# THE ROLE OF MOSQUITO SALIVA ON HOST IMMUNE RESPONSE AND PATHOGENESIS OF WEST NILE VIRUS

by

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To my father, without whom I truly would not be the man I am today.

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West Nile virus is a positive sense single-stranded RNA virus in the family Flaviviridae that emerged globally following the appearance of a more neurotropic subtype. Recently outbreaks of WNV disease have occurred in the Middle East, Europe, Africa, South America, and North America. Vertebrates typically become infected when an infectious mosquito pierces the host epidermis to take a blood meal, depositing virus principally in the extravascular tissue. Accumulating evidence has demonstrated that the mosquito saliva, which carries WNV into the vertebrate, is not simply a transport medium, but can have a profound effect on vertebrate immunity, pathogen transmission efficiency, pathogenesis, and disease course. In the past most small animal models of arbovirus disease have used needle-inoculation, but recent evidence suggests that because of the potential effects of mosquito saliva on the immune system, it is important to re-evaluate the pathogenesis of these infections in the presence of mosquito saliva. A central question of this dissertation project was to determine if mosquito feeding or mosquito saliva could impact WNV disease. To evaluate the potential for mosquito saliva to alter WNV infection, the mouse model of disease employed in this dissertation revealed a potentiation of WNV disease when mosquitoes were allowed to feed at the virus inoculation site immediately before injection of WNV. Further, with higher titer inoculations of WNV subsequent to the feeding of mosquitoes more progressive infection, higher viremia, and accelerated neuroinvasion developed than the mice inoculated with an equivalent titer of WNV alone. To determine the mechanism of this potentiation, the *in vivo* expression of key $T_H1$, $T_H2$, inflammatory, and antiviral cytokines was quantified during peripheral arbovirus infection in the presence or absence of mosquito saliva. Data clearly showed that during early arbovirus infection mosquito salivary proteins down-modulates specific antiviral cytokines while enhancing production of $T_H2$ and immunosuppressive cytokines. To understand the source of these shifts in immune signalling, the *in vitro* response of DCs and macrophages was investigated. Following exposure to *Ae. aegypti* SGE, APCs recently exposed to arbovirus displayed reductions in IFN-β and iNOS expression and transient amplification of IL-10 mRNA levels. Macrophages appeared to be more susceptible to the modulating effects of mosquito saliva than DCs. The influence of mosquito saliva on immune cell migration patterns both into the dermal site of WNV inoculation and the draining lymph node was also evaluated. The principal observation from this study was that the inclusion of mosquito saliva/feeding at the inoculation site of WNV leads to a suppression of

lymphocytes, particularly $CD4^+$ T cells, and a corresponding increase in DCs. Finally, although mosquito exposure and sensitization to it is widespread, the effect of prior exposure to mosquitoes on subsequent arbovirus infection had up until this point been unexplored. Accordingly, the potential for an immune response directed against mosquito salivary proteins to have a protective or confounding effect on naturally transmitted WNV infection was investigated by comparing early WNV pathogenesis in mosquito naïve and sensitized populations of mice. Previous exposure to *Ae. aegypti* feeding results in significantly higher mortality rates associated with elevation of inflammation, APC recruitment, and IL-4 expression concurrent with a decrease in lymphocytes mainly the $CD4^+$ subtype. Mosquito sensitization-mediated amplification of WNV disease is facilitated by the humoral response to mosquito salivary proteins. This dissertation confirms that by ignoring the possible effects of the vector we may misinterpret the early immune response to arboviruses and also possibly aspects of the overall pathogenesis of arboviral infections. The information from the present study provides insight into early host responses to arbovirus infection, and suggests further determinants of WNV virulence.

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| | |
|---|---|
| aa | amino acid |
| Ab | antibody |
| *Ae*. | *Aedes* |
| An. | *Anopheles* |
| ADP | adenoside diphosphate |
| APC | antigen presenting cell |
| ATP | adenoside triphosphate |
| BBB | blood-brain barrier |
| BHK | baby hamster kidney |
| BHQ1 | black hole quencher |
| BSL | biosafety level |
| C | capsid |
| C6/36 | |
| CDC | Centers for Disease Control and Prevention |
| cDNA | complementary DNA |
| $CO_2$ | carbon dioxide |
| CNS | central nervous system |
| cpe | cytopathic effect |
| *Cs*. | *Culiseta* |
| CVV | Cache-Valley virus |
| *Cx*. | *Culex* |
| dI | domain I |
| dII | domain II |
| dIII | domain III |
| DC | dendritic cell |
| DENV | dengue virus |
| DEPC | diethylpyrocarbonate |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| dpe | days post-electroporation |
| dpi | days post-infection |
| dsDNA | double stranded DNA |
| E | envelope |
| EIA | enzyme immunoassays |
| EM | electron microscopy |
| EMCV | encephalomyocarditis virus |
| ER | endoplasmic reticulum |
| EtOH | ethanol |

| | |
|---|---|
| Fig. | figure |
| FFA | focus forming assay |
| g | grams |
| *g* | gravity |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| $H_2O_2$ | hydrogen peroxide |
| h | hour |
| HCV | hepatitis C virus |
| HRP | horseradish peroxidase |
| i.d. | intradermal |
| i.p. | intraperitoneal |
| i.t. | intrathoracic |
| i.v. | intravenous |
| IFA | immunofluorescence assay |
| IFN | interferon |
| Ig | immunoglobulin |
| IHC | immunohistochemistry |
| IL | interleukin |
| IMEM | Iscove's modified Eagle's medium |
| IRES | internal ribosomal entry site |
| JCV | Jamestown Canyon virus |
| JEV | Japanese encephalitis virus |
| kDa | kilodalton |
| L | liter |
| *L.* | *Leishmania* |
| LACV | La Crosse virus |
| LC | Langerhans cell |
| LN | lymph node |
| MAb | monoclonal antibody |
| MeOH | methanol |
| MHC | major histocompatibility complex |
| min | minute |
| ml | milliliter |
| mM | millimole |
| moi | multiplicity of infection |
| MST | median survival time |
| NaCl | sodium chloride |
| NCR | non-coding region |
| ng | nanogram |
| NHS | normal horse serum |
| NK | natural killer |
| NO | nitric oxide |
| NS | non-structural |
| nt | nucleotide |
| NVT | non-viremic transmission |

| | |
|---|---|
| OAS | 2', 5'-oligoadenylate synthetase |
| *Oc*. | *Ochlerotatus* |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| pfu | plaque-forming units |
| PRNT | plaque reduction neutralization test |
| qRT-PCR | quantitative RT-PCR |
| RNA | ribonucleic acid |
| rpm | rotations per minute |
| RPMI | Roswell Park Memorial Institute medium |
| RT | room temperature |
| RT-PCR | reverse transcriptase PCR |
| RVFV | Rift Valley fever virus |
| s.c. | subcutaneous |
| SAT | saliva-activated transmission |
| sec | second |
| SG | salivary gland |
| SGE | salivary gland lysate |
| SGP | salivary gland pair |
| SINV | Sindbis virus |
| TBEV | tick-borne encephalitis virus |
| TCID | tissue culture infectious dose |
| TEM | transmission electron microscopy |
| TEMED | N,N,N',N'-tetramethylethylene diamine |
| TGF | t ransforming growth factor |
| TNF | tumor necrosis factor |
| U | unit |
| UTMB | University of Texas Medical Branch |
| µl | microliter |
| UV | ultraviolet |
| VEEV | Venezuelan equine encephalitis virus |
| VLP | virus-like particle |
| VSV | vesicular stomatitis virus |
| WEEV | Western equine encephalitis virus |
| WHO | World Health Organization |
| WNV | West Nile virus |
| YFV | yellow fever virus |

# CHAPTER 1: AN INTRODUCTION TO MOSQUITO-BORNE VIRUSES

## FLAVIVIRUSES/WEST NILE VIRUS (WNV)

Flaviviruses, members of the family Flaviviridae, genus *Flavivirus*, constitute a broad spectrum of viruses that are typically maintained in natural transmission cycles between arthropod vectors and susceptible vertebrate hosts. Natural flavivirus infections occur primarily in birds and mammals, and most are associated with rash, fever, hemorrhagic or encephalitic syndromes of which the latter can occasionally culminate in a fatal outcome. The range of a member of this family, West Nile virus (WNV), has recently and unexpectedly increased, coincident with what retrospectively appears to be a period of enhanced virulence (Beasley *et al*. 2005), leading to the introduction of WNV to New York City and extension into the New World. Since its introduction into the New World, the WNV outbreak has caused ~22,000 diagnosed human cases, with at least 900 fatalities. Retrospective serological studies recently estimated that in 2003 alone there were 750,000 undiagnosed infections (Busch *et al.* 2006), underscoring the significance of these viruses to human health.

### Genome and Virion Structure

Flaviviruses are small spherical lipid enveloped particles that are approximately 50 nm in diameter. The envelope is composed of two virus encoded proteins, envelope (E) and membrane (M), and the capsid contains a single protein. The E protein facilitates

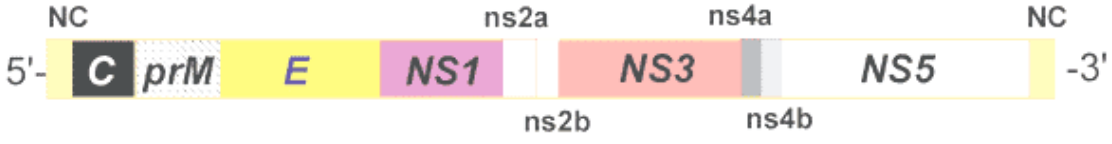


**Figure 1.1 Schematic diagram of the flavivirus virus genome.**

binding and fusion during virus entry, and, as the most prominently exposed surface protein, it is recognised by vertebrate hosts as the major antigenic determinant. These viruses integrate host membrane-derived lipids in their envelope, and contain carbohydrates including glycolipids and glycoproteins. As illustrated in figure 1.1, each virus particle contains a single-stranded positive sense mRNA molecule that is approximately 10.6 kB in length (Chambers *et al*. 1998).

The flavivirus mRNA genome contains a single long open reading frame flanked by 5' and 3'–terminal noncoding regions, and lacks a 3'-terminal poly A tail. Terminal noncoding regions form secondary structures required for virus replication and a 5'-terminal cap that facilitates initiation of translation (Chambers *et al*. 1998).

The long open reading frame produces a large polyprotein. This polyprotein is co- and posttranslationally cleaved into 10 proteins. The polyprotein is composed of seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) located in the C-terminal three-fourths of the molecule that are involved in viral replication and processing (Rice *et al.* 1986). The N-terminal one-fourth of the viral genome is co-linear with the C-terminal, and encodes three major structural proteins (C, prM, E). The capsid (C) protein gene encodes the capsid protein. Although the structure of this protein is unknown, it is clear that it forms a shell around the genomic RNA. The precursor of the M protein (prM) is cleaved by a host signal peptidase following translocation into the endoplasmic reticulum (ER) of the host cell (Lobigs 1993, Amberg *et al.* 1994). The prM protein appears to act as a chaperone for E protein, as the two proteins form a heterodimer shortly after translation and prM is required for proper folding of E (Lorenz *et al.* 2002). The major surface protein of flaviviruses is the E protein, which forms into an icosahedral arrangement and in flaviviruses is often glycosylated (Chambers *et al.* 1998, Ferlenghi *et al.* 2001).

**Attachment, Entry and Uncoating of the Virion**

Flaviviruses enter host cells by binding cellular receptors via a viral surface glycoprotein. The wide host-cell range of flaviviruses suggests that they use receptors,

which are ubiquitous in vertebrates and invertebrates. A variety of cell surface proteins have been suggested as potential receptors, and recently alphaVbeta3 integrin was identified as a functional receptor for WNV cell binding in vertebrates (Lee *et al.* 2006). Additionally, opsonization, or coating, with antibody enhances virus binding of cells expressing Fc receptors, such as antigen presenting cells (APC) (Peiris and Porterfield 1979). Receptor-mediated endocytosis, facilitated by clathrin-coated pits, mediates virus internalization (Gollins and Porterfield 1986b). After internalization, virions are directed into a prelysosomal endocytic compartment where the low pH induces alterations in the E protein that lead to virion fusion with the host membrane and release of the viral nucleocapsid (Gollins and Porterfield 1986a). Studies have consistently demonstrated the requirement for this acidic pH for triggering the virion-host membrane fusion event. Compared to other viruses tick-borne encephalitis (TBE) virus fusion takes place at the unprecedented rate of 40% per second at 37˚C with no measurable lag phase (Heinz and Allison 2000). The flavivirus fusion process thus appears to be the fastest and most efficient known. The E protein interacts with host cell receptors and mediates entry into a target cell. Anti-E glycoprotein antibodies can reduce viral infectivity (Wang *et al.* 2001b) and for some flaviviruses, E amino acid mutations can affect virus binding efficiency, virulence in laboratory animals and infectivity for mosquitoes (Holbrook *et al.* 2001, Hurrelbrink and McMinn 2001). Attachment of a flavivirus to host-cell receptors and subsequent acidification of the environment leads to conformational changes that disrupt the protein-protein interactions of the envelope. These changes are required to drive fusion, and involve a modification in the sites of contact between individual molecules, leading to significant rearrangement of the icosahedral E protein lattice (Gollins and Porterfield 1986b). A variety of techniques have demonstrated that the E proteins are initially dimeric, but exposure to acidic pH causes an irreversible conversion to a homotrimeric interaction (Allison *et al.* 1995). Despite the major shift in E conformation preceding fusion, it appears that the trimeric low-pH form is not responsible; instead the conformational shift is driven by intermediate structures generated during the transition (Corver *et al.* 2000). Replication of flaviviruses occurs

independently of the cell nucleus, and induces cytoplasmic rearrangement in the perinuclear region. The process of the nucleocapsid uncoating is not fully understood. Once released from its coat, the viral RNA is accessible to ribosomes for initiation of translation that leads to production of viral proteins, replication of viral genome, and production of new virions.

## Transcription, Translation and Replication of the Genome

### *Non Structural Proteins*

The flaviviral non-structural proteins and the complementary minus strand of the genome are synthesized from the genomic RNA, which acts as a messenger RNA (mRNA). The negative-strand intermediate is the template for synthesis of a new genomic RNA. Translation of the genomic RNA initiates near the 5' terminus of the genome and proceeds continuously along the genome. The nascent non-structural polyprotein is co- and post-translationally cleaved to generate the polyproteins. The sequential cleavage of the NS proteins is a way by which RNA virus strand synthesis is regulated. NS1 is generally retained within the infected cell, but is also found on the cell surface and secreted from mammalian cells (Mason 1989, Post *et al.* 1991), thereby resulting in the production of anti-NS1 antibodies. Within host cells this protein is important for viral replication and functions at a very early stage of RNA replication (Mason 1989, Post *et al.* 1991). There is no clear function for the extracellular membrane-bound form of this protein, but the secreted form of NS1 has been proposed to be a complement fixing antigen (Brandt *et al.* 1970). NS2A is believed to be involved in the shift between RNA replication and RNA packaging, and cleavage-site mutants had a normal level of RNA replication but made particles that lacked nucleocapsid (Kummerer and Rice 2002). The NS2B protein, a membrane-associated protein, is a necessary cofactor for the serine protease activity in NS3 (Yusof *et al.* 2000). NS3 is a large multifunctional enzyme involved in polyprotein processing and RNA replication, it functions as a serine protease, helicase, NTPase, and RNA triphosphatase (Lindenbach 2003). NS4A is associated with induction of membrane structures convoluted membranes

and paracrystalline arrays (Roosendaal *et al.* 2006), while NS4B has been reported to interfere with the interferon response in host cells by blocking the activation and nuclear translocation of Stat-1 (Munoz-Jordan *et al.* 2005) and recombinant NS4B dissociates ssRNA from NS3 and consequently enhanced the overall helicase activity of NS3 in in vitro assays (Umareddy *et al.* 2006). NS5 is involved with RNA replication, having N-terminal RNA cap-processing activity and C-terminal RNA-dependent RNA polymerase activity (Reed *et al.* 1998).

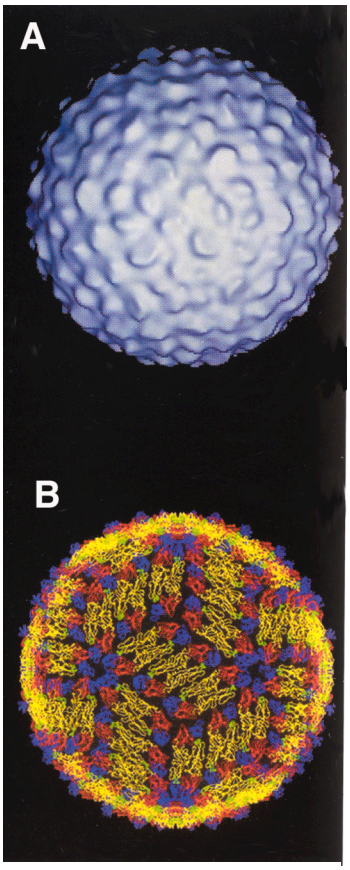


**Figure 1.2 (A) Electron cryomicroscopy image reconstructions, (B) Packing of E on the surface of the virus: The three domains of dengue sE. Domain I is red, domain II is yellow, domain III is blue. Adapted from Kuhn *et al.* 2002.**

***Structural Proteins***

The structural and the NS proteins, are translated as one polyprotein. The new proteins translocate to the endoplasmic reticulum where they are modified by attachment of oligosaccharides and proteolytic cleavages. A host signal peptidase is responsible for the cleavage of the structural proteins (Rice *et al.* 1986). These modifications produce the C, prM and E glycoproteins (Fig. 1.2)

Capsid

The C protein is highly basic and approximately 11 kDa in size (Rice *et al.* 1986), Charged residues at the amino- and carboxy-termini of this highly basic protein are thought to mediate interactions with RNA. The C protein forms the viral nucleocapsid as a complex of multiple C proteins and one copy of genomic RNA. The location of charged regions at either terminus may aid in the interaction of this protein with the viral RNA (Khromykh *et al.* 1996). Internal deletions in this protein lead to decreased flavivirus release (Kofler *et al.* 2002).

Membrane

The prM protein (~26 kDa) is translocated to the endoplasmic reticulum by a hydrophobic region of C, and folds rapidly (Amberg *et al.* 1994, Lorenz *et al.* 2002). The close and immediate association between prM and E proteins and the requirement of prM for proper folding of E suggest that prM has chaperone-like activity for E (Konishi and Mason 1993, Lorenz *et al.* 2002). The cleavage of prM into pr and M occurs in Golgi vesicles by a furin-like enzyme (Stadler *et al.* 1997). The pr segment is believed to protect the E protein in the reduced pH environment of the secretory pathway, while the M protein is found in mature virions (Murray *et al.* 1993).

Envelope

The E protein is the major surface-exposed protein of the mature flavivirus, and as such mediates binding and membrane fusion. This protein is composed of 3 domains (I, II, and III); domain I is the central structural domain which forms a β-barrel. Domain II contains the putative fusion peptide on its distal tip, which mediates association with the host cell membrane (Allison *et al.* 2001). Domain II is the dimerization domain which contains a fusion peptide (aa 98-110) buried between domains I and III. Domain III is an Ig-like domain thought to be the cell-receptor binding domain. The E protein on the outer surface of the virion is arranged as 30 islands of three parallel dimers, with domain III protruding from the surface of the virion (Heinz and Allison 2003).

***5' and 3' non-coding regions***

The non-coding region (NCR) sequences of flaviviruses are variable, but secondary structures formed by these regions, often conserved between flaviviruses, are expected to be more important than the actual genomic sequence (Hahn *et al.* 1987, Brinton and Dispoto 1988). The 5'NCR is believed to participate in viral translation and initiation of positive strand synthesis (Lindenbach 2003), while the flavivirus 3'NCR is involved in replication through interactions with NS3 and NS5 at a conserved stem-loop (Lindenbach and Rice, 2003). A well-conserved sequence upstream of this stem-loop is thought to bind to a complementary sequence in C to mediate cyclization (Lindenbach 2003). With respect to virulence, experiments that examined the predicted secondary

structures of various wild-type and attenuated flavivirus strains and found a correlation between virus virulence and secondary structure (Proutski *et al.* 1997).

**Assembly and Release of New Virions**

Flavivirus RNA replication occurs on host cell membranes in vesicle packets (Fig. 1.3). Ultrastructural studies have demonstrated that flavivirus-infected cells develop an extensive proliferation of intracellular membranes and vacuoles (Lindenbach and Rice 2003). Virion assembly occurs on the surface of the endoplasmic reticulum, and it is generally believed that particle formation occurs by an undefined assembly and budding

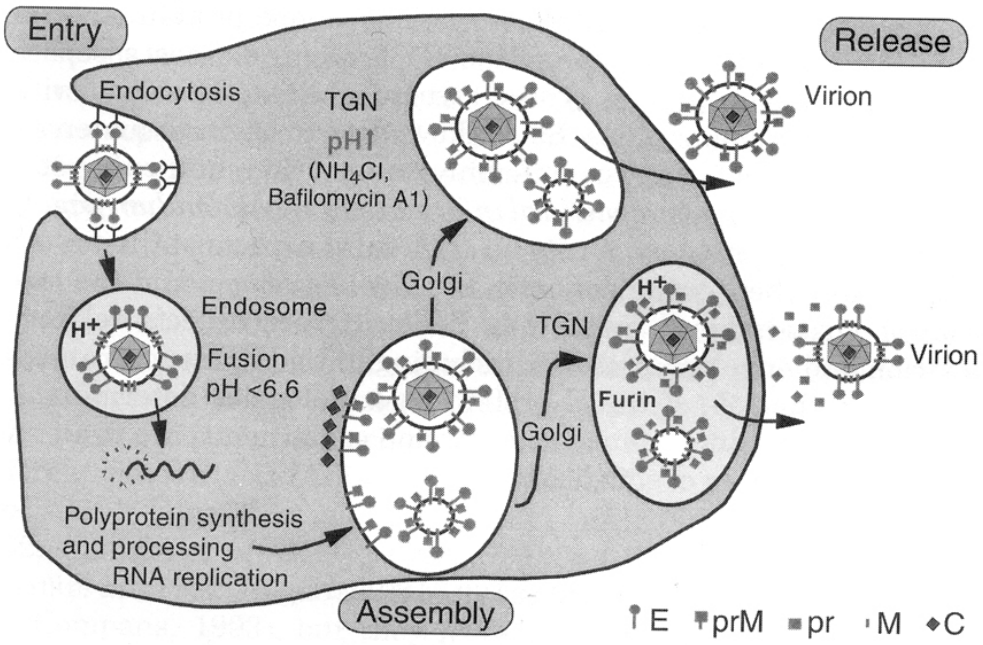


**Figure 1.3 Cellular life cycle of WNV. TGN, trans-Golgi network; SHA, slowly sedimenting hemagglutinin; RSP, recombinant subviral particle (Adapted from Chambers and Monath 2003)**

process on the membrane of the endoplasmic reticulum. Immature viral particles are then transported through the trans-Golgi network, prM is cleaved by furin, and mature virions are released from the cell by exocytosis with a surface composed of 90 E dimers arranged in a complex "herringbone" configuration in which 3 E monomers form an asymmetrical

unit (Gollins and Porterfield 1986b, Chambers and Klowden 1990, Heinz *et al.* 1994, Allison *et al.* 1995, Mackenzie *et al.* 1996, Stadler *et al.* 1997, Mackenzie and Westaway 2001, Lindenbach 2003).

## TRANSMISSION CYCLES OF WNV

Enzootic WNV transmission occurs predominantly between ornithophilic mosquitoes of the genus Culex and wild birds (Fig. 1.4), although WNV can infect a wide variety of vertebrates in nature (Granwehr *et al.* 2004, Hubalek 2004). Within the U.S. 60 of the 174 endemic species of mosquitoes currently recognized in the United States have tested positive for WNV. The vector status and epidemiological significance of many of these species remains unclear (Ahmed *et al.* 1979, Hayes *et al.* 1980, Goddard *et al.* 2002, Andreadis *et al.* 2004, Ebel *et al.* 2005), but Turell *et al.* (2005) evaluated numerous species for their ability to transmit WNV under laboratory conditions and also summarized the results of field isolations and found that many species were competent vectors. Although *Cx. pipiens* was a major epizootic vector of WNV among birds in both the outbreak in New York City and in Bucharest (Campbell *et al.* 2002), its role in transmitting WNV to humans has not been resolved. Currently, *Cx. pipiens quinquefasciatus* has not been implicated in epidemic urban WNV transmission, although the potential for this species to serve as a vector in endemic settings is significant

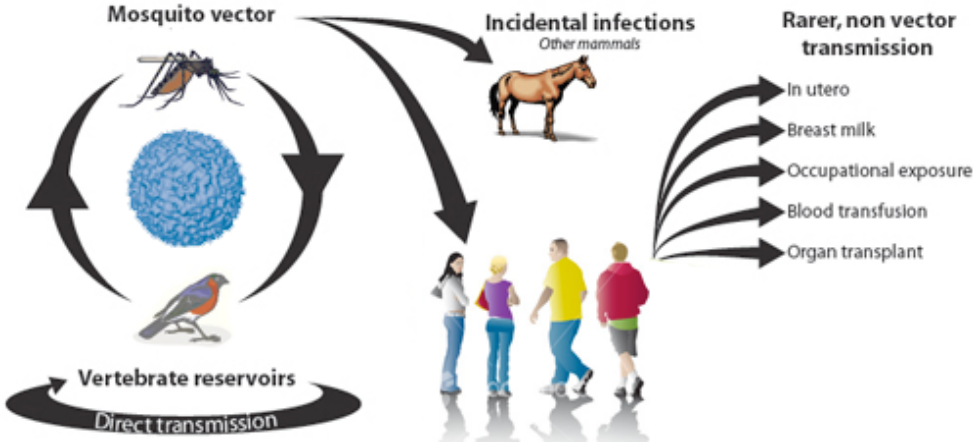


**Figure 1.4 Transmission dynamics of WNV. Adapted from Gould and Fikrig 2004.**

(Sardelis *et al.* 2001). Nonetheless, *Culex* mosquitoes appear to play a major role in human transmission worldwide (Campbell *et al.* 2002). The ornithophilic tendency of *Culex* species dictates that WNV may often rely on mosquitoes with more promiscuous feeding habits to infect vertebrates (Wang 1975). Anthropophilic mosquitoes, such as *Ae. aegypti*, may serve as bridge vectors to humans (Turell *et al.* 2005). Isolates of WNV have also been obtained from other arthropods, including hard and soft ticks (Lawrie *et al.* 2004, Mumcuoglu *et al.* 2005). Ticks, especially nidicolous species, may facilitate maintenance of WNV among avian reservoirs; their role as epidemic vectors is probably minor.

Birds are the natural amplifying host for WNV, with numerous species developing a transient high viremia (>11 $\log_{10}$pfu/ml) that allows transmission of the virus to feeding mosquitoes (Komar *et al.* 2003). Many avian species recover from infection and develop stable immunity, while some species, particularly in the family Corvidae, are exceptionally susceptible to WNV and become ill and die (Komar *et al.* 2003). Migration of viremic birds is an accepted means of WNV dispersal in middle eastern and European countries, and there is a preponderance of evidence to suggest that such a mechanism occurs in the Western hemisphere as well (Rappole *et al.* 2000).

A diverse range of mammalian species is susceptible to WNV infection as demonstrated by experimental infection or detection of natural infection (Campbell *et al.* 2002). Although mammals have traditionally been viewed as dead-end hosts due to their inability to support high-titer viremias, this belief has been challenged recently by experimental evidence that a WNV-infected mosquito can infect uninfected mosquitoes co-feeding on an uninfected mouse (Higgs *et al.* 2005). This so-called "non-systemic transmission" is caused when an infected mosquito inoculates WNV into a host, then prior to replication of the virus in the host tissues, temporally or spatially proximate feeding naïve mosquitoes ingest WNV, thereby becoming infected. The discovery of this mode of transmission challenged the paradigm that arboviruses are transmitted only to mosquitoes feeding on viremic hosts. This phenomenon obviates the requirement for hosts to produce viremias, or even to support viral replication, to contribute to the

maintenance of WNV. The significance of this observation in a natural setting is dictated by the probability of infected and uninfected mosquitoes feeding in proximity. In addition, non-systemic transmission has been demonstrated to occur even in hosts immune to flavivirues (Nuttall *et al.* 1994).

## EPIDEMIOLOGY OF WNV

WNV was first recognized in 1937 subsequent to the isolation of this virus from the blood of a woman in the West Nile district of Uganda (Campbell *et al.* 2002). The patient presented in the setting of a large epidemiologic study of yellow fever virus,

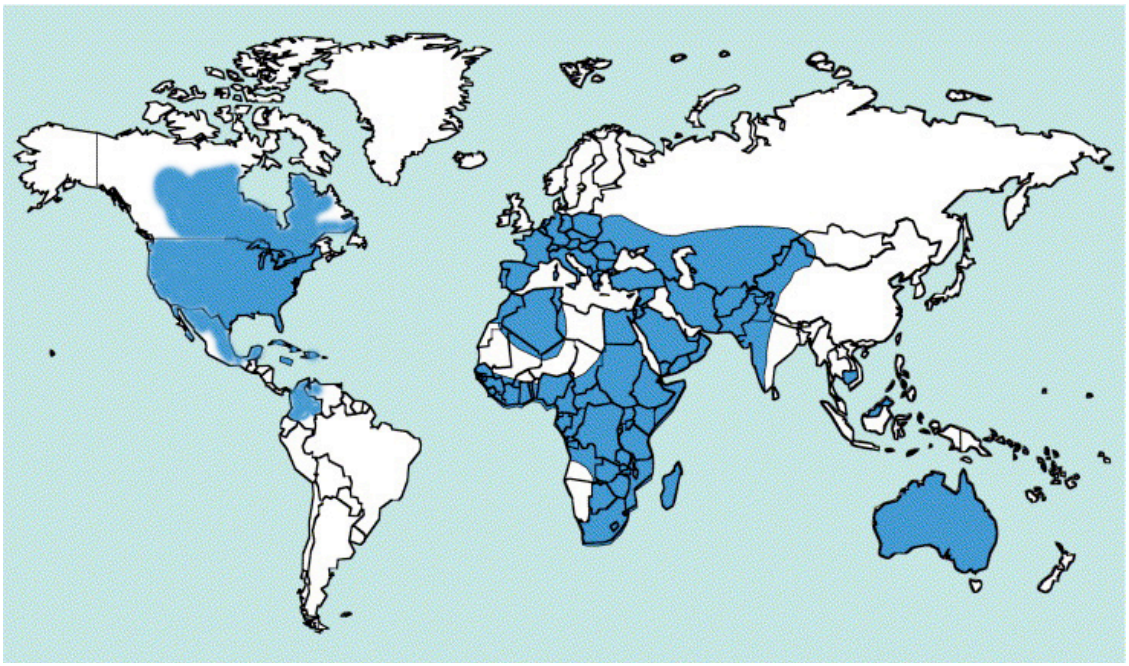

**Figure 1.5 Approximate worldwide distribution of WNV in 2006 (shown in blue). Adapted from Campbell *et al.*, 2002.**

however, inoculations of mice with the patient's serum resulted in the isolation of a virus with physical and pathologic properties similar to those of flaviviruses (Campbell *et al.* 2002). Following this case, WNV was sporadically isolated from febrile patients in North Africa and the Middle East. Several WNV outbreaks in Egypt between 1951 and 1954 led to a more comprehensive understanding of the ecology, epidemiology, and

clinical characteristics of WNV (Murgue *et al.* 2001). On the basis of the detection of WNV in the blood of several children and a high seroprevalence rate among residents of a village north of Cairo in 1950, extended studies of WNV was initiated in 1951 in the upper Nile Delta region (Murgue *et al.* 2001). The studies included human and animal serosurveys; identification of vectors; experimental infection of birds, equines, arthropods, and humans; and ecologic risk assessments. Serosurveys demonstrated that WNV was endemic along the Nile river, with seroprevalence rates approaching 60%. On numerous instances WNV was found to cause serious central nervous system infections in the elderly (Murgue *et al.* 2001), and as early as the 1960s it was identified as an etiologic agent of equine encephalitis in Egypt and France (Murgue *et al.* 2001). This pattern of minor and periodic epidemics in Africa, the Middle East, and southern Europe continued until 1974 when a number of large epidemics occurred with increasing frequency (Fig. 1.5). In 1974, 10,000 human fever cases were reported in South Africa (Murgue *et al.* 2001). Another South African outbreak occurred in 1983, demonstrating that WNV was endemic and widespread on the temperate inland plateau. In 1996 WNV emerged in Romania leading to ~400 cases of human encephalitis, including 16 deaths (Murgue *et al.* 2001). The common house mosquito, *Cx. pipiens*, was identified as a principle vector, and the outbreak was centered in decrepit apartment buildings that had broken window screens, flooded basements, and raw sewage in urban Romania, which exposed people to a large population of WNV-infected mosquitoes. This outbreak was striking because it was the first outbreak of the WNV in which the majority of symptomatic cases involved CNS infection. West Nile virus outbreaks also occurred in Algeria in 1994, Morocco in 1996, the Czech Republic in 1997, and the Democratic Republic of the Congo and Italy in 1998 (Murgue *et al.* 2001). Russia suffered a major outbreak of WNV in late July 1999, with at least 500 cases and 40 deaths in regions such as Volgograd, Astrakhan, and Krasnodar. In 1997, a novel strain of WNV was isolated in Israel that is pathogenic to geese. This same strain emerged in the New York City in 1999 (Roehrig *et al.* 2002).

**WNV in the New World**

The introduction of WNV in North America was followed by progressive spread throughout the United States (Fig. 1.6). In 1999, this cryptogenic WNV outbreak initially presented as a focused event, strictly localized to New York, New Jersey, and Connecticut, with the majority of cases and fatalities found in New York City (Roehrig *et*

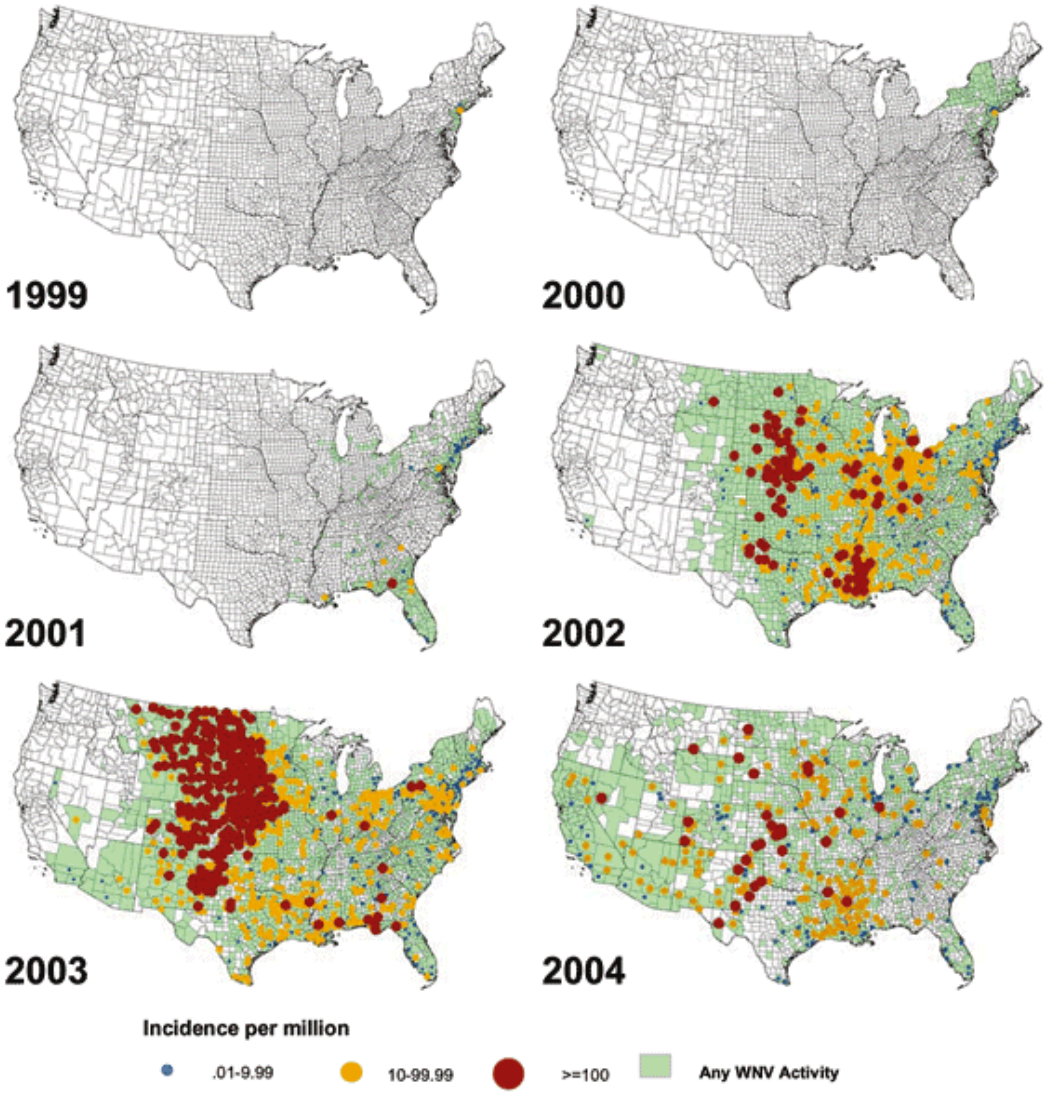


**Figure 1.6 Reported incidence of neuroinvasive West Nile virus disease by county in the United States from 1999–2004. Reported to Centers for Disease Control and Prevention by states as of April 21, 2005. http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.html.**

*al.* 2002). An extensive and exhaustive mosquito-control effort was undertaken to stem the virus in this new environment before it established itself. Efforts prove unsuccessful in early 2000, when WNV was detected in over-wintering mosquitoes captured in the sewers beneath the city (Nasci *et al.* 2001). During the summer of 2000, 21 cases of human WNV illness occurred among 10 counties in the northeastern U.S. The following year, 66 cases were detected among a much more widespread geographic area, involving 38 counties in 10 northeastern States (Roehrig *et al.* 2002). In the summer of 2002, however, the number of WNV cases in North America was unprecedented, becoming the largest outbreak of West Nile meningoencephalitis ever recorded and the largest outbreak of arboviral meningoencephalitis ever documented in the western hemisphere (Roehrig *et al.* 2002). WNV expanded its geographic range from east of the Mississippi River at the conclusion of the 2001 mosquito season to the Pacific Coast by the end of 2002. The number of cases in 2002 was overshadowed in 2003, when the number of reported human cases more than doubled to 9858, causing 262 fatalities (Briese and Bernard 2005). Since its introduction to the United States in 1999, WNV has spread rapidly leading to ~19,000 diagnosed human cases between 1999 and 2005 (http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm#maps) with an estimated 750,000 undiagnosed infections in 2003 alone (Busch *et al.* 2006). The range of WNV now covers all of the continental U.S. and has spread to parts of Canada, Mexico, Central America, the Caribbean, and even South America (Rappole *et al.* 2006).

**Table 1.1 Human WNV disease cases by clinical syndrome, as reported to the Centers for Disease Control and Prevention by April 2005. (Adapted from Hayes *et al.*, 2006)**

| Year | Total cases | Neuroinvasive cases | West Nile fever cases | Other clinical/unspecified | Fatalities |
|---|---|---|---|---|---|
| 1999 | 62 | 59 | 3 | 0 | 7 |
| 2000 | 21 | 19 | 2 | 0 | 2 |
| 2001 | 66 | 64 | 2 | 0 | 9 |
| 2002 | 4156 | 2946 | 1162 | 48 | 284 |
| 2003 | 9862 | 2866 | 6830 | 166 | 264 |
| 2004 | 2539 | 1142 | 1269 | 128 | 100 |
| Total | 16,706 | 7096 | 9268 | 342 | 666 |

## CLINICAL FEATURES OF WNV

In humans, most cases of WNV are transient infections with no overt symptoms (Mostashari *et al.* 2001). The incubation period is typically 2-6 days, but can range from 2-14 days. The clinical syndromes associated with WNV fever are non-specific, and thus diagnosis cannot usually be made on clinical presentation alone (Campbell *et al.* 2002). Acute onset generally begins suddenly with fever, headache, and myalgia. Uncomplicated WNV fever usually lasts about a week, but recovery is prolonged commonly by fatigue. A significant percentage of patients have a generalised roseolar or maculopapular rash that usually clears in the same time frame as the fever. The recent epidemic in the Western hemisphere was distinct in that it caused a reduced frequency of cases reporting rash and lymphadenopathy, and the incidence of meningoencephalitis cases (Campbell *et al.* 2002). The majority of contemporary neurological WNV cases are classified as encephalitis, with the remainder being meningitis (Nash *et al.* 2001). WNV-induced meningitis has a relatively low mortality and progresses akin to typical viral meningitis cases (Ceausu 1997). Similarly, WNV encephalitis clinically resembles other viral encephalitides, with fever, headache, and non-specific symptoms usually preceding encephalitis. Signs and symptoms of encephalitis may include mental status changes, vomiting, depressed deep-tendon reflexes, diffuse muscle weakness, flaccid paralysis, respiratory failure, and in 15% of cases cerebral dysfunction can progress to coma and occasionally death (Campbell *et al.* 2002). Muscle weakness, often proximal muscle weakness, is a very frequent presentation in WNV encephalitis patients, and paresis or paralysis is observed in 10-20% of cases (Nash *et al.* 2001). Onset of paralysis may be isolated to one limb or multiple limbs, is usually asymmetric, and may involve cranial nerves. Rare cases of WNV-associated hepatitis, pancreatitis, myocarditis, cardiac dysrhythmia, rhabdomyolysis, orchitis, uveitis, vitritis, and optic neuritis have been reported (Hayes and Gubler 2006). Some patients may present with a clinical picture of sepsis (hypotension, tachycardia, tachypnea, fever, and rigors) (Hayes and Gubler 2006). Central nervous system (CNS) inflammation is rare and typical cerebrospinal fluid findings are as follows: mild pleocytosis with lymphocytes usually in prominence;

elevated protein levels; and normal glucose concentration (Hayes and Gubler 2006). Magnetic resonance imaging of the brain can be normal or show increased signal density in the leptomeninges, cortex, subcortical white matter, brainstem, cerebellar vermis, thalamus, or deep nuclei such as the substantia nigra (Nash *et al.* 2001). Elevated blood cell counts have been reported, but leucocytosis is evident in less than 50% of encephalitis patients. In recent epidemics, where neurological symptoms were more prominent, overall meningoencephalitis case fatality ratios were 4-14%, although ratios were significantly higher when exclusively encephalitis cases are considered (up to 24% case fatality) (Chowers 2001). The majority of deaths among encephalitis patients were in the elderly and risk of fatal outcome increases with age (Table 1.1) (Campbell *et al.* 2002). Patients surviving meningitis with no focal neurologic deficits usually recover completely, although for patients with encephalitis and flaccid paralysis the prognoses are poor, and severe neurologic deficits often persist for months or are lifelong (Sejvar *et al.* 2003a,b).

## PREVENTION AND PROTECTION

No human vaccine for WNV is available, although several laboratories are currently conducting vaccine research (Campbell *et al.* 2002). Effective prevention of human WNV infections depends on the development of arboviral surveillance and vector mosquito control programs in areas where the virus occurs. Local vectors and breeding sites for all vector mosquito species should be identified. Mosquito surveillance and control should be implemented early in the year to disrupt WNV amplification in birds and mosquitoes. Additionally, an important constituent of any prevention program is public outreach to educate members of the community on how to avoid or decrease the risk of being bitten by potentially infected mosquitoes. Generally, areas where mosquitoes are common should be avoided, and individuals should limit outdoor activity during peak mosquito biting periods.

## DETECTION OF WNV

### Serology

For clinical diagnosis, serology continues to be the dominant method of identifying infections (Campbell *et al.* 2002). Of the available techniques plaque reduction neutralization test (PRNT), although not the most sensitive, remains the more specific of diagnostic tests. Thus, the development of WNV-specific neutralizing antibody between acute and convalescent stages of illness is the most persuasive serological evidence for infection. As with other serological assays, to be conclusive other closely related members of the virus family (in this case Flaviviridae) must be included in the assay for comparison. Besides neutralization tests, antibody-capture enzyme immunoassays (EIA) are frequently used, but positive results with this assay, or immunofluorescent antibody (IFA) tests, must be considered presumptive until verified by PRNT. Hemagglutination-inhibition and complement-fixation tests are not as commonly used for diagnosis as they once were.

### Virus Isolation

When feasible, unambiguous clinical diagnosis of WNV infection can be obtained through direct virus isolation. Generally, samples such as serum, cerebrospinal fluid, or other tissues can be overlaid on cell culture or injected into suckling mice for isolation, followed by specific identification via IFA or other molecular techniques. Nevertheless, it should be pointed out that WNV is rarely isolated from human samples, even with patients at the acute phase of illness. West Nile virus can readily be detected from tissues (principally brain) of fatal WNV cases.

**Real-time reverse-transcription polymerase chain reaction (RT-PCR)-based assays for detection and quantification of WNV**

Over the last decade, propelled by the enhanced diagnostic demand of the North

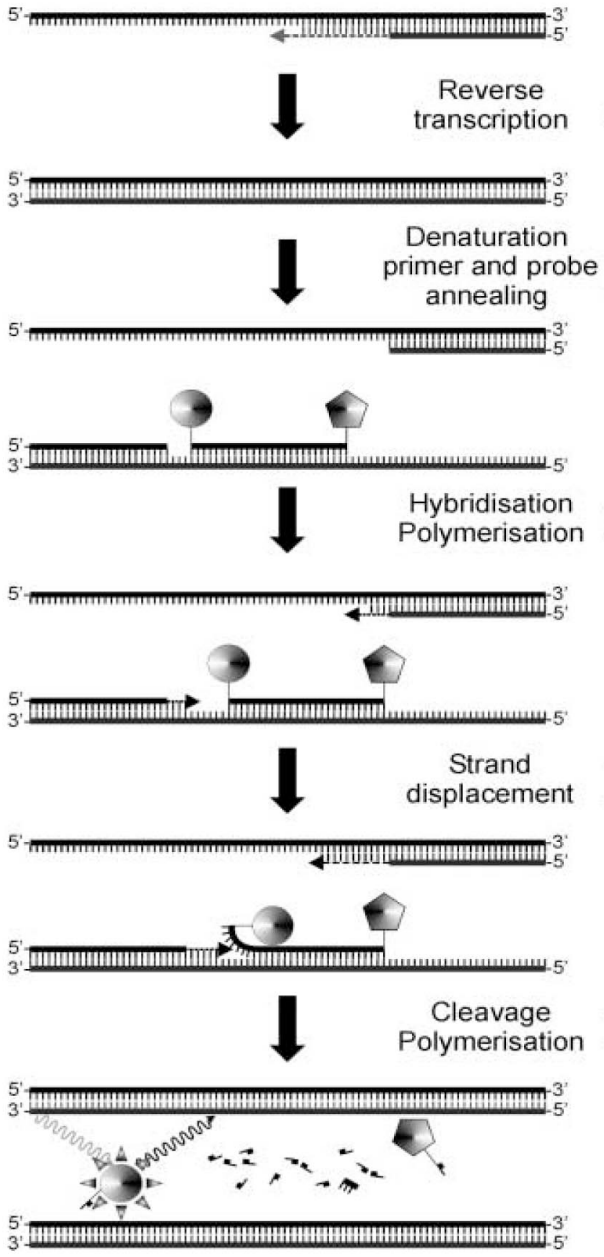


**Figure 1.7 Illustration of the steps of the real-time RT-PCR assay.**

American WNV outbreak, a number of molecular techniques have been developed and optimized for detection of WNV. During this time an assay that modified standard reverse-transcription polymerase chain reaction (RT-PCR) with the addition of fluorescent-labelled oligonucleotides probes demonstrated itself to be highly useful at rapid and accurate detection of WNV in tissues. This assay, called real-time RT-PCR, also offers the advantage of increased sensitivity, specificity, and the potential to quantify the WNV in samples. The increased specificity of this assay compared to other molecular techniques is due to the inclusion of a target-specific internal probe in the reaction. Hybridization of this probe to the target sequence and the stringent requirement that this sequence fall between and in close proximity to the primer sequence for subsequent hydrolysis of the probe, obviate any post-reaction characterization of the amplified DNA. As a result, there is no need to handle amplified DNA in the laboratory, thus reducing the possibility of amplicon contamination. In real-time RT-PCR, RT and PCR amplification reactions are identical to the classical RT-PCR format, except for the addition of the virus-specific oligonucleotides probe, which is dual-labelled with a fluorescent reporter dye and a quencher molecule (Lanciotti *et al.* 2000). When the probe is intact, either free in solution or bound to target DNA, the reporter and the quencher are in close proximity and the emission of fluorescence from the reporter is quenched. The Taq polymerase enzymes used in this assay have a 5' to 3' exonuclease activity, and consequently, during replication of a strand of DNA the polymerase on the target sequence will encounter the bound probe and cleave it (Fig. 1.7). This cleavage results in the reporter dye being released into solution, and its physical separation from the quencher molecule leads to an increase in fluorescence measured by a spectrophotometer. The cycle number at which point the fluorescence in the sample tubes exceeds background fluorescence (as measured in the tubes during the first few cycles) is referred to as the threshold cycle ($C_T$). The $C_T$ value is directly proportional to the original amount of starting target RNA present in the reaction. Therefore, by including quantified RNA standards (or RNA generated from quantified virus samples) of the viral target in the experiment, a standard curve can be generated by plotting the $C_T$ vs. the log of

standard quantity. This standard curve can then be used to estimate the starting quantity of viral RNA in the test specimens. Assuredly, in a laboratory setting, real-time RT-PCR significantly simplifies and accelerates the process of reproducibly quantifying RNA. The combination of amplification with simultaneous hybridization results in high sensitivity without compromising specificity, and furthermore, assays are readily standardised, making comparisons from different samples or tissues more reliable.

## PATHOGENESIS AND VIRULENCE OF WNV IN VERTEBRATES

Most infections with flaviviruses, including those capable of causing encephalitis, are asymptomatic or give rise to a mild febrile illness. Only a small percentage of infections proceed to severe disease and death (Sejvar *et al.* 2003a,b). The pathogenesis of these diseases involves complex interactions between the virus and numerous host factors. Small animal models have been useful tools for understanding how different factors govern the course of vertebrate infection. Rodent models have been used historically, and are particularly useful presently because of the ready availability of knock-out strains of mice. Susceptibility of mice to flaviviruses and WNV has been linked to a number of genetic host factors that are located on chromosome 5 and have been mapped to the oligoadenylate synthetase (OAS) gene cluster (Sangster *et al.* 1994). Presumably, the mechanism of resistance is associated with RNase L, an interferon-induced nuclease which, upon activation, destroys all RNA within the cell (both cellular and viral). RNase L is one of the genes OAS activates, nonetheless the specific connection of this gene cluster to susceptibility has not been determined. It has been suggested that the mechanism of flavivirus resistance may lie within other functions of OAS in cellular response to viral pathology (Samuel 2002). *In vitro* work demonstrated that numerous intracellular proteins interact with untranslated regions of flaviviruses (Brinton 2001), and there is some evidence to suggest that these associations are important for viral replication (Li *et al.* 2002); the differential expression of these proteins may therefore provide an explanation for variable species/cell susceptibility to flaviviruses.

The key question in pathogenesis of encephalitic flaviviral disease concerns conditions that give rise to virus entry from the blood into the central nervous system (CNS), but it is the requisite extraneural replication and pathogenesis that dictates whether CNS infection will occur. As flaviviruses are typically arthropod-borne infections, and in the case of WNV, mosquito-borne, the virions are first inoculated primarily in the extravascular tissue of the skin (Turell *et al.* 1995). Thus, instead of an immediate spread of the virus through the bloodstream, flaviviruses first replicate locally in the dermal tissue followed by spread to draining lymph nodes (LN).

**Peripheral Replication and Cellular Tropisms**

Additional research is necessary to characterize the cellular receptors required for attachment and entry of WNV, although many candidate proteins have been identified including DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and alpha v beta 3 integrin. In vitro data has identified DC-SIGN as a possible receptor (Davis *et al.* 2006). Nonpermissive cell types transfected with this lectin molecule support virus replication and monoclonal antibody specific for DC-SIGN suppressed infection (Davis *et al.* 2006). These results suggest that DC-SIGN or a similar lectin may represent a common attachment receptor molecule for flaviviruses in both vertebrates and arthropods, although some data supports the utilization of different membrane proteins for cellular entry in insects versus vertebrates (Martinez-Barragan and del Angel 2001). An assay using mosquito cell lines and an overlay protein blot identified a 140-kDa, 95-kDa, 70-kDa and 55-kDa plasma membrane-associated molecules that binds WNV (Chu *et al.* 2005). Murine antibodies generated against the 95-kDa and 70-kDa membrane proteins effectively blocked WNV, Japanese encephalitis virus (JEV) and Dengue virus (DENV) serotype 2 infection in C6/36 (*Aedes albopictus*) mosquito cells. It is interesting to note that arbovirus particles produced in mosquito cell lines were more infectious for primary DC cells (Klimstra *et al.* 2003). Studies found that neurovirulent yellow fever virus (YFV) preferentially binds mouse brain membrane-protein preparations (Ni and Barrett 1998), allowing for the possibility that alternate receptors may lead to specific tropism to the CNS. Recently, alpha v beta 3 integrin has

also been associated with flavivirus attachment. Infection of WNV proved to be significantly inhibited in Vero (monkey kidney) cells pretreated with antibodies against alpha v beta 3 integrin and its subunits by receptor competition assay (Chu and Ng 2004). Further, cells pretreated with antibodies against alpha v beta 3 integrin can effectively inhibit JEV and, to a lesser extent, DENV infections. Additionally, gene silencing of the beta 3 integrin subunit in cells resulted in cellular resistance to WNV infection (Chu and Ng 2004). Also, expression of recombinant human beta 3 integrin substantially increased the permissiveness of CS-1 melanoma cells for WNV infection (Chu and Ng 2004). These receptor studies with WNV are largely based on *in vitro* models and, therefore, *in vivo* significance of these receptor proteins is difficult to assess.

Following peripheral inoculation, as occurs in natural transmission, flaviviruses have limited replication in the skin; instead they spread quickly to the draining lymph node probably within immature DCs and Langerhans cells (LC) that are susceptible to WNV infection (Byrne *et al.* 2001). Studies with WNV show that within 24 hours (h) of infection infected LCs migrate from the epidermis to local lymph nodes (Byrne *et al.* 2001). Generally, after antigen acquisition and processing, DCs migrate via lymph vessels to the T-cell areas of regional secondary lymphoid organs where they present major histocompatibility complex (MHC) class I- and II-restricted peptides to naive T-cells. Therefore, the role of DCs in flavivirus infection may be pleiotropic, as their migration to draining lymph nodes promotes antigen presentation, while simultaneously facilitating the spread of the virus. The conflicting roles of DCs and the fate of these cells (whether apoptosis or persistent infection) is particularly important since there is a minimum requirement for activated DCs in an effective T cell response and rapid viral replication demands efficient and appropriate action of DCs (Ludewig *et al.* 1998).

Although primary flavivirus spread and replication in the lymphatic system is apparent, the peripheral sites of replication responsible for the considerable amplification necessary to produce a significant viremia are currently unclear. Flavivirus infections often have no detectable viremia in humans. Studies in humans are limited, but in mouse models WNV is detected in the serum within 24 to 48 h post infection, and peak viremia

occurs between day 2 – 4 pi (Kramer and Bernard 2001a). Concurrently, virus can be detected in visceral tissues. West Nile virus replicates in the spleen, heart, and kidneys of hamsters (Tonry *et al.* 2005) – few clear pathological signs were noted in spleen or heart tissue, although focal peri-tubular inflammation was intermittently observed in the kidneys of some animals. Infection of the vascular endothelium has been observed with some flaviviruses, but these cells have not been implicated as a major source of replication (Liou and Hsu 1998). The length of the incubation period (1 – 2 weeks) suggests that an extraneural phase of viral growth precedes virus entry into the brain, although it is unknown which tissues are important for peripheral replication of flaviviruses.

**Neuroinvasion**

The key to understanding the pathogenesis of encephalitic flaviviral disease lies in elucidating conditions that give rise to virus entry from the blood into the CNS (Fig. 1.8).

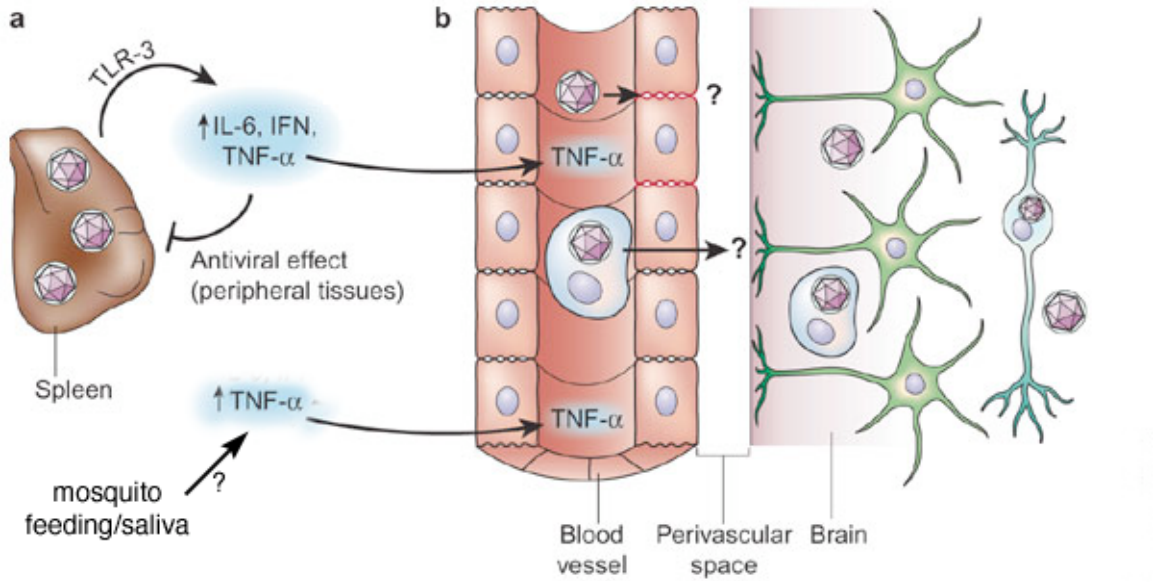


**Figure 1.8 WNV infection in the periphery induces Toll-like receptor-3-dependent secretion of cytokines, including IFN, IL-6, and TNF-α. Mosquito feeding has, likewise, been shown to cause production of TNF-α at early time points. Induction of TNF-α facilitates WNV penetration across the BBB, although the exact mechanism remains unclear. Endothelial cells or their tight junctions appear to be altered by this cytokine making them permissive to WNV or WNV-infected leukocytes. Adapted from Diamond and Klein 2004.**

The rare stochastic event that permits virus in the circulation to breach the blood-brain barrier (BBB) appears to be the crucial step determining severe disease. There are at least four possible routes of flavivirus penetration into the CNS via: 1) infection of peripheral neurons; 2) infection of olfactory neurons; 3) transcytosis through vascular endothelial cells of the brain; and 4) diffusion between capillary endothelial cells during leakiness of the BBB. Previous studies have demonstrated that disruption of the BBB facilitates entry of WNV into the CNS (Lustig *et al.* 1992), therefore it is possible that host factors that alter the permeability of the BBB may affect flavivirus neuroinvasion. Cytokines including interferon (IFN)-γ and tumor necrosis factor (TNF)-α and pathological actions of immune cells may have such an effect (Wang *et al.* 2003a).

In murine models WNV is first detected in the CNS at day 4 post-infection. It is important to note that the route of peripheral inoculation influences the rate of neuroinvasion. Infection via intraperitoneal (i.p.) or intravenous (i.v.) routes leads to earlier infection of the CNS as compared to subcutaneous (s.c.) inoculation (Chambers and Diamond 2003a). Additionally, the timing and amplitude of viremia may be important in determining whether neuroinvasion occurs. Higher, sustained, and/or early viremia is correlated with increased neuroinvasion – enhanced viremia presumably increases the chance that a virion will cross the BBB and early viremia allows for this chance prior to appearance of the adaptive immune response (Huang *et al.* 1963, Albrecht 1968, Monath *et al.* 1986). Flaviviruses are often detected in the olfactory lobe prior to spread into the brain, although WNV can be detected simultaneously at multiple sites in the brain and spinal cord (Diamond *et al.* 2003c). This simultaneous infection of distal sites of the CNS suggests a hematogenous route of dissemination for WNV (Diamond *et al.* 2003c). The olfactory neuroepithelium is particularly vulnerable because it is not protected by the BBB and has a rich supply of capillaries. Histological examination of fatal human cases show viral antigen in neurons, with the greatest involvement in the thalamus and brainstem (Chambers and Diamond 2003a). Despite some evidence that flaviviruses can replicate in oligodendrocytes (Jordan *et al.* 2000) and astrocytes (Chen *et al.* 2000), neurons are the primary target for these viruses (Xiao *et al.* 2001).

Limited evidence of inflammatory response is associated with this flavivirus intrusion into the CNS (Chambers and Diamond 2003a). When an inflammatory response has been detected in the CNS the meningeal layers in the brain substance are implicated, and inflammation takes the form of perivascular lymphocytic accumulation, parenchymal infiltrates, and microglial nodules (Chambers and Diamond 2003a). In response to neuroinvasive WNV infection, macrophages are the predominant inflammatory cell that invade the brain parenchyma, and are generally localized near virus-infected cells (Chambers and Diamond 2003a). Lymphocytes are also observed in the brain following flavivirus infection, with T and B cells mainly seen in perivascular regions. Viral antigen clearance from the brain is temporally associated with activation of the adaptive immune response. Although WNV neutralizing antibody has not been detected in the brain of WNV-infected mice, many studies have demonstrated that a vigorous antibody response is important for recovery from acute encephalitis (Chambers and Diamond 2003b).

Elevated levels of TNF in the serum and CNS, are suggestive of inflammation, and is a poor prognostic indicator for recovery (Ravi *et al.* 1997). West Nile virus-induced mortality is ultimately a result of infection of many neurons and disruption of neurological function (Agamanolis *et al.* 2003). Cytopathic effects are primarily observed in virus-infected neurons, but adjacent, uninfected cells also exhibit pathology (Ceccaldi *et al.* 2004). Cell death is classically associated with degenerative necrosis, although it is unclear whether the majority of infected cells die due to necrosis or apoptosis (Murphy *et al.* 1968). Studies with rodent models demonstrated that neuroadapted DENV and WNV lineage I viruses induce apoptosis in infected and by-stander cells in the CNS (Despres *et al.* 1998, Xiao *et al.* 2001). Additionally, the expression of cloned WNV capsid protein (Yang *et al.* 2002) or DENV E protein (Duarte dos Santos *et al.* 2000) when transfected into cells directly induces apoptosis of neurons. Host-associated mechanisms such as IFN-α-signalling pathways and nuclear factor-κ B (NF-κB) may also provoke apoptosis. Thus, neuron cell death can likely be caused by multiple virus and host related mechanisms.

## IMMUNE RESPONSE TO FLAVIVIRUSES

Flavivirus infections and the immune response directed against them begin in the extraneural tissues. The success of the immune response in limiting virus replication and spread is integral to host survival. As flaviviruses are almost exclusively arthropod-borne (arbo), the first cells to encounter an invading virion are indubitably the cells of the dermis and epidermis, as this is the site to which an arthropod vector feeds and delivers virus.

### Innate immune response

The innate immune system is comprised of the cells and mechanisms that defend the host from infection by viruses and other organisms, in a non-specific manner. Effectors of the innate system recognize, and respond to, pathogens in a generic way. The innate system does not confer long-lasting or protective immunity to the host, but is an immediate reaction to an infection that impede an infection while a specific, adaptive immune response can be initiated. The principle functions of the innate immune system include: the recruitment of immune cells to sites of infection via the production of chemical mediators, including specialized chemical mediators called cytokines; the removal of foreign material, including bacteria, parasites, and viruses in tissues, serum, lymph, by specialized cells; and the activation of the adaptive immune system through antigen presentation.

#### *Cytokines*

Cytokines are non-antibody proteins secreted by cells that act as intercellular mediators (Fig. 1.9) (Thomson *et al.* 1998). They differ from classical hormones in that they are produced by a number of tissue or cell types rather than by specialised glands. Cytokines generally act locally in a paracrine or autocrine rather than endocrine manner, and are known to play a role in host defense against viral infections (Thomson *et al.* 1998). Most vertebrate cell types can produce some cytokines, but lymphocytes, APCs, monocytes, endothelial cells, keratinocytes, and fibroblasts secrete these protein mediators most prominently.

Cytokines are specifically involved in lymphocyte activation, proliferation, and differentiation, as well as inflammation, diapedesis, and anti-viral activities. Expression patterns of cytokine mRNA dictate the specific response of a host to a pathogen. Indeed, early polar shifts in the expression of these messengers can have downstream effects later

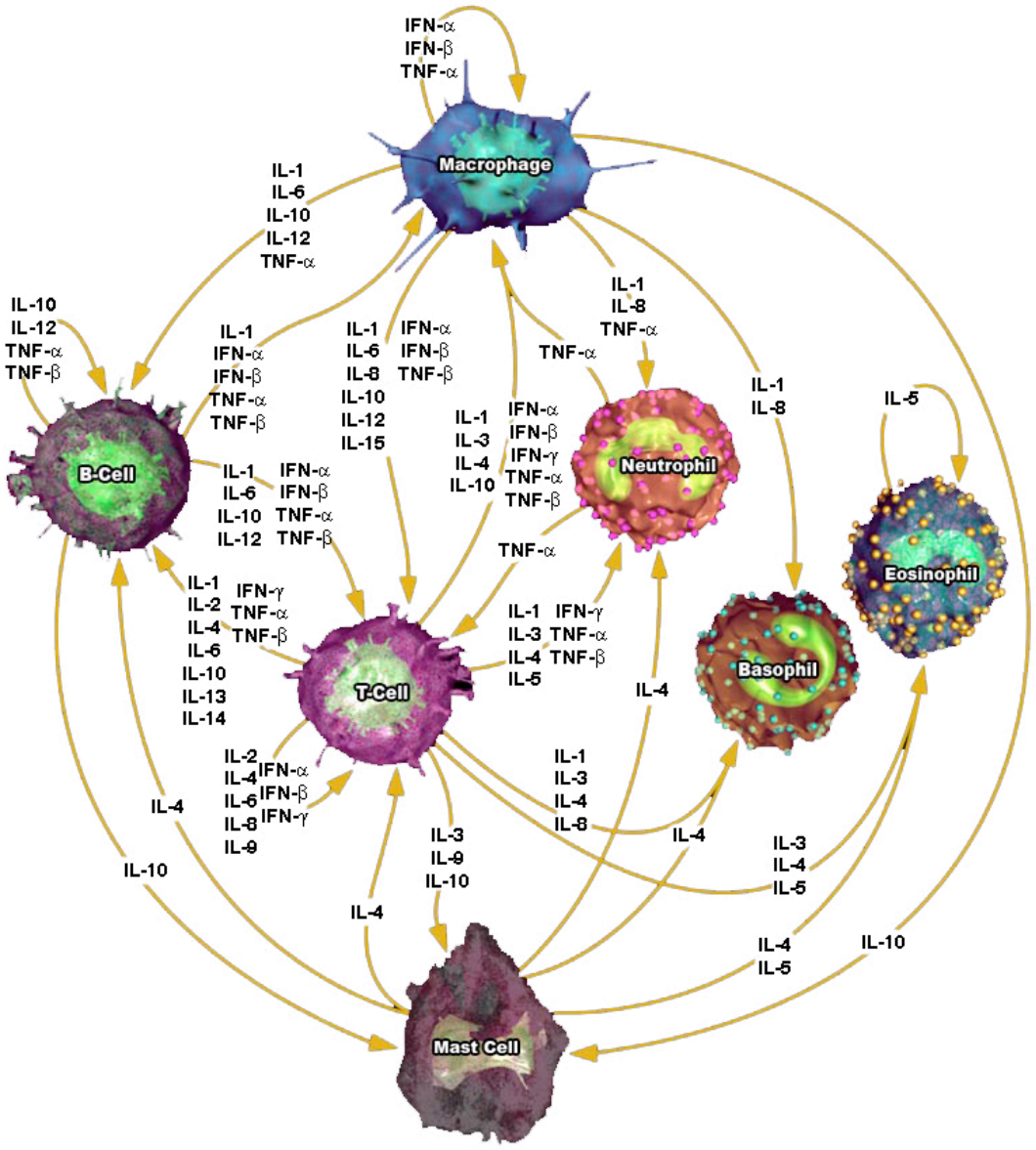


**Figure 1.9 Cytokine signalling between cells of the immune response.**

in infection (Pacsa *et al.* 2000). While cytokines, such as interferon (IFN)-α/β, IFN-γ, and TNF-α, have the potential to trigger the activation of intracellular antiviral pathways after binding to specific receptors on the surface of infected cells, other cytokines, such as IL-1β, IL-2, IL-12, IL-13, and IL-18, contribute to the antiviral response indirectly by modulating various aspects of the immune response, including the autocrine and paracrine up-regulation of IFN-α/β, IFN-γ, and TNF-α (Thomson *et al.* 1998). Most cell types in vertebrates respond to an incoming viral infection by secreting IFNα/β. Alternatively, the production of IFN-γ and TNF-α is largely confined to cells of the immune system, such as natural killer (NK) cells, T-helper ($T_H$) cells, cytotoxic T lymphocytes (CTL), macrophages, and DCs (Thomson *et al.* 1998). Detection of mRNA expression is widely used to investigate the cytokine profiles at the sites of infection and inflammation, since tissue samples for analysis are often too small to permit quantification of cytokines at the protein level. Real-time RT-PCR has emerged as a highly sensitive and accurate method for quantifying cytokine expression.

Type I interferons

The earliest response to flaviviruses is non-specific and includes type I IFN production, including IFN–α and –β, by leukocytes and fibroblasts. These IFNs induce production of approximately 20-30 proteins, and the function of many of these is not fully understood (Chambers and Diamond 2003a). However, three of the proteins (OAS, protein kinase (PKR kinase), and the Mx protein GTPases) that appear to play an important role in the induction of the anti-viral state have been intensively studied (Haller *et al.* 2006). 2'5'Oligo A synthetase, is an enzyme that converts adenosine-triphosphate (ATP) into a unique polymer (2'5' Oligo A) containing 2'- 5'phophodiester bonds (Chambers and Diamond 2003a). Double stranded RNA is required for the activity of this enzyme. The 2'5'Oligo A in turn activates RNAse L which then breaks down viral messenger RNA (mRNA). The second protein is a PKR kinase that, in the presence of double stranded RNA, is autophosphorylated and thereby activated (Chambers and Diamond 2003a). The activated protein kinase in turn phosphorylates the elongation factor eukaryotic initiation factor-2 (eIF-2) and inactivates it. By the action of this IFN-

induced enzyme, protein synthesis is inhibited. The third protein family, Mx protein GTPases, target viral nucleocapsids and inhibit RNA synthesis (Chambers and Diamond 2003a). Although the infected cell may die as a consequence of the inhibition of host protein synthesis, the progress of the infection is stopped since viral protein synthesis is also inhibited. Further, other IFN induced mediators signal surrounding cells to become activated into a virus resistant state. Uninfected cells are not killed by IFN treatment since activation of the two enzymes requires double stranded RNA, which is not produced in normal cells (King and Kesson 1988). IFN can help modulate immune responses by its effects on class I and class II MHC molecules. IFN-α and IFN-β increase expression of class I molecules on all cells thereby promoting recognition by cytolytic T cells, which then destroy virus-infected cells. The importance of this response is demonstrated by the effectiveness of prophylactic human treatment with IFN-α and the increased susceptibility of mice lacking type I IFNs (Brooks and Phillpotts 1999, Johnson and Roehrig 1999, Lucas *et al.* 2003). Administration of IFN-α prevents or suppresses flavivirus infections *in vivo*. *In vitro*, treatment of cells with type I IFNs 4 h prior to or shortly after infection with DENV or St. Louis encephalitis virus (SLE) significantly inhibits viral replication (Crance *et al.* 2003).

Type II interferons

Type II IFN, IFN-γ, also appears to be important in defense against flavivirus infections. IFN-γ is made primarily by cells of the immune system, including natural killer (NK) and T cells. This secreted mediator activates monocytic cells, enhances expression of a set of cytokine, increases MHC class I and II pathways, and induces nitric oxide (NO) production by monocytic phagocytes. Activation of monocytic cells makes them more resistant to infection, apt at destruction of invaders, and enhances their phagocytic activity through increased Fc receptor expression (Rothman and Ennis 1999), while the chemokines induced by IFN-γ aid in directing immune cells to the site of active infection. Fc receptors bind the heavy chain of antibodies and the Clq component of complement facilitating the phagocytic internalization and destruction of opsonized pathogens. As previously mentioned, MHC class I and II pathways help identify infected

cells and viral antigens, respectively, for immune effector cells. Nitric oxide (NO) production by cells presumably disrupts viral RNA and protein synthesis. It has been demonstrated that NO induction by IFN-γ-activated mouse macrophages inhibits JEV replication, and inhibition of NO lead to an increase in mortality (Lin *et al.* 1997, Saxena *et al.* 2001). Contrary to this observation, under certain circumstances NO expression is associated with increased pathogenesis with some flaviviruses (Kreil and Eibl 1996, Andrews *et al.* 1999). In these cases, NO knockout mice had enhanced survival following virus infection. Nonetheless, the potentially deleterious inflammatory response resulting from production of IFN-γ is more than compensated by the antiviral effect of this cytokine and particularly its ability to modulate NO production, class switching of antibody isotypes, and its direct antiviral effects in the CNS (Komatsu *et al.* 1999).

### *Cellular Response to Flavivirus Infection*

NK cells

Natural killer cells are large granular blood lymphocytes derived from the bone marrow. They are thought to contribute to the early response to infectious agents, particularly viruses, and mediate anti-tumoral immunity by their direct cytotoxic action against infected cells, and by secreting cytokines and chemokines that activate and recruit other cells of the immune system (Biron *et al.* 1999). Unlike the highly diverse T and B lymphocytes, NK cells do not express antigen-specific receptors. The diversity of the NK cell repertoire arises from the stochastic expression of inhibitory and stimulatory surface receptors on the target cells (Raulet *et al.* 2001). Studies assessing the role of NK cell activity during WNV infection demonstrated abbreviated cytolytic action against virus-infected cells (Liu *et al.* 1989). This suppression of NK cell activity is associated with enhanced MHC class I expression, suggesting that flaviviruses use a mechanism to evade the host NK cell cytolytic response by up-regulating MHC class I expression. Therefore, these viruses outmaneuver the host immune system, gaining an early advantage, in exchange for enhanced antigen presentation later in infection. Despite the recognized importance of NK function in many viral infections, cytotoxic effector pathways of NK cells may contribute to accelerated and more severe pathogenesis of encephalitic

flavivirus infection (Licon Luna *et al.* 2002). Cytotoxic effector mechanisms may be involved at two stages in the disease process: first, in the events leading to neuroinvasion, and second, in the pathology due to the inflammatory response in the brain. Both the granule exocytosis- and the Fas-mediated pathways of cytotoxicity could contribute to this disease progression. NK cells have been implicated as an important component in an early defence in numerous virus infections, and are induced by a variety of pathogens. Thus far, few studies have attempted to elucidate the role played by these members of the innate immune response in flavivirus infections (Liu and Mullbacher 1988, Diamond *et al.* 2003a, Navarro-Sanchez *et al.* 2005). The cytolytic activity of NK cells is enhanced by type I IFN signalling, which aids in viral clearance from host cells. Additionally, NK cells have been isolated from brains of mice infected with WNV, suggesting that NK cells can be recruited to the site of flavivirus infection *in vivo* (Chambers and Diamond 2003b). Those studies that have considered the role of NK cells suggest that they do not play a significant role in controlling flavivirus infections (Chambers and Diamond 2003a, Shrestha *et al.* 2006a). Adult beige mice that are deficient in the cytolytic function of NK cell showed no increased mortality in Murray Valley encephalitis (MVE) infection (Chambers and Diamond 2003a). With WNV, knocking out the perforin or Fas pathway, key NK cell processes that mediate infected cell lysis, led to no difference in survival as compared to wild-type mice (Shrestha *et al.* 2006a).

Macrophages

Macrophages also play an important role in host defense against infection, through both their direct effects on viruses and their orchestration of the immune response. Flavivirus infection of macrophages promotes production of NO, TNF-$\alpha$, IL-1$\beta$, and IL-8 (Bosch *et al.* 2002, Bouwman *et al.* 2002, Atrasheuskaya *et al.* 2003). The behavior of macrophages is fundamental to the pathogenesis of WNV, although the contribution versus deleterious effects depends on the regulation of macrophage activity (Chambers and Diamond 2003a). Studies have shown that inhibition of phagocytic activity of macrophages results in enhanced viremia and neuroinvasion (Ben-Nathan *et al.* 1996, Chambers and Diamond 2003a). One notable study demonstrated that

macrophage depletion resulted in increased WNV neuroinvasion even when non-neurovirulent strains were used (Ben-Nathan *et al.* 1996). The authors speculate that the lack of macrophages enhanced viremia leading to early invasion of the CNS. Additional data suggest that macrophages exert their protective influence via antigen presentation to B and T cells and cytokine production (Kulkarni *et al.* 1991, Marianneau *et al.* 1999). In the context of antigen presentation, addition of WNV-specific antibodies to macrophages resulted in an increase in their efficiency of presentation, likely due to elevated uptake of antigen by opsonization (Mullbacher *et al.* 2003). Some of the protective effects of macrophages appear to be mediated by production of reactive oxygen intermediates, especially NO. When cells are treated with an exogenous NO donor, decreasing JEV titers correlates with increasing NO levels (Lin *et al.* 1997). Nitric oxide was found to profoundly inhibit viral RNA synthesis, viral protein accumulation, and virus release from infected cells. Interestingly, competitive inhibition of NO by N-nitro-L-arginine methyl ester in mice and cells increases mortality due to JEV (Lin *et al.* 1997). In contrast, inhibition of NO in tick-borne encephalitis virus (TBEV) infection improves survival (Kreil and Eibl 1996). Thus, despite the significant contributions of macrophages to early suppression of flavivirus infection, it is possible that this cell type plays a pleiotropic role due to antibody-mediated enhancement, immunopathology, and their susceptibility to infection (Peiris and Porterfield 1979). Via this mechanism it is hypothesized that a suboptimal level of anti-viral antibody binds to the flavivirus without neutralizing it and enhances the internalization and subsequent infection of macrophages.

***Complement***

Complement is an important nonspecific host effector that aids in the defense against viral invaders. This system assists in the clearance of virus via activation of membrane attack complexes on infected cells, recruitment of monocytes, and opsonization of virions (Ochsenbein and Zinkernagel 2000). Complement is activated *in vivo* by WNV, and mice lacking key parts of this system exhibited enhanced lethality after WNV infection (Mehlhop *et al.* 2005). All pathways of complement activation are important for controlling WNV, as mice deficient in alternative, classical, or lectin

pathway effectors displayed decreased survival. Mice deficient in these molecules demonstrate impaired $CD8^+$ T cell responses, WNV-specific antibody production, and T cell responsiveness.

**Adaptive immune response**

Robust humoral and cellular adaptive immune responses play an important role in protection against acute encephalitis caused by flaviviruses. Lymphocytes, including B cells, $CD4^+$, and $CD8^+$ T cells, as well as virus-specific antibody have been identified as important in defense against flavivirus infection (Chambers and Diamond 2003a).

Following peripheral infection with WNV, neutralizing antibody appears around day 4 – 6 post-infection, with IgM isotype appearing first (Kramer and Bernard 2001a). Flavivirus infections unusually elicit IgM responses that can often persist for extended periods (Roehrig *et al.* 2003), although this initial response typically has variable neutralizing ability and protective capacity (Diamond *et al.* 2003c). Nonetheless, studies with WNV have suggested a critical role for early function of both IgM and complement in controlling extraneural infection. Therefore, the role of the IgM response may be in providing transient flavivirus neutralizing activity while driving complement-dependent T and B cell activation pathways. The neutralizing ability of sera from WNV-infected animals increases substantially concurrent with the appearance of IgG antibody (Chambers and Diamond 2003b). Passive transfer experiments show that IgG can be protective in WNV infection; however the importance of IgG in primary WNV infection may be limited because it does not appear until 6–8 days after infection at a time when peripheral clearance and CNS invasion has already occurred and, therefore, the survival of the animal may already be determined (Diamond *et al.* 2003c).

Antibody responses are primarily directed against the E, NS1, and prM proteins with both neutralizing and non-neutralizing antibody able to confer protection (Putnak and Schlesinger 1990, Diamond *et al.* 2003b). The humoral response can still be effective after CNS infection, as passive transfer of immune antibody improves clinical outcome even after WNV has disseminated into the central nervous system. Antibody asserts its protective effect by direct neutralization of receptor binding (Crill and Roehrig

2001), impeding virus uncoating (Gollins and Porterfield 1986c), and Fc dependent virus clearance. The mechanism of neutralization appears to be caused by inhibition of the intraendosomal acid-catalysed fusion step during flavivirus entry. B cell knock-out (KO) mice (μMT) or B and T cell KO mice (RAG1) showed significantly higher susceptibility to WNV (Engle and Diamond 2003). Passive transfer of immune sera, splenocytes, or B cells restored the ability of these mice to resist severe infection. The factors that drive B cell activation and maturation during WNV infections are unclear and these models cannot rule out the influence of T cell responses (Mullbacher *et al.* 2003). It is possible that B cells could influence $CD4^+$ and $CD8^+$ T cell responses through antigen presentation or other immunoregulatory events, as has been observed with other neurotropic viruses, and thus the possibility requires further exploration (Bergmann *et al.* 2001). Likewise, B cell activation by flaviviruses is at least partially dependent on T cells, unlike vesicular stomatitis virus or polio, which can activate B cells directly (Fehr *et al.* 1998). Therefore, factors that influence T cells can affect the strength of the antibody response in addition to affecting class and isotype switching.

The T cell response is of crucial importance for the recovery from most viral infections, as mice depleted of this cell type develop rapidly progressive flavivirus encephalitis (Cole and Nathanson 1970, Camenga *et al.* 1974) and adoptive transfer of immune splenocytes can protect against encephalitis (Jacoby *et al.* 1980). Furthermore, humans with hematologic malignancies and impaired T cell function have increased risk for neuroinvasive WNV infection (Murray *et al.* 2006). Mice deficient in T cells fail to generate protective immunity after sub-lethal YFV infection, implying that cellular immunity is also involved in the memory response (Bradish *et al.* 1980). Two dominant lineages of peripheral T cells, $CD4^+$ and $CD8^+$, recognize epitopes consisting of a complex of a short flavivirus genome-encoded peptide and a host self- MHC class II or I molecule, respectively. These peptide/MHC complexes are presented to T cells via professional APCs, the successful interaction between these two cell types activates the T cell. All nucleated cells express MHC class I, whereas only certain cells, including DCs, macrophages, B cells, or antigen-primed $CD4^+$ T cells, express MHC class II on their

surface. Some data suggest that for induction of a naïve T cell, DCs may be the only cell type with sufficient qualitative and quantitative stimulatory capacity to trigger activation of both subsets of T cells (Chambers and Diamond 2003b).

Upon recognition of the appropriate MHC class II/peptide complex, $CD4^+$ T cells activate, proliferate, and mature into one of two T helper subsets – $T_H1$ or $T_H2$ - depending on the stimulatory milieu. Flavivirus peptides recognized by MHC class II molecules are generally virion surface-exposed peptides principally from the E protein or NS1 protein (Chambers and Diamond 2003a). In response to most viral infections the cytolytic function resides predominantly in the $CD8^+$ T cells. Rarely do $CD4^+$ T cells differentiate into cytolytic effector cells. The T cell response to flaviviruses is an exception; in DENV infections the bulk of the cytolytic response resides in the $CD4^+$ T cell population, although the reason is unclear and the cytolytic pathway is unknown (Chambers and Diamond 2003a). It is generally understood that $CD4^+$ T cells are critical to host defenses against flaviviruses by facilitating this cytotoxic T lymphocyte (CTL) cell response and a protective antibody response. For the latter, T cells are required to switch B cell immunoglobulin to the IgG isotype. $CD4^+$ T cells also contribute to control of flavivirus infection through $CD8^+$ T cell priming and cytokine production. Depletion of this cell type results in attenuated WNV-specific antibody responses and enhanced lethality (Diamond *et al.* 2003a). However, T cell activation in response to WNV infection does not appear to play a role in CNS inflammatory response, because only $CD8^+$ and NK infiltrates are detected in the CNS (Chambers and Diamond 2003b). A $CD4^+/CD8^+$ cell ratio change in favor of $CD8^+$ cells is observed in response to WNV infection. It is not clear if flavivirus-immune $CD4^+$ T cells are required for induction of $CD8^+$ T cells (Samuel and Diamond 2006).

$CD8^+$ T cells exert their anti-flavivirus effector function in two ways: 1) secretion of cytokines, such as IFN-γ and TNFα, and 2) cellular cytotoxicity via perforin/granzyme or Fas pathways (Klein *et al.* 2005). These functions probably do not reside in the same cell, rather cytokine producing cells appear to far outnumber cytolytic cells. The significance of the induction of IFN-γ in controlling flavivirus infection suggests a need

for a $T_H1$ polarization, though the role of IL-12 in driving this process has not been extensively studied. $CD8^+$ knockout mice ($CD8\alpha^{tm1Mak}$) exhibit a defect in the clearance of flaviviruses and increased mortality after WNV infection (Liu and Chambers 2001, Shrestha and Diamond 2004). These mice had normal humoral responses but higher and persistent WNV burdens in the spleen and CNS. Target cell lysis via perforin and granzyme A and B is generally believed to be the method by which viral spread is limited, with Fas-Fas ligand interactions possibly playing an immunoregulatory role. Granzymes appear to have a more dominant role in control of lineage II WNV, with perforin and Fas having a more limited role in modulating infection (Wang *et al.* 2003b). Distinctively, control of lineage I WNV infections requires perforin as congenic perforin- mice had enhanced CNS burdens and mortality, and, further, adoptive transfer of wild-type but not perforin-deficient $CD8^+$ T-cells reduced CNS viral load and increased survival (Shrestha *et al.* 2006a). Tc cells are readily induced in the spleens of mice infected with WNV, kunjin virus, MVE, or JEV, and Tc cell responses peak 5-6 days post-infection (Chambers and Diamond 2003a). As might be expected, the Tc cell antigenic peptide determinants map to the viral non-structural region, in particular the NS3/NS4A region (Chambers and Diamond 2003b). Some studies with MVE and DENV suggest that cytotoxicity of CTL may contribute to disease pathogenesis (Rothman and Ennis 1999, Licon Luna *et al.* 2002). Relatively prolonged infection times are required (12 to 24 h) for target cells to become sensitive to lysis, suggesting that the MHC class I antigen presenting pathway is relatively inefficient in processing and presenting early flavivirus gene products.

**Central Nervous System Immune Response**

To prevent neurological disease, the immune response to WNV and other flaviviruses must control viral replication in both the periphery and CNS. Flavivirus infections induce an inflammatory response in the CNS, including activation of microglia, astrocytes, and cerebrovascular endothelium, and production of chemokines and inflammatory cytokines. Immune responses in the CNS are especially perilous, because effective resolution of CNS infections requires that virus is cleared with minimal

damage to the high number of critical and non-regenerative cells of this tissue. The early stages of encephalitis involve the expression of TNF-α, IL-1β, and IL-8 by microglia and astrocytes in the CNS (Ravi *et al.* 1997, Liu and Chambers 2001). The induction of this acute non-specific inflammation may be deleterious to the host. High levels of IL-8 are predictive of a fatal outcome, and treatment of mice with iNOS inhibitors during the acute stages of encephalitis diminishes mortality (Andrews *et al.* 1999, Suzuki *et al.* 2000). Elements of the innate immune response appear to play a role, including IFN-α/β that controls replication in the neurons (Keller *et al.* 2006). Expression of soluble mediators including chemokines drives the trafficking of leukocytes into areas of viral infection in the CNS. T cells (both $CD4^+$ and $CD8^+$) have been visualized in the perivascular infiltrate, in cerebral spinal fluid, and in brain parenchyma during human WNV infection (Sampson *et al.* 2000). Both $CD4^+$ and $CD8^+$ T cells appear to be required for viral clearance from the CNS, as evidenced by adoptive transfer experiments (Murali-Krishna *et al.* 1996). The appearance of $CD8^+$ cells in the CNS correlates temporally with clearance of the WNV (Shrestha and Diamond 2004).

## ALPHAVIRUSES

Alphaviruses, members of the family Togaviridae, genus *Alphavirus*, constitute a geographically widespread (La Linn *et al.* 2001) group of viruses that are usually maintained in natural transmission cycles between arthropods and susceptible vertebrates as hosts. Consisting of over 40 ecologically and epidemiologically distinct viruses, Alphavirus infections occur in a wide range of animal species and cause arthritic or encephalitic syndromes in humans which can culminate in death (Tesh 1982, Powers *et al.* 2001). This family of viruses is characterized by its small icosahedral enveloped particles that are approximately 70 nm in diameter. Each virus particle contains a single-stranded positive sense mRNA molecule that is approximately 11.4 kB in length. The mRNA genome contains a 5' terminal cap and a polyadenylated tail at the 3' end. The RNA genome encodes four non-structural proteins (nsP1-4), located in the 5' two-thirds

of the molecule that are involved in viral replication and processing, and three major structural proteins (C, E1, and E2) that are transcribed from a 26S subgenomic promoter.

Sindbis virus (SINV) is one of the most widely distributed of all known Alphaviruses. It was first isolated from *Culex* mosquitoes collected in the village of Sindbis near Cairo in 1952 (La Linn *et al.* 2001). *Aedes*, *Culiseta,* and *Culex* mosquito species contract SINV from birds and transmit it to humans. Virus isolations and serological findings imply that a wide range of wild and domestic vertebrate species are susceptible to SINV infections, nevertheless, there is no evidence of disease in vertebrates other than humans (Frolov 2004). Several species of domestic and wild animals can be infected under laboratory conditions without developing symptoms of the disease, and the biosafety level-2 status of this virus makes it an attractive virus for animal models and viral expression of genes (Lundstrom 2001). Most infections with SINV are subclinical, with the most common symptoms being rash, fever, fatigue, arthralgia, headache, nausea, and retro-orbital pain (Frolov 2004). The incubation time varies from 2 to 10 days and typically the illness begins suddenly. The duration of the disease varies, though in most instances it is brief, and a full recovery is typical. Some symptoms, including arthritis and arthralgia may persist for a longer period (Frolov 2004).

In mouse disease models, alphaviruses induce apoptotic cell death in neurons (Jackson and Rossiter 1997), and for some alphaviruses, the ability to induce apoptosis is directly correlated with neurovirulence in mice (Lewis *et al.* 1996). In alphavirus infection, such as that caused by Sindbis virus (SINV), type I IFN activity is essential to viral clearance. Blocking of IFN-$\alpha/\beta$ signalling hampers the ability of mouse fibroblasts to stop the replication of SINV (Frolov 2004). Also, mouse strains lacking a functioning IFN response have greatly heightened susceptibility to SINV infection, leading to a 5-fold increase in mortality (Byrnes *et al.* 2000, Ryman *et al.* 2000). Mortality caused by alphaviruses is due to infection of neurons leading to acute encephalomyelitis in mice. Antiviral antibody and IFN-$\gamma$ play important roles in the clearance of SINV from the central nervous system of mice, but $CD8^+$ T cells contribute to elimination of viral RNA

(Binder and Griffin 2001). Antibody-independent cytolytic and noncytolytic T cell-mediated control of virus replication occurs in non-neural tissues (Guidotti and Chisari 2000).

## MOSQUITOES

Fossils resembling arthropods first appeared about 600 million years ago, and now arthropods exist in practically every habitat on earth. It is not surprising, given the large number and diversity of arthropods that many have evolved to utilize the rich natural nutrient source of blood (Ribeiro and Francischetti 2003). Mosquitoes are the most important arthropods in relation to human health. Mosquitoes are classified in the order Diptera, family Culicidae. Notable other members of this order are biting midges (Ceratopogonidae), sand flies (Psychodidae), blackflies (Simuliidae), and tsetse flies (Glossinidae). Most mosquitoes fall into three groups: anophelines, culicines, and aedines. They occur on all continents, except for Antarctica, and generally do not live at altitudes exceeding 3,000 meters. Adult female mosquitoes have piercing-siphoning

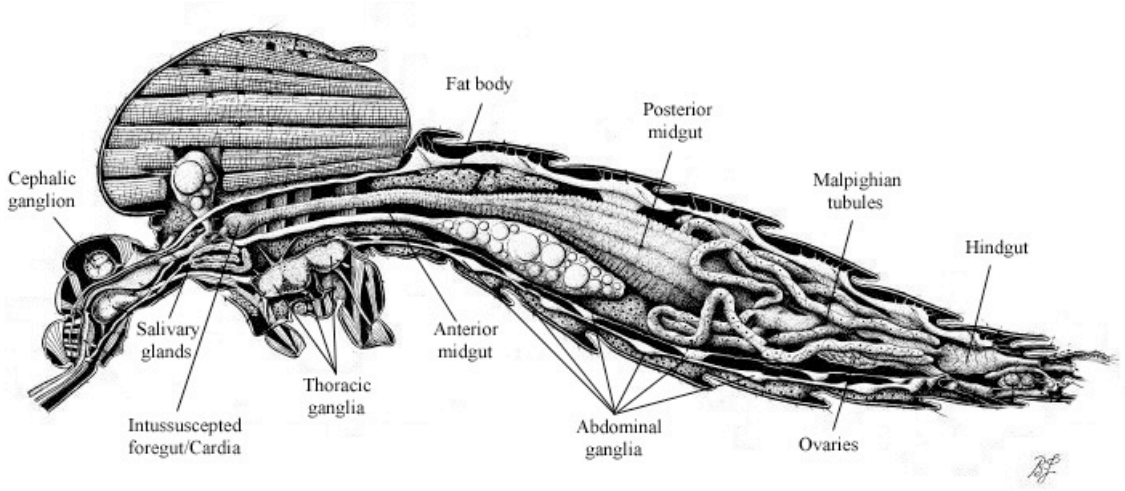


**Figure 1.10 Anatomy of the adult female mosquito. (Reproduced with permission, Wellcome Library, London)**

mouthparts adapted for drawing blood out of vertebrate animals.

**Basic biology**

Mosquitoes are Diptera, and thus, undergo complete metamorphosis, having distinct egg, larvae, pupae, and adult stages. The length of each life stage depends on environmental conditions such as ambient temperature and availability of food sources. Generally, the complete developmental cycle takes approximately 10 days during warmer months (ambient temperature ≈ 27$^{o}$C). All insects have three main body regions, the head, thorax, and abdomen, and adults possess three pairs of legs and a pair of wings attached to the thorax (Fig. 1.10). They possess an open circulatory system within the body cavity called the hemocoel and respiratory system consisting of a series of tracheal tubes (James and Rossignol 1991). Both male and female adult mosquitoes feed on nectar or other carbohydrate sources, while exclusively females feed on the blood of vertebrates to facilitate the high-energy requirement of egg production (Calvo *et al.* 2004). The anatomy of a female adult mosquito is depicted in Fig. 1.12. The most important organ with respect to viral transmission is the mosquito salivary gland.

**Salivary Glands**

Salivary glands, for arthropods, can be defined as organs that synthesize and secrete products that facilitate the acquisition of food. Morphologically (Fig. 1.11), the salivary glands of arthropods can be grouped into two types, tubular and racemose with ducts and acini. The mosquito salivary gland is an example of the former. Mosquitoes have paired salivary glands, which are located in the thorax flanking the oesophagus and are tri-lobed with each lobe connected at the intima. Each lobe has a central duct constituted by a layer of epithelial cells, which are bound by a basal lamina (James and Rossignol 1991). A common lateral salivary duct joins the pair of glands and opens at the base of the hypopharynx. At the base of the mouthparts, a salivary pump forms the junction of the duct and the salivary canal, which opens at the tip of the tubular labrum. The three lobes of each gland include a median lobe and two lateral lobes (James and

Rossignol 1991). In the female mosquito the lateral lobes contain proximal, intermediate, and distal regions, while only a short neck region and a distal region form the median lobe (James and Rossignol 1991). The extracellular apical cavities of the distal regions of the salivary glands are highly dilated with salivary secretions (James and Rossignol 1991). Generally median and distal-lateral lobes are associated with the production of factors necessary for bloodfeeding, while the proximal-lateral lobes produce substances for sugar feeding. It is interesting to note that non-bloodfeeding mosquitoes like *Toxorhynchites brevipalpis* lack the whole median lobe and the intermediate region of the lateral lobes (Watts *et al.* 1982). Three main processes are necessary for the production of saliva (reviewed in James *et al.* 1991): synthesis and secretion of salivary proteins, secretion of saliva, and reabsorption of salts. Cells involved in protein secretion possess abundant rough endoplasmic reticula, plentiful Golgi bodies, and vacuoles containing granules. Fluid-secreting cells typically have an extensive area of plasma membrane with

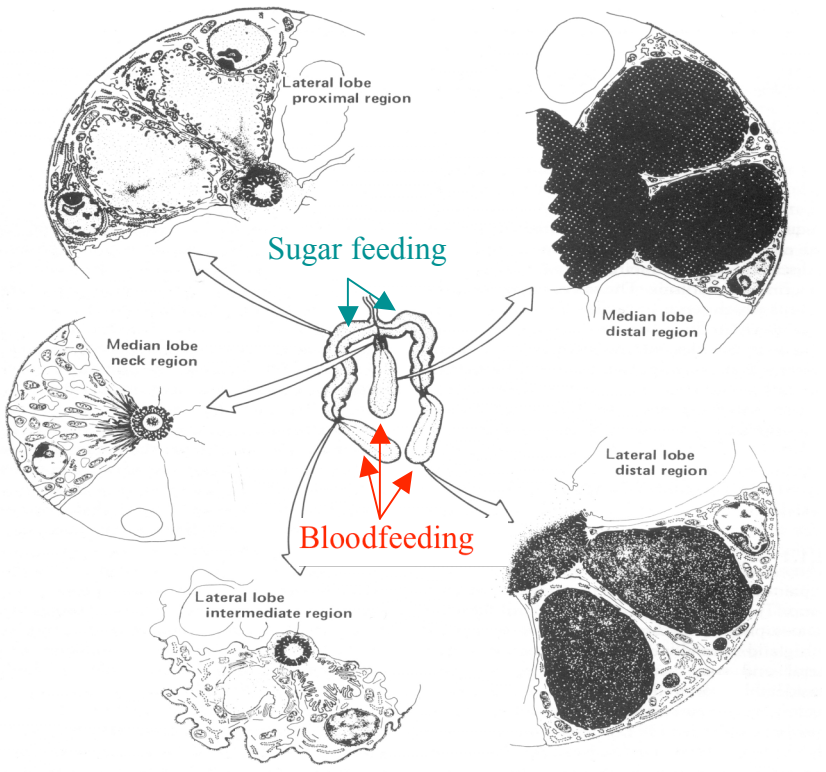


**Figure 1.11 Detail of the structure of the female mosquito salivary gland. Adapted from James and Rossignol, 1991.**

deep invaginations of the apical plasma membrane. Reabsorptive cells display cuboidal or flattened topology.

Due to the divergence in function, male and female mosquito salivary glands display distinct morphology and secretions. Comparatively, female salivary glands are much larger than male glands. Indeed, female glands contain 10 times more protein than their male counterparts (James and Rossignol 1991). Additionally, male salivary glands are very simple, composed of a near homogeneous cell population, while female glands have a complex histology with many cell types. Female mosquitoes are able to salivate differentially depending on the meal to be ingested (Marinotti *et al.* 1990).

The key role of the mosquito salivary gland is to facilitate rapid blood acquisition and digestion. A pair of female salivary glands has an estimated volume of 21.6 nL (Metcalf *et al.* 1945) and contains approximately 4 mg of total proteins (Marinotti *et al.* 1996). Salivary proteins involved in blood-feeding are secreted from the distal lateral lobes, although sialokinin is produced in the median lobe (Champagne and Ribeiro 1994).

**Saliva**

In order for a mosquito to successfully obtain a blood meal it must overcome the complex and redundant physiological responses orchestrated by the vertebrate hemostatic and inflammatory systems that have evolved to prevent an invasion such as blood-feeding. The mosquito, as with all hematophagous arthropods, has evolved mechanisms to effectively block host hemostatic defense with saliva - a complex concoction of secreted proteins and factors. Saliva is a pharmacologic cocktail that can affect vascular constriction, blood clotting, platelet aggregation, inflammation, immunity, and angiogenesis (Ribeiro and Francischetti 2003). Universally, hematophagous arthropod saliva contains at least one anticlotting, one anti platelet, and one vasodilatory substance. Mosquito saliva also contains enzyme s associated with sugar feeding (Grossman and James 1993) and antimicrobial agents to control bacterial growth in the sugar meal (Rossignol and Lueders 1986). The composition of mosquito saliva is relatively simple as it usually contains fewer than 20 dominant proteins (Valenzuela *et al.* 2002) (Calvo *et al.* 2006). Despite the great strides in knowledge of these molecules and their role in

bloodfeeding achieved recently, we still cannot ascribe functions to more than half of the molecules found in arthropod saliva (Valenzuela *et al.* 2002).

Many of the secreted proteins in the saliva of *Ae. aegypti* mosquitoes have been characterized (Valenzuela *et al.* 2002). Full-length cDNA expression library sequencing revealed the presence of an antigen 5-like protein. The role of this protein in bloodfeeding is unknown, although homologous members of this family are composed of mammalian cysteine-rich secretory proteins, the antigen 5 proteins of social wasps and ants, and the group 1 pathogenesis-related proteins of plants (Schreiber *et al.* 1997). The mammalian homologs of this protein have been found in humans and rodents and their expression is androgen-related. Antigen 5 is found in the venom of wasps and fire ants and is believed to be involved in producing an intense, localized inflammatory reaction in mammals (King *et al.* 1978). The plant member of this family is expressed in plant cells in response to stresses, including pathogen infection and may have anti-fungal activity (Niderman *et al.* 1995).

The most abundant mosquito salivary protein in *Ae. aegypti* is a member of the D7 protein family, which is distantly related to the odorant-binding protein superfamily (Calvo and Ribeiro 2006). *Ae. aegypti* secrete at least three D7-like proteins in their saliva, two longer proteins and one shorter protein. These proteins have been found to bind and sequester serotonin with high affinity, as well as other amines, including histamine and norepinephrine and (Calvo and Ribeiro 2006). The short protein is unique in that it contains a KGD (lysine-glycine-aspartic acid) triad flanked by nearby cysteines; this motif is found in disintegrins, which are proteins that interfere with fibrinogen binding to platelets (Scarborough *et al.* 1993). Additionally, a factor Xa (fXa)-directed anticoagulant known as anticoagulant-factor Xa has been identified in *Aedes* saliva (Stark and James 1998). Biochemical analysis of this protein showed that it had specific, reversible, and noncompetitive fXa-inhibitory activity, and kinetic data suggest that this salivary protein interacts with an exosite on fXa and not within the substrate-binding domain (Stark and James 1998). Aedes saliva also contains a 30-kDa salivary allergen (Simons and Peng 2001), which has a C-type lectin signature related to the macrophage

mannose receptor, and a protein with a fibrinogen domain that is homologous to vertebrate angiopoietins (Hackett *et al.* 2000). The function of the 30-kDa protein is unknown, but the protein with the C-type lectin signature may be similar to hemaglutinins, which have been described in the salivary glands of anopheline mosquitoes (Johnson *et al.* 2001), or the C-type lectin recently found to have anticlotting activity in sand fly saliva (Charlab *et al.* 1999). Lectins have also been shown to participate in invertebrate immunity reactions (Ratcliffe *et al.* 1985). The salivary angiopoietin-like proteins shows remarkably high similarity to their mammalian homologues, except the two mosquito angiopoietins are truncated versions that lack 200 residues of the amino terminal. Angiopoietins in vertebrates act either to stimulate or inhibit angiogenesis, the role of the mosquito-secreted forms is unknown (Hackett *et al.* 2000). The apyrase protein in *Ae. aegypti* saliva is a 5'-nucleotidase that dephosphorylates adenosine-diphosphate (ADP) and ATP, but not AMP (Champagne *et al.* 1995). The role of this substance in mosquito saliva appears to be linked to the pro-coagulation and -inflammatory properties of ATP/ADP. Extracellular ATP and AMP, indicators of cell injury, are signals for activation of neutrophils and platelet aggregation, respectively, and ATP also excites nociceptive primary afferents by acting on P2X receptors leading to the sensation of pain (Lakshmi and Joshi 2005). In addition to the proteins mentioned thus far, *Ae. aegypti* saliva contains numerous enzymes (Valenzuela *et al.* 2002): a PAF-acetyl hydrolase, which destroys the platelet aggregating compound PAF; a sphingomyelin phosphodiesterase; a carboxylesterase; an amylase; two glucosidases (Valenzuela *et al.* 2002), which are related to sugar feeding; and a purine hydrolase, previously thought to occur only in plants, protozoa, and prokaryotes (Versees *et al.* 2001). It is thought that the role of the purine hydrolase is to complete the catabolism of host adenosine to hypoxanthine, thereby disassembling a mediator of mast cell degranulation (Tilley *et al.* 2000). Finally, calreticulin, whose secreted form has been shown to have antithrombic and angiogenic activities (Johnson *et al.* 2001), bacterial adhesion proteins, a mammalian testes protein, Sialokinin, a known vasodilator,

and a number of proteins or enzymes associated with invertebrate immunity were identified in *Ae. aegypti* as putative secreted salivary proteins (Valenzuela *et al.* 2002).

**Bloodfeeding**

Bloodfeeding has evolved independently approximately 21 times within the arthropod taxa, and within Diptera in at least nine families (Wheeler and Hynes 2001). It is likely that bloodfeeding evolved from ancestral forms adapted to sucking plant juices. To find a blood meal, female mosquitoes must undergo a series of behaviours that bring them into contact with a host. Carbon dioxide ($CO_2$) is the most universal attractant, but other volatile components, such as lactic acid, acetone, and octenol may also play a role (possible as synergists). Visual cues, warmth, and moisture also appear to be important in host location (Zwiebel and Takken 2004). Some mosquito species preferentially feed on particular groups of vertebrates; for example, *Culex pipiens* have been suggest to be ornithophilic (Wang 1975). Additionally, most mosquitoes have definite circadian rhythms during which specific activity, such as host seeking, occurs (Klowden 1996). The mouthparts are extended into a proboscis that consists of a stylet bundle (fascicle) and the labium that encloses it when the mosquito is not feeding (James and Rossignol 1991). The stylet bundle is formed from six long slender stylets: the labroepipharynx, hypopharynx, and two of both maxillae and mandibles (James and Rossignol 1991). The mandibles end in a sharp point and facilitate the piercing of the epidermis to allow the remaining stylets access. The maxillae have a pointed tip and recurved teeth at the distal ends. They are the main penetrative elements of the mouthparts, which are thrust alternately utilizing the teeth to anchor themselves in the tissues (James and Rossignol 1991). Accordingly, the labroepipharynx (food channel) and hypopharynx (salivary channel) are pulled with them into the tissue. An alternating rotary movement of the head causes the penetration of the stylets into the tissue (Ribeiro *et al.* 1984a). After the female lands on a host, she may probe the skin a few times with the labellae while she searches for a capillary from which to procure blood. Sensilla on the ventral side of the pair of labellae and on the distal part of the labium contain receptors for stimuli that indicate a suitable site for feeding (Zwiebel and Takken 2004). Observations of the

movements of mosquito mouthparts *in vivo* demonstrate that after penetrating the skin, the mosquito thrusts its stylet back and forth before locating a blood vessel and beginning to feed (Labuda and Kozuch 1989). During probing, mosquitoes salivate copiously (Griffiths and Gordon 1952). After successfully puncturing the skin and locating a vessel, the female commences bloodmeal ingestion. While feeding, only the stylet bundle passes into the skin and the labium becomes progressively more bent backwards as the mosquito probes deeper. Two pumps, the cibarial pump and the pharyngeal pump, are the sucking organs.

## HOST IMMUNE RESPONSE TO ARTHROPODS

Bloodfeeding activates a spectrum of host responses, including hemostatic and immunological activity. Mosquitoes feed by physically puncturing the epidermis with specialized mouthparts. The exact source of the blood meal is unclear, though it is likely that mosquitoes sometimes feed directly from capillaries, but the majority of the time from pooled blood. Therefore, it is plausible that mosquito feeding and hence virus inoculation occurs in extravascular spaces of the dermis and activates host hemostatic, inflammatory, and immune responses.

### General response of the skin to mosquito feeding

The initial response of the skin to injury includes pain, hemostatic, inflammatory, and immunologic responses. The pain response in the skin is triggered by mechanical injury. Many molecules, hemostatic mediators, are linked to pain production (Millan 1999). ATP leakage from injured cells, can also cause immediate pain at an affected site (Cook and McCleskey 2002). Serotonin and histamine, which are released by platelets and mast cells, and bradykinin, which is produced by activation of factor XII via tissue-exposed collagen, are also induce pain (Ribeiro 1987). Activation of inflammatory cells, such as neutrophils, is accompanied by production of pain-inducing prostaglandins (Julius and Basbaum 2001). Pain is also induced by chemokines, including IL-1, and bradykinin.

Hemostasis is the term used to describe the host response that controls the loss of blood following injury to a blood vessel and occurs within a few seconds for a small peripheral blood vessel (Champagne 2004). It consists of platelet aggregation, blood coagulation, and vasoconstriction; all of which are redundant phenomena. There are several independent agonists of platelet aggregation (ADP, collagen, thrombin, platelet-activating factor, thromboxane A2), at least two vasoconstrictors are released by platelets (thromboxane A2 and serotonin), and the clotting cascade is a complex system with many points of amplification and control (Champagne and Ribeiro 1994).

Once skin is punctured, an assortment of soluble mediators and cells contribute to the initial response. Polymorphonuclear cells and monocytes are important mediators of inflammation. Soluble mediators released by injured cells recruit neutrophils that accumulate at the site of injury (O'Flaherty and Cordes 1994). Products from the coagulation cascade, such as thrombin, and other proinflammatory mediators, such as platelet-activating factor, also activate neutrophils. Thrombin also causes fibroblast proliferation and neutrophil adhesion, while factor Xa enhances acute inflammation by binding to effector cell protease receptor-1, leading to induction of vascular permeability and leukocyte exudation (Gillis *et al.* 1997). The intrinsic pathway of blood coagulation mediated by bradykinin induces TNF-a release by neutrophils, which in turn stimulates the release of IL-1β and IL-6.

The immune system of the skin is composed of elaborately coordinated responses of innate and adaptive immunity. Antigen presenting cells, such as macrophages and dendritic cells (DC) are attracted to the site of injury, and facilitate both destruction of invaders and commencement of pathogen recognition. Pathogen antigens are processed and presented by APCs to helper $T_H$ lymphocytes in association with MHC class II proteins on the surface of the APC. $T_H$ lymphocytes receive secondary activation signals via co-stimulatory receptor-ligand interactions between themselves and the APC, in addition to immunoregulatory signals in the form of cytokines. $CD4^+$ $T_H$ cells secrete a variety of cytokines involved in immune regulation. $CD4^+$ $T_H$ cells can mature into two subsets according to the signalling environment, and they are characterized by the

specific cytokines they produce. $T_H1$ cells secrete IFN-γ and IL-2, which promote a cell-mediated immune response characterized by expression of complement-fixing and opsonizing antibodies, such as IgG2a and macrophage activation. $T_H2$ cells, in contrast, secrete IL-4, IL-5, and IL-6 that promote the development of selected humoral immune responses including the expression of IgE and of IgG1 in mice. The development of IgG1 and of IgG2a isotypes is dependent on IL-4 and IFN-γ expression, respectively. IL-4 promotes B cell proliferation and the isotype switch from IgM to IgE and IgG1, while IFN-γ stimulates the production of IgG2a and IgG3 and suppresses IgG1 production (Finkelman *et al.* 1990). Absence of co-stimulatory signals results in development of anergy, specific unresponsiveness, rather than activation (Jenkins and Miller 1992).

In the cutaneous space a specialized subset of DCs, known as Langerhans cells (LCs), are involved in initial virus recognition and downstream activation of macrophages and T lymphocytes. These cells are important in directing the immune response to pathogens via production of various cytokines, including IL-1, IL-2, IL-4, IL-6, IL-10, IFN–α, IFN–β, IFN–γ and TNF-α. IFN-γ has effects on the growth and differentiation of many immune cells, induces formation of proteins that inhibit viral mRNA translation and release, and can induce the macrophage nitric oxide response.

LCs are the most peripheral representatives of the host immune system. As described above they are also primary targets for infection by some arboviruses such as WNV (Byrne *et al.* 2001). Residing in the epidermis, LCs capture antigens and migrate to skin-draining lymphoid tissues where their role is to efficiently activate both naive and effector T cells, thus initiating an immune response (Silberberg 1973, Silberberg-Sinakin *et al.* 1976). Their advanced antigen-presenting abilities are, in part, the result of maturational phenotypic changes that arise as they depart the epidermis. The transition from resident epidermal cell type to that of a lymphoid DC is characterized by an enhancement in the expression of cell-surface molecules requisite for T cell stimulation such as class I and II MHC (Cumberbatch *et al.* 1991), the accessory molecule ICAM-1/CD54 (Simon *et al.* 1991), and the co-stimulatory molecules B71/CD80 and B72/CD86. Langerhans cells specifically up-regulate MHC class II, ICAM-1, and the

co-stimulatory molecule, B7-1 in response to WNV infection (Byrne *et al.* 2001). Secretion of IL-1β appears to be important for migration of WNV-induced LC migration from the skin to draining lymph nodes (Byrne *et al.* 2001).

The host reaction to salivary components following insect probing, depends on host and mosquito species, with cutaneous responses as varied as small papules to large pruritic swellings. These responses are often mild in unsensitized hosts, and may become more pronounced if allergic sensitization occurs prior to exposure. The mechanisms include type I (immediate, IgE-dependent) and type-IV (delayed-type hypersensitivity (DTH) cell-mediated) reactions. Both hypersensitivity reactions cause a local increase in blood flow, vascular permeability, and cellular infiltration (Demeure *et al.* 2005). This reaction to mosquito feeding suggests that some components of mosquito saliva are allergenic. Mosquito saliva induces mast cell degranulation, leading to fluid extravasation and neutrophil influx (Demeure *et al.* 2005). IgE is not necessary for this reaction, because it occurs in naïve mice. Mast cells are sentinels of the innate immune system, and are often found intimately connected with DC cells in the dermis (Sueki *et al.* 1993). Such an interaction suggests that these cell types could influence each other as illustrated by DC phenotypic changes following mast cell degranulation (Ioffreda *et al.* 1993). Mast/DC interactions can enhance the immunomodulatory properties inherently endowing this cell type, which exert both stimulatory and inhibitory effects on the adaptive immune response (Hart *et al.* 1998, Villa *et al.* 2001). Indeed, mosquito bites, presumably facilitated by mast cell action, induce DC migration to draining lymph nodes and lymph node hyperplasia (Demeure *et al.* 2005). Soluble mediators released by mast cells are responsible for the wheal and flare reaction, edema, and vascular permeability (McNeil 1996). Specific mediators, including TNF-α and macrophage inflammatory protein (MIP)-2/IL-8 are known to be chemotactic for neutrophils, which enter the bite site (Williams and Galli 2000). Mosquito feeding has been shown to cause a substantial increase (~6-fold) in TNF-α levels within draining lymph nodes 6 h after exposure (Demeure *et al.* 2005). Following *Anopheles stephensi* feeding on mice MIP-2 and IL-10 are selectively increased while IL-13 and IL-4 are marginally increased in the skin and

lymph nodes. With IL-10, heightened levels are detectable approximately 8 h after mosquito exposure and mRNA levels peak at 24 h, and return to pre-feeding levels by 48 h.

**Sensitization to mosquito feeding**

Sensitization to mosquito feeding is associated with specific responses. In an animal model (BALB/c mice) that explored the natural sensitization leading to IgE- and lymphocyte-mediated hypersensitivities, mice were exposed twice a week for 4 weeks to at least 16 *Ae. aegypti* mosquitoes (Chen *et al.* 1998). Following mosquito exposure, sensitized mice develop a wheal 20 min after exposure and a delayed papule 24 h later. Mosquito saliva-specific IgG1 and IgE, but not IgG2a, is increased in sensitized mice, and comparisons show that most of the antigens also elicited human IgE responses (Chen *et al.* 1998). The degree of host response to mosquito saliva is dependent on the duration and intensity of exposure to biting mosquitoes and the immunological profile of the host. The level of circulating antibody generated by mosquito feeding is predictive of the hypersensitivity response – those with low levels of anti-mosquito IgE/IgG have minor skin reactions, while those with relatively higher levels of antibody have more pronounce reactions (Peng *et al.* 1996b). Intriguingly, the immune response to mosquito saliva affects cytokine expression as well. IL-4 production is significantly increased while IFN-$\gamma$ production is decreased in sensitized mice, suggesting that a $T_H2$ immune response predominates following sensitization (Chen *et al.* 1998). Mean lymphocyte proliferation after stimulation with mosquito antigens is higher in mice (Chen *et al.* 1998) and humans (Peng *et al.* 1996b) previously exposed to mosquito bites, implying that lymphocytes are involved in the development of immunological reactions to mosquito saliva. Immunoblotting techniques have been used to characterize the salivary proteins recognized by host antibodies (Billingsley *et al.* 2006). These studies have demonstrated that anti-mosquito antibodies vary with mosquito species and population density, and that the number of salivary antigens recognized increase over the mosquito season (Palosuo *et al.* 1997). Cross-reactivity between the salivary proteins of different mosquito species has been demonstrated, as sera from residents of Canada recognized salivary antigens of

non-local mosquitoes (Peng *et al.* 1998). Immuno-reactive proteins in *An. gambiae* saliva have been matched to 5' nucleotidase, apyrases, calreticulin, D7-related protein, lysozyme, and several unidentified salivary proteins (Billingsley *et al.* 2006). Human desensitization to mosquitoes has been observed naturally during long-term, regular, and high-dose exposure to mosquito bites (Peng *et al.* 1998).

**Modulation of the host immune response by arthropod saliva**

It is now well recognized that the feeding of hematophagous arthropods has an immunomodulatory effect on their hosts. The presence of this activity in vector saliva is a reflection of the inherent overlapping and interconnected nature of the host hemostatic and inflammatory/immunological responses and the intrinsic need to prevent these host defenses from disrupting successful feeding. Duration of feeding varies greatly among bloodfeeders, but even the most rapid feeders must contend with the host hemostasis system, which activates in a matter of seconds (Champagne 2004). Key host targets for arthropod anticoagulation action, such as coagulation factor Xa, also serve to activate anti-microbial responses (Champagne 2004). In addition to hemostasis, arthropods that take a long time to engorge must deal with host inflammatory and immune responses, thus necessitating anti-inflammatory/-immunological factors. It is less clear why quick-engorging arthropods, including sand flies and mosquitoes, which feed in a matter of minutes, have salivary factors that directly suppress or deviate host immune defenses. One possible explanation lies not in looking at the individual sand fly or mosquito, but considering the population of sand flies or mosquitoes feeding on a given host over a prolonged period of time. Immunomodulation would reduce the possibility that a host could develop immune responses that impair the activities of anticlotting and vasodilatory molecules essential for feeding. A second and more convincing reason for immunomodulatory activity in quick-feeding arthropods is the high level of interaction between the host hemostatic, inflammatory, and immune responses. A mosquito by necessity must suppress the hemostasis system. The effect of saliva on vasodilation, coagulation, and platelet aggregation factors result in modulations of inflammation and immune responses.

***Mosquitoes***

The mechanism for mosquito saliva-induced alteration of the host immune response is unclear, but the data has become increasingly convincing that such an effect occurs. Studies looking at the effect that mosquito saliva has on the immune response, including studies published as a result of this thesis research (Schneider *et al.* 2004, 2006) began relatively recently, stimulated by research on the saliva of other arthropods that produced intriguing results (Ribeiro *et al.* 1989, Theodos *et al.* 1993)

Early work described a factor in saliva that directly suppresses TNF-α release, but not antigen-induced histamine secretion, from activated mast cells (Bissonnette *et al.* 1993). A significant amount of research has focused on the effect of mosquito saliva on splenocytes *in vitro*. Experiments by Cross *et al.* (1994) demonstrated that the inclusion of *Ae. aegypti* SGE into naïve cultures led to a suppression of IL-2 and IFN-γ production, while the cytokines IL-4 and IL-5 are unaffected by SGE. Cellular proliferation in response to IL-2 is clearly reduced by prior treatment of cells with SGE (Cross *et al.* 1994). Correspondingly, activated splenocytes isolated from mice fed upon by either *Ae. aegypti* or *Cx. pipiens* mosquitoes produce markedly higher levels of IL-4 and IL-10 concurrent with suppressed IFN-γ production (Zeidner *et al.* 1999). Unexpectedly, this $T_H1$ to $T_H2$ shift in cytokine expression is observed in splenocytes up to 10 d after

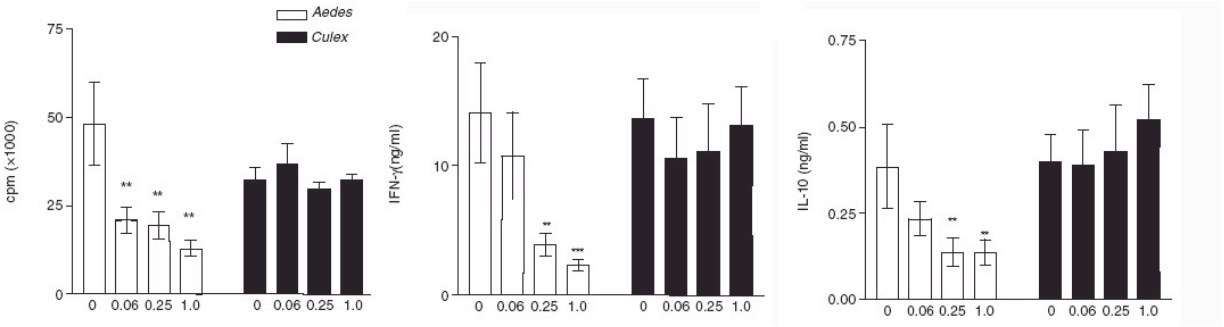


**Figure 1.12 The effects of Aedes and Culex SGE on antigen-specific responses. Cytokine levels were measured by ELISA and Proliferation levels of the cultures were determined by measuring [3H]thymidine incorporation. Data shown are the mean±SD from triplicate cultures. The experiments were independently repeated twice with similar results. **Indicates P<0.01 in comparison to untreated controls. Adapted from Wanasen *et al.* 2004**

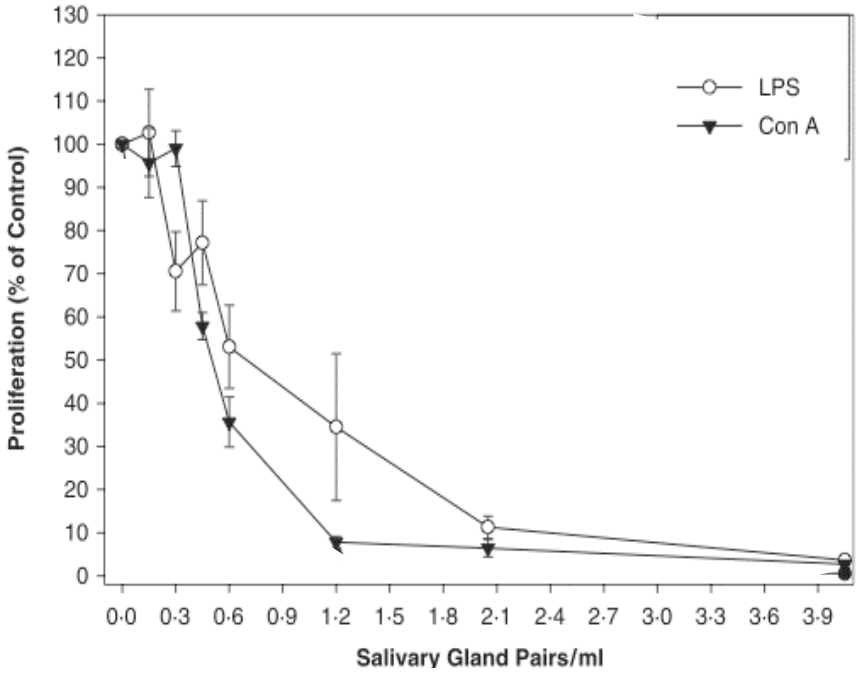


**Figure 1.13 Aedes aegypti SGE suppresses proliferation of Con A-stimulated T-lymphocytes and LPS-stimulated B-cells. Incorporation of [$^3$H]-thymidine was used to determine proliferation. Regression analysis indicates a significant effect ($P < 0{\cdot}01$). Adapted from Wasserman *et al.*, 2004**

mosquito exposure, suggesting that natural feeding of mosquitoes can have a profound, enduring, and systemic effect on T cell functions. Inoculation of the *Ae. aegypti* salivary vasodilator, sialokinin (5-1000 ng) mimics the effect of mosquito (Zeidner *et al.* 1999). Enhancement of IL-10 expression could account for reduction in secretion of other cytokines because it inhibits antigen presentation, IFN–γ expression, and macrophage activation (Thomson *et al.* 1998). IFN–γ is especially important in defense against virus infections. This cytokine causes proliferation and differentiation of many cell types, activates production of cellular proteins that prevent viral mRNA translation, and enhances macrophage nitric oxide production (Ribeiro and Nussenzveig 1993).

Elements of mosquito saliva can directly affect the cell types that migrate to the feeding site. The saliva of *An. stephensi* has been shown to contain a high molecular weight glycoprotein endowed with an intense neutrophil chemotactic activity (Owhashi *et*

*al*. 2001). Moreover, the introduction of saliva of this species into a host causes DC migration and directly causes mast cell degranulation without the facilitation of IgE (Demeure *et al.* 2005). Indirect effects of mosquito salivary anticoagulant proteins, such as *Aedes* anticoagulant-factor Xa (Stark and James 1998) can down-modulate extravasation of some inflammatory cell types and suppress complement pathways. The *Aedes* anticoagulant inhibits fXa, which is required for activation of Hagemen factor, which in turn converts factor XI to its active form XIa and prekallikrein to kallikrein. Kallikrein is responsible for generation of the anaphylatoxin C5a from compliment (Wiggins and Cochrane 1981). A reduction in C5a, as would be expected from inhibition of upstream effectors, could have significant effects at the site of mosquito feeding. Notably, C5a is important for recruitment of antibody, extravasation of complement, homing of polymorphonuclear leukocyte (PMN), and activation of macrophages and neutrophils. Therefore mosquito saliva, by inhibiting factor Xa, decreases the production of C5a, thereby suppressing these downstream effects that hasten the destruction of pathogens. Importantly, a recent study confirmed that complement plays a critical role in controlling WNV infection (Mehlhop and Diamond 2006).

T cell populations are decidedly susceptible to the suppressive effect of mosquito saliva, showing enhanced mortality and decreased division rates (Wanasen *et al.* 2004). This immune suppression appears to be greater in the primary host of the vector species (Fig. 1.12), as *Ae. aegypti* saliva exerts an enhanced suppressive effect on mouse splenocytes than *Cx. pipiens,* an ornithophilic species (although the reciprocal experiment has not been completed, largely due to the deficiency in immunological reagents for avian species). Parallel work by Wasserman *et al.* (2004) demonstrated that T- and B-cell proliferation was inhibited in a dose dependent manner with concentrations as low as 0.15 salivary gland pairs (SGP)/ml (Fig. 1.13) (Wasserman *et al.* 2004). Lower concentrations of salivary gland extract (SGE) inhibited $T_H1$ cytokines (IL-2 and IFN-$\gamma$) and T cell proliferation, while higher concentrations suppressed $T_H1$, $T_H2$ (IL-4 and IL-10), and proinflammatory (GM-CSF and TNF-$\alpha$) cytokines and decreased T cell viability (Fig. 1.12). This pattern suggests that at the immediate feeding site an

immunosuppressive environment predominates, whereas surrounding regions with decreasing saliva concentrations experiences a dysregulation of the immune response. A 387 kDa protein was implicated in the observed activity (Wasserman *et al.* 2004). Depinay *et al.* (2005) observed a suppression of antibody-specific T cell responses mediated by mosquito saliva and dependent on mast cells and IL-10 expression. Naïve mice were exposed to *An. stephensi* bites during a sensitization phase with ovalbumin. Seven days later mice were challenged with ovalbumin , and the DTH response was measured. The ensuing DTH response was reduced by 75% in mice that were exposed to mosquito saliva during the sensitization phase, and the number of leukocytes were also reduced (50%) in this group as compared to controls (Depinay *et al.* 2005). This altered response corresponded with a suppression of IFN-γ and an up-regulation of IL-10.

A recent study suggests that mosquito saliva can also decrease expression of IFN–α/β (Hajnicka *et al.* 2000). The contribution of type I IFN in recovery from infection with arboviruses has been demonstrated *in vivo* by the therapeutic and prophylactic effects of administration of IFN-inducers or IFN (Haahr 1971, Vargin *et al.* 1977, Taylor *et al.* 1980). Mice deficient in IFN–α receptor do not survive infection as compared to 70% survival in wild-type mice after a low-dose infection with MVEV (Lobigs *et al.* 2003). Other vectors, although independently evolved, are useful sources of comparison to aid in elucidating the possible effects of mosquito saliva on host immune response.

***Ticks***

Ticks are in intimate contact with the host immune system for an extended period of time, during which a host can direct a range of innate and adaptive immune responses can be directed against this vector. Therefore, it is not surprising that ticks have developed countermeasures to harmful host immune responses. Tick saliva is correspondingly much more complex that that of mosquitoes with approximately 470 unique proteins (Ribeiro *et al.* 2006). The earliest report of tick-induced immuno-modulation was the observation that *Dermacentor andersoni* infestation significantly reduces *in vitro* proliferation of T-lymphocytes stimulated with concanavalin A, a

lymphocyte mitogen (Wikel 1982). Subsequent to this initial report a variety of tick species have been shown to alter or suppress a broad spectrum of host immune responses. A tick salivary protein was found to block complement activation (Valenzuela *et al.* 2000). Specifically, this protein counters the alternative pathway, inhibiting rabbit erythrocyte lysis by human serum, inhibiting C3b binding, and accelerating factor Bb uncoupling from the C3 convertase.

Tick saliva polarizes T cell responses to a $T_H2$ profile by up-regulating IL-4, enhancing the immunosuppressive cytokine IL-10, and suppressing $T_H1$ cytokines (IL-2 and IFN-γ) (Zeidner *et al.* 1997). Proinflammatory cytokines, IL-1 and TNF-α are also inhibited by tick saliva, with suppression reaching an *in vivo* peak of >90% of controls towards the conclusion of tick engorgement (Ramachandra and Wikel 1992). A recently discovered IL-2 binding protein in *Ixodes scapularis* may explain some of these effects that tick saliva has on lymphocytes (Gillespie *et al.* 2001), as it would provide a simple mechanism for the observed suppression of T cell proliferation. Controversy exists regarding the importance of prostaglandin E2, a factor found in tick saliva, which has established immunomodulatory properties in other systems, as an immunosuppressant in tick saliva (Bowman *et al.* 1996).

Factors in tick saliva can also have direct effects on key inflammatory immune cells, including reduction of the antimicrobial action of macrophages, differentiation of DCs, and adhesion of neutrophils (Kuthejlova *et al.* 2001, Montgomery *et al.* 2004, Cavassani *et al.* 2005). Effects on DC maturation involve a reduction in the population of differentiated cells and a reduction in costimulatory molecule expression (Cavassani *et al.* 2005), while suppression of neutrophil adhesion corresponds with a down-regulation of β2-integrins and decreased efficiency in pathogen uptake and killing (Montgomery *et al.* 2004).

Certain adhesion molecules, such as β2-integrin, are expressed by endothelial cells that interact with corresponding receptors on leukocytes to mediate attachment and migration to sites of tissue damage or infection (Muller *et al.* 2002b, Muller *et al.* 2002a). The saliva of *Ix. scapularis* impairs the adherence of PMNs by decreasing CD18, the

common β subunit of leukocyte function-associated antigen-1 and Mac-1 (Montgomery *et al.* 2004), while the saliva of *D. andersoni* reduces the expression of leukocyte function-associated antigen-1 and very late activation-4 by splenocytes (Macaluso and Wikel 2001). A recent study has shown that these two tick species also down-regulate the expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells (Maxwell *et al.* 2005), which could alter leukocyte migration and reduce antigen presentation by endothelial cells. Furthermore, tick saliva inhibits angiogenesis as shown by a dose-dependent reduction in endothelial cell proliferation that appears to be mediated by a tick saliva-induced degradation of α5β1 integrin (Francischetti *et al.* 2005). Tick saliva, because of its complexity, is a valuable guide for predicting the function of mosquito salivary proteins with unknown activity and a resource for understanding the wide spectrum of host systems with which arthropod saliva can interact.

***Sand flies***

Sand flies are particularly useful comparisons to mosquitoes to facilitate understanding of possible effects that mosquitoes and their saliva may have on a host, due to their similarity in feeding styles and the amount of data compiled on the effect of their saliva on host immune pathways. Sand flies are the prototypical vector with respect to the enhancement of pathogen infection in studies involving modulation of host immune responses, which has important implications for transmission of leishmaniasis (Titus and Ribeiro 1988). Saliva of the Old World sand fly, *Phlebotomus papatasi*, inhibits IFN-γ activation and NO production by macrophages (Hall and Titus 1995). Additionally, *P. papatasi* salivary gland lysate has been shown to down-regulate $T_H1$ and up-regulate $T_H2$ cytokines in mice (Mbow *et al.* 1998), while *Lutzomyia longipalpis* saliva inhibits macrophage presentation of immunogens to specific T-lymphocytes (Theodos and Titus 1993). Salivary gland extract of *L. longipalpis* suppresses both nonspecific and specific antigen-induced proliferative responses of normal mouse splenocytes (Soares *et al.* 1998). Moreover, *L. longipalpis* saliva contains maxadilan, a protein that is the most powerful vasodilator known (Lerner and Shoemaker 1992). In

addition to vasoactive properties, maxadilan is an immunomodulator that inhibits T-lymphocyte activation and DTH (Qureshi *et al.* 1996). Maxadilan increases IL-6 and IL-10 secretion and inhibits TNF-α secretion by macrophages (Bozza *et al.* 1998). Recent reports have established that sand fly saliva contains a highly complex population of proteins (Charlab *et al.* 1999), which means that there are likely to be additional immunomodulatory molecules to be found.

**Arthropod saliva-induced enhancement of disease**

***Mosquitoes***

The mosquito transmits virus in saliva secreted during blood-feeding. As discussed previously, these proteins include apyrases (ATP/ADP diphosphohydrolases) (Champagne *et al.* 1995, Smartt *et al.* 1995), tachykinin peptides which closely mimic endogenous vasodilators (Champagne and Ribeiro 1994, Beerntsen *et al.* 1999), and a number of unidentified immunomodulatory factors (Zeidner *et al.* 1999, Ribeiro and Francischetti 2001, Francischetti *et al.* 2003, Wanasen *et al.* 2004, Wasserman *et al.* 2004). As described above, components of mosquito saliva have immunomodulatory activity, as a consequence of this components of mosquito saliva have the potential to affect immune response and virus/parasite pathogenesis. Indeed, enhanced infection attributable to components of arthropod saliva is an accepted phenomenon (Theodos and Titus 1993, Cupp *et al.* 1998b, Edwards *et al.* 1998, Limesand *et al.* 2000, Nuttall and Labuda 2004). Studies with mosquitoes have demonstrated that *Ae. triseriatus* saliva can potentiate vesicular stomatitis New Jersey virus infection in mice, causing previously resistant mice to become infected (Limesand *et al.* 2000). Also, *Ae. triseriatus* salivary gland treatment of mouse fibroblast cells resulted in a significant increase in viral growth compared to untreated controls (Limesand *et al.* 2003).

Adult mice are typically resistant to La Crosse virus (LACV) administered subcutaneously by needle, although passive viremias have been reported in mice at 12 h post-subcutaneous inoculation of large doses of LACV (Pekosz *et al.* 1995). However, when LACV-infected mosquitoes feed upon adult mice, many mice die of encephalitis (Higgs *et al.* unpublished data). Fatal infection also results when adult mice are infected

s.c. with LACV-infected salivary gland suspensions. Unlike mice inoculated with virus alone, mice infected in the presence of mosquito saliva developed a viremia of 1 – 3 d duration and die of LACV encephalitis within 6 – 12 d following mosquito feeding (Table 1.2). A dose response effect was noted; the more LAC-infected mosquitoes that fed, the earlier clinical symptoms appear and longer the duration and higher the titer of viremia. Additional studies were then conducted to determine if LACV-infected salivary glands alone could potentiate murine infection. The mortality rate in mice equaled that induced by feeding of infected vectors (Table 1.3). In contrast, s.c. challenge of mice with LACV, derived from a 10% brain suspension from a lethal mouse infection or from *Ae. albopictus* cell culture, resulted in the death of only one mouse (Higgs *et al.* unpublished data). Another study (Osorio *et al.* 1996) investigated the potential for mosquito feeding to influence the disease course and reservoir competence of wild

**Table 1.2 Feeding by LACV-infected mosquitoes results in fatal infection of adult mice, as compared to asymptomatic infection caused by needle inoculated LACV. [a] number of mosquitoes feeding, [b] (died/exposed), [c] $log_{10}$ 50% tissue culture infective dose ($TCID_{50}$)**

| Exposure | | Mice % Mortality | | Viremia Days | Titer[c] | Antibody % positive | Days to death |
|---|---|---|---|---|---|---|---|
| Mosquitoes | 1-5[a] | 100 | (7/ 7)[b] | 0-2 | 1-2 | 100% | 9-11 |
| | 6-10 | 100 | (4/4) | 1-2 | 1-2 | 100% | 7-8 |
| | 25+ | 100 | (20/20) | 2-3 | 1-3 | 100% | 6-7 |
| Subcutaneous | | 11 | (1/9) | ND | ? | 11% | ? |

mammals that naturally occur in regions where LACV is prevalent. Despite the recognition that white-tailed deer constitute 65% of *Ae. triseriatus* blood meals, needle-inoculation models of LACV had previously assumed that due to low viremias detected in deer, these animals did not play an important role in the maintenance of LACV in nature. Subsequent experiments using infected mosquitoes to transmit LACV demonstrated that mosquito inoculation of LACV leads to increased viremia titer and duration. A parallel study with chipmunks observed that chipmunks infected via mosquito as compared to needle-inoculation developed viremias that were 3 log higher in (Osorio *et al.* 1996).

**Table 1.3 Comparison of disease induced by feeding of LACV-infected mosquitoes, co-inoculation of LACV and salivary glands, inoculation of infected mosquito salivary glands, and needle-inoculated LACV. $log_{10}$ 50% tissue culture infective dose = ($TCID_{50}$)**

| Treatment | % Mortality | | Virus Dose ($log_{10}TCID_{50}$) |
|---|---|---|---|
| Subcutaneous LAC | 11 | (1/9) | 5 - 5.5 |
| Subcutaneous LAC and noninfected salivary glands | 17 | (1/6) | 5.5 |
| Subcutaneous LAC-infected salivary glands | 100 | (9/9) | 5.5 |
| Feeding by LAC infected mosquitoes | 89 | (42/47) | < 3.5 |

Mice generally do not become infected with Cache-Valley virus (CVV), an arthropod-borne bunyavirus, following needle-inoculation of the virus (Edwards *et al.* 1998). However, injection of CVV into sites of mosquito feeding results in production of viremia and anti-CVV antibody by 2 wk post-exposure. This observed enhancement of CVV infection resulted after feeding by either *Ae. triseriatus*, *Ae. aegypti*, or *Cx. pipiens* and occurred when virus injection was delayed up to 4 h after mosquito feeding, but it was not observed when virus inoculation was performed at a site distant from mosquito feeding. The results of this study suggest that arbovirus infection can be enhanced by mosquito-vertebrate host interactions and that factors in arthropod saliva instead of replication in arthropod vectors is the required factor for increased virulence of infections mediated by infected mosquitoes.

Studies with VSV further demonstrate the potential for the act of mosquito feeding or mosquito saliva to potentiate viral disease (Limesand *et al.* 2000). Pathogenesis of VSV is age dependent: mice <3 weeks old infected with VSV by peripheral needle-inoculation develop encephalitis and die, while older mice similarly infected show almost no signs of viral replication with less than 15% of animals producing neutralizing antibody. Following mosquito inoculation of VSV, 94% of 3-week old mice and 73% of adult mice seroconverted to VSV. It is interesting to note that in a supplementary experiment where mice were continuously exposed to mosquito feeding, a lower rate of seroconversion was observed, perhaps suggesting that factors in

mosquito saliva suppressed antibody production. Follow-up *in vitro* studies suggested that the mechanism of VSV enhancement might be attributed to mosquito saliva-induced suppression of type I IFN expression (Limesand *et al.* 2003). The ability of mosquito factors to facilitate establishment and disease development of a pathogen also extends to protozoan parasites. Indeed, *Plasmodium berghei* sporozoites delivered into mice via mosquito bites are more infectious than needle inoculated sporozoites (Fig. 1.14)

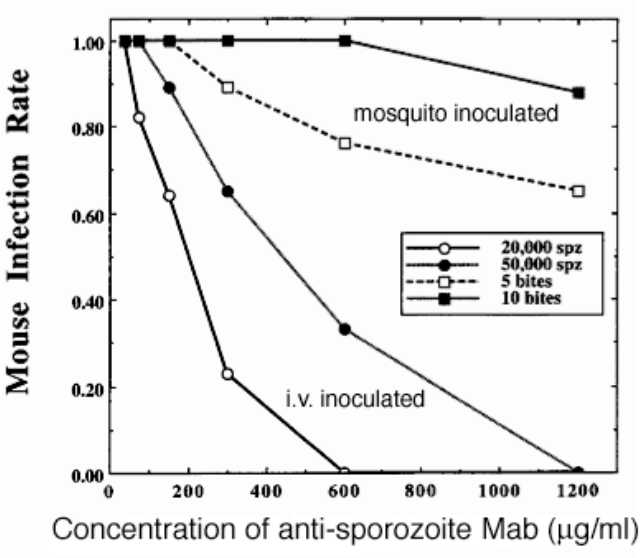


**Figure 1.14 Comparative infectivity of sporozoites (spz) to immunized mice when sporozoites were delivered by either infectious mosquito bite (approximately 15 spz/bite) or syringe inoculation into tail vein. Adapted from Vaughan *et al.* 1999.**

(Vaughan *et al.* 1999). In a study by Vaughan *et al.* (1999), parasite inoculations as high as 50,000 sporozoites were unable to cause infection in vaccinated mice, whereas less than half of similarly immunized mice were protected against sporozoites delivered by infectious mosquito bite; blood-stage infections occurred in 65% and 88% of mice exposed to the bites of 5 and 10 mosquitoes, respectively. The significant deviation in immunization efficacy between needle and naturally inoculated infections points to a powerful modulating effect of mosquito saliva. Additionally, based on this study the 50% infectious dose ($ID_{50}$) for intravenous-delivered sporozoites can be estimated to be between 1,700 and 11,250, whereas the feeding of 4 mosquitoes led to the infection of

50% of unvaccinated mice. Given that the actual number of sporozoites transmitted to a host during bloodfeeding ranges from 6 to 11 sporozoites (Vaughan *et al.* 1999) and not all gland-infected mosquitoes inoculate sporozoites, even a relatively generous estimate of 60 sporozoites transmitted by 4 mosquitoes would mean that mosquito-transmission of malaria requires 1-2 orders of magnitude (~$1/25^{th}$ – $1/200^{th}$) fewer sporozoites than needle inoculation to infect a host.

***Ticks***

Tick-borne diseases also appear to utilize the unique environment created by arthropod saliva to amplify the chances of successfully establishing an infection. Zeidner *et al.* (Zeidner *et al.* 2002) demonstrated that 100- to 1000- fold fewer *Borrelia burgdorferi* spirochetes were required to infect mice when the bacteria were co-inoculated with tick saliva. Notably, this effect appeared to be augmented when SGE from the natural vector species of the spirochete was used, suggesting a complex co-evolution between parasites and their vectors. Additional studies with other species of *Borrelia* confirmed a pathogen enhancing effect of tick saliva; Pechova *et al.* (Pechova *et al.* 2002) demonstrated that injection of *Borrelia* spirochetes together with SGE increases the level of bacteremia and accelerates the appearance of bacteria in the urinary bladder, compared with the injection of spirochetes alone. Additionally, more *Ix. ricinus* nymphs became infected when feeding on mice inoculated with *B. afzelii* plus SGE (Pechova *et al.* 2002). Analysis of cytokines produced by cells of draining lymph nodes from these *Borrelia* infected-, SGE-treated mice showed a suppression of proinflammatory cytokines IFN-γ, IL-6 and GM-CSF in comparison to the control mice infected without SGE (Pechova *et al.* 2002). Parallel work by Machackova *et al.* (Machackova *et al.* 2006) found that *B. burgdorferi* spirochete levels also were 10-fold higher when SGE was added to inoculum. These effects appear to be at least partly mediated by SGE-induced inhibition of the of murine macrophage microbicidal activity (Kuthejlova *et al.* 2001). Besides affecting early parasite viability, tick SGE also increases the susceptibility of cells to *Theileria parva* parasite infection by 30 to 60% (Shaw *et al.* 1993). This study demonstrated that short-term preincubation of

inflammatory cell types or cell types that would typically be found in the skin with *Rhipicephalus appendiculatus* SGE causes a broader spectrum of cell types to attach and internalize parasites.

Studies designed to elucidate the mechanism of non-viremic transmission (NVT) of arboviruses, a form of transmission between two arthropods feeding on an uninfected host, concluded that factors in tick saliva are essential for this method of horizontal transmission and cause an enhanced viremia (Nuttall and Labuda 2004). When ticks were fed on guinea pigs inoculated with TBEV a significant increase in the tick-to-tick transmission rate is observed if virus is inoculated with SGE (Labuda *et al.* 1993a, Labuda *et al.* 1993b). The effect was not tick species specific; SGE from *R. appendiculatus, Ix. ricinus* and *D. reticulatus* are equally effective. Inoculation of SGE from partially fed ticks resulted in a 4-fold increase in co-feeding tick infection rate, when compared with inoculation of SGE from unfed ticks. Salivary gland extract coinoculated with TBEV also increased the level of viremia in mice. In NVT experiments with Thogoto virus (Jones *et al.* 1992b, Jones *et al.* 1992a), no effect was seen using mosquito SGE. The components of tick saliva involved in this so-called "salivary-activated transmission" (SAT) are uncertain (Nuttall *et al.* 1994), although a protein/peptide seems to be involved, since SAT activity is destroyed if SGE is treated with pronase or proteinase-k (Jones *et al.* 1990). In their recent review of NVT of arboviruses, Nuttall and Labuda (2004) concluded, that SAT plays a critical role in the life cycle of TBEV. Observations in support of this hypothesis include: 1) successful transmission of TBE virus between co-feeding infected and uninfected ticks, is independent of a detectable viremia in the host (Alekseev and Chunikhin 1990, Labuda *et al.* 1993c); 2) NVT can be mimicked by syringe inoculation of TBEV, when it is mixed with tick saliva/salivary gland extract (Alekseev *et al.* 1991, Labuda *et al.* 1993b). It is now recognized that the basic mechanism of NVT is the ability of TBEV to exploit the pharmacological activities of tick saliva at the feeding site (Nuttall and Labuda 2004). A possible mediator of this effect is suggested by the demonstration that tick SGE can directly inhibit the action of IFN, thereby promoting virus replication (Hajnicka *et al.*

2000). Thus, research with emphasis of the role of tick saliva on disease pathogenesis has led to the discovery of numerous intriguing vector-host-pathogen systems that are affected by saliva. Similar systems with sand flies have also revealed the profound effect that vector saliva can have on disease development.

***Sand flies***

The discovery that sand fly saliva can enhance *Leishmania* infection was the first evidence that vector saliva can have a significant impact on pathogenesis. Sand flies transmit *Leishmania* to a mammalian host while probing for a vessel and inject the parasites in their saliva. When *Leishmania major* is inoculated with salivary gland extracts of sand flies, parasite burden, lesion size, and disease outcome are all amplified

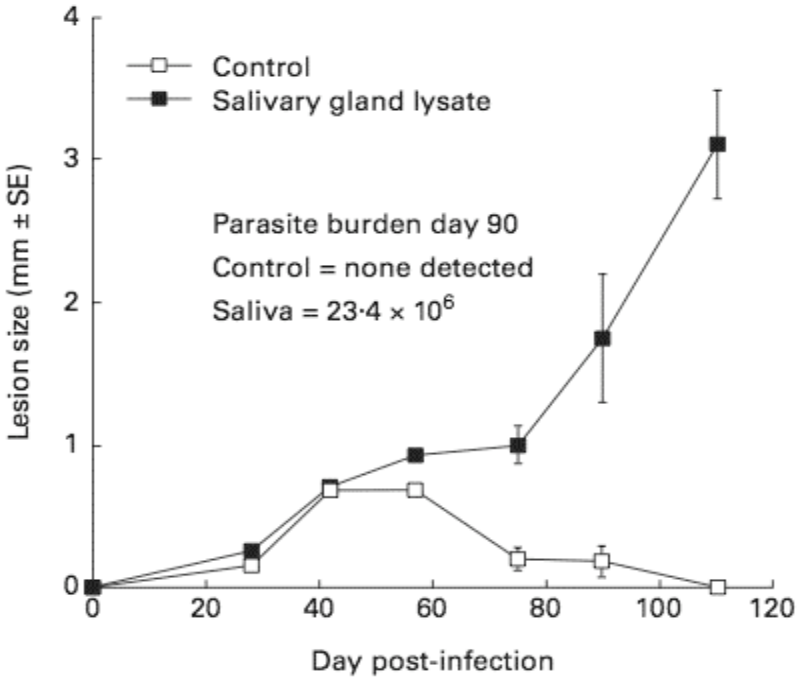


**Figure 1.15 Co-inoculation of sand fly salivary gland extract with *L. braziliensis* leads to an exacerbation of disease, such that lesion is enlarged and persistent while parasite burden is amplified. (Used with permission from Gillespie *et al.*, 2000, *Parasite Immunology*)**

(Titus and Ribeiro 1988). In this seminal research, Titus and Ribeiro (1988) found that co-inoculation of sand fly SGE lead to a parasite burden that was 5580-fold greater than in control groups inoculated with parasites alone. Additionally, in leishmaniasis the killing and antigen-presentation ability of macrophages is inhibited in the presence of saliva (Theodos and Titus 1993). More recent studies reveal that sand fly saliva exacerbates infection with all species of *Leishmania* (Samuelson *et al.* 1991, Theodos *et al.* 1991, Warburg *et al.* 1994, Lima and Titus 1996, Norsworthy *et al.* 2004). The most decisive results are seen with *L. braziliensis* (Fig. 1.15), which by itself induces only a transient infection in mice, but when paired with *L. longipalpis* saliva is capable of inducing lifelong lesions and enormous parasite loads in the host (Lima and Titus 1996). Leishmania disease exacerbation is associated with an early increase in the frequency of epidermal cells that produce $T_H2$ cytokines, particularly IL-4 (Belkaid *et al.* 1998). As it stands to reason that humans are probably exposed to uninfected sand fly bites prior to being fed upon by an infectious sand fly, studies were carried out to evaluate the effect of prior exposure to vector feeding on disease development. Predictably, previous exposure to sand fly SGE or feeding completely abrogates the enhancing effect of SGE (Belkaid *et al.* 1998, Kamhawi *et al.* 2000). Previously exposed mice exhibit strong delayed-type hypersensitivity reaction to sand fly feeding, experience enhanced IFN-γ concurrent with depressed IL-4 production, and produce antibodies that presumably neutralized the action of the salivary proteins. A major sand fly salivary protein, called maxadilan, has since been characterized; this protein produces comparable potentiation of Leishmania disease and thus appears to be the principle factor in sand fly saliva that enhances infection. The exacerbating result of sand fly saliva on Leishmania disease exemplifies the effect a rapid-feeding hematophagous insect can have on the ability of the host immune response to combat infection.

## SPECIFIC AIMS OF PROJECT

Specific Aim 1. To examine the effect of mosquito feeding and saliva on arbovirus infection of mice. The specific hypothesis to be tested is that the presence of

mosquito saliva during arbovirus infection causes enhanced murine viremia, while decreasing survival rate and median survival time. Previous studies have demonstrated that *Ae. triseriatus* saliva can potentiate VSV New Jersey virus infection in mice, causing normally resistant mice to become infected (Limesand *et al.* 2000). Additionally, LACV viremias in deer are increased in load and duration when the host is infected via mosquito instead of by needle inoculation (Osorio *et al.* 1996). This aim will definitively resolve whether mosquito saliva potentiates arbovirus infection using WNV as a representative.

Specific Aim 2. To evaluate the effect of mosquito saliva on leukocyte migration at the initial site of arbovirus delivery. The specific hypothesis to be tested is that mosquito saliva alters levels of murine macrophage, LC, and T cell populations in the epidermis and draining lymph nodes during arbovirus infection. Mosquito salivary gland extract causes a decrease in proliferation and an increase in apoptosis of $CD4^+$ cells in vitro (Wasserman *et al.* 2004), as well as a deviation in secretion (Wanasen *et al.* 2004) and expression of cytokines important for directing immune cell activity (Schneider *et al.* 2004). This aim will be accomplished using multi-color flow cytometry to compare influx of cells in the skin and draining lymph node during WNV infection in the presence or absence of mosquito saliva.

Specific Aim 3. To determine if mosquito saliva alters cytokine expression at the site of infection and in APC during arbovirus infection. The specific hypothesis to be tested is that the presence of mosquito saliva during arbovirus infection/transmission causes down-regulation of $T_H1$ and anti-viral cytokines and up-regulation of $T_H2$ cytokines at the site of infection with equivalent dysregulation of cytokine expression in APCs. Specific cytokine secretion, especially by macrophages and dendritic cells (Yang *et al.* 2001), is essential for initiation of the cellular immune response. Zeidner *et al.* (1999) demonstrated that *in vitro* stimulated lymphocytes of C3H/HeJ mice fed upon by *Ae. aegypti* mosquitoes had decreased levels of IFN-γ and increased levels of IL-4 and IL-10. This aim will be achieved using real-time RT-PCR to measure the expression of key cytokines within primary sites of replication, both *in vivo* and *in vitro*, during the early events of arbovirus infection in the presence or absence of mosquito saliva.

## IMPETUS FOR PROJECT

Arboviruses are an increasing health concern due to a continuous resurgence and emergence of epidemic arboviral diseases affecting both humans and domestic animals. While immunomodulatory effects on vertebrate hosts have been well characterized in ticks, far less research has focused on mosquitoes. Although several studies have focused on mosquito saliva and its effect on the immune response, the majority of research in this area is disproportionately shifted towards *in vitro* work using isolated splenocytes and lymphocytes. Knowledge is lacking in the area of saliva-skin cell interactions as they relate to arbovirus pathogenesis. In the past most small animal models of arbovirus disease have used needle-inoculation (Chambers and Diamond 2003), but recent evidence suggests that because of the potential effects of mosquito saliva on the immune system, it is important to re-evaluate the pathogenesis of these infections in the presence of mosquito saliva.

The long-term goal of this project is to determine the role of mosquito saliva in enhancement of arbovirus disease and to develop an understanding of the immunomodulatory activity of mosquito saliva by elucidating the underlying mechanism of mosquito induced immunomodulation. The overall hypothesis of this proposal is that **the presence of mosquito saliva during viral inoculation influences the disease course and pathogenesis of arboviral infection**. To evaluate this hypothesis, we designed experiments to first determine if mosquito saliva alters the course of disease in a murine model, and then address the mechanism of alteration by examining the effect of mosquito saliva on cell migration and cytokine production in the skin. Understanding the pharmacological activity of mosquito saliva and its resultant effect on virus transmission and pathogenesis will allow better prevention of arbovirus infections, through superior design of arboviral vaccines, counteraction of saliva-induced modulation, and enhanced design of mosquito-borne disease models.

This project was primarily designed to ascertain the mosquito saliva-induced modulation of the immune response and its effects on virus infection. The results from

these studies will allow a better understanding of cytokine expression and immune cell function during mosquito-transmitted viral infection.

Specific aim 1 was designed to determine if co-inoculation of mosquito saliva can alter the course or outcome of arboviral infection, allowing a more thorough understanding of the natural pathogenesis of an arbovirus. Further work will elucidate the effect of prior exposure to mosquitoes on subsequent mosquito-transmitted WNV infection. Specific aim 2 was constructed to ascertain the role of immune cell migration in mosquito saliva-induced alterations of the response to an invading arbovirus, while specific aim 3 endeavored to establish the effect of mosquito saliva on APCs, key mediators of early immune response, and their early response to arbovirus. Overall this research enhances our understanding of the mechanisms through which natural vector feeding can influence virus transmission and disease progress.

Until recently few studies of the influence of mosquito saliva on host immune activity have included *in vivo* research and only two have incorporated virus (Limesand *et al.* 2003, Ader *et al.* 2004). Much of the research with mosquito saliva involves *in vitro* and mitogen stimulation of immune cells, such as splenocytes, and little *in vivo* examination. Work focusing on immune cells of the skin, such as macrophages and DCs is almost entirely absent. Particularly lacking, due to the requirement of specialized laboratories, is research that combines mosquito saliva with arboviruses. Due to the importance of understanding natural arbovirus transmission, merging these two areas of research is essential.

As demonstrated by the unanticipated introduction of WNV into the United States and the resurgence of DENV hemorrhagic disease and JEV, arboviruses have become a significant human health concern (Gubler 2002). Without an understanding of the effects of mosquito saliva on host immune systems and resultant consequences on pathogenesis, many of our viral disease models may be flawed. Most vaccines rely upon a robust antibody and T cell response (Davis *et al.* 2004, Cheng *et al.* 2005, Harari and Pantaleo 2005), areas that are likely to be influenced by mosquito saliva (Gillespie *et al.* 2000). Knowledge of the immunology of the natural transmission through appropriate models

will allow investigators to design more effective vaccines for arboviruses. Furthermore, study of the immune modulation affected by factors in mosquito saliva could eventually lead to the discovery of novel molecules that alter the immune responses, as in the case of Maxadilan, a protein in sandfly saliva with immunomodulatory effects and potent vasodilatory activity (Lerner and Shoemaker 1992, Qureshi *et al.* 1996). Also, prior exposure of mice to bites of uninfected sand flies confers powerful protection against *L. major* (Kamhawi *et al.* 2000), and similarly reversal of *Ix. scapularis* saliva cytokine modulation protects mice from *B. burgdorferi* infection (Zeidner *et al.* 1997). Therefore, it may be possible that immunity to vector saliva could protect against arbovirus infection (Wong and Opdebeeck 1989) (Wikel 1988, Wong and Opdebeeck 1993).

Previous work concerning mosquito saliva potentiation of virus infection has inappropriately compared needle-inoculated to mosquito-inoculated routes of infection. Such comparisons are inherently flawed due to disparities in virus concentration and phenotype generated by the divergent sources of virus. Indeed, research in this lab has suggested that individual mosquitoes transmit from 1 to 5 logs of virus to mice (Vanlandingham *et al.* 2004). Studies using mosquito-transmitted virus contain considerable internal variability due to the larger range of virus injected. None of the preceding studies on vector potentiation of disease have compared the viruses between needle-inoculated and mosquito-inoculated groups to determine whether confounding differences exist, despite the fact that the selective differences in the environmental conditions provided by insect and mammalian cells has been shown to produce incongruent phenotypes of virus (Novella *et al.* 1995). Therefore these studies cannot rule out the possibility that the deviation observed is simply due to inherent differences between viruses from different sources.

This study bridges the gap between descriptive research on mosquito saliva and mechanistic research on arbovirus pathogenesis. The project is significant because the innate and early adaptive immune responses have enormous influence over the downstream immune reaction to and pathogenesis of viral infections. By ignoring the possible effects of the vector we may not only misinterpret the early immune response to

arboviruses, but also possibly aspects of the overall pathogenesis of arboviral infections. This is clearly illustrated with LACV, where a vertebrate reservoir for the virus was excluded based on the limited viremia produced by needle-inoculation and subsequently studies utilizing mosquito transmission demonstrated that the reservoir could sustain sufficient viremias (Osorio *et al.* 1996). The information from the present study will provide insight into early host responses to arbovirus infection, and may shed light on determinants of WNV pathogenesis.

# CHAPTER 2: THE POTENTIATION OF WEST NILE VIRUS ENCEPHALITIS IN A MURINE MODEL BY THE FEEDING OR SALIVA OF *AEDES AEGYPTI*


## ABSTRACT

Mosquitoes infect humans with arboviruses while taking a blood meal, inoculating virus with their saliva. Mosquito saliva contains compounds that counter host hemostatic, inflammatory, and immune responses. Modulation of these crucial defensive responses may influence virus infection. Using a mouse model we explored the potential for mosquitoes to impact the course of WNV disease by determining if differences in pathogenesis occurred in the presence or absence of mosquito saliva. Mice inoculated intradermally with $10^4$ pfu of WNV subsequent to the feeding of mosquitoes developed more progressive infection, higher viremia, and accelerated neuroinvasion than the mice inoculated with WNV alone. At a lower dose of WNV ($10^2$ pfu), mice fed upon by mosquitoes had a higher mortality rate. This study suggests that mosquito feeding and factors in mosquito saliva can potentiate WNV infection, and offers a possible mechanism for this effect via accelerated infection of the brain.

## INTRODUCTION

The introduction and rapid spread of WNV in the United States, affecting humans, birds, and domestic animals, highlighted the importance of arboviruses to public health. Clinically, human infection can range from asymptomatic with seroconversion to severe meningitis, encephalitis, acute flaccid paralysis, or death (Huhn *et al.* 2003). The determinants of disease development and severity, however, are not well defined. Human infection occurs when an infectious mosquito takes a blood meal, injecting WNV with its saliva. Mosquito saliva contains factors that actively modulate host hemostatic, inflammatory, and immune responses (Calvo *et al.* 2006). WNV is maintained in nature via transmission between susceptible vertebrate hosts by mosquitoes, including those of the genera *Aedes*, *Anopheles*, *Culex*, and *Ochlerotatus* (Bernard *et al.* 2001). In particular, *Ae. aegypti* are well adapted to feed on mammals, and previous work has demonstrated that their saliva has immunomodulatory activity (Wanasen *et al.* 2004). Although these mosquitoes are not the principal WNV vector, this species has been found to be infected with this virus in the field, they are competent vectors of WNV (Turell *et al.* 2001), and may act as bridging vectors between the avian-*Culex* cycle and mammalian hosts (Turell *et al.* 2005).

### Potentiation of pathogen transmission by arthropod saliva

Enhanced vertebrate infection attributable to components of arthropod saliva is a recognized phenomenon (Titus and Ribeiro 1988). Mosquito saliva can affect both immune responses and virus pathogenesis. For example, saliva of *Oc. triseriatus* potentiates vesicular stomatitis New Jersey virus infection in mice, causing resistant mice to become infected (Limesand *et al.* 2000), and treatment of mouse fibroblast cells with *Oc. triseriatus* SGE significantly increases viral replication compared to untreated controls (Limesand *et al.* 2003). Studies with other types of vectors are insightful with respect to vector-induced disease potentiation. Zeidner *et al.* (2002) observed enhanced spirochete loads in target organs when *Borrelia burgdorferi* spirochetes were co-

inoculated with tick saliva. Correspondingly, the percentage of guinea pigs with detectable viremia more than doubled when the inoculation of tick-borne encephalitis virus contained tick SGE (Labuda *et al.* 1993a). Co-injection of *Leishmania* parasites with sandfly SGE significantly enhances parasite burden, lesion size, and disease progression in immunologically naïve mice (Belkaid *et al.* 1998).

**Shortcoming of previous work assessing role of mosquito saliva in pathogen transmission**

Previous work concerning mosquito saliva potentiation of viral infection has compared two routes of infection: needle-inoculation and mosquito-inoculation (Reisen *et al.* 2000). Such comparisons are subject to variation in virus concentration and phenotype generated by the divergent sources of virus. Mosquitoes transmit a range of viral titers (Hurlbut 1966 Vanlandingham *et al.* 2004); for example, individual mosquitoes transmit from 1.0 to 5.0 $\log_{10}$ pfu of WNV (Vanlandingham *et al.* 2004). Studies that utilize mosquitoes to transmit virus may therefore be difficult to interpret because of differences in the virus titer delivered. Despite the fact that the selective differences in the environmental conditions provided by insect and mammalian cells have been shown to produce incongruent viral phenotypes (e.g. virulence or tropism) (Novella *et al.* 1995), previous studies on vector potentiation of disease have ignored the potential influence of different inoculation methods. Therefore one cannot rule out that the differences noted in these studies are due to intrinsic differences of the viral inoculum. To determine if mosquito saliva or feeding has an effect on WNV pathogenesis, we performed studies to compare disease development and progression following infection with known titers of well-characterized WNV infection in the presence or absence of mosquito saliva.

Based on known immunomodulatory activity of mosquito saliva (Wanasen *et al.* 2004, Wasserman *et al.* 2004, Demeure *et al.* 2005, Billingsley *et al.* 2006, Depinay *et al.* 2006) and the potentiation of diseases by the saliva of other vectors (Jones *et al.* 1989), we postulated that the dysregulation of key immune mediators by mosquito saliva could impact WNV disease. A mouse model of infection (Beasley *et al.* 2005) was used in

which a low passage WNV isolate was inoculated intradermally (i.d.) to mimic natural infection. Treatment groups were evaluated by comparing viremia, display of symptoms, and viral titer in selected organs.

## MATERIALS AND METHODS

### Virus

WNV strain 114 (GenBank accession numbers AY187013 and AY185907) (Girard *et al.* 2004) is genetically and phenotypically identical to WNV-NY99 (Davis *et al.* 2004). Virus was inoculated intradermally at a concentration of either $10^2$ or $10^4$ plaque-forming units, levels that are within the range of virus naturally secreted by mosquitoes (Vanlandingham *et al.* 2004).

### Mice

Female, 4-week old Swiss Webster mice were obtained from Harlan (Indianapolis, Indiana), and housed in a biosafety level-3 animal facility. This strain of mice is an outbred line and therefore their response to pathogens and vectors are more variable.

Mice were initially divided into 4 groups (n = 6) by the inoculum they received: PBS (negative control), WNV alone ($WNV^{alone}$), WNV with SGE ($WNV^{SGE}$), and WNV following the feeding of uninfected mosquitoes ($WNV^{mos}$). Later experiments lacked the group concomitantly injected with WNV and SGE due to the greater deviation of the group inoculated with WNV following the feeding of mosquitoes in the first trial of this study (Fig. 2.1). An inoculation containing either $10^2$ or $10^4$ pfu of WNV in a volume of 20 μl, was administered intradermally (i.d.) in the lower abdomen (Fig. 2.1). Mice were observed twice daily for progression of symptoms, including ruffled fur, lethargy, hunching, eye discharge, and paralysis. Three mice per group were alternately bled every other day for nine days, and blood was allowed to clot at room temperature (30 min) prior to centrifugation (8 min at 850 × g). Following separation of serum, samples were stored at −80°C until serum viremia was determined via titration.

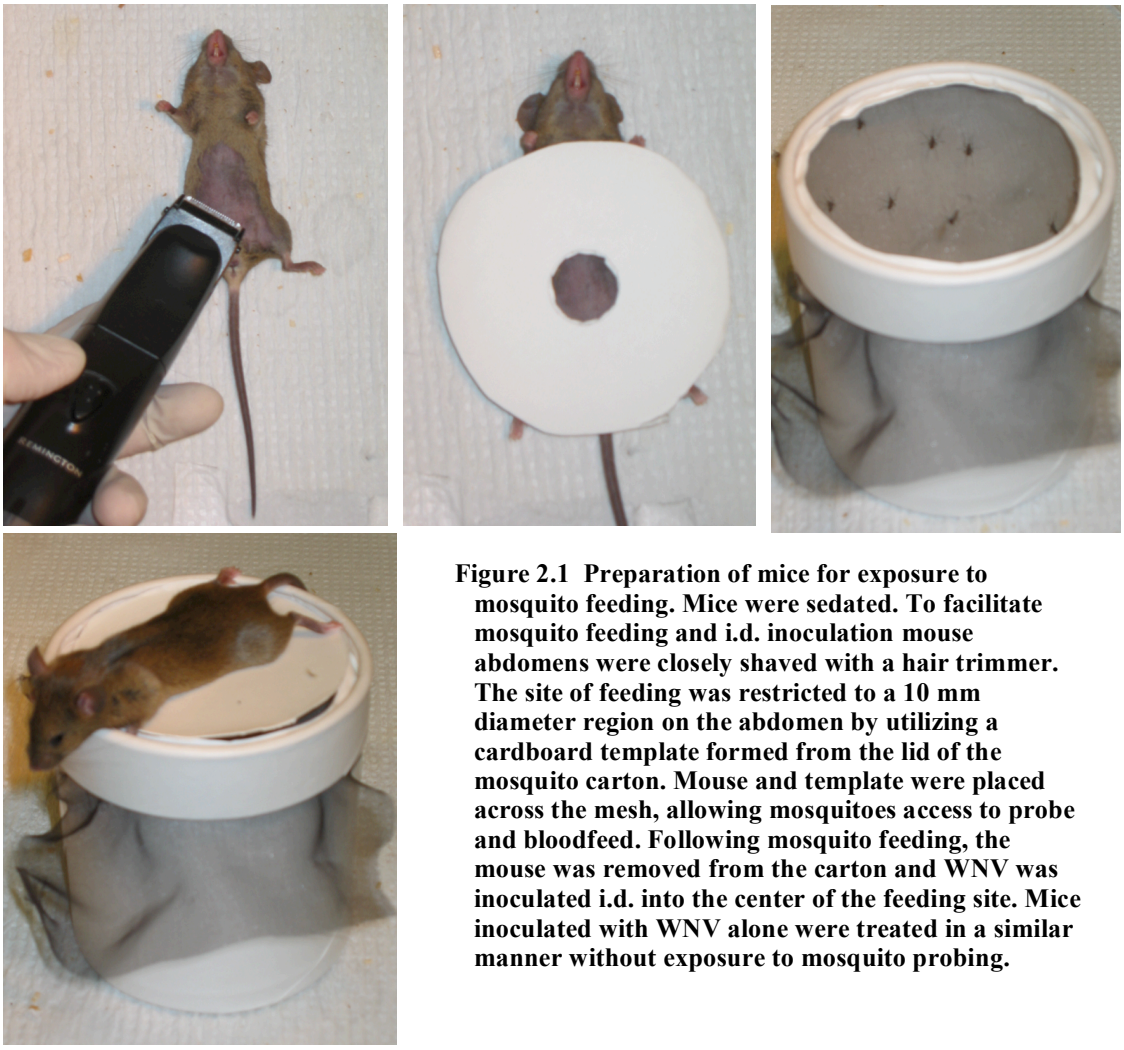

**Figure 2.1 Preparation of mice for exposure to mosquito feeding. Mice were sedated. To facilitate mosquito feeding and i.d. inoculation mouse abdomens were closely shaved with a hair trimmer. The site of feeding was restricted to a 10 mm diameter region on the abdomen by utilizing a cardboard template formed from the lid of the mosquito carton. Mouse and template were placed across the mesh, allowing mosquitoes access to probe and bloodfeed. Following mosquito feeding, the mouse was removed from the carton and WNV was inoculated i.d. into the center of the feeding site. Mice inoculated with WNV alone were treated in a similar manner without exposure to mosquito probing.**

## Virus Titration

Serum samples and inoculums were titrated as serial 10-fold dilutions on Vero cells as previously described (Higgs *et al.* 2005). One hundred microliters of each sample was loaded in duplicate into the first wells of a 96-well plate, and the samples were diluted 10-fold into L-15 across the plate (total volume in each well = 100 μl). Following the dilution series, 100 μl of Vero cells in L-15 was added to each well (1/6 of cells from a confluent 150 $cm^2$ per plate). The plates were sealed and stored in a secondary container at 37$^oC$. Seven days post-infection wells were scored for cytopathic

effect (cpe) to calculate the tissue culture infectious dose 50% endpoint titers ($log_{10}TCID_{50}$). Viral titers were calculated by the method of Reed and Muench (1938) and are reported in $log_{10}TCID_{50}$ per milliliter.

**Neutralization Assay**

Neutralization tests were performed by mixing three-fold dilutions of sera (beginning at a dilution of 1/50) with WNV virus-like particles (VLPs), generated by a modification of the methods of Scholle *et al.* (Scholle and Mason 2005), encoding firefly luciferase gene between the coding regions of the C and NS1 proteins (Fig. 2.2). These particles are equivalent to WNV particles except for their inability to exit a cell once they have infected it. Following a 60 min incubation at 37°C, the antibody/VLP mixtures were then allowed to infect Vero cells grown in a black-walled 96-well plate for 24 h. The supernatant fluid was then removed from the wells, and the cell layers were lysed in a luciferase reporter buffer and read in a luminometer. Values are reported as the serum dilution that reduced the yield of photons by 80%.

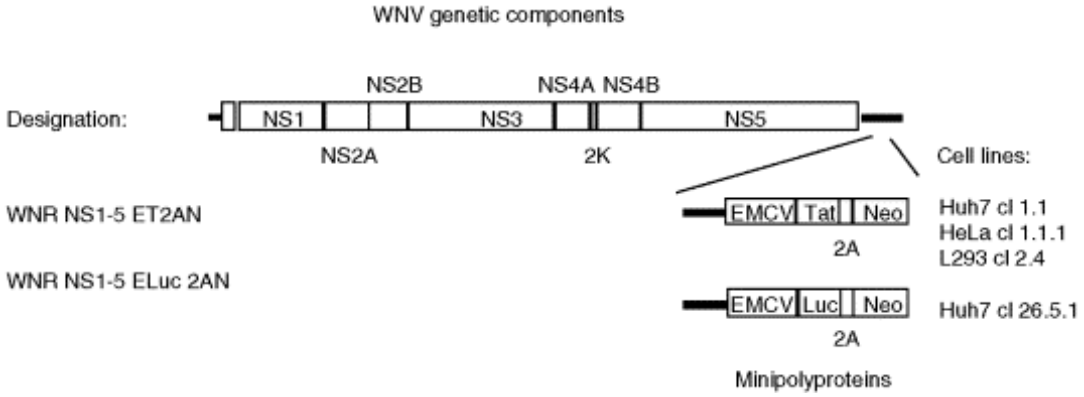


**Figure 2.2 Structure of WNV replicons (VLP) used in this study of neutralization assay. WNV replicons are deleted for the region encoding the structural proteins C, prM, and E with the exception of a small portion of C, containing a cis-acting RNA replication signal and the transmembrane region of E serving as the signal sequence for NS1. The 3′ UTRs contain a minipolyprotein coding cassette consisting of the EMCV internal ribosomal entry site (IRES), a reporter gene (HIV tat or firefly luciferase), followed by the autocatalytic FMDV 2A protease and neomycin phosphotransferase. Huh7 cl 1.1 and L293 cl 2.4 cell lines were cloned from cultures transfected with in vitro transcribed replicon RNA. HeLa cl 1.1.1 cells were cloned from cultures infected with replicons transpackaged out of Huh7 cl 1.1 cells (Scholle *et al.*, 2004). Huh7 cl 26.5.1 cells were selected after electroporation of Huh7 cultures with total RNA isolated from BHK cl 26 cells (Rossi *et al.*, 2005).**

**Organs**

Four-week old female Swiss Webster mice were divided into groups (n = 6) receiving injections of: $WNV^{alone}$ or $WNV^{mos}$. On 4 and 7 dpi, under sedation, 3 of the mice from each group were euthanized, perfused with PBS (pH 7.4), and organs were surgically removed and half was placed in RNAlater (Qiagen, Valencia, California) overnight, then liquid was removed and samples were held at -80$^{o}$C until RNA isolation. As described below RNA was isolated and RNA levels of WNV were quantified by real-time RT-PCR (Vanlandingham *et al.* 2004), normalized to the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, and then multiplied by 10,000 for convenience in interpretation of results. Viral titers resulting from this quantitative method are expressed as equivalent pfu ± standard error. The remaining half of each organ was fixed, embedded, and processed by immunohistochemistry for detection of WNV antigen using VECTASTAIN Elite Avidin/Biotinylated Enzyme Complex Kit (Vector Laboratories, Burlingame, CA) and primary polyclonal antibody reactive against WNV as previously described (Girard *et al.* 2004). .

**RNA extraction**

Tissues were thawed quickly at 37$^{o}$C, a small slice of each tissue of approximately 20 mg was isolated and placed in a 2.0 ml secure-lock eppendorf tubes, 600 µl of RLT (with 1:100 β-mercaptoethanol; RNeasy Mini Kit, Qiagen, Valencia, California) was added to each tube, and one 5mm grade-25 steel bead (GlenMills, Clifton, NJ) was added to each tube. Samples were homogenized via a Polytron homogenizer (Daigger, Vemon Hills, IL) at 30 cycles/sec for 2 min. Following tissue disruption, beads were removed without making contact with liquid by passing a magnet up the outside of the tube. Samples were spun at high speed for 5 min to remove indigestible material and supernatant was transfered to a clean tube. RNA was extracted from the tissues using the RNeasy Mini Protocol for Isolation of Total RNA from Animal Tissues (RNeasy Mini Handbook, p 50-55). Six hundred microliters of 70% EtOH were added to each tube, and each sample was transferred to a RNeasy mini column. Columns were centrifuged for 15 sec at 8,000 g, and the flow-though was discarded. The

remainder of each sample was added to the column and the column was again centrifuged for 15 seconds at 8,000 g. Seven hundred microliters of Buffer RW1 were added to each column, and the columns were centrifuged for 15 sec at 8,000 g. The column was then transferred to a new collection tube, and 500 µl of Buffer RPE were added to each sample and centrifuged for 15 sec at 8,000 g. The flow-through was discarded, another 500 µl of Buffer RPE were added to the column, and the samples were centrifuged for 2 min at 8,000 g. The columns were transferred to a 1.5 ml tube, and 50 µl RNase free water was pipetted directly onto the membrane. The samples were centrifuged for 1 min at 8,000 g, and the flow-through was collected, reapplied to the column, and centrifuged for an additional minute at 8,000 g. Samples were stored at -80$^{o}$C until analyzed.

**Creation of Standard Curve**

With real-time RT-PCR generation of a standard curve is uncomplicated due to the linear response of fluorescence over a large dynamic range (Fig. 2.3). Quantification of mRNA transcripts can be either relative or absolute, and both methods require utilizing a standard curve.

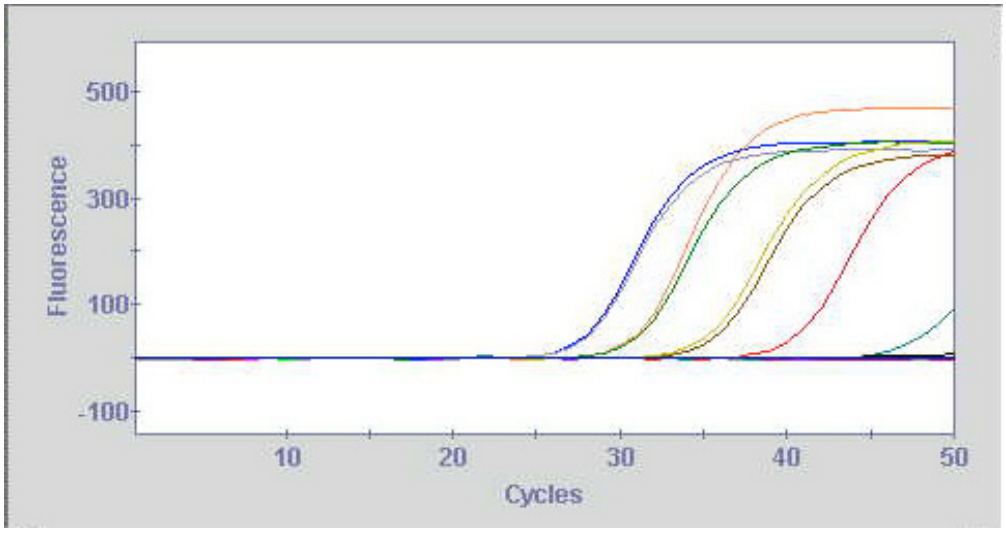


**Figure 2.3. Amplification curves generated by the polymerization of WNV standards. Fluorescence increases with each subsequent cycle, $C_T$**

To absolutely quantify a sample, the standard curve must be created from a sample of known quantity. Traditionally, this involves subcloning the target sequence behind a T7 or SP6 RNA polymerase promoter in a plasmid vector. An *in vitro*-transcribed sense RNA transcript is generated, the sample is digested with DNAse and the RNA is quantified accurately via UV-spectrophotometry. Mathematically, the target copy number per μg of RNA can be determined and thus, with a standard curve created using this RNA, either the copy number or μg of RNA can be determined for unknowns (Fig. 2.3). For samples such as virus the copy number or "μg of RNA" are of less utility than a value that is more naturally relevant such as plaque forming units (pfu) or $\log_{10}TCID_{50}$. As with all methods of quantifying live virus, use of real-time RT-PCR requires that certain assumptions be made. Specifically, one must assume that within tissues to be compared the level of non-viable virus or cellular viral-RNA remains at a constant level compared to RNA associated with viable virus. Such an assumption requires no greater leap of faith than presuming that visualization of monkey kidney cell mortality in response to virus produced from a wide variety of cell types and organisms is

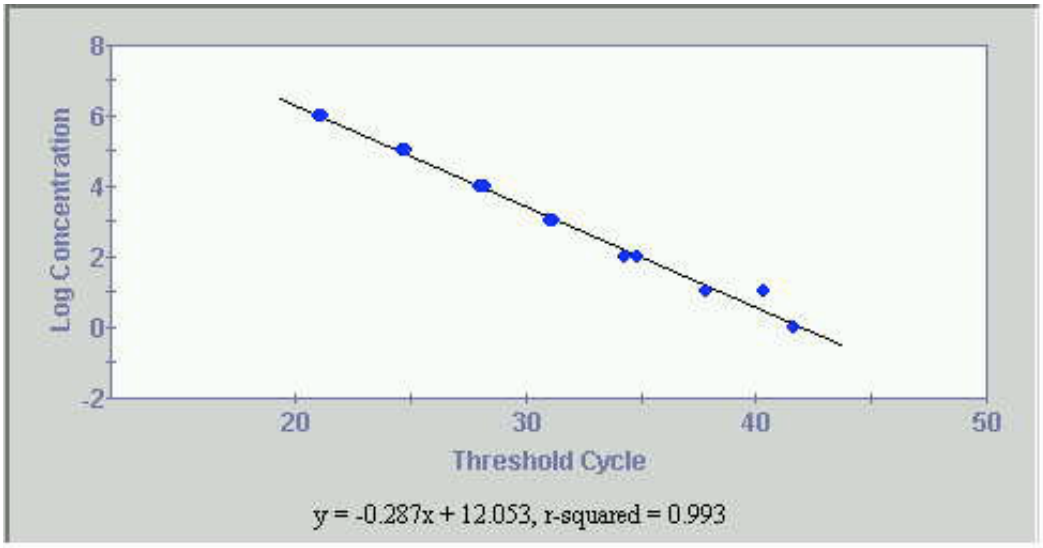


**Figure 2.4. Standard curve created for evaluating the level of WNV RNA in samples. R-squared value (0.99) shows high level of agreement between standards and values.**

a reliable measure. One effective method to create a standard curve that estimates viable viral load, is to extract RNA from a sample of known infectivity (e.g. pfu or $log_{10}TCID_{50}$). Serial dilutions can be made from the resulting RNA and to estimate the starting level of virus, a line equation is generated with the $C_T$ values of the standard curve and the log of the viral concentration (Fig. 2.4). $C_T$ values from the unknown samples are determined and plugged into the line equation, resulting in an estimated value that can be expressed as "$log_{10}TCID_{50}$ equivalents". This method of viral titer quantification is used for the experiments described herein. Often, viral titers are normalized to constitutively-expressed or "housekeeping" genes to normalize samples. In these cases viral load values are expressed as "$log_{10}TCID_{50}$ equivalents relative to GAPDH". Standard curves for GAPDH and other host genes are quantified relatively, as described below.

Relative quantification determines the comparative shifts in RNA in relation to another experimental group or a calibrator. The exact calibrator concentration does not need to be determined. As long as the dilution is known and the range of $C_T$ values fall at the extremes of sample level, the relative level of experimental samples can be established. A calibrator is used to create a dilution series, a standard curve, with arbitrary units, and during RT-PCR the $C_T$ value of the sample is compared to the $C_T$ values of the dilution series. To estimate the starting level of target RNA, a line equation is generated with the $C_T$ values of the standard curve. $C_T$ values from the unknowns are then plugged into the line equation, producing values that allow for comparison between samples. Therefore the exact number that is generated by the equation is unimportant, but the relation between values is informative. This method of comparing samples was utilized to assess the differences in host immune gene expression.

**Mosquitoes**

*Ae. aegypti* mosquitoes (RexD strain) were reared and maintained in an insectary at 27$^{o}$C and 80% relative humidity, as previously described (Higgs *et al.* 2005). Uninfected female *Ae. aegypti* mosquitoes 8- to 12-days post-eclosion were allowed to feed on the specified groups of mice as approved by the University of Texas Medical

Branch Institutional Animal Care and Use Committee. Individual mice were ventrally shaved and placed on top of mesh covering 1-quart cartons containing 15 mosquitoes that had been deprived of sucrose for 12 h to encourage feeding. The site of feeding was restricted to a circular area (1.0 cm in diameter) on the lower abdomen of an anaesthetized mouse utilizing an appropriately sized cardboard template, which was located between the mouse and mosquitoes (Fig. 2.1). Following a 30 min feeding period, WNV was inoculated i.d. into the centre of the feeding site. Mosquitoes from each mouse were chilled and the number of engorged females was noted; an average of 11.5 mosquitoes fed on each mouse with a range of ±1.5 mosquitoes.

**Salivary Gland Extract**

To obtain SGE, salivary glands were dissected from female *Ae. aegypti* mosquitoes (7-10 days post-eclosion), as previously described (Schneider *et al.* 2004), and was mixed with WNV (see above) to create a 20 μl inoculum containing an equivalent of 1.0 salivary gland pair.

**Blood-Brain Barrier Permeability Assays**

Assays were performed to assess if the feeding of mosquitoes could directly affect the permissiveness of the BBB. Swiss Webster (Harlan) mice were sedated with pentobarbital (Nembutal®; 50 mg/kg IP) and exposed or not to the feeding of *Ae. aegypti* (n = 50) for approximately 30 minutes. No less than 30 mosquitoes fed on each mouse, as assessed by direct observation of bloodmeals. At 1, 4, and 14 h post-exposure mice were inoculated intraperitoneally with 800 μl of 1% (w/v) Evans Blue dye. Under normal conditions Evans Blue dye is restricted from crossing the BBB, and thus the presence of this dye in the brain following perfusion is indicative of vascular permeability in this tissue.

***Perfusion Protocol***

One hour after injection of Evans Blue dye mice (n=3/group) were euthanized by an over-dose of Nembutal®. Mice were placed on their backs on a dissection tray with the tray on a slight incline. An abdominal Y-incision was made with surgical scissors to

expose the ribcage. Residual dye was washed off with PBS and chest cavity was exposed via lateral cuts bisecting ribs on both sides of the mouse. The heart was uncovered, a blunt 18-gauge needle was inserted into the left ventricle, and using scissors the right atrium was pierced to allow the blood to flow out. Mice were perfused with approximately 15 – 25 ml of PBS until fluid flowing from the atrium was clear and liver, eyelids, and tongue appeared white.

***Evans Blue dye Detection***

Brains were excised following surgical removal of the skull and the extent of Evans Blue dye leakage was macroscopically assessed. This assay was replicated twice. Subsequent to visual inspection additional techniques were utilized to determine BBB permeability and hence brains were fixed in 4% formal saline for 24 h, placed in 70% ethanol, and processed for 8-10-micron sagittal sections. Unstained brain sections were visualized under fluorescent microscopy, as Evans Blue glows red in these conditions, but dye was not apparent in these thin sections. Therefore, sections were processed by IHC using the protocol described above with a primary antibody directed against murine albumin.

Another set of mice (n=3/group) was exposed to the same conditions, with the exception of perfusion. The specimens were instead extracted in 3 ml pure formamide (Sigma, St. Louis, Missouri) for 72 h and the optical density of the extracted Evans Blue dye measured at 620 nm.

**Statistics**

The Student's unpaired *t*-test was used to assess significance between tissue titers of WNV. Analysis was performed utilizing SigmaPlot (Systat Software, Inc., Point Richmond, California). Wilcoxon signed ranks test was performed, using Analyse-it General 1.71 (Analyse-It Software, Ltd., Leeds, England), and two-tailed *p* values were generated, to compare survival curves. Fisher's exact tests were used to compare the proportion of tissues with detectable WNV. In all statistical tests, values of *p* that were less than 0.05 were considered significant.

## RESULTS

### Mosquito saliva enhanced progress of disease and mortality

To determine if mosquito saliva alters the course of WNV infection, mice were inoculated i.d. with WNV alone (WNV $^{alone}$) or concomitantly with mosquito saliva which was provided either via addition of SGE to viral inoculum (WNV$^{SGE}$) or via direct feeding of mosquitoes on mice (WNV$^{mos}$). Survival curves between groups varied (Fig.

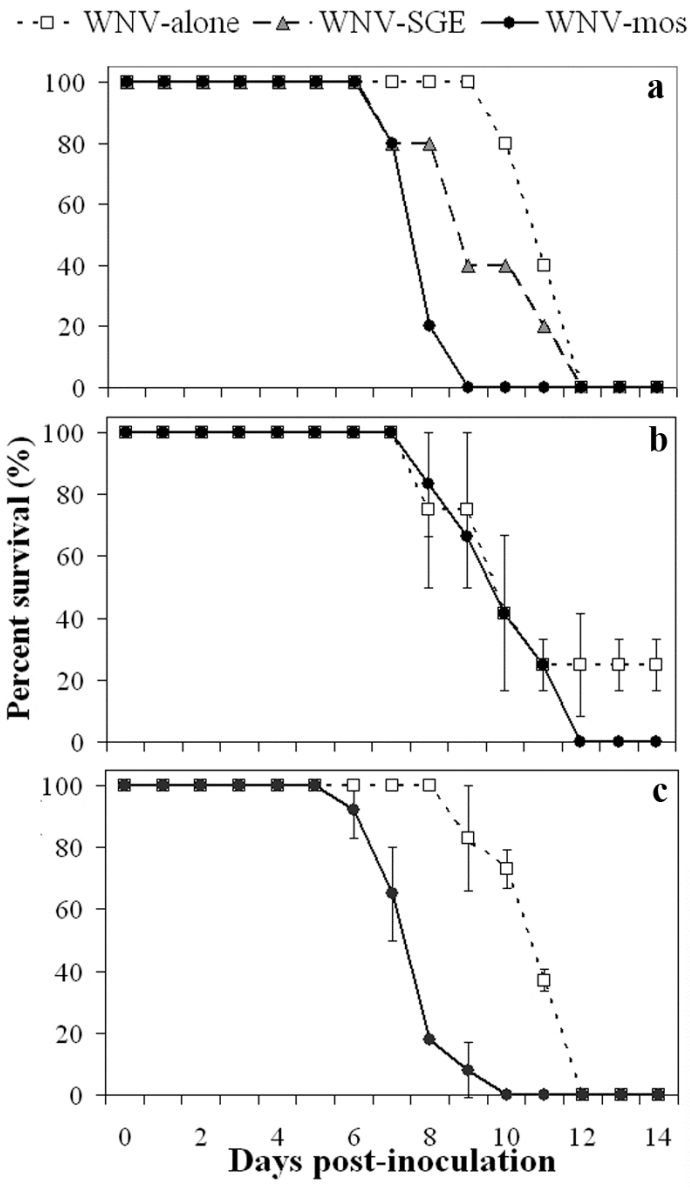


**Figure 2.5. Mice exposed to mosquito feeding prior to WNV inoculation have accelerated and increased mortality. (a) Survival of mice inoculated i.d. with $10^4$ pfu of WNV alone, concurrently with *Ae. aegypti* mosquito salivary gland extract (SGE), or following the feeding of mosquitoes (mos). (b-c) Average survival of mice following inoculation with (b) $10^2$ or (c) $10^4$ pfu of WNV. Viral injection was administered alone or subsequent to the feeding of *Ae. aegypti* mosquitoes. Results reflect the average of 3 trials (n = 6). At each dose survival curves are statistically divergent ($10^2$ pfu: $p = 0.03$; $10^4$ pfu: $p = 0.03$). Error bars above and below points indicate standard error.**

2.5a-c). The inoculum of $10^2$ pfu of WNV resulted in similar survival curves between groups, but differences in overall survival rates. All $WNV^{mos}$ mice died, but on average, 24.9% of mice injected with $WNV^{alone}$ survived (Fig. 2.5b). This suggests that when low amounts of virus were inoculated, factors in mosquito saliva altered the disease in a manner that enhanced the likelihood of a fatal outcome.

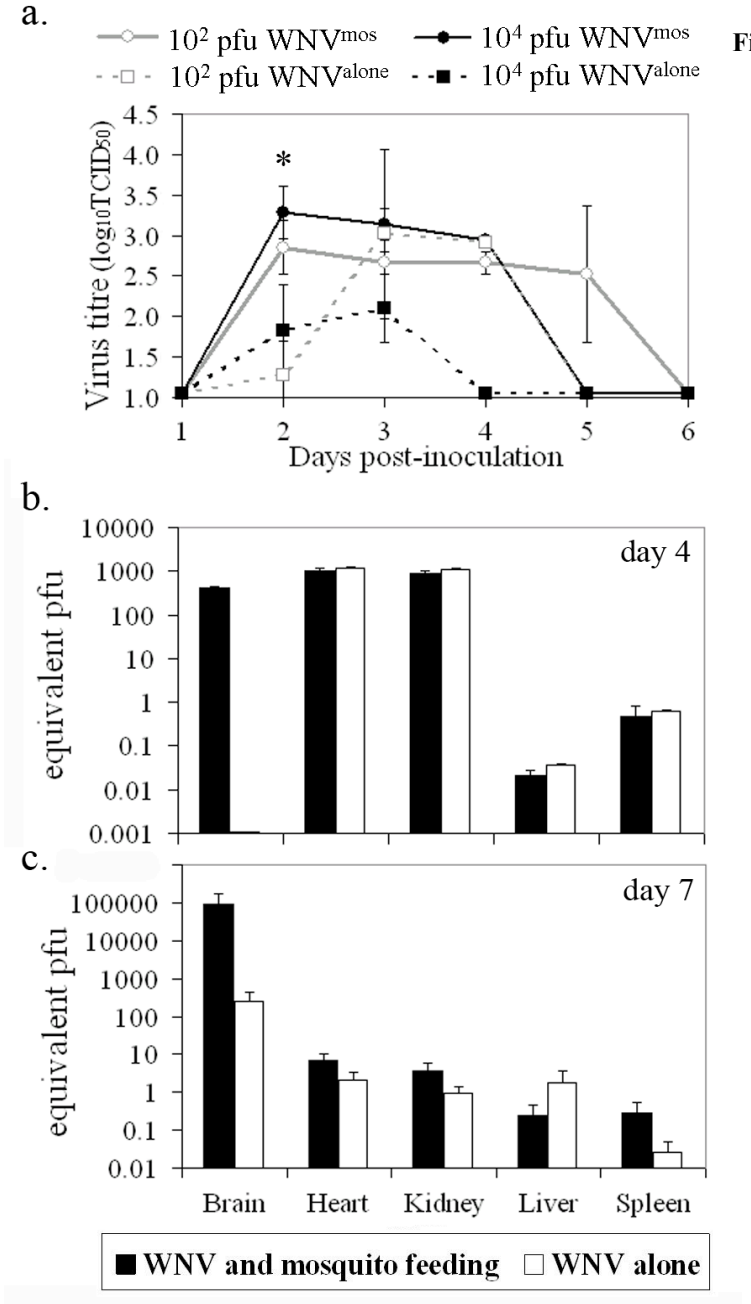


**Figure 2.6. Enhanced WNV serum and tissue titre in $WNV^{mos}$ mice. (a) Average viremia following i.d. inoculation of WNV, as determined by titration on Vero cells. N=6 mice per time-point per group, and level of detection by this method is 1.05 $\log_{10}TCID_{50}$. (b-c) Viral load of WNV in selected tissues at (b) 4 dpi and (c) 7 dpi with $10^4$ pfu of WNV. Mice were inoculated i.d. into the feeding site of *Ae. aegypti* mosquitoes or without mosquito exposure. Real-time RT-PCR values of WNV were divided by corresponding GAPDH values and then multiplied by 10,000. Error bars above bars indicate standard error and * denotes $p<0.05$.**

Infection with $10^4$ pfu of WNV resulted in 100% mortality in all experimental groups (Fig. 2.5a,c). Mice inoculated with $10^4$ pfu of WNV subsequent to the feeding of mosquitoes ($WNV^{mos}$) succumbed earliest to the infection [mean survival time (MST): 7.5 days], followed by the $WNV^{SGE}$ (MST: 8.6 days), and finally the $WNV^{alone}$ group (MST: 10.6 days). These findings support the hypothesis that mosquito feeding accelerates the progression of viral infection. The intermediate MST of the $WNV^{SGE}$ group suggests that this modulation is due to saliva constituents, rather than to the mechanical act of mosquito feeding.

The reduced potentiation activity of SGE may be an effect of the extraction process or the inclusion of cellular components absent from saliva. Certainly during isolation of SGE it is possible that enzymes are released that could compromise the potency of the saliva. The effect of saliva in SGE may also be diluted by the presence of cellular proteins. Interestingly, at 9 days post inoculation, all of the $WNV^{mos}$ mice had died, whereas all of the $WNV^{alone}$ mice remained alive (Fig. 2.5a). All trials with $10^4$ pfu of WNV demonstrated parallel observations (Fig. 2.5b). At both WNV doses, the $WNV^{mos}$ and $WNV^{alone}$ curves differed significantly ($p = 0.03$). Survival curves did not differ significantly between the $WNV^{alone}$ group inoculated with $10^4$ pfu and the $WNV^{mos}$ group inoculated with $10^2$ pfu, suggesting that an inoculum of $10^2$ pfu of WNV with mosquito saliva was equivalent to an inoculum of $10^4$ pfu of $WNV^{alone}$ with respect to pathogenesis.

**Mosquito feeding prior to virus inoculation increased viremia**

To investigate the mechanism by which mosquito feeding enhanced WNV disease progression, we measured viremia levels for 7 dpi. Virus levels peaked at 2 and 3 dpi. On 2 dpi, mice treated with $10^2$ and $10^4$ pfu of $WNV^{alone}$ had comparatively low titers of 1.3±0.42 and 1.8±0.56 $\log_{10}TCID_{50}$/mL, respectively (Fig. 2.6a). In contrast, $WNV^{mos}$ mice inoculated with $10^2$ and $10^4$ pfu of WNV had relatively high titers of 2.9±0.33 and 3.3±0.33 $\log_{10}TCID_{50}$/mL (Fig. 2.6). Mice in the $WNV^{mos}$ groups generally had higher viremias compared to other treatment groups (2 dpi: $p = 0.003$), and longer durations of viremia (3.0±0.3 days), compared to mice in the $WNV^{alone}$ groups (2.1±0.2 days).

At early time points, mice in the $WNV^{mos}$ group had consistently higher viremias than did the $WNV^{alone}$ group (Fig. 2.6a), suggesting rapid amplification of viral replication. To evaluate localized replication, at 1 and 3 dpi, RNA was isolated from skin samples at the inoculation site and from the draining lymph nodes. Mice in the $WNV^{mos}$ group consistently had more WNV in the skin (6.4- and 15.8- fold more, at 1 and 3 dpi, respectively) and in the draining lymph node (25.9- and 3.4- fold more, at 1 and 3 dpi, respectively) compared to mice inoculated with $WNV^{alone}$. Although these results were not significantly different ($p = 0.07$), they are consistent with the trend of enhanced viral replication in the presence of mosquito saliva. These data suggest that mosquito saliva promotes WNV replication at the primary infection sites, thereby accelerating disease development.

Levels of neutralizing antibody to WNV were determined to ascertain whether observed *in vitro* inhibition of B cells by mosquito saliva (Wanasen *et al.* 2004) correlated with effects on the in vivo humoral response to WNV. Low neutralizing antibody titers detected in all groups at 7 dpi, were not statistically different ($WNV^{alone}$: 180±11; $WNV^{SGE}$: 530±130; $WNV^{mos}$: 260±78).

**Mosquito feeding accelerated neuroinvasion**

Due to the significance of CNS infection in disease outcome, viral titers in the

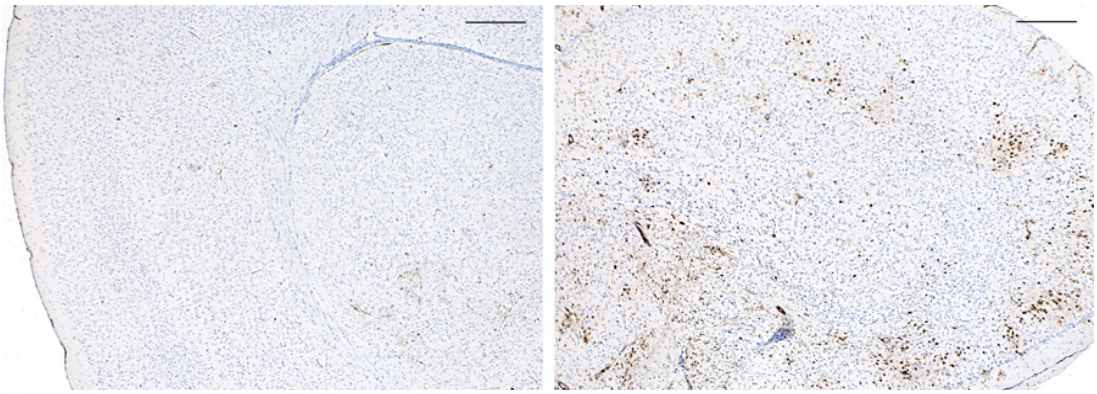

**Figure 2.7. Amplified levels of WNV antigen in the brains of $WNV^{mos}$ mice at 7 dpi as detected by IHC in the forebrain. The $WNV^{alone}$ (left panel) section contains a single small focus while the $WNV^{mos}$ (right panel) section displays widespread staining. Scale bar = 50 microns.**

brain were determined at two time-points to investigate if mosquito saliva influences viral entry or replication in the brain. At 4 dpi, significantly more virus was detected real-time RT-PCR in brains of $WNV^{mos}$ mice as compared to $WNV^{alone}$ mice ($p = 0.01$). WNV was only detected in the brains of $WNV^{mos}$ mice (3/6 mice; average of 410 equivalent pfu) (Fig. 2.6b), suggesting that mosquito saliva accelerates infection of the brain. Pathologic changes were not observed in haematoxylin and eosin (H&E)-stained sections of brain, and WNV antigen was undetectable by IHC. The absence of viral antigen and pathology at this early time point is consistent with previous reports (Xiao *et al.* 2001).

At 7 dpi, brain tissues of $WNV^{mos}$ mice had an average viral titer 350 times higher than the $WNV^{alone}$ group ($8.4 \times 10^4 \pm 7.9 \times 10^4$ equivalent pfu vs. $2.5 \times 10^2 \pm 1.7 \times 10^2$ equivalent pfu) by real-time RT-PCR (Fig. 2.6c). 100% (10/10) of $WNV^{mos}$ mice had detectable WNV in brain tissue, compared to only 80% (8/10) 0f $WNV^{alone}$ brain tissue samples, again supporting the proposition that mosquito saliva enhances CNS infection. As previously reported (Xiao *et al.* 2001), we observed minimal perivascular cuffing and contracted large neurons in deep layers of the cerebral cortex at 7 dpi in both groups (data not shown). WNV antigen was detected by IHC in the brains of 2 out of 6 mice in the $WNV^{alone}$ group. Antigen distribution was limited to small foci of neurons within the striatum (Fig. 2.7). In the $WNV^{mos}$ group, WNV antigen was detected in the brains of 4 out of 6 mice within parietal cells of the hippocampus, neurons at the ventricular surface, glial cells of the white matter, and neurons within the striatum and cerebral cortex. Staining was intense and widespread compared to the $WNV^{alone}$ group (Fig. 2.7). Greater than 10 foci were observed in two of the brain samples, whilst in the other two samples, staining was too extensive to enumerate foci. In contrast, only one focus per antigen-positive brain was identified in day 7 samples from the $WNV^{alone}$ group.

**WNV titers in tissues were higher in mice exposed to mosquitoes**

The virus titers of selected tissues were determined at two time-points to investigate the influence of mosquito saliva on virus tropisms and/or tissue-specific replication (Fig. 2.6b and 2.6c). By real-time RT-PCR, no differences between treatment groups were found in virus titers of the spleen, liver, heart, and kidney samples at 4 dpi

(Fig. 2.6b), although virus was detected in all tissues. At 7 dpi, tissues from the $WNV^{mos}$ mice tended to have higher viral loads than $WNV^{alone}$ mice (Fig. 2.6c). The use of an inbred mouse strain may have reduced variability between individuals; however our preference for outbred mice more accurately represents the diversity of natural populations. The greater variation may have reduced the power to detect significant differences between groups, thus reproducible, albeit not statistically significant, differences must be considered carefully. All heart samples contained detectable levels of WNV, yet the average titer of WNV in the $WNV^{mos}$ group was 3.4 times higher than that of the $WNV^{alone}$ group. Correspondingly, in kidneys, titers in the $WNV^{mos}$ group were 4.1 times greater than the average titers in the $WNV^{alone}$ group. Similar differences were observed in spleens (Fig. 2.6c), although only 43% (3/7) of $WNV^{alone}$ spleens had detectable WNV as compared to 100% (10/10) of $WNV^{mos}$ spleens ($p = 0.02$). Overall, these observations suggest that exposure to mosquito feeding promotes infection of specific tissues, and/or impairs the ability of tissues to clear the viral infection.

To evaluate the effect of mosquito feeding on BBB permeability, we used an Evans Blue dye exclusion assay. At 4 and 15 h post-exposure, intense Evans Blue staining was only observed in the brains of mice exposed to mosquito feeding (Fig. 2.8). Replicates of this assay failed to produce supportive results. Additionally, sections stained by IHC with antibody against murine albumin and spectrophotometric analysis of Evans Blue dye content of the brain was similarly inconclusive.

## DISCUSSION

Results of this study suggest that mosquito feeding potentiates WNV infection in a mouse model. Mice exposed to mosquitoes succumbed to infection more quickly when infected with $10^4$ pfu of WNV, had higher mortality at the lower dose of WNV ($10^2$ pfu), and had higher viremia during early stages of infection. These differences may be explained by mosquito feeding-, or saliva-induced accelerated neuroinvasion of WNV and amplified average virus titers within the brain.

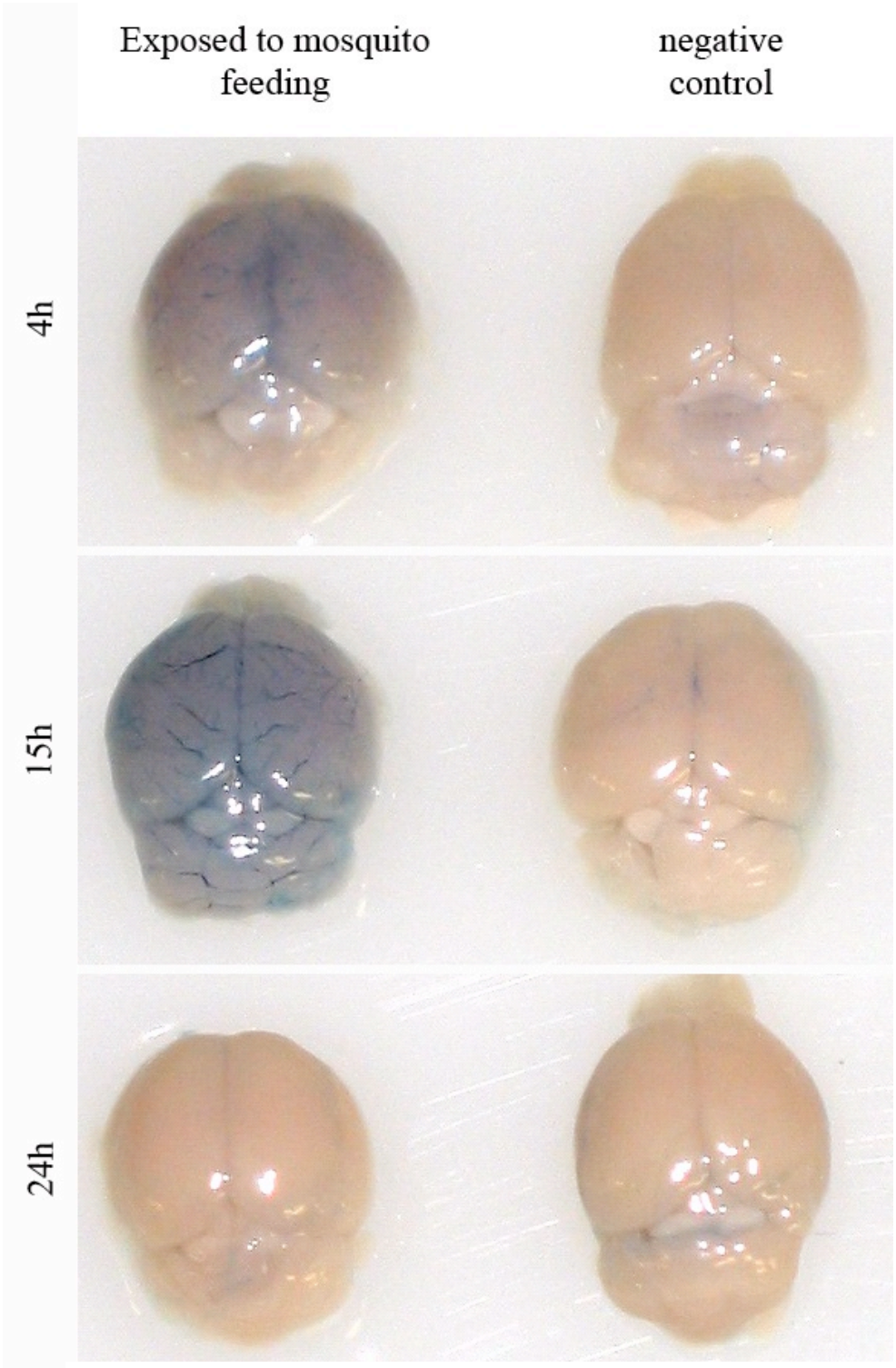


**Figure 2.8. Evans blue staining in the brains of mice exposed or not to uninfected mosquito feeding. Staining is indicative of BBB permeability.**

This study clarifies and extends previous studies that have described mosquito-associated potentiation of arbovirus infection. Limesand *et al.* (Limesand *et al.* 2000) demonstrated that 94% of the 3-week-old mice fed on by mosquitoes infected with vesicular stomatitis New Jersey virus developed antibody, while antibody was detected in only 13% of needle-inoculated mice. In a related study mice did not become infected after injection of Cache Valley virus alone, however, injection of the virus into sites of mosquito feeding resulted in detectable viremia and production of anti-CVV antibody (Edwards *et al.* 1998). Previously, La Crosse virus caused high levels of mortality in mice when injected by infected mosquitoes or inoculated into the site of intense mosquito feeding, whereas needle-inoculated virus, even at high titer, rarely caused mortality (Stephen Higgs & Barry Beaty, unpublished data). Additionally, a dose effect was noted - as the number of mosquitoes fed per mouse increased, mice developed clinical symptoms and succumbed to infection more rapidly.

**Pathogenesis of needle-inoculated WNV**

The mechanisms of WNV pathogenesis are not completely understood; of what is known, possible targets of salivary modulation can be inferred. In mice, WNV induces a systemic infection, resulting in encephalitis and death (Wang *et al.* 2001a). Type-1 IFNs and humoral immunity provide immediate defense against the dissemination of WNV (Anderson and Rahal 2002 Diamond *et al.* 2003, Diamond *et al.* 2003a Diamond *et al.* 2003, Lucas *et al.* 2003 Diamond *et al.* 2003). Recent studies have shown that co-inoculation of *Ae. aegypti* saliva with an arbovirus leads to over 50% inhibition of the local type-1 and type-2 IFN response of the host at crucial early time-points in infection, when compared to inoculation of the virus alone (Schneider *et al.* 2004). This saliva-induced suppression of interferon corresponds with other studies (Hajnicka *et al.* 2000, Limesand *et al.* 2003). Since IFN–$\alpha$ and $\beta$ have demonstrated protective activity against WNV (Anderson and Rahal 2002), their suppression could impair the ability of the innate immune response to resist viral infection while the adaptive response establishes, thereby permitting replication and dissemination of the virus early in infection. Cellular immunity also participates in recovery of the host from WNV infection (Wang *et al.*

2003a, Wang *et al.* 2003b), and cytotoxic T lymphocytes and a robust $T_H1$ response have proved to be important in host defense against flaviviruses infection (Chambers and Diamond 2003b). Indirect inhibition of antiviral cytokines and the $T_H1$ response may be precipitated by a substantial increase in $T_H2$ cytokines observed following mosquito feeding which have an inhibitory effect on natural killer cells, IFN–γ production, and antigen presentation (Zeidner *et al.* 1999, Schneider *et al.* 2004). Mosquito saliva has the potential to affect both innate and adaptive immune defenses against WNV infection, and these modulations may, at least in part, determine the course of viral pathogenesis. It is important to note that effects of mosquito saliva on systemic host immune response have been observed for up to 14 days after mosquito feeding (Zeidner *et al.* 1999). Differences observed in this study between experimental groups as early as 1 and 2 dpi suggest that the effect of mosquito saliva may be due to modulation of host innate immune responses, although previous studies have also suggested effects of saliva on adaptive immune response (Zeidner *et al.* 1999, Wanasen *et al.* 2004).

**Possible mechanisms of accelerated WNV mortality due to mosquito feeding**

The accelerated and increased death rate of mice infected with WNV following the feeding of mosquitoes may be attributable to earlier and enhanced infection of the brain, especially considering that fatal arboviral infection in vertebrates involves CNS pathology including lethargy, decreased responsiveness, confusion, and paralysis (Ceccaldi *et al.* 2004). The enhancement of neurological infection observed in $WNV^{mos}$ mice may be explained by direct effects of mosquito feeding or indirectly via immune response to mosquito saliva (Schneider *et al.* 2004) and enhanced viremia. Previous research demonstrated that tumour necrosis factor–α (TNF-α) levels were enhanced ~6-fold by mosquito feeding (Demeure *et al.* 2005). Wang *et al.* (Wang *et al.* 2004) showed that TNF-α receptor 1 signalling is instrumental in enhancement of blood-brain barrier permeability to WNV infection. Also the experimental effect of mosquito feeding on disease development is particularly significant given that, in a natural setting, hosts are continually fed upon by mosquitoes and often at high densities.

Higher viremia early in infection could also contribute towards accelerated pathogenesis since a high viremia enhances the potential for the brain to become infected (Ben-Nathan *et al.* 1996). Higher virus levels in the serum of $WNV^{mos}$ mice at 2 dpi may facilitate viral dissemination and entry into the CNS prior to establishment of a protective immune response. This early divergence between groups suggests that the innate immune response is affected by mosquito feeding. The finding of enhanced WNV viremia in mosquito-exposed mice concurs with a study that demonstrated higher and longer viremias following exposure to La Crosse virus if infection was via *Oc. triseriatus* mosquitoes versus a needle (Osorio *et al.* 1996). Studies (Edwards *et al.* 1998) also observed enhancement of CVV viremia caused by mosquito feeding. These studies along with the present research suggest a complex role for mosquito saliva not only in disease pathogenesis, but also in the natural cycle of arboviruses.

Neutralizing antibody titer did not vary significantly between groups at 7 dpi, despite higher viremia in the groups of mice exposed to mosquitoes. The higher viral titers in the $WNV^{mos}$ group might be expected to stimulate the production of elevated serum antibody titers (Shinzato *et al.* 1993); therefore our data may indicate suppression of antibody response by mosquito saliva. Previous studies reported an inhibitory effect of saliva on B cell proliferation *in vitro*, although they did not investigate the response during an active viral infection (Wanasen *et al.* 2004). Additional research is needed to determine whether differences exist at earlier time-points or in the CNS.

## CONCLUSIONS

Results from this study suggest that the potentiation of WNV infection induced by mosquito feeding is linked to factors in mosquito saliva, as opposed to the mechanical act of feeding. Potentiation of infection in the $WNV^{mos}$ group cannot be explained by enhanced hematogenous virus dissemination due to the physical puncturing of capillaries by mosquito probing at the site of virus inoculation given that WNV infection was similarly potentiated in the $WNV^{SGE}$ group.

Our data demonstrate that mosquito feeding can potentiate infection of an important emerging arbovirus, and suggest that this potentiation is due to factors in mosquito saliva. Importantly, this potentiation can lead to differences in the survival rates and disease course of the host. The effect of mosquito saliva alters early pathogenesis, such as amplified viral titer proximal to the inoculation site and serum, and later pathogenesis, including accelerated neuroinvasion and time/rate of death. Further work in this area will allow for determination of specific factors in vector saliva that affect disease course and may thereby lead to enhancements in prophylactics for arthropod-borne diseases.

# CHAPTER 3: *AEDES AEGYPTI* SALIVARY GLAND EXTRACTS MODULATE ANTI-VIRAL AND $T_H1/T_H2$ CYTOKINE RESPONSES TO ARBOVIRUS INFECTION


## ABSTRACT

Vector-borne viruses are naturally transmitted when a vector salivates during feeding on a vertebrate host. Most laboratory studies of infection disregard the role that the vector plays in the pathogenesis of the virus. In this study, intradermal inoculations of *Ae. aegypti* SGE and SINV were used to investigate the effect of mosquito feeding on the vertebrate immune response to infection with an arthropod-borne virus. Murine cytokine expression in the skin was quantified by means of real-time RT-PCR. In response to co-inoculation of SINV with SGE, IFN-β expression at 24 and 72 h post inoculation was significantly reduced by 2.2- and 2.3-fold, respectively, when compared to injection of virus alone. IFN-γ expression in response to SINV infection was significantly decreased by 1.6-fold at 24 h post inoculation when SGE was co-inoculated. In contrast, IL-4 expression was significantly up regulated when SGE was co-inoculated at 24 h post inoculation becoming a 3.3-fold increase by 72 h post inoculation. Compared to expression in the presence of SINV alone, IL-10 expression showed a 7.6-fold increase by 72 h post inoculation in mice receiving SGE concurrently with SINV. This study suggests that the response to virus is significantly different when an infection is initiated in the presence of mosquito salivary factors, and we identify a possible mechanism for potentiation of viral infections initiated by the natural mosquito vector or in the presence of mosquito saliva.

## INTRODUCTION

Arthropod-borne (arbo) viruses are of growing concern to human health (Gubler 1998). These viruses are maintained in nature through transmission between susceptible vertebrate hosts by hematophagous arthropods, most notably mosquitoes. Arboviruses are a significant cause of morbidity and mortality worldwide (Gratz 1999). For example, Japanese encephalitis virus is the most common cause of viral encephalitis world-wide, and mosquito-transmitted Dengue viruses causes 100 million cases of Dengue fever a year. In 2003 alone approximately 9,000 Americans had confirmed cases of WNV, and over 200 of these cases were fatal.

### *Modulation of Host Immune Response by Arthropod Feeding*

Human infection occurs when an infected mosquito takes a blood meal, inoculating virus with its saliva (Osorio *et al.* 1996). Although mosquitoes may feed extravascularly or directly from the vascular system, it appears that a majority of saliva is deposited extravascularly in the dermis during the probing and feeding of the mosquito (Turell *et al.* 1995). It is now well established that arthropod saliva actively counters host hemostatic, inflammatory, and immune responses (Ribeiro 1987). The effect of vector saliva on the immune response is diverse and varies among vectors - saliva of black flies decreases the expression of MHC class II (Cupp *et al.* 1998a), while *Ae. aegypti* saliva causes suppression of IL-2, interferon (IFN)-γ, and T-lymphocyte proliferation *in vitro* (Cross *et al.* 1994). Mosquito saliva contains potent antihemostatic, anti-inflammatory, and immunosuppressive factors. Identified within saliva are apyrases (Ribeiro *et al.* 1984b Ribeiro and Francischetti 2003, Smartt *et al.* 1995, Calvo *et al.* 2004, Reno and Novak 2005), tachykinin peptides (Champagne and Ribeiro 1994, Francischetti *et al.* 2003), anti-thrombin proteins (Ribeiro and Francischetti 2003, Valenzuela *et al.* 2003), and a number of unidentified immuno-modulatory factors (Ribeiro and Francischetti 2003, Calvo *et al.* 2004). Clearly, elements of mosquito saliva have the potential to cause dramatic effects on immune responses and virus pathogenesis. For example, studies have

demonstrated that *Ochlerotatus triseriatus* saliva can cause genetically resistant mice to become infected with the New Jersey serotype of VSV (Limesand *et al.* 2000). After SGE-treatment VSV is potentiated in cell lines that have an innate IFN-α/β response, as compared to cells that lack this response (Limesand *et al.* 2003). Similarly, when *Leishmania* parasites are inoculated with salivary gland extracts of sand flies, parasite burden, lesion size, and disease outcome are all amplified (Donnelly *et al.* 1998, Norsworthy *et al.* 2004). In addition, sandfly saliva inhibits the ability of macrophages to present antigen and kill the *Leishmania* parasite (Theodos *et al.* 1991). This enhancement of pathogen burden is caused by other haematophagous arthropods, such as ticks; significantly higher spirochete loads were detected in target organs of mice that were coinoculated with *Borrelia* spirochetes and tick salivary gland lysates (Zeidner *et al.* 2002). There may be epidemiological implications, also. For example, the magnitude and duration of La Crosse virus viremias in deer are increased when the host is infected via mosquito versus by needle inoculation (Osorio *et al.* 1996). Recently, *Ae. aegypti* SGE was shown to suppress antigen-specific proliferation of splenocytes, enhance splenocyte cell death, and decrease T cell replication in cell culture (Wanasen *et al.* 2004). Cytokines, the key modulators of the immune response, appear to be affected by factors present in mosquito saliva. Zeidner *et al.* demonstrated that *in vitro* stimulated splenocytes of C3H/HeJ mice fed upon by *Ae. aegypti* produced decreased levels of IFN-γ, and increased levels of IL-4 and IL-10 (Zeidner *et al.* 1999). Shifts in these secreted low-molecular-weight proteins could be partially responsibly for the observed differences in immune response and microbial pathogenesis.

While significant work has been performed on mosquito saliva and its effect on the immune response, detailed research in this area is disproportionately shifted towards other vectors and *in vitro* work. In addition, the majority of research lacks focus on the site of initial natural infection, the skin, and fail to investigate the interaction of mosquito saliva and virus on the host. SINV is commonly used as a model arbovirus, and is especially practical due to its bio-safety level-2 classification and ability to be transmitted by *Ae. aegypti*, a mosquito previously shown to cause immunomodulatory effects on

mammals. This study sought to enhance our understanding in this area through the use of this model arbovirus and *in vivo* quantification of cutaneous cytokine mRNA levels.

## MATERIALS AND METHODS

### Virus

Sindbis virus strain H55K70 was used for infection of mice. This virus strain has cell attachment and mouse virulence phenotypes identical to those of the AR339 prototypical SINV strain (Ryman *et al.* 2000). Virus was diluted to appropriate concentration in PBS subsequent to inoculation.

### Salivary Gland Extract

Mosquitoes were reared and maintained in an insectary at 27$^{o}$C and 80% relative humidity, as previously described (Higgs *et al.* 2005). Mosquitoes were supplied with a cotton wool pad soaked in 10% sucrose solution *ad libitum*. Female *Ae. aegypti* mosquitoes 7- to 10-days old were immobilized by chilling, surface-sterilized by brief immersion in 70% ethanol, and salivary glands were collected in PBS. Salivary glands were pelleted by brief centrifugation (500 rpm for 1 min), PBS was decanted, and salivary glands were stored dry at –80$^{o}$C until use. For preparation of SGE, salivary glands were thawed and resuspended in the appropriate volume of PBS. The solution was sonicated and centrifuged at 13,000 rpm for 10 min at 4$^{o}$C to release salivary proteins and remove residual cellular debris.

### Mice

Female C3H/HeJ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine), and housed in a biosafety level 2 animal facility. This strain of mouse has a mutation in the Toll-like receptor-4, therefore effects of SGE in this model were not affected by potential bacterial contamination from non-sterile mosquitoes (Poltorak *et al.* 1998). Mice (six per group) were injected with PBS, SINV alone ($10^4$ PFU), SGE alone (two salivary gland pair equivalents), or SINV plus SGE. Previous data has suggested that $10^4$ PFU of virus is a quantity that is relevant to natural transmission of arboviruses (Vanlandingham *et al.* 2004). All solutions were freshly prepared immediately prior to

inoculation. Mice were injected intradermally in the pinna of each ear in a total volume of 10 μl using a 30-gauge needle. This study was repeated twice. All protocols were approved by the Animal Care and Use Committee of the University of Texas Medical Branch.

**RNA Isolation**

At 24 and 72 h post-inoculations three mice per group were sedated with Halothane (Halocarbon laboratories, River Edge, New Jersey) inhalation and euthanized. Approximately 6.0 mm diameter circular sections including the inoculation site of the ears were dissected and immediately placed in RNAlater (Qiagen, Valencia, California). Samples remained at -80$^{o}$C until processing, when they were homogenized via bead disruption in a Polytron (Daigger, Vernon Hills, Illinois) and RNA was extracted using Qiagen's RNAeasy kit (Valencia, California) following the manufacturer's protocol. Subsequent to RNA isolation, samples were treated with DNA-free DNase (Ambion, Austin, Texas). Effective removal of residual DNA was confirmed by performing real-time PCR on samples without reverse transcriptase.

**Removal of genomic DNA contamination**

Residual genomic DNA was removed from samples using DNA-free™ DNase treatment and removal reagents (Ambion, Austin, Texas). Samples were generally treated directly after RNA isolation, but occasionally frozen samples were thawed quickly and treated. To each RNA sample 5.0 μl of 10× DNase I Buffer and 1.0 μl of DNase I (2 units) were added. Samples were mixed gently and tubes incubated at 37$^{o}$C for 20 min in a waterbath. Following incubation the DNase Inactivation Reagent was warmed to room temperature and resuspended by vortexing. Five microliters of DNase Inactivation Reagent slurry were added to each tube and mixed well. Tubes were incubated for 2-3 minutes at room temperature and each tube mixed twice during incubation period to re-disperse the DNase Inactivation Reagent. Tubes were centrifuged at 10,000 × g for 1 min to pellet the DNase Inactivation Reagent, and supernatant was

removed to a fresh RNase-free eppendorf tube. In this condition samples were frozen for future quantification of cytokine.

**Cytokine Quantification**

Skin RNA from individual mice was analyzed by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). For real-time amplification of mRNA, three oligonucleotides were designed for each target cytokine gene, a forward and reverse primer pair and a FAM (6-carboxyfluorescein) labeled probe, where FAM was the reporter dye and a black hole quencher (BHQ1), was attached to the 3'-guanidine residue. Primer-probe sets were created for murine IL-4, IL-10, IL-12 p40, IFN-β, IFN-γ, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) utilizing Primer Express (Applied Biosystems, Foster City, California), and optimized to the indicated cycling conditions. The forward, reverse, and FAM-labeled probes were listed in Table 1. Amplification of RNA used 2.5ml of template RNA, 25 pmol FAM-labeled probe, 100 pmol of each primer, in a total volume of 25 μl per reaction. Cycling conditions were 2 min at 50$^{o}$C, 10 min at 95$^{o}$C followed by 15 sec at 95$^{o}$C, and 1 min at 60$^{o}$C for a total of 50 cycles. Each experimental sample was run in triplicate wells, and the amount of template RNA was normalized by amplifying the constitutively expressed housekeeping gene, GAPDH, in parallel with each cytokine gene evaluation to normalize for differences based on sample size, extraction, and reverse-transcriptase efficiency. The level of cytokine mRNA normalized to GAPDH amplification was then expressed as an average for each sample.

**Generation of primer-probe sets**

Successful quantification by real-time RT-PCR requires the annealing of three oligonucleotides to the cDNA. Two template specific primers determine the end-points of the amplicon and provide the first level of specificity, and detection of the amplicon is supplied by the use of an additional oligonucleotide – the FAM-labeled probe. The probe provides an additional level of specificity, because fluorescent emission requires the fluorescent molecule must be released from the quencher. For this to occur, the probe

must be complementary to amplicon sequence between the primer binding sites. If no amplicon complementary to the probe is amplified during the PCR, the probe remains unbound and the fluorescence quenched. A more detailed description of real-time RT-PCR is provided in Chapter 2.

The sites chosen for amplification were highly conserved regions, while the size of the amplicons were designed to be no longer than 100 base pairs (bp). This size restriction was imposed because logically shorter amplicons amplify more efficiently and are more tolerant of reaction conditions (they are more likely to be denatured during the 95$^{o}$C step of PCR), which allows the probes and primers to compete more successfully for binding to their complementary sequence. Short amplicons have the added benefit of requiring short polymerization times, decreasing reaction time, which reduces amplification of genomic DNA contaminants and increases specificity by requiring that sequences complementary to primer and probe be in close proximity. For real-time RT-PCR primer-probe set design, emphasis was placed on designing the oligonucleotides to meeting the specific cycling conditions, instead of designing sets and then optimizing the conditions.

Primer Express (Perkin Elmer, Wellesley, Massachusetts) was used to facilitate design of primer-probe sets (Table 3.1), although inadequacies in the program required that additional selection criteria be applied to select a successful set from the candidates produced by the program. Specifically, the length of primers chosen was between 15-20 bases and the G/C content was kept between 30% and 70%. To minimize non-specific primer binding stretches of GC content were limited to no more than 5 bases and no more than 2 C's or G's were allowed in the last 5 bases on the 3' end. Secondary structure was determined to be minimal in all primers and primers were designed to be within a couple of bases of the probe. The melting temperature ($T_m$) was kept around 60$^{o}$C.

Similar conditions were considered for the probe design, with a couple of added constraints. The probe was generally between 20 and 30 bases, had a $T_m$ of 65 to 67$^{o}$C, had more C's than G's (otherwise the antisense strand sequence was used) and the 5' end

of the probe never ended in a G residue (a guanosine residue adjacent to the reporter dye quenches some of the reporter fluorescence even after cleavage).

**Table 3.1 Cytokine primers and dual-labeled probes (6-carboxyfluorescein and black-hole quencher), utilized in this study.**

| | Forward Primer (5' to 3') | Reverse Primer (5' to 3') | FAM-labeled Probe (5' to 3') |
|---|---|---|---|
| GADPH | TCACTGGCATGGCCTTCC | TCTCCAGGCGGCACGT | TTCCTACCCCCAATGTGTCCGTCGT |
| IFN-b | CCATCATGAACAACAGGTGGAT | GAGAGGGCTGTGGTGGAGAA | CTCCACGCTGCGTTCCTGCTGTG |
| IFN-g | TCAGCTGATCCTTTGGACCC | TCTCAGAGCTAGGCCGCAG | AGGAGAAGCCCAGAACTTCTGTCTCAAGTCAG |
| IL-12 (p40) | TCAGTGTCCTGCCAGGAGG | CAGTTCAATGGGCAGGGTCT | TGTCACCTGCCCAACTGCCGAG |
| IL-4 | TCATCGGCATTTTGAACGAG | TTTGGCACATCCATCTCCG | GCATGGCGTCCCTTCTCCTGTGA |
| IL-10 | ACAGCCGGGAAGACAATAACTG | CCGCAGCTCTAGGAGCATG | ACCCACTTCCCAGTCGGCCAGAG |
| IL-2 | CCTGAGCAGGATGGAGAATTACA | TCCAGAACATGCCGCAGAG | CCCAAGCAGGCCACAGAATTGAAAG |
| iNOS | CAGCTGGGCTGTACAAACCTT | CATTGGAAGTGAAGCGTTTCG | CGGGCAGCCTGTGAGACCTTTGA |

### Statistics

Cytokine mRNA levels were graphically rendered using SigmaPlot 8.0 (SPSS Science, Chicago, Illinois), and differences in cytokine mRNA production were determined by Student's *t*-test. Values of $p<0.05$ were considered significant.

## RESULTS

To determine the expression of cytokines in the injection site at different time points of experimental inoculums, total RNA was extracted from isolates of both ears and quantified using real-time RT-PCR. The level of selected cytokine mRNA in groups that received SINV and SGE was compared to the level in groups that received SINV alone. The cytokine mRNA levels of the remaining groups were compared when significant differences were observed.

### Effect of SGE on expression of pro-inflammatory and $T_H1$-type cytokines

Following experimental inoculation RNA was isolated from tissue at 24 and 72 h, and the level of pre-inflammatory and $T_H1$-type cytokine mRNA was quantified. IFN-β mRNA levels were significantly reduced by 2.2 to 2.3-fold on day 1 and 3 post-injection in groups that received a co-inoculation of SGE along with SINV (Figure 3.1A). The dermal level of IFN-γ mRNA at the site of inoculation was significantly reduced at 24 h post-injection by 1.6-fold (Fig. 3.1B) with the addition of SGE to the inoculum of SINV ($p = 0.006$). IL-12 p40 mRNA levels (Fig. 3.1C) were significantly increased in the skin of co-inoculated mice as compared to groups receiving virus alone (day 1: 5.9-fold, day 3: 4.8-fold; $p < 0.05$). Experimental inoculum groups receiving PBS or SGE had approximately equivalent expression of IFN-β, IFN-γ, and IL-12. Thus, the inoculation of SGE did not stimulate the expression of pro-inflammatory or $T_H1$-type cytokines observed in this study. SINV alone stimulated the expression of IFN-β and IFN-γ, but only slightly affected the expression of IL-12 p40 at 24 and 72 h post inoculation.

### Effect of SGE on expression of $T_H2$-type cytokines

Following experimental inoculums RNA was isolated from tissue at day 1 and day 3, and the level of $T_H2$-type cytokine mRNAs was quantified. In contrast to

expression of type-1 and type-2 IFNs, IL-10 and IL-4 mRNA levels were significantly up regulated with the co-inoculation of SGE ($p < 0.005$). IL-4 mRNA levels were 1.8 and 3.3-fold at 24 and 72 h post-inoculation, respectively, in the skin of mice co-inoculated with virus and SGE as compared to SINV alone (Fig. 3.2A). Inoculation of SINV alone led to a rise in IL-4 expression at day 1 post inoculation, but this was no longer evident at day 3 post-inoculation. By 72 h post-inoculation IL-10 mRNA in the injection site of

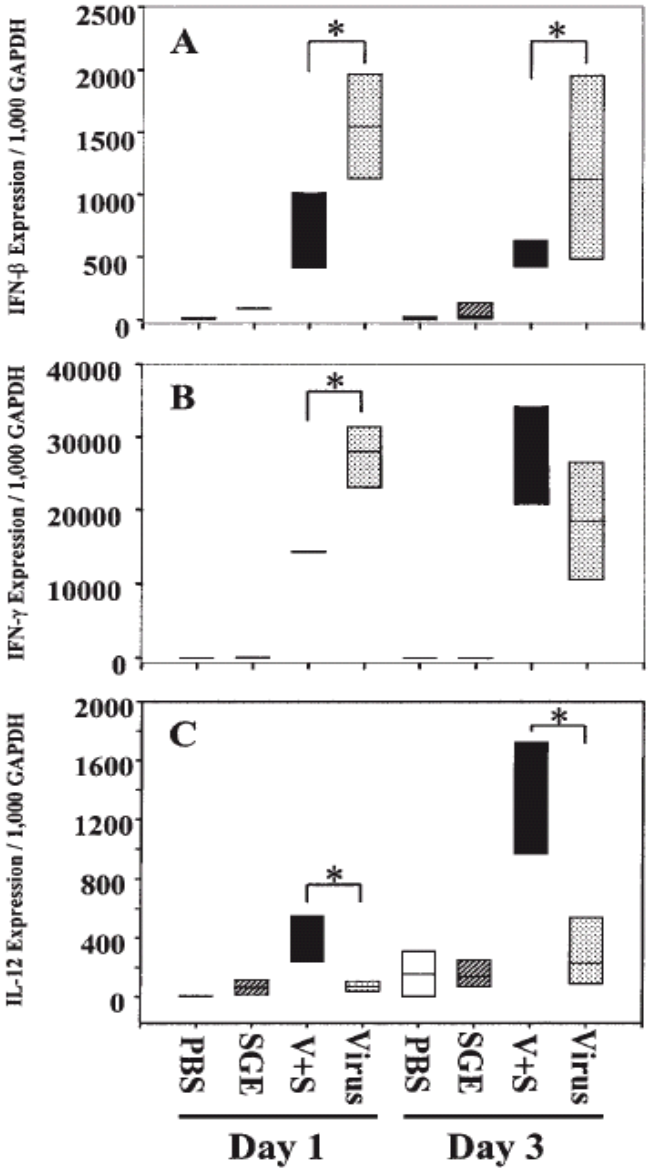


**Figure 3.1. $T_H1$-type and antiviral cytokine expression in the skin harvested from the inoculation site of C3H/HeJ mice. Skin (ear) was harvested from individual mice on the days indicated, and cytokine mRNA levels were then quantified. Samples were normalized by dividing by the mRNA level of GAPDH and multiplied by 1000. * indicates statistically significant ($P < 0.05$) differences. PBS: Control phosphate-buffered saline inoculated group, SGE: salivary gland extract inoculated group, V+S: group inoculated with Sindbis virus and SGE, Virus: group inoculated with Sindbis virus alone. A) IFN-β; B) IFN-γ; C) IL-12. Bars represent 75 to 25% confidence levels and the middle line corresponds to the median.**

mice co-inoculated with SGE was 7.6-fold that of mice inoculated with SINV alone (Fig. 3.2B). Expression of IL-10 was not stimulated by the inoculation of SINV alone. Whereas inoculation of PBS or SGE alone caused low expression of pre-inflammatory or $T_H1$-type cytokines, this was not the case for IL-4 and IL-10 mRNA expression, which was enhanced by the inoculation of SGE alone, leading to a 4.4-fold rise in IL-4 mRNA levels ($p < 0.005$). IL-10 mRNA levels were enhanced by 3.9 and 1.8-fold, at 24 and 72 h respectively, by inoculation of SGE alone as compared to injection of PBS ($p < 0.01$).

## DISCUSSION

The study presented here suggests that the vertebrate immune response to virus is significantly different when an infection is initiated in the presence of mosquito salivary factors. Our data indicate that SGE has considerable effects on dermal and/or epidermal cell expression of cytokines during early infection with SINV. The shift in cytokine expression was observed following a single injection of SGE concurrent with viral infection. Co-inoculation of SGE with SINV significantly down regulates IFN-γ and IFN-β, while significantly up-regulating $T_H2$ cytokines IL-4 and IL-10, as compared to mRNA levels observed with inoculation of SINV alone.

### Cytokines

Cytokines are secreted most prominently by lymphocytes, antigen-presenting cells, monocytes, endothelial cells, keratinocytes, and fibroblasts, which play a central role in modulation of the immune response (both innate and adaptive). Specifically they are involved in lymphocyte activation, proliferation, and differentiation, as well as inflammation, diapedesis, and anti-viral activity. Patterns of expression of cytokine mRNA dictate the specific response of a host to a pathogen. Indeed, early polar shifts in the expression of these messengers can have profound downstream effects later in infection (Pacsa *et al.* 2000). Detection of mRNA is widely used to investigate the cytokine profiles at the sites of infection and inflammation, since tissue samples for analysis are often too small to permit quantification of cytokines at the protein level.

Real-time RT-PCR has emerged as a highly sensitive and accurate method for quantifying mRNA.

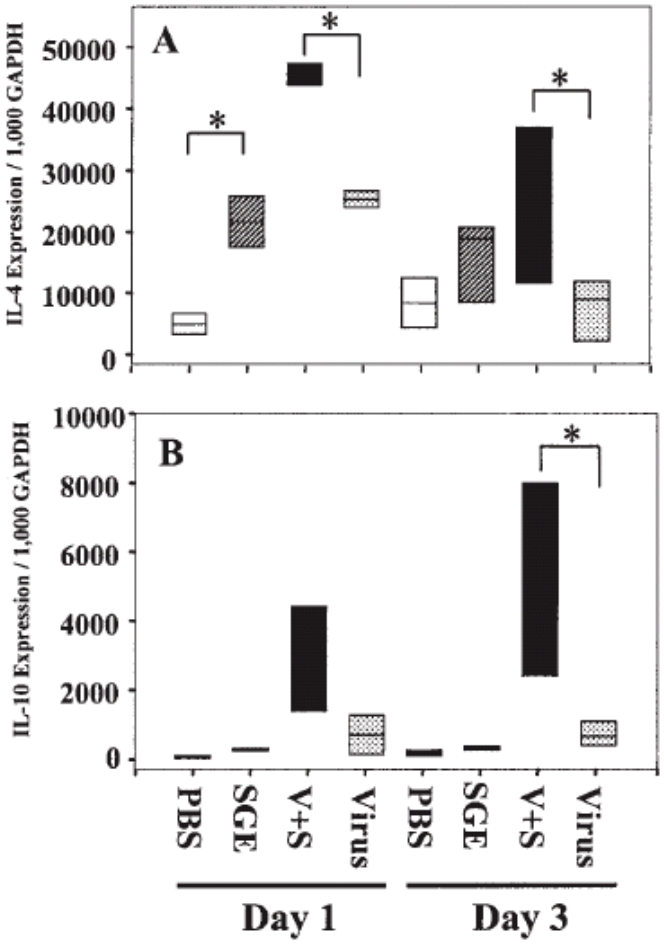


**Figure 3.2. $T_H2$-type cytokine expression in the skin harvested from the inoculation site of C3H/HeJ mice. Samples were normalized by dividing by the mRNA level of GAPDH and multiplied by 1000. * indicates statistically significant ($P < 0.05$) differences. PBS: Control phosphate-buffered saline inoculated group, SGE: salivary gland extract inoculated group, V+S: group inoculated with Sindbis virus and SGE, Virus: group inoculated with Sindbis virus alone. A) IL-4; B) IL-10. Bars represent 75% to 25% confidence levels and the middle line corresponds to the median.**

***Type I Interferons***

The immediate host response to viral infection is non-specific and involves many cytokines, including IFN-β. Secretion by cells activates antiviral mechanisms in an autocrine and paracrine manner, leading to a degradation of viral RNA, inhibition of viral translation, and up-regulation of MHC class I molecules. Previous studies have suggested that mosquito saliva can suppress this response (Limesand *et al.* 2003); salivary gland treatment of mouse fibroblast cells culminated in significant increases in viral growth, whereas treatment of cell types that lack the IFN α/β response did not cause differences in viral growth. Our study provides strong evidence that the co-inoculation of SINV with SGE can significantly reduce the expression of IFN-β in response to SINV, supporting the hypothesis that mosquito saliva significantly reduces the expression of IFN-β and, therefore, elements of the type I IFN response to virus. Indeed, the contribution of IFN-β in recovery from infection with arboviruses has been demonstrated *in vivo* by the therapeutic and prophylactic effects of administration of IFN-inducers or IFN (Haahr 1971, Vargin *et al.* 1977, Taylor *et al.* 1980). Mice deficient in IFN-α receptor had 100% mortality as compared to 30% in wild-type after a low-dose infection with Murray Valley encephalitis virus (Lobigs *et al.* 2003). Similar results are seen with IFN-α/β-deficient mice infected with SINV (Ryman *et al.* 2000). A reduction in expression of these key proteins caused by SGE in this study could allow significant enhancement of viral replication and dissemination. The IFN-β response is exceedingly important as a first-line defense against viral infection, and may be critical to initial establishment and subsequent pathogenesis of a viral infection.

***Type II Interferons***

The type II IFN, IFN-γ, plays a central role in immunoregulatory functions and is primarily made by natural killer and T cells (Boehm *et al.* 1997). This $T_H1$ cytokine functions by up-regulating MHC class I molecules, activating anti-microbial activity of lymphocytes, and stimulating the synthesis of adhesion factors on endothelial cells. Production of IFN-γ is instrumental in the successful defense against many viruses (Mullbacher *et al.* 2003, Wang *et al.* 2003a). Hence an early suppression of IFN-γ levels

after exposure to virus, as seen in mouse groups co-inoculated with SINV and SGE, has the capacity to radically shift the immune response to and downstream effects of an infection by decreasing production of MHC class I molecules on cell surfaces, diminishing the anti-viral activity of early immune cells, and reducing the recruitment of lymphocytes to the site of virus inoculation. Whereas the effect was short lived (with significant differences only detectable at 24 h post inoculation) under these conditions, SGE significantly reduced the transcription of IFN-γ at key, early points in viral infection. These data are in agreement with earlier studies, which found that *Ae. aegypti* feeding or SGE exposure significantly down-regulated IFN-γ production in murine spleen lymphocytes (Chen *et al.* 1998, Cross *et al.* 1994, Zeidner *et al.* 1999).

***Interleukin-12***

IL-12 is a critical immunoregulatory cytokine that promotes $T_H1$ differentiation, activates NK-cells positive for CD56, and stimulates the synthesis of IFN-γ in lymphocytes. Surprisingly, our results show a striking increase in the level of IL-12 p40 mRNA in groups co-inoculated with SGE and SINV, as compared to groups inoculated with SINV alone, suggesting that SGE could modulate cell-mediated immunity via different mechanisms. For example, it has been reported that the homodimeric form of the p40 subunit can actually suppress the biological activities of IL-12 p70, which is composed of two disulfide-bound subunits p35 and p40. However, this possibility warrants further investigation, and future quantification of the p35 subunit and the p70 heterodimer will clarify the exact nature of the modulation of this cytokine.

***Interleukin-4***

IL-4 is produced principally by the $T_H2$ subpopulation of activated T-cells. This prototypical $T_H2$ cytokine promotes proliferation and differentiation of B-cells and inhibits natural killer cell activation. In contrast to an earlier study (Cross *et al.* 1994), which found that Concanavalin-A driven lymphocyte IL-4 production was unaffected by *Ae. aegypti* SGE, we demonstrated that IL-4 levels were enhanced with inoculation of SGE (as compared to PBS inoculation) or co-inoculation of SGE with SINV (as compared to SINV alone). The observation that SGE inoculated *in vivo* causes a

different effect on cytokine expression than *in vitro* is not surprising given the complexity of cell types and interactions in a living animal. Furthermore, stimulation via active viral infection, instead of mitogenesis, more closely mimics a natural response. Work by Zeidner *et al.* also showed a significant up regulation of IL-4 following feeding by *Ae. aegypti* and *Cx. pipiens* (Zeidner *et al.* 1999). Additionally, a separate group found that repeat exposure to *Ae. aegypti* caused a sequential increase in IL-4 levels in activated murine splenocytes (Chen *et al.* 1998). Our results confirm the validity of these observations in vivo and show that a similar shift is present following virus exposure. A robust cellular immune response is important in limiting arbovirus infection and dissemination (Diamond *et al.* 2003a). Significant enhancement of IL-4 levels early in an infection could have profound effects on viral establishment and pathogenesis by polarizing the host immune response towards a predominantly $T_H2$ response, and thus away from essential factors such as IFN-γ.

***Interleukin-10***

Traditionally, IL-10 has many immunomodulatory effects *in vivo*. IL-10 inhibits the synthesis of a number of cytokines such as IFN-γ, IL-2 and tumor necrosis factor β. (Brady *et al.* 2003). In human monocytes IL-10 and IFN-γ antagonize the function of the other. In addition, IL-10 has been revealed also to be a physiologic antagonist of IL-12. Therefore, an increase in the level of IL-10 could profoundly weaken or discontinue the $T_H1$ response, in spite of continued expression of $T_H1$ cytokines. It also down regulates MHC class II antigen expression by monocytes and inhibits antigen presentation by several types of APC, including epidermal Langerhans cells (Enk *et al.* 1993, Macatonia *et al.* 1993). Our results demonstrate that the addition of SGE into a viral inoculum leads to a 7.6-fold rise in the IL-10 mRNA levels in the inoculation site by 72 h post injection. Viruses such as Epstein-Barr virus , cytomegalovirus, Yaba-like disease poxvirus, and orf virus, whose genomes code for IL-10 homologs, reveal the potential advantage that enhanced IL-10 levels could give to an invading virus (Moore *et al.* 1990, Kotenko *et al.* 2000, Griffiths 2002, Salek-Ardakani *et al.* 2002). Studies on long-term IL-10 therapy in chronic hepatitis C virus (HCV) cases have demonstrated the

proviral activity of this cytokine (Nelson *et al.* 2003). In one study, IL-10 caused a decrease in the number of HCV-specific $CD4^+$ and $CD8^+$ IFN-gamma secreting T cells, alterations cytokine production towards a $T_H2$ dominant profile, and an increase in viral burden. Avery and Hoover's work with lentivirus suggested a role for heightened levels of IL-10 during the permissive stage of a viral infection (Avery and Hoover 2004). The results of our study suggest that mosquito saliva can up regulate the production of IL-10.

## CONCLUSION

Whether the observed changes in cytokine mRNA levels are directly caused by factors in mosquito saliva or through shifts in extravasated cell types, antigen presentation, or effectiveness in the cellular control of viral infection, remains to be determined. The study by Wanasen *et al.* (2004) supports the likelihood that cytokine levels are affected by the capacity of SGE to suppress T cell proliferation (Wanasen *et al.* 2004). Except for species level benefits for suppression of host immune response, there is a lack of obvious necessity for individual mosquitoes to alter the immune response of their hosts, given the short feeding time of a mosquito. Nonetheless, anticoagulants and vasodilators already identified in mosquito saliva may, in addition, affect important immune functions. For example, tachykinins have been shown to modulate macrophage activation and antigen presentation (Champagne and Ribeiro 1994, Beerntsen *et al.* 1999, Zeidner *et al.* 1999). Furthermore, thrombin is known to be chemotactic for leukocytes; thus, thrombin inhibitors already identified in mosquito saliva could decrease emigration of these important immune cells (Ribeiro and Francischetti 2001, Andersen *et al.* 2003, Valenzuela *et al.* 2003). It is possible that the shift in the immune response in the presence of salivary factors is caused not by specific action of factors in mosquito saliva, but by distraction of the early immune response with the amalgam of proteins injected concurrently with the virus. The fact that effects of whole saliva can be partially mimicked by the inoculation of the salivary protein Sialokinin alone implies that the effect is due to specific compounds within saliva (Zeidner *et al.* 1999). Studies using non-salivary tissue lysates from the vector as a control show an absence of immuno-

modulatory effects attributable to these tissue extracts, suggesting that our observed effects are due to specific factors in mosquito saliva or salivary glands that are not present in the normal cellular milieu (Swist *et al.* 2002).

In summary, this study suggests a possible mechanism for potentiation of viral infections initiated by the natural mosquito vector in the presence of mosquito saliva. To our knowledge this is the first report of an *in vivo* effect of mosquito salivary gland extracts on the cutaneous immune response to a viral infection. The results of this study extend and clarify previous studies that relied on *ex vivo* techniques and artificial/non-viral stimulation of immune cells. Down regulation of innate antiviral cytokines and a polarization from a $T_H1$ to a $T_H2$ response early in viral infection as a result of factors in mosquito saliva could provide arboviruses a significant advantage in initial establishment of infection and alter viral pathogenesis. The observed differences between virus injected alone and in the presence of SGE could have significant implications to other injection-based models of mosquito-borne diseases.

# CHAPTER 4: AEDES AEGYPTI SALIVARY GLAND EXTRACTS ALTER CYTOKINE SIGNALING BY ANTIGEN PRESENTING CELLS


## ABSTRACT

Macrophages and dendritic cells are critical early responders in host defense against WNV infection, through both their direct effect on invaders, their antigen presenting abilities, and their orchestration of the immune response. Antigen presenting cells are significant in early arbovirus infection not just because of their role in orchestrating the immune response to the virus, but they are also important sites of early peripheral replication of virus. Early disruption of these proteins can have downstream repercussions that can alter the outcome of infections. The specific signals sent by these antigen presenting cells, via soluble mediators, can have a profound effect on the immune response to the an invading arbovirus and the course of the virus infection. Some cytokines trigger the activation of intracellular antiviral pathways, while others contribute to the antiviral response broadly by regulating the response directed against it. Arboviruses are transmitted to the host intimately associated with arthropod saliva, which has been shown to have immunomodulatory activity. Sand fly saliva inhibits TNF-α production, whilst enhancing IL-6, IL-10, while mosquito saliva suppresses the production of cytokines by splenocytes. To evaluate whether mosquito saliva can alter antigen presenting cell signalling in the presence of arbovirus, *in vivo* experiments were performed primary cultures of dendritic cells and macrophages. Results suggest that during active arbovirus infection mosquito saliva decreases the expression of IFN-b and iNOS by macrophages, and to a lesser extent dendritic cells, while transiently enhancing IL-10 expression. These results enhance our understanding of how mosquito saliva can affect host immune response and WNV pathogenesis.

## INTRODUCTION

Vertebrate reactions to salivary components following insect probing, depends on host and mosquito species. Cutaneous responses vary from small papules to large pruritic swellings. Hypersensitivity reactions cause a local increase in blood flow, vascular permeability, and cellular infiltrate. The immune system of the skin is composed of elaborately coordinated responses of innate and adaptive immunity. Antigen presenting cells, such as macrophages and DCs are attracted to the site of injury, and facilitate both destruction of invaders and commencement of pathogen recognition. Pathogen antigens are processed and presented by APCs to helper $T_H$ lymphocytes in association with MHC class II proteins on the surface of the APC. $T_H$ lymphocytes require secondary activation signals via co-stimulatory receptor-ligand interactions between themselves and the APC, in addition to immunoregulatory signals in the form of cytokines. The specific signals sent by APCs, via soluble mediators, can have a profound effect on the immune response to the invader and the course of the infection. Antigen presenting cells are significant in early arbovirus infection not just because of their role in orchestrating the immune response to the virus, but they are also important sites of early peripheral replication of virus. Indeed, with WNV infection, dendritic cells are accepted as the primary site of viral replication (Byrne *et al.* 2001).

Macrophages are important in host defense against WNV infection, through both their direct effect on invaders and their orchestration of the immune response. Flavivirus infection of macrophages promotes production of NO, TNF-$\alpha$, IL-1$\beta$, and IL-8 (Bosch *et al.* 2002, Bouwman *et al.* 2002, Atrasheuskaya *et al.* 2003). The behavior of macrophages is fundamental to the pathogenesis of flaviviruses, although their benefit versus deleterious effects depends on the regulation of their activity (Chambers and Diamond 2003a). Studies have demonstrated the significance of macrophages in WNV disease. One study showed that inhibition of phagocytic activity of macrophages results in enhanced viremia and neuroinvasion during WNV infection (Ben-Nathan *et al.* 1996).

A similar study demonstrated that macrophage depletion resulted in increased WNV neuroinvasion remarkably even when non-neurovirulent strains were used (Ben-Nathan *et al.* 1996). This suggests that the lack of the early antiviral activity of macrophages enhanced viremia thereby leading to early invasion of the CNS. Additional data suggest

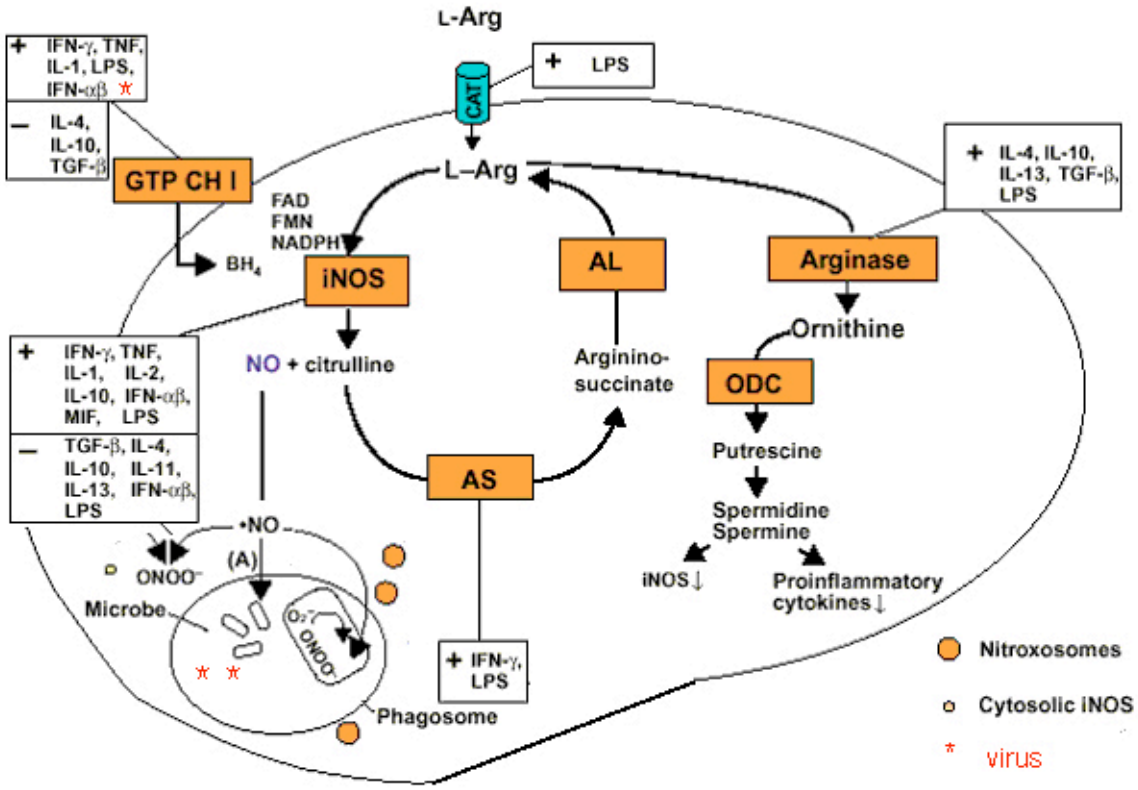


**Figure 4.1 Regulation and function of iNOS pathways in mouse macrophages. The activity of iNOS is regulated by cytokines and microbial products, which influence the uptake of L-arginine (L-Arg) by (1) amino acid transporters, (2) the synthesis of cofactors, such as $BH_4$ by GTP cyclohydrolase I (GTP-CH I), (3) the expression of iNOS mRNA and protein, (4) the enzymatic recycling of citrulline to arginine, and (5) the depletion of arginine by arginase. Products of the arginase−ODC pathway, act as immunosuppressants and can further downregulate the production of NO. The antimicrobial activity of iNOS can result from NO radicals or from peroxynitrite ($ONOO^-$) formed by the reaction of NO with $O_2$. Abbreviations: AL, argininosuccinate lyase; AS, argininosuccinate synthetase; MIF, macrophage migration inhibitory factor; ODC, ornithine decarboxylase; OAT, ornithine aminotransferase. Adapted from Bogdan, C, *Nature* Immunology, 2001.**

that macrophages exert their protective influence via antigen presentation to B and T cells and via cytokine production (Kulkarni *et al.* 1991, Marianneau *et al.* 1999). Some of the protective effects of macrophages appear to be mediated by production of reactive oxygen intermediates, especially NO (Fig. 4.1). When murine macrophage-like RAW 264.7 cells were treated with an exogenous NO donor decreasing JEV titers correlated with increasing NO levels (Lin *et al.* 1997). Nitric oxide was found to profoundly inhibit JEV RNA synthesis, viral protein accumulation, and virus release from infected cells (Lin *et al.* 1997). Competitive inhibition of NO by N-nitro-L-arginine methyl ester in mice and in cells, increased mortality due to JEV (Lin *et al.* 1997). Despite the significant contributions of macrophages to early suppression of flavivirus infection, it is possible that this cell type plays a duplicitous role due to antibody-mediated enhancement, immunopathology, and their susceptibility to infection (Peiris and Porterfield 1979).

Dendritic cells, by virtue of their expression of DC-SIGN-like attachment molecules, are believed to be initial targets for WNV replication after skin inoculation (Byrne *et al.* 2001). Studies assessing the effect of WNV infection on DCs give an incomplete picture of their role, therefore data from studies looking at the results of DC infection by other flaviviruses are informative. The infection of DCs by DENV induces production of TNF-α and IFN-α, but not IL-6 and IL-12 (Ho *et al.* 2001). A concurrent study found that exposure to live DENV led to maturation and activation of both the infected and surrounding uninfected DCs, and corroborated observation of the production of TNF-α and IFN-α (Libraty *et al.* 2001). In contrast, this group detected low-level release of IL-12 p70, a key cytokine in the development of cell-mediated immunity, following DENV infection. Interestingly, activation of the DENV-infected DCs is blunted compared to the surrounding, uninfected DCs (Libraty *et al.* 2001). Upon the addition of IFN-γ, there is enhanced activation of DENV-infected DCs and enhanced DENV-induced IL-12 p70 release. This data suggest that when DCs are the early, primary target of virus, the vigor of the subsequent cell-mediated immunity is modulated by the relative presence or absence of key cytokines in the microenvironment surrounding the virus-infected DCs.

The main mediators used by APCs to direct early immune response against a viral invader are a variety of cytokines. Cytokines produced by APCs are known to play a role in host defense against viral infections (Thomson *et al.* 1998), likely via their effect on lymphocyte activation, proliferation, and differentiation, and via inflammation, diapedesis, and anti-viral activity. Expression patterns of cytokine mRNA dictate the specific response of a host to a pathogen. Early disruption of these proteins can have downstream repercussions that can alter the outcome of infection (Pacsa *et al.* 2000). While cytokines, such as IFN-α/β, IFN-γ, and TNF-α, trigger the activation of intracellular antiviral pathways after binding to specific receptors on the surface of infected cells, other cytokines, such as IL-1β, IL-2, IL-12, IL-13, and IL-18, contribute to the antiviral response indirectly, by modulating various aspects of the immune response, including the autocrine and paracrine up-regulation of IFN-α/β, IFN-γ, and TNF-α (Thomson *et al.* 1998). Antigen presenting cells, similar to other cells in the body, respond to an incoming viral infection by secreting IFNα/β (Shirato *et al.* 2006). Activation cascades triggered by these proteins are the first line of defense for a cell against viral infection. Expression of IFNs suppress WNV replication within the infected cells, while also leading to a reduction in susceptibility of uninfected by-stander cells and specific anti-viral activation of nearby macrophages (Thomson *et al.* 1998). Pretreatment of DCs with IFN-γ, IFN-α, or IFN-β drastically reduced the susceptibility of these cells to WNV (Shrestha *et al.* 2006b).

The protective effects of macrophages and DCs appear in large part to be related to their ability to direct the antiviral immune response and limit their own infection. Clearly, the early signalling activities of these APCs, including the production of regulatory cytokines, are essential to successful defense against arbovirus infection. Consequently, any alteration of their function can exacerbate disease outcome.

Since the early stage of arbovirus pathogenesis is a period of time that vector saliva is in contact with APCs, saliva may thus have an impact on the early infection environment. Currently there has been no research to assess the effect of mosquito saliva on host APCs, however studies with other arthropods confirm that disease vectors can

affect APC function and suggest the potential for this characteristic in mosquito saliva. For example, *Ix. ricinus* SGE inhibits the killing of *B. afzelii* spirochetes by murine macrophages, and also reduces the production of two major defense molecules of phagocytes, superoxide and NO (Kuthejlova *et al.* 2001). Tick saliva additionally alters DC characteristics by reducing the expression of co-stimulatory molecules on their surface (Cavassani *et al.* 2005). *P. papatasi* saliva contains a potent inhibitor of protein phosphatase 1 and protein phosphatase 2A of murine macrophages (Waitumbi and Warburg 1998), and, reduces expression of the iNOS mRNA and NO production (Waitumbi and Warburg 1998). The authors suggest that the *P. papatasi* salivary protein phosphatase inhibitor interferes with the ability of activated macrophages to transmit signals to the nucleus, thereby preventing activation of iNOS. Sand fly saliva was also shown to inhibit the ability of macrophages to present leishmanial antigens to parasite-specific T cells (Theodos *et al.* 1993) and TNF-α production, while enhancing IL-6, IL-10, and intracellular cyclic-AMP accumulation in macrophages (Soares *et al.* 1998). Recently even the saliva of biting midges, *Culicoides sonorensis*, was shown to suppress NO secretion from peritoneal macrophages (Bishop *et al.* 2006). The potential for mosquito saliva to alter APC function is suggested in its demonstrated effect on the immunosuppressive cytokine IL-10. Interleukin-10 is significantly increased following the feeding of mosquitoes (Zeidner *et al.* 1999), and it inhibits antigen presentation, IFN−γ expression, and macrophage activation (Thomson *et al.* 1998). Reduced IFN−γ levels could also affect APCs since it activates production of cellular proteins that prevent viral mRNA translation and enhances macrophage NO production (Ribeiro and Nussenzveig 1993). This collection of research clearly establishes the ability of vector saliva to affect several important functions of APCs.

Mosquito-borne viruses are transmitted within mosquito saliva, and consequently the first cells to come into contact with these viruses are simultaneously exposed to salivary proteins. Cells at the site where mosquito saliva is deposited include DCs and macrophages. These APCs are the most peripheral components of the host immune response. Therefore, considering that the saliva of related arthropods have recognized

effects on APC function, and given the fact that early dysregulation of APC antiviral and signalling activities can alter the severity or outcome of viral disease, it is important to assess the effect that mosquito saliva has on these primary regulators of the immune response. The following experiments were undertaken to address this gap in our understanding of mosquito-borne virus transmission by determining if the presence of mosquito saliva during initial exposure of APCs to virus alters their expression of important immune mediators. Primary cultures were exposed to WNV and, to assess specificity of the results, SINV in the presence or absence of mosquito saliva, followed by real-time RT-PCR quantification of cytokine expression.

## MATERIALS AND METHODS

### Virus

WNV strain 114 (GenBank accession numbers AY187013 and AY185907) (Girard *et al.* 2004) is genetically and phenotypically identical to WNV-NY99 (Davis *et al.* 2004). Virus was overlaid on cells at a multiplicity of infection (moi) of 1 – 5 diluted in the cell-type specific medium appropriate for the particular assay.

Sindbis virus strain H55K70 was used for infection of cells. This virus strain has cell attachment and mouse virulence phenotypes identical to those of the AR339 prototypical SINV strain (Ryman *et al.* 2000). Virus was diluted to appropriate concentration in medium subsequent to inoculation. Virus was overlaid on cells at a moi of 1 – 5.

### Salivary Gland Extract

*Ae. aegypti* were reared and maintained in an insectary as previously described (Higgs *et al.* 2005). Salivary glands were isolated as described in Chapter 2. For preparation of SGE, salivary glands were thawed and resuspended in the appropriate volume of PBS. The solution was sonicated and centrifuged at 13,000 rpm for 10 min at 4$^{o}$C to release salivary proteins and remove residual cellular debris. Unless noted otherwise, primary cells were treated with a concentration of 1 salivary gland pair/ml.

**Isolation of Peritoneal Murine Macrophages**

Macrophages isolated from the peritoneal cavity are suitable primary cells for studying the activation properties of this cell type. The intraperitoneal space provides an accessible site for the harvest of either inflammatory or non-activated ("resident") macrophages. While a significant larger yield of inflammatory macrophages can be obtained from peritoneal cavities following inoculation with sterile inflammatory agents, such as thioglycollate, the number of resident macrophages able to be obtained from untreated mice is still sufficient for small scale experiments. Resident peritoneal macrophages have the advantage of not being activated and being a more homogeneous population; therefore in this study it was desirable to isolate macrophages from noninflamed peritoneums.

To collect resident peritoneal macrophages mice were euthanized by halothane or carbon dioxide asphyxiation. Abdomens were soaked with 70% ethanol to minimize the possibility of contamination and to aid in dissection. A midline incision was made beginning at the lower abdomen (about the level of the bladder), with care taken to avoid puncturing the peritoneum. Abdominal skin was retracted to expose the intact peritoneal membrane. A 30-cc syringe was attached to a 19-gauge needle and filled with 30 ml of harvest medium (Dulbecco's minimum essential medium with 5% sterile, endotoxin-free fetal bovine serum). Any air bubbles were cleared from the syringe. With the beveled end of the needle facing up, the needle was inserted through the central line of the peritoneal wall. A small amount of medium was expelled as the needle penetrated the membrane to ensure that the needle would not puncture the intestines. Ten milliliters of harvest medium was then injected into the peritoneum of the mouse, and then the peritoneal fluid was slowly withdrawn into the syringe. The fluid was withdrawn with the needle beveled end down and raised slightly to tent the peritoneum membrane. Tenting of the membrane reduces the chance of organs blocking the flow of fluid into the needle. This process was repeated with the same syringe and medium for a total of 3 mice per syringe. The needle was then removed from the syringe and the pooled peritoneal fluid was dispensed into a 50-ml polypropylene, conical centrifuge tube and

held on ice. Peritoneal lavage fluid was centrifuged for 10 min at 400 × g at 4°C to pellet resident peritoneal macrophages. Supernatant was discarded and cells were gently resuspended in culture medium.

For most experiments macrophages were resuspended in supplemented Eagle minimal essential medium (Eagle minimal essential medium, 2mM glutamine, 15mM HEPES buffer, 0.02% (w/v) sodium bicarbonate, 100 IU/ml penicillin, and 100 μg/ml streptomycin), although in environments where a carbon dioxide incubator was unavailable macrophages were grown in supplemented Leibowitz-15 medium (10% fetal bovine serum, 2mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin). Cells were plated overnight at $5 \times 10^5$ per well in 24-well plates, and non-adherent cells were washed off (of remaining cells over 85% were identified as macrophages by Diff-Quik staining) and experiment commenced the following morning.

Depending on the age and size of the mice used, approximately $1 – 3 \times 10^6$ cells were harvested from each of these untreated mice. The cells initially harvested from the peritoneal lavage were approximately 50-65% macrophages. The purity was greatly enhanced by overnight incubation followed by washing to remove nonadherent cells. Mice chosen for these experiments are specifically nonreactive to endotoxin, therefore removing the possibility that results are due to the impurity of mosquito salivary glands. This had to be considered because macrophages are acutely sensitive to endotoxin contamination.

**Isolation and Harvesting of Bone Marrow Derived Dendritic Cells**

Large numbers of DCs can be generated from mouse bone marrow progenitor cells. This method of obtaining DCs is critical for their study *in vitro*, as DCs are rare populations in all organs. Isolation procedures are time consuming and cell yields are low. $1 \times 10^4 – 10^5$ cells can be isolated from the skin and spleen of one mouse, respectively, whereas bone marrow precursors treated with granulocyte-macrophage colony-stimulating factor (GM-CSF) can produce as many as $4 \times 10^8$ DCs per mouse in about 9 days. This culture method reproducibly provides typical DCs, characterized by

nonadherence, large size, irregular shape, motility, and abundant surface CD11c and MHC class II.

To isolate bone marrow progenitors, C3H/HeJ mice were ordered from Jackson Laboratories (Bar Harbor, Maine) were allowed to rest for at least 5 days. Allowing mice to rest was important as stress (and thus corticosteroid releases) and dehydration can reduce all yields. Mice were kept under specific pathogen free conditions and were approximately 6 – 10 week old female mice. All tools and reagents used for this procedure were sterilized and proper sterile technique was utilized throughout. Mice were sedated via halothane inhalation and euthanized by cervical dislocation. Mice were surface-sterilized with 70% ethanol and an incision was made from the abdomen, down the hind leg, terminating at the paw. Intact femurs were surgically removed and placed in washing medium (Iscove's Modified Dulbecco's Medium (IMDM) with 1mM sodium pyruvate, 50 μM β-mercapethanol, 50 μg/ml gentamycin, and 100 U/ ml penicillin) on ice, until all femurs were removed. Muscle was removed from the bones using a sterile gauze pad, and the cleaned bones were placed in a Petri dish filled with 70% ethanol for 2 – 5 min for disinfection. Following sterilization, bones were washed with PBS and placed into a dish containing IMDM. Both ends (epiphyses) of each bone were cut with dissection scissors, and using a 26-gauge needle attached to a 3-ml syringe femurs were flushed with IMDM into a fresh Petri dish containing about 10 ml of IMDM. Medium was flushed through either ends of the femur until the bone appeared white upon visual inspection. Epiphyses were also held over the fresh IMDM and marrow was forced out with a stream of IMDM. This process was repeated until all bones (generally 6-10) were completed. Clusters within the marrow suspension were disintegrated by vigorous pipetting, and then collected into 15-ml conical tubes. Cells were pelleted and washed once by centrifugation for 10 minutes at 280 × g and 4$^{o}$C, followed by resuspension in complete IMDM (IMDM with 1mM sodium pyruvate, 50 μM β-mercapethanol, 50 μg/ml gentamycin, 10% filtered, endotoxin-free fetal bovine serum and 100 U/ ml penicillin). Cells were enumerated with a hemocytometer and 1:1 trypan blue solution – erythrocytes were excluded from count by size and shape. Bone marrow progenitor concentration was

adjusted to $10^6$/ml, and I ml of cells was combined with 9 ml of complete IMDM in untreated 100-mm Petri dishes (Falcon, Fisher Scientific). Cultures contained 20 ng/ml of recombinant GM-CSF (Becton Dickinson Biosciences). Plates were maintained in a humidified 37°C, 5% $CO_2$ incubator. Three days after seeding the cultures 5 ml of fresh IMDM (containing 20 ng/ml of GM-CSF) were gently added to the side of each plate. From this time point forward, care was taken to avoid jarring or excessive movement of the cells, as shifts in the environment of these cells can lead to an increased spontaneous activation state. Cultures were monitored daily to access progress and morphology of cells. At day six, 9 ml of medium were removed from each plate and 10 ml of fresh IMDM containing GM-CSF (20 ng/ml) were added. Refreshing of the medium allows for the removal of contaminating cell types, including granulocytes that develop often as nonadherent balls of very round cells and residual lymphocytes. By day 4 aggregates of growing DCs attached to the adherent stroma can be visualized. The aggregates rise above the monolayer, in contrast to the flattened macrophage colonies, and DCs are distinguishable by their dendrites (spiky processes), which extend from the periphery of the aggregates. By day 6, the wells were usually covered with numerous clumps of proliferating DCs. At day 8 bone marrow DCs were removed from culture plates by gently pipetting IMDM complete medium over the adherent stroma. Cells from many plates were pooled, centrifuged (10 min at 280 × g, at 15°C), and resuspended. Cells were counted as mentioned previously and the yield of DCs was accessed by either flow cytometry or visual enumeration of large irregular shaped cells using a hemocytometer. Flow cytometry was performed as detailed elsewhere (Chapter 5) using antibodies specific for CD11c and CD86. Bone marrow DCs were plated in 500 μl of complete IMDM at a level of $5 \times 10^5$ DCs/well in a 24-well overnight and used for experiments the following day.

**RNA Isolation**

At 24 and 48 h post-infection, three wells per group were sampled. Medium was removed from wells and replaced with 350 ml of RLT lysis buffer (Qiagen); Cell supernatant was centrifuged at 400 × g for 5 minutes. During this time, cells were

homogenized in RLT by agitation of the 24-well plate (it is the experience of this researcher that single cell suspensions lysed with this minimal treatment). Following centrifugation, supernatant was reserved for later use, and lysed cells in RLT were placed in their corresponding tubes and vortexed for 15 sec to facilitate lysis of any cells that were pelleted. Samples remained at -80$^{o}$C until processing, when they were quickly brought to room temperature with a water bath and vortexed. RNA was extracted using Qiagen's RNAeasy kit following the manufacturer's protocol as detailed previously. Subsequent to RNA isolation, samples were treated with DNA-free DNase (Ambion). Effective removal of residual DNA was confirmed by performing real-time PCR on samples without reverse transcriptase.

**Removal of genomic DNA contamination**

Residual genomic DNA was removed from samples using TURBO DNA-free™ DNase treatment and removal reagents (Ambion). Samples were generally treated directly after RNA isolation, but occasionally frozen samples were thawed quickly and treated. To each RNA sample 5.0 μl of 10× TURBO DNase I Buffer and 1.0 μl of TURBO DNase (2 units) were added. Samples were mixed gently and tubes incubated at 37$^{o}$C for 20 min in a water bath. Five microliters of resuspended DNase Inactivation Reagent slurry were added to each tube and mixed well. Tubes were incubated for 2-3 minutes at room temperature and each tube mixed twice during incubation period to re-disperse the DNase Inactivation Reagent. Tubes were centrifuged at 10,000 × g for 1 min to pellet the DNase Inactivation Reagent, and supernatant was removed to a fresh RNase-free eppendorf tube. In this state samples were frozen for future quantification of cytokines.

**Cytokine Quantification**

Cytokine expression of primary cells was determined by real-time RT-PCR. For real-time amplification of mRNA, three oligonucleotides were designed, as described previously, for each target cytokine gene, a forward and reverse primer pair and a FAM (6-carboxyfluorescein) labeled probe, where FAM was the reporter dye and a quencher,

was attached to the 3'-guanidine residue. Primer-probe sets utilized (Table 4.1) included murine IL-4, IL-10, IL-12 p40, IFN-β, IFN-γ, GAPDH and WNV. Amplification of RNA used 2.5ml of template RNA, 25 pmol FAM-labeled probe, 100 pmol of each primer, in a total volume of 25 μl per reaction. Cycling conditions were 2 min at 50°C, 10 min at 95°C followed by 15 sec at 95°C, and 1 min at 60°C for a total of 50 cycles. Each experimental sample was run in duplicate wells, and the amount of template RNA was normalized by amplifying the constitutively expressed housekeeping gene, GAPDH, in parallel with each cytokine gene evaluation to normalize for differences based on extraction and reverse-transcriptase efficiency. The level of cytokine mRNA normalized to GAPDH amplification was then expressed as an average for each sample.

**Table 4.1 Primer-probe sets used with specific primary cell cultures. SINV levels were assessed by virus titration instead of real-time RT-PCR.**

| Primary cell type | Cytokines | Constitutively-expressed genes | virus |
|---|---|---|---|
| **Bone marrow-derived DCs** | IFN-γ, IFN-β, IL-12 p40, IL-10, IL-4 | GAPDH | WNV |
| **Resident peritoneal macrophages** | IFN-γ, IFN-β, IL-12 p40, IL-10, IL-1β iNOS | GAPDH | WNV |

## Virus Titration

Serum samples and inoculums were titrated as serial 10-fold dilutions on Vero cells as previously described (Higgs *et al.* 2005). One hundred microliters of each sample was loaded in duplicate into the first wells of a 96-well plate, and the samples were diluted 10-fold into L-15 across the plate (total volume in each well = 100 μl). Following the dilution series, 100 μl of Vero cells in L-15 was added to each well (1/6 of cells from a confluent 150 $cm^2$ per plate). The plates were sealed and stored in a secondary

container at 37°C. Seven days post-infection wells were scored for cytopathic effect (cpe) to calculate the tissue culture infectious dose 50% endpoint titers ($log_{10}TCID_{50}$). Viral titers were calculated by the method of Reed and Muench (1938) and are reported in $log_{10}TCID_{50}$ per milliliter.

**Statistics**

Cytokine mRNA levels were graphically rendered using SigmaPlot 8.0 (SPSS Science, Chicago, Illinois), and differences in cytokine mRNA production were determined by Student's *t*-test. Values of $p<0.05$ were considered significant.

## RESULTS

The direct effect of *Ae. aegypti* saliva on the response of resident peritoneal macrophages and bone marrow-derived DCs to arbovirus was evaluated. Specifically the expression of key cytokines and immune mediators was investigated. For DCs IFN-γ, IFN-β, IL-2, IL-4, IL-10, and IL-12 mRNA was quantified. Likewise, for macrophages IFN-γ, IFN-β IL-1β, IL-10, IL-12, and iNOS mRNA was quantified. Additionally, GAPDH was quantified to normalize samples and virus titers were determined by titration or real-time RT-PCR. For each cell type at least 3 replicates were completed.

### Effect of Mosquito SGE on Resident Peritoneal Macrophages in the Context of Arbovirus Infection.

#### *West Nile Virus*

To assess the effect of exposure to mosquito saliva on macrophage function during WNV infection, we examined changes in cytokine expression and virus titers at 24 h and 48 h post-infection.

West Nile virus infection of macrophages stimulated a high level of type I IFN expression, whereas little to no IFN was detected in uninfected control macrophages or uninfected macrophages treated with SGE alone. At 24 h post infection macrophages treated with SGE (1.0 salivary gland pair (SGP)/ml) displayed mRNA levels of IFN-β that were approximately 33% less than those produce by infection with WNV alone (Fig. 4.2). At 48 h post-infection levels of IFN-β were still significantly lower in wells treated

with SGE (Fig. 4.3). At this time-point there was a dose-dependent decline in IFN-β expression correlating with levels of SGE from 0.25 SGP – 1.0 SGP/ml. At the highest level of SGE, IFN-β mRNA was reduced by over 50% (Fig. 4.3). One set of samples was pretreated with SGE for 1 hour prior to inoculation of WNV, in this group IFN-β levels were reduced further. In one replicate of this experiment, no difference in IFN-β expression levels was noted, although it is possible that improper culturing conditions accounted for this. No difference in type II IFN mRNA levels was detectable in any of the replicates.

Expression of the pro-inflammatory cytokine IL-1β could not be detected in either WNV-infected or uninfected groups.

At 24 h post-infection, WNV alone led to only minimal levels of IL-10 production by macrophages (Fig. 4.4). In contrast, treatment with SGE alone or in concert with

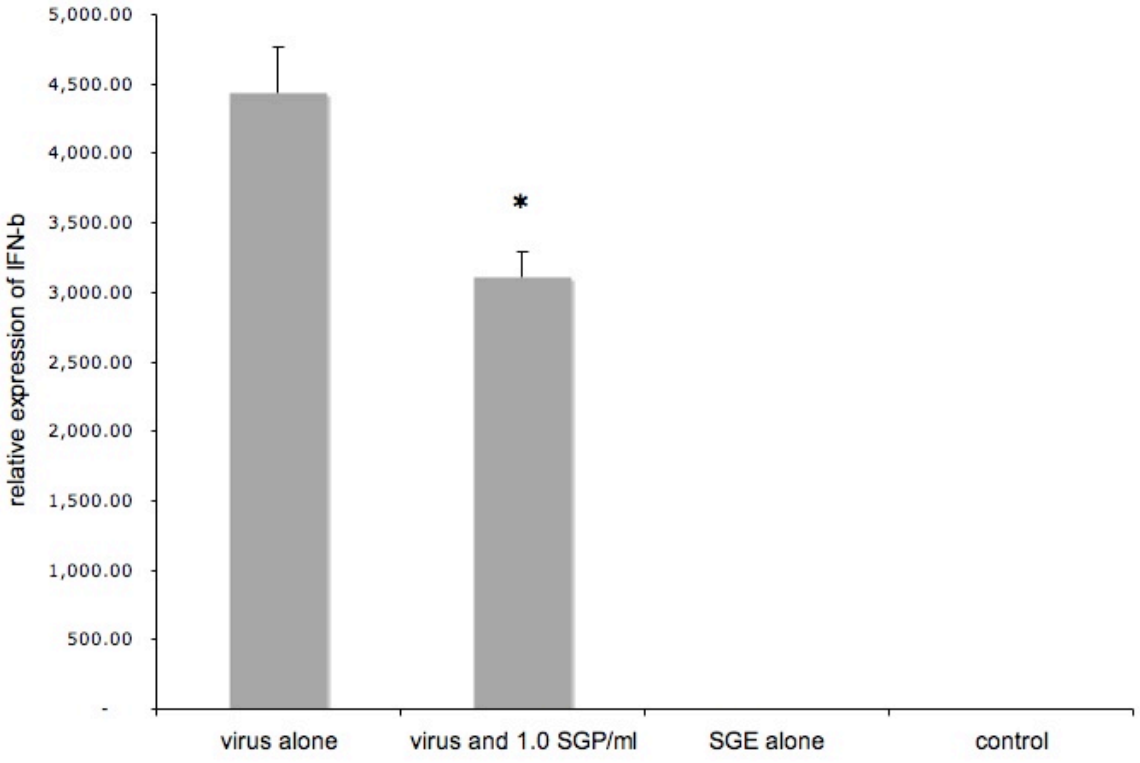


**Figure 4.2 IFN-β expression in macrophages 24 h post-infection with WNV. * p<0.05**

WNV caused relatively high levels of IL-10 to be expressed at 24 h post-infection. Due to the large variation between wells, this difference was not statistically significant, but the trend of enhancement was observed for all replicates of this experiment. By 48 h post-infection IL-10 could be detected in most groups. Surprisingly, in two of the four replicates IL-10 mRNA levels were decreased in the group treated with SGE and WNV relative to the group treated with WNV alone (Fig. 4.5). This observation was not statistically significant and, having occurred in only half of the replicates, probably is an artifact of variability between samples that are not different.

Expression of IL-12 in macrophages varied according to treatment group and could be detected in all groups. At 24 h post inoculation, groups treated with SGE had the lowest level of IL-12 mRNA (Fig. 4.6). The group co-treated with WNV and SGE had the lowest level of IL-12 expression ($p < 0.05$) with a reduction of over 68% as compared to infection with WNV alone. At 48 h post-infection there was no discernible difference in IL-12 mRNA levels between groups (data not shown).

Inducible nitric oxide synthase expression was observed in all treatment groups. The group infected with WNV alone demonstrated the highest level of iNOS expression (Fig. 4.7), with levels in the group co-exposed to WNV and SGE indistinguishable from control groups. Expression of iNOS in the group treated with WNV and SGE was 30.4% that of expression levels in the group infected with WNV alone ($p<0.05$). At 48 h post-inoculation, wells co-treated with SGE tended to have lower levels of iNOS mRNA that WNV alone groups, but the difference was not significant (Fig. 4.8).

Viral loads increased between 24 h to 48 h post-infection from an average of approximately $1.20\times10^5$ to $1.34\times10^6$ $TCID_{50}$ (Fig. 4.9). Between WNV-infected groups virus titers were identical, suggesting that any effect mosquito saliva may have does not alter viral replication in peritoneal macrophages.

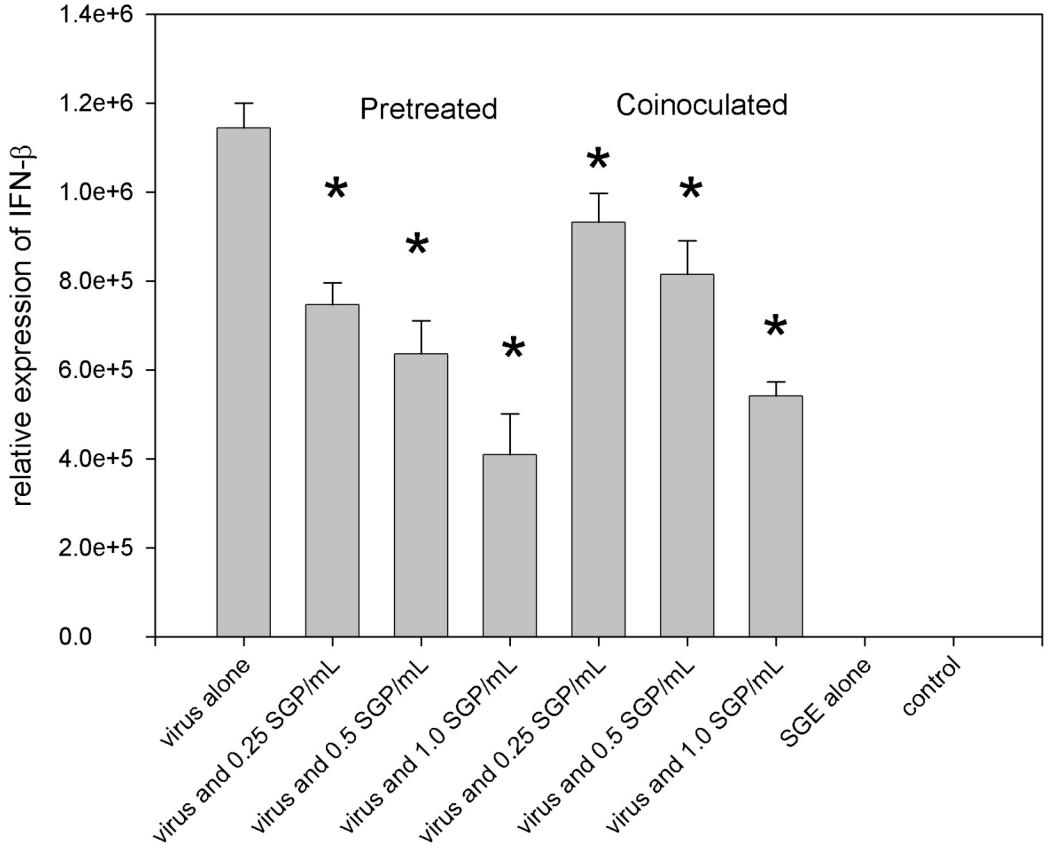


**Figure 4.3 IFN-β expression in macrophages 48 h post-infection with WNV. * $p<0.05$**

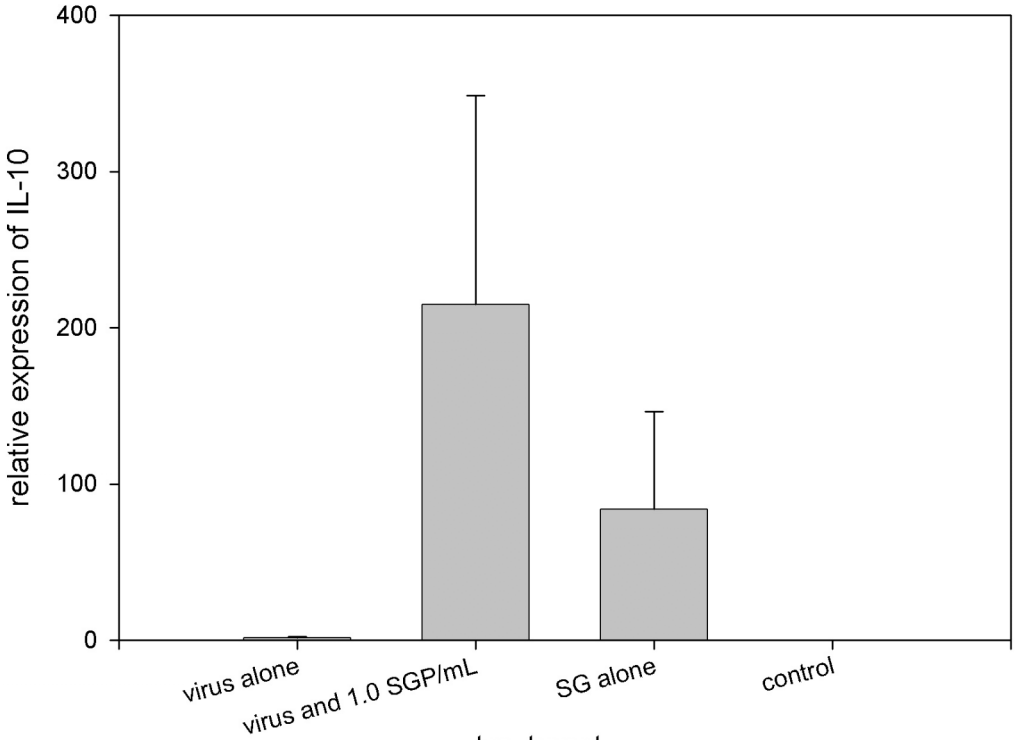


**Figure 4.4 IL-10 expression 24 h post-infection with WNV.**

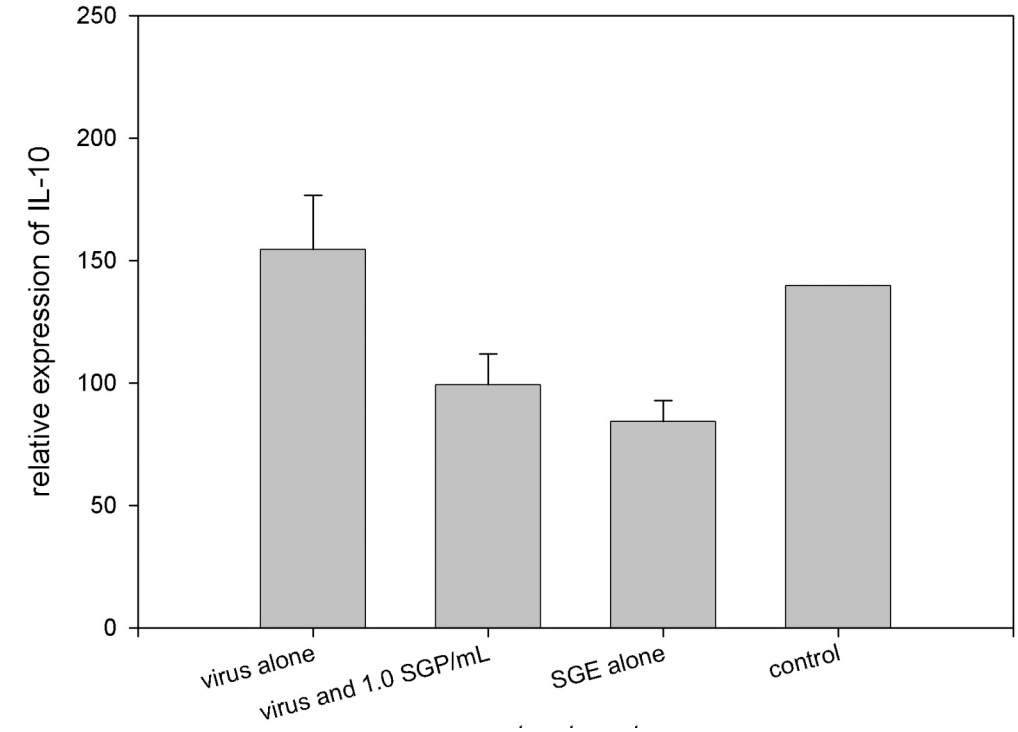


**Figure 4.5 IL-10 expression in macrophages 48 h post-infection with WNV.**

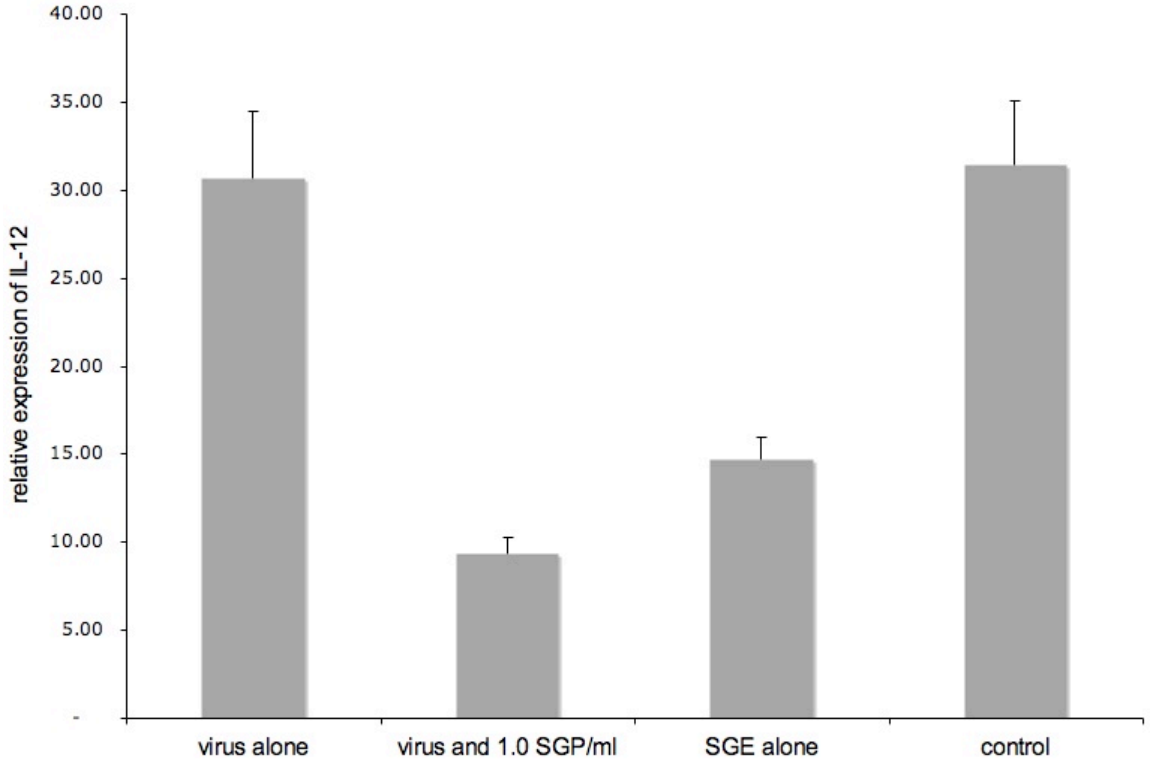


**Figure 4.6 IL-12 expression in macrophages 24 h post-infection with WNV.**

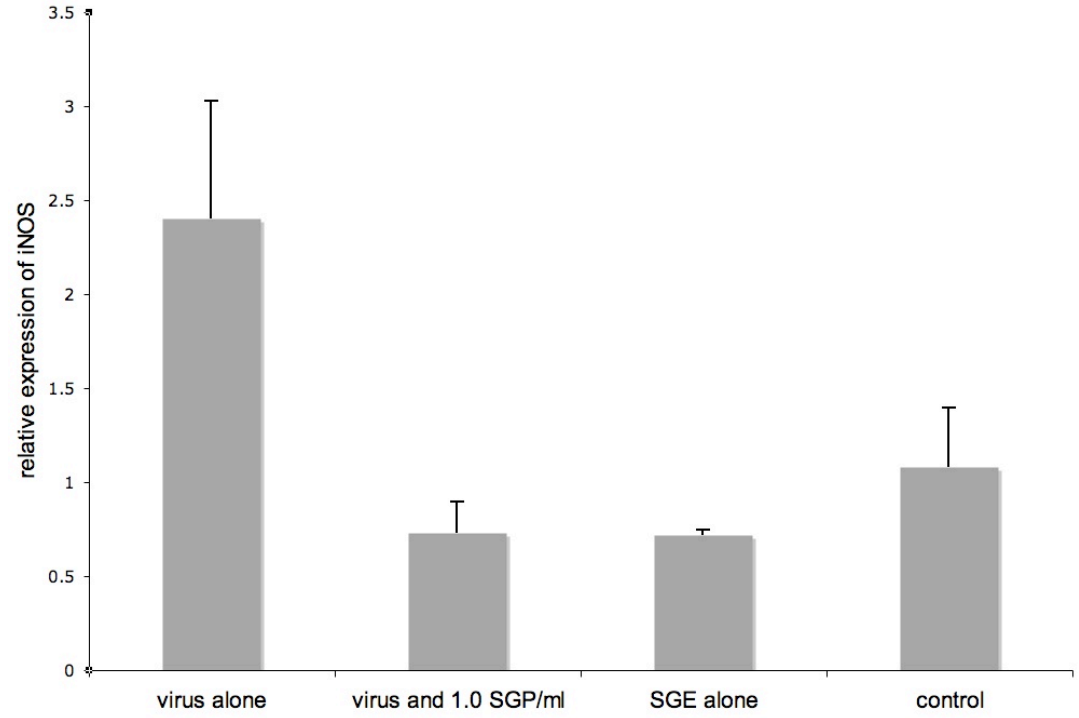


**Figure 4.7 iNOS expression in macrophages 24 h post-infection with WNV.**

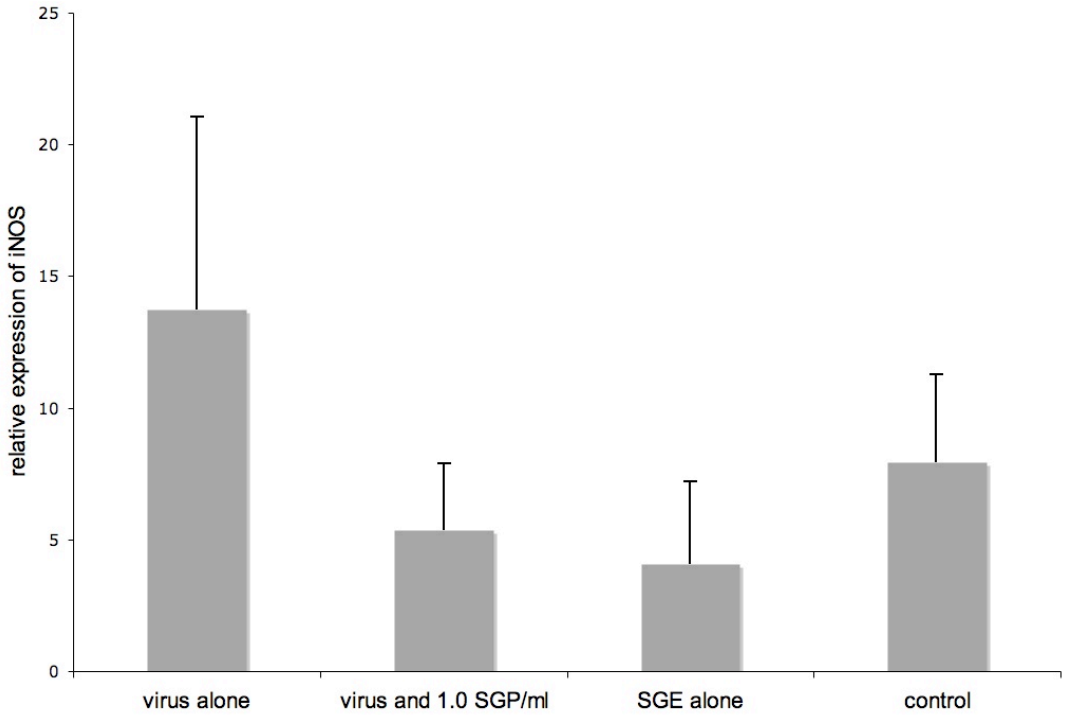


**Figure 4.8 iNOS expression in macrophages 48 h post-infection with WNV**

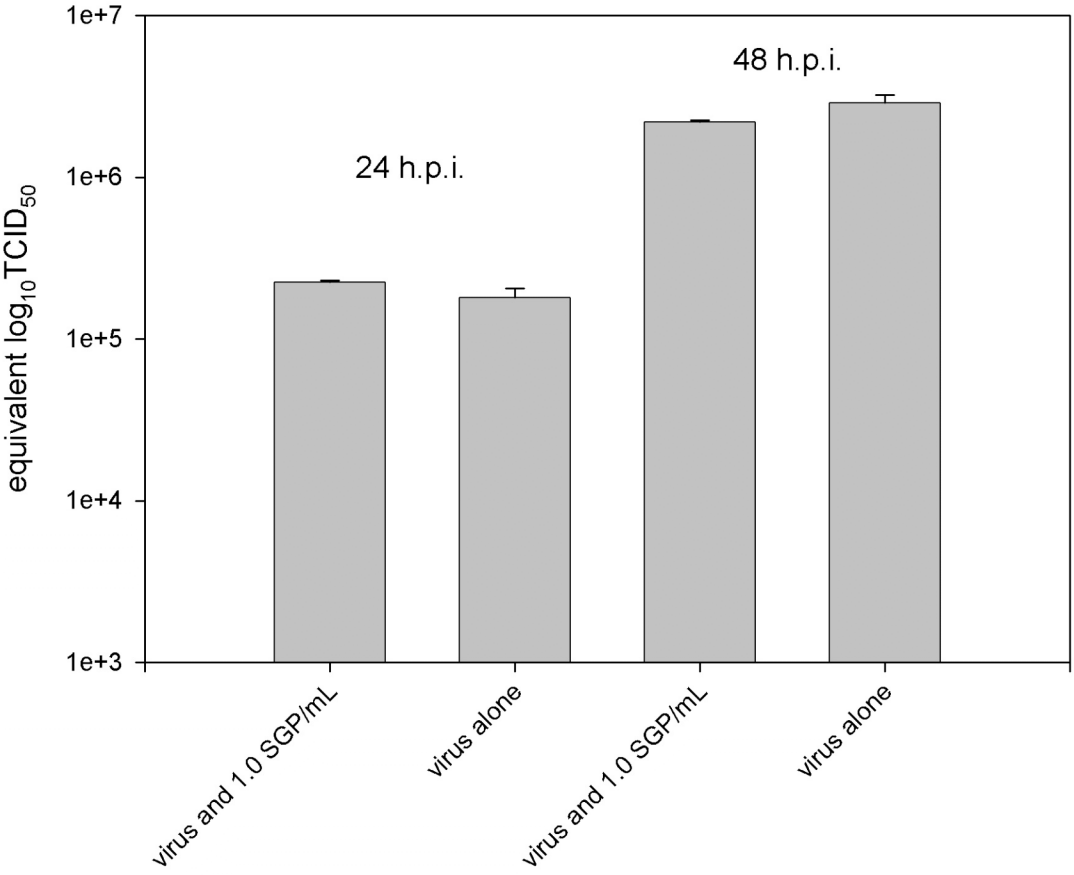


**Figure 4.9 Titer of West Nile virus in macrophages at 24 and 48 h post-infection.**

***Sindbis virus***

To determine if the effect of mosquito saliva on macrophages during arbovirus infection is unique to WNV-infected macrophages, the effect of exposure to mosquito saliva on macrophage function during SINV infection was examined. Levels of cytokine expression at 24 h and 48 h post-infection were determined.

Interferon type II expression was detected in all groups infected with SINV (Figs. 4.10,4.11). The level of IFN-β mRNA was significantly ($p$<0.05) decreased at 24 h post-infection by 39.7% in groups exposed to SGE concurrent with SINV infection (Fig. 4.10). By 48 h post-infection, this trend continued, but the suppression was only marginally significant ($p$=0.05; Fig. 4.11). As with WNV infection of macrophages, the

levels of IFN-γ mRNA did not vary significantly between groups at either time point (data not shown).

At 24 h post-infection, SINV alone induced relatively low levels of IL-10 production by macrophages (Fig. 4.12). Contrastingly, exposure to SINV with SGE caused a 50.2% increase in mRNA levels of IL-10 at the same time-point. The difference in expression of IL-10 between SINV-infected groups with or without SGE was statistically significant. By 48 h post-infection IL-10 was detected in all groups, but levels in groups infected with SINV were elevated. Between infected groups, those infected with SINV in the presence of SGE demonstrated lower IL-10 expression levels than those infected with virus alone (Fig. 4.13). This observation was statistically significant ($p<0.05$) and occurred over two dilutions of SGE.

Expression of IL-12 in macrophages did not vary according to treatment group,

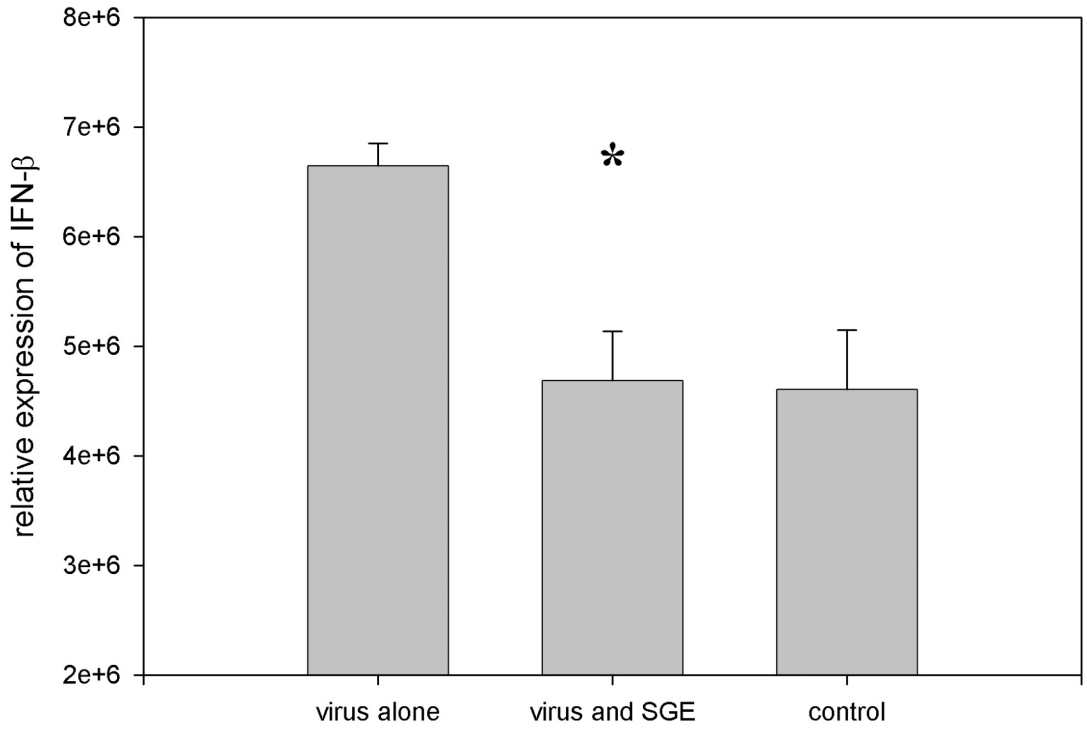


**Figure 4.10 IFN-β expression in macrophages 24 h post-infection with SINV. * p<0.05.**

although its expression could be detected in all groups. At 24 h and 48 h post-infection there was no discernible difference in IL-12 mRNA levels between groups (data not shown).

Inducible nitric oxide synthase expression was detected in all treatment groups except for uninfected controls. The group infected with SINV alone had the highest level of iNOS expression at 24 h post-infection (Fig. 4.14), with levels in the groups exposed to WNV and SGE, both 0.5 and 1.0 SGP/ml, reduced by 41.2% and 48.5%, respectively ($p<0.05$). Expression of iNOS at 48 h post-infection was also suppressed in the group treated with WNV and SGE by 64.7% as compared to the group infected with WNV alone (Fig. 4.15; $p<0.05$).

Viral loads increased from 24 h to 48 h post-infection from an average of approximately 4.7 to 5.2 $\log_{10}TCID_{50}$. Between SINV-infected groups virus titers were equal, suggesting that any effect mosquito saliva may have does not alter viral replication in peritoneal macrophages.

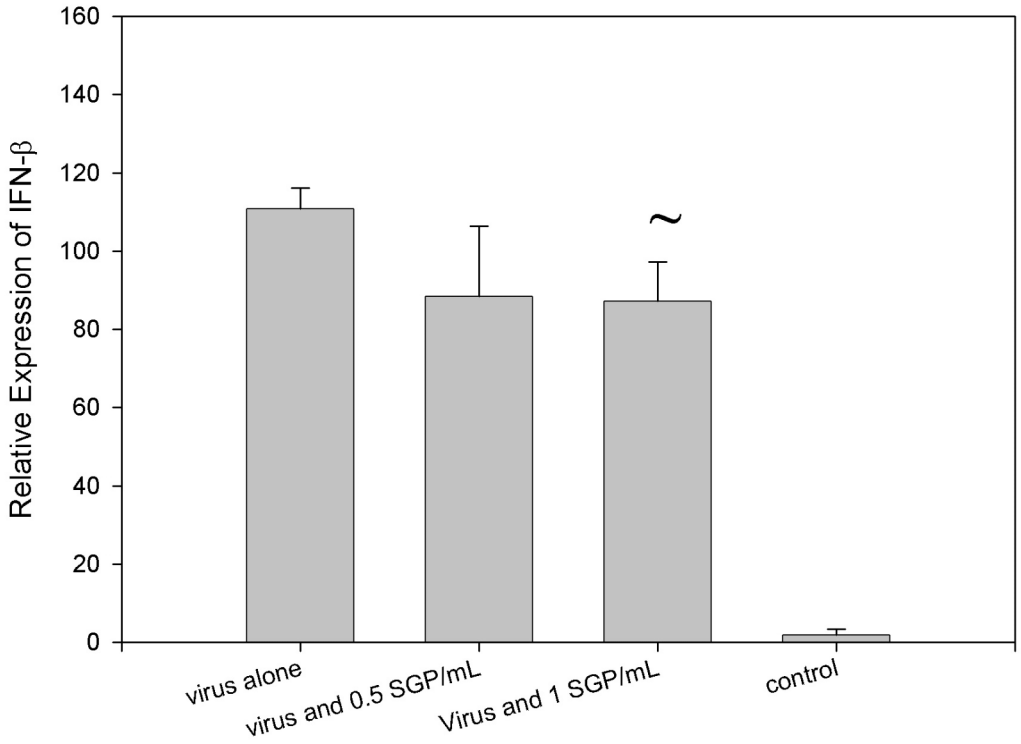


**Figure 4.11 IFN-β expression in macrophages 48 h post-infection with SINV. ~ p=0.05**

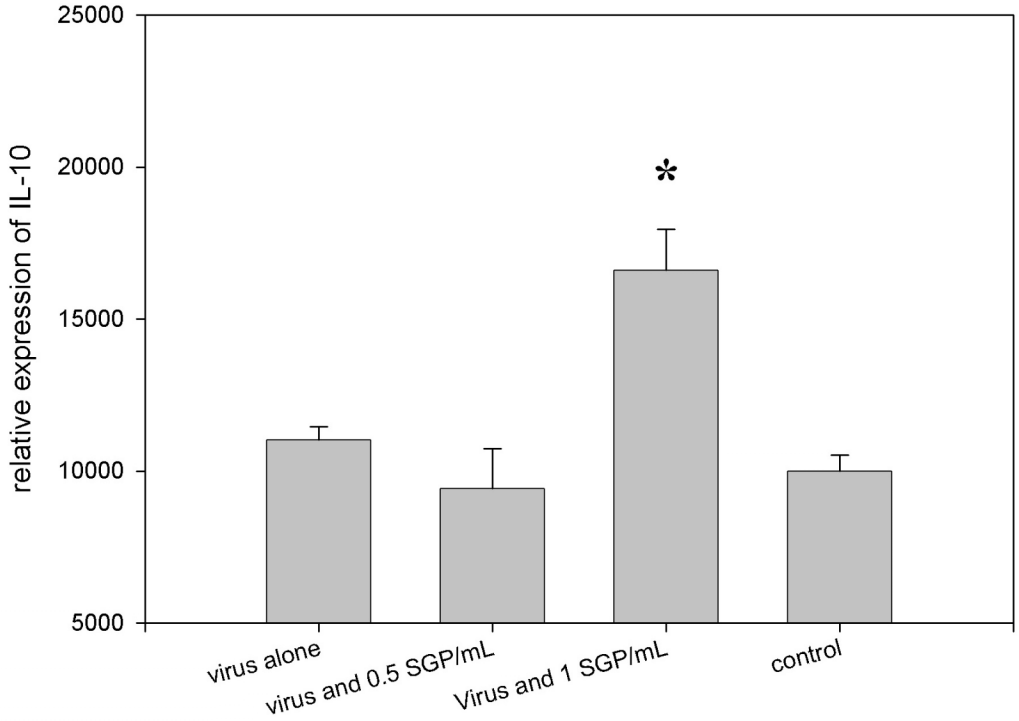


**Figure 4.12 Expression of IL-10 in macrophages 24 h post-infection with SINV. * p<0.05.**

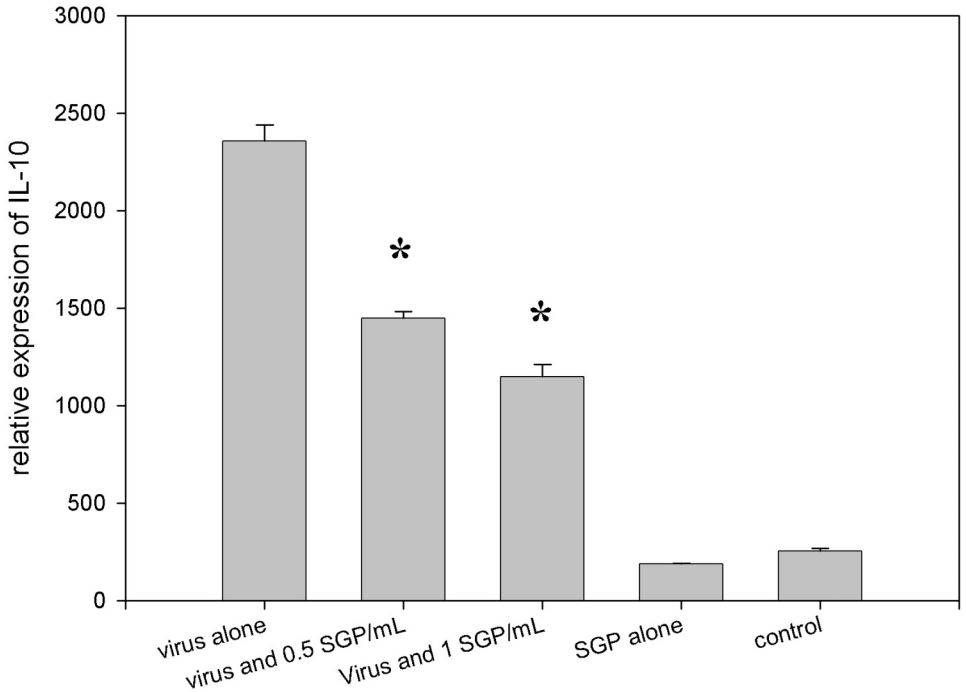


**Figure 4.13 Expression of IL-10 in macrophages 48 h post-infection with SINV. * p<0.05.**

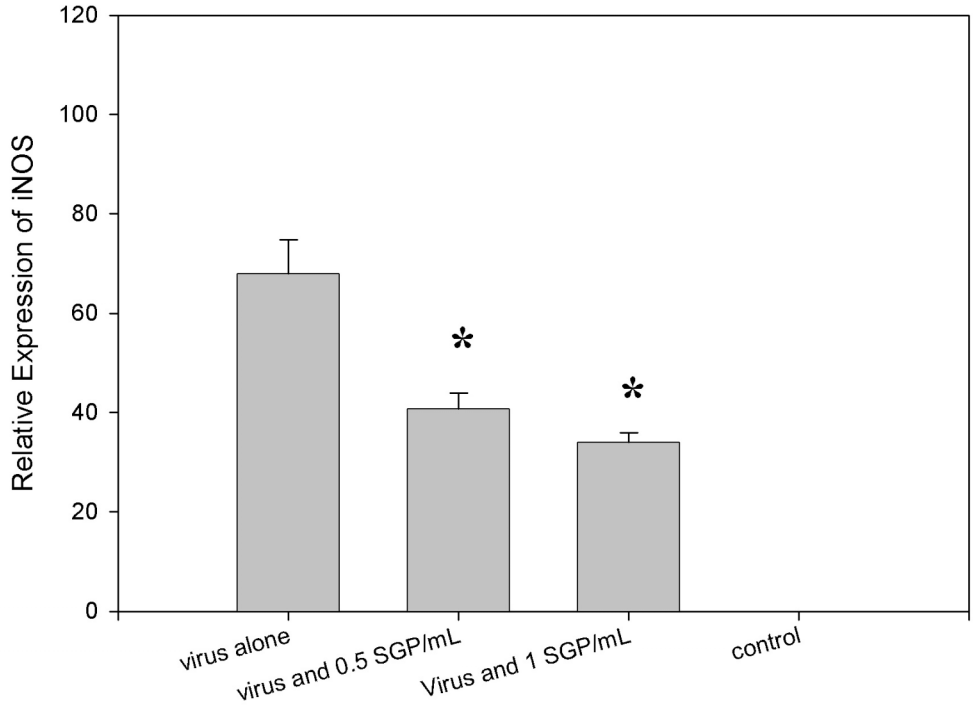


**Figure 4.14 Expression of iNOS in macrophages 24 h post-infection with SINV. * p<0.05.**

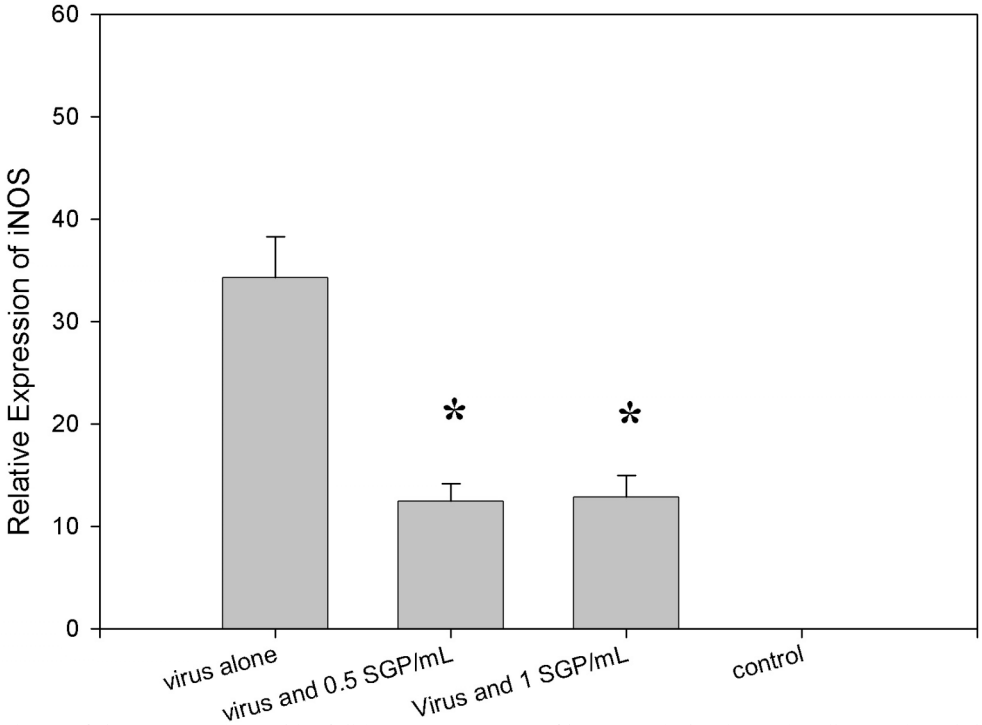


**Figure 4.15 Expression of iNOS in macrophages 48 h post-infection with SINV. * p<0.05.**

**Effect of Mosquito SGE on Bone Marrow-Derived Dendritic Cells**

To examine the effect of exposure to mosquito saliva on DC function during WNV infection, we investigated the expression of cytokines in bone marrow-derived DCs at 24 h and 48 h post-infection.

West Nile virus infection of DCs led to a high level of type I IFN expression, with little to no IFN detectable in uninfected control macrophages or macrophages treated with SGE alone. At 24 h and 48 h post-infection infected DCs treated with SGE displayed mRNA levels of IFN-β that were less than those produce by infection with WNV alone (Figs. 4.16, 4.17). Despite this tendency, this pattern was only identified in 2 of 3 replicates and, due to well-to-well variation, significance could not be established. Expression of the type II IFN, IFN-γ, was undetectable in all replicates and groups of DCs.

Expression of the $T_H2$ cytokine IL-4 was statistically equivalent in both WNV-

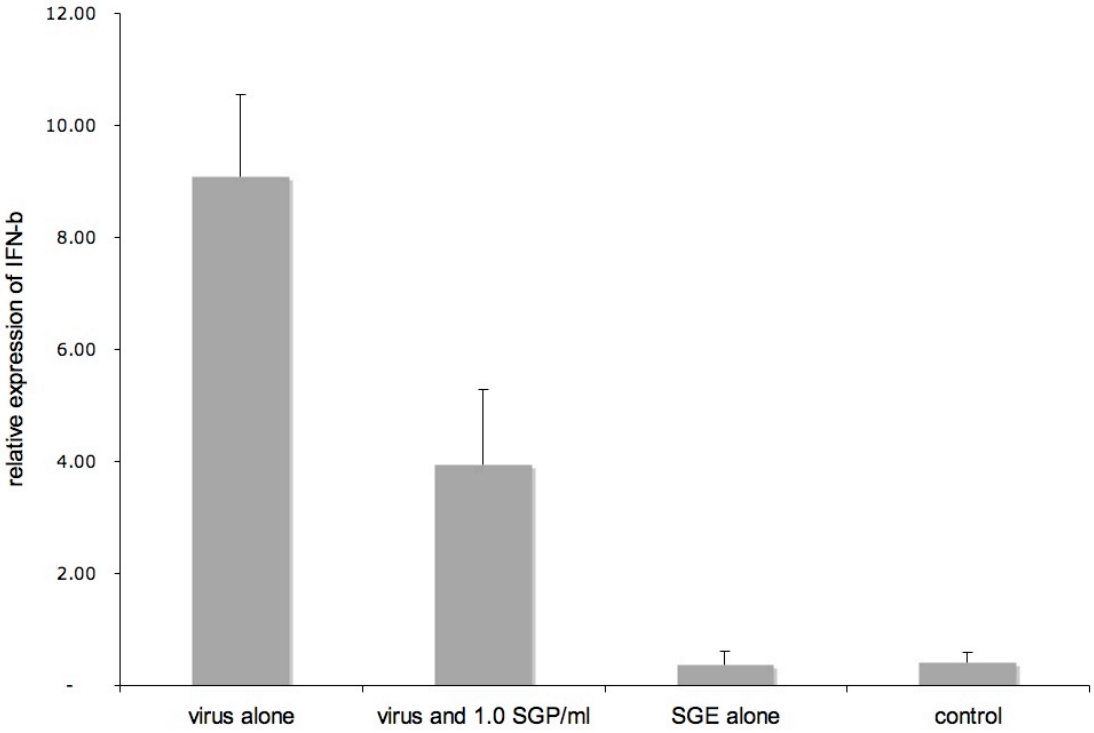


**Figure 4.16 Expression of IFN-β in dendritic cells 24 h post-infection with WNV.**

infected and uninfected groups at both time points (data not shown).

In contrast to what was observed in macrophages, at 24 h post-infection infection with WNV alone or with SGE led to increased levels of IL-10 production by DCs (Fig. 4.18). In contrast, treatment with SGE alone or medium control caused minimal levels of IL-10 to be expressed at this same time-point. By 48 h post-infection IL-10 could be detected at slightly higher levels than at 24 h in infected groups (Fig. 4.19).

Except for one replicate where DCs coexposed to WNV and SGE showed a reduction ($p$=0.08) as compared to the WNV alone group, expression of IL-12 in DCs did not vary between WNV-infected groups at either 24 or 48 h post-infection (data not shown).

Viral loads increased slightly from 24 h to 48 h post-infection from an average of approximately $1.88\times10^5$ and $2.17\times10^5$ to $2.24\times10^5$ and $2.20\times10^5$ $TCID_{50}$, respectively, for groups treated with WNV alone and WNV together with SGE.

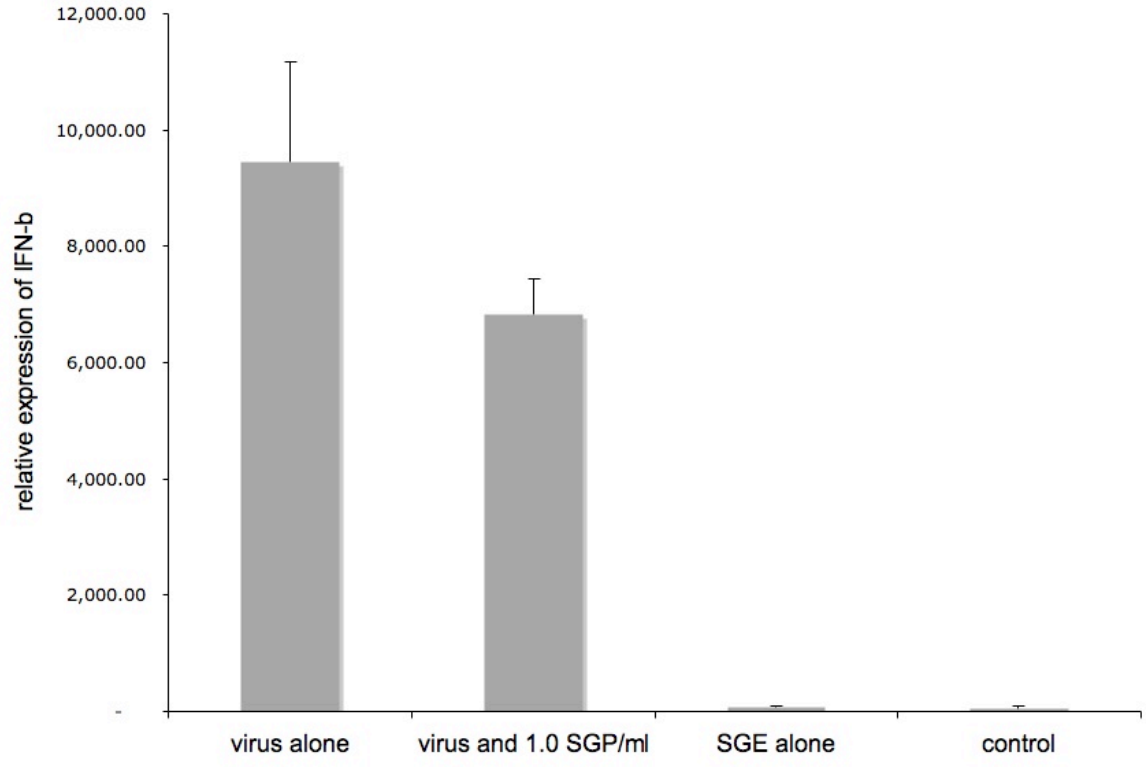


**Figure 4.17 Expression of IFN-β in dendritic cells 48 h post-infection with WNV.**

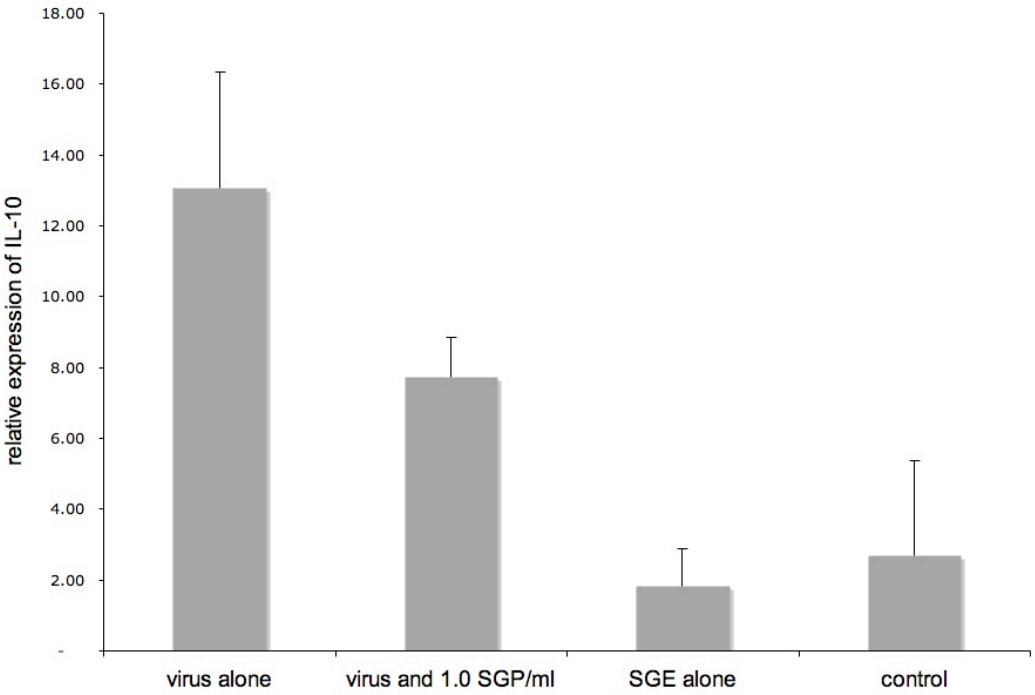


**Figure 4.18 Expression of IL-10 in dendritic cells 24 h post-infection with WNV.**

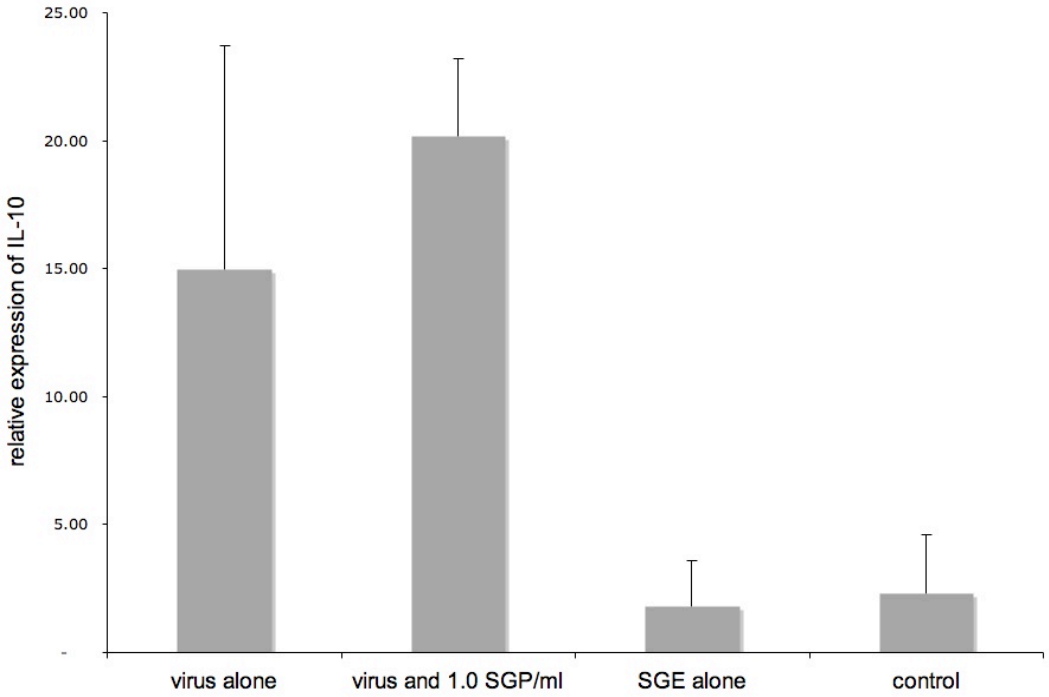


**Figure 4.19 Expression of IL-10 in dendritic cells 48 h post-infection with WNV.**

Between WNV-infected groups virus titers were identical, suggesting that any effect mosquito saliva may have does not alter viral replication in dendritic cells.

## DISCUSSION

The aim of this study was to determine if mosquito saliva could affect the response of macrophages and/or DCs to WNV infection and, importantly, whether altered function of APCs could help to explain the mosquito-induced modulations observed in WNV pathogenesis. This question is of considerable significance given that these cells are simultaneously the primary site of infection and the most peripheral components of the host immune response. Furthermore their first contact with an arbovirus is probably in association with arthropod salivary proteins. Mosquito saliva has been shown to have powerful effects on immune cells (Wanasen *et al.* 2004, Wasserman *et al.* 2004) and saliva of related arthropods have suppressive effects on the microbicidal activity of macrophages. It is therefore particularly important to evaluate the effect of mosquito saliva on APCs during arbovirus exposure.

Dendritic cells are assumed to be the primary site of replication for WNV (Byrne *et al.* 2001), and therefore their immediate response both as a regulator of immune response and to limit infection within themselves is undoubtedly very important in the development of an effective host response to WNV infection. The role of macrophages in host control of WNV infection is more conclusively affirmed than the role of DCs. Inhibiting the phagocytic activity of macrophages results in enhanced viremia and neuroinvasion during WNV infection, while depleting macrophages results in increased WNV (Ben-Nathan *et al.* 1996).

Antigen presenting cells process and present viral antigen to $T_H$ lymphocytes in association with MHC class II proteins on the surface of the APC. $T_H$ lymphocytes require secondary activation signals including immunoregulatory signals in the form of cytokines. The nature of the signals sent by APCs, via cytokines, can have a powerful effect on the immune response to a virus and the course of the infection.

As shown by this study, one of the most prominent and consistent effects that mosquito saliva has on APCs, particularly macrophages, is a suppression of iNOS expression. The catalytic activity of iNOS produces the reactive oxygen intermediate NO, which has a range of functions including smooth muscle relaxation, inhibition of platelet activation, and direct and indirect immune responses (Marletta 1994). The expression of iNOS is associated with a broad variety of transcription factors, including NF-κB, STAT-1α, IFN regulatory factor-1 (IRF-1), and nuclear factor IL-6 (Bogdan 2001). The expression of NO itself exerts a dual effect on iNOS transcription: low levels of NO enhance iNOS expression, whereas higher levels suppress iNOS (Bogdan 2001). It is unclear how mosquito saliva might suppress iNOS expression in cells, but a number of regulatory mechanisms exist that could be involved. For example, transforming growth factor β (TGF-β) and any factor that blocks L-arginine, the cofactor of iNOS, suppresses the production of NO in macrophages (Bogdan 2001). Additionally, data suggest that the $T_H2$ cytokines IL-4 and IL-13 suppress iNOS transcription (Bogdan 2001).

Mosquito saliva-induced suppression of iNOS could be a mechanism by which mosquito feeding exacerbates arbovirus disease, because some of the protective effects of macrophages appear to be mediated by NO production. For example cells treated with an exogenous NO donor had suppressed JEV titers (Lin *et al.* 1997). This antiviral effect is via the ability of NO to strongly inhibit viral RNA synthesis, protein accumulation, and virus release from infected cells (Lin *et al.* 1997). A recent study suggests that the inhibitory effect of NO on DENV infection is partly due to inhibition of the RNA-dependent RNA polymerase activity, which then downregulates viral RNA synthesis (Takhampunya *et al.* 2006). Nitric oxide also acts as an immune signalling molecule affecting such processes as differentiation, proliferation, and apoptosis of immune cells; the production of cytokines; and the expression of co-stimulatory and adhesion molecules (Bogdan 2001). Interestingly, NO inhibits the adhesion of leukocytes to the endothelium and transmigration into tissue (Grisham *et al.* 1998). Due to the molecular nature of NO, its lack of defined receptors, its intra- and extra-cellular activity, and its pathogenic and

beneficial effects during disease the effect of modulations in iNOS mRNA levels are hard to predict. Presumably, early in virus infection and in the periphery where cells would come into contact with mosquito saliva the effects of NO are more beneficial due to the antiviral activity within immune cells. Therefore a reduction in NO could be advantageous to the virus, particularly as it may reduce a cell's ability to control viral replication. It is also possible, given the broad activities of this molecule, that a decrease in necessary signalling functions of NO early in WNV infection could be partially responsible for the amplification of infection observed after mosquito feeding.

In addition to effects that a suppression of iNOS might have on early arbovirus infection, a reduction in IFN-β expression is clearly a disadvantage to a host infected with virus. Type I IFNs comprise an important innate immune system control against viral infections (Pestka *et al.* 2004). In general, IFN-β induces an antiviral state within cells through the upregulation and activation of antiviral proteins, including RNA-activated protein kinase, RNaseL, and Mx (see Chapter 1) and by modulating adaptive immune responses (Pestka *et al.* 2004). Pretreatment of cells in vitro with IFN-α/β potently inhibits flaviviruses (Samuel and Diamond 2005). However, the inhibitory effect of IFN is significantly attenuated after viral replication has begun (Samuel and Diamond 2005). Research suggests that WNV nonstructural proteins specifically inhibit IFN-α/β signaling by preventing JAK1 and Tyk2 phosphorylation and IFN-β gene transcription (Guo *et al.* 2005). Therefore the antiviral effects mediated by IFN during flavivirus infection primarily benefits uninfected cells in the vicinity of infected cells. This supports our observation there was no concomitant decrease in WNV or SINV titers in cells despite a reduction in IFN-β expression in APCs. Furthermore, the protective effect that IFN has on surrounding cells suggest that a reduction of IFN production by cells exposed to virus as observed to occur in the presence of mosquito saliva could enhance the susceptibility nearby cells. If such an effect were to occur early in infection, when mosquito saliva is in proximity to infected cells, one would predict that the survival and spread of virus could be enhanced. In this manner it is possible that subtle changes in

APC cytokine expression could in some measure account for differences in WNV pathogenesis that occur following mosquito feeding.

The early upregulation of IL-10 in arbovirus infected APCs in the presence of mosquito saliva is also noteworthy. This cytokine is usually considered immunoregulatory or immunosuppressive depending on the context, but a recent study has identified profound effects of IL-10 expression early in infection (Brooks *et al.* 2006). Studies with peripheral blood leukocytes found that early after DENV infection cells produced mainly $T_H1$ cytokines, but at later points cytokines such as IL-10 were detected. (Chaturvedi *et al.* 1999). This group found that increased IL-10 production early during virus infection induces lasting T cell inactivation and decreases control of virus infection temporally associated with the establishment of effector T cell responses, even when high viral titers exist before the onset of T-cell activity. Moreover, *in vivo* antibody blocking of the IL-10 receptor completely prevented viral persistence when administered shortly after infection (Ejrnaes *et al.* 2006). Therefore, since IL-10 can induce T-cell unresponsiveness when present during T-cell activation, the interaction of immunosuppressive IL-10–producing APCs with T cells may lead to the loss of T-cell responsiveness and allow for an enhancement of viral replication and blunted adaptive immune response. Even a transient enhancement of IL-10 expression by APCs, as observed in the present study to be caused by mosquito saliva, could delay or suppress T cell activation, and thus initiation of the adaptive immune response.

## CONCLUSION

In conclusion, changes in APC immune mediator expression levels in response to mosquito saliva could have a significant effect on the immune response to an arbovirus and WNV pathogenesis. Suppression of antiviral molecules or enhancement of immunosuppressive cytokines, even restricted to the first 48 h or to the primary site of replication, could favor the replication and spread of the virus while dulling the protective responsiveness of the host. With a virus such as WNV, where specific virulence factors are not well understood and alterations in primary viremia could turn an asymptomatic

infection into encephalitis, even seemingly subtle changes brought about by mosquito saliva at peripheral sites cannot be ignored.

# CHAPTER 5: MOSQUITO FEEDING AFFECTS LEUKOCYTE RECRUITMENT INTO THE SITES OF INTITIAL WEST NILE VIRUS REPLICATION


## ABSTRACT

West Nile virus is transmitted during the bloodfeeding of a mosquito. Mosquitoes feed from or around the capillaries and small vessels of the dermis. Therefore the first cells to come into contact with WNV are the cells of the skin, followed by those of the draining lymph node. The feeding of uninfected mosquitoes has the demonstrated ability to alter the cell content of these two compartments. For example, the saliva of *An. stephensi* has been shown to contain a high molecular weight glycoprotein endowed with an intense neutrophil chemotactic activity, while the secretion of saliva of this species into a host causes dendritic cell migration and mast cell degranulation. Indirect effects of mosquito salivary anticoagulant proteins, such as *Aedes* anticoagulant-factor Xa may down-modulate extravasation of some inflammatory cell type. Due to the recognized ability of mosquito feeding to affect cell influx into the feed site, studies were undertaken to investigate whether alterations in cell recruitment exist between WNV inoculated alone or in the presence of mosquito saliva. Flow cytometry was used to identify infiltrating cell types in the skin and draining lymph nodes. Results indicate that the predominate effect of mosquito feeding is to reduce the level of $CD3^+$ lymphocytes, concomitantly with accelerated influx of $CD11b^+$ and $CD11c^+$ leukocytes. These data help to explain the enhanced WNV disease mediated by mosquito feeding.

## INTRODUCTION

The skin and draining lymph nodes represent the first areas to come into contact with arboviruses. The skin is a complex structure composed of a dermis and epidermis, the latter of which acts as a physical and chemical barrier that is constantly renewed through the proliferation and differentiation of keratinocyte stem cells (Leigh *et al.* 1994). The epidermis is also populated by two main immunocompetent cells of hemopoietic origin: a subset of γδ T lymphocytes and LCs, the peripheral representatives of DCs (Leigh *et al.* 1994). Whilst the dermis is made up of blood and lymphatic vessels that flow through an extracellular matrix where fibroblasts, macrophages, DCs, and mast cells reside (Nickoloff and Turka 1993). A subset of $CD8^+$ and $CD4^+$ T lymphocytes transiently recirculates through the normal dermis back to the draining lymph nodes via afferent lymph (Picker 1994).

If skin tissue is damaged, as occurs during mosquito feeding, an assortment of soluble mediators and cells contribute to the initial response. Polymorphonuclear cells and monocytes are important mediators of inflammation. Soluble mediators released by injured cells activate neutrophils that accumulate at the site of injury (O'Flaherty and Cordes 1994). Products from the coagulation cascade, such as thrombin, and other proinflammatory mediators, such as platelet-activating factor, also activate neutrophils. Thrombin also causes fibroblast proliferation and neutrophil adhesion, while factor Xa enhances acute inflammation by binding to effector cell protease receptor-1, leading to induction of vascular permeability and leukocyte exudation (Gillis *et al.* 1997). Thus, once the skin equilibrium is disrupted via injury or infection an inflammatory response is initiated characterized by the migration of varying waves of cells into the dermis. Concurrent with neutrophil accumulation, other cells migrate from both the epidermis (LCs and γδ T cells) and the blood (granulocytes, monocytes, NK cells, and CD4/CD8 T-cells).

The role of epidermal LCs is to capture antigens and migrate to draining lymph nodes where they activate both naïve and effector T cells, which initiate the immune

response (Silberberg 1973). The maturational phenotypic changes that occur as LCs migrate are believed to be a prerequisite to their enhanced antigen-presenting capabilities (Cumberbatch *et al.* 1991). This transformation is characterized by an increase in cell surface markers, including class I and II MHC, ICAM-1, and the co-stimulatory molecules B7-1/CD80 and B7-2/CD86 (Rattis *et al.* 1996). Although the exact event necessary to initiate LC migration is not clearly understood, there is evidence that engagement of MHC class II antigens on the LC cell surface and modulation of adhesion molecule expression (LFA-1, ICAM-1, $\alpha$4 integrin, E-cadherin) is associated with decreases in epidermal LC density and increase DC numbers in draining lymph nodes (Aiba *et al.* 1993, Scheynius *et al.* 1993, Ma *et al.* 1994, Schwarzenberger and Udey 1996). Various cytokines and chemokines have also been implicated in this response, including TNF-$\alpha$, IL-1, and CC chemokines receptor 7 (Saeki *et al.* 1999).

Both virus infection and mosquito saliva have the capacity to affect the migration of immune cells (Byrne *et al.* 2001, Demeure *et al.* 2005, Maxwell *et al.* 2005). Previous research has shown that cutaneous WNV infection leads to a decrease in LC cell density in the epidermis with a concomitant increase in LC concentration in draining lymph nodes (Johnston *et al.* 2000). Further data revealed that IL-1$\beta$, but not TNF-$\alpha$, is required for LC migration, as a significant suppression of LC migration was only observed while knocking out IL-1$\beta$ (Byrne *et al.* 2001). Neutrophils were observed to be one of the first responders to mosquito feeding, and their homing to the site of bloodfeeding appeared to be regulated by mast cell degranulation (Demeure *et al.* 2005). Elements of mosquito saliva can directly affect the cell types that migrate to the feeding site. The saliva of *An. stephensi* has been shown to contain a high molecular weight glycoprotein endowed with an intense neutrophil chemotactic activity (Owhashi *et al.* 2001). Moreover, the secretion the saliva of this species into a host causes DC migration and directly triggers mast cell degranulation without a requirement for IgE (Demeure *et al.* 2005). Indirect effects of mosquito salivary anticoagulant proteins, such as *Aedes* anticoagulant-factor Xa (Stark and James 1998) may down-modulate extravasation of some inflammatory cell type, as well as suppressing complement pathways. The *Aedes*

anticoagulant inhibits fXa, which is required for activation of Hagemen factor, which in turn converts factor XI to its active form XIa and conversion of prekallikrein to kallikrein. Kallikrein is responsible for generation of the anaphylatoxin C5a from compliment (Wiggins *et al.* 1981). A reduction in C5a, as would be expected from inhibition of upstream effectors, could have significant effects at the site of mosquito feeding. Notably, C5a is important for recruitment of antibody, extravasation of complement, homing of PMNs, and activation of macrophages and neutrophils. Importantly, a recent study confirmed that complement plays a very critical role in controlling WNV infection (Mehlhop *et al.* 2006). Therefore mosquito saliva, by decreasing the production of C5a, could suppress these effects that hasten the destruction of pathogens and affect cell migration to the site of arbovirus inoculation.

It is possible that mosquito saliva could alter cell extravasation by affecting the facilitators of this movement. Cell entry into sites of inflammation is mediated by the differential expression of chemokines and adhesion molecules. For example, specific adhesion molecules, such as β2-integrin, are expressed by endothelial cells that interact with corresponding receptors on leucocytes to mediate attachment and migration to sites of tissue damage or infection (Muller *et al.* 2002a). Although effects on this system have not been demonstrated with mosquito saliva, related arthropod saliva has been shown to affect expression of some of these factors. The saliva of *Ix. scapularis* impairs the adherence of PMNs by decreasing CD18, the common β subunit of leucocyte function-associated antigen-1 and Mac-1 (Montgomery *et al.* 2004). The saliva of *D. andersoni* reduces the expression of leucocyte function-associated antigen-1 and very late activation-4 by splenocytes (Macaluso and Wikel 2001). Recently it was demonstrated tick saliva also suppresses the expression of ICAM-1 and VCAM-1 on endothelial cells (Maxwell *et al.* 2005), which could decrease leucocyte migration from the blood. Furthermore, factors in sand fly saliva have a demonstrated chemotactic effect on murine monocytes *in vitro* (Anjili *et al.* 1995). The evidence from other hematophagous arthropods suggests that mosquito saliva may affect cell migration, and this may be mediated by alteration of host adhesion or chemokines molecules.

In addition to effect on inflammatory cells, T cell populations are particularly susceptible to suppressive effects of *Ae. aegypti* saliva, showing enhanced mortality and decreased division rates (Wanasen *et al.* 2004). This immune suppression appears to be superior in *Ae. aegypti* saliva. Contemporary work by Wasserman *et al.* (2004) described inhibition of T- and B-cell proliferation in a dose dependent manner with concentrations as low as 0.15 salivary gland pairs/ml.

Studies have suggested that independently mosquito feeding and virus inoculation can affect dermal cell migration, but have not investigated the effect of mosquito saliva on cell migration at the initiation of an arbovirus infection (Byrne *et al.* 2001; Demeure *et al.* 2005). The objective of this study was to investigate the influence of mosquito saliva on immune cell migration patterns both into the dermal site of infection and out to the draining lymph node following arboviral infection. This was accomplished using multi-color flow cytometry. Due to the importance of LCs in early response to arbovirus infection (Byrne *et al.* 2001, Johnston *et al.* 2000, Johnston *et al.* 1996), LCs were investigated with respect to saliva-induced deviations in migration from the site of inoculation to the draining lymph node via flow cytometry. We hypothesized that mosquito saliva could alter levels of murine macrophage, LC and T cell populations in the epidermis and draining lymph nodes during arbovirus infection. Mice infected intradermally (i.d.) with or without the addition of saliva (via mosquito feeding at the inoculation site) were compared for differences in cell infiltration at the site of inoculation, LC migration to draining lymph nodes, and cell populations within the lymph node. As no study has explored the effect of mosquito saliva on migration of immune cells during active virus infection, this study is necessary to determine if there are deviations in leukocyte migrations caused by saliva. Establishing the environments confronted by an arbovirus when injected in the presence or absence of saliva will provide an enhanced understanding of the early pathogenesis of naturally acquired infections.

## MATERIALS AND METHODS

### Virus

A low passage WNV was used, as previously described (see Chapter 2) for these experiments.

### Mice

Female, 4-week old Swiss Webster mice were obtained from Harlan (Indianapolis, Indiana), and housed in a biosafety level-3 animal facility. Mice were divided into 4 groups (n = 6) based on the inoculum they received: 1) PBS (negative control), 2) WNV alone, 3) mosquito feeding alone, and 4) WNV following the feeding of uninfected mosquitoes. After mice were allowed to equilibrate for a period of one to two weeks they were sedated and exposed or unexposed (as dictated by the experimental group) to mosquitoes and/or an inoculation containing $10^4$ pfu of WNV in a volume of 10 μl of PBS. Virus was administered i.d. in the pinna of the right ear. Mice assigned to a group that required mosquito exposure were exposed to the feeding of approximately 10 mosquitoes. Mosquito probing was restricted to the ear using a cardboard template surrounding a soft latex sleeve. The latex sleeve allowed immobilization of the ear without inducing any tissue damage or irritation that could alter results. Mice were maintained in this position over a carton with *Ae. aegypti* mosquitoes for approximately 30 minutes or until the requisite number of mosquitoes were observed to have engorged. At 24 and 48 h post infection, three mice per group were sedated and euthanized. Tissues were processed for flow cytometry and occasionally a subset of tissues was reserved for confirmation of observations by microscopy or IHC. Mouse experiments were repeated 3 times and the Institute of Animal Care and Use Committee at the University of Texas Medical Branch approved all protocols.

### Flow Cytometry

#### *Isolation of tissue*

Mice were deeply sedated and euthanized via halothane inhalation. Death was assured by cervical dislocation and limbs were immobilized on a dissection board. Ears

were surface sterilized with 70% ethanol, tape-stripped by repeated applications of cellophane tape to remove stratum corneum, surgically removed with clean dissection scissors, and placed individually in the wells of a 24-well plate filled with 70% ethanol for 15 min. For lymph node isolation a Y-shaped incision was made beginning at the upper chest, forking at the chin, and terminating near the cheekbone. The skin of the sub-mandibular region was pulled back, stretched and affixed to the dissection board with 25-gauge needles to reveal lymph nodes draining the ear. Individual nodes were teased from surrounding fat and collected in 15 ml conical tubes on ice with Iscove's modified Eagle's medium (IMEM). Lymph nodes from each mouse within a group were kept separate in some replicates and pooled in others.

***Preparation of single-cell suspensions***

Ears

After incubating intact ears in ethanol for 15 minutes, ears were removed and, using fine forceps, split ventrally along the cartilage into dorsal and ventral 'leaflets' and placed into 2ml of Roswell Park Memorial Institute (RPMI; Sigma-Aldrich) medium supplemented 1:100 with Liberase CI (10 mg/ml; Roche, Indianapolis, Indiana) in 24-well plates for 30 min at 37$^{o}$C. Following incubation, Ears were removed from medium and placed individually into medicons (Becton Dickinson, Franklin Lakes, New Jersey) with 500 μl of their own medium and run in a Medimachine™ (Becton Dickinson) for 5 minutes. Medicons are disposable polyethylene chambers containing an immobile stainless steel screen with approximately 100 hexagonal holes. Around each hole are 6 microblades designed for efficient cutting of hard and soft tissues. The Medimachine™ works together with the medicons and operates at a constant speed of approximately 80 rpm; it is a standardized sample preparation system for the automated, mechanical disaggregation of solid animal tissues for flow cytometric analysis. Subsequent to the 5 min processing, the top of the medicon was removed and, using 10-ml syringes, 10 ml of DNase medium (RPMI complete (10% FCS with 1:100 each L-glutamine, penicillin, streptavidin) and 0.05% DNase25 (Sigma, St. Louis, Missouri)) was simultaneously added to the top while being withdrawn from the bottom, effectively flushing the unit.

The 10 ml of medium that was extracted from the medicon was then gently forced through a 70μm-filicon (Becton Dickinson) into a 10-ml conical tube, and cells were kept on ice for remainder of preparation. Cells were then pelleted with a 10 min centrifuge at 1,400 rpm and 4°C. The cell pellet was then resuspended in 10 ml of FACS staining buffer (0.09% sodium azide, 1% heat-inactivated fetal calf serum, Dulbecco's PBS without $Mg^{2+}$ or $Ca^{2+}$ adjusted to pH 7.4-7.6 and filter through 0.2 μm pore membrane) and pelleted again by centrifugation for 3 min at 4°C and 1,400 rpm. This wash step was completed twice to remove residual non-cellular material, cells were resuspended in 1ml FACS staining buffer, and a total viable cell count was performed using a hemocytometer and trypan blue (0.4%).

Lymph nodes

Lymph nodes and 10 ml IMDM medium were poured into a 70 μm nylon mesh cell strainer in a Petri dish. Using a 3-cc syringe plunger, lymph nodes were gently tapped to separate cells. Cells were passed through filter 3 more times, then pipetted into a 15 ml conical tube, and held on ice. Tubes were centrifuged at 1,400 rpm and 4°C for 10 minutes. Depending on assays to follow, cells were resuspended in 3 ml IMDM or FACS staining buffer (0.09% sodium azide, 1% heat-inactivated fetal calf serum, Dulbecco's PBS without $Mg^{2+}$ or $Ca^{2+}$ adjusted to pH 7.4-7.6 and filter through 0.2 μm pore membrane) and a total viable cell count was performed using a hemocytometer and trypan blue (0.4%).

***Cell Staining and Detection***

One million cells were plated per well in 96-well V-bottom plates and pelleted by a 3 min spin at 1,400 rpm and 4°C. Cells were resuspended with 100 μl of purified 2.4G2 antibody (1 μg/well) specific for murine FcγII/III receptors (Fc block, BD Biosciences) in FACS staining buffer. Fc block is used to prevent non-specific staining by fluorochrome-conjugated antibodies binding to mouse Fc receptors. Cells were blocked for 20 minutes at 4°C in the dark, followed by centrifugation at 1,400 rpm for 3 min at 4°C. Pelleted cells were then resuspended in 50 μl with the appropriate amount of fluorochrome-

conjugated monoclonal antibody specific for cell-surface antigens. Generally peridinin chlorophyll protein-cyanine dye (PerCP-Cy5.5), allophycocyanin (APC), Alexa Fluor® 488, and phycoerythrin (PE) were used at a 1:100 dilution of stock, whereas fluorescein isothiocyanate (FITC) was used at 1:50. Multicolor staining was performed for 30 min at 4°C in the dark, followed by centrifugation at 1,400 rpm for 3 min at 4°C to pellet cells and resuspension in 250 μl/well with staining buffer. In this manner, cells were washed twice to remove residual, unbound antibody. If only extracellular proteins were stained then, subsequent to final wash, cells were resuspended in 300 μl of FACS staining buffer and transferred to flow cytometry tubes (Daigger). For intracellular staining, cells were thoroughly resuspended in 100 μl per well of Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4°C. Following fixation, cells were washed twice in 250 μl 1× BD Perm/Wash solution and cells were pelleted each time with centrifuge conditions as noted above. The intracellular antigen langerin was stained in 50 μl BD Perm/Wash solution containing a 1:100 dilution of Alexa Fluor® 488-conjugated antibody for 30 min at 4°C in the dark. Cells were pelleted as before, washed in 250 μl of BD Perm/Wash solution, and repeated. Following the two wash steps cells were resuspended in ~300 μl of FACS staining buffer and transferred to flow cytometry tubes for cytometric analysis.

## RESULTS

To further assess the role of mosquito saliva/feeding in WNV pathogenesis, the effect of mosquito feeding on cellular infiltrate into the skin and draining lymph nodes in the context of WNV replication was investigated. On visual inspection at the exposure site, inoculation of WNV cause minimal changes beyond a slight redness of the skin, while mosquito feeding caused a more discernable response, characterized by minor inflammation and occasionally discrete hemorrhages of capillaries. Cell populations in the skin and draining lymph nodes at early time points (24 and 48 h) post-exposure were examined via flow cytometry to characterize the initial cellular environment encountered by WNV in the presence or absence of mosquito feeding.

**Cell infiltration**

Salivary constituents that alter hemostasis and inflammatory responses of the host may alter the migratory properties of both resident-skin and blood-borne cell types during early WNV infection. We investigated whether mosquito saliva modifies cellular content of the skin and lymph node. The cellular response to WNV inoculation and/or mosquito feeding was characterized by the accumulation of a variety of leukocytes.

As compared to untreated mice, mice in any of the exposure groups could be recognized firstly by an increase in neutrophils (Ly-6c/g positive cells). At 24 h post-exposure, the level of neutrophil influx was highest in groups of mice that were inoculated with WNV with or without exposure to mosquito feeding (Fig. 5.1). Although one replicate of the experiment showed a relative suppression of neutrophils in the group injected with WNV after mosquito feeding, a subsequent replicate did not confirm this. Neutrophils were detected in untreated control samples (Fig. 5.1 and 5.2). While this is not unusual, it is likely due to the fact that skin tissues were collected as whole, unperfused ears. Since these contain some blood, non-infiltrating neutrophils may have also been detected. By 48 h post-exposure the level of neutrophils found in the skin increased to much higher levels in groups exposed to mosquitoes alone, mosquitoes and WNV, or WNV alone as compared to control tissue (Fig. 5.2), with 10- to 100-fold higher levels in skin exposed to experimental conditions. Concurrently, ears that had been exposed to mosquito feeding (with or with out WNV inoculation) tended to have more neutrophils than skin that was solely inoculated with WNV (Fig. 5.1 and 5.2).

At 24 h post-exposure there was a reduction in the percent of the skin population made up of macrophages (F4/80 positive cells) in mice that had been exposed to a treatment as compared to controls (Fig. 5.1). This alteration could be a reflection of the number of other cell types infiltrating the exposed tissues (thus a cell population that remains the same in number would appear to decrease), but calculations to estimate the actual number of macrophages in the skin continued to indicate a decrease in their concentration. This decrease in macrophage concentration suggests that this cell type was migrating out of the tissue, presumably after activation by antigen. An increase in

CD11b and F4/80 positive cell concentration within these same groups at this time point (Figs. 5.3, 5.4, 5.12) in the draining lymph nodes would appear to support this notion. At 48 h post-exposure the proportion and actual numbers of macrophages in the skin decreased slightly in WNV-exposed groups, however at this point levels were higher than in the control group (Fig. 5.2). Notably, at both 24 and 48 h post-exposure the group of mice that had been exposed to mosquito feeding prior to WNV inoculation tended to have a lower concentration of detectable macrophages in the skin.

Mosquito feeding or saliva appears to increase the level of LCs in the skin, as demonstrated by the consistently higher number of langerin-expressing cells detected in groups fed upon by mosquitoes, particularly in the group also inoculated with WNV (Figs. 5.1 and 5.2). A corresponding slight reduction in the proportion of cells expressing langerin in the draining lymph nodes at 24 h post-exposure suggests that, at this early time point, fewer LCs are migrating to the draining lymph node. By 48 h post-exposure, the number of LCs in the skin are still higher in the group of mice that were exposed to mosquitoes in addition to WNV (Fig. 5.2), although, at this time point, LC numbers are also higher in the draining lymph node within this group of mice (Fig. 5.5 and 5.6).

Perhaps the most important observation was that within the skin, mosquito saliva exposure leads to a reduction in T cells ($CD3^+$ cells), both the in the fraction of the cell population comprised of T cells and in the actual number of T cells per ear (Fig. 5.7). Exposure of mice to mosquito feeding alone decreased the number of $CD3^+$ cells in the skin as compared to unexposed control mice. Additionally, among mice inoculated with WNV, mice exposed to mosquito feeding had a reduced level of $CD3^+$ cells in the skin. This effect was observed at both 24 h and 48 h post-exposure (Fig. 5.7 and 5.8). In one replicate of the experiment $CD4^+$ cell levels in the skin were reduced in infected mice when mosquitoes were allowed to feed at the inoculation site, but this observation was not confirmed in the subsequent replicate. No corresponding decrease in $CD3^+$ cells could be observed in the draining lymph nodes (Fig. 5.9), although mice fed on by mosquitoes alone had significantly reduced levels of T cells, particularly $CD4^+$ T cells as compared to control mice at 24 h post exposure.

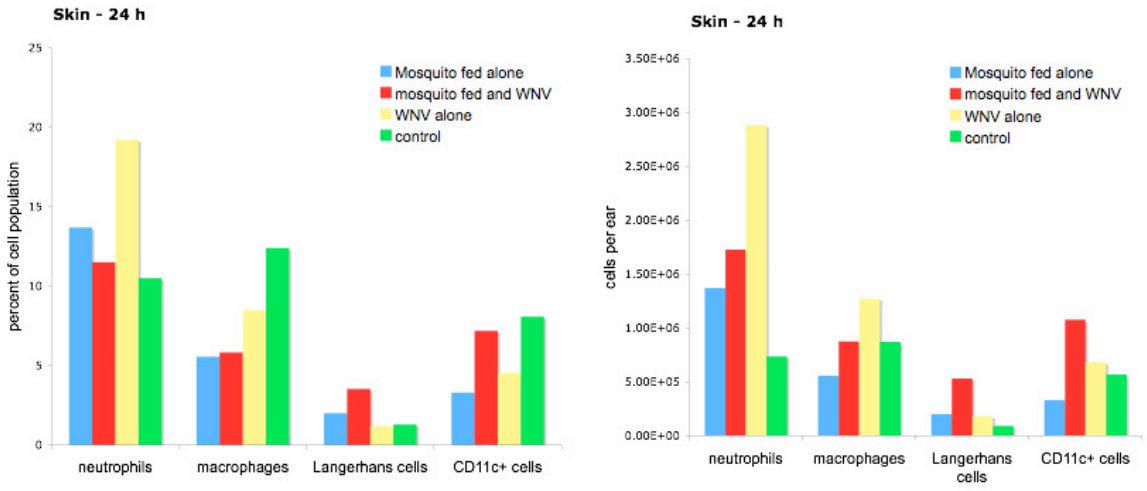


**Figure 5.1 Leukocytes present in the skin at 24 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates.**

Infection with WNV leads to an influx of $CD11b^+$ cells into the skin. $CD11b^+$ cells are predominantly macrophages, but can also be DCs and, to a lesser extent neutrophils. At 24 h post exposure mice that were concomitantly exposed to mosquito feeding showed an elevated level of this cell population in the skin that decreased by 48 h post exposure, whereas mice inoculated with WNV alone had a relatively lower level of $CD11b^+$ cells in the skin at 24 h post exposure that eventually surpassed the level of the mosquito exposed group at 48 h post infection (Figs. 5.10 and 5.11). The enhanced influx of $CD11b^+$ cells at 48 h post infection in the group inoculated with WNV alone was associated with a increase in CD11b/CD11c dual-positive cell population increase (Fig. 5.11). At 24 and 48 h post infection both groups infected with WNV displayed enhanced levels of $CD11b^+$ cells (Figs. 5.12 and 5.13). An observation that was likely associated with the temporal variation in the CD11b+ population was the relative increase in MHC class II expressing cells at 24 h post infection in mice exposed to mosquitoes and WNV, along with the subsequent increase in at 48 h in the group inoculated with WNV alone (Figs. 5.14 and 5.15). An increase in E-cadherin expressing cells in both WNV-infected groups is symptomatic of the migration of Langerhans cells from the epidermis to the draining LN following virus infection (Figs. 5.16 and 5.17). West Nile virus infection is also associated with an increase of NK cell and CD86+ cells to the draining

LN. At 48 h post infection the level of $CD11c^{+}$ cells in the LN is considerably higher in mice exposed to mosquitoes and WNV as compared to the level in mice inoculated with WNV alone (Fig. 5.18).

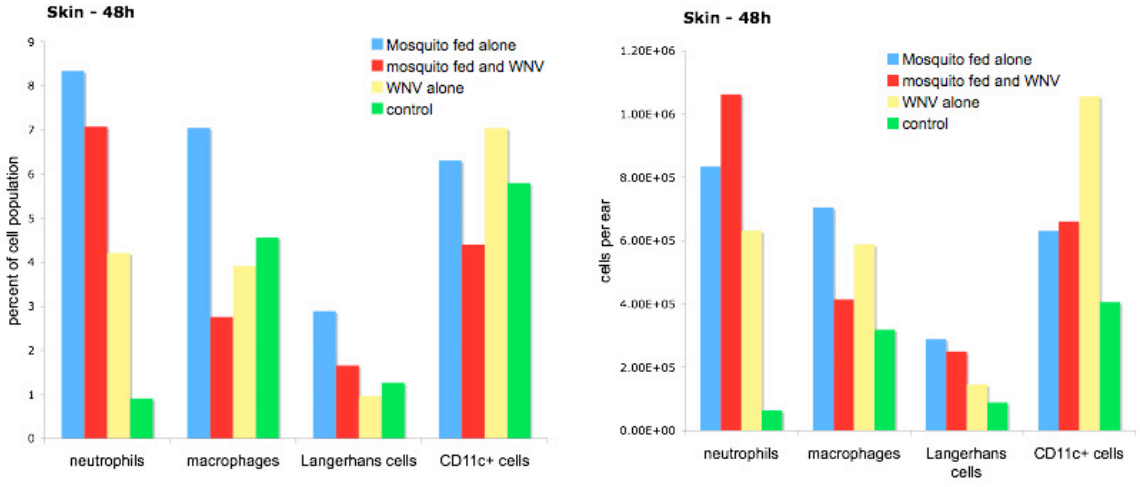


**Figure 5.2 Leukocytes present in the skin at 48 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

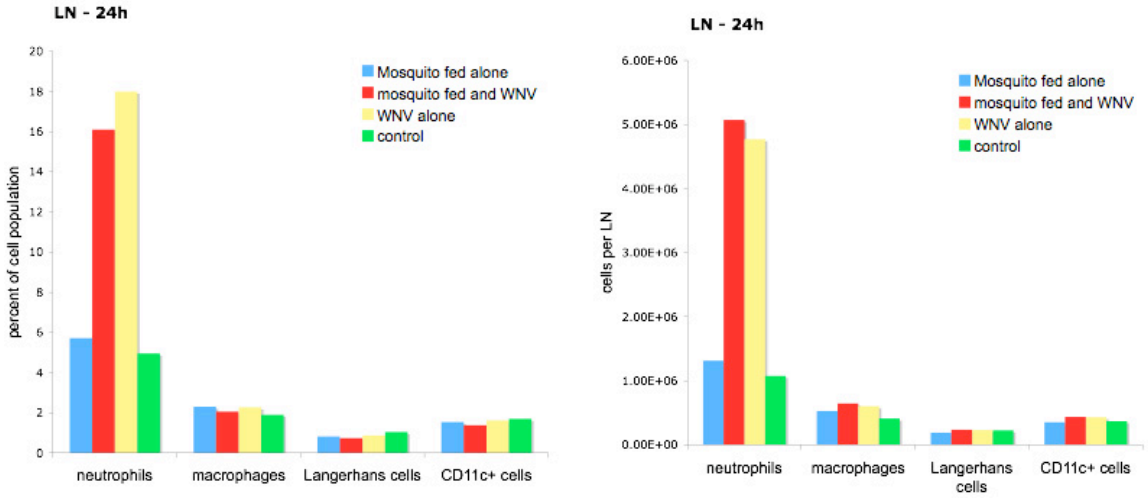


**Figure 5.3 Leukocytes present in the LN at 24 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

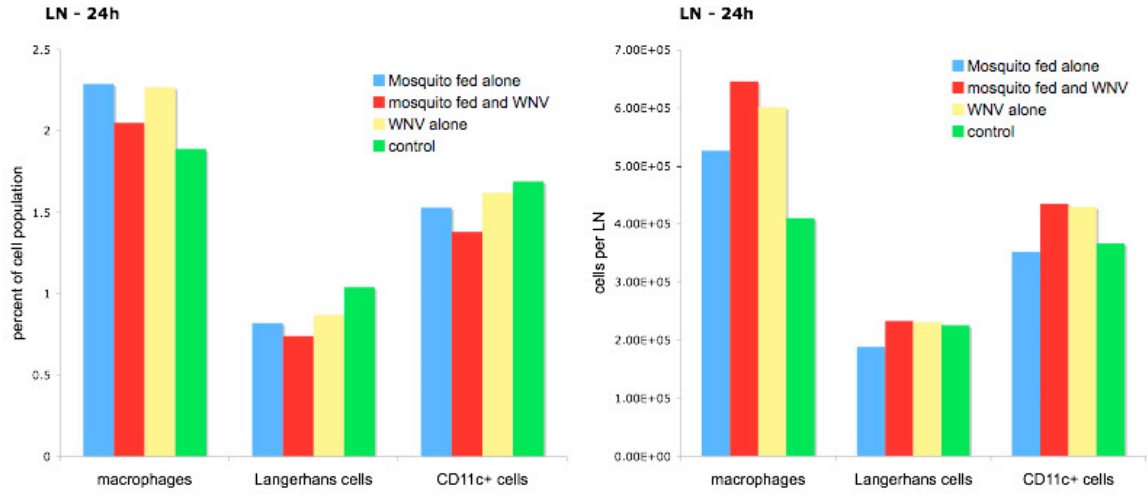


**Figure 5.4 Leukocytes present in the LN (close-up of Fig 5.3) at 24 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

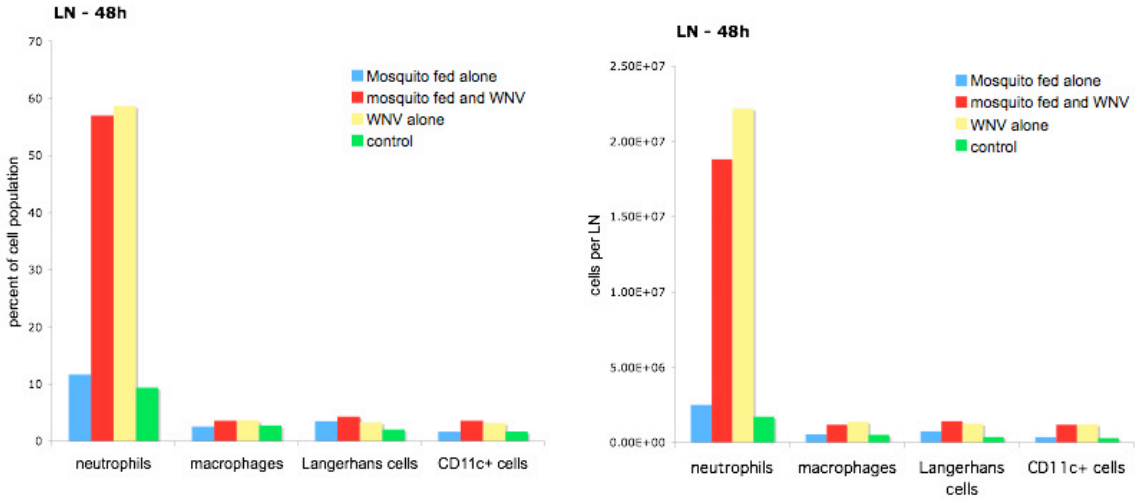


**Figure 5.5 Leukocytes present in the LN at 48 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

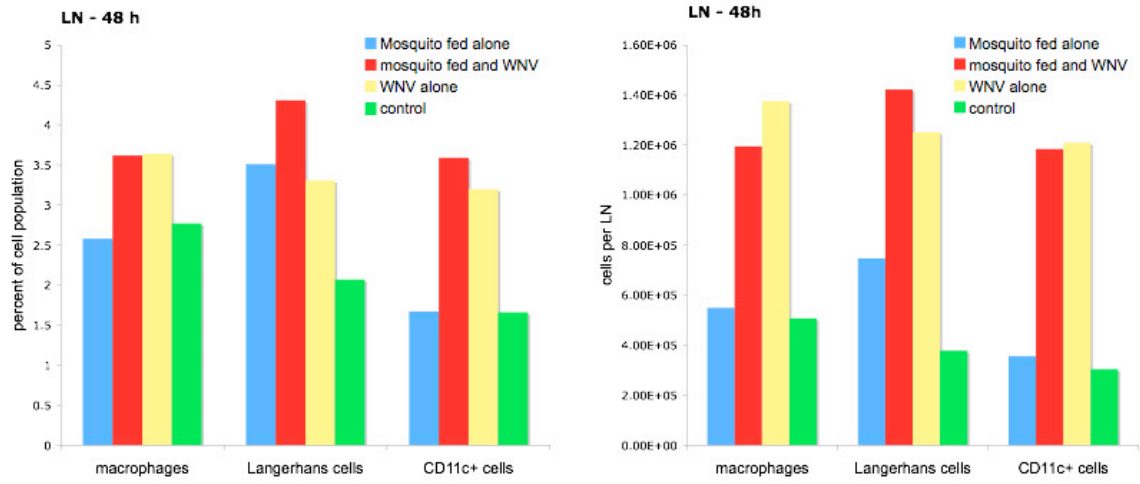


**Figure 5.6 Leukocytes present (close-up of Fig. 5.5) in the LN at 48 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

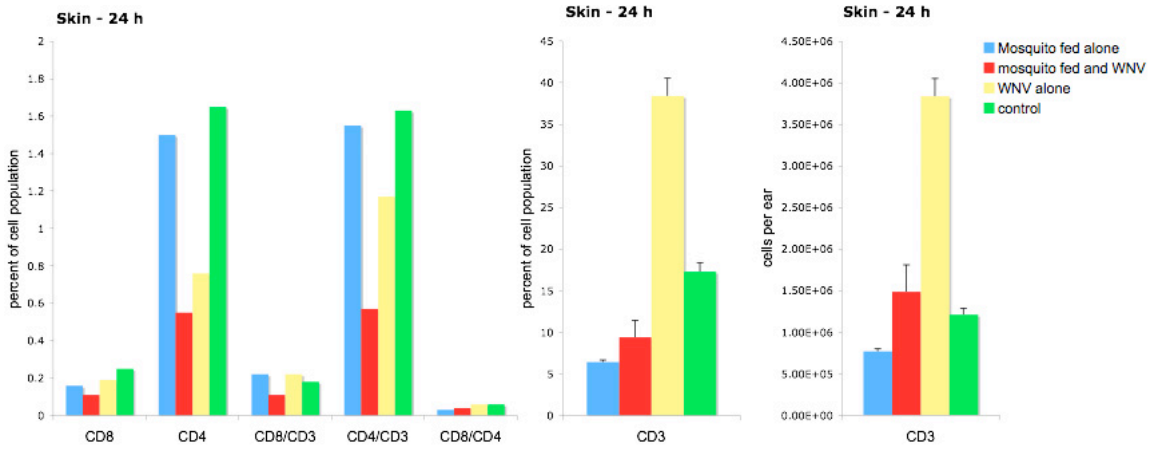


**Figure 5.7 CD4/CD8/CD3$^{+}$ Leukocytes present in the skin at 24 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

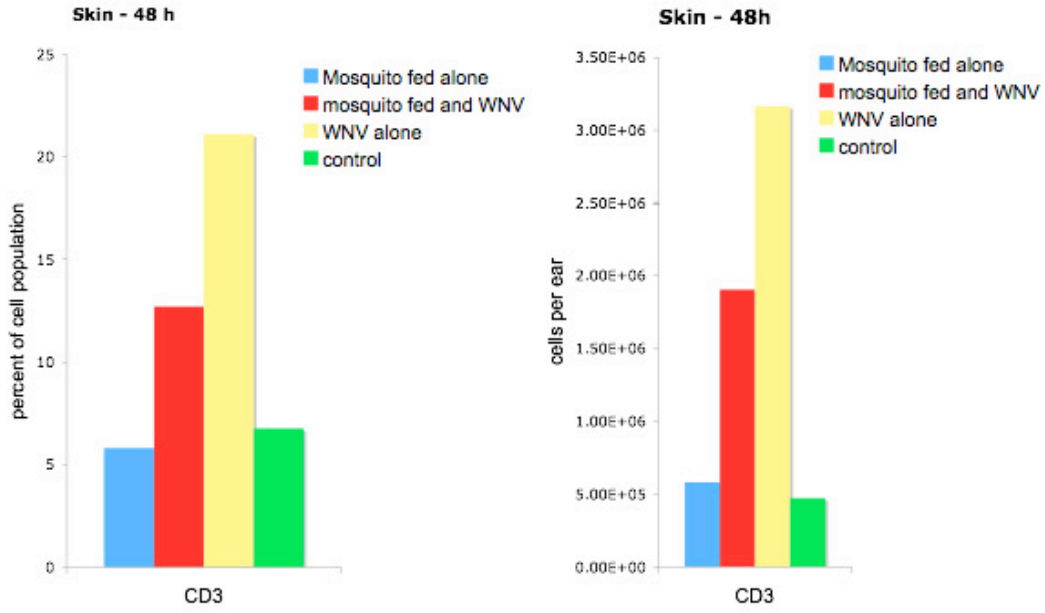


**Figure 5.8 CD3+ cells present in the skin at 48 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

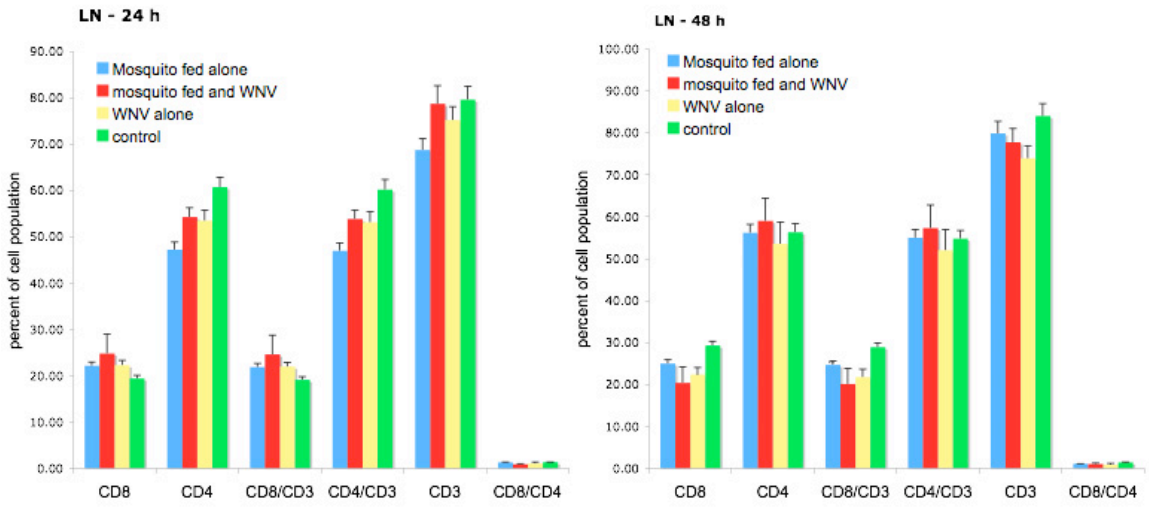


**Figure 5.9 CD4/CD8/CD3+ cells present in the LN at 48 h post-exposure to indicated conditions; percent of population are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

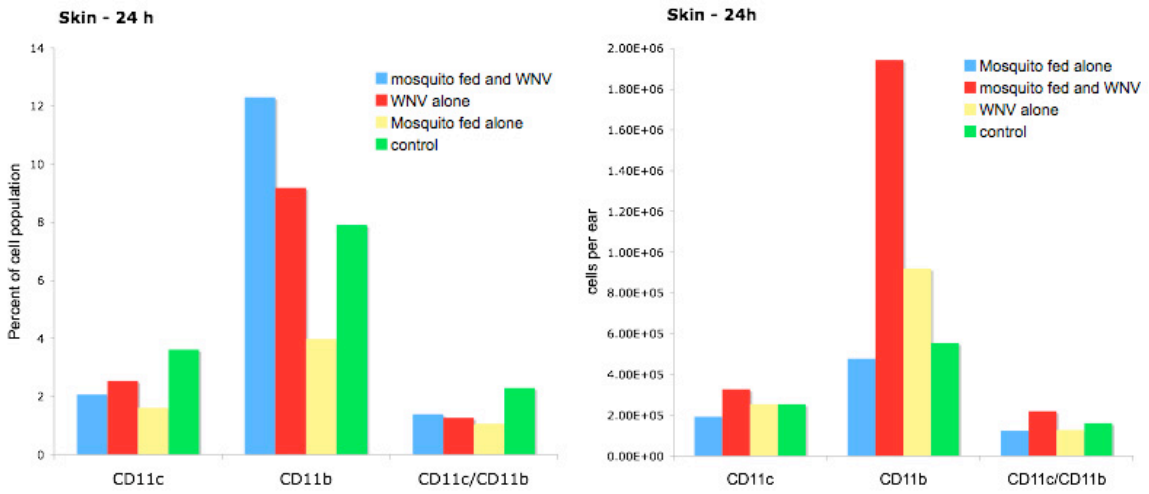


**Figure 5.10 CD11c+, CD11b+, and CD11c/CD11b dual positive cells present in the skin at 24 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

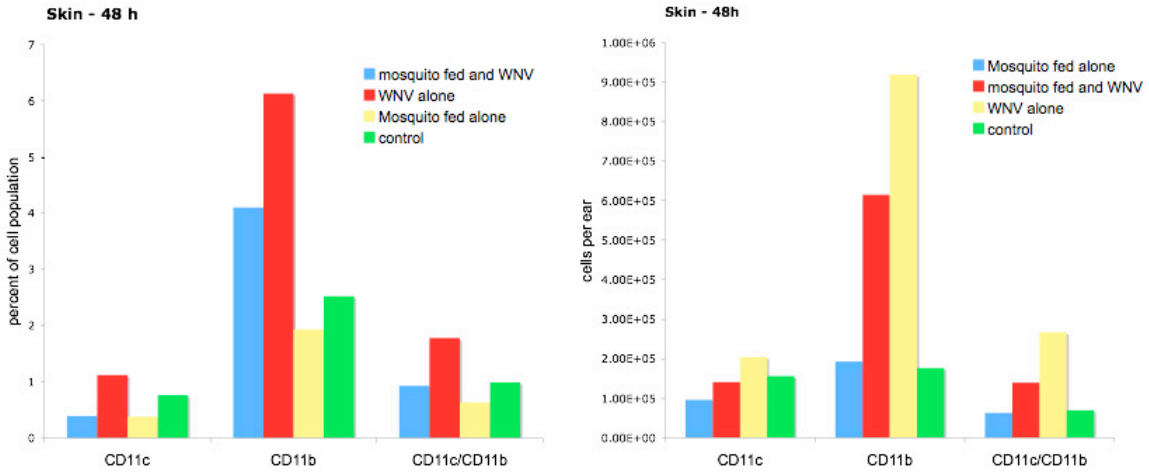


**Figure 5.11 CD11c+, CD11b+, and CD11c/CD11b dual positive cells present in the skin at 48 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

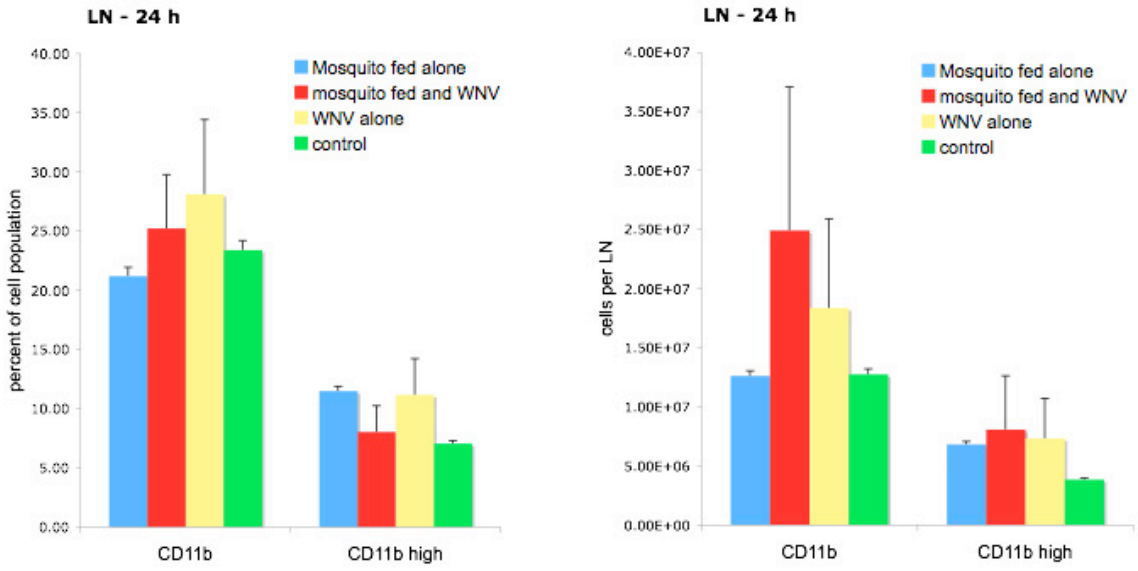


**Figure 5.12 CD11b+ cells present in the LN at 24 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

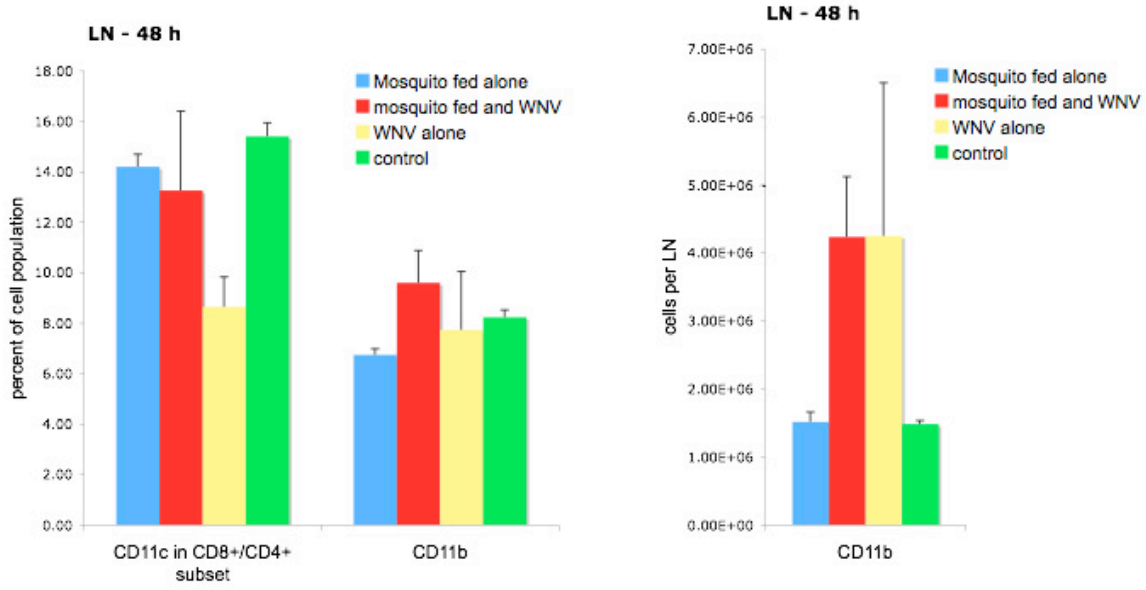


**Figure 5.13 CD11c expression in the CD8+/CD4+ subset and CD11b+ cells present in the LN at 48 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

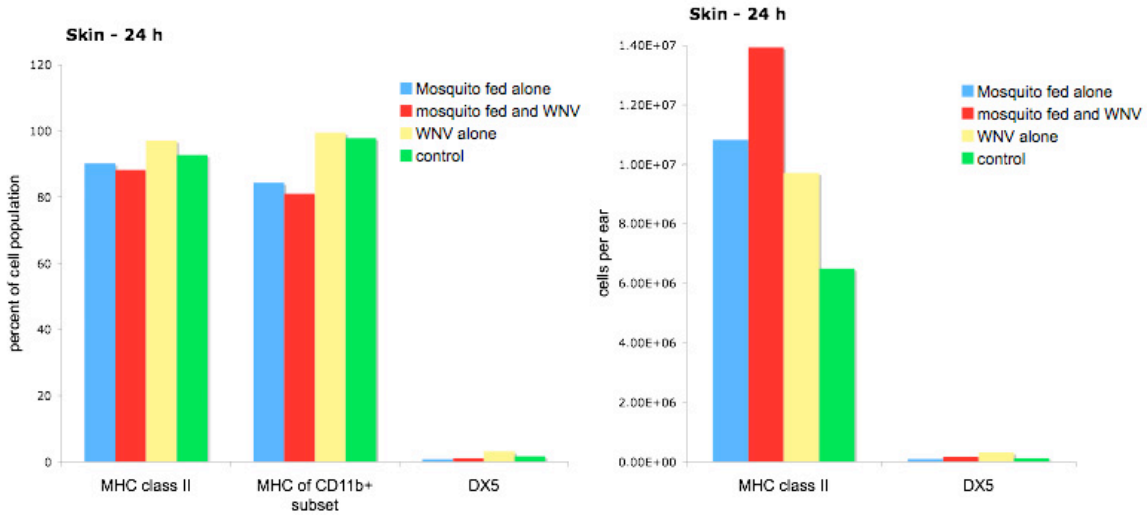


**Figure 5.14 MHC class II+ cells, MHC class II expression in CD11b+ subset, and NK (DX5+) cells present in the skin at 24 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

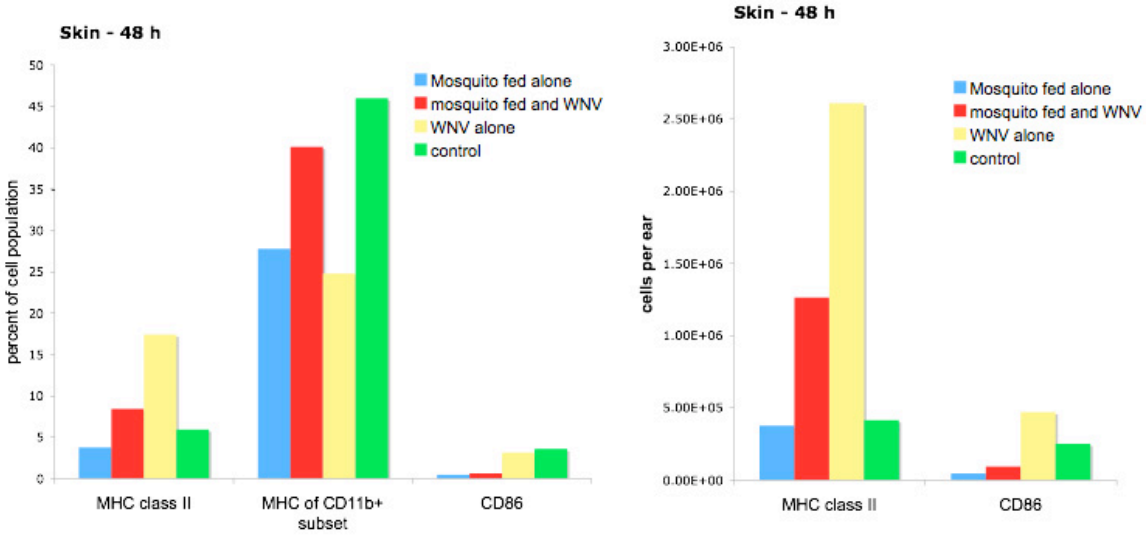


**Figure 5.15 MHC class II+ cells, MHC class II expression in CD11b+ subset, and NK (DX5+) cells present in the skin at 48 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

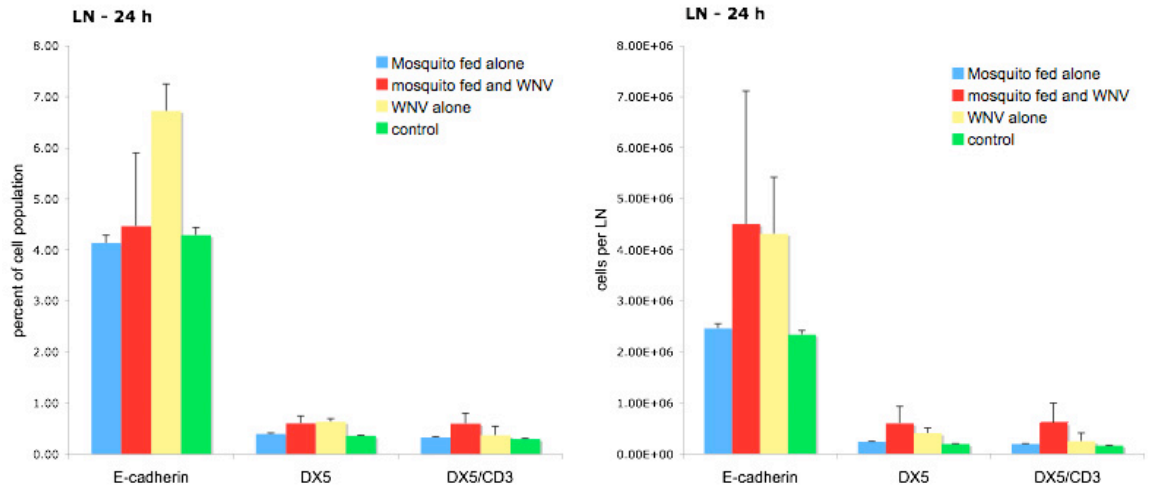


**Figure 5.16 E-cadherin+ cells, NK (DX5+) cells, and NK T cells (DX5+/CD3+) cells present in the LN at 24 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

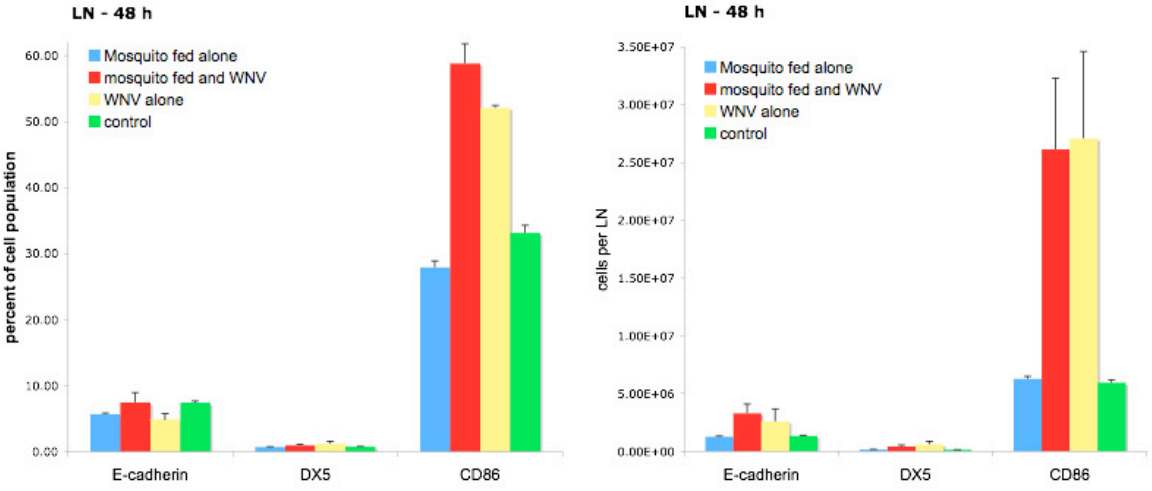


**Figure 5.17 E-cadherin+ cells, NK (DX5+) cells, and CD86-expressing cells present in the LN at 48 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

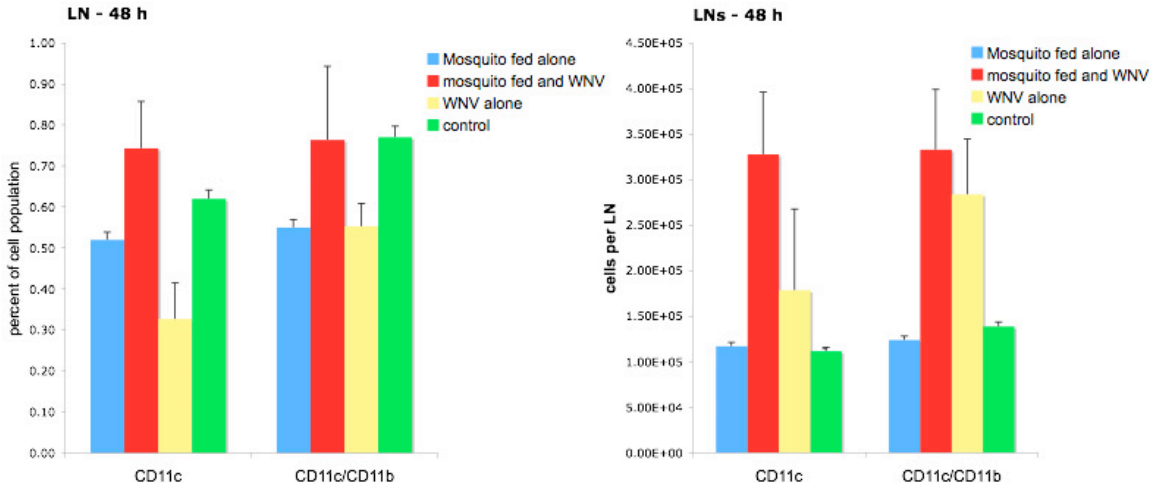


**Figure 5.18 CD11c+ and CD11c/CD11b dual positive cells present in the LN at 48 h post-exposure to indicated conditions; both percent of population and actual numbers are shown and are representative of at least 2 replicates. Results for each group are from pooled cells of 3 mice.**

## DISCUSSION

This study was conducted to enhance our knowledge of the early cellular events following natural WNV transmission and, in addition, to evaluate the role of mosquito saliva in cell influx into the inoculation site and draining LNs. To accomplish this, mice infected i.d. in the presence of absence of mosquito feeding were compared for differences in cell infiltration at the site of inoculation and within the LN. Additional groups were exposed to mosquitoes alone or were naïve to aid in interpretation of results. The characteristic inflammatory response was observed following experimental treatment of the skin. The response was distinguished by the migration of varying waves of cells into the dermis and subsequently into the lymph node.

Neutrophils were observed to be one of the first responders to mosquito feeding, which concurs with a previous study looking at the effect of mosquito feeding alone (Demeure *et al.* 2005). At early time points, neutrophil influx was greater in groups

exposed to WNV infection, but by 48 h post exposure the group exposed to mosquitoes alone had a relatively high level of neutrophils in the skin.

Demeure and associates (2005) also noted that *A. stephensi* feeding alone leads to a migration of DCs to the draining LN, which peak at 48 h post exposure. Perhaps this effect of mosquito feeding alone helps to explain the higher levels of LCs found in groups exposed to mosquito feeding (either with or without WNV inoculation). These researchers established that the DC migration was facilitated by the degranulation of mast cells by mosquito feeding, and showed that mast cell activation is followed by hyperplasia of the draining lymph node due to the accumulation of $CD3^+$, $B220^+$, $CD11b^+$, and $CD11c^+$ leukocytes. (Demeure *et al.* 2005). Interestingly, mast cell degranulation caused by mosquito feeding appears to result in an immune-inhibitory environment, mediated by IL-10 (Demeure *et al.* 2005). It is also clear that WNV infection in the skin in the absence of mosquito feeding leads to DC migration to draining LNs (Johnston *et al.* 2000). Johnston and colleagues (2000) showed that this migration of DCs was associated with an increase in MHC class II-expressing cells in the draining LN. Similarly, we observed an augmentation of MHC class II-expressing cells following WNV infection. Our data suggests that this migration occurs earlier in groups where mosquitoes fed in addition to virus inoculation. Byrne and associates (2001) observed that 18 h after cutaneous WNV infection there is a significant increase in LC numbers in the draining LNs that corresponds to decreased in epidermal LCs, as well as LN hyperplasia. This migration of LCs to LNs appears to be IL-1β-dependent (Byrne *et al.* 2001). Although our data demonstrates an increase in LCs in the LN, in agreement with Byrne and others observations, we were unable to show a concurrent decrease in LC numbers in the skin. It is possible that in our model, LCs are replenished via the blood at a rate rapid enough to prevent dips in their population. Nevertheless, our data does show a reduction of macrophage numbers in the skin following mosquito feeding and/or WNV infection corresponding to a rise in this cell population in the draining LN, suggesting that these conditions cause macrophages to become activated and migrate to the LN.

Comparing WNV-infected groups suggests that there may be an enhancement of macrophage migration in the group exposed to mosquito feeding.

A prominent observation was the relative decrease in $CD3^+$ cells in the skin after mosquito feeding. This constitutes *in vivo* evidence in support of previous studies that found that *Ae. aegypti* saliva significantly suppresses T cell activities at lower concentrations and enhances T cell death at higher concentrations (Wanasen *et al.* 2004; Wasserman *et al.* 2004). Wasserman and colleagues found that very dilute levels of SGE caused an inhibition of T-lymphocyte proliferation. Dose-dependent suppression of cytokine secretion, particularly $T_H1$ and proinflammatory cytokines, also resulted from SGE treatment of T-lymphocytes (Wasserman *et al.* 2004). Alterations in cytokine production by T cells presumably could alter cell influx. Both groups independently found that *Ae. aegypti* SGE causes a significant decrease in T cell viability. Corresponding to our data, the enhanced cell death, and subsequent decline in tissue level, was more pronounced in $CD4^+$ T cells.

While there are no global shifts in the cell populations or migration with the addition of mosquito feeding to the site of WNV inoculation, clear differences are evident. These include reduced $CD3^+$ populations, enhanced levels of LCs, and earlier peak of $CD11b^+$ cell populations. It is possible that many of the $CD3^+$ cells detected in the skin are γδ T cells (Wang *et al.* 2006). Although this T cell subset comprises a minority of $CD3^+$ cells in lymphoid tissues, they are well represented in the epidermis and peripheral blood. Gamma-delta T cells provide an immediate response to peripherally inoculated WNV, limiting viral replication and invasion of the CNS in the first few days of infection (Wang *et al.* 2003). Enhanced levels of these cells in the blood and recruitment to sites of viral infection have been documented in both human (Agrati *et al.* 2001b, Agrati *et al.* 2001a) and murine (Rakasz *et al.* 1999) infections. Therefore the activity of γδ T cells is keenly important following cutaneous viral inoculation, and they are believed to help bridge the gap between innate and adaptive immune response (Wang *et al.* 2006). The effect of mosquito saliva has not been established for this subset of T cells, but it is not improbable that γδ T cell activity and viability, like that of αβ T cells,

could be similarly suppressed. A recent study has suggested that *Ae. aegypti* saliva may directly induce apoptosis of γδ T cells (Donald Champagne, personal communication). Further studies will be necessary to definitively identify the $CD3^+$ cells in the skin and how they react to mosquito saliva, particularly in the context of arbovirus infection. The reduction in $CD4^+$ cell numbers brought about by mosquito feeding could have an effect on WNV infection. A recent study with WNV (Sitati and Diamond 2006), illustrated the important role that $CD4^+$ T cells play; suppression or deficiency of this subpopulation during WNV infection in mice resulted in prolonged CNS infection and uniform lethality. Additionally, mice lacking $CD4^+$ T cells had reduced IgG production and, later in infection, WNV-specific $CD8^+$ T cell activation and trafficking to the CNS were compromised (Sitati and Diamond 2006). Mice that lack $CD8^+$ T cells have higher CNS viral burdens and increased mortality rates after infection with WNV (Shrestha *et al.* 2004).

Given the potent molecules present in mosquito saliva and their demonstrated effect on cytokine expression, adhesion molecules, and regulators of the inflammatory response, more extreme alterations in cell populations might be predicted. It is possible that the method of inoculation of virus might have caused significant tissue trauma, thereby masking discrete changes that may occur with the minimally invasive route by which a virus is delivered to the host via a mosquito. Relative to a mosquito proboscis and the minute amount of saliva injected by this vector, a 30-gauge needle and 10 μl of fluid seem like an indelicate and rough manner to introduce a virus. Unfortunately, fluctuations in the amount of virus delivered can alter the inflammatory response to a site, and therefore the coarser, but measurable, method of viral inoculation had to be employed. Hitherto techniques for inoculation of miniscule volumes of fluid have not been perfected in our laboratory, and required equipment for such procedures was unavailable.

## CONCLUSION

This study expands our understanding of the early events subsequent to WNV infection. Additionally, data is presented that suggests that mosquito feeding can affect the early inflammatory response to an arbovirus infection. The *in vivo* observations of altered T-lymphocyte levels in the presence of mosquito saliva supports and clarifies *in vitro* data with *Ae. aegypti* SGE. In a viral infection, such as WNV, that is generally asymptomatic, but unpredictably and rapidly can progress to life-threatening neurological disease, understanding the factors that contribute to pathogenesis is critical.

# CHAPTER 6: PRIOR EXPOSURE TO UNINFECTED MOSQUITO BITES ENHANCES MORTALITY TO NATURALLY-TRANSMITTED WEST NILE VIRUS ENCEPHALITIS – A ROLE FOR THE IMMUNE RESPONSE TO THE VECTOR IN ARBOVIRUS INFECTIONS


## ABSTRACT

Following peripheral inoculation by mosquitoes, WNV replicates in the skin and then spreads quickly to the draining lymph node probably within immature dendritic cells that are susceptible to infection. There is a close relationship between early peripheral virus burden and proclivity towards neuroinvasion and death with WNV encephalitis. Numerous studies have demonstrated that early factors such as timing and magnitude of viremia, integrity of innate immune response, activity of type I IFNs, and early replication efficiency of virus are acutely important in the survival of the host. Generally, individuals are exposed to the bites of uninfected mosquitoes prior to exposure to an infected mosquito. Humans and rodents develop an immune response to mosquito salivary proteins, characterized by IgE/IgG reactivity, immediate wheal and flare reaction, and/or delayed type hypersensitivity. There is reason to believe that pre-exposure to a disease vector may influence disease outcome. Mice pre-exposed to the sand flies followed by fly-transmitted infection with *Leishmania major* had a fivefold increase in infiltrating leukocytes in the skin, augmented IFN-γ, decreased IL-4, and, remarkably, were protected against the severe disease. Therefore the potential for an immune response directed against mosquito salivary proteins to have a protective or confounding effect on naturally transmitted WNV infection was investigated by comparing early WNV pathogenesis and WNV disease in mosquito naïve and sensitized populations of mice. Pre-exposure to mosquito feeding profoundly affected subsequent WNV disease. Mice previously-exposed to *Ae. aegypti* feeding developed significantly

higher mortality associated with elevation of inflammation, APC recruitment, IL-4 expression in the draining LNs, and LN hyperplasia concurrent with a decrease in lymphocytes mainly of the $CD4^+$ subtype. This study expands our understanding of natural exposure to arboviruses and early immune responses that follow.

## INTRODUCTION

West Nile virus is a positive sense single-stranded RNA virus in the family Flaviviridae that emerged globally following the appearance of a more neurotropic subtype. Recently outbreaks of WNV disease have occurred in the Middle East, Europe, Africa, South America, and North America (Deardorff *et al.* 2006). Since its introduction to the United States in 1999, WNV has spread rapidly leading to ~19,000 diagnosed human cases between 1999 and 2005 (http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm#maps) with an estimated 750,000 undiagnosed infections in 2003 (Busch *et al.* 2006). WNV infection in most cases is a mild febrile illness, which occasionally leads to neuroinvasive disease characterized by cognitive impairment, muscle weakness, paralysis, and periodically death (Chambers and Diamond 2003a). Vertebrates typically become infected when an infectious mosquito pierces the host epidermis to take a blood meal, depositing virus principally in the extravascular tissue (Turell *et al.* 1995). Accumulating evidence has demonstrated that the mosquito saliva, which carries WNV into the vertebrate, is not simply a transport medium, but can have a profound effect on vertebrate immunity, pathogen transmission efficiency, pathogenesis, and disease course (Edwards *et al.* 1998, Limesand *et al.* 2000, Gillan and Devaney 2004, Schneider *et al.* 2004, Wasserman *et al.* 2004, Billingsley *et al.* 2006, Schneider *et al.* 2006).

Following peripheral inoculation, WNV replicates in the skin and then spreads quickly to the draining lymph node probably within immature DCs and LCs that are susceptible to infection (Byrne *et al.* 2001). Studies with WNV show that within the first day or two of infection, infected LCs migrate from the epidermis to local lymph nodes (Byrne *et al.* 2001). The role of these cells in flavivirus infection may be pleiotropic, as their migration to draining lymph nodes promotes antigen presentation, while simultaneously facilitating the spread of the infection. The signalling environment where LCs come into contact with antigen and the activation state of the LCs can affect the ability of these APCs to preset antigen and activate proliferation of T cells (Byrne *et al.*

2001). At the early stages of virus infection changes in DC or LC abilities can tip the balance in favor of a virus, potentially allowing for augmentation of virus replication prior to activation of the adaptive immune response. There is a close relationship between early peripheral virus burden and proclivity towards neuroinvasion and death (Chambers and Diamond 2003b). Numerous studies have demonstrated that early factors such as timing and magnitude of viremia, integrity of innate immune response, activity of type I IFNs, and early replication efficiency of virus are acutely important in the survival of the host (as reviewed in Chambers and Diamond 2003b).

**Sensitization to arthropod feeding and salivary proteins**

Generally, individuals are exposed to the bites of uninfected mosquitoes prior to exposure to an infected mosquito. Humans and rodents develop an immune response to mosquito salivary proteins, characterized by IgE/IgG reactivity, immediate wheal and flare reaction, and/or delayed type hypersensitivity (Chen *et al.* 1998). The degree of host response to mosquito saliva is dependent on the duration and intensity of exposure to biting mosquitoes and the immunological profile of the host (Peng *et al.* 1996b). The level of circulating antibody generated by mosquito feeding is predictive of the hypersensitivity response – those with low levels of anti-mosquito IgE/IgG have minor skin reactions, while those with relatively higher levels of antibody have more pronounce reactions (Peng *et al.* 1996b).

Mice pre-exposed to the SGE of sand flies followed by infection with *Leishmania major* in the presence of SGE had a fivefold increase in infiltrating leukocytes in the skin, augmented IFN-γ, decreased IL-4, and, remarkably, were protected against the severe disease (Belkaid *et al.* 1998, Kamhawi *et al.* 2000). In response to mosquito salivary antigens sensitized mice show a significantly increased lymphocyte proliferation response, but, conversely, have an enhanced IL-4 production and decreased IFN-γ production suggesting that a $T_H2$ immune response predominates despite the development of the delayed skin reaction (Chen *et al.* 1998). Mean lymphocyte proliferation after stimulation with mosquito antigens was higher in mice (Chen *et al.* 1998) and humans (Peng *et al.* 1996b) previously exposed to mosquito bites, implying

that lymphocytes are involved in the development of immunological reactions to mosquito saliva. Immunoblotting techniques have characterized the salivary proteins recognized by host antibodies (as reviewed in Billingsley *et al.* 2006), and have demonstrated that anti-mosquito antibodies vary with mosquito species, population density, and season (Palosuo *et al.* 1997).

Belkaid and associates (2000) suggested that such an immune response generated by hematophagous arthropods actually may facilitate acquisition of a bloodmeal and promote survival, instead of disrupting feeding. They found that in both humans and mice, the DTH response was associated with increased blood flow, and proposed that the increased lymph in the affected tissues might ease penetration of the proboscis in the skin.

In many natural settings, exposure to mosquitoes is the norm instead of the exception. The possibility that pre-exposure to mosquito saliva might modulate infection with any pathogen has not been explored. Whereas sensitization to mosquito saliva causes recognized alterations in immune response to subsequent mosquito feeding and similar anti-arthropod reactions have led to modifications of pathogen transmission, exploring this phenomenon in the context of mosquito-borne virus is important to our understanding of arbovirus pathogenesis. Therefore the potential for an immune response directed against mosquito salivary proteins to have a protective or confounding effect on naturally transmitted WNV infection was investigated by comparing early WNV pathogenesis and WNV disease in mosquito naïve and sensitized populations of mice. Survival studies were paired with analysis of the cellular and humoral response shortly after mosquito transmitted WNV infection.

## MATERIALS AND METHODS

### Virus

The WNV isolate used to infect mosquitoes was identical to that used for all experiments herein and was isolated from a liver homogenate of an infected blue jay, *Cyanocitta cristata*, collected in Houston, TX in 2002. This strain is similar to WNV-

NY99 with respect to genotype, plaque morphology, and virulence in mice (Girard *et al.* 2005). The virus was propagated in Vero (green monkey kidney) cell culture. Stock virus was produced following a single passage on Vero cells and harvested as a tissue supernatant and stored at -80$^{o}$C until used in mosquito infections.

**Mice**

Four-week old female BALB/c mice (Harlan) were used in all experiments. At least six mice were used for each experimental group, and in those replicates where flow cytometric analysis was performed 10 mice were included in each group. Mice were divided into two main groups that were further sub-divided depending on the requirements of the experiment. The three main groups were: control mice never exposed to mosquitoes or WNV; mice that were previously naïve to mosquito exposure prior to the feeding of an infected mosquito; and mice that were exposed to uninfected mosquitoes before being exposed to a WNV-infected mosquito. The latter group generally was exposed four times to mosquitoes, but in one replicate of the experiment both two and four exposures were investigated. Exposures to uninfected mosquitoes occurred once a week for 4 weeks or twice biweekly for mice receiving four and two exposures, respectively. Mice were sedated with Nembutal® (50 mg/kg i.p.) and exposed or not to the feeding of *Ae. aegypti* or *Cx. pipiens* (n = 20) for approximately 30 minutes. At least 10 mosquitoes fed on each mouse, as assessed by direct observation of bloodmeals within abdomens. Feeding was restricted to the left ear using a cardboard template surrounding a soft latex sleeve. One week after the final mosquito exposure all experimental groups were exposed to the feeding of a single infected mosquito. For this mice were sedated and the feeding of infected *Ae. aegypti* or *Cx. pipiens* was restricted to the right ear. Following infection mice were returned to cages, bled periodically, and observed for symptoms of disease. In some experiments a subset of three mice were taken at 36 h post infection and tissues isolated for flow cytometric and immunohistochemical analysis.

**Mosquitoes**

Mosquitoes used for pre-exposure were uninfected females 7-10 days post-eclosion that were deprived of sucrose for 24 h to encourage feeding. For pre-exposure *Ae. aegypti* or *Cx. pipiens* mosquitoes were allowed to feed on the ears of sedated mice for a period of ~0.5 h.

Mosquitoes are usually infected with viruses in two ways: orally (*per os*), by using artificial membrane feeders, for example, and parenterally via intrathoracic inoculation. Whereas *per os* methods most closely approximate natural infections, they often do not lead to 100% infection rates in mosquitoes. Although WNV orally infects several species of arthropod (Turell *et al.* 2005), the relative efficiency varies considerably (Schiefer and Smith 1974). On the other hand, parenteral infections effectively provide a method by which most mosquitoes can be infected. This method circumvents the midgut infection barrier, usually achieves a 100% infection rate with WNV, and leads to rapid systemic infection of mosquitoes. Due to these considerations and the importance of ensuring that all mosquitoes are infected, mosquitoes were infected by intrathoracic inoculation. Mosquitoes were inoculated into the thorax using glass needles made from capillary (hematocrit) tubes. The tubes were drawn into a fine tip over a flame or prepared using an apparatus made for this purpose (Narishege PB-7 puller and EG-4 beveller). Divisions were stamped onto the needles to provide a measure of the inoculum quantity. Needles were held in the glass holder supplied with the tubes and this was attached to a syringe with a three-way valve via plastic tubing. The valve allowed for easy control of the inoculation and reduced the risk of reflux into the syringe. Seven- to ten-day old *Ae. aegypti* or *Cx. pipiens* vectors were immobilized by brief chilling in a refrigerator and maintained in immobilized state during inoculation with a chill table. After inoculation mosquitoes were transferred to cartons before recovery.

For infectious exposure, mosquitoes were infected with WNV by intrathoracic inoculation. Seven days later infected mosquitoes (1 per mouse) were fed on mice. Individual mosquitoes were separated into cartons covered with mesh, mice were sedated and restrained, and the site of feeding was restricted. Following feeding RNA was

isolated from each mosquito and WNV load was detected via real-time RT-PCR to confirm infection status. RNA was extracted from mosquitoes as previously detailed, with minor modifications. Mosquitoes were frozen at –80°C until processing and were then added to 350 μl of RLT lysis buffer (Qiagen) in secure-lock tubes with one steel ball. Methodology continued from here forward as detailed previously.

An additional group of infected mosquitoes was used to determine if prior exposure to mosquitoes influenced the quantity of WNV salivated by the mosquitoes into mice. For this experiment groups of 20 mosquitoes were allowed to feed on previously-exposed mice (4× mosquito feeds), mosquito-naïve mice, or not allowed to feed. Immediately following feeding salivary glands were extracted from both fed and unfed mosquitoes. Individual glands were placed into 350 μl of RLT lysis buffer (Qiagen) in separate tubes. Due to the simple structure of mosquito salivary glands, bead disruption of tissues was not necessary, instead tubes were vigorously vortexed for 15 sec each, 350 μl of 70% ethanol was added, and RNA isolation proceeded normally. Viral titer of each salivary gland pair was quantified via real-time RT-PCR, and all titers from fed mosquito salivary glands were divided by the average titer from unfed mosquito salivary glands to give an estimate of percent of WNV salivated into host.

**Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) of SGE**

To assess the reactivity of mouse sera to mosquito salivary proteins electrophoresis was performed on *Ae. aegypti* or *Cx. pipiens* SGE for use in Western blots. The gel electrophoresis glass was washed with NaOH (not with detergent) and allowed to air dry. Spacers were placed between glass plates and fixed in place with clamps on the casting stand (Mini-PROTEAN II electrophoresis system; Biorad, Hercules, California). A separating gel was prepared in a volume of 30 ml: 7.5 ml 40% acrylamide:Bis (30:1), 15 ml 1.5 M Tris-HCl (pH 8.9), 7.5 ml $H_2O$, 0.3 ml 10% SDS (Biorad), and 0.3 ml 10% $NH_4S_2O_8$. Five milliliters of this solution was reserved in a separate tube, and 10 μl of N,N,N',N'-tetramethylethylene diamine (TEMED) was mixed into remaining solution and poured between glass plates. After polymerization (5-10 min), 25 μl of TEMED was added to the rest of the separating gel solution, mixed and

poured between the glass plates, leaving space for wells and concentrating gel. The gel was covered with 1 ml of 0.1% SDS in water to facilitate the catalytic reaction. The concentrating gel was prepared in a 10 ml volume: 1 ml 40% acrylamide:Bis (30:1), 2.5 ml 0.5 M Tris-HCl (pH 6.8), 6.5 ml $H_2O$, 0.1 ml 10% SDS, and 0.1 ml 10% $NH_4S_2O_8$. Once the separating gel was polymerized, 10 µl of TEMED was added to concentrating gel solution, mixed, poured on top of separating gel, and the comb was inserted. Subsequent to polymerization of concentrating overlay, the comb was removed and the gel was placed onto the gel sandwich clamp assembly, affixed to the inner cooling core, and then into the Mini-PROTEAN II electrophoresis cell lower buffer chamber (Biorad). Both the lower and upper chambers were filled with running buffer, the combs were removed and wells were washed with running buffer. Approximately 30 salivary gland pairs were suspended in 300 µl of PBS then the solution was sonicated and centrifuged at 13,000 rpm for 10 min at 4$^{o}$C to release salivary proteins and remove residual cellular debris. Protein loading buffer was added (1:4), and the sample and protein markers were heated at 95 $^{o}$C for 5 min. Sample tubes were cooled, vortexed, and then spun briefly. The equivalent of about 1 salivary gland pair was added to each well and empty wells were loaded with 1× loading buffer. Electrophoresis was run at 50 V until the samples/dye lined up at the separating gel, then a constant 20 mA current was set and sample was run until the dye reached the bottom of the gel.

**Western Blot with mouse serum**

Transfer buffer (1× NuPage) was prepared with 20% methanol and cooled to 4 $^{o}$C. A 83×60 mm Hybond ELC nitrocellulose membrane and two 89×66 mm 3MM CRH Whatman filter sheets were cut and soaked in transfer buffer. Four sponge pads were soaked in transfer buffer and all air bubbles were forced out by pressing with gloved fingers. The concentrating gel was removed from the separating gel, transfer buffer was poured over gel and then the 3MM filter paper was placed over the gel (making contact with the middle first and then laying down the ends to avoid air bubbles). The gel and 3MM filter paper were flipped upside-down and the gel was gently detached from the glass plate with a blade or spatula. The gel and 3MM paper were placed onto the sponge

pads on the negative electrode with the gel facing up. Transfer buffer was poured over the gel and the nitrocellulose membrane was place onto the gel (again from middle to ends). Any bubbles between gel and membrane were pressed out with a spatula, more transfer buffer was poured on top, and the remaining 3MM filter paper was applied to the top of the membrane. Two well-soaked sponge pads were placed on top of the filter paper and a generous amount of transfer buffer was poured over the top. This was then covered with the positive electrode, pieces were secured together by placing in the tank, and the chamber was filled with fresh transfer buffer. Transfer was performed in the 4$^{o}$C refrigerator at 30 V for 1 h.

Proteins were fixed on membrane (0.25 g Ponceau C, 0.5 ml HAc, and 50 ml $H_2O$) for 15 min with agitation. Membranes were then fixed several times with Milli-Q water, and the quality of transfer was confirmed. Membranes were blocked (5% dry milk, 1 mg/ml bovine serum albumin, 0.5% Tween20 in PBS; 10 ml per membrane) over-night with agitation at 4$^{o}$C.

Pooled mouse serum (400 μl) was applied to separated regions of the membrane using the Mini-PROTEAN® II Multiscreen Apparatus (Biorad), diluted 1:100 (10% blocking buffer in 0.5% Tween20 in PBS), for 1 h at room temperature. Unbound antibody was then removed with 3 washes (20 mM Tris-HCl pH 7.5, 0.5 M LiCl, and 0.1% Tween20 in $H_2O$) of 10 minutes. The membrane was then incubated with the peroxidase-labeled anti-mouse IgG, diluted 1:5000 (10% blocking buffer in 0.5% Tween20 in PBS), for 1 h at room temperature. Unbound secondary antibody was then removed with 3 washes (20 mM Tris-HCl pH 7.5, 0.5 M LiCl, and 0.1% Tween20 in $H_2O$) of 10 minutes. Excess liquid was then wicked from the membrane by placing it for a moment on 3MM filter paper. Membranes were then treated with ECL plus Western Blotting Detection System (Amersham Biosciences, Piscataway, New Jersey) for 30 sec, surplus reagent was removed by touching the membrane's corner to 3MM filter paper, and membrane was placed on Saran wrap and the wrap folded to prevent leakage of reagent. The membranes were then placed into an exposure cassette, and Hyperfilm ECL

was then exposed to membranes for 0.5 – 2.0 min. Film was developed and serum antibody binding to mosquito salivary proteins assessed.

**Apyrase Assay**

To determine whether antibodies against specific mosquito salivary proteins can hinder protein function, an enzymatic assay was used to assess the activity of apyrase (Fig. 6.1), a key mosquito salivary protein (Champagne *et al.* 1995), in the presence of mouse serum. A stock solution of both *Ae. aegypti* and *Cx. pipiens* SGE was prepared for use throughout assays. Solutions were created by isolating salivary glands from 30 mosquitoes into 300 μl of 0.1 M NaOH. Salivary proteins were solublized and used in the assay at a concentration of 0.1 salivary gland pair/reaction. Reactions were prepared in a 96-well plate and when used, murine serum pools were from groups (exposed or unexposed to *Ae. aegypti* or *Cx. pipiens*) were diluted 1:20 in buffer, tested in triplicate, and allowed to incubate on the plate with SGE prior to addition of (adenosine triphosphate) ATP. This dilution of serum from mice sensitized to *An. stephensi* was previously shown to cause inhibition of greater than 90% of salivary gland apyrase activity, while titers of 1:160 retained the ability to inhibit more than 50% of apyrase activity (Mathews *et al.* 1996). Salivary solutions were added to the plate and to them were added pooled serum, 0.1 NaCl, or nothing. Samples were allowed to incubate with SGE for 30 min. Reaction buffer (0.05 M Tris-HCl pH 9.0, 0.1 M NaCl, 5 mM $CaCl_2$, and 20 mM β-mercaptoethanol added just before reaction) was mixed with ATP stock to give a 2 mM concentration of ATP, and was preheated directly before use to 37$^{o}$C. One hundred microliters of reaction buffer were added to each well and the plate was incubated at 37$^{o}$C for 10 minutes. While the incubation was ongoing, $PO_4^-$ standards were made with serial dilutions of sodium phosphate stock (1, 0.5, 0.25, 0.125, 0.0625, 0.031 mM). Following the incubation period, standards were added to the plate. To each well was added 25 μl acid molybdate (1.25 g ammonium molybdate dissolved in 100 ml 2.5 N sulphuric acid) and 3 μl reducing mixture (0.2 g 1-amino-2-napthol-4-sulfonic acid, 1.2 g sodium bisulfate, 1.3 g sodium sulfite; this was stored in the dark, and 25 mg was mixed with $H_2O$ just prior to use), and samples were incubated at 37$^{o}$C for 20 min. Plates

were read with an enzyme-linked immunosorbent assay (ELISA) plate reader at 660 nm. Standards were used to create a line equation that yielded mM of $PO_4^-$ from values ELISA values. As all wells were treated with the same level of ATP, higher levels implies increased apyrase activity, whereas lower levels would indicates inhibition of apyrase activity.

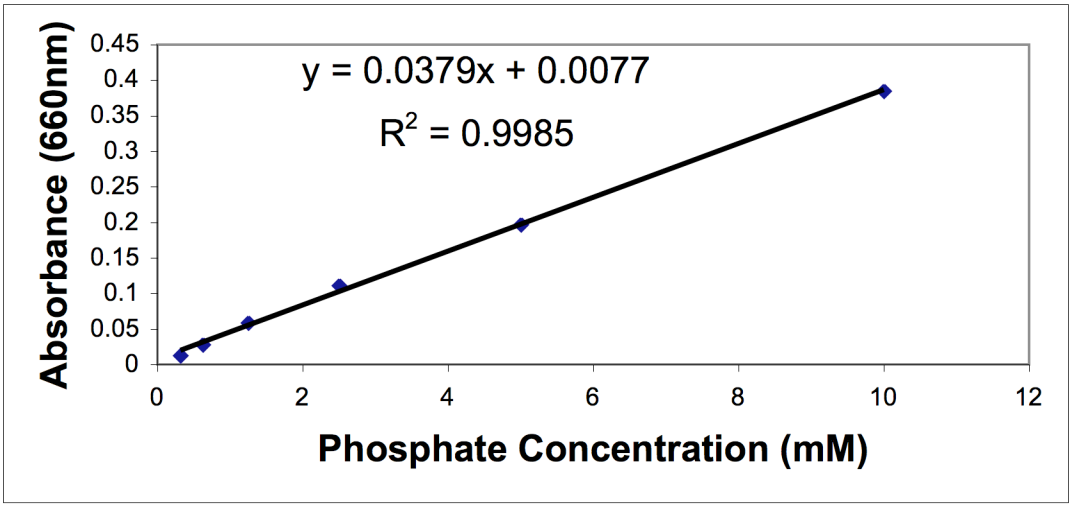


**Figure 6.1. Apyrase activity standard curve**

**Silver Staining of Gels for Visualization of Mosquito Salivary Proteins**

For staining of SGE GelCode® Color Silver Stain Kit (Pierce, Rockford, Illinois) was utilized. This lit allows for highly sensitive (less than 1 ng of protein is detectable) staining of proteins electrophoresed in polyacrylamide gels. Proteins stain in distinct colors against an amber background.

Clean glass trays and ultrapure water were used for all procedures, and all washes/staining was performed at room temperature on a platform shaker to ensure efficient and uniform washing and staining. Following SDS-PAGE (described above) of SGE, gels were fixed overnight (50% ethanol and 5% acetic acid solution), and then washed with four changes of ultrapure water (once every 30 minutes) for a total of 120 min. During washes stain reagent working solutions were prepared as follows: Silver Working Solution: 10 ml of Silver concentrate into 140 ml water; Reducer Aldehyde

Working Solution: 10 ml of Reducer Aldehyde Concentrate into 65 ml water; Reducer Base Working Solution: 10 ml of Reducer Base Concentrate into 65 ml water; and Stabilizer Base Working Solution: 10 ml of Stabilizer Base Concentrate into 440 ml water. Subsequent to wash step, gels were incubated in Silver working solution for 30 min, followed by a water rinse of 10 sec. The reducer working solution was prepared immediately before use by combining equal volumes of reducer aldehyde and reducer base working solutions, and gels were incubated in reducer working solution for 4 min. Gels were momentarily rinsed with water, and then added to the stabilizer working solution for 30 min. After stabilization, the gels were immediately photographed by digital camera with rear illumination.

**Real-time RT-PCR to Detect Shifts in Cytokine mRNA in LN and Skin**

To determine whether prior exposure to mosquitoes alters cytokine signalling after initial WNV transmission real-time RT-PCR was performed on primary sites of WNV replication. Following isolation of cells from ears and lymph nodes, approximately $10^7$ cells per tissue (less for ears) were reserved for RNA isolation. Cells were pelleted by centrifugation and resuspended in 600 μl of RLT lysis buffer (Qiagen), vortexed for 15 sec, and then RNA was isolated as previously described.

Samples were analyzed for variation in cytokine expression, predominantly assessing IL-2, IL-4, IL-10, IL-12, and IFN-γ mRNA levels. Protocols for running real-time RT-PCR, normalizing between samples, and analyzing results have been discussed previously.

**Passive Transfer of Mouse Anti-Mosquito Saliva Antibodies**

To assess the role of antibody directed against mosquito saliva in the divergent WNV disease course observed in mosquito naïve and mosquito exposed mice, serum from *Ae. aegypti*-sensitized BALB/c mice was used for passive transfer experiments. Blood was collected from uninfected mice exposed at four weekly intervals to *Ae. aegypti* feeding, and was centrifuged at 500 × g for 5 min. Serum samples were pooled, and 200 μl was inoculated undiluted i.p. into mosquito naïve mice. Twelve hours later mice were

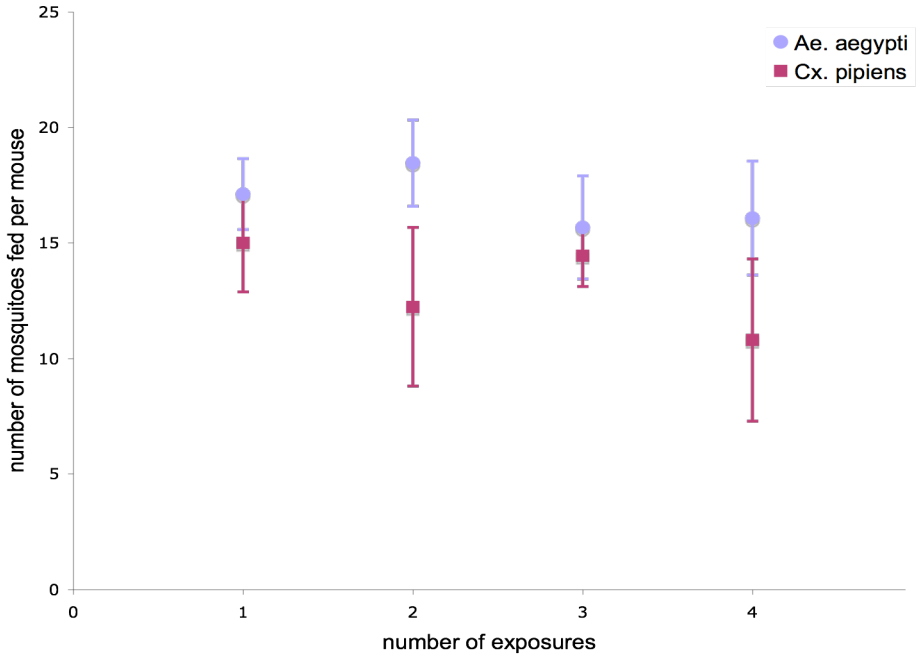


**Figure 6.2 The number of mosquitoes that fed on each mouse, did not vary significantly between species or exposures.**

challenged via the feeding of a single infected mosquito. Vital status was observed twice daily.

## RESULTS

### Preexposure of Mice to Uninfected Mosquitoes

For each preexposure 20 *Ae. aegypti* or *Cx. pipiens* mosquitoes were allowed to feed on each sedated mouse. An overall average of 17 and 13 mosquitoes fed per exposure per mouse for *Ae. aegypti* or *Cx. pipiens*, respectively (Fig. 6.2). No significant differences in feeding success were noted between exposures. Visually, the intensity of the inflammatory response appeared to increase at the feed site with each subsequent exposure.

### Infection of Mosquitoes

For infectious exposure of mice, mosquitoes were infected with WNV by intrathoracic inoculation. Seven days later infected mosquitoes were fed individually on

mice, and each mosquito was saved for confirmation of infection. To demonstrate that the individual mosquitoes used for mouse infection were indeed infected, RNA from fed mosquitoes was analyzed by real-time RT-PCR. All mosquitoes were verified to be infected. Infectious mosquitoes had an average titer of $1.19\times10^{9}\pm0.03\times10^{9}$ equivalent $\log_{10}TCID_{50}$ (Fig. 6.3), and mosquito titers did not significantly vary between groups.

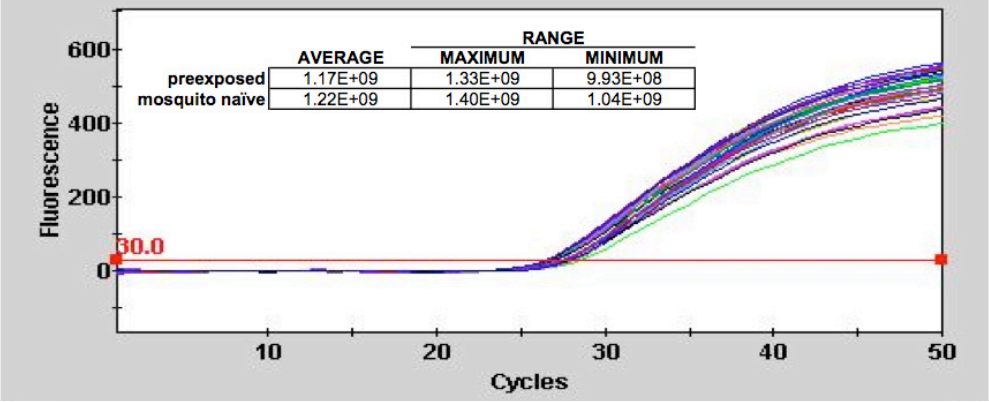


**Figure 6.3 Real-time RT-PCR Amplification of WNV in mosquitoes used for infectious feed on mice.**

## The Effect of Preexposure to Mosquito Feeding on the Course of WNV Infection

Due to the likelihood that individuals who are at risk for flavivirus infection are preexposed to the bites of uninfected mosquitoes, we investigated whether this exposure might modify the host response to mosquito-transmitted WNV. Mice were sensitized twice or four times, two weeks or one week apart, respectively, by the feeding of mosquitoes. They were challenged by the bite of a single WNV-infected mosquito, which demonstrated high body titers for all groups (Fig. 6.3; $1.2\times10^{9}\pm0.03$ equivalent $\log_{10}TCID_{50}$). As illustrated in Fig. 6.4A, preexposure to mosquito feeding exacerbated WNV disease as shown by decreased survival within the group previously exposed to mosquitoes. Consistently, groups of mice exposed to *Ae. aegypti* mosquitoes prior to an infectious feed by the same mosquito species displayed an increased mortality rate, with as few as two prior exposures to mosquito feeding sufficient to cause this deviation in survival. This effect of mosquito sensitization-induced aggravation of WNV infection

could not be shown when *Culex pipiens* acted as the sensitizer and vector (Fig. 6.4B).

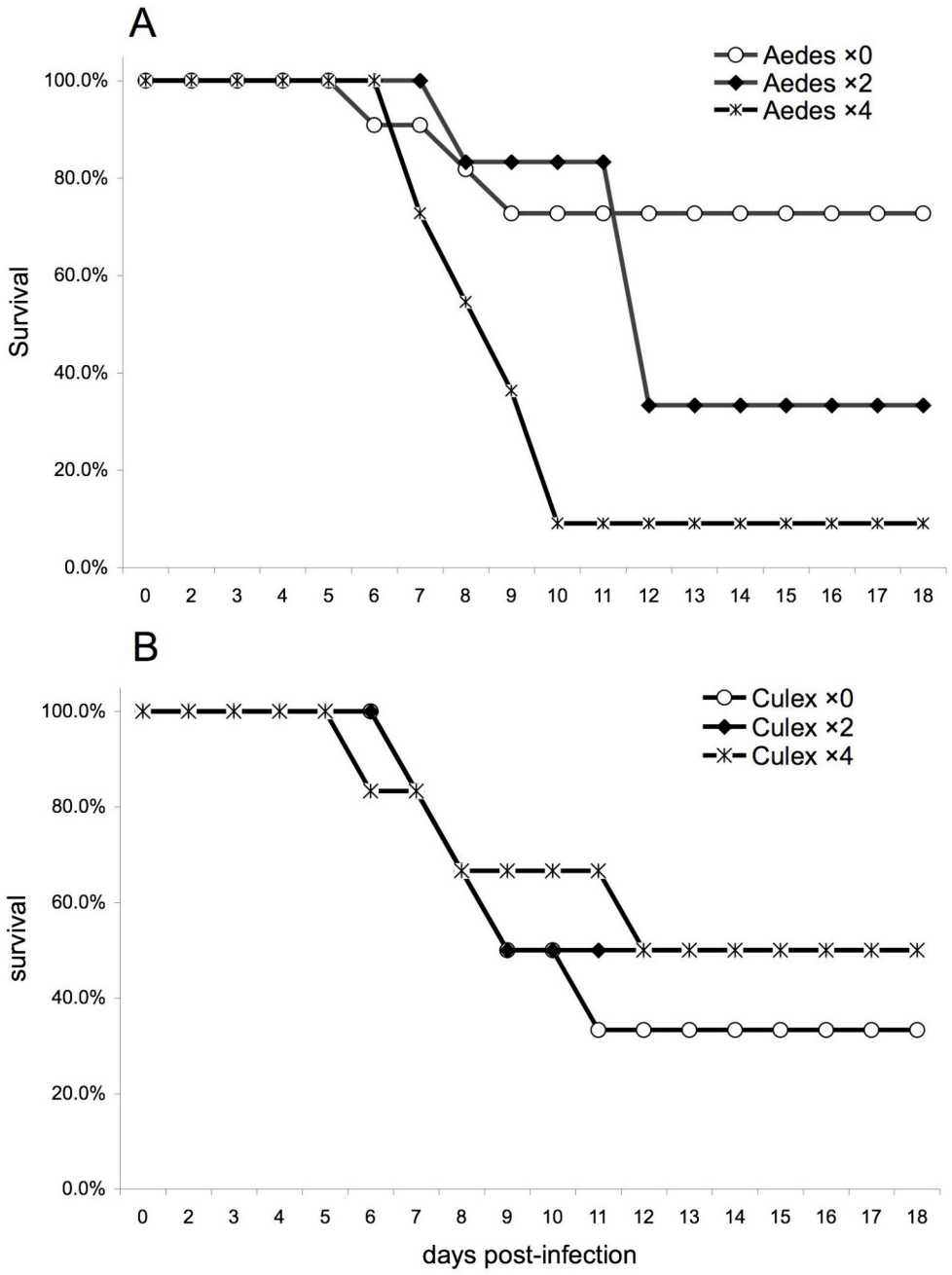


**Figure 6.4 Survival of mice infected via the bite of a single WNV-infected *Ae. aegypti* (A) or *Cx. pipiens* (B) mosquito. Curves indicate the survival of mice previously exposed to mosquitoes 0, 2, or 4 times. (A) contains pooled data from three replicates ($n \geq 5$ mice/group/replicate), and end survival is significantly different by Fisher's exact text ($p < 0.05$). (B) n = 6 mice/group.**

**Analysis of Anti-saliva Antibodies in Mosquito-sensitized Mice and Their Role in WNV Disease Exacerbation.**

Anti-salivary protein antibodies were readily detected in mice that had been previously exposed to mosquito feeding. A Western blot analysis of the serum showed 6 major bands recognized by the serum of *Ae. aegypti*-exposed mice (Fig. 6.5) and 3–4

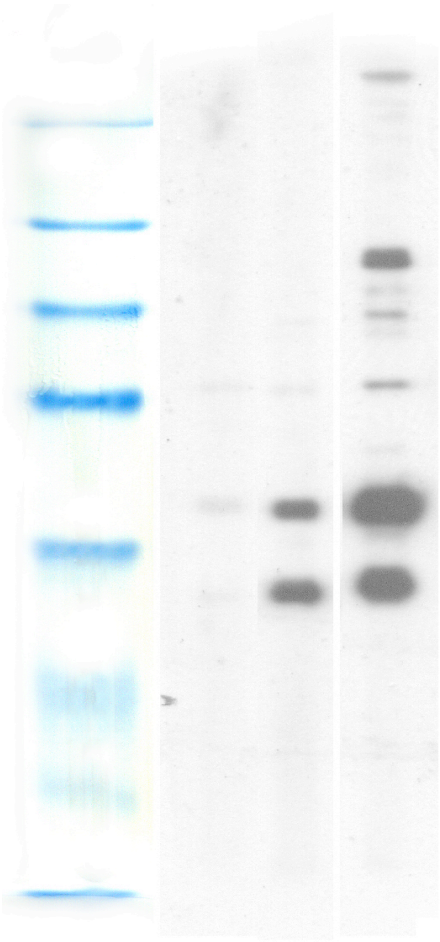

**Figure 6.5 Western blot analysis of *Ae. aegypti* salivary glands sera from unimmunized BALB/c mice (lane 2), mice exposed twice to mosquito feeding (lane 3), and mice exposed 4 times to mosquito feeding (lane 4). Protein ladder is shown in lane 1; bands correspond (from top to bottom) to 175, 83, 62, 47.5, 32.5, 25, 16.5, and 6.5 kDa.**

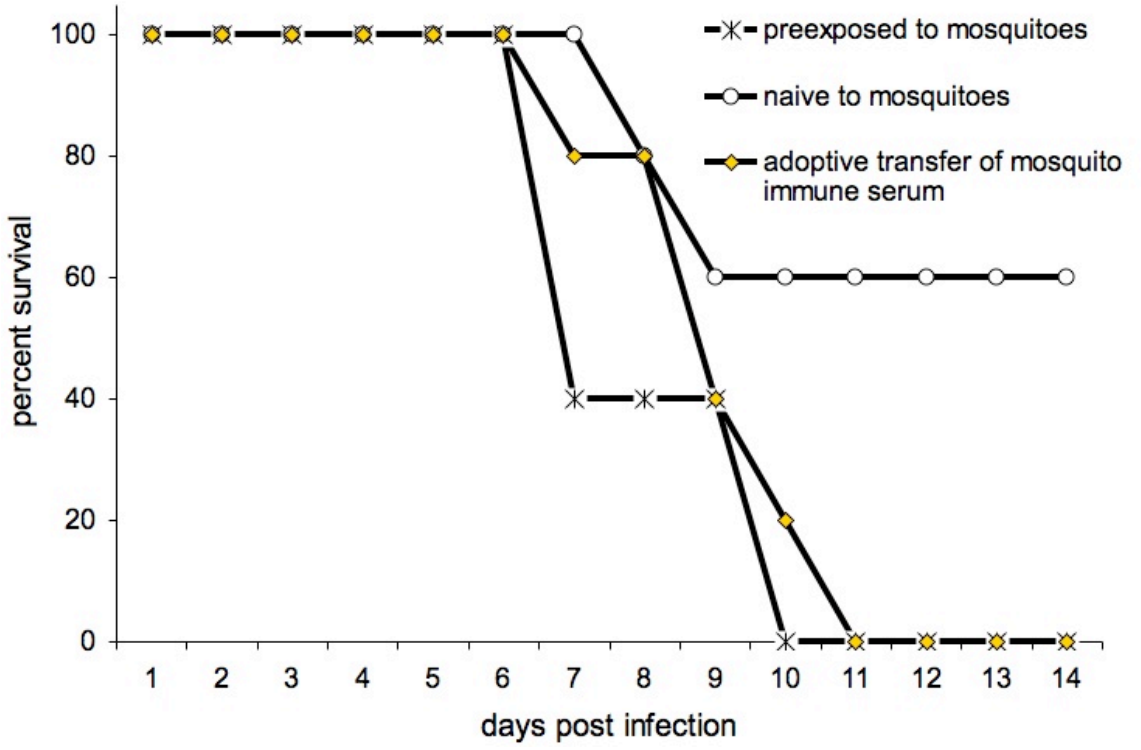


**Figure 6.6 Survival of BALB/c following WNV infection in pre-exposured (4× to *Ae. aegypti*), naïve, and mice inoculated with mosquito-immune serum. Mortality was significantly different.**

bands recognized by the serum of *Cx. pipiens*-exposed mice (data not shown). These data indicate that mosquito bites can stimulate mouse B cells to secrete specific IgG antibodies

**Passive Transfer of Mosquito-Immune Serum.**

To assess the role of these antibodies in the observed enhancement of WNV disease, serum from mosquito-exposed mice was inoculated i.p. into age-matched, naïve mice. Passive transfer of *Ae. aegypti*-sensitized serum (4× exposed) to naïve mice caused these mice to develop higher mortality, characteristic of the group previously exposed to mosquito feeding (Fig. 6.6).

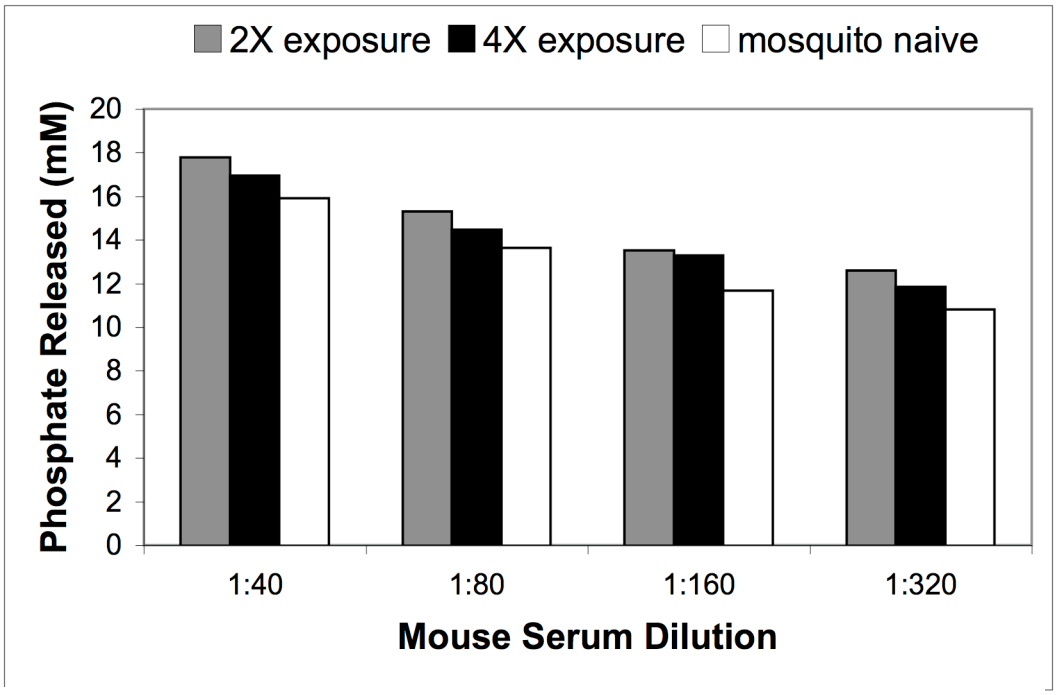


**Figure 6.7 Apyrase catalytic activity as determined by phosphate release from ATP. No difference was observed.**

**Effect of Anti-Mosquito Antibodies on the Function of a Salivary Protein**

To determine if anti-mosquito saliva antibodies could disrupt the function of salivary proteins, a biochemical assay was employed to assess the activity of a representative mosquito protein apyrase. Apyrase is one of the most common proteins secreted by a mosquito into its host. Given the ubiquity of this protein in hematophagous arthropods it is presumably important for successful acquisition of a bloodmeal, and reduction of salivary apyrase activity is associated with increased probing time (Rossignol 1984). Previous studies have shown that the serum of mice repeatedly exposed to *An. stephensi* feeding drastically inhibits the activity of apyrase. Treatment of both *Cx. pipiens* and *Ae. aegypti* SGE with serum from mice exposed to these mosquito species caused no reduction in apyrase activity (Fig. 6.7).

**Effect of Mosquito Sensitization on Amount of WNV inoculated by Mosquitoes.**

To establish whether differences between *Ae. aegypti* pre-exposed and naïve groups could be due to a deviation in the amount of virus inoculated into mice previously exposed or not to mosquitoes, the quantity of virus inoculated into mice was approximated. Salivary glands were dissected from mosquitoes immediately following feeding on mosquito immune and non-immune mice and compared using unfed mosquito salivary glands as a baseline. Approximately $1.1\times10^{7}$ equivalent $\log_{10}TCID_{50}$ was detected in mosquitoes that fed on naïve or mosquito-sensitized mice. No difference was evident between these two groups (Fig. 6.8).

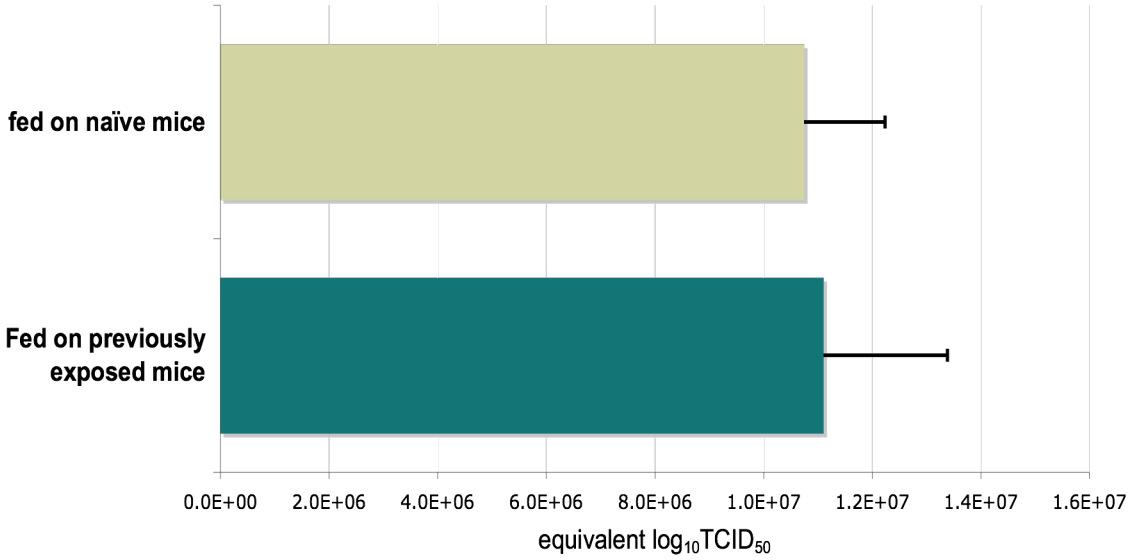


**Figure 6.8 Level of WNV detected in *Ae. aegypti* salivary glands immediately after feeding on preexposed or naïve BALB/c mice.**

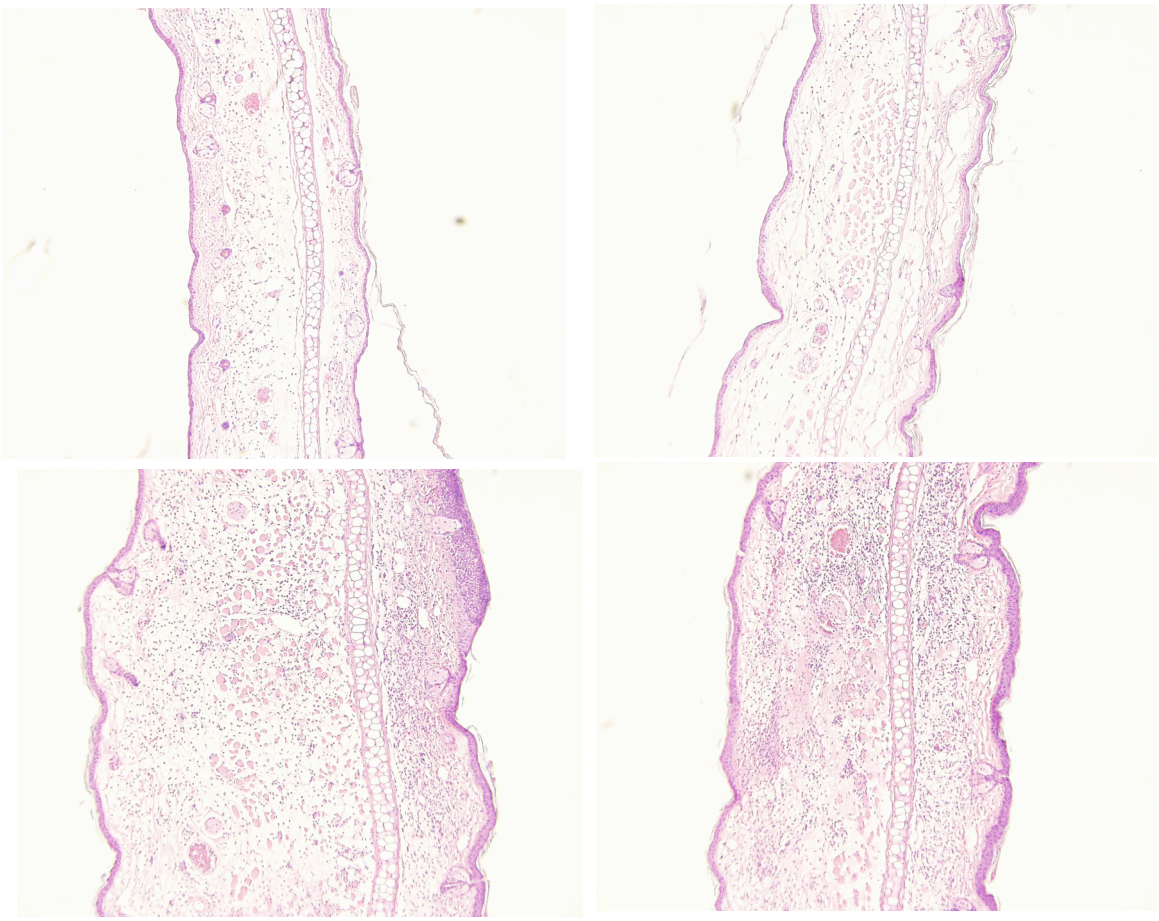

**Figure 6.9 Sites of *Ae. aegypti* feeding have enhanced inflammation and cell influx in mosquito-sensitized mice (bottom) at 24 h post-exposure, as compared to mice previously naïve to mosquito feeding (top).**

## Analysis of Cell Influx After WNV Challenge in Naïve Mice and Mice Sensitized to *Ae. aegypti* Feeding

One possible mechanism for the deviation observed in sensitized mice is that prior exposure to mosquito saliva leads to a memory response to the salivary proteins, which in turn alters cell influx and/or recruits more cell types susceptible to infection. On histological inspection of the site of challenge the preexposed group indeed has a different response (Fig. 6.9). Amplified inflammation in the sensitized group, characterized by mononuclear cell (plasma and histocytic cells) and neutrophil infiltrate, lead to swelling of the tissue to over twice the size of unexposed mice and areas of mild

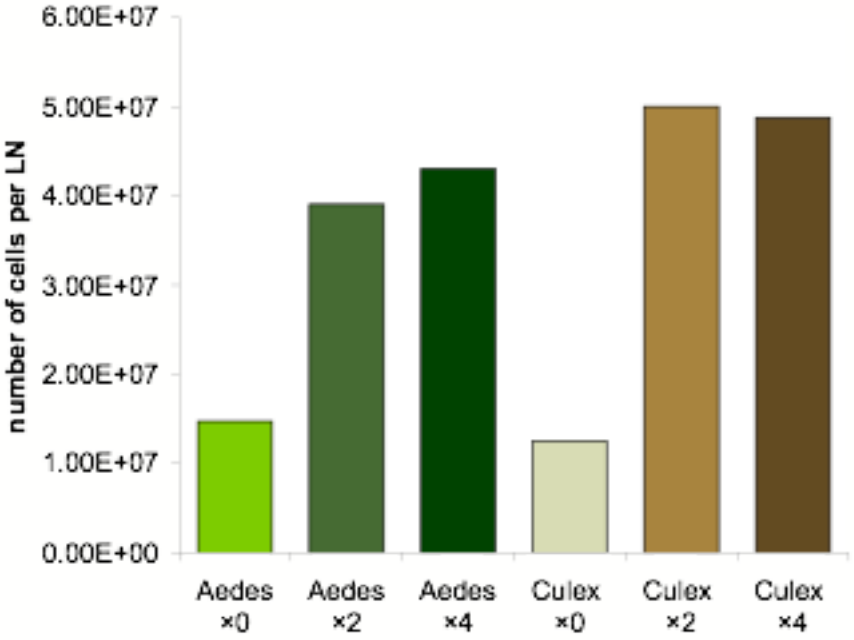


**Figure 6.10 Number of cells per retromaxillar lymph node 36 h after infection with WNV. Mice were previously exposed to uninfected mosquitoes 0, 2, or 4 times.**

necrosis. Lymph nodes in the preexposed group were also noticeably larger and cell counts were significantly higher (Fig. 6.10). Both *Ae. aegypti* and *Cx. pipiens* preexposed mice had significantly more cells in retromaxillar LNs, two to three times that of naïve mice. As few as two exposures to mosquito feeding was sufficient to achieve this enhanced lymphadenopathy. The enhanced cellularity of draining LNs suggests that lymphocytes are involved in the development of immunological reactions to mosquito saliva.

**Flow Cytometric Analysis of the Early Dermal Reactivity after Challenge in Naïve Mice and Mosquito-Sensitized Mice.**

The mechanism by which preexposure to mosquitoes enhances mosquito-transmitted WNV infection is unclear. One possibility is that the immune response to salivary proteins promotes cellular recruitment into the skin, including DCs, allowing a

higher proportion of invading virions to find a susceptible and mobile cell for infection within the inoculation site. To investigate this possibility, the cells in the skin were identified with multi-color flow cytometry after mosquito inoculation of WNV. Cell populations were isolated and stained with a variety of antibodies.

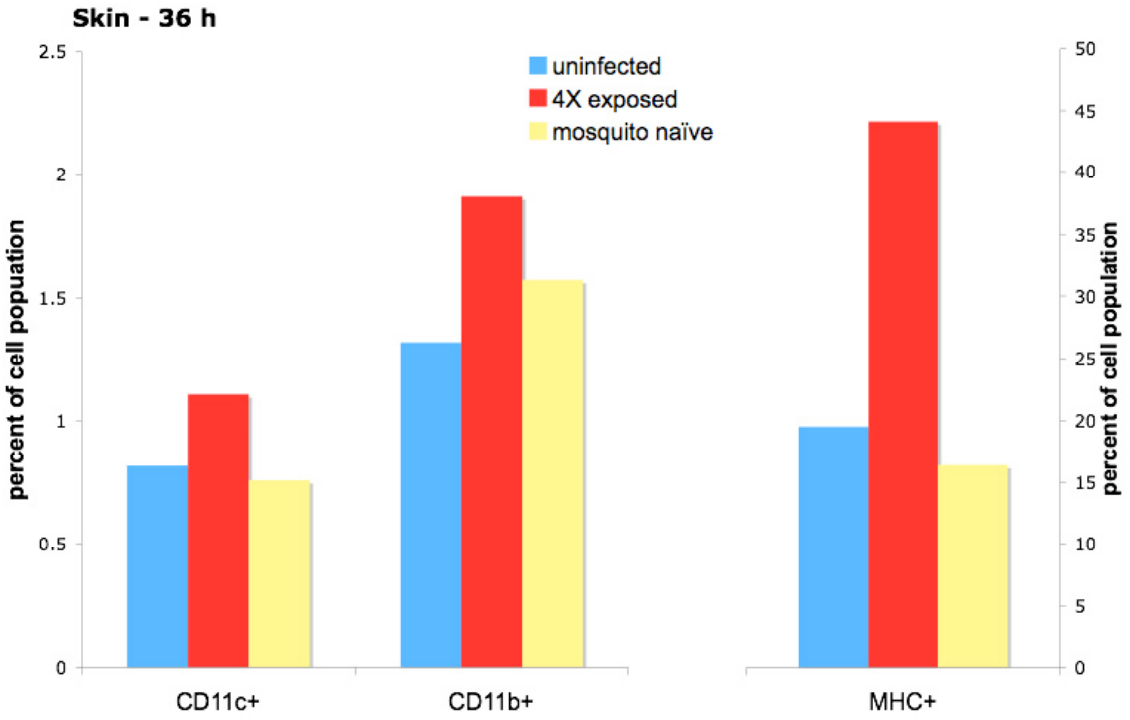


**Figure 6.11 Proportion of skin cell population made up of CD11c, CD11b, and MHC class II positive cells 26 h after WNV inoculation by *Ae. aegypti* as assessed by flow cytometry. Mice were preexposed or not to *Ae. aegypti* feeding.**

All groups of mice inoculated with WNV (*Ae. aegypti* and *Cx. pipiens* naïve, 2× preexposed, 4× preexposed) showed an inflammatory response to viral replication. By comparison, in mice preexposed to mosquito feeding, the inflammatory response was significantly altered. The number of leukocytes recruited was approximately doubled, and was composed mainly of mononuclear phagocytes and neutrophils (Fig. 6.9). The kinetics of this response are indicative of a DTH response to salivary components in preexposed mice. The considerable autofluorescence, which is characteristic of epidermal and dermal cells, complicated analysis of the skin populations as compared to

cells of the LNs, but clear patterns were obvious (Fig. 6.11). Following WNV infection, the proportion of MHC class II expressing cells was more than doubled in mice previously exposed to *Ae. aegypti* feeding. Class II MHC is chiefly present on monocytes, macrophages, and dendritic cells with B cells expressing lower levels on their surface. Cells expressing CD11c and CD11b were also higher after infection in the skin of mice sensitized to *Ae. aegypti* feeding. These surface proteins are associated with APCs, CD11c predominantly being expressed on DCs and CD11b being expressed mainly on macrophages but also NK and granulocytic cells.

**Flow Cytometric Analysis of Lymph Node Reactivity after Challenge in Naïve and Mosquito-Sensitized Mice.**

Following any infection inflammatory cells, especially DCs, that have homed to the site, migrate or flow in the lymph fluid to the draining LN. The LNs proximal to an infection are the principal sites where the innate immune response interfaces with the adaptive immune response. The cell population and signalling environment are not just a view into the particular localized response, but are critical to the success or failure of host defenses. To further assess the effect of mosquito sensitization on cell migration following naturally transmitted WNV infection, LN cells were reduced to single-cell suspensions and identified with multi-color flow cytometry after mosquito inoculation of WNV. Cell populations were isolated and stained with a variety of antibodies as detailed in the methods section.

Groups of mice inoculated with WNV (*Ae. aegypti* and *Cx. pipiens* naïve, 2× preexposed, 4× preexposed) produced an inflammatory response to viral replication. In contrast, mice preexposed to mosquito feeding had an enhanced inflammatory response, as determined by the size of draining LNs. Predictably, the actual numbers of all cell subpopulations evaluated increased in mice sensitized to mosquito feeding corresponding with the augmented inflammatory response (Fig. 6.12). Determination of the relative abundance of subpopulations within LNs, reveals a dominant pattern: following WNV inoculation by mosquitoes, mice that were preexposed to mosquitoes show an enhanced leukocyte level, including CD11c+ and CD11b+ cells, concurrent with diminished

lymphocyte populations, as revealed by CD3+ cells (Fig. 6.12). Of the lymphocyte population, CD4+ cells are lowest in the groups sensitized to mosquitoes (Fig. 6.13 - 6.16).

In mice preexposed to *Ae. aegypti* feeding before WNV infection CD3+ cells comprised 69.2% of LN cells in mice exposed 2× and 64.5% of LN cells in mice exposed 4×, compared to 73.6% of cells in mice previously unexposed to mosquitoes (Fig. 6.13 and 6.14). Cells expressing CD4 on their surface decreased from 53.6% of the LN population in naïve mice to 50.3% and 45.7% in mice previously exposed to *Ae. aegypti* 2× and 4×, respectively. Less of a reduction was apparent with the CD8+ subpopulation (Fig. 6.13 and 6.14), making up 19.2%, 18.1%, and 17.8% of the LN population in naïve, 2×-, and 4×-exposed mice, respectively. Class II MHC positive cells were more

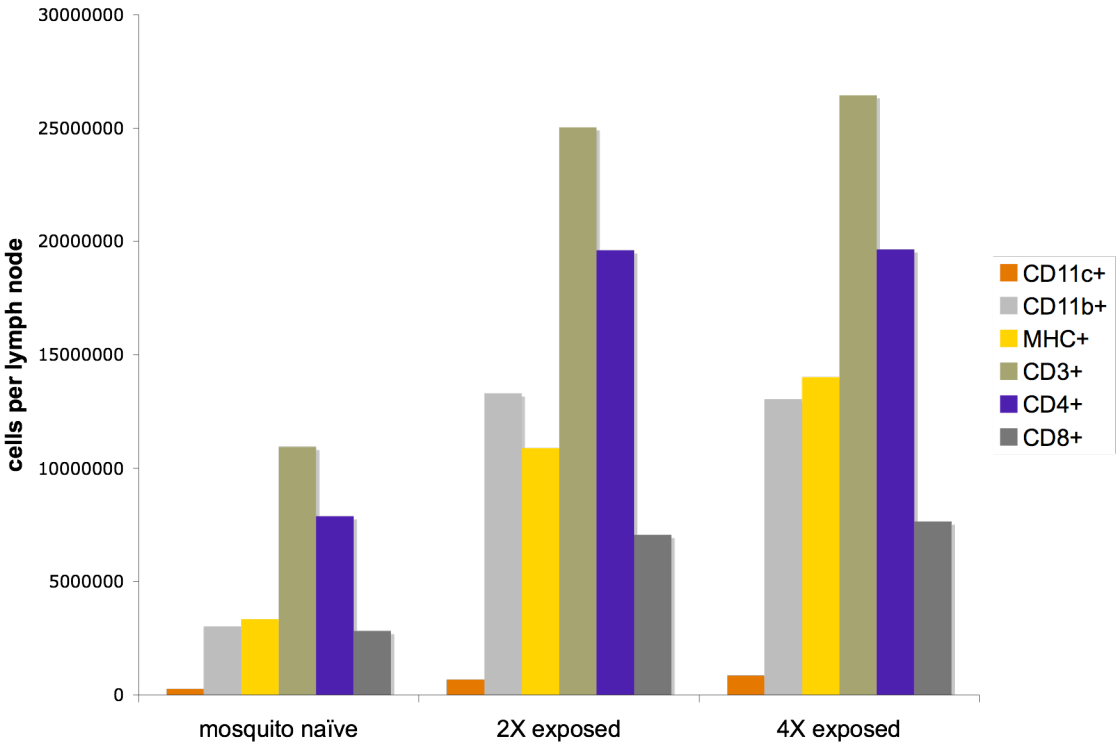


**Figure 6.12 leukocytes in the LN 36 h post-infection with WNV, characterized by expression of surface proteins, in naïve, 2× exposed, and 4× exposed to *Ae. aegypti* mosquitoes; pools of 3 mice.**

abundant in mice previously exposed to mosquitoes (Fig. 6.17 and 6.19), making up 27.9% and 32.6% of the LN population, juxtaposed with the 22.7% observed in naïve mice (Fig. 6.17). Similarly, 13.3% and 9.7% more of the total LN population was made of CD11b+ cells in 2×- and 4×-exposed mice, than in naïve mice (Fig. 6.17 and 6.18). Interestingly, cells expressing DX5, a protein associated with NK cells are found at a higher level in previously exposed mice (Fig. 6.21). Within the DX5+ group the percent of cells expressing CD3+ is significantly higher in the mosquito-sensitized group; DX5/CD3 dual positive cells are generally considered NK T cells.

Similar trends continued with mice previously exposed to *Cx. pipiens*, but were less pronounced. CD3+ cells decreased from 75.4% of the LN population of naïve mice, to 68.6% and 67.6% in mice preexposed 2× and 4×, respectively (Fig. 6.15). Fifty-five percent of LN cells in naïve mice expressed CD4, in contrast to 51.1% in 2× and 49.7% in 4× preexposed mice (Fig. 6.16). The CD8 subpopulation was minimally altered during WNV infection by previous exposure to *Cx. pipiens* (Fig 6.16; 19.5% for naïve, 16.7% for 2× preexposed, and 17.4% for 4× preexposed). As with *Ae. aegypti* preexposure, sensitization with *Cx. pipiens* lead to an enrichment of MHC class II positive cells in the draining LN following WNV infection (Fig. 6.17). The LNs of preexposed mice had 4.9% to 6.9% more MHC class II positive cells than LNs from naïve mice (Fig. 6.17). Likewise, CD11b positive cells were more abundant in mice previously exposed to mosquitoes, making up 24.6% and 26.6% of the LN population, compared to the 22.1% observed in naïve mice (Fig. 6.20). Dissimilar to mice sensitized to *Ae. aegypti*, mice sensitized to *Cx. pipiens* showed no significant alteration in DX5+ cells (Fig. 6.22).

The trend that appears in the LNs of mice previously exposed to mosquito saliva is, following WNV inoculation by mosquitoes, an enhanced leukocyte level, including CD11c+ and CD11b+ cells, concurrent with diminished lymphocyte populations, as revealed by CD3+ cells (Fig. 6.23).

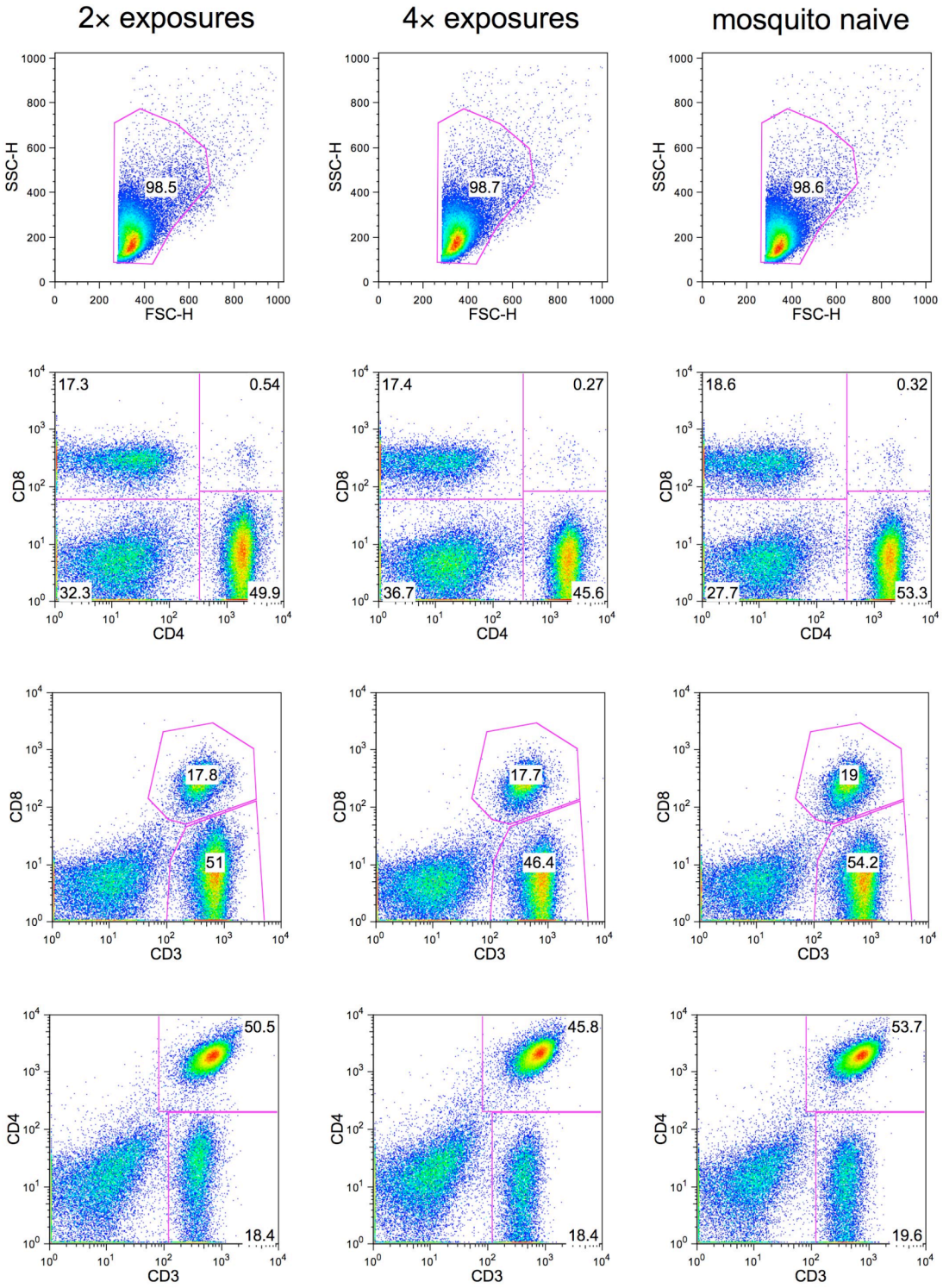


**Figure 6.13 Flow cytometry scatter plots for CD4/CD8/CD3-labeled LN cells isolated from mice (pools of 3 mice) exposed or not to *Ae. aegypti* feeding. Tissues were isolated 36 h post-infection with WNV.**

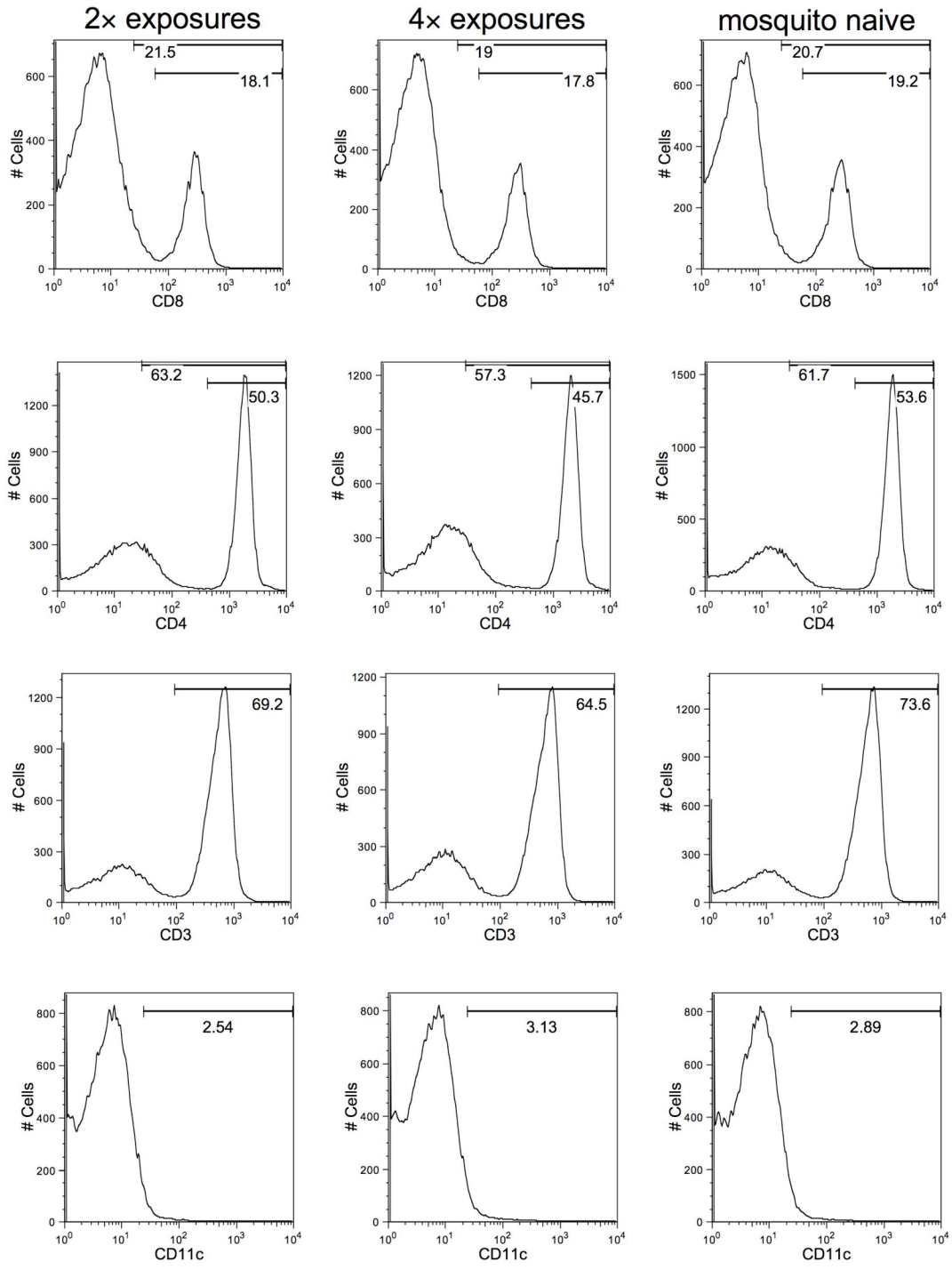


**Figure 6.14 LN Cells positive for CD4, CD8, or CD3 at 36 h post-WNV infection. Cells were isolated from mice (pools of 3 mice) exposed or not to *Ae. aegypti* feeding.**

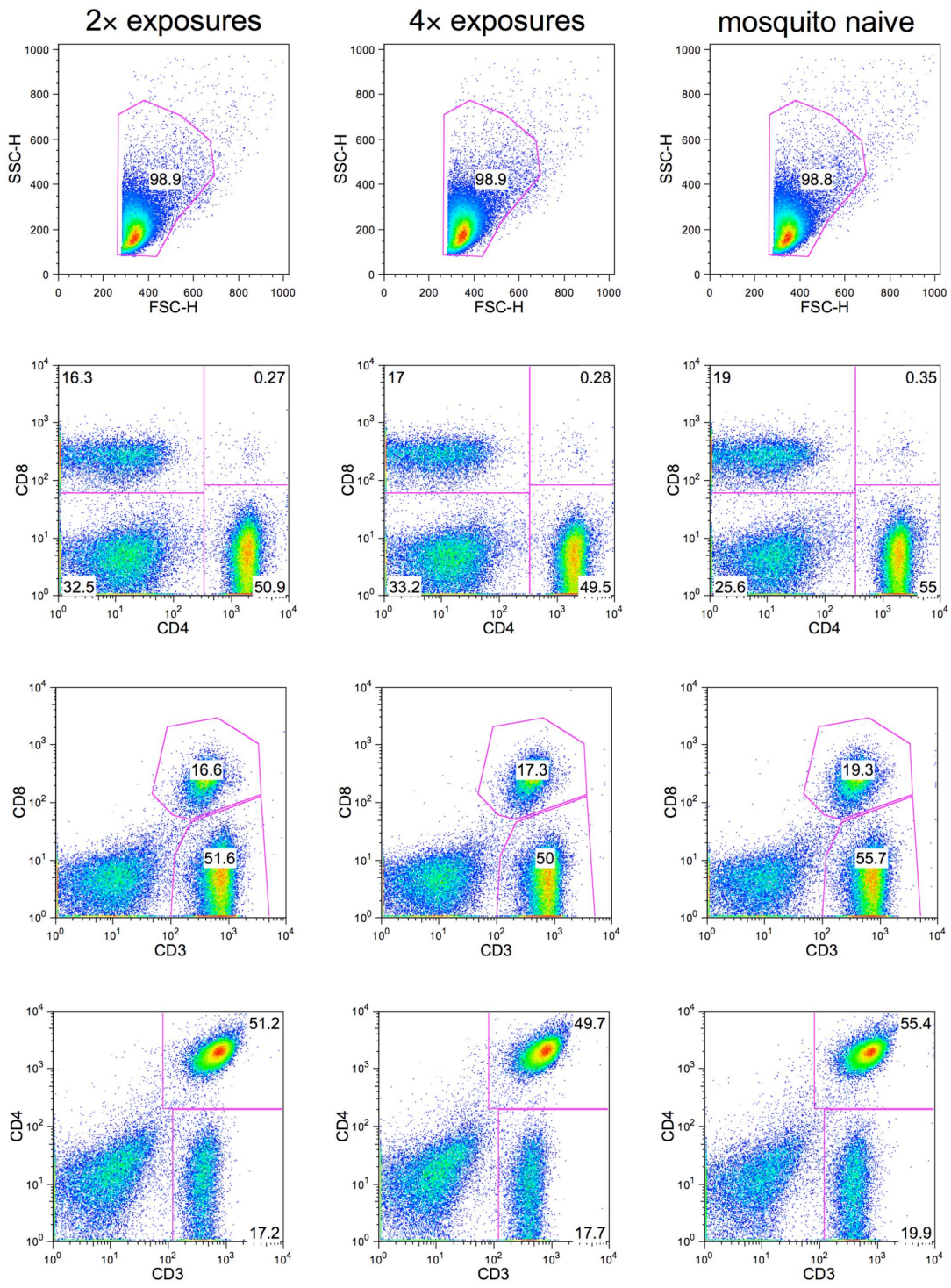


**Figure 6.15 Flow cytometry scatter plots for CD4/CD8/CD3-labeled LN cells isolated from mice (pools of 3 mice) exposed or not to *Cx. pipiens* feeding. Tissues were isolated 36 h post-infection with WNV.**

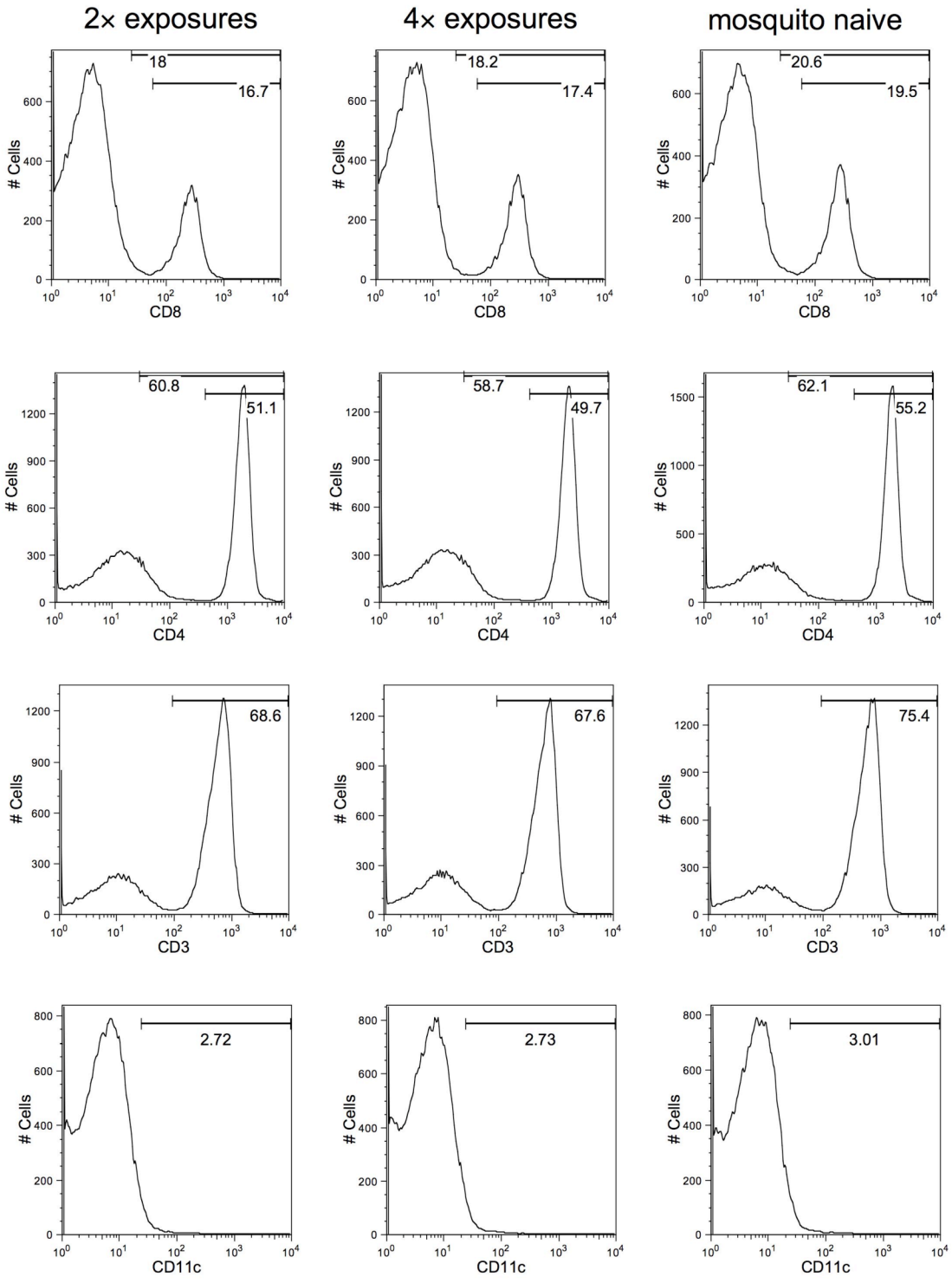


**Figure 6.16 Cells positive for CD4, CD8, or CD3 at 36 h post-WNV infection. Cells were isolated from mice (pools of 3 mice) exposed or not to *Cx pipiens* feeding. Representative of 2 runs.**

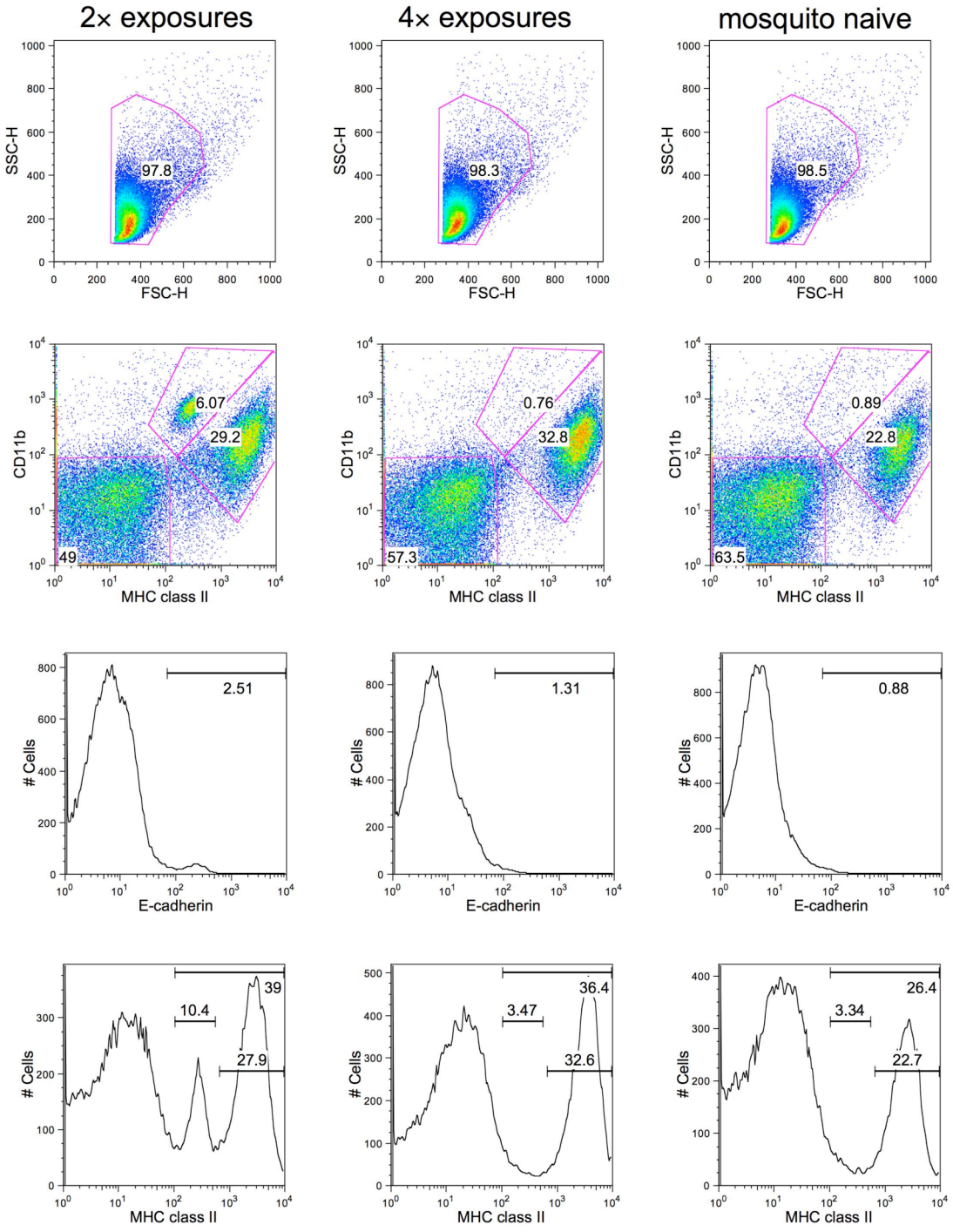


**Figure 6.17 Surface expression of CD11b, MHC class II and E-cadherin as assessed by flow cytometry 36 h post-infection with WNV. Prior to infection mice were exposed or not to *Ae. aegypti* feeding. Analysis was completed with cells pooled from 3 mice and are representative of 2 runs.**

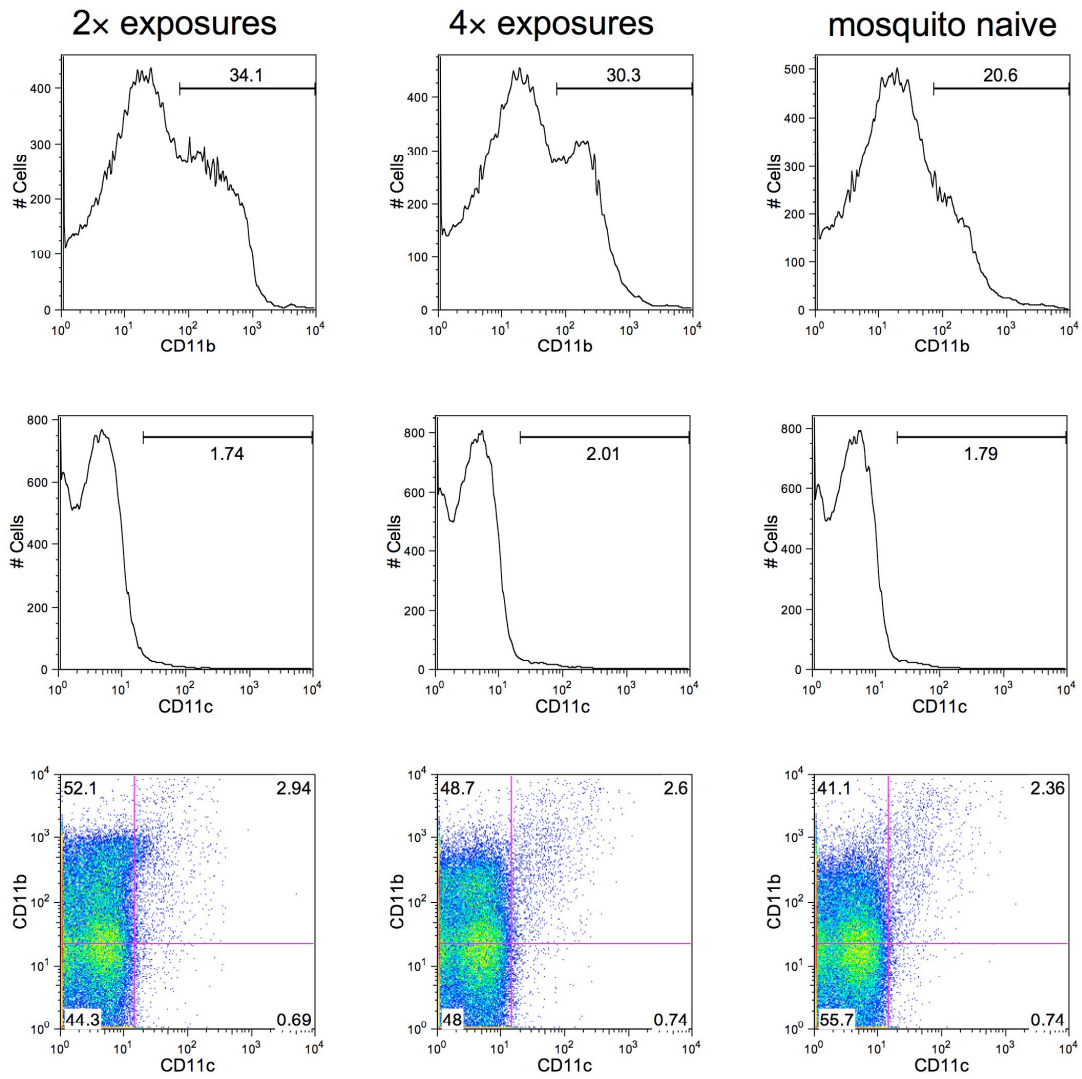


**Figure 6.18 Surface expression of CD11c, and CD11b as assessed by flow cytometry 36 h post-infection with WNV. Prior to infection mice were exposed or not to *Ae. aegypti* feeding. Analysis was completed with cells pooled from 3 mice and are representative of 2 runs.**

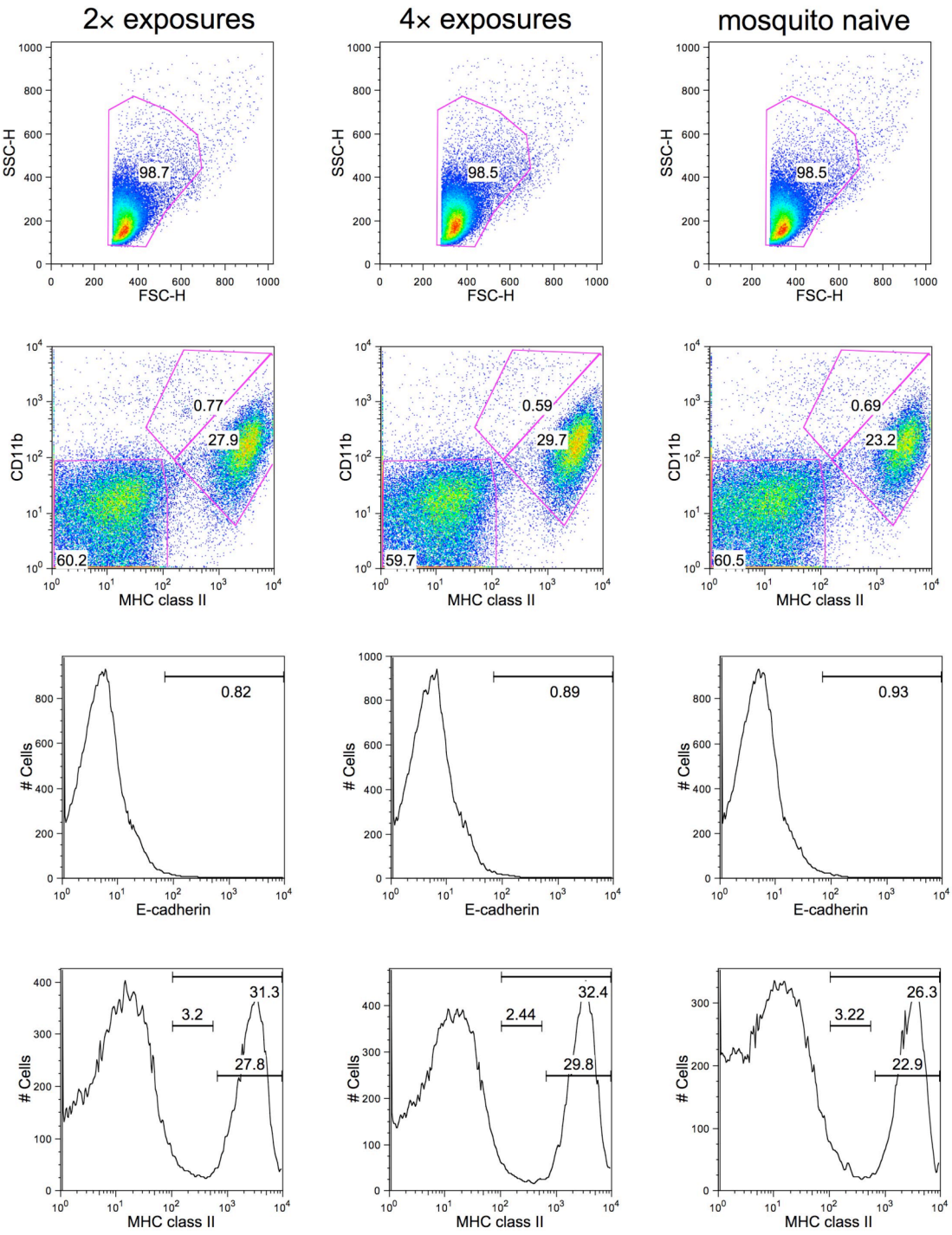


**Figure 6.19 Surface expression of CD11b, MHC class II and E-cadherin as assessed by flow cytometry 36 h post-infection with WNV. Prior to infection mice were exposed or not to *Cx. pipiens* feeding. Analysis was completed with cells pooled from 3 mice and are representative of 2 runs.**

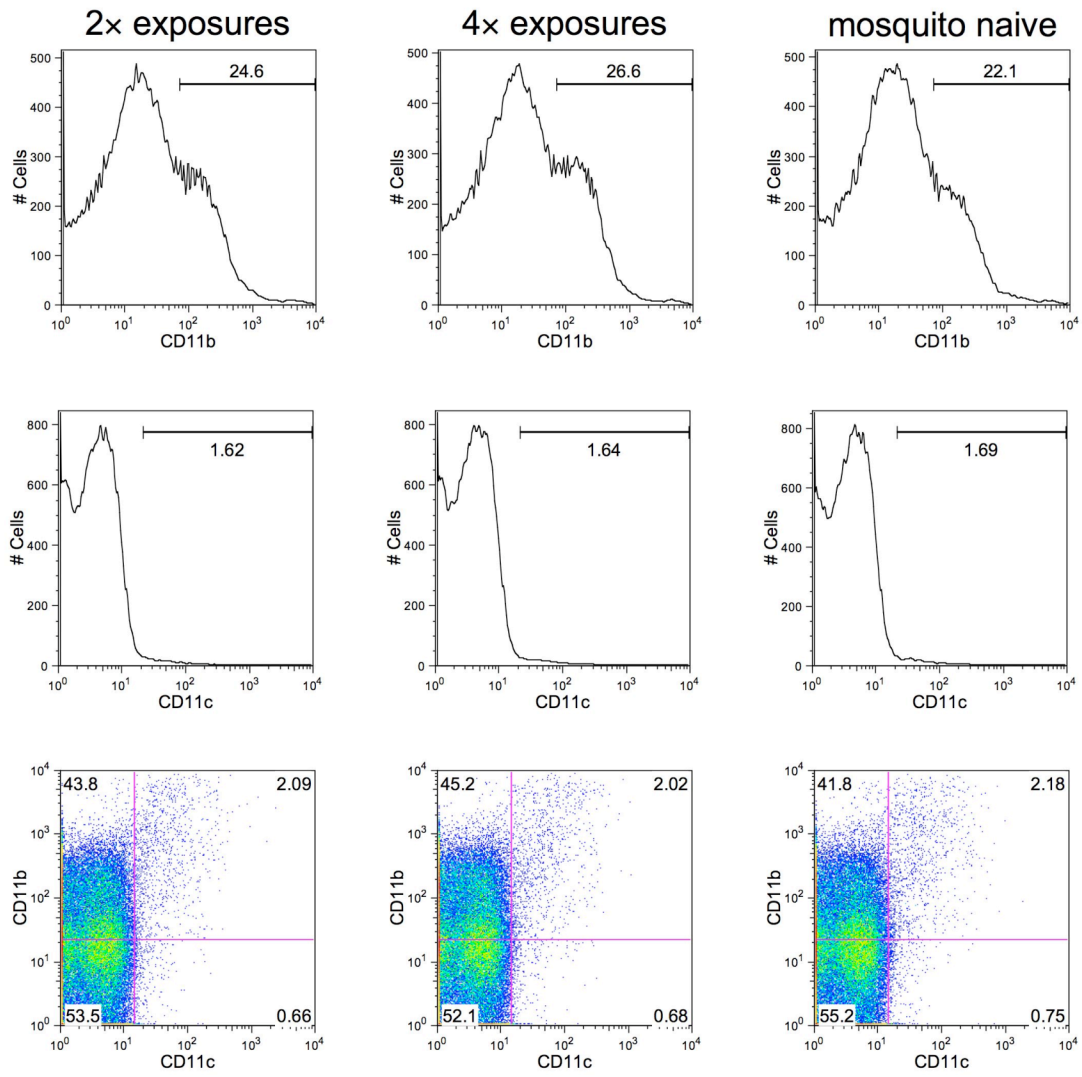


**Figure 6.20 Surface expression of CD11c, and CD11b as assessed by flow cytometry 36 h post-infection with WNV. Prior to infection mice were exposed or not to *Cx. pipiens* feeding. Analysis was completed with cells pooled from 3 mice and are representative of 2 runs.**

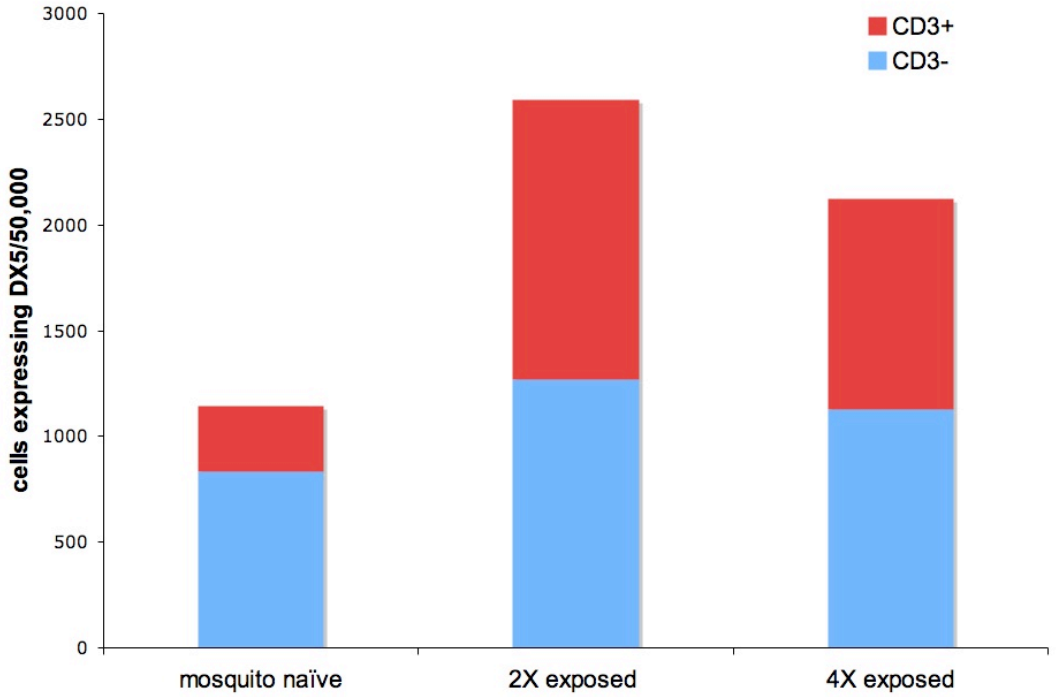


**Figure 6.21 CD3 expression in DX5$^{+}$ subpopulation in the lymph nodes of mice 36 h after being infected via the feeding of *Ae. aegypti***

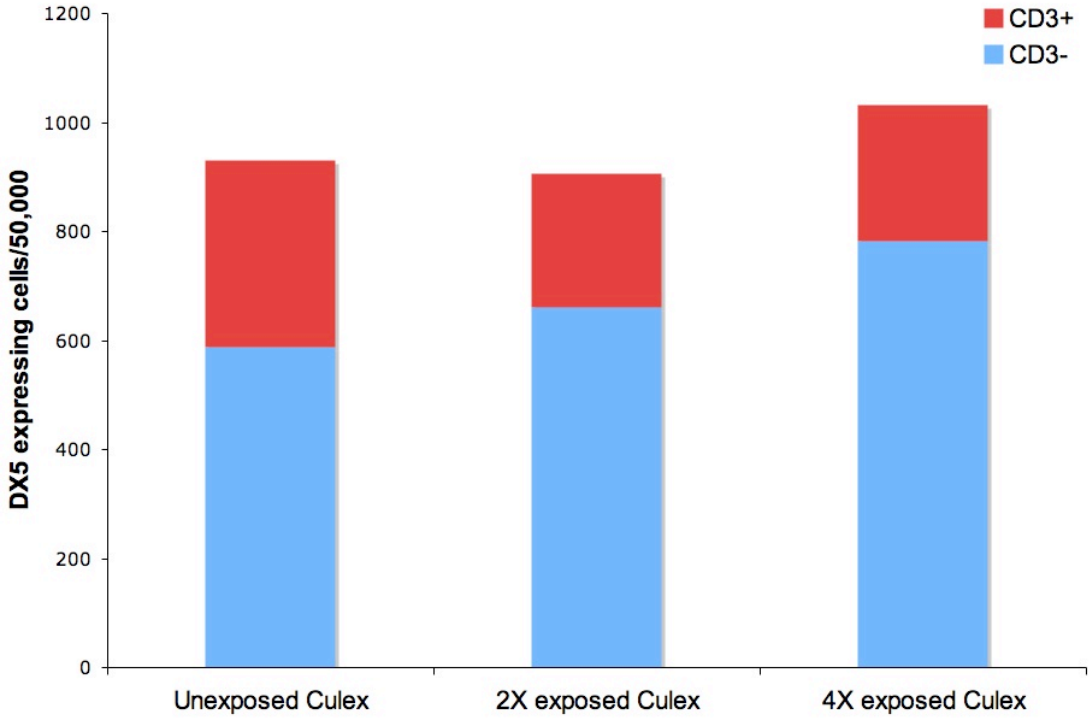


**Figure 6.22 CD3 expression in DX5$^{+}$ subpopulation in the lymph nodes of mice 36 h after being infected via the feeding of *Cx. pipiens***

## DISCUSSION

In an attempt to mimic the circumstances of natural transmission of an arbovirus, we established a murine model of WNV encephalitis, initiated by the feeding of an infected mosquito. The importance of including mosquito saliva in animal models of arbovirus transmission and disease has been clearly demonstrated by an increasing number of groups (Osorio *et al.* 1996, Edwards *et al.* 1998, Zeidner *et al.* 1999, Limesand *et al.* 2000, Valenzuela *et al.* 2002, Limesand *et al.* 2003, Schneider *et al.* 2004, Wanasen *et al.* 2004, Wasserman *et al.* 2004, Depinay *et al.* 2006, Schneider *et al.* 2006, Titus *et al.* 2006). The rationale for initiating the infection in the skin using mosquitoes to transmit the virus, as opposed to classical infection strategies such as i.p. inoculation, is that in addition to targeting the tissue environment in which the early host-arbovirus

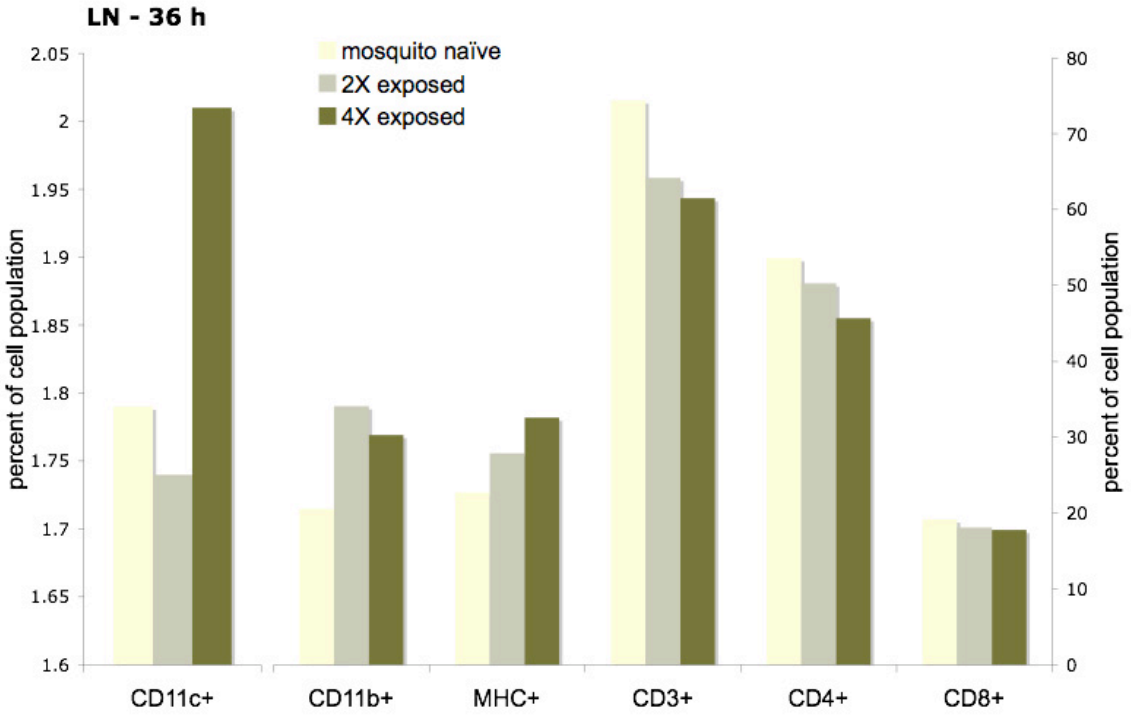


**Figure 6.23 Percent of LN cells expressing CD11c, CD11b, MHC class II, CD3, CD4, and CD8. Cells were isolated from 3 mice/group at 36 h post-infection. Results are representative of 2 runs.**

encounters normally occur, mosquito saliva has a demonstrated ability to affect cell types found in the skin or draining lymph nodes (Wanasen *et al.* 2004, Wasserman *et al.* 2004). Additionally, the skin has a very unique set of effector responses and cells absent from other tissue compartments. Naturally, WNV infections are inoculated by mosquitoes into the skin of hosts that are usually not naïve to mosquito exposure. West Nile virus minimum infection rates in mosquitoes are generally below 0.8% (Bernard *et al.* 2001, Kramer and Bernard 2001b). Even in the most conservative of logical estimations, the average host must be bitten by dozens if not hundreds of mosquitoes prior to being fed upon by a WNV-infected mosquito. Undoubtedly, exposure to mosquitoes or activities that increase a person's exposure to mosquitoes is the most fundamental risk factor for mosquito-borne diseases (Campbell *et al.* 2002). Given this information and the fact that mosquito saliva is mostly composed of proteins, thus potential antigens, it is straightforward to predict that repeat exposure to mosquito feeding will illicit an immune response and that this response may have some influence on subsequent viral infections delivered into the host in saliva.

Indeed, immune responses to mosquito feeding are common and this phenomenon has been observed for a variety of hosts and mosquitoes (Reunala *et al.* 1994, Mathews *et al.* 1996, Peng *et al.* 1996a, Palosuo *et al.* 1997, Chen *et al.* 1998, Wang *et al.* 1999, Peng *et al.* 2004, Shigekiyo *et al.* 2004, Waitayakul *et al.* 2006). The scale of a host response to mosquito saliva is dependent on the duration and intensity of exposure to mosquito feeding (Chen *et al.* 1998). Also, the level of circulating antibody to mosquito saliva is predictive of the strength of the hypersensitivity response directed at the feeding site (Chen *et al.* 1998). Previous research has shown that the immune response to mosquito saliva is associated with an enhancement of IL-4 production concurrent with a decline in IFN-γ production, suggesting that a $T_H2$ response predominates in the reaction to mosquito saliva in mosquito sensitized individuals (Chen *et al.* 1998). Such an immediate polarization of the immune response away from $T_H1$, the proper response to a viral infection, would be highly disadvantageous to a host.

The goal of the study described herein was to determine if prior exposure to the feeding of mosquitoes would have an impact on arbovirus infection. To this end, naïve or mosquito-exposed mice were subjected to the feeding of a single WNV-infected mosquito. Disease course was observed and a spectrum of assays employed to identify any deviations.

Preexposure to *Ae. aegypti*, but not *Cx. pipiens*, significantly modified the course of subsequent WNV infection. Nearly 100% mortality was observed in mice infected with WNV following four exposures to *Ae. aegypti*. While survival was higher in the group of mice exposed only twice to *Ae. aegypti* feeding, mortality was, nonetheless, still greater than in the mosquito naïve group. There was no significant difference as compared to naïve mice in level of mortality within the groups of mice previously exposed to *Cx. pipiens* (exposed either 2 or 4 times).

A number of mechanisms could be responsible for the observed differences between preexposed and nave mice. One possibility is that the acquired immunity to mosquito salivary proteins inhibits their activity, decreasing the success of bloodfeeding, requiring mosquitoes to probe the skin more, thereby inoculating more virus into the host. One can imagine that the inhibition of a number of salivary proteins could have this effect. For example, suppressing the function of a salivary vasodilator would have the effect of decreasing the ability of a mosquito to locate a vessel, since vasodilators make vessels larger and therefore easier to locate, and slow or abrogate its ability to imbibe blood once a vessel is located. Blocking the activity of an anticoagulant, would obviously have the undesired effect of allowing the host to develop a clot and hinder feeding. The mosquito salivary protein apyrase is involved in suppressing the host hemostatic system. Previous research has suggested that a decrease in salivary apyrase activity enhances probing time and pathogen inoculation (Rossignol *et al.* 1984). To determine whether anti-saliva antibodies affect apyrase catalytic activity, SGE was incubated with dilutions of mosquito-sensitized serum and then the phosphohydrolase ability of apyrase was assessed. No significant difference in catalytic activity was observed across a range of dilutions that in a different model was shown to cause a 60% –

90% reduction in activity (Mathews *et al.* 1996), suggesting that humoral response to *Ae. aegypti* and *Cx. pipiens* saliva does not neutralize the activity of apyrase. Apyrase is one of the most abundant proteins in mosquito saliva and the ubiquity of this activity among blood feeders implies that its role is keenly important. Apyrase function is not altered by the anti-mosquito immune response, but we cannot rule out that the activity of other salivary proteins is unaffected. The number of proteins in mosquito saliva for which uncomplicated activity assays exist is limited and thus an exhaustive search for such an effect is beyond the scope of this study.

In an effort to investigate the succeeding part of this theory – functional decline of saliva in preexposed mice increases probing and thus virion inoculation – an attempt was made to estimate the amount of virus that is inoculated into naïve or preexposed mice. Approximations of the amount of saliva deposited into a host have been made by quantifying saliva or salivary activity in fed and unfed mosquitoes (Ribeiro *et al.* 2001). A similar approach was utilized to roughly predict the quantity of virus inoculated. Immediately following mosquito feeding, mosquito salivary glands were dissected and the viral load was compared between mosquitoes that fed on naïve and preexposed mice. The average titer of these two groups was nearly indistinguishable, suggesting that the deviation seen between groups was not due to an alteration in the virus inoculum.

An alternative explanation for the altered disease course in mice previously exposed to mosquitoes is that the immune response elicited by salivary proteins leads to the recruitment of cell types that are susceptible to WNV infection. Such an influx of permissive cell types, together with the increase in cell movement as a result of the inflammatory response could enhance early WNV replication and dissemination. Additionally, it is possible that migration or levels of cells advantageous to the host during viral infection are decreased. To evaluate these possibilities, flow cytometry was employed to identify cell populations at the inoculation site and draining LNs. It was immediately clear that the level of cell influx was augmented both at the site of inoculation and the draining lymph node, suggesting that there was a cellular component to the anti-saliva response and, possibly, the altered pathogenesis in sensitized mice.

In both the inoculation site and the draining LN MHC class II expressing cells, a surface-protein distinctive to macrophages and DCs, made up a higher proportion of the population. CD11c and CD11b expressing cells, also found on the surface of APCs, were also at a higher level in the inoculation site. This consistent pattern of elevated APC recruitment in preexposed mice supports the prospect that the increased availability and movement of susceptible cell types early in infection aids the spread of WNV. Attempts to detect WNV positive cells in these tissues with indirect fluorescent labeling failed, likely due to the low level of infected cells and cellular antigen at this early time point. In the draining LNs an intriguing pattern of enhanced APC levels concomitant with decreased lymphocyte levels developed in preexposed mice. Moreover, this suppression of T cells appeared to influence CD4+ cells more acutely. The selective alteration of a particular subpopulation of T cells dispels the possibility that the decrease in the proportion of lymphocytes is just an artifact of the increased fraction of the LN being taken up by DCs. A recent study with WNV (Sitati *et al.* 2006), illustrated the essential role that CD4+ T cells play. Suppression or deficiency of this subpopulation during WNV infection in mice resulted in prolonged CNS infection and uniform lethality. Additionally, mice lacking CD4+ T cells had suppressed IgG production and later in infection WNV-specific CD8+ T cell activation and trafficking to the CNS were compromised (Sitati *et al.* 2006). Mice exposed to *Cx. pipiens* for sensitization and WNV-infection, showed similar trends as above-mentioned observations with *Ae. aegypti*-exposed mice, but overall the divergence between naïve and preexposed mice were less pronounced.

One observation that was unique to mice preexposed to *Ae. aegypti* was a enhancement of NK cells, particularly NK T cells. The role of NK cell activity during WNV infection appears to be limited, as NK cells demonstrated blunted cytolytic activity against virus-infected cells (Liu *et al.* 1989). Furthermore, the cytotoxic effector pathways of NK cells may contribute to accelerated and exacerbated pathogenesis of WNV, either in the events leading to neuroinvasion or by enhancing pathogenic inflammation in the CNS (Licon Luna *et al.* 2002).

It is possible that the enhanced mortality in *Ae. aegypti*-sensitized mice is due to a dysregulation of the initial immune response to WNV. Chen and associates (1998) demonstrated that the immune response to mosquito saliva causes an enhancement of IL-4 production and a decline in IFN-γ production, suggesting that a $T_H2$ response could outweigh a $T_H1$ response in mosquito sensitized mice even in the presence of a virus infection. Such an immediate polarization of the immune response away from $T_H1$, even a moderate shift, could be an advantage to the virus. Real-time RT-PCR on LN RNA suggests an upregulation of IL-4 in mosquito-exposed groups, but no suppression of IFN-γ (data not shown). This shift in a potent $T_H2$ response mediator or a similar dysregulation of immune cell signalling could help to explain the altered disease course in preexposed mice.

Exposure to both *Ae. aegypti* and *Cx. pipiens* induced anti-saliva antibodies, although *Ae. aegypti* feeding produced antibodies reactive against more salivary proteins than *Cx. pipiens* feeding. Qualitatively, the antibody response appeared more robust following *Ae. aegypti* feeding, although an ELISA was not performed and Western blots, especially with disparate protein targets, are generally poor quantitative guides. As demonstrated by the experiment where *Ae. aegypti*-immune serum was passively transferred to naïve mice, the humoral response to mosquito salivary proteins likely is associated with the phenotypic differences in WNV disease course. It is important to point out the fact that passive transfer of anti-saliva antibody mimicked the effect of preexposure to mosquitoes thereby precluding the possibility that enhanced WNV disease was due to the repeated stress of mosquito feeding.

Surprisingly, the results of this study with mosquito preexposure and virus infection are in direct contrast to a similar study with sand fly preexposure and parasite infection (Kamhawi *et al.* 2000). Except for the vector and pathogen used in these experiments, conditions were almost identical. Preexposure to sand flies causes a reversal to the parasite-enhancing effects of saliva, presumably by inhibiting the activity of the immune response-altering factor in sand fly saliva. Additionally, preexposure to sand fly saliva mediates an early strong inflammatory reaction in the skin. The deviation

in observations between these two models may not relate to differences in salivary protein function or immune response to these proteins, but rather in the dissimilarity between WNV and *Leishmania*. Viruses are relatively simple entities that minimally respond to their environment, whereas parasites are relatively complex organisms that constantly react to their surroundings. The transition between an arthropod vector and vertebrate host is almost certainly a vulnerable time for the parasite, as it has to adjust to a completely different environment. Given the parasite's obligatory transition and the fact that the typical inoculation site is minimally reactive to their introduction (Belkaid *et al.* 1998), the dramatic increase in inflammation in sensitized mice would present a hugely unfavorable environment to a parasite. Furthermore, macrophages, a cell type generally permissive to *Leishmania* infection, recruited to the site and activated by the potent inflammatory signally would probably prove more resistant to the parasites. Conversely, the increased cell influx following mosquito sensitization may be less of a hindrance to inoculated virus, which readily infects numerous types of inflammatory cells and whose ability to do so could be enhanced by the activation of these cells (Cardosa *et al.* 1986).

The relevance of the observation that prior exposure to mosquito feeding exacerbated WNV disease is heightened by the almost ubiquitous history of exposure to mosquitoes among land-dwelling vertebrates. The effects of cross reactivity between the salivary proteins of different species cannot be predicted, but would have to be taken into account to achieve a thorough understanding of the natural results of mosquito-sensitization in the context of WNV infection. The observation in one population that antibody to mosquito saliva decreased through childhood and adolescence suggests that there may be a natural cycle of desensitization to mosquito saliva (Peng *et al.* 2004). If this occurs then the negative effects may diminish as one is increasingly exposed to mosquito bites. The level of exposure necessary for desensitization and the effect of exposure to divergent salivary proteins from various mosquito species remains to be determined. The intensity of mosquito saliva-reactive antibodies has been shown to wax and wane in parallel with seasonal highs and lows of mosquito density (Palosuo *et al.*

1997). The enhanced disease course following mosquito sensitization could add to the factors that boost risk of symptomatic arboviral disease for children in and recent migrants to endemic areas. Defining this phenomenon with arboviruses, such as DENV, whose progression from mild to severe is more precariously contingent on subtle shift in the immune response may prove fruitful.

# CHAPTER 7: CONCLUSIONS

The aim of this dissertation was to determine the role of mosquito saliva in enhancement of arbovirus, specifically WNV, disease and to develop an understanding of the immunomodulatory activity of mosquito saliva by elucidating the underlying mechanism of mosquito induced immunomodulation both *in vivo* and *in vitro*. West Nile virus in particular has become a serious concern in the United States where it has recently caused over 22,000 diagnosed cases and probably over one million undiagnosed infections (Busch *et al.* 2006). Only in the last few decades has the role of the arthropod been considered in disease pathogenesis. In this regard, the immunomodulatory effects on vertebrate hosts have been relatively well characterized in sand flies and in ticks, far less research has focused on mosquitoes. Even fewer studies have focused on the effect of mosquito saliva on the *in vivo* immune response or mosquito saliva's role in arbovirus pathogenesis. Knowledge is lacking in the area of saliva-skin cell interactions as they relate to arbovirus pathogenesis. In the past most small animal models of arbovirus disease have used needle-inoculation (Chambers and Diamond 2003), but recent evidence suggests that because of the potential effects of mosquito saliva on the immune system, it is important to re-evaluate the pathogenesis of these infections in the presence of mosquito saliva.

Delineation of the role of mosquito saliva in the pathogenesis of arbovirus infections is crucial to understanding the natural transmission and infection dynamics of diseases like WNV. The information gained from this dissertation project aids in understanding the effect that mosquito saliva has on WNV disease and constitutes a strong addition to our knowledge of the immunological mechanisms underlying observations of arbovirus disease exacerbation. Defining the vector-induced changes in host immune response to infection may help us to understand why mosquitoes are such potent vectors of pathogens, and in doing so, perhaps gain insight into methods of counteracting the mechanism and lessening human infection.

A central question of this dissertation project was to determine if mosquito feeding or mosquito saliva could impact WNV disease. A number of studies solidly

suggest that we could anticipate an effect that would promote the virus at the cost of host health. For example, experiments using infected mosquitoes to transmit LACV demonstrated that mosquito inoculation of LACV leads to increased viremia titer and duration, while chipmunks infected via mosquito as compared to needle-inoculation develop viremias that were 3 log higher in (Osorio *et al.* 1996). Similarly, injection of CVV into sites of mosquito feeding results in production of viremia and anti-CVV antibody in previously insusceptible hosts (Edwards *et al.* 1998). The most tantalizing evidence was unpublished data that asymptomatic LACV infection could become a fatal infection when mosquitoes, instead of a needle, inoculated the virus (Steve Higgs personal communication).

To evaluate the potential for mosquito saliva to alter WNV infection, the mouse model of disease employed in this dissertation (Chapter 2) revealed a potentiation of WNV disease when mosquitoes were allowed to feed at the virus inoculation site immediately before injection of WNV. Further, with higher titer inoculations of WNV subsequent to the feeding of mosquitoes more progressive infection, higher viremia, and accelerated neuroinvasion developed than the mice inoculated with an equivalent titer of WNV alone. At a lower dose of WNV, mice fed upon by mosquitoes had a higher mortality rate. These results supported the hypothesis that mosquito feeding and factors in mosquito saliva can potentiate WNV infection, and additionally revealed a possible mechanism for this effect via accelerated infection of the brain.

The observation of accelerated WNV neuroinvasion in groups fed upon by mosquitoes is likely associated with the increased progression to death, because mortality in WNV encephalitis is strongly correlated with the timing and amplitude of CNS infection (Shrestha and Diamond 2004). A possible direct mechanism for mosquito feeding to augment WNV seeding into the CNS by increasing the permeability of the BBB was explored. Since TNF receptor signalling proved to be instrumental for WNV to cross the BBB (Wang *et al.* 2004), it was hypothesized that enhanced TNF-$\alpha$ levels produced by mosquito feeding (Demeure *et al.* 2005) would increase the permissiveness

of the BBB. Although the results were suggestive of a direct effect of mosquito feeding on BBB permeability, the data were inconclusive.

Undoubtedly, the higher viremia observed early in infection in the group of mice exposed to mosquito feeding could contribute towards accelerated CNS pathogenesis, as it has previously been determined that amplified viremia enhances the potential for the brain to become infected (Ben-Nathan *et al.* 1996). Variation in local expression of cytokines following arbovirus inoculation might explain this deviation in disease course mediated by mosquito saliva. While there exists some disagreement in the specifics, a growing number of groups have observed dysregulation of cytokine production, usually in the form of suppression, in response to mosquito saliva (Bissonnett *et al.* 1993, Depinay *et al.* 2006, Limesand *et al.* 2003, Wanasen *et al.* 2004, Wasserman *et al.* 2004, Zeidner *et al.* 1999). To determine if these immunomodulatory effects of mosquito saliva are relevant in the context of active virus replication, the *in vivo* expression of key $T_H1$, $T_H2$, inflammatory, and antiviral cytokines was quantified during peripheral arbovirus infection (Chapter 3) in the presence or absence of mosquito saliva. Data clearly showed that during early SINV infection mosquito salivary proteins down-modulates specific antiviral cytokines while enhancing production of $T_H2$ (IL-4) and immunosuppressive (IL-10) cytokines. Of particular significance was the observation that *Ae. aegypti* saliva suppressed mRNA levels of IFN-β, a critical cytokine that mediates the initial host defense against virus infections. This data both supported and extended the results of Limesand *et al.* (2003), who showed a similar reduction in type I IFN mediated by mosquito saliva. Mice lacking IFN-β or its products have increased WNV loads in their draining lymph nodes, sera, and spleens, which is associated with early viral entry into the CNS and higher viral burden in neuronal tissues (Samuel et al 2006), suggesting that this one effect of mosquito saliva could largely explain the observations of WNV disease exacerbation. The mosquito saliva-mediated increase in IL-10 expression is important, because increased IL-10 production early during virus infection has been demonstrated to induce lasting T cell inactivation and diminished control of virus infection (Brooks *et al.*

2006). Based on our data, the effect of mosquito saliva on cytokines may therefore facilitate WNV infection and intensify arbovirus disease.

To understand the source of these shifts in immune signalling, the *in vitro* response of DCs and macrophages was investigated (Chapter 4). Following exposure to *Ae. aegypti* SGE, DCs and macrophages recently exposed to either WNV or SINV displayed reductions in IFN-β expression and transient amplification of IL-10 mRNA levels. Macrophages appeared to be more susceptible to the modulating effects of mosquito saliva than DCs, although this observation could be an artifact of the divergent methods in which these cells were isolated. Resident peritoneal macrophages were isolated as primary cultures while DCs were generated from bone marrow progenitors, a technique known to produce cells that are less reactive and mature than those naturally-occurring in the vertebrate skin. Additional information that resulted from this *in vitro* study was the observation that iNOS expression was also affected by mosquito saliva. The expression of iNOS in infected macrophages was reduced in a dose-dependent manner by increasing concentrations of *Ae. aegypti* SGE. A less pronounced reduction was seen in DCs. The saliva of other arthropods, including sand flies (Mbow *et al.* 1998) and ticks (Gwakisa *et al.* 2001), have also been shown to have NO-reducing activity. Nitric oxide has demonstrated antiviral action. Since some of the protective abilities of macrophages appear to be mediated by NO production, suppression of NO may have adverse effects for a host during arbovirus infection by allowing increased replication of virus. This antiviral effect may be mediated either through the ability of NO to strongly inhibit viral RNA synthesis, protein accumulation, and virus release from infected cells (Lin *et al.* 1997) or through its recognized immune signalling action that affects such processes as differentiation, proliferation, and apoptosis of immune cells; the production of cytokines; and the expression of co-stimulatory and adhesion molecules (Bogdan 2001).

An alternative explanation for the mosquito-mediated enhancement of WNV disease relates to the effect that mosquito saliva may have on cell influx into the virus inoculation site. Studies have suggested that independently mosquito feeding and virus

inoculation can affect dermal cell migration (Byrne *et al.* 2001; Demeure *et al.* 2005). However, these studies have not investigated the effect of mosquito saliva on cell migration at the initiation of an arbovirus infection. To address this gap in our knowledge, the influence of mosquito saliva on immune cell migration patterns both into the dermal site of WNV inoculation and the draining lymph node was evaluated (Chapter 5). The principal observation from this study was that the inclusion of mosquito saliva/feeding at the inoculation site of WNV leads to a suppression of lymphocytes, particularly $CD4^+$ T cells, and a corresponding increase in DCs. T-cells were reduced both in the fraction of the cell population comprised of T cells and in the actual number of cells per ear. Further, exposure of mice to mosquito feeding alone decreased the number of lymphocytes in the skin as compared to unexposed control mice. These results constitute *in vivo* evidence in support of previous *in vitro* studies demonstrating that *Ae. aegypti* saliva significantly suppresses T cell activities at lower concentrations and enhances T cell death at higher concentrations (Wanasen *et al.* 2004; Wasserman *et al.* 2004). In addition, our data demonstrate that the anti-lymphocyte activity observed for mosquito saliva may translate into actual alterations during WNV infection. Wasserman and colleagues (2004) found that very dilute levels of SGE caused an inhibition of T-lymphocyte proliferation and a significant decrease chiefly in CD4+ T cell viability. This decline in CD4+ T cells is significant because a deficiency of this subpopulation during WNV infection in mice resulted in prolonged CNS infection and uniform lethality (Sitati and Diamond 2006). The reduction in CD4+ lymphocytes caused by mosquito saliva could therefore help to explain the more severe progression of WNV disease observed with co-inoculation of virus and *Ae. aegypti* saliva.

Finally, although mosquito exposure and sensitization to it is widespread, the effect of prior exposure to mosquitoes on subsequent arbovirus infection had up until this point been unexplored. In many natural settings frequent exposure to mosquitoes is the norm instead of the exception. Since sensitization of a vertebrate to mosquito saliva causes recognized alterations in immune response to subsequent mosquito feeding, and also because similar anti-arthropod reactions have led to modifications of pathogen

transmission, exploring this phenomenon in the context of mosquito-borne virus was important to improve our understanding of arbovirus pathogenesis. Accordingly, the potential for an immune response directed against mosquito salivary proteins to have a protective or confounding effect on naturally transmitted WNV infection was investigated by comparing early WNV pathogenesis and WNV disease progression in mosquito naïve and sensitized populations of mice (Chapter 6). Pre-exposure to mosquito feeding profoundly affected subsequent WNV disease. Previous exposure to *Ae. aegypti* feeding results in significantly higher mortality rates associated with elevation of inflammation, APC recruitment, IL-4 expression in the draining LNs, and LN hyperplasia concurrent with a decrease in lymphocytes mainly the $CD4^+$ subtype. The enhanced disease severity is thus hypothesized to be caused by a combination of an increase availability of susceptible cell types, dysregulation of immune signalling, suppression of immune recognition of virus due to decreased CD4+ T cells, and enhanced spread of WNV due to destabilization of tissue integrity mediated by inflammation. Mosquito sensitization-mediated amplification of WNV disease is facilitated by the humoral response to mosquito salivary proteins. In support of this conclusion passive transfer of mosquito-immune serum to naïve mice mimicked the effect seen in pre-exposed mice. The immune response to mosquito saliva could therefore play a role in the unpredictable progression of WNV infections in WNV encephalitis. Further research into the role of immune response to mosquito antigens in arbovirus disease pathogenesis will aid in advancing our understanding of mosquito-transmitted disease.

This project was primarily designed to investigate the mechanisms of mosquito saliva-induced modulation of the immune response and its effects on virus infection. The results from these studies will allow a better understanding of cytokine expression, immune cell migration, and, broadly, the early events of mosquito-borne viral infection.

Knowledge of the immunology of the natural transmission through appropriate models will allow investigators to design more effective vaccines for arboviruses. The observations of this project demonstrate that the effect of the vector should be included in studies assessing the efficacy of vaccines. Furthermore, this study bridges the gap

between descriptive research on mosquito saliva and mechanistic research on arbovirus pathogenesis, thereby verifying the arbovirus-potentiating effects of mosquito saliva. This dissertation confirms that by ignoring the possible effects of the vector we may misinterpret the early immune response to arboviruses and also possibly aspects of the overall pathogenesis of arboviral infections. The information from the present study provides insight into early host responses to arbovirus infection, and suggests further determinants of WNV virulence.

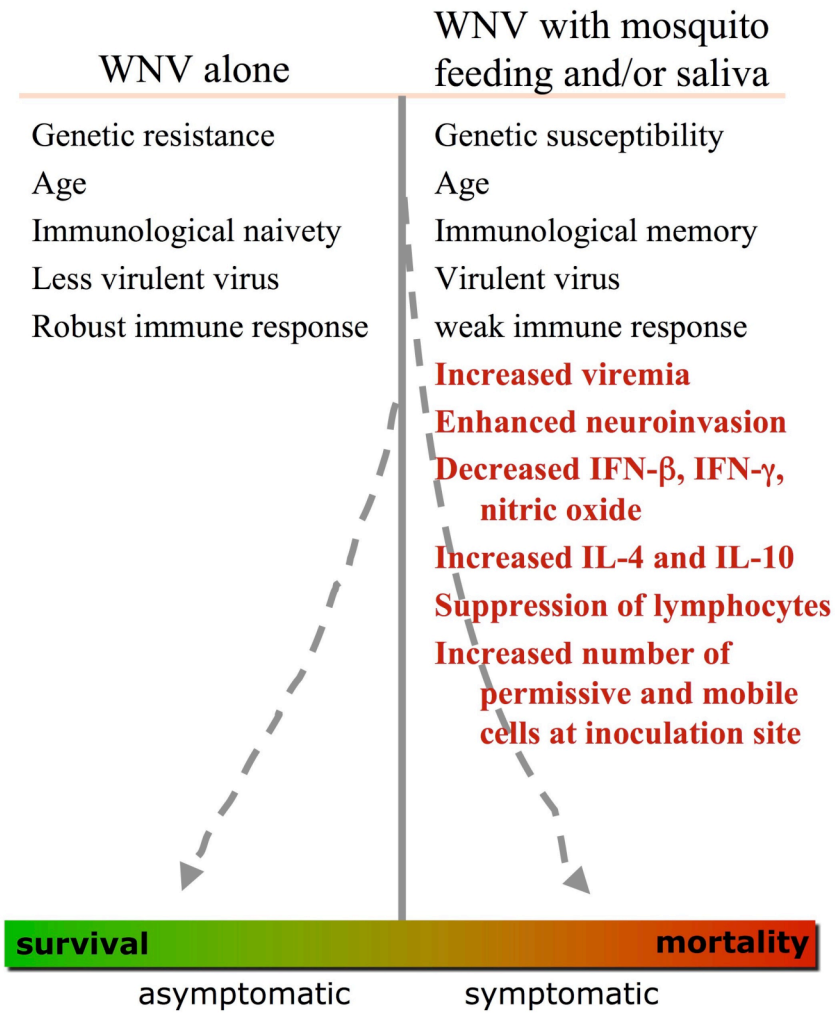


**Figure 7.1 Factors influencing the pathogenesis of WNV infection. Mosquito saliva-induced modulations of pathogenesis and the immune response of the host favor the arbovirus at the detriment of the host. The affects of mosquito feeding and mosquito saliva are indicated in red.**

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**Zwiebel, L. J., and W. Takken. 2004.** Olfactory regulation of mosquito-host interactions. Insect Biochem Mol Biol 34: 645-52.

# VITA

Bradley Scott Schneider was born on September 10, 1973 in Fullerton, California. He grew up in Newport Beach, where he attended Corona del Mar High School. Having discovered a strong interest in biological sciences, he obtained a joint degree in molecular, cellular, developmental biology and biopsychology from the University of Colorado at Boulder. Subsequent to completion of his bachelors degree, he was granted an Emerging Infectious Disease fellowship in the Division of Vector-Borne and Zoonotic Diseases at the Centers for Disease and Prevention in Fort Collins, Colorado. During his three years there, he worked on tick-borne diseases. In August 2002, he entered the University of Texas Medical Branch in Galveston, Texas to study arboviruses. At Galveston, he received the Centers for Disease Control Vector-Borne Disease Training grant to study the role of mosquitoes in the pathogenesis of West Nile virus.

**GRANTS AND HONORS**

Granted a three-year post-doctoral fellowship from the Pasteur Foundation, Department of Parasitology, Early Responses to Parasites and Immunopathology Unit, Institut Pasteur, Paris, France.

Accepted from an international pool of applicants to participate in the prestigious Biology of Disease Vectors Course, Bangkok, Thailand with tuition/accommodations supported by UNICEF-UNDP-World Bank-WHO Special Programme for Research and Training in Tropical Diseases and John D. and Catherine T. MacArthur Foundation, June 2005.

Awarded “Who’s Who Among Students in American Universities”, January 2006.

Centers for Disease Control and Prevention’s Vector-Borne Infectious Disease Fellowship Training Grant, 2003-2006.

Awarded the Zelda Zinn Casper Merit Based Grant, University of Texas Medical Branch, 2005-2006.

Cumulative grade point average of 4.0 for graduate education (2002-2006)

Manuscript awarded Charles C. Shepard Science Award for excellence in science achievement by authors of outstanding scientific papers, Centers for Disease Control, Atlanta, Georgia. June 22, 2005.

Awarded the James H. Nakano Citation for outstanding scientific publication, National Centers for Infectious Disease, 2005.

Elected into The Honor Society of Phi Kappa Phi, 2004-2006.

Honor Award: Employee of the Quarter. Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Disease. 2001.

Manuscript nominated for Health and Human Services' Shepard Award, 2001 (An Analysis of Spirochete Load, Strain, and Pathology in a Model of Lyme Borreliosis).

Partners in Public Health Award. Centers for Disease Control and Prevention / Agency for Toxic Substances and Disease Registry Honor Awards, 2000.

**POSITIONS HELD**

Chair, Experimental Pathology Student Government Association, 2004-2005

Committee Member, Institutional Animal Care and Use Committee, 2004-2006

Committee Member, Curriculum and Academic Planning Committee, 2005-2006

Participant, Basic Biomedical Science Curriculum new student recruitment 2004-2006

Team Member, Bioterrorism Response Deployable Laboratory Unit, Centers for Disease Control and Prevention, National Centers for Infectious Disease. 2002

**PEER-REVIEWED RESEARCH PUBLICATIONS**

Wicker, JA, DWC Beasley, MC Whiteman, CT Davis, S Zhang, **BS Schneider**, S Higgs, RM Kinney, and ADT Barrett. 2006. A single amino acid substitution in the central portion of the WNV NS4B protein confers a highly attenuated phenotype in mice. Virology. 349(2):245-53.

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Dolan, MC, **BS Schneider**, NS Zeidner, KC Stafford III, GO Maupin. 2004. Control of Immature *Ixodes scapularis* (ACARI:IXODIDAE) on Rodent Reservoirs of *Borrelia burgdorferi* in a Residential Community of Southeastern Connecticut. Journal of Medical Entomology. 41(6): 1043-1054.

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**SUBMITTED**

Fair J, **BS Schneider**, CT Davis, RB Tesh, S Higgs, CJ Peters. A Real-Time Reverse-Transcriptase PCR to Simultaneously Detect and Distinguish West Nile Virus and St. Louis Encephalitis Virus in a Single Tube.

Zeidner, NS, JS Rutherford, GB Schoeler, **BS Schneider**, MC Dolan, R Kimsey, SK Wikel. Neutralization of IL-4 and IL-5 Counteracts Tick Modulation of Host

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