with anti-Fc RII/III mAb (rat IgG2b anti-mouse CD16/CD32, BD Bioscience, San Jose, CA), and surface proteins and intracellular cytokines were stained with antibodies purchased from BD Biosciences (San Jose, CA): anti-CD3-peridinin chlorophyll protein-cyanine

dye CyTM5.5 (Percp-Cy5.5; 17A2), anti-CD8α-allophycocyanin (APC; 53-6.7), anti-IFN-γ-fluorescein isothiocyanate (FITC; XMG1.2), anti-IL-2-phycoerythrin (PE; JES6-5H4), anti-TNF-phycoerythrin-cyanine dye Cy7TM (PE-Cy7; MP6-XT22), anti-CD90.1-peridinin chlorophyll protein (Percp-Cy5.5; OX-7), anti-CD90.1- phycoerythrin (PE ;OX-7), anti-MHC class II alloantigens-phycoerythrin (PE, M5/114.15.2) and anti-CD80 fluorescein isothiocyanate (FITC, 16-10A1). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Sigma-Aldrich Inc. (St. Louis, MO). Intracellular cytokines secreted by $CD8^+$ T cells in spleens from immunized mice were stained with internalized antibodies using a BD Cytofix/CytopermTM Kit (BD Bioscience, San Jose, CA) according to the kit instructions. Data were acquired on a BD LSRII Fortessa (BD Biosciences, San Jose, CA) at the UTMB Flow Cytometry Core Facility and analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis. Statistical differences for B lymphocyte assays, serum titer and frequency and quantity of different cell compartments were determined using Student t test (unpaired) or ANOVA with the Tukey post test as appropriate. Values for $p<0.05$ were considered significant. All calculations were performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA).

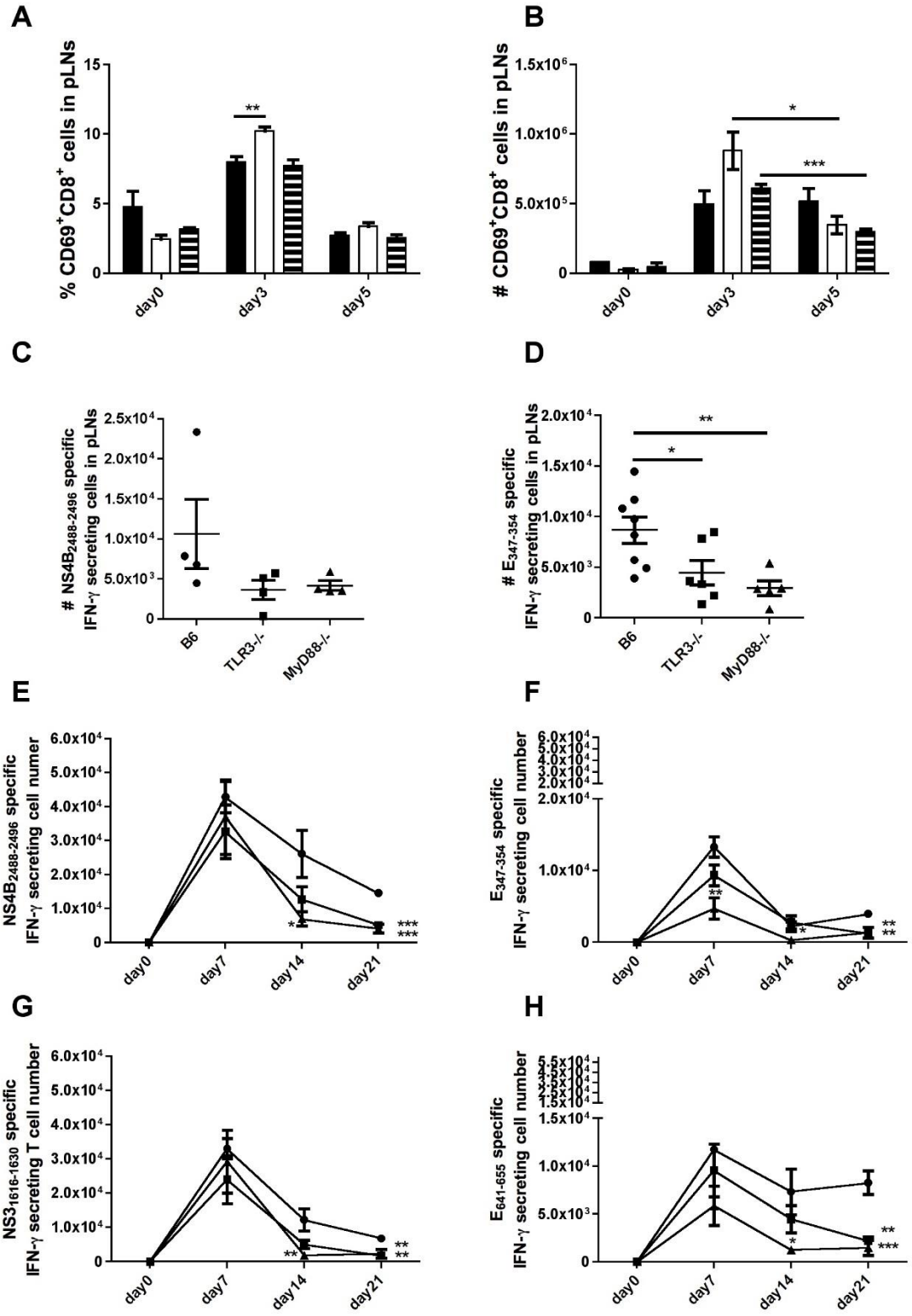
RESULTS.

The absence of TLR3- and MyD88-dependent signaling impaired $CD8^+$ T cell activation.

Given that both TLR3- and MyD88-dependent signaling are important in the development of T cell responses (Quinn, et al. 2013)**;** (Davies, et al. 2014), we examined the importance of these signaling pathways in influencing T cell activation following immunization with the single cycle vaccine, RepliVAX WN. To study T cell activation,

the expression level of the early activation marker CD69 on $CD8^+$ T cells was determined. Following RepliVAX WN immunization of foot pads, the frequency of $CD69^+CD8^+$ T cells in pLNs of RepliVAX WN immunized B6 mice peaked on 3 dpi and decreased through the end of the study on 5 dpi (**Fig 2.2 A**).

Figure 2.2


A
% CD69+CD8+ cells in pLNs
day0
day3
day5
B
CD69+CD8+ cells in pLNs
C
NS4B2488-2496 specific IFN-γ secreting cells in pLNs
B6
TLR3-/-
MyD88-/-
D
E347-354 specific IFN-γ secreting cells in pLNs
E
NS4B2488-2496 specific IFN-γ secreting cell numer
day7
day14
day21
F
E347-354 specific IFN-γ secreting cell number
G
NS31616-1630 specific IFN-γ secreting T cell number
H
E641-655 specific IFN-γ secreting cell number

Impaired $CD8^+$ T cell activation and impaired WNV-specific T cell response kinetics following RepliVAX WN inoculation in TLR3-/- and MyD88-/- mice. The expression of CD69 on $CD8^+$ cells and WNV-specific IFN-γ secreting $CD8^+$ T cells from popliteal lymph nodes (pLNs) of B6 (filled bars), TLR3-/- (open bars) and MyD88-/- (striped bars) mice previously s.c. immunized with RepliVAX WN by foot pad injection was detected. The frequency (A) and the total number (B) of $CD69^+CD8^+$ were measured on 3 and 5 dpi. CD69 expression on naïve $CD8^+$ T cells were determined on the day of immunization. The quantity of $NS4B_{2488-2496}$ (C) (n=4) and $E_{347-354}$ (D)(n=8) specific IFN-γ secreting T cells were quantified on 5 dpi by ELISPOT. WNV-specific IFN-γ $CD8^+$ and $CD4^+$ T cells from spleens of B6 (n=5, ●), TLR3-/- (n=5, ■) and MyD88-/- (n=5, ▲) mice immunized i.p. with 10^6 IU RepliVAX WN were quantified on 0, 7, 14, and 21 dpi. Anti-$NS4B_{2488-2496}$ (E) and anti-$E_{347-354}$ (F) IFN-γ secreting cells represented WNV-specific $CD8^+$ T cells. Anti-$NS3_{1616-1630}$ (G) and anti-$E_{641-655}$ (H) IFN-γ secreting cells represented WNV-specific $CD4^+$ T cells. Naïve cells that were *ex vivo* cultured in medium and stimulated with predominant peptides and the immunized cells that were *ex vivo* cultured in medium were used as control. The results shown are from a representative experiment of 2 performed. Data are presented as the mean quantity of IFN-γ secreting cell number ± SEM. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) compared to the same time point for B6 mice.

However, the total number of $CD69^+CD8^+$ T cells in draining lymph nodes (**Fig 2.2 B**) remained at a nearly constant level from 3 through 5 dpi in B6 mice suggesting a well-maintained $CD8^+$ T cell activation status. Although MyD88-/- mice displayed a similar pattern of $CD69^+CD8^+$ T cell frequency as B6 mice, the total number of $CD69^+CD8^+$ T cells significantly decreased (unpaired student t test, p=0.0002) on 5 dpi compared with that on 3 dpi suggesting an impaired maintenance of $CD8^+$ T cell activation in the absence of MyD88. Compared with B6 mice, $CD69^+CD8^+$ T cells in TLR3-/- mice peaked at a significantly higher frequency (ANOVA test, p=0.0023) on 3 dpi but decreased to a level similar to B6 mice on 5 dpi. Although the total number of $CD69^+CD8^+$ T cells was consistently but not significantly higher in TLR3-/- compared to B6 mice on 3 dpi, the number of these cells significantly decreased on 5 dpi to a lower level (unpaired student t test, p=0.0122) than that in B6 mice suggesting that the TLR3 deficiency also compromised the maintenance of $CD8^+$ T cell activation status.

We tested whether the number of functional RepliVAX WN-activated $CD8^+$ T cells was diminished in TLR3-/- and MyD88-/- mice compared to B6 mice. WNV-specific, anti-$NS4B_{2488-2496}$ (**Fig 2.2C**) and anti-$E_{347-354}$ (**Fig 2.2D**), IFN-γ secreting T cells in pLNs were quantified during the primary response on 5 dpi. Compared with B6 mice, both TLR3-/- and MyD88-/- mice generated a consistently lower quantity of $NS4B_{2488-2496}$ specific effector $CD8^+$ T cells although the difference did not reach significance and a significantly lower quantity of $E_{347-354}$ specific effector $CD8^+$ T cells on 5 dpi (ANOVA test, p=0.014).

The deficiency of either TLR3 or MyD88 impacted the development of WNV-specific T cell responses following RepliVAX WN immunization.

Given that the deficiency of TLR3- and MyD88-dependent signaling decreased the magnitude of the anti-WNV $CD8^+$ T cell response on 5 dpi following RepliVAX WN immunization, it was possible that the absence of either TLR3 or MyD88 altered the kinetics of T cell responses. To test if the response kinetics was impaired in TLR3-/- or MyD88-/- mice, the quantity of WNV-specific, $NS4B_{2488-2496}$ (**Fig 2.2E**) and $E_{347-354}$ (**Fig 2.2F**), IFN-γ secreting $CD8^+$ T cells and WNV-specific, $NS3_{1616-1630}$ (**Fig 2.2G**) and $E_{641-655}$ (**Fig 2.2H**), IFN-γ secreting $CD4^+$ T cells in B6, TLR3-/- and MyD88-/- mice were determined on 0, 7, 14, 21 dpi following RepliVAX WN immunization. In B6 mice, anti-WNV $CD4^+$ and $CD8^+$ T cells proliferated and reached peak levels on 7 dpi. The quantity of WNV-specific $CD4^+$ and $CD8^+$ T cells decreased through to the endpoint of the study on 21 dpi. Compared with B6 mice, the quantity of $NS4B_{2488-2496}$, $NS3_{1616-1630}$ or $E_{641-655}$ specific IFN-γ secreting T cells in MyD88-/- mice on 7 dpi was not significantly decreased. However, the quantity of $E_{347-354}$ specific IFN-γ $CD8^+$ T cells in MyD88-/- mice was significantly decreased on 7 dpi (ANOVA test, p=0.0044) (**Figure 2.2 E-H**) suggesting MyD88-dependent signaling may influence $CD8^+$ T cell proliferation. MyD88

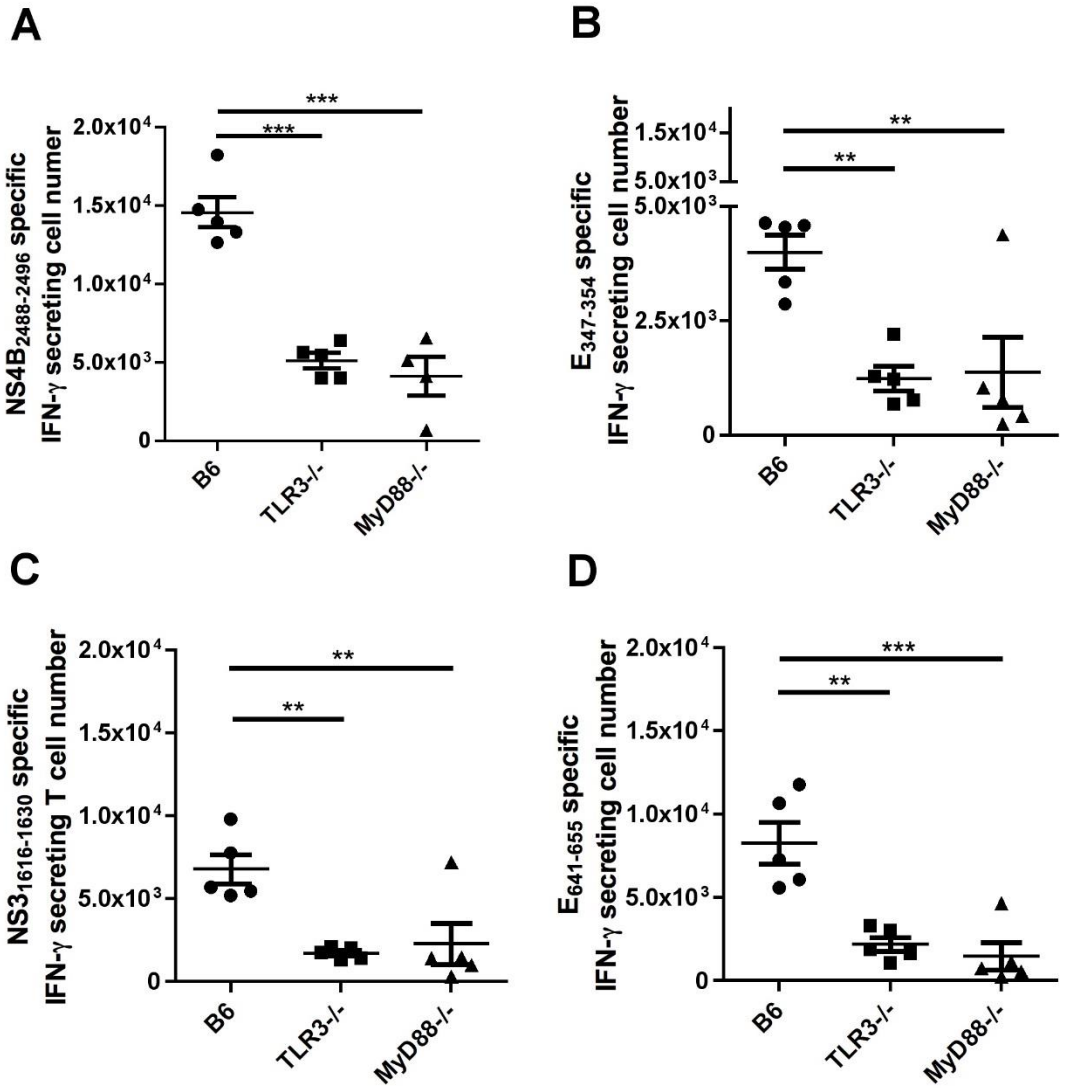
deficiency also resulted in a significantly decreased quantity of $NS4B_{2488-2496}$ (ANOVA test, p=0.0379; ANOVA test, p<0.0001) and $E_{347-354}$ (ANOVA test, p=0.0268; ANOVA test, p=0.0039) specific IFN-γ secreting $CD8^+$ T cells and $NS3_{1616-1630}$ (ANOVA test, p=0.0104; ANOVA test, p=0.0030) and $E_{641-655}$ (ANOVA test, p=0.0115; ANOVA, p=0.0003) specific IFN-γ secreting $CD4^+$ T cells on 14 and 21 dpi suggesting that MyD88-dependent signaling also played an important role in antiviral $CD4^+$ and $CD8^+$ T cell differentiation. TLR3 deficiency impaired the peak level of $E_{347-354}$ specific IFN-γ $CD8^+$ T cells on 7 dpi, although the difference did not reach significance, suggesting the less important role of TLR3 compared with MyD88-dependent signaling in modulating $CD8^+$ T cell proliferation. Although TLR3 deficiency did not significantly impair the quantity of WNV specific $CD4^+$ and $CD8^+$ T cells on 7 and 14 dpi, the quantity of anti-WNV $CD4^+$ and $CD8^+$ T cells was significantly decreased (statistic method and p value were shown in the next section) on 21 dpi compared with B6 mice, suggesting a comparatively more important role of TLR3 signaling in T cell differentiation than proliferation. Taken together, MyD88-dependent signaling was more involved in influencing anti-WNV $CD4^+$ and $CD8^+$ T cell proliferation than was TLR3 signaling although both signaling pathways were important in T cell differentiation into $CD4^+$ and $CD8^+$ memory T cells. The lower level of virus-specific T cells detected in MyD88-/- and TLR3-/- mice reflected the differences in proliferation and differentiation compared to B6 mice rather than a shift in response kinetics.

Deficiency of either TLR3 or MyD88 resulted in a reduced quantity of WNV specific memory $CD4^+$ and $CD8^+$ T cells.

A successful vaccine candidate will be required to elicit vigorous memory T cell responses. As shown above, TLR3- and MyD88-dependent signaling influenced the T cell expansion and differentiation phases of the T cell response. We tested the role of

TLR3- and MyD88-dependent signaling in influencing the quantity and quality of memory T cells that develop following RepliVAX WN immunization. To test the role of these signaling pathways in determining the magnitude of the memory T cell response, WNV-specific memory $CD4^+$ and $CD8^+$ T cells recognizing dominant WNV epitopes ($NS4B_{2488-2496}$, $E_{347-354}$, $NS3_{1616-1630}$ and $E_{641-655}$) were quantified in B6, TLR3-/- and MyD88-/- mice on 21 dpi. The results showed that the number of WNV-specific $CD8^+$ ($NS4B_{2488-2496}$ (ANOVA test, $p<0.0001$), $E_{347-354}$ (ANOVA test, $p=0.0039$)) (**Fig 2.3 A,B**) and $CD4^+$ ($NS3_{1616-1630}$ (ANOVA test, $p=0.0030$) and $E_{641-655}$(ANOVA, $p=0.0003$)) (**Fig 2.3 C,D**) memory T cells in TLR3-/- mice were significantly reduced on 21 dpi, compared with B6 mice.

Figure 2.3



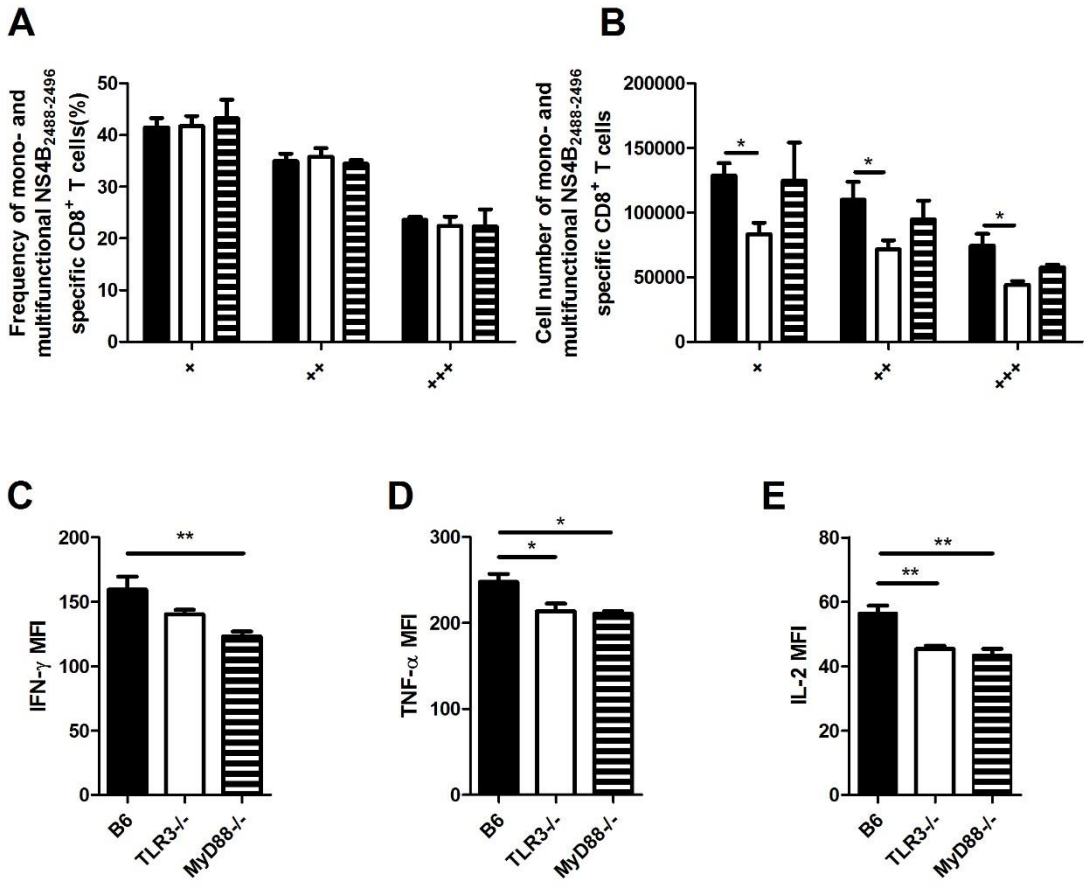
Decreased quantity of WNV-specific IFN-γ $CD8^+$ and $CD4^+$ memory T cells in TLR3-/- and MyD88-/- mice. WNV-specific IFN-γ $CD8^+$ and $CD4^+$ T cells from spleens of B6 (n=5, ●), TLR3-/- (n=5, ■) and MyD88-/- (n=5, ▲) mice immunized i.p. with 10^6 IU RepliVAX WN were quantified by ELISPOT on 21 dpi. Anti-$NS4B_{2488-2496}$ (A) and anti-$E_{347-354}$ (B) IFN- γ secreting cells represented WNV-specific $CD8^+$ T cells. Anti-$NS3_{1616-1630}$ (C) and anti-$E_{641-655}$ (D) IFN- γ secreting cells represented WNV-specific $CD4^+$ T cells. The results shown are from a representative experiment of 2 performed. Data are presented as the mean quantity of IFN-γ secreting cell number ± SEM. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) compared to B6 mice.

A significantly decreased quantity of anti-WNV $CD4^+$ and $CD8^+$ memory T cells was also detected in MyD88-/- mice compared with B6 mice. These results suggested that both TLR3- and MyD88-dependent signaling were important in influencing the differentiation of $CD4^+$ and $CD8^+$ T cells into memory T cells. This result was consistent with the result that the absence of either TLR3- or MyD88-dependent signaling impaired the response kinetics by compromising T cell differentiation(**Fig 2.2 E-H**).

The role of TLR3- and MyD88-dependent signaling in development of effector function by anti-WNV $CD8^+$ T cells.

We next determined whether deficiency of TLR3- or MyD88-dependent signaling also impaired the effector function potential of RepliVAX WN-induced memory T cells. Polyfunctionality of T cells is referred to as the capacity of effector T cells to simultaneously produce multiple cytokines or factors, e.g. IFN-γ, granzyme B, TNF-α, IL-2 (Klatt, et al. 2011) and polyfunctional effector T cells have been shown to be more likely to differentiate into memory T cells. (Harari, et al. 2006) To study the functionality of T cells induced by RepliVAX WN immunization, we measured the expression of IFN-γ, TNF-α and IL-2 by WNV specific $CD8^+$ T cells that were *ex vivo* stimulated with the immunodominant $CD8^+$ epitope peptide, $NS4B_{2488-2496}$, at the time of the peak primary response (7 dpi). In B6 mice, on 7 dpi, the frequency (**Fig 2.4A**) and quantity (**Fig 2.4B**) of effector T cells decreased as they became more polyfunctional (monofunctional (IFN-γ^+ TNF-α^-IL-2^-) T cells> dual functional (IFN-γ^+TNF-α^+IL-2^-) T cells > triple functional (IFN-γ^+TNF-α^+IL-2^+) T cells).

Figure 2.4



Altered effector cytokine secretion of WNV-specific $CD8^+$ T cells in RepliVAX WN-immunized TLR3-/- and MyD88-/- mice. Splenocytes from B6 (filled bars, n=3), TLR3-/- (blanked bars, n=3) and MyD88-/- (striped bars, n=3) mice immunized with 10^6 IU RepliVAX WN. The frequency (A) and total number (B) of mono- and poly-functional T cells on 7 dpi were measured following *ex vivo* stimulation with $NS4B_{2488-2496}$. Naïve splenocytes *ex vivo* stimulated with $NS4B_{2488-2496}$ or immunized splenocytes cultured with medium were used as control. The monofunctional anti-WNV $CD8^+$ T cells that only secreted IFN-γ are shown as "+". The polyfunctional anti-WNV $CD8^+$ T cells that secreted IFN-γ and TNF-α are shown as "++" while the ones that secreted IFN-γ, TNF-α and IL-2 are shown as "+++". The secretion of effector cytokines, IFN-γ (C), TNF-α (D) and IL-2 (E), by total $NS4B_{2488-2496}$ $CD8^+$ T cells were detected following *ex vivo* stimulation with $NS4B_{2488-2496}$. The production level of cytokines was measured as the mean fluorescence intensity (MFI) of each fluorescence detection antibody. The results shown are from a representative experiment of 2 performed. Data on polyfunctionality are presented as the mean frequency (A) and quantity (B) of mono- and poly-functional T cell number ± SEM. Data on effector cytokine secretion are presented as the mean MFI of IFN-γ (C), TNF-α (D) and IL-2 (E) ± SEM. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) compared to B6 mice.

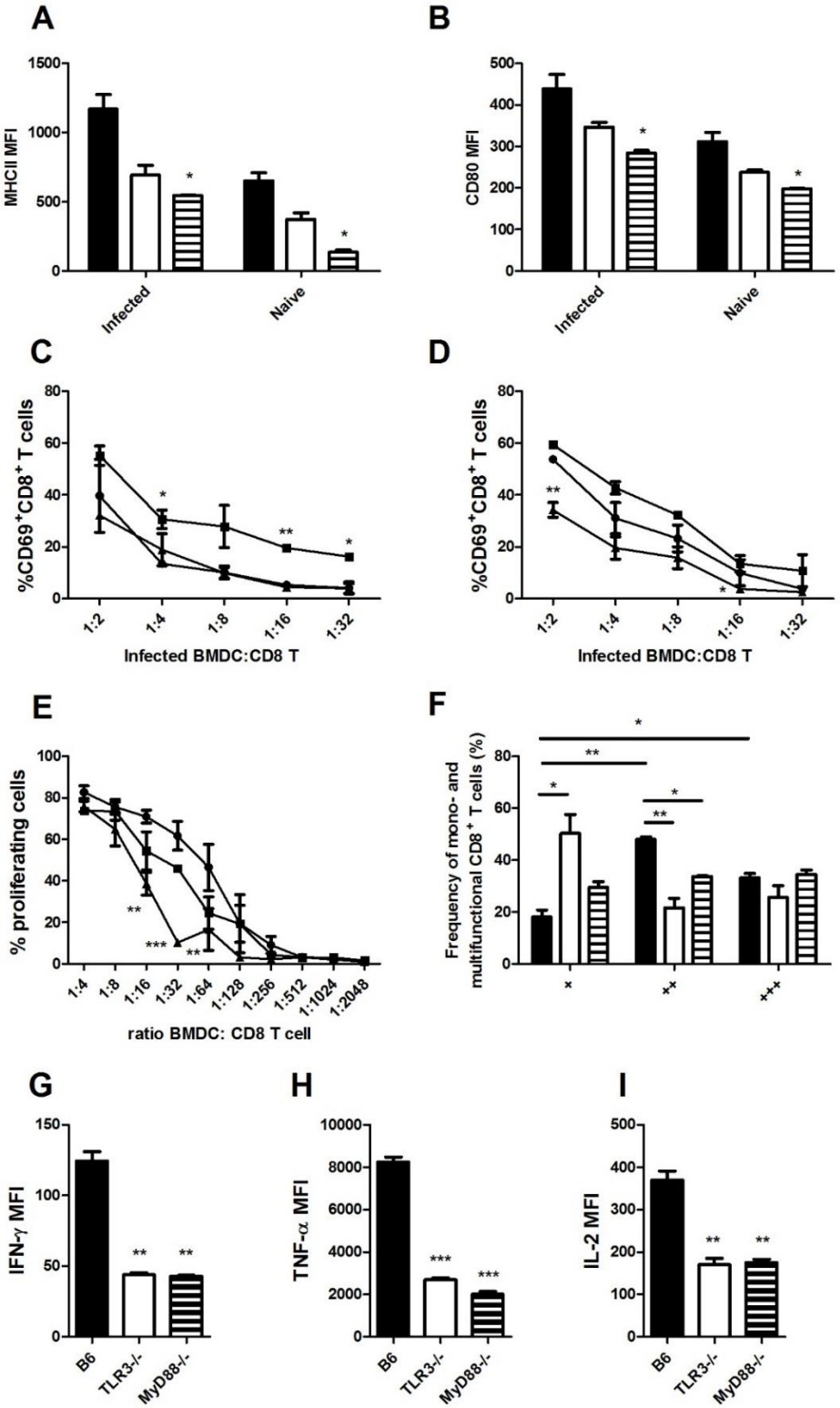
Compared with B6 mice, the frequency of multifunctional T cells (IFN-γ^+TNF-α^+IL-2^+ and IFN-γ^+TNF-α^+IL-2^-) was not impacted by TLR3 deficiency (**Figure 2.4B**). However, TLR3 deficiency significantly impaired the total number of $NS4B_{2488-2496}$ specific multifunctional T cells (**Figure 2.4C**) (student t test, $p<0.05$) but not the total number of $NS4B_{2488-2496}$ specific IFN-γ secreting cells (**Figure 2.2E**) suggesting that TLR3 plays a role in influencing the polyfunctionality of $CD8^+$ T cells in the context of TLR signal strength. Compared with B6 mice, neither the quantity nor frequency of multifunctional T cells (IFN-γ^+TNF-α^+IL-2^+ and IFN-γ^+TNF-α^+IL-2^-) was impaired in MyD88-/- mice. Therefore, these results provide evidence that TLR3 signaling, but not MyD88-dependent signaling, was involved in influencing the polyfunctionality of WNV specific $CD8^+$ T cells. To more fully understand the effector function potential of wild type and knockout cells, the effector function of $NS4B_{2488-2496}$ specific $CD8^+$ T cells was also determined by quantifying the total amount of IFN-γ, TNF-α and IL-2 produced by activated cells (quantified as the mean fluorescence intensity). Compared with B6 mice, TLR3 deficiency significantly impaired TNF-α (ANOVA test, $p<0.05$) and IL-2 (ANOVA test, $p<0.01$) production by $NS4B_{2488-2496}$ specific $CD8^+$ T cells. IFN-γ secretion by $NS4B_{2488-2496}$ specific $CD8^+$ T cells was slightly but not significantly impaired in TLR3-/- mice. (**Fig 2.4C-E**) In MyD88-/- mice, the quantity of IFN-γ, TNF-α and IL-2 produced by $NS4B_{2488-2496}$ specific $CD8^+$ T cells was significantly impaired. (**Figure 2.4C-E**) In summary, TLR3 but not MyD88 deficiency impaired both the polyfunctionality and effector function of anti-WNV effector $CD8^+$ T cells. Although MyD88 deficiency did not impair the polyfunctionality of WNV-specific $CD8^+$ effector T cells, MyD88 deficiency significantly reduced the production of IFN-γ (ANOVA test, $p<0.01$), TNF-α (ANOVA test, $p<0.05$) and IL-2 (ANOVA test, $p<0.01$) by $CD8^+$ T cells.

Inefficient antigen presentation of RepliVAX WN epitopes to naïve T cells by bone marrow derived ($CD11b^+CD11c^+$) dendritic cells deficient in TLR3- or MyD88-dependent signaling.

Naïve $CD8^+$ T cells are primed for proliferation and differentiation by interacting with activated antigen presenting cells (APCs). In viral infections or vaccination, dendritic cells (DCs) act as the major APCs (Banchereau and Steinman 1998). Previous studies have shown that DC-intrinsic TLR3- and MyD88-dependent signaling were essential for activating DCs and upregulating the expression of MHC molecules and costimulatory molecules (e.g. CD80 and CD86) (Oosterhoff, et al. 2013) that are critical for inducing naïve T cell proliferation and differentiation (Welten, Melief and Arens 2013). As shown in the proceeding sections, TLR3- and MyD88-dependent signaling influenced T cell activation, proliferation and functional pattern *in vivo*. Therefore, we questioned whether TLR3 or MyD88 signaling acted at the level of the APC to enhance APC function and enable $CD8^+$ T cell activation, expansion and effector cell differentiation. To provide a uniform population of naïve $CD8^+$ T cells, we cultured naïve OVA specific OT-I $CD8^+$ T cells with BMDCs infected with an OVA-peptide-expressing SCFV (OT-I-SCFV) (**Figure 2.1**). Thus, we could test the APC function of BMDC lacking MyD88 or TLR3 signaling for the ability to activate a uniform population of naïve $CD8^+$ T cells. We first measured the maturation of B6, TLR3-/- and MyD88-/- BMDCs following OT-I-SCFV infection *in vitro* by detecting the expression level of the activation markers MHC II and CD80. Compared with uninfected BMDCs (data not shown), OT-I-SCFV inoculated BMDCs developed an increased expression of the DC activation markers, MHC II and CD80, at 24 hpi. Compared with B6 BMDC, TLR3-/- and MyD88-/- BMDCs had a decreased expression level of CD80 and MHC II while MyD88 deficiency caused a significant impairment (ANOVA test, $p<0.05$) (**Figure 2.5**

A, B) suggesting that MyD88-dependent signaling is more important in the activation of BMDCs following OT-I-SCFV infection.

Figure 2.5


A
MHCII MFI
1500
1000
500
0
Infected
Naive
B
CD80 MFI
500
400
300
200
100
0
Infected
Naive
C
%CD69+CD8+ T cells
80
60
40
20
0
1:2
1:4
1:8
1:16
1:32
Infected BMDC:CD8 T
D
%CD69+CD8+ T cells
Infected BMDC:CD8 T
E
% proliferating cells
100
80
60
40
20
0
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
1:1024
1:2048
ratio BMDC: CD8 T cell
F
Frequency of mono- and multifunctional CD8+ T cells (%)
80
60
40
20
0
+
++
+++
G
IFN-γ MFI
150
100
50
0
B6
TLR3-/-
MyD88-/-
H
TNF-α MFI
10000
8000
6000
4000
2000
0
B6
TLR3-/-
MyD88-/-
I
IL-2 MFI
500
400
300
200
100
0
B6
TLR3-/-
MyD88-/-

Impaired capacity of TLR3-/- and MyD88-/- myeloid DCs to prime naïve $CD8^+$ T cells. BMDCs generated from B6(● or filled bars), TLR3-/- (■ or open bars) and MyD88-/- (▲ or striped bars) mice were infected with OT-I-SCFV and co-cultured with $CD8^+$ T cells as described in Materials and Methods. Naïve BMDCs and $CD8^+$ T cells cocultured with naive BMDCs were used as control. The expression of DC activation markers, MHC II (A) and CD80 (B), were measured as the MFI of each fluorescence detection antibody from BMDCs at 24 hours after OT-I-SCFV infection. The frequency of $CD69^+CD8^+$ T cells co-cultured with serially diluted BMDCs was measured on day 1.5 (C) and day 3 (D) during the coculture. T cell proliferation (E) was assessed by measuring the frequency of $CFSE^-$ cells (represented the progenitor $CD8^+$ T cells) of $CD8^+$ T cells co-cultured with serially diluted BMDCs. The polyfunctionality of $CD8^+$ T cells (F) was shown as the frequency of mono- and poly-functional $CD8^+$ T cells from the coculture BMDCs and T cells at the ratio 1:4. The monofunctional $CD8^+$ T cells that only secreted IFN-γ were shown as "+". The polyfunctional $CD8^+$ T cells that secreted IFN-γ and TNF-α were shown as "++" while the ones that secreted IFN-γ, TNF-α and IL-2 were shown as "+++". The production level of effector cytokines, IFN-γ (G), TNF-α (H) and IL-2 (I), by $CD8^+$ T cells were measured as the MFI of each fluorescence detection antibody from the coculture of BMDCs and T cells at the ratio 1:4. The results shown are from a representative experiment of 2 performed. The mean and SEM were achieved from the duplicates in each experiment. Data are presented as the mean frequency (C) of $CFSE^-$ T cells ± SEM and the mean frequency (F) of mono- or polyfunctional T cells ± SEM. Data on effector cytokine secretion and DC activation are presented as the mean MFI of MHC II (A), CD80 (B), IFN-γ (G), TNF-α (H), and IL-2 (I) ± SEM. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) compared to B6 mice.

As shown in **Fig 2.2A and B**, the expression of the early activation marker CD69 by $CD8^+$ T cells *in vivo* was modulated by TLR3- and MyD88-dependent signaling. Therefore, we tested whether this result might reflect a lack of MyD88- or TLR3-signaling in mDCs that could negatively influence CD69 expression on $CD8^+$ T cells. As shown in **Figure 2.5C and D**, the results indicated that OT-I SCFV inoculated B6 BMDCs activated $CD8^+$ T cells and upregulated the frequency of CD69-expressing T cells from day 1.5 to day 3 after coculture. The frequency of $CD69^+$ T cells decreased as the quantity of B6 BMDCs was reduced by serial dilution At the early time point, day 1.5 after coculture (**Fig 2.5C**), TLR3-/- BMDCs induced a significantly higher frequency of $CD69^+CD8^+$ T cells, compared to B6 BMDCs. Similarly, on day 3, the frequency of B6 BMDC-primed $CD69^+CD8^+$ T cells was similar to the level of TLR3-/- BMDC-primed

$CD8^+$ T cells. The frequency of MyD88-/- BMDC-primed $CD69^+CD8^+$ T cells maintained at a similar level as that on day 1.5 but was significantly lower than that of $CD69^+CD8^+$ T cells primed by B6 BMDCs on day 3. This suggested that MyD88-/- BMDCs failed to activate $CD8^+$ T cell normally as compared to B6 BMDCs. Taken together, these results highlighted the important role of TLR3- and MyD88-dependent signaling in maturing mDCs for activating naïve $CD8^+$ T cells.

To further test whether TLR3- and MyD88-dependent signaling were involved in modulating antigen presentation activity, naïve $CD8^+$ OT-I-T cells that were stimulated with serial dilutions of OT-I-SCFV-inoculated B6, TLR3-/- or MyD88-/- BMDCs and APC function was measured as the ability of limiting number of DCs to induce proliferation of the naïve $CD8^+$ T cells as a readout. DC populations with efficient APC activity should elicit T cell proliferation even at high dilution while DC populations with weak APC activity would lose APC activity at higher dilution. Therefore, the endpoint dilution can be used as a measure to compare APC activity among DC from the different mouse strains. The results showed that $CD8^+$ T cells displayed a vigorous proliferation in the presence of $1.25x10^4$ activated B6 BMDCs (**Figure 2.4E**) compared with the naïve BMDC control (data not shown). Following the serial dilution of activated B6 BMDCs, the proliferation of $CD8^+$ T cells gradually decreased and reached to an endpoint at the BMDC: $CD8^+$ T cell ratio of 1: 512. Compared with B6 BMDCs, TLR3-/- BMDCs primed $CD8^+$ T cell proliferation following the same pattern but TLR3 deficiency caused a slightly decreased magnitude of $CD8^+$ T cell proliferation although the endpoint dilution of APC function was also 1:512. However, MyD88-/- BMDCs significantly compromised $CD8^+$ T cell proliferation before BMDC: T ratio achieved 1:128 (**Figure 2.4E**). The results suggested that TLR3- and MyD88-dependent signaling impacted $CD8^+$ T cell proliferation by influencing the antigen presenting function of mDCs and that mDC intrinsic MyD88-dependent signaling was more important than TLR3 signaling. We also studied whether mDC intrinsic TLR3- and MyD88-dependent signaling also

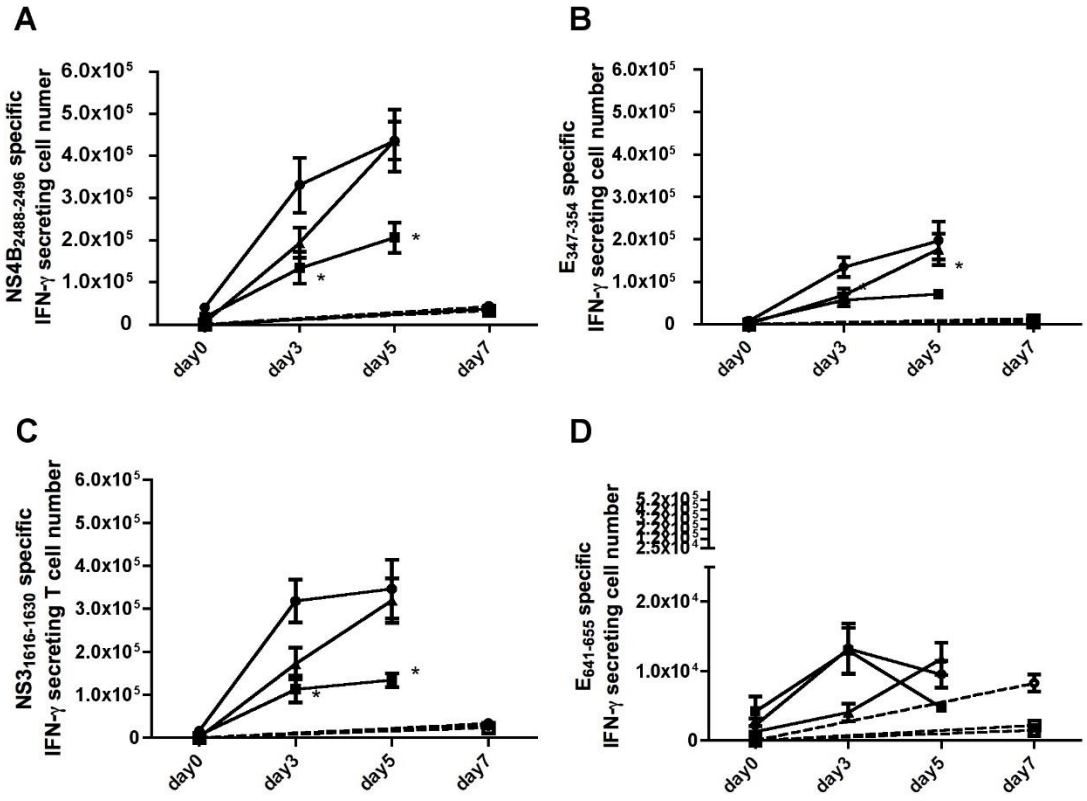
influenced the pattern of effector function of $CD8^+$ T cells as shown previously *in vivo* (**Fig 2.4**). $CD8^+$ T cells primed with the control naïve BMDCs showed an almost non-detectable production of effector cytokines (data not shown) while $CD8^+$ T cells that were primed by inoculated B6 BMDCs displayed a vigorous production of effector cytokines, IFN-γ, TNF-α and IL-2, on day 3 after coculture.(**Fig 2.5 G-I**) Compared with the B6 BMDCs group, the absence of either TLR3- or MyD88-dependent signaling from BMDCs resulted in a significantly decreased production of IFN-γ, TNF-α and IL-2 by $CD8^+$ T cells (**Figure 2.5 G-I**). This was consistent with the impaired cytokine production by WNV specific $CD8^+$ T cells in TLR3-/- and MyD88-/- mice *in vivo* (**Figure 2.4 C-F**). With regards to the polyfunctionality, $CD8^+$ T cells primed by B6 BMDCs developed a significantly higher frequency of dual functional T cells than that of monofunctional and triple functional T cells.(**Figure 2.5 F**) Compared with B6 BMDCs, antigen presentation by TLR3-/- BMDCs resulted in a significantly higher frequency of monofunctional $CD8^+$ T cells but a significantly lower frequency of dual functional $CD8^+$ T cells. $CD8^+$ T cell primed by MyD88-/- BMDCs showed a significantly decreased frequency of dual functional T cells but no alteration in the frequency of mono- and triple functional fractions. These results suggested that the DC-intrinsic TLR3- and MyD88-dependent signaling endowed antigen presenting BMDCs with optimal APC function for optimal activation and differentiation of naïve $CD8^+$ T cells. Additionally, TLR3 signaling in mDCs was more important in influencing the multifunctionality of $CD8^+$ T cells and this result was consistent with the results of the *in vivo* study (**Fig 2.3A and B**).

Deficiency in either TLR3 or MyD88 deficiency resulted in diminished recall responses by memory T cell responses.

Memory T cells stay quiescent in the absence of specific antigens but rapidly expand and provide an antiviral response once re-exposed to the same pathogen.

Successful vaccination requires induction of a specific and vigorous memory T cell response. However, the roles of TLR3- and MyD88-dependent signaling in the recall response of memory T cells are unknown. Therefore, the magnitude of memory T cell responses in B6, TLR3-/- and MyD88-/- mice following re-challenge with RepliVAX WN was determined by quantifying WNV specific IFN-γ secreting $CD8^+$ and $CD4^+$ T cells via ELISPOT on day 0, 3, 5 post-rechallenge. Compared with primary T cell responses (shown as dotted lines), re-challenging immunized mice resulted in a more rapid and vigorous T cell response (shown as solid lines) shown as a significantly increased number of IFN-γ secreting T cells through day 0 to 5 post-rechallenge.(**Fig 2.6**)

Figure 2.6



Memory $CD4^+$ and $CD8^+$ T cell responses were differentially impaired in RepliVAX WN-immunized TLR3-/- and MyD88-/- mice. RepliVAX WN immunized B6 (●), TLR3-/- (■) and MyD88-/- (▲) (n=4 mice/group) mice were re-challenged i.v. with 10^7 IU RepliVAX WN. $CD8^+$ (A, B) and $CD4^+$ (C, D) WNV specific IFN-γ secreting T cells from spleens of B6 (●), TLR3-/- (■) and MyD88-/- (▲) mice (n=5 mice/group) were quantified by ELISPOT assay on day 0, 3, and 5 post-rechallenge (solid lines). WNV-specific ($NS3_{1616-1630}$- and $E_{641-655}$-specific) $CD4^+$ and WNV-specific ($NS4B_{2488-2496}$- and $E_{347-354}$-specific) $CD8^+$ IFN-γ secreting T cells in primary responses (dotted lines) following RepliVAX WN immunization of B6, TLR3-/- and MyD88-/- on 7 dpi are also shown. Data are presented as the mean number of IFN-γ secreting T cells per spleen ± SEM. The results shown are from a single experiment performed. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) compared to the same time point for B6 mice.

The quantity of anti-WNV ($NS4B_{2488-2496}$- and $E_{347-354}$-specific) $CD8^+$ T cells (**Fig 2.6A and B**) and ($NS3_{1616-1630}$-specific) $CD4^+$ T cells (**Fig 2.6C**) in B6 mice increased

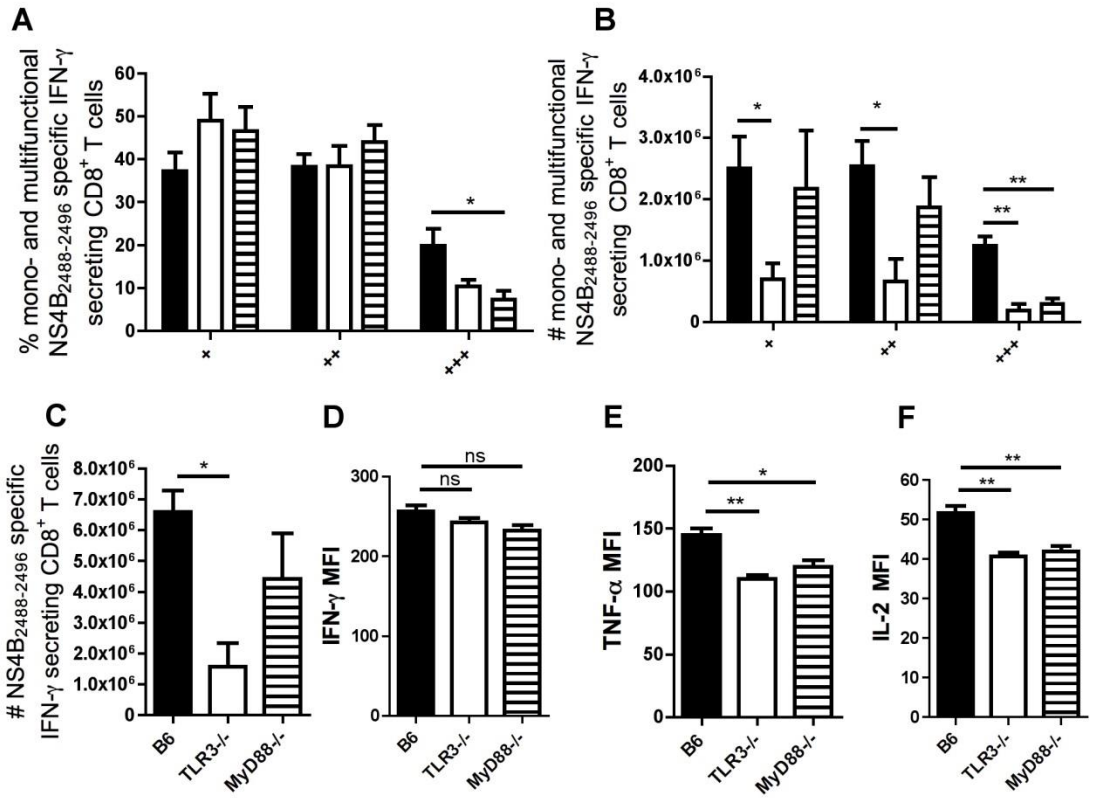
significantly from the time of re-challenge through day 5 post-rechallenge. Although MyD88 deficiency resulted in a consistent, but not significant, reduction in the quantity of anti-WNV $CD8^+$ and $CD4^+$ T cells on day 3 post-rechallenge compared with B6 mice, the number of antiviral T cells in MyD88-/- mice reached a similar level as that in B6 mice on day 5 post-rechallenge. The absence of TLR3 signaling caused a significantly (ANOVA test, $p<0.05$) decreased number of anti-WNV effector $CD8^+$ and $CD4^+$ T cells on both day 3 and 5 post-rechallenge compared with B6 mice. Given that TLR3-/- and MyD88-/- mice developed a similar quantity of anti-WNV memory $CD4^+$ and $CD8^+$ T cells in the primary response, these results suggest that TLR3 signaling played a more important role in modulating the reactivation or proliferation of memory T cells and thus made a stronger impact on the magnitude of recall responses. MyD88-dependent signaling was less involved in determining the development of secondary $CD4^+$ and $CD8^+$ T cell responses. Unexpectedly, MyD88 signaling impaired the quantity of $E_{641-655}$-specific $CD4^+$ T cells on day 3 post-rechallenge.(**Fig 2.6D**) The quantity of $E_{641-655}$-specific $CD4^+$ T cells in TLR3-/- was lower than B6 and MyD88-/- mice on day 5 post-rechallenge. Compared with the primary $E_{641-655}$-specific $CD4^+$ T cell response, the magnitude of $E_{641-655}$-specific $CD4^+$ T cell recall response is not as vigorous as that of other epitope peptide specific responses. It suggested that the role of TLR3- and MyD88-dependent signaling in modulating specific T cell secondary response might be different according to the immunodominance of different epitope peptides.

Deficiency of either TLR3- or MyD88-dependent signaling impaired the effector function of $CD8^+$ memory T cell responses.

Following the identification of the role of TLR3- and MyD88-dependent signaling in modulating the magnitude of secondary T cell responses, we also tested whether these signaling pathways were involved in developing a superior quality (effector function

pattern) of recall memory $CD8^+$ T cell responses by measuring the polyfunctionality and effector function of $NS4B_{2488-2496}$ specific $CD8^+$ memory T cells in B6, TLR3-/- and MyD88-/- mice on day 3 post-rechallenge. The results indicated that $CD8^+$ T cells with dual cytokine function were detected at a similar frequency and quantity as the monofunctional $CD8^+$ T cells in B6 mice.(**Fig 2.7A and B**) The increased proportion of dual functional T cells compared to that in the primary response (**Fig 2.4B**) suggested a better polyfunctionality of $CD8^+$ T cells in the recall response. Compared with B6 mice, TLR3-/- mice developed a higher frequency of monofunctional but a lower frequency of triple functional $NS4B_{2488-2496}$ specific $CD8^+$ T cells suggesting an impaired $CD8^+$ T cell polyfunctionality in the absence of TLR3 signaling. TLR3 deficiency also resulted in a significantly lower quantity of mono-, dual and triple functional T cells than B6 mice.(**Fig 2.7B**) This might be caused by the significantly decreased total number of IFN-γ secreting $NS4B_{2488-2496}$ $CD8^+$ T cells (**Fig 2.7C**) and the impaired polyfunctional fraction (**Fig 2.7B**) in TLR3-/- mice.

Figure 2.7


A
% mono- and multifunctional NS4B2488-2496 specific IFN-γ secreting CD8+ T cells
B
mono- and multifunctional NS4B2488-2496 specific IFN-γ secreting CD8+ T cells
C
NS4B2488-2496 specific IFN-γ secreting CD8+ T cells
D
IFN-γ MFI
E
TNF-α MFI
F
IL-2 MFI
B6
TLR3-/-
MyD88-/-
ns

Altered polyfunctionality and effector cytokine secretion of WNV specific $CD8^+$ T cells in TLR3-/- and MyD88-/- mice during secondary responses. RepliVAX WN immunized B6, TLR3-/- and MyD88-/- mice were re-challenged i.v. with 10^7 IU RepliVAX WN. Splenocytes from B6 (filled bars, n=3), TLR3-/- (open bars, n=3) and MyD88-/- (striped bars, n=3) were harvested on day 3 post-rechallenge. The frequency (A) and total number (B) of mono- and polyfunctional $CD8^+$ T cells were measured following *ex vivo* stimulation with $NS4B_{2488-2496}$. The monofunctional anti-WNV $CD8^+$ T cells that only secreted IFN-γ are shown as "+". The polyfunctional anti-WNV $CD8^+$ T cells that secreted IFN-γ and TNF-α are shown as "++" while the ones that secreted IFN-γ, TNF-α and IL-2 are shown as "+++". The total number of IFN-γ secreting cells (C) was measured by intracellular cytokine staining. The production of effector cytokines, IFN-γ (D), TNF-α (E) and IL-2 (F), by total $CD8^+$ T cells were detected following *ex vivo* stimulation with $NS4B_{2488-2496}$. Naïve splenocytes *ex vivo* stimulated with $NS4B_{2488-2496}$ and rechallenged splenocytes cultured with medium were used as control. The production level of cytokines was measured as the mean fluorescence intensity (MFI) of each fluorescence detection antibody. The results shown are from a single experiment performed. Data on polyfunctionality are presented as the mean frequency (A) and quantity (B) of mono- and poly-functional T cell number ± SEM. Data on the total number of IFN-γ secreting cells (C) are presented as the mean cell number ± SEM. Data on effector cytokine secretion are presented as the mean MFI of IFN-γ (C), TNF-α (D) and IL-2 (E) ± SEM. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) compared to B6 mice.

MyD88 deficiency caused a significantly decreased frequency (ANOVA, $p<0.05$) and quantity (ANOVA, $p<0.01$) of triple functional $CD8^+$ T cells compared with that of B6 mice suggesting an important role of MyD88-dependent signaling in determining the polyfunctionality of effector $CD8^+$ T cells derived from memory T cells. The effector function by $CD8^+$ T cells was also detected by measuring the level of IFN-γ, TNF-α and IL-2 by $NS4B_{2488-2496}$ produced by $CD8^+$ T cells in B6, TLR3-/- and MyD88-/- mice on day 3 post-rechallenge. The results showed that antiviral $CD8^+$ T cells in the three strains of mice produced a similar level of IFN-γ suggesting neither TLR3 nor MyD88 deficiency impaired the production of IFN-γ by secondary $CD8^+$ T cells. However, either TLR3 or MyD88 deficiency caused a significantly diminished TNF-α and IL-2 production by $CD8^+$ T cells compared to B6 mice.(ANOVA test, $p<0.05$ or $p<0.01$) In summary, the absence of TLR3 or MyD88-dependent signaling impaired the functional

pattern of secondary effector $CD8^+$ T cells by defecting the generation of triple functional $CD8^+$ T cells and the production of TNF-α and IL-2.

DISCUSSION.

Studies have shown that both TLR3- and MyD88-dependent signaling pathways played an important role in protecting against WNV infection in the mouse infection model. (Daffis, et al. 2008); (Szretter, et al. 2010) In this present study, we showed that TLR3- and MyD88-dependent signaling are involved in shaping anti-WNV T cell responses by influencing the magnitude and quality of the antiviral T cell responses. We showed that the absence of TLR3 caused a significantly decreased quantity of multifunctional $CD8^+$ T cells, a significantly diminished expression level of TNF-α and IL-2 by WNV-specific $CD8^+$ T cells and a significantly decreased quantity of memory $CD4^+$ and $CD8^+$ T cells. The absence of MyD88 resulted in a significantly deficient primary T cell expansion and differentiation to memory $CD4^+$ and $CD8^+$ T cells and a significantly impaired expression of IFN-γ, TNF-α and IL-2 by effector $CD8^+$ T cells. Additionally, anti-WNV $CD8^+$ T cells have been shown to be essential for viral clearance in the mouse model. (Shrestha and Diamond 2004) These impairments in $CD8^+$ T cell quantity and function suggest that TLR3- and MyD88-dependent signaling may protect hosts against WNV infection by maintaining efficient $CD8^+$ and $CD4^+$ T cell responses. TLR3 signaling is more likely to be involved in modulating the quality and effector function of $CD8^+$ T cells while MyD88-dependent signaling tends to shape both the magnitude and effector function of the primary effector $CD8^+$ T cells. Additionally, TLR3 signaling is involved in determining both the magnitude and quality of $CD8^+$ T cell memory responses. MyD88-dependent signaling is important in influencing the effector function of $CD8^+$ memory T cell responses.

T cell response development can be divided into several steps: T cell activation, T cell expansion, T cell differentiation into memory T cells, and T cell contraction. In this study, I focused on evaluating the role of TLR3- and MyD88-dependent signaling in T cell expansion and differentiation into memory T cells.

MyD88-dependent signaling rather than TLR3 signaling was more important in T cell expansion. A previous study showed that the activation of either TLR3- or MyD88-dependent signaling by adjuvants could promote HIV-specific T cell expansion; however, the TLR3 ligand induced more vigorous proliferation. By contrast, in our observations, the absence of MyD88-dependent signaling caused a more impaired T cell expansion compared with the lack of TLR3. This result might be attributed to the involvement of MyD88-dependent signaling in multiple PRR and cytokine signaling pathways. However, this significant impairment was only observed in $E_{347\text{-}354}$ but not $NS4B_{2488\text{-}2496}$ epitope specific $CD8^+$ T cell expansion. In our study, compared with $E_{347\text{-}354}$, $NS4B_{2488\text{-}2496}$ is more predominant WNV epitope. (Brien, Uhrlaub and Nikolich-Zugich 2007) Therefore, it is possible that besides MyD88-dependent signaling, differences in TCR signaling might also influence T cell expansion. The activation of TLR (TLR3- or MyD88-dependent) signaling by TLR ligands and the stimulation of TCRs with anti-CD3 antibodies or antigen-pulsed APCs were both shown to promote specific $CD8^+$ T cell expansion and response. (Tabiasco, et al. 2006); (Cottalorda, et al. 2006) Therefore, our study results are consistent with the notion that the immunodominance of T cell epitope and TLR signaling are both important and synergize in determining the magnitude of T cell expansion. Additionally, the *in vitro* coculture study showed that the impaired proliferation of $CD8^+$ T cells expressing identical $OVA_{257\text{-}264}$ specific TCRs could not be observed in the presence of sufficient antigen presenting TLR3-/- or MyD88-/- mDCs. This suggests that the quantity of activated APCs is also important in determining the magnitude of $CD8^+$ T cell proliferation. Therefore, activating sufficient APCs presenting predominant T cell epitopes and triggering innate signaling during vaccination are likely

to play roles in the optimal proliferation of effector $CD8^+$ T cells. However, although $CD8^+$ T cell proliferation can be achieved in the presence of a sufficient quantity of TLR3 or MyD88 deficient APCs, effector function and multifunctionality of $CD8^+$ T cells are still apparent.

Although MyD88-dependent signaling plays an important role in $CD8^+$ T cell expansion, our results indicate that the proliferation of anti-WNV $CD4^+$ T cells was more likely to be MyD88-independent. This suggests that other signals (TCR signals, cytokines, etc) may play a role in influencing $CD4^+$ T cell responses. Although $CD4^+$ T cells provide essential help for $CD8^+$ T response development, the unimpaired $CD4^+$ T cells response suggests that the significantly decreased $CD8^+$ T cell expansion occurred even in the presence of $CD4^+$ T cell help. Moreover, our *in vitro* proliferation assay results (**Fig 2.5E**) showed that in the absence of $CD4^+$ T cells the proliferation of $CD8^+$ T cells primed by WT, TLR3-/- and MyD88-/- BMDCs followed the same pattern that was observed *in vivo*. That is, MyD88 rather than TLR3 deficiency caused a more impaired $CD8^+$ T cell proliferation. This result strongly suggests that the significantly impaired $CD8^+$ T cell expansion in RepliVAX WN immunized MyD88-/- mice was attributed to dysfunctional APCs rather than $CD4^+$ T cells.

Vaccines should induce the development of sufficient memory T cells to protect against infections. Our results showed that the induction of anti-WNV memory T cells by RepliVAX WN immunization was also shaped by innate TLR3- and MyD88-dependent signaling. A significantly decreased quantity of anti-WNV CD8+ and CD4+ memory T cells were observed in the absence of either TLR3-/- or MyD88-/-. The decreased quantity of anti-WNV memory T cells in RepliVAX WN-immunized MyD88-/- mice probably resulted from the compromised T cell expansion we demonstrated *in vivo*. Therefore, it suggests that T cell expansion is an important determinant of memory cell development.

However, different from MyD88-deficiency, TLR3 deficiency also caused a similarly decreased quantity of memory $CD4^+$ and $CD8^+$ T cells although the T cell expansion phase appeared less affected. Previous studies have shown that autocrine activity of IL-2 prolonged the longevity of T cells and promoted their differentiation into memory T cells. (Li, et al. 2001); (Kondrack, et al. 2003) Additionally, multifunctional CD8+ T cells are more likely to differentiate into memory T cells compared with monofunctional T cells. (Harari, et al. 2006) Given that TLR3 deficiency also impaired CD8+ T cell functionality both *in vivo* and *in vitro*, TLR3 signaling might influence memory T cell differentiation by influencing the quality of $CD8^+$ T cells following RepliVAX WN immunization. Compared with TLR3 signaling, MyD88 signaling impacted the development of memory T cells by manipulating both the expansion and the quality of T cell responses. Taken together, our results suggested that the development of memory T cells is determined by both the initial expansion of primary T cells and the quality of activated T cells. Our study using an *in vitro* APC assay emphasized the important role of mDCs intrinsic TLR3- and MyD88-dependent signaling in determining T cell expansion and quality. In the mDC and $CD8^+$ T cell coculture (**Fig 2.5**), the absence of TLR3- and MyD88-dependent signaling limited the number of DC that could functionally activate naïve $CD8^+$ T cells. This diminished APC activity may be responsible for the diminished numbers of WNV-specific T cells we observed *in vivo* in TLR3-/- and MyD88-/- mice. This also suggests that the fate of T cells that differentiate into memory cells is determined as early as T cell activation and that innate signaling is involved in the decision.

The T cell recall response to RepliVAX WN challenge was also impacted by TLR3- and MyD88-dependent signaling. Interestingly, different from their role in influencing primary responses, TLR3- rather than MyD88-dependent signaling play a more important role in determining the magnitude of secondary anti-WNV T cell responses. Unexpectedly, MyD88 deficiency did not result in a decreased expansion of

secondary WNV specific IFN-γ secreting $CD8^+$ T cells. This result suggests that differentiation of activated T cells to memory T cells is more sensitive to the absence of MyD88 signaling than is memory T cell differentiation to effector cells upon secondary antigen exposure.

Given that by the end of primary responses TLR3 and MyD88 deficiency resulted in a similar quantity of memory T cells, it suggests that the magnitude of the secondary T cell response is determined by factors other than simply influencing the quantity of memory cells (e.g. the environment for $CD8^+$ memory T cell reactivation). Cognate antigens, TLR ligands (especially TLR3 ligands) and inflammation in the environment where secondary responses occur have been shown to influence memory T cell reactivation. Several studies have shown that cytokines (e.g. IL-12, IL-18, IL-15, type I IFN) and TLR3 ligand (poly I:C) promote memory $CD8^+$ T cell reactivation by facilitating their expansion, IFN-γ secretion and cytotoxicity. (Berg, et al. 2003); (Kamabayashi, et al. 2003); (Liu, et al. 2002); (Kohlmeier, et al. 2010) Additionally, the milieu for $CD8^+$ memory T cell reactivation was shown to include functional activated innate immune cells (e.g. monocytes, dendritic cells, mononuclear phagocytic cells). (Soudja, et al. 2012); (Suarez-Ramirez, et al. 2011); (Narni-Mancinelli, et al. 2007) Therefore, our results highlight the potentially essential role of TLR3- and MyD88-dependent signaling in modulating the milieu necessary for optimal development of secondary T cell responses. The quality of the secondary $CD8^+$ T cell responses in TLR3-/- and MyD88-/- mice was also impaired shown as increased frequency of monofunctional $CD8^+$ T cells and decreased frequency of multifunctional $CD8^+$ T cells. This implies that secondary T cell responses are influenced by these signaling pathways as priming T cell responses.

Interestingly, we found an upregulated expression of CD69 in activated $CD8^+$ T cells in TLR3-/- but not MyD88-/- mice. These results suggest the possibility of a different migration manner of $CD8^+$ T cells in TLR3-/- mice. CD69 upregulated

expression has been shown to be correlated with the failure of the development of T_{CM}s (Hess Michelini, et al. 2013) or the facilitation of peripheral tissue T_{RM}s development (Mackay, Rahimpour, et al. 2013). Therefore, more $CD69^+$ T cells in TLR3-/- mice suggested fewer T_{CM}s were developed in spleen or more effector or memory T cells were developed in the peripheral blood or tissues. This is consistent with the observation that memory $CD8^+$ T cell in TLR3-/- mice was significantly impaired. This is also related with the observed impaired CD8+ T cells multifunctionality which indicates a deficient development of central memory T cells. Additionally, the overexpression of CD69 has been shown to be present in autoimmune disease. (Gessl and Waldhausl 1998) Therefore, the overexpression of CD69 suggests that there might be a distinguished Treg or TGF-β regulatory pattern in TLR3-/- mice compared with wild type mice. The upregulated expression of CD69 suggested a unique mechanism by which TLR3 but not MyD88-dependent signaling modulate T cell responses.

Taken together, TLR3- and MyD88-dependent signaling pathways influence the primary $CD8^+$ T cell responses by manipulating the function of APCs (mDCs). TLR3 signaling mainly impacts the quality of $CD8^+$ T cells while MyD88-dependent signaling impacts both the proliferation and quality of $CD8^+$ T cells. However, these two signaling pathways influence the differentiation of memory $CD8^+$ T cells to a similar extent and loss of either pathway results in a diminished quantity of memory $CD8^+$ T cells. For secondary responses, TLR3 signaling plays a more important role in determining the response magnitude suggesting a different milieu in TLR3-/- mice for memory $CD8^+$ T cell reactivation compared with MyD88-/- mice.

Chapter 3. The role of TLR3- and MyD88-dependent signaling in the development of B cell responses induced by RepliVAX WN.[1]

INTRODUCTION.

RepliVAX WN has been shown to elicit vigorous B cell responses. Like WNV infection, immunization with RepliVAX WN induces IgG responses specific to the antigen targets, NS1 and SVP. (Nelson, et al. 2010) RepliVAX WN-induced B cell responses were shown to be shaped by type I IFN signaling. (Winkelmann, et al. 2012) The absence of type I IFN signaling has been shown to cause an impaired NS1 and SVP specific IgM production suggesting the important role of type I IFN signaling in B cell activation. In these studies, lack of type I IFN resulted in a higher antigen load which presumably would induce a higher IgG response in IFNAR-/- mice. However, the absence of type I IFN signaling resulted in a significantly decreased or similar magnitude of anti-NS1 and anti-SVP IgG responses. This suggests that type I IFN signaling influences the kinetics of B cell responses. Furthermore, IgG1 but not IgG2c is expressed in the absence of type I IFN suggesting the important role of type I IFN signaling in influencing the antibody response following RepliVAX WN immunization. Therefore, in this chapter, a study was performed based on the hypothesis that TLR3- and MyD88-dependent signaling pathway differentially influence B cell response development following RepliVAX WN immunization in mice.

SIGNIFICANCE.

[1] This chapter includes the sections (material and method, results and discussion) which were published in the Journal of Virology. American Society for Microbiology (ASM) grants the author (Jingya Xia) the right to republish/adapt portions of the article in her any other publication. The cited portion is from the article, Xia J, Winkelmann ER, Gorder SR, Mason PW, Milligan GN. TLR3- and MyD88-dependent signaling differentially influences the development of West Nile virus-specific B cell responses in mice following immunization with RepliVAX WN, a single-cycle flavivirus vaccine candidate. J Virol. 2013. 87(22):12090-101.

In this study, I identified and compared the different mechanisms by which TLR3- and MyD88-dependent signaling pathways were involved in B cell response development following RepliVAX WN immunization. As demonstrated in Chapter 2, the study will: 1. identify the essential role of TLR3- and MyD88-dependent signaling in B cell response and memory development; 2. Determine the role of these signaling pathways in the immunogenicity of the vaccine candidate, RepliVAX WN; and 3. Develop a model for the potential of different TLR ligands to adjuvant WNV vaccines.

MATERIALS AND METHOD.

Mice. C57BL/6J (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TLR3 deficient (TLR3-/-) and MyD88 deficient (MyD88-/-) mice on a B6 background were obtained from Dr. Michael Diamond (Washington University, St. Louis) and maintained as a breeding colony at the Association for Assessment and Accreditation of Laboratory Animal Care- approved animal research center of the University of Texas Medical Branch. Animals were age and sex matched for all experiments. All animal research was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch with oversight of staff veterinarians.

Viruses and WNV Antigen. RepliVAX WN was generated in BHK (VEErep/Pac-Ubi-C*) cells as previously described (Widman, Ishikawa, et al. 2008). Firefly luciferase-expressing SCFV particles (FLUC-SCFV) containing a WNV replicon genome expressing a humanized FLUC gene were generated in BHK (VEErep/C*-prM-E-Pac) cells as previously described (Gilfoy, Fayzulin and Mason 2008). RepliVAX WN and FLUC-SCFV were titrated using Vero cells as previously described (Rossi, Zhao, et al. 2005). WNV subviral particles (SVPs) used as ELISPOT and ELISA capture antigens were produced by infecting Vero cells with RepliVAX WN. The cell culture supernatant

containing the released WNV SVPs was harvested and concentrated using centrifugal filtration, and SVPs were purified on a sucrose gradient. WNV NS1 antigen was harvested and purified from transfected VEErep-bearing BHK cells as previously described (Widman, Ishikawa, et al. 2008).

Immunization with RepliVAX WN. For quantifying WNV-specific serum IgM and IgG antibody titers and WNV-specific antibody secreting cells (ASC), mice were immunized by the intraperitoneal (i.p.) route with 10^6 IU RepliVAX WN. To assess the level of viral gene replication and evaluate early B cell activation in secondary lymphoid tissues, mice were immunized by subcutaneous (s.c.) food pad (FP) injection with 10^6 IU RepliVAX WN or FLUC-SCFV. Inocula were delivered in L-15 medium containing 10mM HEPES and 0.5% FBS.

Serological analysis. An Enzyme-linked immunosorbent assay (ELISA) was used to titrate anti-NS1 and anti-SVP IgM and IgG antibodies as described previously (Nelson, et al. 2010). Briefly, the capture antigens, purified WNV SVPs and purified NS1 proteins, were absorbed on ELISA plates (Corning Incorporated, Corning, NY) overnight at 4°C. Plates were blocked and serial dilutions of serum from B6, TLR3-/- and MyD88-/- immunized and naïve mice were added on the coated plates. Plate-bound IgG was developed with HRP-IgG (Southern Biotechnology Associates, Inc., Birmingham, AL), HRP-IgM (Southern Biotechnology Associates, Inc.) or with biotinylated anti-mouse IgG1 or IgG2c (BD Pharmingen, San Diego, CA) followed by incubation with streptavidin peroxidase (Sigma-Aldrich, St. Louis, MO). Normalized OD readings at 490nm (OD_{490}) obtained from serial dilution of serum were analyzed by non-linear regression. The end point titer was defined as the serum dilution resulting in an OD_{490} value equivalent to three standard deviations above OD_{490} values from sera of mock-vaccinated animals.

Avidity was measured by a modified ELISA test (Kneitz, et al. 2004) in which serum samples were plated on NS1- or SVP-coated plates for one hour followed by a 10 minute wash with 4M, 6M, or 8M urea, or saline as control. Plates were developed as described previously (Nelson, et al. 2010) and OD_{490} readings for each serum sample were obtained for urea-treated wells (OD_{490} urea) and saline-treated wells (OD_{490} saline). The avidity index for NS1- and SVP-specific IgG was calculated as: OD_{490} urea/OD_{490} saline.

Enzyme-linked immunospot assay (ELISPOT). ELISPOT assays for antibody secreting cells (ASCs) were performed as described previously (Nelson, et al. 2010) using microtiter filter plates (Millipore corporation, Billerica, MA) coated with purified WNV NS1 protein or purified WNV SVPs. Ag specific ASCs were quantified using an ImmunoSpot reader and analyzed with ImmunoSpot software (Cellular Technology Ltd, Cleveland, OH).

Flow cytometry analysis and antibodies. Popliteal lymph nodes (pLNs), Inguinal LNs (iLNs) and spleens were collected from immunized and naive B6, TLR3-/- and MyD88-/- mice. Single cell suspensions were blocked with anti-Fc RII/III mAb and surface stained with antibodies purchased from BD Biosciences (San Jose, CA): anti-CD19-phycoerythrin (PE; 1D3), anti-CD69-peridinin-chlorophyll proteins-Cy5.5 (Percp-Cy5.5; H1.2F3), and anti-CD86-fluorescein isothiocyanate (FITC; GL1). Anti-PNA-fluorescein isothiocyanate was purchased from Sigma-Aldrich (St. Louis, MO). Data were acquired on a BD LSRII Fortessa (BD Biosciences, San Jose, CA) at the UTMB Flow Cytometry Core Facility and analyzed using FlowJo software (Tree Star, Ashland, OR).

***In vivo* imaging (IVIS).** The posterior half of B6, TLR3-/- and MyD88-/- mice was shaven prior to s.c. immunization in the FP with 10^6 IU FLUC-SCFV. At 14 hours post immunization (hpi) and at 1, 2, 3, 4, 6, 8, and 10 days post immunization (dpi), real-time *in vivo* imaging was performed using a Xenogen IVIS 200 (Caliper LS, Hopkinton, MA) on D-Luciferin (Caliper LS) -treated and anesthetized mice (Winkelmann, et al. 2012). Images were analyzed by defining FP regions with FLUC activity and measuring total flux (photons per second; p/s). Data were acquired using Living Image 4.0 software (Caliper LS) and reported as the average total flux from FP of all mice in an experimental group.

Neutralization assay. Day 28 pi serum was pooled from groups of RepliVAX WN-immunized B6 (n=35), TLR3-/- (n=35), and MyD88-/- (n=34) mice and the endpoint titer of each serum pool calculated by ELISA. Serum pools were normalized by dilution of B6 and TLR3-/- pools to achieve an endpoint titer equivalent to the MyD88-/- serum pool. Equivalent endpoint titers were confirmed by serum titration curves on SVP ELISA plates (See Fig 3.7A for example). Neutralization titers of the normalized serum pools were determined using luciferase-expressing RepliVAX WN (FLUC-SCFV) as described previously (Widman, Ishikawa and Winkelmann, et al. 2009); (Widman, Ishikawa, et al. 2008). The 50% neutralization titer was determined by nonlinear regression using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA).

Statistical analysis. Statistical differences for B lymphocyte assays, serum titer and frequency and quantity of different cell compartments were determined using Student t test (unpaired) or ANOVA with the Tukey post test as appropriate. Values for $p<0.05$ were considered significant. All calculations were performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA).

RESULTS.

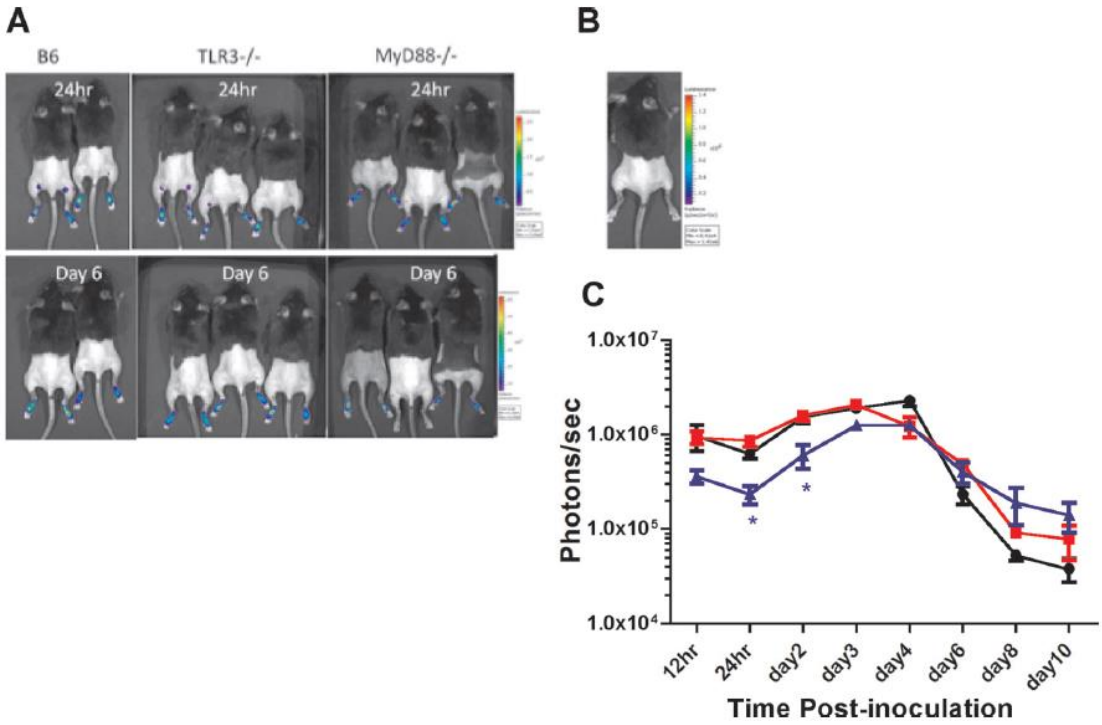
Diminished antibody response to RepliVAX WN in the absence of either TLR3- or MyD88-dependent signaling.

Stimulation of TLRs initiates innate immune responses including the production of type I IFN, which limited viral gene expression and therefore altered availability of viral proteins for induction of adoptive immunity. We therefore tested whether SCFV gene expression would be increased in TLR3-/- or MyD88-/- mice immunized with RepliVAX WN. B6, TLR3-/- and MyD88-/- mice were immunized with FLUC-SCFV, and FLUC gene expression at the FP site of injection was quantified between 14 hpi and 10 dpi using an *in vivo* imaging system (IVIS). Expression of SCFV-encoded FLUC was readily detected in FP of all infected mice as early as 14 hpi (**Fig. 3.1A**) but was not detected in uninoculated mice (**Fig. 3.1B**). In B6 mice, FLUC bioluminescence was maintained at a high level through 4 dpi and diminished thereafter to low levels on 10 dpi (Fig. 3.1C). Similar patterns of expression of the SCFV-encoded FLUC gene were detected in both TLR3-/- and MyD88-/- mice. The FLUC intensity in MyD88-/- mice was initially approximately two-fold lower at 2 dpi before increasing to levels nearly equivalent to B6 mice on 3 dpi. FLUC expression was maintained at levels equivalent to or slightly higher than B6 mice on days 4 through 10 pi. FLUC expression in TLR3-/- mice was very similar to B6 mice on all days tested.

TLR signaling directs the production of type I IFN and proinflammatory cytokines that were shown to modulate the development of adaptive immune responses to several viruses (Winkelmann, et al. 2012); (Takaqi, et al. 2011). Accordingly, I investigated if the TLR signaling pathways induced by the SCFV vaccine RepliVAX WN influenced the development of the WNV-specific antibody response. Groups of B6,

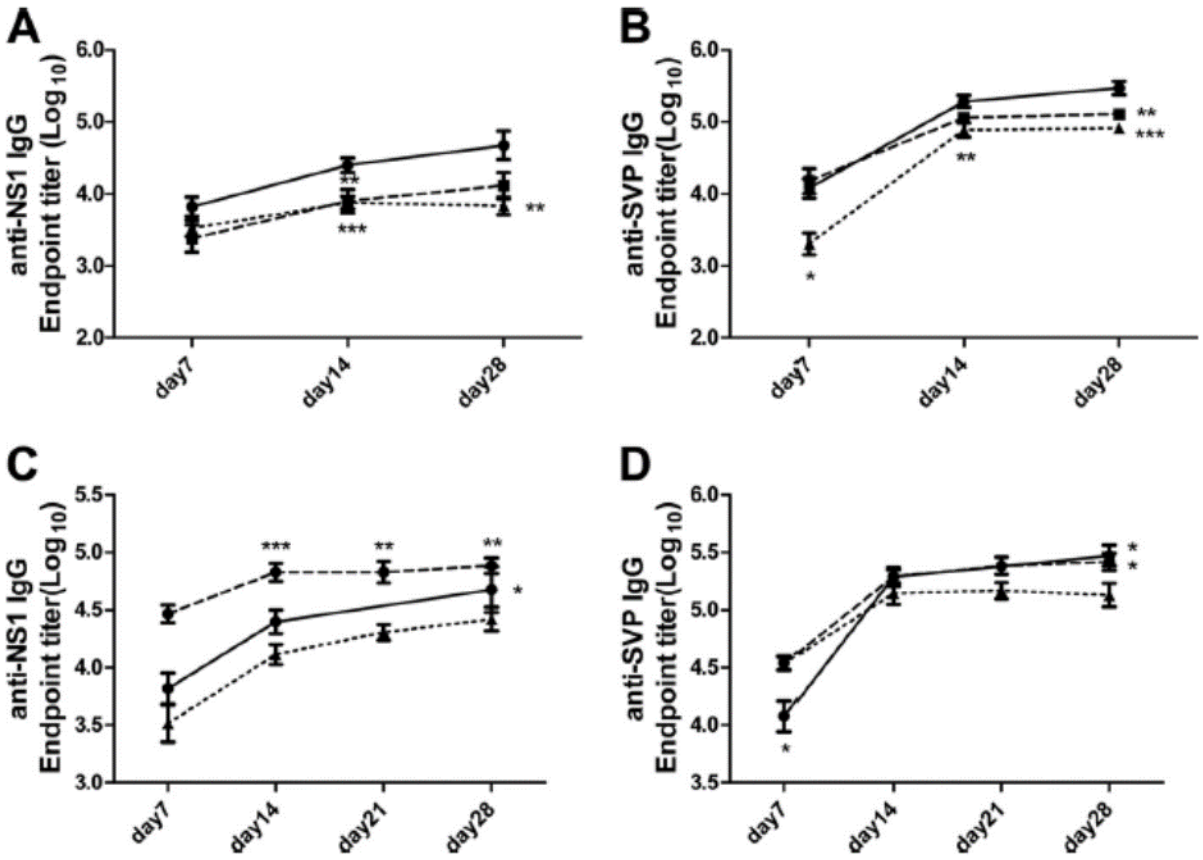
TLR3-/-, and MyD88-/- mice were immunized i.p. with 10^6 IU RepliVAX WN and WNV-specific serum IgG responses were quantified on 7, 14, and 28 dpi. Vigorous anti-NS1 (**Fig. 3.2A**) and anti-SVP (**Fig. 3.2B**) IgG antibody titers were detected in B6 mice. In comparison, both TLR3-/- and MyD88-/- mice produced significantly less NS1-specific IgG (**Fig. 3.2A**) and SVP-reactive IgG (**Fig. 3.2B**) on 14 and 28 dpi. Although loss of either innate signaling pathway impaired the RepliVAX WN-specific humoral response, WNV antigen-specific serum IgG levels were most compromised in MyD88-/- mice. Specific antibody responses in MyD88-/- mice inoculated with a ten-fold higher dose of RepliVAX WN (10^7 IU) were still lower than in B6 mice inoculated with 10^6 IU RepliVAX WN (**Fig 3.2C, D**) strongly suggesting that the diminished response in MyD88-/- mice was the result of a cellular defect and not the result of an initially lower antigen load in MyD88-/- mice (**Fig 3.1C**).

Figure 3.1: SCFV gene expression in B6, TLR3-/-, and MyD88-/- mice.



Groups of B6 (black●) or TLR3-/- (red ■) or MyD88-/- (blue▲) mice were inoculated s.c. in both rear FP with 10^6 IU FLUC-SCFV and imaged at the indicated time points as described in Materials and Methods. Results are expressed as the mean bioluminescence from FP from each group. Error bars represent the standard error of the mean (SEM) from individual mice. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) compared to the same time point for B6 mice. The limit of detection for the assay was 10^4 photons/sec.

Figure 3.2: Diminished WNV-specific serum IgG titers in TLR3-/- and MyD88-/- mice.



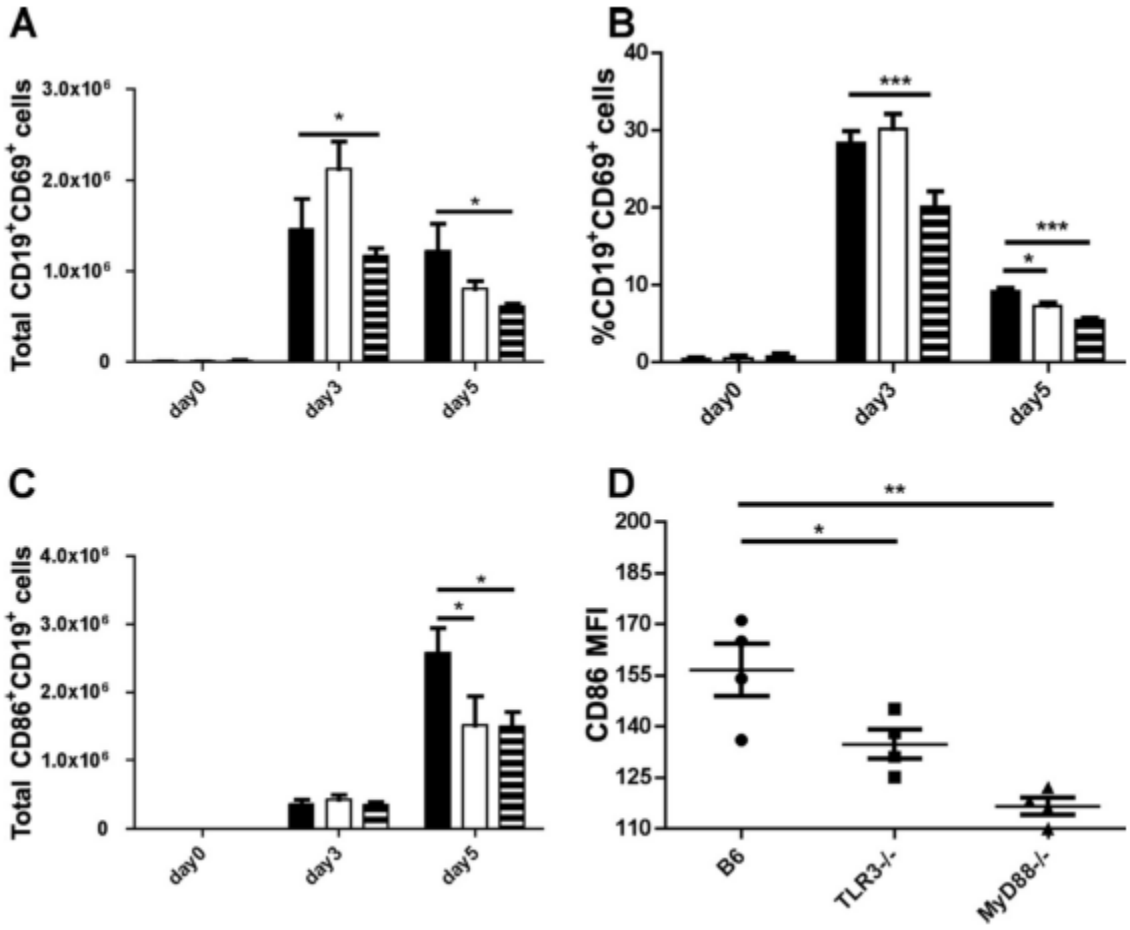
Diminished WNV-specific serum IgG titers in TLR3−/− and MyD88−/− mice. B6 (n = 10) (●), TLR3−/− (n = 10) (■), and MyD88−/− (n = 9) (▲) mice were immunized i.p. with 106 IU RepliVAX WN. Anti-NS1 (A) or anti-SVP (B) serum IgG was measured by ELISA on 7, 14, and 28 dpi. Results are pooled from 2 experiments. Data are presented as the mean titer (log 10) ± SEM. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$ (compared to results at the same time point for B6 mice). (C and D) Anti-NS1 (C) or anti-SVP (D) serum IgG titers from B6 (n = 5) (●) and MyD88−/− (n = 5) (▲) mice immunized i.p. with 10^6 IU (solid line) or 10^7 IU (dashed lines) RepliVAX WN. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$ (compared to results at the same time point for 10^6 IU RepliVAX WN-immunized B6 mice).

Diminished B cell activation following RepliVAX WN immunization of MyD88-/- and TLR3-/- mice in the adjacent lymphoid tissues around the immunization loci.

I investigated which cellular events during development of a WNV-specific B cell response were affected by loss of these signal pathways. Following events such as cross-linking of BCRs with antigen, binding of TLR ligands, or exposure to type I IFN, B cells become activated and express the early activation markers CD69 and CD86 (Minquet, Dopfer and Pollmer, et al. 2008); (Swanson, et al. 2010); (Purtha, Chachu, et al. 2008); (Daniel 2005). To determine if B cell activation was impaired by the lack of either TLR3- or MyD88-dependent signaling, we measured CD69 expression on B cells ($CD19^+$ cells) at 0, 3, and 5 dpi (**Fig. 3.3A**). In the pLNs of B6 and TLR3-/- mice, the frequency of $CD69^+$ $CD19^+$ cells peaked by 3 dpi before diminishing at 5 dpi. In contrast, the frequency of $CD69^+$ $CD19^+$ cells in MyD88-/- mice peaked at a significantly lower level on 3 dpi ($p<0.001$, ANOVA). The total number of $CD69^+$ $CD19^+$ B cells in pLN of B6 mice rose on 3 dpi and was maintained at high levels through 5 dpi. The number of activated B cells in TLR3-/- mice was similar to B6 mice on days 3 and 5 pi but was significantly reduced in MyD88-/- mice on both days (**Fig. 3.3B**; $p<0.05$, ANOVA). A similar pattern of expression was also observed for the activation marker CD86. There was a gradual increase in the frequency and quantity of $CD86^+$ $CD19^+$ cells in pLNs on 3 and 5 dpi in all three mouse strains. TLR3-/- and MyD88-/- mice showed a significant reduction in the quantity of $CD86^+$ $CD19^+$ cells on 5 dpi (**Fig. 3.3C**; $p<0.05$, ANOVA). Further, although the quantity of $CD86^+$ $CD19^+$ cells did not significantly differ among B6, TLR3-/- and MyD88-/- mice on 3 dpi, the level of CD86 expression measured as the mean fluorescence intensity (MFI) was significantly reduced on pLN B cells of TLR3-/- and MyD88-/- mice compared to B6 mice (**Fig. 3.3D**; $p<0.05$, ANOVA).

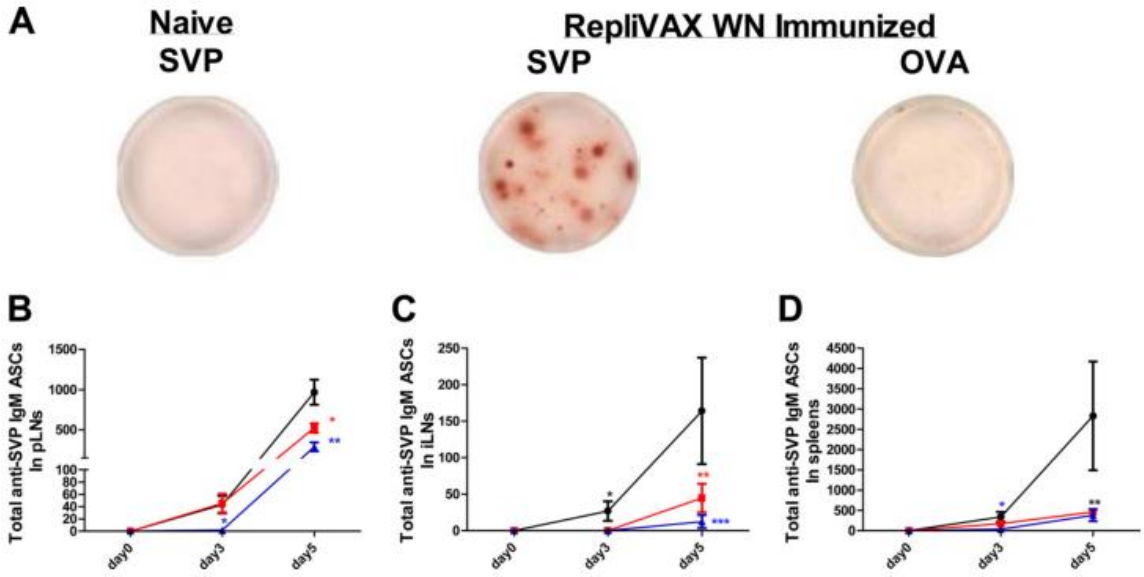
To determine if the lack of TLR3- and MyD88-dependent signaling resulted in early functional deficits, mice were immunized s.c. in the FP with 10^6 IU RepliVAX WN and anti-SVP IgM ASC were quantified in pLNs, iLNs, and spleens on 0, 3 and 5 dpi. IgM ASCs from immunized mice were readily detected on SVP-coated wells but not on ovalbumin-coated wells demonstrating the antigen-specificity of the ASCs (**Fig 3.4A**). SVP-reactive IgM ASCs were detected at all three lymphoid tissues of RepliVAX WN-immunized B6 mice on 3 dpi and the number increased through 5 dpi (**Fig. 3.4 B, C, D**). SVP-reactive IgM ASCs were detected in the pLN and spleen of TLR3-/- mice on day 3 and numbers increased through 5 dpi. In MyD88-/- mice, SVP-reactive IgM ASCs were not detected at any site on 3 dpi but were detected at low levels at all three sites on 5 dpi. Additionally, the number of anti-SVP IgM ASC was significantly reduced on 5 dpi in both TLR3-/- and MyD88-/- mice ($p<0.001$, ANOVA).

Figure 3.3: Decreased expression of B cell activation markers following RepliVAX WN immunization of TLR3-/- and MyD88-/- mice.



B6 (n=4, filled bar), TLR3-/- (n=4, open bar) and MyD88-/- (n=4, striped bar) mice were inoculated s.c. in the FP with 10^6 IU RepliVAX WN. (A) Frequency of $CD19^+$ B cells expressing CD69 in pLNs from B6, TLR3-/-, and MyD88-/- mice. B. Total number of $CD69^+CD19^+$ B cells in pLNs from B6, TLR3-/-, and MyD88-/- mice. C. Total number of $CD86^+CD19^+$ B cells from pLNs of immunized mice. D. The mean fluorescence intensity (MFI) of CD86 expression on activated B cells ($CD69^+CD19^+$) in pLNs from B6 (●), TLR3-/- (■) and MyD88-/- (▲) mice on 3 dpi. Results are from a representative experiment of 2 performed and are expressed as the mean ± SEM (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Figure 3.4: The SVP-specific IgM antibody secreting B cell response to RepliVAX WN immunization is diminished TLR3-/- and MyD88-/- mice.



(A). Specificity of ELISPOT for detecting SVP-reactive IgM ASC. Representative ELISPOT wells coated with SVP (left, middle) and OVA (right) for detection of ASC from naive (left) and RepliVAX WN immunized mouse (middle, right). SVP-specific IgM ASC response in pLNs (B), iLNs (C) and spleens (D) of RepliVAX WN immunized mice (10^6 IU RepliVAX WN s.c. in FP). SVP-specific IgM ASCs from B6 (●, n=8), TLR3-/- (■, n=8) and MyD88-/- (▲, n=6) immunized mice were quantified by ELISPOT. The results shown are from a representative experiment of 2 performed. Data are presented as the mean ± SEM. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the same time point for B6 mice).

Altered GC cellularity, WNV-specific serum IgG antibody, and ASC responses to RepliVAX WN in TLR3-/- and MyD88-/- mice.

The GC is the specialized site in lymphoid follicles for antigen-specific B cell proliferation, maturation, and differentiation into LLPCs and MBCs (McHeyzer-Williams, et al. 2011). To determine if lack of TLR3- or MyD88-dependent signaling pathways influenced the development, cellularity or maintenance of the GC, we quantified $CD19^+$ PNA^+ GC B cells from RepliVAX WN-immunized mice by flow cytometry on 7, 14, and 21 dpi in B6, TLR3-/-, and MyD88-/- mice. As shown in Fig. 3.5A, the number of GC B cells in B6 mice peaked by 14 dpi and diminished by 21 dpi as the GC devolved (Jing, et al. 2008). The development of GC cells in TLR3-/- mice exhibited similar kinetics and achieved a similar response magnitude as in B6 mice. By contrast, the number of PNA^+ GC B cells in MyD88-/- mice was significantly reduced on 14 dpi compared with B6 mice ($p<0.001$, ANOVA).

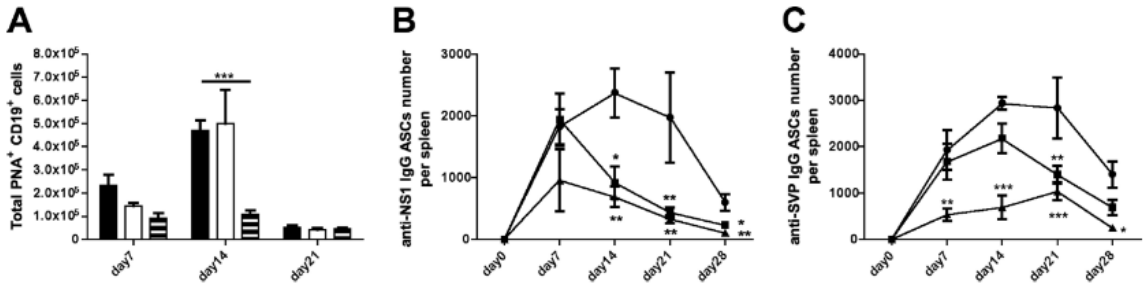
As an additional approach to assessing the influence of TLR3- and MyD88-dependent signaling on GC, we quantified NS1- and SVP-specific ASC on 7, 14, 21, and 28 dpi by ELISPOT (**Fig. 3.5B, C**). In B6 mice, the quantity of anti-NS1 and anti-SVP ASC peaked on 14 dpi and gradually decreased thereafter through 28 dpi. Both the NS1- and SVP-specific ASC responses of TLR3-/- mice rose rapidly and peaked at day seven (NS1-specific ASC) or 14 dpi (SVP-reactive ASC) at levels similar to B6 mice. However, the anti-NS1 response dropped rapidly and was significantly lower than B6 mice 14, 21 and 28 dpi ($p < 0.05$, $p<0.01$) and the anti-SVP ASC response was significantly lower beginning day 21 ($p<0.01$). Compared to B6 mice, MyD88-/- mice showed a significant deficiency in the number of both NS1- and SVP-specific IgG ASC from 7 to 28 dpi.

Previous studies using TLR3-/- and MyD88-/- mice have shown an increased IgG1 component of the serum IgG antibody response to infection with Neospora caninum or immunization with poly I:C adjuvanted OVA (Mineo, Oliveira, et al. 2010); (Kumar, Koyama, et al. 2008). To determine the influence of TLR3- and MyD88-dependent signaling on IgG subclass utilization, we determined the titers of NS1- and SVP-specific IgG2C and IgG1 antibody in serum of immunized mice on 28 dpi. As shown in **Fig. 3.6A** anti-NS1 IgG2C titers were significantly lower in TLR3-/- and MyD88-/- mice compared to B6 mice. By contrast, IgG1 titers were readily detectable in TLR3- and MyD88-deficient mice but anti-NS1 IgG1 antibody was undetectable in B6 mice (**Fig. 3.6B**). Using the limit of IgG1 detection as a conservative titer for B6 mice, the IgG2c/IgG1 ratio was 3.6 in B6 mice compared to 2.3 and 2.3 for TLR3-/- and MyD88-/- mice, respectively (**Table 3.1**). For the anti-SVP antibody response, the titers of IgG1 produced by all three mouse strains were similar (**Fig. 3.6C**) resulting in a more comparable IgG2c/IgG1 ratio (**Table 3.1**). Neutralization titers were determined to assess potential differences in antibody function. The 50% neutralization titer of pooled serum from RepliVAX WN-immunized B6 mice was 1/ 1890 compared to 1/ 1124 for TLR3-/- mice and 1/ 982 for MyD88-/- mice. To compare the neutralizing activity of comparable amounts of antibody from each strain, pooled serum from B6 and TLR3-/- mice was diluted to achieve a SVP-specific titer equivalent to the MyD88-/- serum pool and the endpoint titers were confirmed by ELISA. As shown in Table 3.1, there was no significant difference in the neutralization activity of serum from the three mouse strains.

To assess antibody affinity, B6 and TLR3-/- serum pooled from 34-35 immunized mice was normalized by dilution to achieve an anti-SVP IgG titer equivalent to that of a MyD88-/- serum pool (N=34 mice). The endpoint titration curves of these serum samples are nearly super-imposable (**Fig 3.7A**) strongly suggesting equivalent affinities among the mouse strains. To assess antibody avidity, we determined the avidity index for serum

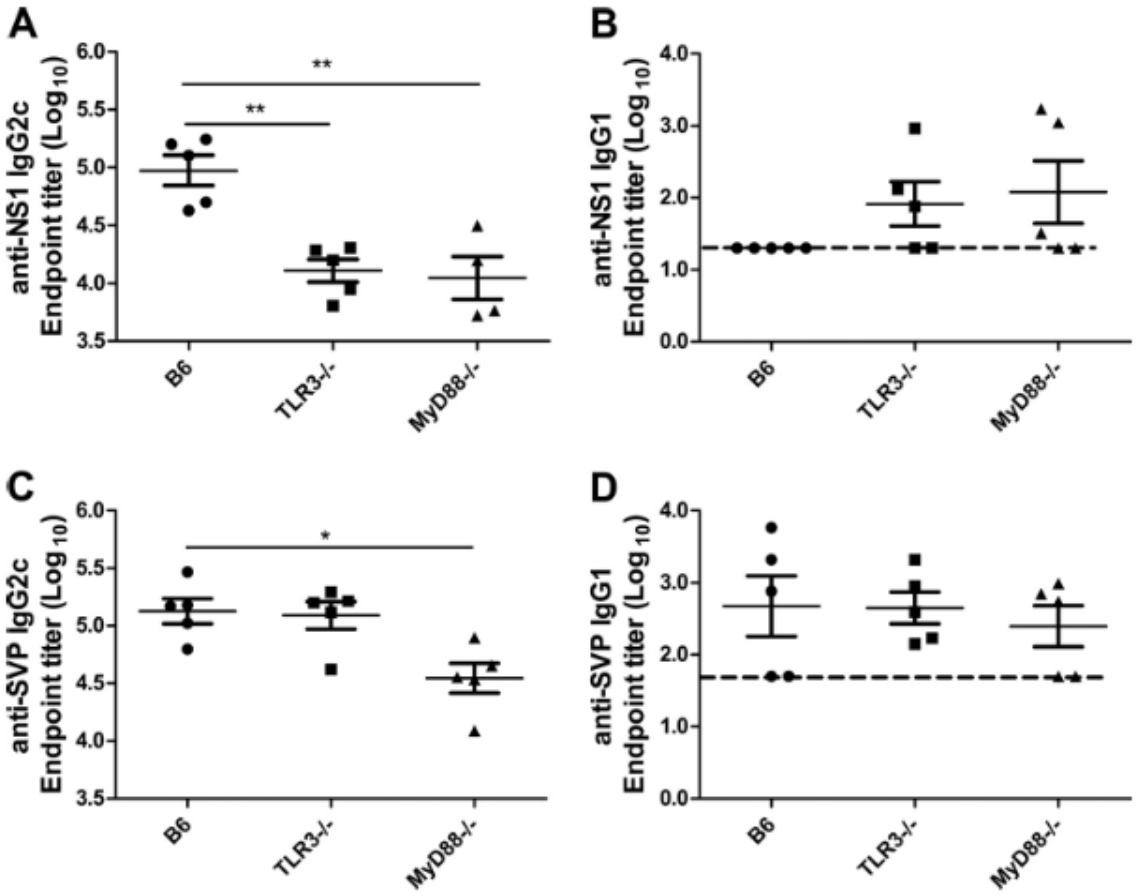
from individual mice of each strain using a urea-inhibition assay. The avidity index of anti-NS1 IgG antibodies was not different among the three mouse strains over a range of urea wash concentrations (**Fig 3.7B**). The index was significantly different ($p < 0.05$) between B6 and MyD88-/- mice for the SVP response on day 28 at the highest urea dose only, suggesting only minor differences in antibody avidity between the 2 strains (**Fig 3.7D**). As expected, the avidity index for the serum samples from each mouse strain increased between day 7 and day 28 as the NS1-specific (**Fig 3.7C**) and SVP-specific (**Fig 3.7D**) antibody responses matured. Together these data indicate that the affinities and avidities for NS1- and SVP-reactive antibodies were very similar among strains.

Figure 3.5: Altered GC cellularity, and WNV-specific ASC responses to RepliVAX WN in TLR3-/- and MyD88-/- mice.



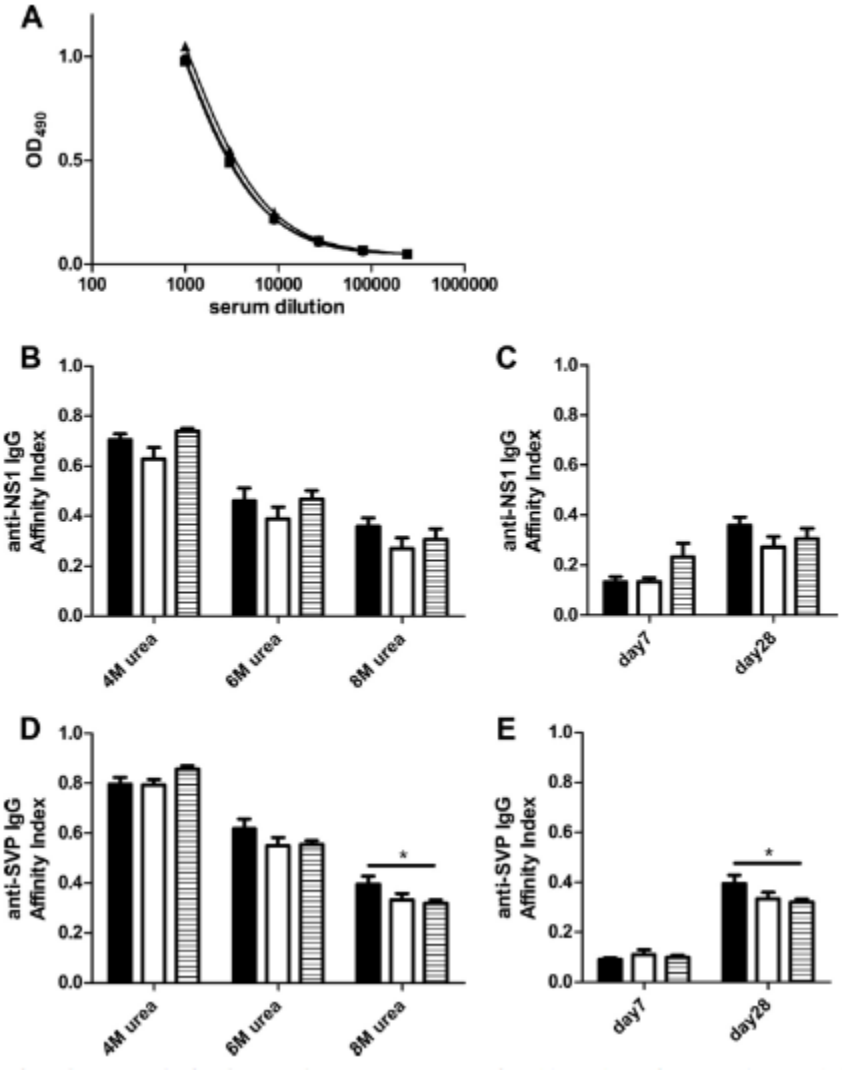
(A) Decreased cellularity of GC in RepliVAX WN-immunized MyD88-/- mice. B6 (n=5, filled bar), TLR3-/- (n=5, open bar) and MyD88-/- (n=5, striped bar) mice were immunized i.p. with 10^6 IU RepliVAX WN. Spleen cells were harvested and the number of GC B cells, measured as PNA^+ $CD19^+$ cells, was quantified by flow cytometry on 7, 14 and 21 dpi. Anti-NS1 (B) and anti-SVP (C) IgG ASCs from spleens of B6 (n=5, ●), TLR3-/- (n=5, ■) and MyD88-/- (n=5, ▲) mice immunized i.p. with 10^6 IU RepliVAX WN were quantified on 7, 14, 21, and 28 dpi. The results shown are from a representative experiment of 2 performed. Data are presented as the mean quantity of GC cells (A), or ASC number (B, C) ± SEM. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) compared to the same time point for B6 mice.

Figure 3.6: Decreased IgG2c and increased IgG1 expression by NS1-specific IgG antibodies on 28 dpi in B6, TLR3-/- and MyD88-/- RepliVAX WN-immunized mice.



Mice were immunized i.p. with 10^6 IU RepliVAX WN. The endpoint titer of anti-NS1 serum IgG2c antibody (A) and IgG1 antibody (B) and of anti-SVP serum IgG2c antibody (C) and IgG1 antibody (D) were calculated as described in Methods. Data are presented as the mean endpoint titer ± SEM. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) compared to the same time point for B6 mice.

Figure 3.7: Similar affinities and avidities for IgG antibodies from RepliVAX WN-immunized B6, TLR3-/-, and MyD88-/- mice.



(A) Day 28 pi sera was pooled from groups of RepliVAX WN-immunized B6 (●, n=35), TLR3-/- (■, n=35), and MyD88-/- (▲, n=34) mice and the endpoint titer of each serum pool calculated by ELISA. B6 and TLR3-/- serum pools were diluted to achieve a titer equivalent to the MyD88-/- serum pool. Serum titration curves of the normalized serum pools generated by SVP- ELISA, are shown. (B, D) Avidity indices of serum IgG from 28 dpi from individual B6 (filled bar, n=5), TLR3-/- (open bar, n=5), or MyD88-/- (striped bar, n=5) under the indicated urea wash conditions. (C, E) Avidity indices of serum IgG from day 7 and 28 dpi from B6 (filled bar, n=8), TLR3-/- (open bar, n=8), or MyD88-/- (striped bar, n=5) under 8M urea wash conditions. The results shown are from a representative experiment of 2 performed. (* $p<0.05$) compared to B6 mice.

Table 3.1: Properties of NS1- or SVP-specific serum IgG antibodies from B6, TLR3-/- and MyD88-/- mice immunized with RepliVAX WN.

	B6	TLR3$^{-/-}$	MyD88$^{-/-}$
IgG_{2c}/IgG_1 ratio NS1	3.6 ± 0.4[a]	2.3 ± 0.7	2.3 ± 0.9
IgG_{2c}/IgG_1 ratio SVP	2.1 ± 0.8	2.0 ± 0.4	2.0 ± 0.6
50% Neut. Titer[b]	2934 ± 776	1654 ± 598	4126± 1294

[a] IgG2c/IgG1 ratios were calculated for the individual mice shown in Fig. 3.7. The results shown represent the mean ± SEM for 5 mice.
[b] Serum pooled from groups of 34-35 RepliVAX WN-immunized B6, TLR3-/-, or MyD88-/- mice and normalized to equivalent ELISA titer. Results are expressed as the mean titer ± SEM of 5 separate experiments.

Reduced LLPC responses in RepliVAX WN-immunized TLR3-/- and MyD88-/- mice.

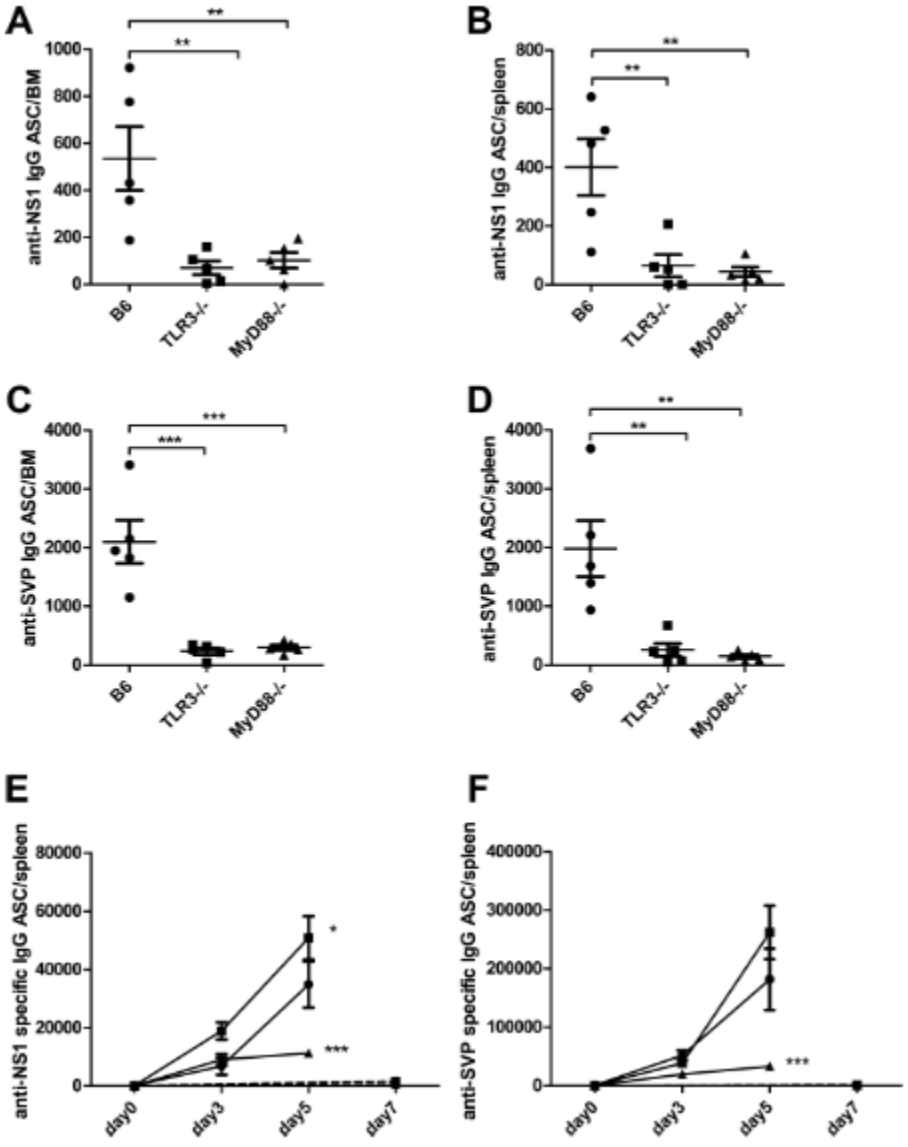
Persistent humoral protection involves constitutive antibody secretion by LLPCs located predominantly in the bone marrow and the generation of quiescent MBCs located primarily in secondary lymphoid tissues (McHeyzer-Williams, et al. 2011); (Good-Jacoboson and Tarlinton 2012). The generation of LLPCs and MBCs has been shown to be closely associated with the GC response (McHeyzer-Williams, et al. 2011); (Good-Jacoboson and Tarlinton 2012). Given the impact of TLR3- and MyD88-deficiency on the GC and ASC responses, we investigated whether B cell memory developed normally in TLR3-/- and MyD88-/- mice. We quantified antigen-specific LLPCs localized in spleens and bone marrow of TLR3-/- and MyD88-/- immunized mice on 56 dpi by ELISPOT. The absence of either TLR3- or MyD88-dependent signaling caused a significant reduction in the quantity of anti-NS1 and anti-SVP ASC in the bone marrow

(**Fig. 3.8A,C**) and spleen (**Fig. 3.8B,D**) suggesting that both TLR3- and MyD88-dependent signaling played a role in the generation of WNV-specific LLPCs.

Reduced MBC responses in RepliVAX WN-immunized MyD88-/- but not TLR3-/- mice.

Antigen-specific MBCs reside long-term primarily in secondary lymph organs (McHeyzer-Williams, et al. 2011). To test if TLR3- and MyD88-dependent signaling pathways also played an important role in the generation of MBCs, we evaluated secondary ASC responses after re-challenge of immunized mice with RepliVAX WN. Anti-SVP and anti-NS1 plasma cells were quantified by ELISPOT following the RepliVAX WN re-challenge. As expected, recall ASC responses in immunized B6 mice, indicative of MBCs, were more rapid and vigorous than primary B cell responses which generated only ~2000 anti-NS1 ASC and ~2000 anti-SVP ASC per spleen only on day 7 (**Fig. 3.8E, F**). By contrast, immunized B6 mice developed numerous specific ASC (~7,000 anti-NS1 ASC and ~50,000 anti-SVP ASC/ spleen) as early as day 3 after re-challenge. ASC numbers continued to expand quickly and reached ~35,000 anti-NS1 ASC and ~200,000 anti-SVP ASC/ spleen on day 5 after re-challenge. In contrast, in MyD88-/- mice, there was a significantly reduced number of anti-NS1 or anti-SVP ASC on both day 3 and 5 post-rechallenge ($p<0.001$, ANOVA). Compared with B6 mice, TLR3-/- mice showed a similar quantity of antigen-specific ASC following re-challenge, indicating that the lack of TLR3 signaling did not compromise development of WNV-specific MBCs.

Figure 3.8: Reduced MBC responses in RepliVAX WN-immunized MyD88-/- but not TLR3-/- mice.



The development of LLPCs and MBCs is differentially impaired in RepliVAX WN-immunized TLR3-/- and MyD88-/- mice. NS1-(A, B) and SVP-(C, D) specific LLPCs from bone marrow (A,C) and spleens (B,D) of B6 (●), TLR3-/- (■) and MyD88-/- (▲) mice (n=5 mice/group) were quantified by ELISPOT assay on 56 days after i.p. immunization with 10^6 IU RepliVAX WN. (E,F) The recall response to RepliVAX WN re-challenge by MBCs from B6, TLR3-/-, and MyD88-/- mice. RepliVAX WN immunized B6 (n=4), TLR3-/- (n=4) and MyD88-/- (n=4) mice were re-challenged i.v. with 10^7 IU RepliVAX WN. Anti-NS1 (E) and anti-SVP (F) IgG ASCs from B6, TLR3-/- and MyD88-/- mice were quantified by ELISPOT assay on the indicated days. WNV-specific ASCs in primary responses (dotted lines) following RepliVAX WN immunization of B6 (n=5), TLR3-/- (n=5) and MyD88-/- (n=5) on 7 dpi are also shown. Data are presented as the mean number of ASCs per spleen ± SEM. The results shown are from a representative experiment of 2 performed. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) compared to the same time point for B6 mice.

DISCUSSION.

WNV-specific antibody is an important component in immune protection against WNV disease as shown by WNV infection of B cell deficient mice and by passive transfer of WNV-specific IgM and IgG antibodies (Diamond, Sitati, et al. 2003); (Diamond, Pierson and Fremont 2008); (Hofmeister, et al. 2011); (Dunn, et al. 2010). Therefore, in addition to generating polyfunctional T cell responses, effective WNV vaccines will need to elicit vigorous and durable antibody responses to protect against WNV disease. We have previously shown that immunization with the candidate vaccine RepliVAX WN results in the development of strong antibody responses to the E glycoprotein and the nonstructural protein NS1 (Nelson, et al. 2010), which represent important targets for a protective antibody response (Diamond, Pierson and Fremont 2008). These previous studies also showed that signaling through the type I IFN receptor influences the nature of the antibody response (Winkelmann, et al. 2012). RepliVAX WN is intrinsically adjuvanted with ligands for several PRRs including RIG-I, TLR3, TLR7/8, and MDA-5. In these studies we focused on the roles of the TLRs and specifically on TLR3- and MyD88-dependent signaling on the development of the WNV-specific B cell response to the single cycle vaccine RepliVAX WN.

There are conflicting conclusions about the role of TLR3 signaling resulting from WNV infection. In a study by Daffis, et al (Daffis, et al. 2008), TLR3 played a protective role against WNV infection and TLR3-/- mice displayed higher virus titers in the brain and experienced increased mortality relative to wild type controls. By contrast, Wang, et al (Wang, Town, et al. 2004) concluded that TLR3 signaling lead to high lethality and facilitated neuroinvasion of virus following WNV infection. Studies with MyD88-deficient mice have clearly demonstrated a protective role for MyD88-dependent signaling in preventing WNV invasion of the central nervous system (Szretter, et al. 2010). WNV titers in the brain were significantly increased in MyD88-/- mice although titers in peripheral tissues remained generally very similar to wild type mice in the early stages of infection. Although these previous studies provide evidence for TLR3- and MyD88-dependent signaling pathways in protection, the increased susceptibility and high mortality to WNV infection by MyD88-/- and TLR3-/- mice at early times after infection (Daffis, et al. 2008); (Szretter, et al. 2010) complicates a comprehensive examination of the roles of these pathways in development of long-term adaptive immune responses to WNV. Our results using a SCFV vaccine demonstrated that the IgM ASC response was significantly lower at early times after immunization and that the serum IgG response to both SVP and NS1 antigens was significantly lower in RepliVAX WN-immunized TLR3-/- and MyD88-/- mice. Interestingly, studies by others showed that the early WNV-specific IgM and IgG responses to wild type WNV infection (day 6-10) were less affected by lack of MyD88 (Szretter, et al. 2010) or TLR3 (Daffis, et al. 2008) than was the response to the SCFV RepliVAX WN in the present study. While the single cycle particle RepliVAX WN initiates a WNV infection identical to wild-type virus and induces a vigorous anti-WNV humoral response (Nelson, et al. 2010), it is possible that the presence of high expression of PAMPs, increased antigen loads, and heightened inflammatory responses during infection with fully infectious WNV may provide redundant stimulatory signals to the developing B cell response rendering it less

dependent on specific TLR-signaling than is the response to the SCFV RepliVAX WN. While the roles of TLR3- and MyD88-dependent signaling in B cell responses to infectious WNV remain uncertain, the results of the present study clearly demonstrate the important role of intrinsic PAMPs in development of optimal antibody responses under the more modest inflammation and antigen availability conditions following immunization with a single-cycle vaccine. Further, these results demonstrate the dependence of B cell memory development on the intrinsic adjuvanting by RepliVAX WN-associated PAMPs.

The development of antibody responses can be divided into several steps including B cell activation, B cell expansion and affinity maturation in the GC, and the development of the B cell memory response. TLR3- and MyD88-dependent signaling pathways have been shown to be pivotal in the development of antiviral humoral responses (Guay, et al. 2007); (Hou, et al. 2011); (Delgado, et al. 2009). Injection of various TLR agonists has been shown to impact B cell activation and/or proliferation. For example, injection of TLR3 agonists, in synergy with BCR signaling and CD40L, aids in B cell activation and proliferation whereas TLR7 agonists promote development of antibody secreting cells (Boeglin, et al. 2011). Previous studies in virus infection models have shown that early B cell activation in draining lymph nodes as manifested by surface expression of CD69 and CD86 occurs within the first few days after infection, is polyclonal, and transient (Purtha, Chachu, et al. 2008); (Coro, Chang and Baumgarth 2006). Similarly, we observed a transient increase in the frequency and total number of $CD19^{+}$ B cells from the pLN of B6 mice that expressed increased CD69 and CD86 levels at three days after RepliVAX WN inoculation. In agreement with the findings of Purtha et al (Purtha, Chachu, et al. 2008) following WNV infection, the number of CD69 expressing B cells detected in RepliVAX WN inoculated MyD88-/- mice was moderately, but significantly, lower than that of wild type mice. Additionally, the mean fluorescence intensity of CD86 expression by MyD88-/- and TLR3-/- B cells was

significantly decreased on 3 dpi and the total number of $CD86^+$ B cells was significantly lower at 5 dpi in these strains. Consistent with the diminished activation phenotype of B cells in MyD88-/- and TLR3-/- mice, there was a significant reduction of anti-SVP IgM-secreting cells in the draining LN and spleens of these mice over the initial 5 dpi. The detection of IgM ASCs in these mice strains on 3 dpi in the current study compared to the detection at 7 dpi by Purtha, et al (Purtha, Chachu, et al. 2008) using a WNV infection model may reflect a higher assay detection sensitivity due to the higher availability of epitopes or different epitopes expressed by the WNV SVP capture antigen in our studies compared to recombinant E protein used in the previous studies (Purtha, Chachu, et al. 2008). Although the diminished serum IgG responses in MyD88-/- mice in response to high dose RepliVAX WN inoculation strongly suggest an overall defect in the B cell response, it is also possible that the early B cell activation events in MyD88-/- mice may have reflected a lower initial antigen load. This caveat does not hold for TLR3-/- mice as the IgM ASC response in TLR3-/- mice was significantly diminished although the antigen load appeared equivalent to B6 mice.

The GC is the site of B cell proliferation, isotype class-switch, affinity maturation, and differentiation of antigen-specific B cells into MBCs or LLPCs. In the present study using SCFV particles as a vaccine immunogen, the cellularity of the GC reaction was significantly impaired in MyD88-/- mice. The involvement of MyD88-dependent signaling in development or maintenance of the GC reaction is apparently pathogen-dependent. No defects in GC response development were observed following infection of MyD88-/- mice with influenza VLPs (Kang, et al. 2011), murine polyomavirus (Guay, et al. 2007), or Salmonella typhimurium (Neves, et al. 2010). However, our observations of reduced GC cellularity in RepliVAX WN-immunized MyD88-/- mice are consistent with those of others following immunization of MyD88-/- mice with inactivated RSV (Delgado, et al. 2009) or 2009 Pandemic Influenza split vaccine (Jeisy-Scott, et al. 2012), murine gamma herpesvirus 68 infection (Gargano, Moser and Speck 2008), or infection

with the murine gammaretrovirus, Friend virus (Browne 2011). As an additional assessment of GC development in TLR3-/- and MyD88-/- mice, we quantified WNV antigen-specific ASC responses during the initial 28 dpi. Consistent with the evidence of diminished GC cellularity in RepliVAX WN-immunized MyD88-/- mice, the number of antigen-specific IgG ASCs was consistently, significantly lower compared to B6 mice. These results are similar to those reported by Jeisy-Scott, (Jeisy-Scott, et al. 2012) following infection of MyD88-/- or TLR7-/- mice with influenza and suggest that MyD88- dependent signals are important early for development of the GC response and antigen-specific ASC response. Interestingly, in immunized TLR3-/- mice, although the total number of PNA^+ $CD19^+$ B cells in the GC was unaltered, the WNV-specific ASC response was initially equivalent to B6 mice but was not maintained and diminished thereafter to levels significantly lower than in wild type mice. These results are consistent with the notion that TLR3-dependent signals were necessary for optimal development or maintenance of the ongoing ASC response in the GC. In support of this idea, the presence of TLR7 ligand in bacteriophage QB VLP immunogens was shown to rescue the development of GC responses in IL-21-deficient mice indicating that TLR ligands can cooperate in association with other immune products to facilitate strong GC responses (Bessa, Kopf and Bachmann 2010).

The impact of the lack of TLR3- or MyD88-dependent signaling was manifested also in the development of B cell memory. Given the relatively weak GC response and ASC response of RepliVAX WN-immunized MyD88-/- mice, the detection of significantly diminished numbers of NS1- and SVP-specific LLPC in the bone marrow and spleen on 56 dpi in these mice was not surprising. Similarly, the recall IgG ASC response to intravenous high dose RepliVAX WN challenge, reflecting stimulation of memory B cells, was significantly smaller in MyD88-/- mice. Consistent with these results, diminished development of LLPC and MBC has been observed previously in

TLR7-/- and MyD88-/- mice following immunization with inactivated influenza vaccines (Jeisy-Scott, et al. 2012); (Kang, et al. 2011).

The role of TLR3 in development of antibody responses has not been studied as extensively. In the present studies, while diminished LLPC responses to NS1 and SVP were observed in spleen and bone marrow of immunized TLR3-deficient mice, the recall IgG ASC response by MBC was nearly equivalent in magnitude and kinetics to wild-type mice. This differential effect of TLR3-deficiency on development of LLPC and BMC may reflect the predisposition of antigen-specific B cells produced early in the GC response to differentiate to MBC whereas those B cells produced later in the GC differentiate primarily into LLPCs (Shlomchik and Weisel 2012). Such a model is consistent with the developed, then rapidly diminishing IgG ASC response we detected in the spleens of RepliVAX WN-immunized TLR3-/- mice in the present studies.

The skewing of IgG subclass profile has been reported previously in various infection and immunization models using MyD88-/- or TRIF-/- mice (Mineo, Oliveira, et al. 2010); (Kumar, Koyama, et al. 2008) and may reflect diminished class switch DNA recombination resulting from decreased type I IFN responses (Bekeredjian-Ding, et al. 2005); (Finkelman, et al. 1991); (Le Bon, Schiavoni, et al. 2001) or with decreased availability of synergistic TLR/ BCR signals (Pone, Zan, et al. 2010); (Pone, Xu, et al. 2012). In the present study, we detected decreased levels of serum anti-NS1 IgG2c and increased levels of anti-NS1 IgG1 antibodies in RepliVAX WN-immunized MyD88-/- and TLR3-/- mice. However, it is interesting that the IgG2c/IgG1 ratio was less affected for the SVP-specific response among mouse strains. The reason for this difference is unclear but may reflect differences in the epitope density or bioavailability between SVP and NS1 antigens *in vivo*. In this light, it is possible that enhanced BCR signaling due to the multivalent nature of SVP particles (Minquet, Dopfer and Schamel 2010) may require less TLR-dependent signaling to drive switch of antibodies from the IgG1 subclass whereas class switching of B cells in response to the less complex antigen NS1 (Winkler,

et al. 1988); (Crooks, et al. 1994) may be more dependent on TLR-signaling. Serum antibody from all mouse strains demonstrated virus neutralization activity and the affinities and avidities of the serum IgG antibodies appeared very similar among strains. While perhaps less important for the ability of antibody to neutralize virus, the expression of appropriate IgG subclasses is important for protection manifested through complement-dependent or Fcγ Receptor-dependent mechanisms known to play a role in protection against WNV (Chung, Thompson, et al. 2007); (Vogt, Dowd, et al. 2011).

Taken together, the results of the current studies indicate that both TLR3- and MyD88-dependent signaling play important roles in shaping the development of humoral responses to the single cycle vaccine, RepliVAX WN. MyD88-dependent signaling affects the development of humoral responses by impacting B cell activation, development of the GC reaction and the generation of LLPCs and MBCs whereas GC responses develop, but are not maintained, in the absence of TLR3-signaling which ultimately reduces differentiation of WNV-specific B cells into LLPCs. The results of this study enhance our understanding of the link between specific TLR pathways and developing B cell response. Specifically, they have important implications for the role of individual PAMPs on intrinsic adjuvanting of single-cycle vaccines as it relates to development of vaccine-specific B cell memory. This understanding will be important for the rational development of new vaccines as well as for improving the efficacy of existing vaccines.

Chapter 4. Discussion and future direction.

The present work on the mechanisms of RepliVAX WN immunogenicity showed that this intrinsically adjuvanted vaccine candidate elicited a vigorous adaptive immune response and memory immunity at least in part via TLR3- and MyD88-dependent

signaling. However, these two signaling pathways modulate adaptive immune responses in different ways.

The role of TLR3- and MyD88-dependent signaling in modulating the reactivation of memory T and B cells.

The present study mainly focused on how TLR3 and MyD88-dependent signaling modulate primary adaptive immune responses. However, TLR3/Trif and TLR7/MyD88 pathways are both stimulated at the surface of cell membrane or endosome. No study has investigated the role of the signaling pathways activated by cytosolic innate sensors (e.g. RIG-I and MDA-5) in the development of RepliVAX WN-induced adaptive immune responses. Therefore, in the future, the development of anti-WNV T and B cell response should be studied in RepliVAX WN-immunized RIG-I-/- or MDA-5-/- mice. The viral gene expression, the quantity of IFN-γ secreting $CD4^+$ and $CD8^+$ T cells, and anti-NS1 and anti-SVP plasma cells in RIG-I-/- and MDA-5-/- mice through the primary response would be detected and compared with that in B6 mice. Anti-WNV LLPCs, MBCs, memory $CD4^+$ and $CD8^+$ T cells would be quantified and the protection by T and B cell memory immune responses against WNV challenge in RIG-I-/- and MDA-5-/- immunized mice would be compared with that in B6 mice. Meanwhile, the subclass switch, affinity, and neutralizing capacity of anti-WNV IgG and the functional pattern of anti-WNV $CD4^+$ and $CD8^+$ T cells in the three strains of mouse would be detected and compared. Additionally, although not dramatically impaired, the affinity of anti-SVP antibody in TLR3-/- mice was lower than that in B6 mice. Therefore, BCR would be sequenced during B cell responses development (especially during GC reaction) and the mutation frequency, mutation loci and CDR length in KO mice and B6 mice would be studied and compared.

In the present work, we have studied the role of TLR3- and MyD88-dependent signaling in the recall T and B cell response. However, more studies are also required to

fully address the nature of memory T and B cells and the reactivation of memory responses following RepliVAX WN immunization. In the present study, reinoculation experiments were performed to indirectly show the decreased quantity of memory cells in TLR3-/- or MyD88-/- mice. To study how TLR3- and MyD88-dependent signaling modulate the reactivation of WNV-specific memory cells is important to show whether immunocompetent immunized hosts utilize TLR3- or MyD88-dependent signaling to generate protective memory T and B cell responses against WNV infection. Additionally, addressing and comparing the role of TLR3- and MyD88-dependent signaling in activating memory cells help to understand how to optimize memory responses following the immunization strategies with multiple immunizations.

Since we have compared the quantity of memory B cells by indirectly quantifying the plasma cells following re-inoculation, another approach would be used to confirm the result by stimulating the purified $CD19^+$ B cells with antigen and CD40L and quantifying MBCs via *in vitro* limiting dilution assay. The culture supernatant would be harvested to test the affinity and neutralizing capacity of MBC secreted antibodies. The result (the fold change of reactivated MBCs, shown as antibody-secreting cells, in TLR3-/- and MyD88-/- mice) of the *in vitro* limiting dilution assay would be compared with the result from the *in vivo* reinoculation experiment to confirm the quantity of MBCs. The affinity and neutralizing capacity of WNV-specific antibodies in the serum from the 3 mouse strains would be tested. To further identify the quantity of LLPCs and MBCs developed in the recall response (or following the rechallenge with wild type WNV) in the 3 mouse strains, ELISPOT and *in vitro* limiting dilution assay would be utilized at the endpoint of secondary responses.

In the present work, the expansion of WNV-specific $CD8^+$ and $CD4^+$ memory T cells and the effector functional of $NS4B_{2488\text{-}2496}$-specific memory T cells were characterized. In future studies, the functional pattern of $NS3_{1616\text{-}1630}$- and $E_{641\text{-}655}$-specific $CD4^+$ T cells and $E_{347\text{-}354}$-specific $CD8^+$ would be characterized. To study the cytotoxic

function of memory T cells, an *in vivo* CTL assay would be performed in which the lysis of WNV- or RepliVAX WN-pulsed target cells which are transferred into the immunized 3 strains of mice would be tested.

Our studies examined the recall response to the SCFV, RepliVAX WN. The recall response to replicable WNV would likely provide additional information about the roles of these PRR signaling pathways. Immunized TLR3-/- and MyD88-/- mice would be rechallenged with wild type WNV. The viral gene expression, the recruitment of APCs, the disease score or survival rate would be compared with rechallenged B6 mice. WNV infected $CD11c^+$ DCs have been shown to reactivate WNV-specific $CD4^+$ and $CD8^+$ memory T cells via IL-1R-mediated signaling. (Durrant, Robinette and Klein 2013) We also showed that $CD11c^+$ DCs modulate T cell responses by TLR3- and MyD88-dependent signaling. Therefore, it is possible that immunocompromised (TLR3 or MyD88 deficient) WNV-infected $CD11c^+$ DCs fail to reactivate vigorous memory responses. To further study the involvement of $CD11c^+$ DCs intrinsic TLR3- and MyD88-dependent signaling in reactivating the memory response, WNV- or RepliVAX WN infected $CD11c^+$ DCs from the 3 mouse strains would be adoptive transferred into immunized B6 mouse, the quantity and function of activated WNV-specific memory T cells would be identified. Meanwhile, the migration of $CD11c^+$ DCs from the 3 mouse strains to the loci where memory cells are localized would also be compared.

The immunomodulatory role of TLR3- and MyD88-dependent signaling in different cell types.

The entire list of cell types (e.g. DCs, T cells, B cells) in which TLR3- and MyD88-dependent signaling acts to modulate the development of adaptive immune responses is not completely clear. To further identify the responsibility of TLR3- and MyD88-dependent signaling in different cell types, conditional depletion transgenic mice could be generated and immunized with RepliVAX WN and adaptive immune response

would be recorded. The conditional depletion transgenic mice are generated with the purpose of knocking out a gene from a specific cell type while gene function is maintained in other types of cells as normal. The transgenic mice are generated via site-specific recombinase mediated Cre-Lox recombination. Cre recombinase specifically combines a pair of short target Lox sequences and deletes the internal gene. The conditional depletion is achieved by crossing two transgenic mouse strains. One is LoxP-flanked mouse strain which has the loxP sequence inserted at both sides of the target chromosomal DNA sequence. In the other strain, Cre recombinase DNA sequence is inserted in the transcriptional region of a certain cell marker gene and thus only the cells with this marker express Cre recombinase. Therefore, by crossing the two strains, we could achieve Cre-induced depletion of target gene from the certain cell type in offsprings. For example, if we aim to deplete MyD88 gene from $CD11c^+$ cells, LoxP sequence will be flanked on both sides of the MyD88 gene to generate $MyD88^{fl/fl}$ transgenic mice. The Cre sequence will be inserted in CD11c gene transcriptional region to create Cd11c-Cre mice. By crossing $MyD88^{fl/fl}$ mice with Cd11c-Cre mice, in F1 generation the conditional depleted transgenic mice $MyD88^{fl/fl}$Cd11c-Cre mice will be bred.

In chapter 2, TLR3- and MyD88-dependent signaling in $CD11c^+$ DCs was found to be important in directing the proliferation and multifunctionality of anti-WNV $CD8^+$ T cells *in vitro*. However, whether $CD11c^+$ DC intrinsic TLR3- and MyD88-dependent signaling play the same role *in vivo* and whether the signaling pathways in other cell types ($CD4^+$ T cells, $CD8^+$ T cells) are also involved in modulating adaptive immune response development is still poorly understood. Moreover, in chapter 3, it is still unknown which type of cells utilize TLR3- and MyD88-dependent signaling modulate B cell response development. In this way, the transgenic mice with a conditional depletion of TLR3 or MyD88 from $CD11c^+$ cells, $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells

could be immunized with the same dosage of RepliVAX WN and the development of adaptive immune responses could be recorded.

The role of TLR3- and MyD88-dependent signaling in aged mouse model.

A symptomatic WNV infection of humans is most likely to occur in immunocompromised or aged populations. However, the aged immune system responds differently compared to young adults and requires a specialized model to allow relevant studies to be performed. One study showed that multiple immunizations with RepliVAX WN in aged mice (18-22 months old) induce a protective immune response against WNV. (Uhrlaub, et al. 2011) However, we found that the kinetics of T and B cell responses in TLR3-/- and MyD88-/- mice (11~12 months old) showed no significant impairment compared with age-matched WT mice following a single dose immunization with RepliVAX WN.(data not shown) This result suggests that a more complex and different immunogenic mechanism following RepliVAX WN immunization may occur in more aged mouse model. Therefore, we would questioned whether TLR3- and MyD88-dependent signaling are also differentially required by the aged population for RepliVAX WN-induced immune protection. The study in this model could be initiated by detecting the viral replication, innate recognition of RepliVAX WN and innate immune responses in aged mice. This may include the study of the cellular tropisms, viral particle uptake and the pattern of innate responses. After determining the pattern of innate immune responses, antigen load, APC maturation and the recruitment of innate immune cells, more research on T and B cell responses would be performed. Immunization with RepliVAX WN multiple times would also be applied to study the role of TLR3- and MyD88-dependent signaling in the aged immunized model.

Appendices.

Appendix A Virus-specific memory at peripheral sites of Herpes Simplex type 2 (HSV-2) infection in guinea pigs.[2]

In Chapter 2 and 3, I focus on the role of innate PRR signaling (TLR3- and MyD88-dependent signaling) in the development of T and B cell responses in lymphoid tissues. This immune response is mainly responsible for developing central memory immunity. As another important branch, resident memory immunity in peripheral tissues is newly discovered and requires more investigation. Therefore, besides my dissertation work, I also investigated the nature of resident memory T and B cell immunity in the peripheral tissues (genital tract and sensory ganglia) which is shown in this appendix section.

INTRODUCTION.

HSV-2 infection is widespread globally with an estimated 23.6 million new infections occurring each year (Looker, Garnett and Schmid 2008). Although disease associated with HSV-2 infection is often limited, more severe manifestations unfortunately also occur. HSV-2 present in the birth canal of infected mothers may be passed to neonates during vaginal delivery resulting in serious morbidity and mortality (Brown, et al. 1987); (Whitley, et al. 1991). Vigorous cell-mediated responses are normally responsible for diminishing the severity and duration of HSV-2 disease and unfortunately immune compromised individuals are more likely to experience severe complications resulting from HSV-2 infection (Whitley and Lakeman 1995); (Hull, et al. 1984). HSV-2 infection also has been shown to increase the risk of HIV infection and

[2] This appendix section adapted the content from the article published in PLoS One. PLOS requires no permission from the author (Jingya Xia) to reuse the article. The cited article is Xia J, Veselenak RL, Gorder SR, Bourne N, Milligan GN. Virus-Specific Immune Memory at Peripheral Sites of Herpes Simplex Virus Type 2 (HSV-2) Infection in Guinea Pigs. PLoS One. 2014. 9(12):e114652. doi: 10.1371/journal.pone.0114652.

increased HIV shedding is often observed during an active HSV-2 infection (Holmberg, Stewart and Gerber 1988); (Wald and Link 2002).

HSV-2 has co-evolved with humans and is an extremely successful pathogen, capable of residing long-term in its host and ready transmission to uninfected individuals. Genital HSV-2 infection of the epithelia ultimately results in lifelong latent infection of the innervating sensory ganglia and spinal cord (Cook and Stevens 1973); (Koelle and Corey 2003); (Ohashi, et al. 2011). Once thought to reactivate only occasionally from latency, it is now generally held that reactivation events for most individuals are quite frequent (Schiffer, Wald, et al. 2011) and result in virus shedding often in the absence of overt clinical symptoms. Further, clinical evidence suggests that the period of virus shedding following reactivation is most often of relatively short duration (Mark, et al. 2008); (Schiffer, Abu-Raddad, et al. 2009) due perhaps to the clearance of virus by HSV-specific T cells residing at the site of previously infected skin (Zhu, Koelle, et al. 2007); (Peng, Zhu, et al. 2012). Similar populations of HSV-specific $CD4^+$ and $CD8^+$ T cells have been found in latently infected trigeminal ganglia of humans (Verjans, et al. 2007); (Van Velzen, et al. 2013); (Held, et al. 2012) and in mice following ocular HSV-1 infection (Liu, Tang and Hendricks 1996); (Divito, Cherpes and Hendricks 2006). However, the long term presence and immune function of virus-specific T cells in neural tissues following genital HSV-2 infection has received much less study and less information is available. Sacral ganglia-resident memory cell populations are not currently amenable to study in humans.

Infection of mice with fully virulent HSV-2 commonly results in encephalitis and death, precluding easy analysis of the magnitude, phenotype and function of virus specific ganglia- and spinal cord-resident memory T cells in this animal model. The guinea pig model of genital HSV-2 infection represents a unique system to address the nature of both genital-resident and neural tissue-resident immune memory. Genital infection of guinea pigs results in a self-limiting vulvovaginitis with neurologic manifestations mirroring

those found in human disease. Virus is transported by retrograde transport to cell bodies in the sensory ganglia and autonomic neurons in spinal cords (Ohashi, et al. 2011). During this phase of infection, the virus also establishes a latent infection and, similar to humans, the animals undergo spontaneous, intermittent reactivation of virus. HSV-2 recurrences may manifest as clinically apparent disease with erythematous and/or vesicular lesions on the perineum or as asymptomatic recurrences characterized by shedding of virus from the genital tract.

Candidate prophylactic vaccines against HSV-2 have been capable of inducing high levels of systemic, HSV-specific immune responses but have failed to prevent HSV-2 infection or HSV disease (Corey, et al. 1999); (Stanberry, Spruance and Cunningham 2002). A different vaccine approach is needed to impact HSV-2 disease, perhaps involving development of strong local immune responses to HSV-2 infection. Tissue resident memory immune cells are strategically located to protect against re-infection or interfere with HSV-2 shedding following release of virus after reactivation from latency. The potential for protection conferred by these tissue-resident cell populations has profound implications for HSV-2 vaccine strategies, but the nature of tissue-resident memory immune cells in neural tissues following genital HSV-2 infection, the distribution of immune B and T cells within the reproductive tract, the nature of tissue-resident HSV-specific ASCs and the functional activity of locally-produced antibody is not well understood. We utilized a genital HSV-2 infection of guinea pigs to address these issues and demonstrate the utility of this approach in assessing the nature of tissue-resident immune cell populations in an animal model that most effectively recapitulates human HSV-2 infection.

MATERIALS AND METHODS.

Virus. HSV-2 strain MS stocks were prepared on Vero cell monolayers and stored at -80°C as described previously (Bourne, et al. 1999). The replication-defective strain HSV-2 dl 5-29 deleted of the HSV DNA replication protein genes UL5 and UL29

and the complementary cell line V529 expressing the UL5 and UL29 proteins (Da Costa, et al. 2000) were a kind gift of Dr. David Knipe (Harvard Medical School, Boston, MA). Virus stocks were prepared by infection of V529 cells with HSV-2 dl5-29 at an MOI of 10. Following development of cytopathic effect (typically 24 hours), cells were subjected to three cycles of freeze-thaw, cell debris pelleted by centrifugation, and the supernatant was aliquoted and frozen at -80°C.

Guinea pigs. Female Hartley guinea pigs were purchased from Charles River (Burlington, MA) and Strain 13 guinea pigs were obtained from Dr. Marisa St. Claire, National Institutes of Allergy and Infectious Disease, NIH. Female guinea pigs weighing 275 to 300 g were infected by intravaginal (ivag) inoculation with 200 ml of a suspension containing 10^6 PFU of HSV-2 strain MS as described previously (Bourne, et al. 1999). Animals were observed daily and primary disease severity and frequency of spontaneous recurrent disease were scored daily as described previously (Valencia, Veselenak and Bourne 2013).

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Guinea pigs were maintained under specific pathogen free conditions and were supplied with food and water ad libitum at the Association for Assessment and Accreditation of Laboratory Animal Care-approved animal research center of the University of Texas Medical Branch. All animal research was humanely conducted and approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch with oversight of staff veterinarians.

Lymphocyte Isolation from genital tracts and neuronal tissues. Genital tract and neuronal tissues (spinal cord and sensory ganglia) were collected from guinea pigs and cut into fine pieces. Genital tract tissue was successively digested with DNase I and Dispase II (Roche Diagnostics, Mannheim, German) for 45 min followed by Collagenase (Roche Diagnostics), Hyaluronidase (Sigma-Aldrich, Inc. St. Louis, MO) and DNase I

digestion for 60 min at 37°C. Neuronal tissues were digested with Liberase (Roche Diagnostics) and DNase I for 45 min at 37°C. Lymphocytes from genital tract and neuronal tissues were isolated by centrifugation over OptiPrep Density Gradients and Percoll Gradients, respectively.

Polyclonal stimulation of B cells. Enriched lymphocyte populations from the spleen, bone marrow, lower genital tract (vagina and cervix), and neuronal tissues (spinal cord, sensory ganglia) were cultured at $4x10^6$ cells/mL in T cell medium alone or with 4.0 μg/mL LPS and 1.0 μg/mL CpG for 72 hrs. Preliminary studies indicated maximum recovery of antibody secreting cells at 72 h of culture. HSV-specific ASCs were quantified on HSV-glycoprotein-coated ELISPOT plates.

Titration of anti-HSV-2 immunoglobulins. HSV-specific IgA, IgG, IgG1 and IgG2 titers from immune serum or B cell culture supernatants were obtained by ELISA as described previously (Milligan and Bernstein 1995). Plate-bound immunoglobulins were detected by addition of polyclonal goat anti-guinea pig IgG (#A60-110A) or polyclonal rabbit anti-guinea pig IgA (#A60-105) (Bethyl Laboratories, Inc., Montgomery, TX) followed by polyclonal HRP-rabbit anti- goat IgG (#A50-100P) or polyclonal HRP-goat anti-rabbit IgG (#A120-101P; Bethyl laboratories, Inc., Montgomery, TX), respectively. IgG1 and IgG2 titers were detected by incubation of plate bound HSV-specific antibodies with polyclonal biotinylated goat anti-guinea pig IgG1 (#ABIN457757) or polyclonal biotinylated goat anti-guinea pig IgG2 (#ABIN457760; antibodies-online Inc., Atlanta, GA) followed by streptavidin peroxidase (Sigma-Aldrich, St. Louis, MO). Normalized optical density readings at 490 nm (OD490) obtained from serial dilution of serum or culture supernatants were analyzed by non-linear regression. The end point titer was defined as the serum dilution resulting in an OD_{490} value equivalent to three standard deviations above OD_{490} values from naive sera or medium only. For determination of glycoprotein specificity, ELISA plates were coated with HSV-2 recombinant

glycoprotein D (rgD2) and recombinant glycoprotein G (rgG2) purchased from Meridian Life Science, Inc., Memphis, TN.

Neutralization Assay. Neutralizing antibody titers from serum or B cell culture supernatant were determined by a modification of the technique described previously (Milligan, Bernstein and Bourne 1998). Neutralizing antibody in supernatants was also demonstrated by adding a series of two-fold dilutions of virus to undiluted ASC supernatant containing Low Tox M rabbit C (Accurate Chemical and Scientific, Westbury, NY) at a final dilution of 1/15. Following incubation at 37 ˚C for one hour, HSV-2 in each sample was quantified by plaque assay on Vero cell monolayers. Vero cells (American Type Culture Collection number CCL-81) were obtained originally from the laboratory of Dr. Lawrence Stanberry, Columbia University School of Medicine, New York, NY).

Enzyme-linked immunospot assay (ELISPOT). Assays to quantify HSV-specific ASCs were performed as described previously (Milligan and Bernstein 1995) on HSV-glycoprotein-coated plates. To quantify HSV-specific T cells, lymphocytes isolated from spleen, genital tract, or neuronal tissues of uninfected or HSV-2 infected guinea pigs were stimulated by 48 h culture with splenocyte- or mesenteric lymph node cell-antigen presenting cells infected with HSV-2 dl5-59 and pulsed with UV-killed HSV-2 to ensure that both MHC class I and class II antigen presenting pathways were engaged to stimulate both virus-specific $CD8^+$ and $CD4^+$ T cells. Lymphocytes were treated with medium as a control. Cultures were incubated in the presence of recombinant human IL-2 (eBioscience, San Diego, CA) on anti-IFN-γ antibody-coated plates. HSV-specific IFN-γ secreting T cells were detected using monoclonal antibody V-E4 as the capture antibody and biotinylated anti-guinea pig IFN-γ antibody N-G3 as the detection antibody (Schafer, et al. 2007) (both antibodies a kind gift of Dr. Hubert Schäfer, Robert Koch Institute, Berlin, Germany). Spots were visualized by incubation with streptavidin peroxidase and AEC (3-Amino-9-ethylcabazole) substrate (Sigma-Aldrich, St. Louis, MO). HSV-

specific ASCs and IFN-γ secreting cells were quantified using an ImmunoSpot reader and analyzed with ImmunoSpot software (Cellular Technology Ltd, Cleveland, OH).

Enrichment of $CD4^+$ and $CD8^+$ cells. Single cell suspensions of genital tract lymphocytes and neural tissue lymphocytes were blocked with anti-Fc RII/III mAb (#553142; BD Biosciences, San Jose, CA) and surface stained with monoclonal mouse anti-guinea pig CD8- fluorescein isothiocyanate (FITC; #MCA752F; clone CT6) or monoclonal mouse anti-guinea pig CD4- phycoerythrin (PE; #MCA749PE; clone CT7; AbD Serotec, Oxford, UK), labeled with anti-FITC or anti-PE microbeads, respectively, (Miltenyi Biotec, Bergisch Gladbach, Germany) and applied to a magnetic column (Miltenyi Biotec) according to the manufacturer's protocol. The phenotype of column-bound and column-flow through populations was assessed by flow cytometry. Data were acquired on a BD FACSCanto II (BD Biosciences) at the UTMB Flow cytometry Core Facility and analyzed using FlowJo software (Tree Star, Ashland, OR). Column enrichment of $CD4^+$ T cells commonly resulted in greater than 90% $CD4^+$ cells (0.64% $CD8^+$ cells). Column enrichment of FITC-labeled $CD8^+$ T cells was less efficient (typically 12–29% of collected cells were $CD8^+$) although less than 0.5% of obtained cells were $CD4^+$.

Statistical analysis. Statistical differences for B lymphocyte assays, T lymphocyte assays, and antibody titers were determined using Student's t test. Differences in cell frequency were determined using the Chi square test. Values for $p<0.05$ were considered significant. All calculations were performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA).

RESULTS.

Tissue Location of HSV-specific Antibody Secreting Cells (ASC)

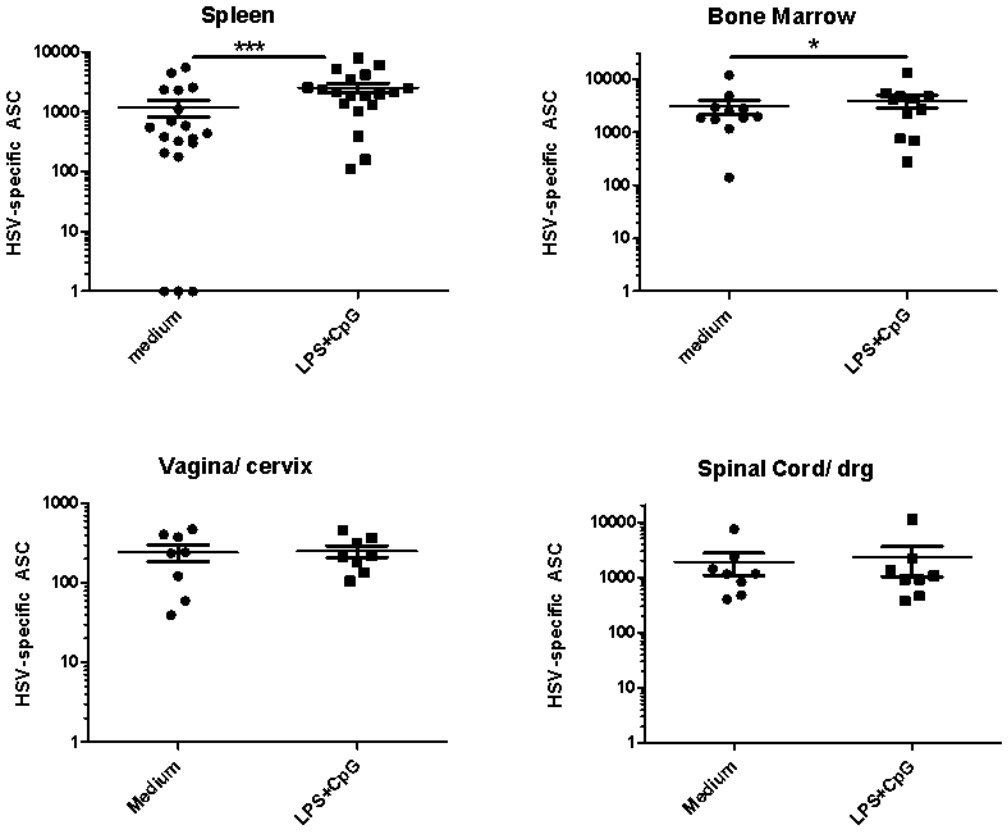
We previously demonstrated that HSV-specific ASC were detected for long periods of time in the sensory ganglia site of HSV latency and lower female genital tract

site of virus shedding in guinea pigs infected intravaginally with HSV-2 (Milligan, Meador, et al. 2005). We hypothesized that these ASC represented long-lived plasma cells existing in survival niches at these sites of chronic inflammation although an alternative explanation is that these ASC derived from tissue-resident memory B cells as a result of periodic exposure to HSV antigens released during reactivation events. Memory B cells, but not plasma cells, become activated and differentiate into ASC upon stimulation with polyclonal activating agents such as TLR-agonists (Poeck, et al. 2004); (Walsh, et al. 2013). The presence of memory B cells in the culture can therefore be detected as a TLR-agonist-induced increase in the number of antigen-specific, ASCs. To test for the presence of memory B cells at peripheral tissues, we stimulated lymphocyte populations isolated from bone marrow, spleen, vagina/cervix, or spinal cord/ sensory ganglia with a combination of LPS and CpG oligonucleotides or medium alone as a control and quantified HSV-specific ASC by ELISPOT. Hartley guinea pigs were infected with HSV-2 strain MS. Fourteen of the 19 animals used for assessment of HSV-specific ASC were scored for both primary and recurrent HSV-2 disease to document HSV disease. Primary HSV-2 disease was observed in 12 of 14 animals with a mean disease score of 5.4±1.1 and at least one recurrent lesion was detected in 12 of 14 infected animals between days 15 PI to the day of euthanasia resulting in a mean recurrent disease score of 1.2±0.26. Tissues were harvested from individual animals between days 49–70 after infection. Less than five HSV-specific ASCs were detected in all tissues from uninfected control animals. However, as shown in **Figure 4.1**, HSV-specific ASC were detected in all tissues following control treatment. Significantly greater numbers of HSV-specific ASC were detected in LPS/CpG-stimulated splenocyte cultures compared to medium-stimulated cultures ($p<0.0001$, $p< 0.05$ respectively, Student t test) indicating the presence of HSV-specific memory B cells. Additionally, a smaller but still significant increase in ASC number was also detected in LPS/CpG-stimulated bone marrow cells compared to stimulation with medium alone ($P<0.05$,

Student's t test). In contrast, there was no difference in HSV-specific ASC number following medium- or LPS/CpG-stimulation of lymphocytes isolated from the vagina/cervix or spinal cord/sensory ganglia. Therefore, HSV-specific memory B cells were readily detectable in the spleen and to a lesser extent in the bone marrow of HSV-2 infected guinea pigs but not at the peripheral sites of HSV-2 infection. These results suggest that the vast majority of HSV-specific ASC present in the lower genital tract, lumbosacral ganglia and adjacent spinal cord on days 49-70 post infection represented a persisting population of plasma cells.

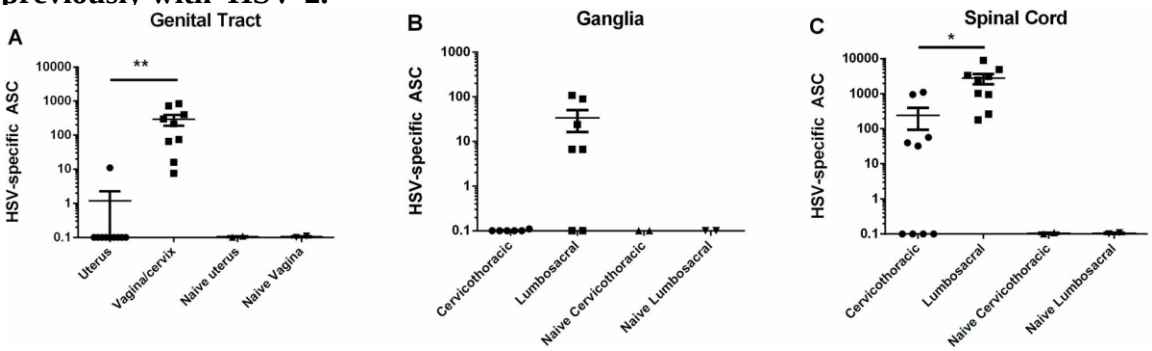
To more precisely define the tissue distribution of HSV-specific ASC, lymphocytes from uterus, vagina/cervix, cervicothoracic sensory ganglia, cervicothoracic spinal cord, lumbosacral sensory ganglia and the adjacent spinal cord were isolated from individual animals between 49–70 days post infection and HSV-specific ASC quantified by ELISPOT. As shown in **Figure 4.2**, HSV-specific ASC were detected in lymphocyte populations isolated from tissues of infected- but not uninfected-guinea pigs. The presence of genital tract-resident, HSV-specific ASC was limited mainly to the vagina and cervix as only a very low number of HSV- specific ASCs were isolated from the uterus of a single infected animal (**Figure 4.2A**). HSV-2 genomes were detected at this time period in the vagina/cervix tissue from two of four infected animals which most likely represented HSV-2 shedding; however, no virus genomes were detected in the uterus. Similarly, HSV-specific ASCs were detected in the lumbosacral ganglia innervating the genital tract of five of seven animals, but not in the cervicothoracic ganglia of infected guinea pigs (**Figure 4.2B**). HSV-specific ASCs were detected in spinal cord tissue isolated from the cervicothoracic region in five of ten infected animals whereas significantly higher numbers of HSV-specific ASC were detected in the lumbosacral region of the spinal cord in nine of ten infected animals ($P<0.05$, Student's t test, **Figure 4.2C**).

Figure 4.1: HSV-specific ASC residing long-term in the female genital tract, lumbosacral ganglia and adjacent spinal cord are predominantly plasma cells.



Hartley guinea pigs were infected ivag with HSV-2 strain MS. Lymphocytes from the spleen, bone marrow, vagina/cervix, and lumbosacral ganglia and adjacent spinal cord of individual animals were harvested between days 49–70 after infection and stimulated with medium or LPS/CpG and HSV-specific ASC quantified by ELISPOT. Each data point represents results from an individual animal. Results shown include tissues from three experiments for spleen and bone marrow and two experiments for vagina/cervix and spinal cord/ganglia. HSV-specific ASCs from tissues of uninfected animals were always less than five ASC/tissue. (* $P<0.05$; *** $P<0.0001$, Student's t test).

Figure 4.2: Location of HSV-specific, tissue-resident ASCs in guinea pigs infected previously with HSV-2.



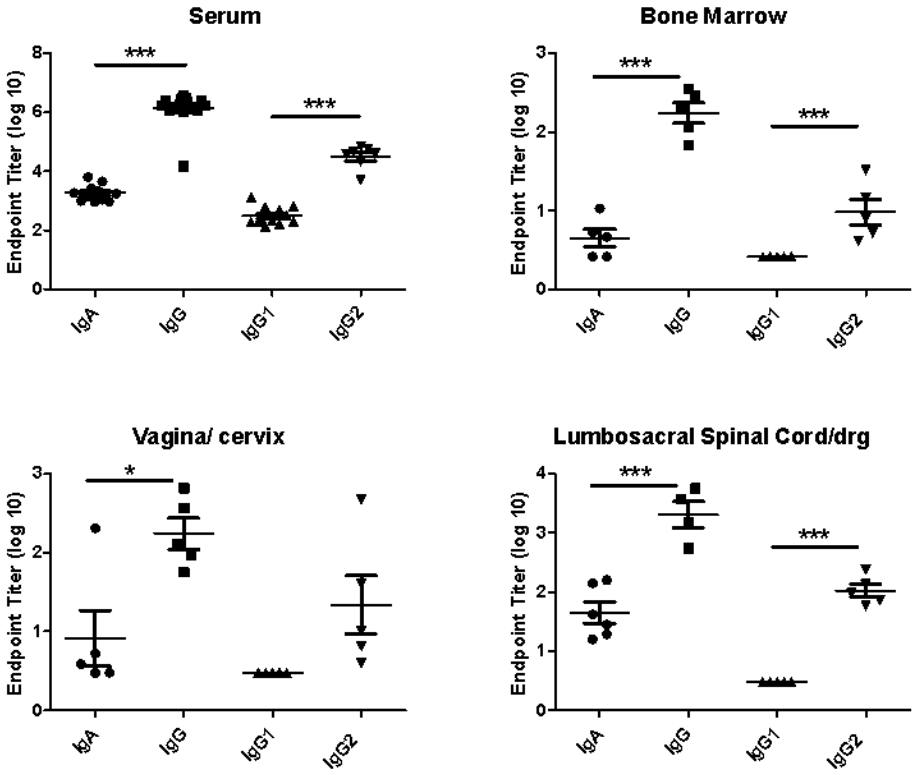
Hartley guinea pigs were infected ivag with HSV-2 strain MS and lymphocytes isolated from the indicated tissues on days 48–57 post infection. HSV-specific ASCs were quantified by ELISPOT on HSV-2 glycoprotein-coated plates. Each data point represents results from an individual animal. (* $P<0.05$; ** $P<0.01$, Student's t test).

Characteristics of HSV-specific antibody secreted by ASC isolated from lymphoid and peripheral tissues of HSV-2 infected guinea pigs.

To characterize the antibody produced by tissue-resident ASC and to determine if the isotype and IgG subclass profile of antibody from HSV-specific ASC isolated from peripheral sites of HSV-2 infection were similar to HSV-2-specific antibodies in immune serum, ASC were isolated from selected tissues and the antibody-containing culture supernatant was harvested after three days of culture for analysis by ELISA (**Figure 4.3**). In sera from immune animals, titers of HSV- specific IgG were significantly higher than IgA titers ($P<0.001$, Student's t test) and the IgG2 subclass predominated the response. A similar isotype profile was apparent for HSV-specific antibody produced by bone marrow-, vagina/cervix-, and spinal cord/ganglia ASC. Antibody was also tested for binding to recombinant HSV-2 glycoproteins representing known targets of antibodies in convalescent sera. As shown in **Fig. 4.4**, HSV-specific serum IgG bound at a nearly equivalent level to full-length or truncated recombinant HSV-2 glycoprotein D (rgD2) while binding to recombinant HSV-2 glycoprotein G (rgG2) was detected at a significantly lower level. The same binding pattern was observed for IgG produced by

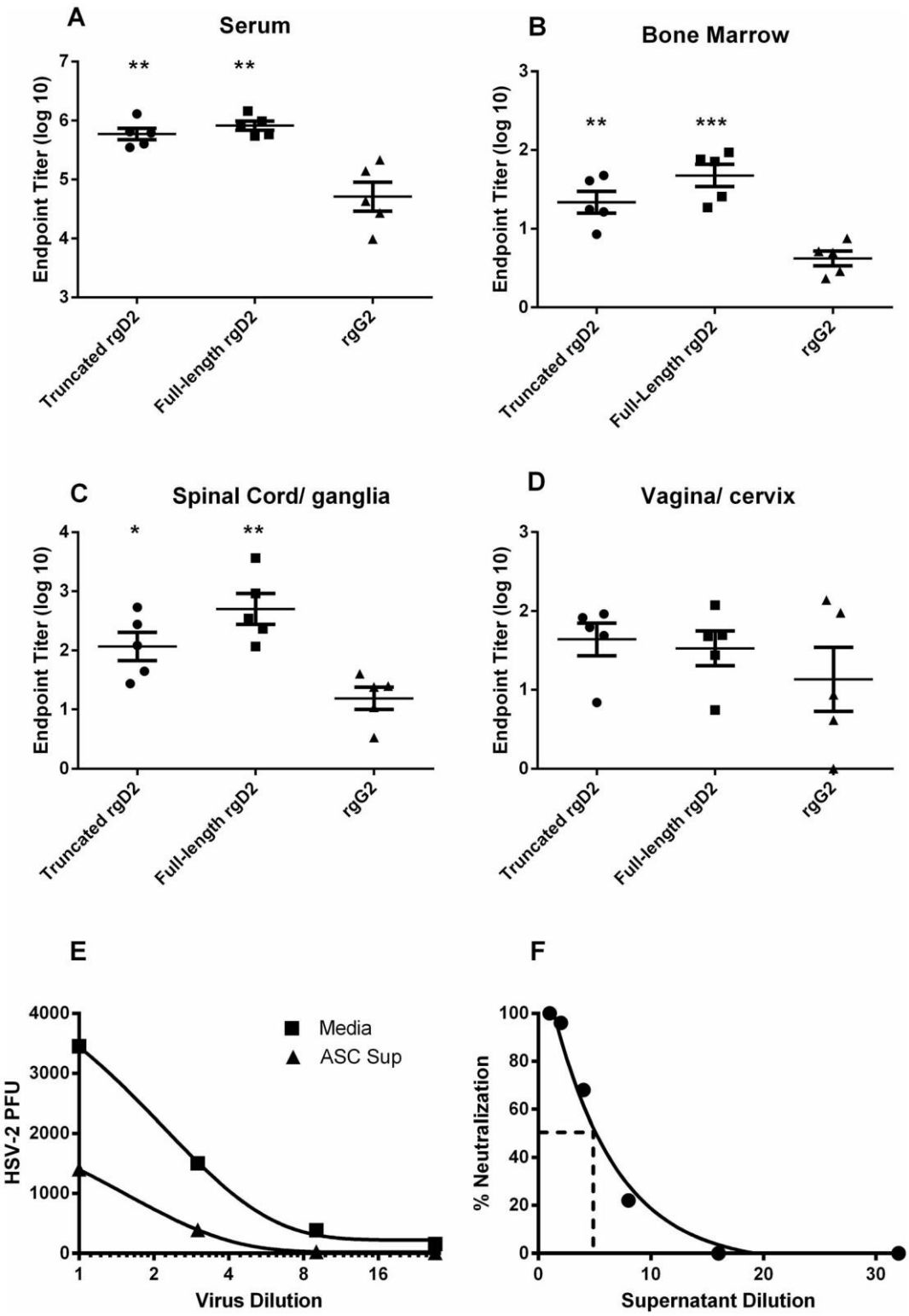
bone marrow- and spinal cord/ganglia- ASC (**Fig. 4.4B, C**). The level of IgG produced by ASC from the vagina/cervix was more variable and therefore binding to the two forms of rgD2 and rgG2 was not significantly different (**Fig. 4.4B**). HSV-2 neutralizing antibody was present at a titer of 1:1023±196 in the serum of HSV-2 infected guinea pigs. Functional HSV-2-neutralizing antibody was also produced by ASCs cultured from the lumbosacral ganglia and spinal cords of HSV-2 infected animals (**Fig. 4.4E, F**). Incubation of HSV-2 with supernatants of spinal cord/ganglia-resident ASC decreased viral titers over a range virus inoculums compared to the medium control (**Fig. 4.4E**). Additionally, ASC supernatant could be diluted approximately 5-fold and retain the ability to neutralize 50% of input virus (**Fig. 4.4F**). Neutralizing antibody was not detected in supernatants of ASC from genital tracts perhaps due to an approximately 10-fold lower number of HSV-specific ASC isolated from genital tract ASC compared to lumbosacral spinal cord ASC (**Fig. 4.2A** compared to **Fig. 4.2C**). To our knowledge, these studies represent the first characterization of HSV-specific antibodies produced by neural tissue-resident ASC during HSV latency.

Figure 4.3: Isotype and IgG subclass of HSV-specific antibodies produced by ASCs isolated from lymphoid tissue and non-lymphoid sites of HSV-2 infection.



Hartley guinea pigs were infected ivag with HSV-2 strain MS, serum was collected and lymphocytes were isolated from the indicated tissues on days 48–57 post infection. Supernatants from ASC cultures were collected on day three after ASC isolation. The immunoglobulin isotype and IgG subclass of HSV-specific antibodies from serum and ASC supernatant were determined by ELISA. Each data point represents ASC supernatant from an individual animal. (* $P<0.05$; ***$P<0.0001$, Student's t test).

Figure 4.4: IgG antibody from tissue-resident ASC isolated from HSV-2-infected guinea pigs is reactive with HSV-2 glycoproteins and neutralizes HSV-2.



Hartley guinea pigs were infected ivag with HSV-2 strain MS, serum was collected and lymphocytes were isolated from the indicated tissues between days 48–57 post infection. Supernatants from ASC cultures were collected on day three after isolation and the endpoint titer of antibodies reactive with full- length recombinant HSV-2 gD (rgD2), truncated rgD2, or truncated recombinant HSV-2 rgG (rgG2) was determined by ELISA. (*P<0.05; ** P<0.01; *** P<0.001 compared to HSV-2 gG2 titer). Virus neutralization was detected by incubating a constant virus titer in serial dilutions of ASC supernatant to determine a 50% neutralizing titer (E) or by incubating serial dilutions of HSV-2 virions in undiluted ASC supernatant (F). Results shown are from a representative experiment of three performed.

HSV-specific, tissue-resident memory T cells present at the sites of HSV-2 latency and virus shedding.

The activation and infiltration of HSV-specific T cells producing IFN-γ at the peripheral sites of acute HSV infection in the genital tract and sensory ganglia has been observed in murine models of genital HSV-2 infection (Milligan, Bernstein and Bourne 1998); (Iijima, Linehan, et al. 2007); (Johnson, Chu and Milligan 2008) and from HSV lesion-derived T cells (Koelle, Corey and Burke, et al. 1994); (Koelle, Abbo, et al. 1994). To further characterize T cell immunity in the genital tract, lumbosacral ganglia and spinal cord, Strain 13 guinea pigs were inoculated ivag with HSV-2 strain MS. All animals experienced primary HSV disease resulting in a mean disease score of 5.7±1.1. On day 7 post infection, HSV-specific, IFN-γ-secreting T cells from the spleen, vagina/cervix, and lumbosacral ganglia and the adjacent spinal cord were quantified using a pair of previously described monoclonal antibodies specific for guinea pig IFN-γ (Schafer, et al. 2007) in an IFN-γ ELISPOT assay. Lymphocytes from the indicated tissues were stimulated by culture with stimulator cells infected with HSV-2 dl5-29 and pulsed with UV-inactivated HSV-2 to ensure that both MHC class I and class II antigen presenting pathways were engaged to stimulate both $CD4^+$ and $CD8^+$ T cells. As shown in Fig. 4.5A-B, IFN-γ secreting cells (SC) were readily detected in tissues from HSV-infected, but not uninfected guinea pigs. HSV-specific IFN-γ SCs detected in the spleen,

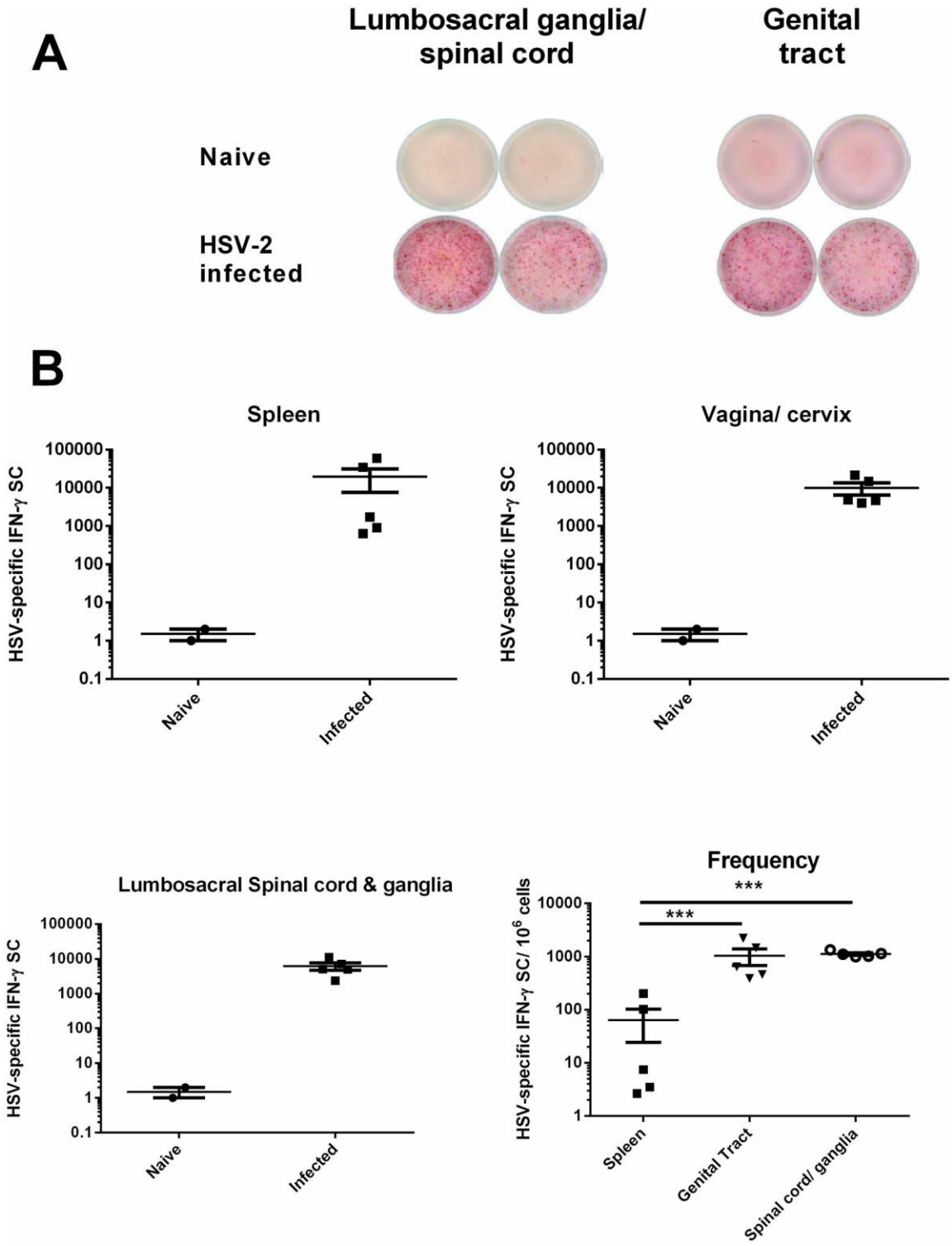
vagina/cervix, and spinal cord/lumbosacral ganglia of HSV- infected guinea pigs were over four logs greater than in uninfected animals (**Fig. 4.5B**). Although the total number of HSV-specific, IFN-γ SCs was similar in all three tissues, the frequency of these cells isolated from the spleen was somewhat variable among individual animals and significantly lower overall than the frequencies of HSV-specific, IFN-γ SC isolated from the vagina/cervix or spinal cord/ganglia ($P<0.0001$, Chi square).

The IFN-γ ELISPOT assay was modified for use in outbred animals and used to detect and quantify HSV-specific memory T cells from tissues of HSV-2-infected Hartley guinea pigs. Animals were inoculated ivag with HSV-2 strain MS and the primary and recurrent disease were scored. Ten of the 11 animals utilized for memory T cell analysis experienced primary HSV disease (Mean score 6.7±1.0) and 10 of 11 animals experienced at least one recurrent lesion (Mean score 5.2±0.7) between day 15 and the time of euthanasia (day 99–150). Tissues were harvested from individual animals between days 99–150 post infection. Cells from spleen, vagina/cervix, uterus, lumbosacral ganglia, cervicothoracic ganglia and the corresponding adjacent region of spinal cord were stimulated with stimulator cells infected with HSV-2 dl5-29 and pulsed with UV-inactivated HSV-2 to stimulate both $CD4^+$ and $CD8^+$ memory T cells. As shown in **Fig. 4.6A-B**, HSV-specific memory T cells were readily detected in the spleen of HSV-2 infected animals (mean 291,426±72,324 IFN-γ SC/spleen). HSV-specific memory T cells were also detected in both the upper and lower genital tract of all animals tested (**Fig. 4.6C, D**). Although present at significantly higher frequency in the vagina/cervix than in the uterus ($P<0.0001$, Chi square; **Fig. 4.6C**), overall, the total number of HSV- specific memory T cells was not different between these tissues (**Fig. 4.6D**) reflecting differences in the yield of lymphocytes isolated from these tissues. HSV-specific memory T cells were detected over the entire length of the spinal cord. However, the frequencies of tissue-resident, memory T cells detected in lumbosacral region spinal cord and cervicothoracic region spinal cord were significantly different ($P<0.0001$, Chi

square; **Fig. 4.6E**) and the total number of HSV-specific memory T cells was significantly greater in spinal cord tissue from the lumbosacral region ($P<0.05$, Student's t test). Similarly, HSV-specific memory T cells were detected in both the lumbosacral and cervicothoracic ganglia. Importantly, virus-specific memory T cells were also detected at significantly higher frequency in the lumbosacral compared to cervicothoracic ganglia ($P<0.0001$, Chi square; **Fig. 4.6G**) and the total number of HSV-specific memory T cells was significantly greater in lumbosacral ganglia compared to cervicothoracic ganglia ($P<0.01$, Student's t test, **Fig. 4.6H**).

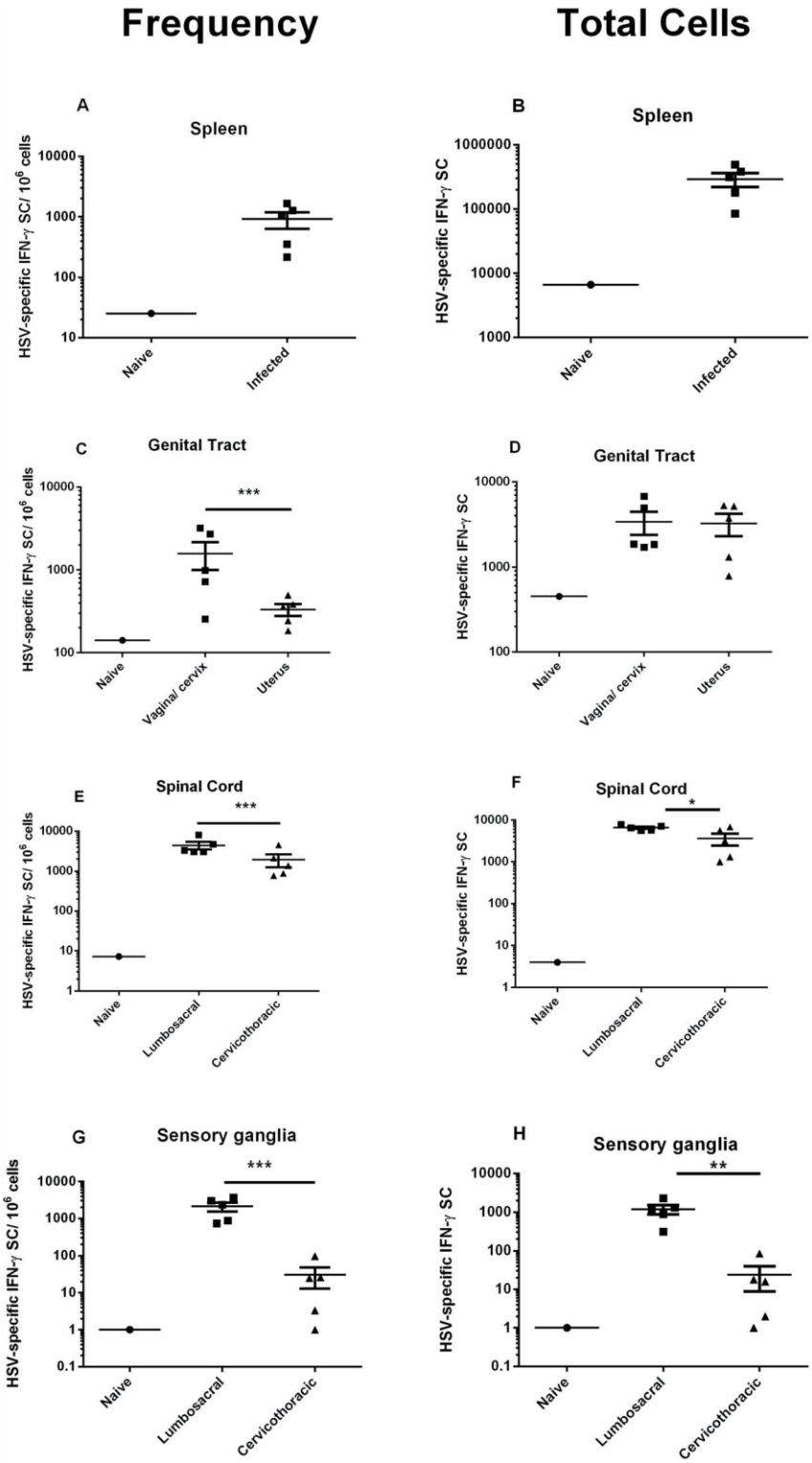
Lymphocytes were harvested from peripheral tissues of individual infected animals between days 99-150 post infection and passed over selection columns to enrich for $CD4^+$ or $CD8^+$ subsets to further characterize the T cell memory response. $CD4^+$ enriched populations were stimulated with stimulator cells pulsed with UV-inactivated HSV-2 while $CD8^+$ enriched populations were stimulated with stimulator cells infected with HSV-2 dl5-29. HSV-specific memory T cells representing both $CD4^+$ and $CD8^+$ subsets were readily detected in the vagina/ cervix of HSV-2-infected guinea pigs at 2-6 months post infection (**Fig. 4.7**). HSV- specific $CD4^+$ T cells were detected in the vagina/cervix at significantly lower total cell number compared to HSV-specific $CD8^+$ T cells ($P<0.05$, Student's t test). Both $CD4^+$ and $CD8^+$ HSV-specific memory T cells were maintained in the sensory ganglia/spinal cords of HSV-2 infected guinea pigs (**Fig. 4.7**). The total number of ganglia/spinal cord-resident, HSV-specific $CD4^+$ and $CD8^+$ T cells was not different demonstrating the maintenance of both subsets of virus specific memory T cells at the sites of HSV-2 latency during the initial months after HSV-2 infection.

Figure 4.5: Detection and quantification of HSV-specific IFN-γ secreting cells in spleen, genital tract and neuronal tissues of HSV-2 infected guinea pigs.



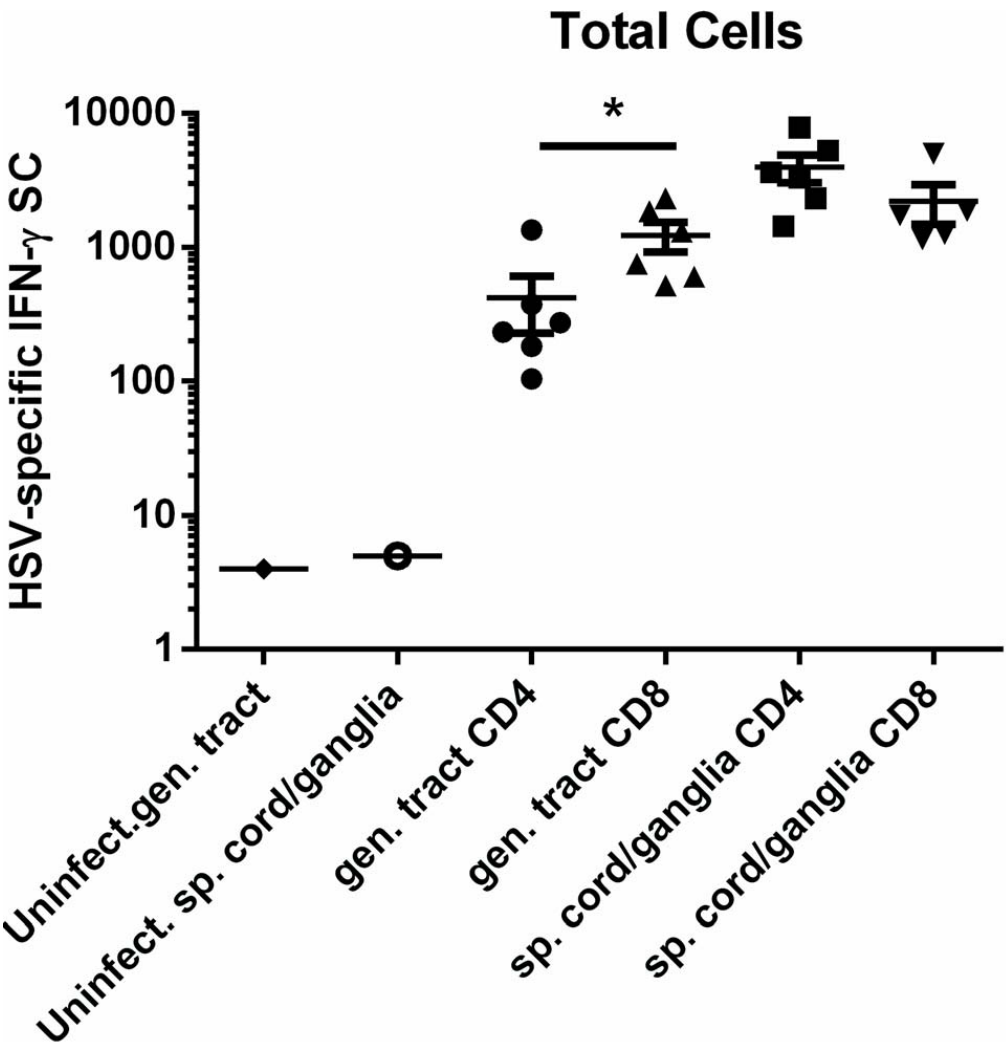
Lymphocytes from the indicated tissues of HSV-2-infected Strain 13 guinea pigs (n=5) were harvested on day 7 post infection. A) IFN-γ ELISPOT of lymphocytes harvested from sensory ganglia/spinal cord and genital tract. B) Total number and frequency of HSV-specific IFN-γ SC in the spleen, genital tract, and sensory ganglia/spinal cord of HSV-2 infected guinea pigs. Each data point represents results from an individual animal. *** $P<0.0001$, Chi square.

Figure 4.6: Tissue distribution of HSV-2 specific memory T cells following genital HSV-2 infection of guinea pigs.



Hartley guinea pigs were infected ivag with HSV-2 strain MS and lymphocytes isolated from the indicated tissues on days 99-150 post infection. HSV-specific, IFN-γ SCs were detected and quantified by IFN-γ ELISPOT. The frequency of HSV-specific, IFN-γ SC per 10^6 cells is given in A,C,E,G and the total number of HSV-specific, IFN-γ SC is given in B,D,F,H for each tissue. Each data point represents results from an individual animal. (* P<0.05; ** P<0.01, Student's t test; *** P<0.0001, Chi square).

Figure 4.7: Presence of $CD4^+$ and $CD8^+$ HSV-specific memory T cells in genital tracts and neuronal tissue of HSV-2 infected guinea pigs.



Hartley guinea pigs were infected ivag with HSV-2 strain MS and lymphocytes isolated from the indicated tissues on days 99–150 post infection. Enriched populations of $CD4^+$ and $CD8^+$ T cells were obtained using a magnetic-based kit. HSV-specific, IFN-γ SCs were detected and quantified by IFN-γ ELISPOT. Each data point represents results from an individual animal. (* P<0.05, Student's t test).

DISCUSSION.

HSV-specific, tissue-resident $CD4^+$ and $CD8^+$ memory T cells have been detected in genital skin at the site of previous HSV-2 infection (Peng, Zhu, et al. 2012); (Zhu, Peng, et al. 2013); (Tang and Rosenthal 2010) and at the site of HSV latency in the trigeminal ganglia of HSV-1-infected humans and mice (Verjans, et al. 2007); (Van Velzen, et al. 2013); (Held, et al. 2012); (Liu, Tang and Hendricks 1996); (Divito, Cherpes and Hendricks 2006). The maintenance of memory T cell populations in the lumbo-sacral ganglia following genital HSV-2 infection has not been as well characterized. Using a guinea pig model of HSV-2 genital infection, we show here that a full complement of adaptive immune cells including HSV-specific B cells, $CD4^+$ T cells, and $CD8^+$ T cells resides at the genital tract site of HSV-2 infection and at the site of latency in the sensory ganglia. Additionally, consistent with the recent observation that genital HSV-2 infection results in virus infection of autonomic neurons in the spinal cord (Ohashi, et al. 2011), we show here that the development and maintenance of all these HSV-specific memory immune cells extends also to this site of HSV-2 infection in the spinal cord. These tissue resident populations most likely play an important role in modulation of HSV-2 reactivation and control virus shedding. The induction of these tissue-resident memory cells by immunization would most likely be an important contributor to the successful immunization strategies against HSV.

The cellular components of B cell memory are quiescent memory B cells and antibody-secreting, long lived plasma cells (LLPC). Memory B cells normally reside in secondary lymphoid tissue although they have been detected at peripheral sites of previous virus infection (Onodera, et al. 2012). Since immune reagents that distinguish memory B cells are not yet available for guinea pigs, we utilized the ability of memory B

cells to become activated and secrete antibody in response to polyclonal stimulation to detect these cells. TLR agonist-stimulated memory B cells were readily detected in the spleen and bone marrow of infected animals but not in lymphocytes from genital or neural tissues. While the assay may have lacked the sensitivity to absolutely exclude the presence of memory B cells in these tissues, the results strongly suggest that the majority of the HSV-specific ASC detected in these tissues represent LLPC. However, it remains possible that because the guinea pig experiences spontaneous reactivation of HSV-2 from latency, some of the HSV-specific ASC we detected in genital and neural tissues may have resulted from antigen stimulation of tissue-resident memory B cell populations. LLPC reside primarily in lymphoid tissues such as the spleen or bone marrow and are responsible for maintaining serum antibody titers. However, populations of LLPCs have also been detected at sites of chronic inflammation including the nervous systems of humans infected with polio (Esiri 1980) or animals persistently infected with coronaviruses (Tschen, Bergmann, et al. 2002); (Tschen, Stohlman, et al. 2006). The influence of intermittent exposure to HSV antigen following spontaneous HSV-2 reactivation on these ASC populations is not yet known. However, the ASC population is most likely maintained in an antigen-independent fashion in survival niches at sites of chronic HSV infection in which survival stimuli such as IL-6 or CXCL12 are consistently present (Wilson and Trumpp 2006).

Both IgG and IgA have been detected in serum and vaginal secretions of guinea pigs following intravaginal infection with HSV-2 (McBride, et al. 1988) or Chlamydia (Rank, Batteiger and Soderberg 1988). Although HSV-specific IgA was consistently detected, IgG antibody represented the predominant isotype produced by genital tract resident ASC as reported in both mice and humans (Parr, Bozzola and Parr 1998); (Johansson and Lycke 2003). Additionally, the predominance of IgG2 expression by antibodies derived from peripheral tissue ASC mirrored that of serum antibody and bone marrow derived ASC. Antibody produced by genital- and neural-resident ASC

recognized rgD2 and rgG2 and HSV-specific antibody produced by neural tissue-resident ASC was capable of neutralizing HSV-2. Together these results demonstrate the production of functionally relevant antibody by these cells at sites of chronic HSV infection.

IgG may enter vaginal secretions by transudation from the serum or by receptor-mediated transport across the vaginal epithelium by FcRN (Li, et al. 2011) and does not require the presence of genital-resident ASC. However, IgG production by genital-resident, virus-specific ASC would increase the local concentration of virus-specific IgG resulting in increased movement of this antibody into vaginal secretions via simple diffusion or FcRN-mediated transport. The role for local antibody in protection of humans against HSV-2 infection or HSV disease is uncertain. The presence of HSV-specific maternal antibody in vaginal secretions decreases the risk of HSV-2 acquisition by neonates (Brown, et al. 1987). By analogy, locally- produced HSV-specific antibodies in mucosal secretions might prevent or limit virus infection. It has been shown that re-infection of ganglia is extremely difficult to achieve in previously infected animals due in part to the presence of pre- existing HSV-specific antibody (McKendall, Klassen and Baringer 1979); (Stanberry, Bernstein, et al. 1986). Locally-produced antibody in neural tissues may limit virus access to neurons, limit spread and acute replication of virus within the ganglia, and, upon reactivation from latency, interfere with virus spread from infected nerve ending to epithelial cells (R. Klein 1980); (Simmons and Nash 1985); (Mikloska, Sanna and Cunningham 1999). Plasma cells are also a source of cytokine production in inflamed tissues and B cell-derived cytokines such as IL-6, IL-12, TGF-β, and IL-10 that may play a role in regulating the inflammatory response in sites chronically infected with HSV-2. Given these functions of HSV-specific B cells, the populations of HSV-specific ASC we detected long term in genital tract, latently-infected sensory ganglia and spinal cords seem strategically located to play an active role in

modulation of recurrent disease and shedding or in protection of genital and neural tissue against re- infection upon subsequent HSV-2 exposure.

In humans, HSV-specific $CD8^+$ T lymphocytes have been isolated from HSV lesion material and from genital skin at the dermal-epidermal junction after HSV-2 infection (Zhu, Koelle, et al. 2007); (Peng, Zhu, et al. 2012); (Zhu, Peng, et al. 2013). HSV-specific $CD4^+$ T cells have also been detected in the vaginal epithelium long-term after HSV-2 genital infection of mice (Milligan, Bernstein and Bourne 1998); (Iijima, Linehan, et al. 2007); (Milligan and Bernstein 1995) and have also been isolated from HSV lesion material from humans (Koelle, Corey and Burke, et al. 1994); (Koelle, Abbo, et al. 1994). The development of genital tract-resident memory T cells in guinea pigs as a result of genital HSV-2 infection is apparently very similar and it is of note that a strong IFN-γ response is detected upon stimulation of these cells as has been detected in both mice and humans (Milligan, Bernstein and Bourne 1998); (Zhu, Peng, et al. 2013); (Tang and Rosenthal 2010). Prophylactic vaccination to induce genital tract-resident memory T cells would theoretically provide protection against the initial infection whereas therapeutic vaccination to enhance a genital tract- resident memory T cell population might aid in modulating virus shedding from the genital tract of those individuals already infected.

HSV-specific, IFN-γ secreting effector T cells were detected in the lumbosacral ganglia and spinal cord during acute HSV-2 infection. Importantly, large populations of HSV-specific, $CD4^+$ and $CD8^+$ memory T cells were detected at these sites up to 150 days after HSV-2 infection. The maintenance of memory T cell populations at the sites of HSV-2 latency following genital HSV-2 infection has not been extensively studied previously. Sacral ganglia-resident T cell populations are not readily accessible in humans and infection of mice with HSV-2 frequently results in mortality complicating long-term study of memory T cells. We know from ocular HSV-1 infection of mice that HSV-specific $CD8^+$ T cells surround HSV-1 infected neurons in the trigeminal ganglia of

mice (Divito, Cherpes and Hendricks 2006); (Liu, Khanna, et al. 2001) and may be involved in the maintenance of HSV latency via non-lytic mechanisms involving IFN-γ and release of granzyme B (Liu, Tang and Hendricks 1996); (Knickelbein, Khanna, et al. 2008). Similarly, HSV-2 –specific $CD4^+$ T cells have been shown to play a role in resolution of an acute virus infection of the lumbosacral ganglia neurons following genital HSV-1 infection (Johnson, Chu and Milligan 2008). The role of these neural tissue-resident memory T cells in modulating HSV-2 reactivation and how the population dynamics of these populations is influenced by chronic exposure to HSV-2 is currently unclear. Utilization of this animal model in which spontaneous reactivation of HSV-2 occurs should help resolve these issues.

In contrast to the tissue location of HSV-specific ASC, the HSV-specific memory T cells were more broadly dispersed in both the genital tract and neural tissues. These results suggest differences in either the inflammatory milieu or antigen load between the T and B cell experiments or different sensitivities of our assays to detect HSV-specific memory T and ASC populations. Our results also demonstrate that beyond the ganglia site of HSV-2 latency, HSV-specific resident memory T cells become established in the spinal cord following HSV-2 genital infection. Ohashi et al. (Ohashi, et al. 2011) detected the greatest quantities of latent HSV-2 genomes in the sacral region of the spinal cord. Consistent with this finding, although HSV-specific memory T cells were detected throughout the entire spinal column, the highest frequency and the greatest total number of HSV-specific memory T cells were detected in the lumbosacral region. The current study therefore extends previous results regarding the nature of immune memory in the sacral ganglia and spinal cord sites of latency following genital HSV-2 infection.

Guinea pigs infected ivag with HSV-2 experience intermittent, spontaneous reactivation events resulting in recurrent virus shedding and development of clinical symptoms remarkably similar to humans. A roadblock to studying immune control of HSV-2 in this model has been the lack of reagents for immune response proteins. We

previously isolated genital tract resident lymphocytes to detect HSV-specific ASC in the genital tracts and neural tissue of HSV-2-infected guinea pigs for time periods up to 8 months after genital infection (Milligan, Meador, et al. 2005). Our current studies extend these previous results and demonstrate that ASC releasing functionally protective, HSV-specific antibody are present at the sites of HSV-2 latency and sites of virus shedding. The nature of immune memory at sites of HSV-2 infection and latency was further clarified by development of an IFN-γ ELISPOT to detect and quantify HSV-specific effector and memory T cells and demonstrate vigorous HSV-specific memory T cell responses at these epithelial and neural sites. Future studies utilizing the guinea pig HSV-2 infection model will allow characterization of important properties of these cells and test immunization regimens to induce genital tract resident T cell populations in the context of virus reactivation and shedding that most effectively models human HSV-2 infection.

Future direction.

The function of peripheral resident T and B cell response in HSV-2 chronic infection.

In Appendix, we characterized, for the first time, the nature of persistent tissue resident, HSV-specific T and B cells in latently infected guinea pigs. However, the exact function of each individual memory cell type in protection is not clear. Additionally, the protective role of resident memory cells induced by prophylactic and therapeutic immunization also requires further investigation for optimizing immunization strategies. Many studies have identified the important role of T and B cell responses in protection of mice from HSV-2 infection. $CD4^+$ rather than $CD8^+$ T cells have been shown to be more protective for mice following HSV-2 infection. Both B and T cell response have been detected locally at genital tract in the guinea pig model. At present, manipulation of B cell responses or depleting B cells in guinea pigs is not possible which complicates the

study of B cell function. However, other models suggest a predominant role of T cells in protection. Development of ELISPOT assays to detect HSV-specific T cells opens the possibility of performing similar studies in guinea pigs. By depleting $CD4^+$ or $CD8^+$ T cells with anti-guinea pig CD4 or anti-guinea pig CD8 monoclonal antibodies, the protective role of $CD4^+$ and $CD8^+$ T cells will be determined. Reagents for these studies are currently under development.

Additionally, HSV-2 infection in guinea pigs with spontaneous HSV-2 reaction mimics HSV-2 infection in human. Our guinea pig model should allow future studies to examine the role of resident memory cells in preventing the initial HSV-2 infection in naïve hosts reflecting the efficiency of prophylactic immunization. Meanwhile, this model could also be helpful to study the role of resident memory cells in modulating viral shedding in HSV-2 recurrent diseases and test the efficiency of therapeutic immunization or medicines.

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VITA

Jingya Xia was born in Tianjin, P.R. China in 1986. She is the only child of Yushuang Wang and Guoyue Xia. She received her Bachelor of Science degree in Biological Science from Nankai University, P.R. China in 2009. With a strong interest in antiviral immunology, she came to University of Texas Medical Branch (UTMB), Galveston, TX, as a graduate student in 2009. After several rotations, it was her honor to join Dr. Gregg Milligan's lab to study the development of antiviral adaptive immune responses. During her Ph.D. training, she had two projects focusing on 1. The role of TLR3- and MyD88-dependent signaling in the development of adaptive immune responses against RepliVAX WN, a successful WNV vaccine candidate; 2. The development of adaptive immune responses and the nature of peripheral resident T and B memory cells in HSV-2 acute and chronic infection.

EDUCATION

B.S.(09/2005-07/2009), in Biological science, Nankai University, Tianjin, P.R.China.
Graduate student (09/2009 till now), Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, U.S.A.

PROFESSIONAL WORK HISTORY and RESEARCH ACTIVITIES:

1. Identify the function of P311 gene in the lung development. Laboratory of biochemistry and molecular biology laboratory, Department of Biochemistry and Molecular Biology, College of Life Sciences, Nankai University (03/2007 - 06/2009) Advisor: Dr. Zhinan Yin and Dr. Liqing Zhao

2. Identify the cytokine profile of 12j macrophage infected by WNV mutant strain TWN1111 and TX1153. Host innate immunology Lab, Microbiology & Immunology, UTMB (05/2010 - 07/2010) Advisor: Dr. Tina Wang

3. Understanding the role of TLR3 and MyD88-dependent signaling in the development of adaptive immune responses in the mice model immunized by RepliVAX WN. (supported by James W. McLaughlin predoctoral fellowship) (07/2010 -05/2013) Lab: Viral immunology Lab, Microbiology & Immunology, UTMB. Mentor: Dr. Gregg N. Milligan

4. Identifying the development of central, peripheral and local virus-specific immunity in the guinea pig HSV-2 infection model. (supported by Sealy Center for Vaccine Development (SCVD) predoctoral fellowship, UTMB) (05/2013 till 09/2014) Lab: Viral immunology Lab, Microbiology & Immunology, UTMB. Mentor: Dr. Gregg N. Milligan

MEMBERSHIP IN SCIENTIFIC SOCIETIES/PROFESSIONAL ORGANIZATIONS

The American Association of Immunologist: 2013.

HONORS/AWARDS

2005 – 2006: Excellent Undergraduate Scholarship of Nankai University, Nankai University, P.R.China.

2006 – 2007: Excellent Undergraduate Scholarship of Nankai University, Nankai University, P.R.China.

2008: Second Prize as an excellent project in the "100 Projects" of Creative Research for the Undergraduates of Nankai University. Project Name: Identification the expression pattern of SPC in the lung tissues using in situ hybridization.

2011 – 2013: James W. McLaughlin predoctoral fellowship, UTMB

2012: Sealy Center for Vaccine Development (SCVD) travel award, UTMB

2012: John Stanton Scholarship, UTMB

2013: Sealy Center for Vaccine Development (SCVD) predoctoral fellowship, UTMB

2013: Zhou Sisters Great Expectations Scholarship, UTMB.

PUBLICATION

A. ARTICLES IN PEER-REVIEWED JOURNALS:

1. **Jingya Xia**, Evandro R. Winkelmann, Summer R. Gorder, Peter W. Mason, Gregg N. Milligan. TLR3- and MyD88-dependent signaling differentially influence the development of West Nile virus-specific B cell responses in mice following immunization with the single-cycle flavivirus (SCFV) Replivax WN. 2013. J Virol. 87(22):12090-101.

2. **Jingya Xia**, Evandro R. Winkelmann, Summer R. Gorder, Peter W. Mason, Gregg N. Milligan. TLR3- and MyD88-dependent signaling differentially influence the development of West Nile virus-specific T cell responses by facilitating the function of myeloid DCs. In preparation.

3. **Jingya Xia**, Ronald L. Veselenak, Summer R. Gorder, Nigel Bourne, and Gregg N. Milligan. Virus-specific memory at peripheral sites of Herpes simplex type 2 (HSV-2) infection in guinea pigs. PLoS One. 2014. 9(12):e114652.

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B. Abstracts:

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Permanent address: Qianjin Community 35-7-401, Shengli Street, Dagang, Binhai New Area, Tianjin, P. R. China, 300270

This dissertation was typed by Jingya Xia.