THESIS

MODELING DENGUE VIRAL INFECTION, INSECT TRANSMISSION AND MULTIPLEX PCR IN HUMANIZED MICE

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ABSTRACT

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Due to the restricted host specificity of dengue virus, up until now no ideal animal model has sufficiently mimicked key aspects of viral pathogenesis and immune response. In this regard, new advances with humanized mice(hu-mice), constructed by engrafting human tissues and cells and harboring a functioning human immune system, have been of great value in advancing research with human viruses Here we sought to utilize hu-mice to understand natural dengue pathogenesis and immune response. RAG1$^{-/-}$ $\gamma_c^{-/-}$ or RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice are immune-compromised and do not reject foreign grafts. This allows engraftment of human hematopoietic stem cells via intrahepatic injection into newborn mice, forming the RAG-hu mouse model. Bone marrow, liver, thymus (BLT) mice are an improved version of these mice as they harbor a more robust human immune cell repertoire. BLT mice were prepared by transplanting seven week old RAG1$^{-/-}$ $\gamma_c^{-/-}$ or RAG2$^{-/-}$ $\gamma_c^{-/-}$ strain mice with fragments of human liver and thymus under the kidney capsule, followed by an intravenous injection of autologous hematopoietic stem cells. After construction, both models were injected subcutaneously and intraperitoneally with dengue virus and monitored for the presence of infection. Our results showed that RAG-hu and BLT mice were fully susceptible to dengue virus infection as evidenced by viremia, and generation of dengue specific human IgM and IgG antibodies. Additionally, the presence of IgM and IgG that are capable of virus neutralization illustrated the functionality of the human immune system reconstituted in these mice.

To expand on the utility of this hu-mouse model, we also evaluated if dengue virus could be transmitted via mosquitoes to humanized mice, modeling the natural route of dengue transmission. Dengue infected mosquitoes were allowed to feed on BLT and RAG-hu mice and mice were monitored for the presence of dengue viremia. We achieved successful insect mediated transmission of dengue virus as evidenced by viremia and dengue specific antibody production in exposed mice. This achievement permits many novel experiments on vector competence and vector based viral intervention. Lastly, a multiplex quantitative real time PCR (qRT-PCR) assay capable of detecting and differentiating all four dengue viral serotypes was also established. This assay was able to detect each of the viral serotypes in a single reaction allowing for detection of virus in mixed infection studies. Taken together, these studies characterized a human immune competent hu-mouse model capable of supporting dengue viral infection with all four serotypes, as well as illustrating dengue-meditated disease and virus-specific immune responses.

ACKNOWLEDGEMENTS

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being able to fully conduct experiments methodically and my ability to learn and conduct new techniques and protocols.

Through this work I have realized that my passion and drive are in being part of a research team, and my future is to be in a research lab at a university, biotech company, or government agency.

I would like to thank my masters committee of Ramesh Akkina, Gerald Callahan, and Paul Laybourn for their time, support, and critical review of my work.

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CHAPTER 1

LITERATURE REVIEW

1.1 Discovery and Impact of Dengue Viruses

Dengue virus (DENV) is a member of enveloped, positive-strand RNA viruses of the flaviviridae family. The flaviviridae family also includes West Nile virus, yellow fever virus, Japanese encephalitis virus, hepatitis C virus, and tick-borne encephalitis virus. Flaviviruses are transmitted to humans by arthropod vectors such as mosquitoes or ticks (Gubler 1998; Thomas, Strickman et al. 2003). DENV is found in tropical and subtropical areas (Figure 1.1) throughout the world, with prevalence in both urban and suburban areas (Wang, Ni et al. 2000). DENV is endemic in more than one-hundred countries with more than two-and-a-half billion people, or

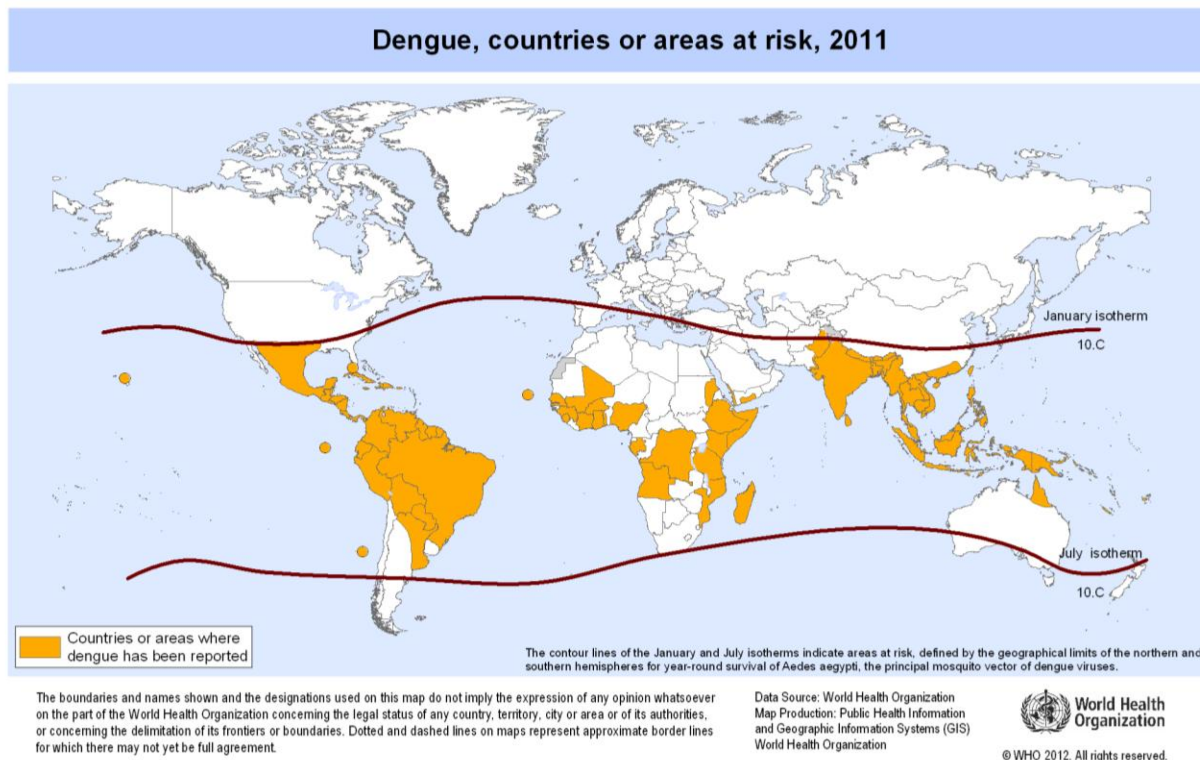

Figure 1.1 Geographical Distribution of Dengue. Areas that dengue have been reported shown in orange and contour lines indicate geographic limits of *A.aegypti*. Over 2.5 billion people are living in areas at risk for infection (WHO 2012).

forty percent of the world's population living in areas at risk for infection. The World Health Organization (WHO) estimates that there are between fifty and one-hundred million DENV infections each year, causing hospitalization of five-hundred thousand people, and a death rate of two-and-a-half percent (WHO 2012). The earliest report of disease with dengue-like symptoms dates back to a Chinese encyclopedia of disease symptoms and remedies that was published from 265 to 420 A.D during the Chin Dynasty (Nobuchi 1979). It is speculated that DENV was the etiological agent during disease outbreaks in the French West Indies in 1635, in Panama in 1699, and the Philadelphia epidemic of 1780 (Carey 1971; Howe 1977; McSherry 1982). Reported cases of dengue disease were seen in 1779 and 1780 in Africa, Asia, and North America (Rush 1789; Hirsch 1883). The first verified dengue epidemic occurred from 1953 to 1954 in the Philippines. This outbreak was believed at the time to be among other hemorrhagic fevers, but confirmed to be dengue in 1958 through serological testing. DENV activity spread and increased in the 1970s in Asia, the Pacific Islands, and the Americas (Causey and Theiler 1958; Aaskov, Buzacott et al. 2006). In the 1980s and 1990s, DENV continued to expand, and reached areas with mosquito vectors (Gubler 1998). Factors believed to cause increase in dengue epidemics are population growth and urbanization, deterioration in water quality, suboptimal waste management, the lack of effective mosquito control, and human air and ship travel (Gubler 1998).

1.2 Dengue Viral Structure

There are four phylogenetically and genetically distinct, but antigenically related serotypes classified as DENV-1, DENV-2, DENV-3 and DENV-4 (Mackenzie, Gubler et al. 2004). The dengue virion is a spherical particle existing as either a 50nm diameter immature

particle or a mature 60nm diameter particle with a lipopolysaccharide envelope. The 11kb dengue genome has a single open reading frame that encodes three structural proteins: capsid (C), membrane (M), and envelope (E) (Figure 1.2). In addition to these three structural proteins are seven viral encoded non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Halstead 2008). The viral particle consists of the RNA genome surrounded with C proteins and forms the inner core (Figure 1.3). The structural proteins E and M are surface proteins on the

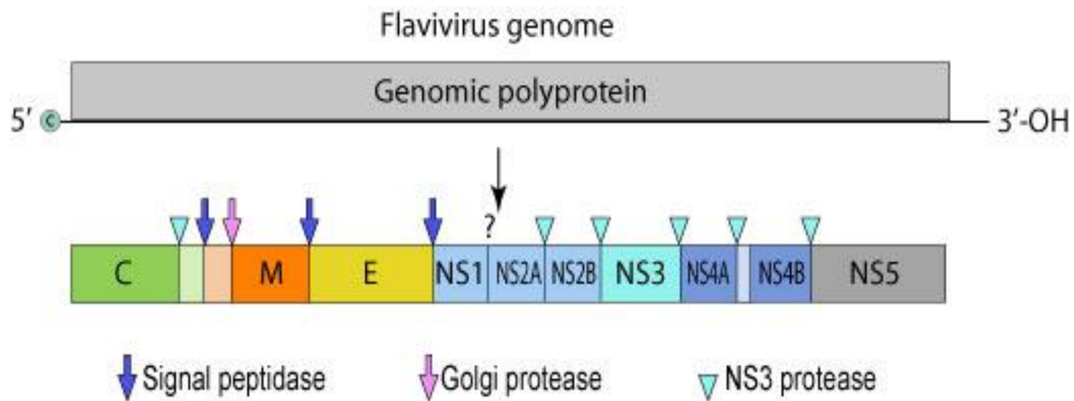

Figure 1.2 Schematic Representation of the Dengue Genome. The dengue genome has a single open reading frame encoding three structural proteins(C, M and E) and seven non-structural proteins(NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Zone 2012).

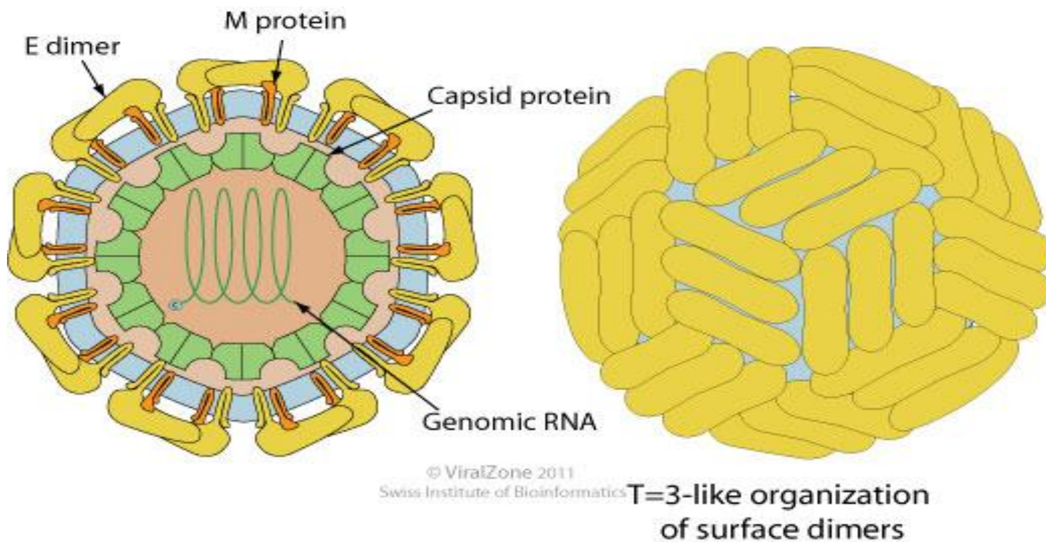

Figure 1.3 Dengue Envelope and Virion Structure. The dengue spherical particle consists of the genomic RNA, surrounded by the capsid, then the envelope with E and M proteins bound (Zone 2012).

virion envelope and the conformations of these proteins are used to distinguish between immature and mature virus. The immature virus is referred to as “spiky” as M proteins bound to a precursor membrane protein (pr) form heterodimers with E proteins that appear as ‘spikes’ on the viral surfaces (Figure 1.4). In mature virions the soluble pr is cleaved from M protein by furin, anchoring the M proteins and causing the pr protein to be absent in the mature viral membrane (Pokidysheva, Zhang et al. 2006; Yu, Zhang et al. 2008).

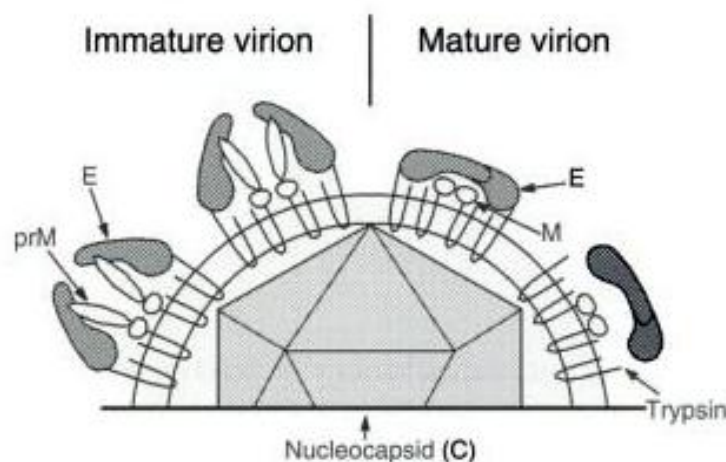

Figure 1.4 Envelope Spikes on Immature and Mature Virions. Representation of the “spiky” immature virion and the mature virion (Heinz F.X 2004).

1.3 Mosquito Transmission of Dengue Virus

DENV infected *Aedes aegypti* and *Aedes albopictus* mosquitoes transmit virus to a human host (Howe 1977). The principal vector *A. aegypti* is a small black and white mosquito, with tropical and subtropical geographic distribution. *A. aegypti* are domesticated and prefer to lay eggs in various artificial containers found in or near homes, resulting in high production of mosquitoes in these areas. Adult *A. aegypti* rest indoors and prefer to feed during the day on human hosts. Peak feeding times are just after dawn and right before dusk, but have been observed feeding throughout the day. The same mosquito can feed on multiple people during the

same blood meal, thus having the ability to spread pathogens to multiple hosts (Gubler and Rosen 1976; Putnam and Scott 1995; Platt, Linthicum et al. 1997; Scott, Naksathit et al. 1997). Naïve *A. aegypti* mosquitoes become infected when they feed on an infected human host, taking up DENV in a blood meal through its proboscis. The virus moves with the ingested blood into the esophagus and into the midgut. Dengue viral particles can resist the mosquitos' stomach digestive juices and break through the stomach wall barrier infecting midgut epithelial cells. DENV rapidly replicates and disseminates into the hemocoel and travels to, infects and becomes concentrated at the salivary glands completing the five-to-seven day cycle. DENV can then be transmitted to a new human host during a blood feed, through the proboscis and infected salivary glands of the mosquito (Halstead 2008). After a human host is bitten by an infected mosquito, the virus undergoes an incubation period of three-to-fourteen days after which dengue disease symptoms may occur (Siler 1926; Teichmann, Gobels et al. 2004).

1.4 Dengue Viral Replication

The risk of DENV infection depends on age, viral serotype, genotype, and genetic background of the host. DENV enters a variety of different cells including macrophages, monocytes, and dendritic cells. DENV enters though cell receptor-mediated endocytosis. The viral RNA is processed, replicated and the virus assembled and budded off (Figure 1.5) (Mackenzie, Gubler et al. 2004). In the human host, dengue viremia ranges in titer from 10^3 to over $10^{8.5}$ (Gubler, Suharyono et al. 1981). Viremia peaks around the onset of symptoms, and can be detected from three-to-fourteen days post-infection (Eram, Setyabudi et al. 1979).

1.4a Viral Entry

After a DENV-infected *A. aegypti* mosquito feeds on and transfers dengue to a naïve human host, the transmitted virus infects susceptible cells through the attachment of the virus to a cell surface receptor. For example, during a primary infection the E protein of DENV binds to the dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) receptor. With DENV bound to DC-SIGN, receptor mediated endocytosis and pH dependent fusion is used for the virus to enter the cell (Wu, Grouard-Vogel et al. 2000). Other recognized cell surface receptors are heparin sulfate, a mannose receptor on CD14+ monocytes, and GRP78/Bip on hepatic cells (Germi, Crance et al. 2002; Jindadamrongwech, Thepparit et al. 2004; Miller, de Wet et al. 2008).

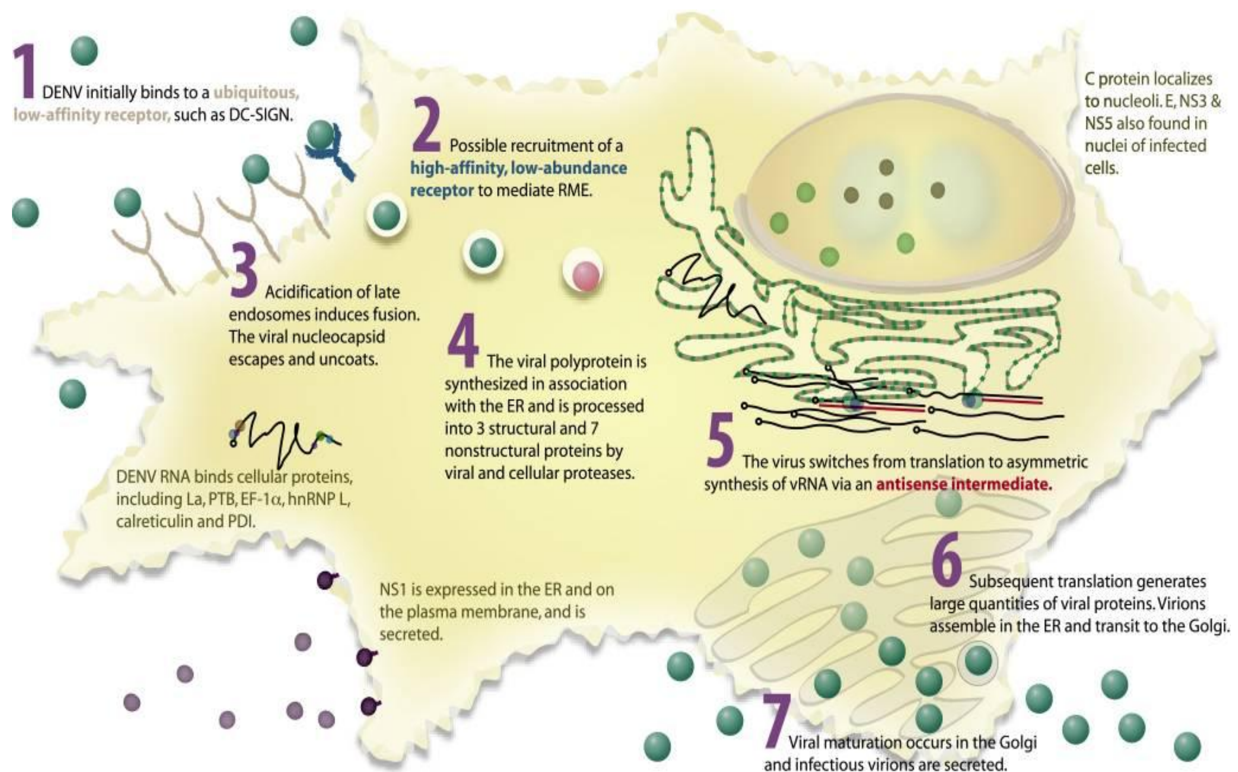

Figure 1.5 Dengue Replication Cycle. Dengue enters a susceptible cell through receptor-mediated endocytosis. In endosomic vesicles, dengue virions are uncoated and release the genome into the ER. Viral RNA is translated into a polypeptide and processed to form viral proteins. Replication and viral assembly occurs in the ER, and the virions travel to the Golgi for modification and is exported via exocytic vesicles (Clyde, Kyle et al. 2006).

1.4b Post Viral Entry Processing

After dengue virions are enclosed in endosomic vesicles they are exposed to acidic pH levels triggering structural modifications of the viral envelope E proteins. This conformational change leads to the exposure of a fusion peptide, which interacts with and bends the endosomic membrane towards the viral membrane, inducing the two membranes to fuse (Gollins and Porterfield 1984). Cellular lysosomal proteases within the endosomic vesicle uncoat the virion and digest the envelope, releasing the dengue genome into the ER (Heinz, Auer et al. 1994; Heinz, Stiasny et al. 2004). The 5' end of the genomic sequence contains several initiation codons that are regulated by the 5' conserved hairpin stem loop structure (Chiu, Kinney et al. 2005). Once initiated, ribosomes translate the positive viral RNA strand through the cellular translation machinery to form a polypeptide. This polypeptide is post-translationally cleaved by cellular proteases into structural and non-structural proteins (Harris, Holden et al. 2006). Host proteases cleave between C-prM and prM-E; NS1 and NS2A; and E-NS1 and NS4-NS4B. The NS2B/NS3 protease cleaves between NS2A and NS2B; NS2B and NS3; NS3 and NS4A; and NS4B and NS5 (Falgout, Miller et al. 1993; Lobigs 1993; Amberg, Nestorowicz et al. 1994). Following cleavage by the host protease, both types of proteins are arranged in different sides of the Endoplasmic Reticulum (ER) membranes. prM, E and NS1 proteins are translocated to the lumen of the ER and modified post-translationally in the Golgi apparatus through the addition of carbohydrate moieties (Yamshchikov and Compans 1993). Nonstructural proteins, such as NS3 and NS4A, induce invaginations in the ER. Inside these invaginations RNA replication takes place.

1.4c Viral Genome Replication

The 5'-UTR region of DENV genomes are about 100-nucleotides long, and contain a hairpin stem loop structure and a 7-methyl guanylate cap (Clyde, Kyle et al. 2006). The 3'-UTR region is 450-nucleotides long, contains a conserved 3' stem-loop needed for efficient replication, a conserved sequence CS1 important for cyclization of the genome, and lacks a poly(A) tail (Brinton, Fernandez et al. 1986; Zeng, Falgout et al. 1998; Alvarez, Lodeiro et al. 2005; Elghonemy, Davis et al. 2005; Yu and Markoff 2005). To make the viral polymerase, DENV RNA is translated in the cytoplasm using host cell machinery. This RNA-dependent RNA polymerase binds to the 5' stem-loop structure and interacts with the 3'-end through cyclization of the entire genome, generating the negative or template strand of the genome. In the Golgi apparatus, the template strand is used to transcribe the positive strand genome (Filomatori, Lodeiro et al. 2006). ER membrane invaginations provide a membrane-bound microenvironment required for RNA synthesis and viral assembly. Replication into many positive viral RNA stands are linked to the ER membrane invaginations that enfold around the active replication complexes (RCs) and form membrane-bound viral replication compartments. Membrane compartments separate viral RNAs and proteins from the cellular components allowing for efficient RNA synthesis and viral assembly (Jain 2005).

1.4d Viral Assembly and Budding

On the ER of infected cells DENV is rapidly being packaged and assembled into the virion. The ER facilitates the orientation of the capsid, allowing for the association between the viral RNA and the C protein to generate nucleocapsid complexes (Markoff, Falgout et al. 1997).

The nucleocapsid complex, with the addition of prM and E proteins in the ER, form the heterodimer complex.

This complex moves to the Golgi apparatus for post-translational modification with the addition of sugar residues. The complex buds through the ER lumen, with a prM-E-lipid envelope. While the virus is being exported via exocytic vesicles, mature M proteins form by furin-mediated cleavage of prM and causes E proteins to conformationally change to its homodimer form (Wang, He et al. 1999).

1.5 Immune Response to Dengue Infection

DENV initially infects human Langerhans and dermal/interstitial dendritic cells, which migrate to lymph nodes to present viral antigens to T-cells, initiating cellular and humoral immune responses (Wu, Grouard-Vogel et al. 2000). DENV also infects monocytes and macrophages, resulting in T-cell activation of DENV-specific $CD4^+$ memory T cells and $CD8^+$ T lymphocytes (Chareonsirisuthigul, Kalayanarooj et al. 2007). These activated T cells release cytokines and chemical mediators to further activate the immune system. The infected monocytes and macrophages produce tumor necrosis factor, interleukin-1α, interleukin-1β, interleukin-6, and platelet-activating factor. These factors along with complement activation products (C3a and C5a) and histamine correlate with the severity of illness due to playing a role in increased vascular permeability, plasma leakage, and shock (Malasit 1987; Lei, Yeh et al. 2001). To form acquired immunity to the infecting serotype of DENV, antibodies that are primarily directed against the E and M glycoproteins are produced. First exposure to DENV infection, known as primary infection, is characterized by a slow and low-titer antibody response (Figure 1.6). Immunoglobulin M (IgM) antibodies are the first isotype to appear. IgM antibodies

first appear within three-to-ten days and levels peak around two weeks after the onset of fever and decline to undetectable levels over the next few months. Immunoglobulin G (IgG) antibodies are first detectable at low titer by one week after the onset of fever and slowly increase over time (Halstead 1992; Guzman and Kouri 1996). Each dengue serotype produces unique immunity to the specific serotype. However, cross-protective immunity to other serotypes can occur within the first couple months post-infection. Therefore, infection of all four dengue serotypes can occur within a single individual (Gubler 1988). During a second exposure to DENV a secondary antibody response occurs. During this infection, IgG antibodies are produced in the acute phase and titers rise dramatically to high levels in the following two weeks. IgM antibody levels are significantly lower throughout a secondary DENV infection. Immunoglobulin A (IgA) and

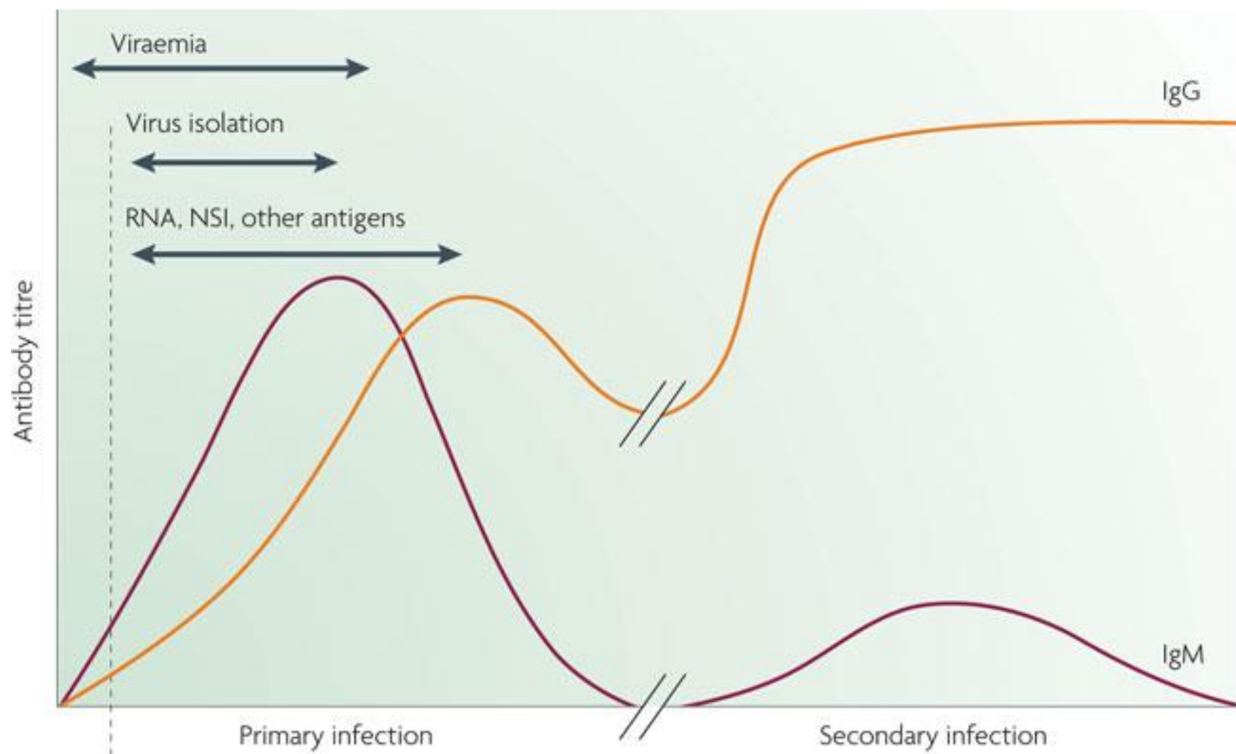

Figure 1.6 IgM and IgG levels in Response to Primary and Secondary Dengue Infection. In a primary dengue infection, IgM levels first appear within 3 to 10 days after the onset of symptoms, peak, then decrease over time. IgG levels are detectable one week post onset of symptoms and increase over time. In a secondary infection, IgM levels are low, while IgG levels rise dramatically (Peeling, Artsob et al. 2010).

Immunoglobulin E (IgE) responses to DENV have also been seen but currently are not used as markers for DENV infection (Halstead, Venkateshan et al. 1984). If an individual has a secondary DENV infection with a heterologous serotype, they are at a great risk for severe disease due to antibody-dependent enhancement (ADE). In ADE the preexisting heterologous dengue antibody specific for the virus serotype from the primary infection recognizes the new DENV serotype during secondary infection, forming an antigen-antibody complex that is non-neutralizing. On the cell surface, Fc receptors bind to the Fc region of the antigen-antibody complex allowing the virus to bind. The virus is subsequently internalized by the cell, allowing the virus to replicate (Mackenzie, Gubler et al. 2004; Dejnirattisai, Jumnainsong et al. 2010).

1.6 Dengue Pathology

Dengue disease in humans range from very mild symptoms that go undetected to dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) and may lead to death. Infection by any of the four serotypes of DENV results in similar symptoms but may vary in severity. The severity of disease depends on the serotype and strain of the virus, and the age, genetic background, and immune status of the individual human host (Gubler 1998).

1.6a Dengue Fever

Primary infection results in dengue fever in older children and adults. Symptoms of dengue fever include fever with severe joint pain, headache, body aches, rashes, weakness, nausea and vomiting (Siler 1926; WHO 1986; Waterman and Gubler 1989; Hayes and Gubler 1992). Individuals infected with DENV may also experience symptoms such as loss of appetite, constipation, diarrhea, respiratory distress, a mild sore throat, and an altered sense of taste. A

fever may rise to 102°F to 105°F and last for two-to-seven days with symptoms of pharyngitis, conjunctivitis (pink eye), and lymphadenopathy (swollen lymph nodes). Up to fifty percent of infected individuals develop a rash and a second rash may appear later on the body and spread to extremities. After body temperatures return to normal levels, petechiae (broken capillary blood vessels) seen as red or purple spots and intense pruritus (itching) may appear. Mild to severe hemorrhagic manifestations, including petechiae and purpura (bleeding under the skin seen as red or purple spots) may occur, along with gastrointestinal hemorrhages/bleeding, bleeding gums, menorrhagia (heavy menstrual bleeding), epistaxis (nosebleed), hematuria (bloody urine), and jaundice (yellow pigmentation of the skin) (Hayes and Gubler 1992; Gubler 1998; Germi, Crance et al. 2002). Dengue fever is rarely fatal and has an acute phase of illness that lasts for only three-to-seven days with a prolonged convalescent (recovery) phase. The convalescent phase may last for weeks with symptoms of weakness and depression (Gubler 1998).

1.6b Dengue Hemorrhagic Fever

Dengue Hemorrhagic Fever (DHF) is a disease primarily seen in young children (Dietz, Gubler et al. 1996). A more virulent or infective isotype of DENV increases the potential to cause serious disease with high levels of viremia correlating to the development of DHF. The greatest risk for severe disease is ADE through secondary DENV infection with a heterologous serotype. Host factors that increase the risk of progression to severe disease include being female, type AB blood, several human leukocyte antigen class I alleles, a single-nucleotide polymorphism in the tumor necrosis factor gene, and a promoter variant of the DC-SIGN receptor genes (Stephens, Klaythong et al. 2002; Fernandez-Mestre, Gendzekhadze et al. 2004; Sakuntabhai, Turbpaiboon et al. 2005; Kalayanarooj, Gibbons et al. 2007). Host factors that

reduce the risk of progression to severe disease during a secondary DENV infection include race and polymorphisms in the vitamin D receptor and Fcγ receptor genes (Thisyakorn and Nimmannitya 1993; Loke, Bethell et al. 2002; de la, Kouri et al. 2007). DHF initially resembles a primary DENV infection and is characterized by the sudden onset of fever for two-to-seven days with no symptoms representative for DHF. However, after the fever remits plasma leakage is a clinical symptom representative for DHF (Gubler 1998). The critical stage in DHF is when fever subsides with signs of circulatory failure, hemorrhagic manifestations, thrombocytopenia (decrease of platelets in the blood), and hemo-concentration (increased level of red blood cells). Hemorrhagic manifestations are seen through symptoms of skin hemorrhages such as purpuric lesions at the site of intravenous access, petechiae and purpura on the torso and limbs, epistaxis, gastrointestinal hemorrhage, bleeding gums, and hematuria with ground-coffee looking vomit and melena (black tarry feces). The melena is associated with iron oxidation from increased hemoglobin in the gastrointestinal tract. Without early diagnosis, some patients experience a mild or severe shock from blood loss and decreased blood flow (Eram, Setyabudi et al. 1979; Sumarmo, Wulur et al. 1983).

1.6c Dengue Shock Syndrome

In dengue shock syndrome (DSS), fever and other nonspecific signs and symptoms are followed by the sudden deterioration of the patient's condition. When the fever subsides in these patients skin may become cool, blotchy, and congested. Other symptoms are circumoral cyanosis, a rapid and weak pulse, restlessness, abdominal pain, and rapid progression into a critical stage of shock due to plasma leakage (Sumarmo, Wulur et al. 1983; WHO 1986). Other symptoms of DSS are similar to those of DHF and can result in death. Antishock therapy can

help recovery if used quickly after signs of DSS evolve (Eram, Setyabudi et al. 1979; Sumarmo, Wulur et al. 1983; Gubler and Clark 1995).

1.7 Vector Control and Dengue Vaccine

Currently there is no vaccine readily available for use against DENV and tools to prevent DENV infection including mosquito control are limited (Mackenzie, Gubler et al. 2004). Vector control methods, such as insecticides and bed nets, can help to reduce mosquito-mediated transmission. With people at risk for infection on the rise and no treatments for active disease, vaccine development or protective methods are urgently needed.

1.7a Vector Control

To reduce or prevent DENV transmission, environmental management and chemical control methods, including larvicides and adulticide space sprays, have been used with some effect (Gubler 1997). Other methods of control are cleaning of stagnant water in containers that are near homes and public areas and the use of mosquito nets (Rigau-Perez, Gubler et al. 1994). Additionally, more effective vector control methods may provide additional reduction in viral transmission. These include use of window curtains and water container covers treated with long-lasting insecticides, and control released larvicides that provide several months of application (Rodier, Gubler et al. 1996).

1.7b Dengue Vaccine Development

The rising spread and outbreaks of DENV infection and disease show the importance for developing a vaccine against DENV. Developing a vaccine for DENV has been in the works for

several decades but progress has been slow due to the complex pathology of dengue and the need to simultaneously target all four viral serotypes (Hombach 2007). Disease progression to DHF and DSS due to secondary infection elaborates the need for a vaccine to not only protect naïve individuals but also those that have previously had dengue (Hombach, Cardosa et al. 2007). The ideal DENV vaccine should not have reactogenicity, should induce life-long protection against infection with all of the four DENV serotypes and be affordable (Whitehead, Blaney et al. 2007). Vaccine trials should be evaluated with diverse at-risk populations due to differences in DENV transmission intensity and circulation (Hombach 2007).

There is currently no commercially available DENV vaccine, but several types of partially effective vaccines have been developed. Of these, live attenuated viruses, and chimeric viruses using yellow fever vaccine and attenuated DENV as backbones have been approved for clinical trials (Edelman, Wasserman et al. 2003; Durbin, Whitehead et al. 2005; Guirakhoo, Kitchener et al. 2006; Raviprakash, Apt et al. 2006). Additionally, genetically engineered DENV vaccines targeting either prM, E or NS1 genes have been tested with some promising results (Konishi 2011).

1.7c Live Attenuated Viruses

A live attenuated virus vaccine for DENV prevention is the most affordable type of vaccine, which if successfully created, would meet the needs of those in developing countries. Two tetravalent live attenuated dengue vaccines were developed and provided efficient seroconversion for all four DENV serotypes after a few doses in clinical trials (Sabchareon, Lang et al. 2002; Edelman, Wasserman et al. 2003; Sun, Edelman et al. 2003; Sabchareon, Lang et al. 2004; Simasathien, Thomas et al. 2008). However, during these clinical trials an imbalanced

immune response resulting in disease severity was seen and trials were quickly stopped (Stephenson 2005). In response, a new formulation was developed (formulation17) and was tested in phase I clinical trials. This formulation demonstrated less reactivity in volunteers with tetravalent neutralizing antibody responses in sixty-three percent of people after only a couple doses. This vaccine is still undergoing trials (Sun, Cunningham et al. 2009).

1.7d Chimeric Viruses

A chimeric virus contains genes from one virus with the substitution of specific genes from another virus. The yellow fever virus strain 17D is commonly used as a vector for constructing new vaccines against other flavivirus (Chambers, Nestorowicz et al. 1999; Huang, Butrapet et al. 2000). In developing a chimera for DENV, the prM and E structural genes from all four serotypes replace the homologous genes on the vector virus. Using 17D, a DENV chimeric vaccine has been developed and shown to produce high levels of neutralizing antibodies, deriving protection against DENV challenge in non-human primates. This chimeric vaccine was also successful in phase I clinical trials and is currently in Phase II and III clinical trials in various countries (Guirakhoo, Arroyo et al. 2001; Guirakhoo, Pugachev et al. 2002; Guirakhoo, Pugachev et al. 2004; Guirakhoo, Kitchener et al. 2006; Barban, Munoz-Jordan et al. 2012). A chimeric tetravalent vaccine using a DENV-2 strain as a backbone also produced high levels of neutralizing antibodies against all four DENV serotypes, and protected against a DENV challenge in AG129 mice (Huang, Butrapet et al. 2003). This vaccine is currently in phase I clinical trials, but there are concerns regarding the possibility of genetic recombination with virulent viruses (Seligman and Gould 2004; Stephenson 2005).

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1.8 Animal Models of Dengue Viral Infection

DENV infection is restricted to human and primate species and primate species do not have full disease progression as in humans, causing animal model studies to be limited. An ideal animal model would be able to sustain DENV infection using either a low viral injection dose or through mosquito-mediated transmission and once infected would display key features of dengue pathogenesis as well as mount an immune response. Upon secondary infection the animal model should have antibody-dependent enhancement of host cell viral entry.

1.8a Non-Human Primate Models

Primates are natural hosts for DENV but are not an effective model to study DENV replication and pathogenesis since they generally show no sign of disease. Subcutaneous injection of DENV in non-human primates (NHP) replicate virus at low levels, and are only in lymphoid-rich tissues (Marchette, Halstead et al. 1973). The use of an immunosuppressive drug, cyclophosphamide, enabled DENV infection of monocytes in Rhesus monkeys for prolonged periods (Marchette, O'Rourke et al. 1980). An intravenous injection with a higher dose of DENV in Rhesus macaques induced signs of hemorrhage and coagulopathy (Onlamoon, Noisakran et al. 2010). Even though NHPs do not develop any clinical signs of disease, they do develop an antibody immune response similar to that seen in humans (Marchette, Halstead et al. 1973). After a secondary dengue infection, viremia increases in NHPs suggesting that ADE may increase viral load through cross-reactive antibodies (Halstead, Shotwell et al. 1973). Even though viremia can be demonstrated in a NHP model, this model does not show dengue pathology resembling DHF/DSS, and thus is unsuitable for detailed DENV infection studies.

1.8b Small Animal Models

Wild-type mice show either a low level or lack of replication of DENV with no clinical symptoms. High-dose intracranial DENV injections into suckling mice and adult immunocompetent mice only induced neurological disease and paralysis, which is not generally observed in humans (Raut, Deolankar et al. 1996). Interferon-deficient mice lacking both IFN-α/β and -γ receptors in a 129 background (AG129) show severe infection and viremia after an intravenous and subcutaneous infection with DENV, but do not truly reproduce human disease (Shresta, Kyle et al. 2004). AG129 mice, though partially immunocompromised, develop a broadly cross-reactive and long-lasting antibody response to DENV (Johnson and Roehrig 1999). Through passive transfer of anti-DENV monoclonal antibodies, cross-reactive immune serum, or diluted homotypic serum prior to infection in AG129 mice, ADE has been seen (Goncalvez, Engle et al. 2007). Wild-type immunocompetent mice, such as A/J, BALB/c and C57BL/6 mice, show some level of DENV replication and limited pathogenesis (Shresta, Kyle et al. 2004). DHF-like disease has been seen in BALB/c and C57BL/6 mice injected with high doses of an adapted strain (Yen, Chen et al. 2008). Severe combined immunodeficiency (SCID) mice are deficient in B and T cell development. These mice can be engrafted with human stem cells or derived tumor cells. When infected with DENV, the SCID tumor mouse model does show sustained infection and viral replication. However, DENV in these mice spreads to the brain and induces paralysis (An, Kimura-Kuroda et al. 1999). Some of these models harbor viral replication, but none developed severe dengue disease.

1.8c Humanized Mice Models

The production of humanized mice, through the engraftment of human hematopoietic stem cells, provides the ability to study dengue pathogenesis and immune response (Kuruvilla, Troyer et al. 2007; Mota and Rico-Hesse 2011). Using this small animal model with human cells and tissues provides a more cost-efficient way to study DENV than a larger animal model. Previous immunodeficient mouse models have been useful for DENV infection studies with viral replication and limited antibody responses (Raut, Deolankar et al. 1996; An, Kimura-Kuroda et al. 1999; Johnson and Roehrig 1999; Shresta, Kyle et al. 2004; Yen, Chen et al. 2008). One of these commonly used humanized mouse models is NOD/SCID mice. NOD/SCID mice are made through a cross between a severe combined immunodeficient (SCID) and a non-obese diabetic (NOD) mouse derived from BALB/c strain mice. BALB/c scid mice are homozygous for the autosomal recessive mutation (scid) in the Prkdc gene that encodes for a protein kinase DNA-activated catalytic polypeptide. This mutation results in the lack of somatic recombination, which is the mechanism of genetic recombination in the early stages of immunoglobulin and T cell receptor production. Due to this immunodeficiency, NOD/SCID mice are unable to reject foreign engraftment (Dorshkind, Keller et al. 1984). Engraftment of human hematopoietic $CD34^+$ stem cells in these mice allowed for the development of human immune cells and thus the humanized mouse (huNOD/SCID) (Bente and Rico-Hesse 2006). Following a DENV infection the huNOD/SCID mice show viremia levels along with fever, erythema and thrombocytopenia. However, these mice do not show any signs of severe disease and do not generate significant antibody production (Mota and Rico-Hesse 2009; Mota and Rico-Hesse 2011). Also, these mice have a short life span, due to the high incidence of lymphomas, making long-term experimentation in this model difficult (Bente, Melkus et al. 2005). NOD/SCID mice have also

been further developed to include an interleukin-2 receptor γ-chain knockout (*IL2rγ^{null}*) deriving the NOD-*SCID IL2rγ^{null}* (NSG) mouse model. The IL2 γ-chain is a cytokine receptor subunit common on IL receptors. IL receptors recognize cytokines that are used in signaling to a variety of cells to induce growth, differentiation, and activation. A defect in the IL2 γ-chain results in X-linked SCID causing decreased levels of B cells, T cells, and natural killer cells (Ohbo, Suda et al. 1996). These mice sustain higher levels of engraftment but do not have a functional immune system. RAG1$^{-/-}$ $\gamma_c^{-/-}$ or RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice lack the common gamma chain IL2Ry genes (γ_c) and either recombination activating genes RAG 1 or 2, respectively. These mice have multilineage hematopoiesis leading to a partially functional immune system (Traggiai, Chicha et al. 2004; Berges, Wheat et al. 2006). A knockout in either the RAG1 or RAG2 gene affects the mechanism of genetic recombination in the early stages of immunoglobulin and T cell receptor production, resulting in B and T cell receptors that cannot fully form. Humanized RAG1$^{-/-}$ $\gamma_c^{-/-}$ or RAG2$^{-/-}$ $\gamma_c^{-/-}$ (RAG-hu) mice have been reported to allow higher engraftment and pathogen specific antibody production (Berges, Wheat et al. 2006). When infected with DENV, symptoms of fever and viremia develop along with virus-specific antibody production; however, viral neutralization is limited (Kuruvilla, Troyer et al. 2007).

In this regard, mice that have human engraftment of Bone marrow cells, Liver, and Thymus produce higher human cell engraftment and more complete multi-lineage hematopoiesis, termed BLT. This BLT model was first established using the NOD/SCID background (NOD/SCID-hu BLT) and is developed by surgically implanting human fetal liver and thymus under the kidney capsule of seven-week old mice, followed by an intravenous injection of autologous bone marrow stem cells. NOD/SCID-hu BLT mice were shown to have a partial-human immune system through the development of the major functional cells of the

human adaptive immune system; T-cells, B-cells, dendritic cells and macrophages (Shultz, Ishikawa et al. 2007). The NOD/SCID-hu BLT mouse model has been shown to mount an effective adaptive immune response against toxic shock syndrome toxin 1 and Epstein-Barr virus. Extensive work has also established this model as ideal for human immunodeficiency virus studies (HIV). These works illustrate the use of these mice for viral infection studies (Melkus, Estes et al. 2006; Sun, Denton et al. 2007). A BLT model has also been made from the NSG (NSG-BLT) mouse and showed antibody production and signs of dengue disease in response to a DENV infection (Mota and Rico-Hesse 2011; Jaiswal, Pazoles et al. 2012). The RAG-hu model is susceptible to DENV infection, develops fever, and has antibody production. Therefore, we used this mouse strain to develop the BLT-RAG-hu mouse model (from here referred to as BLT). This model is the primary focus for the studies conducted through this thesis.

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CHAPTER 2

CULTIVATION OF DENGUE VIRUS IN TISSUE CULTURE AND VIRUS TITRATION

2.1 Introduction

Prior to modern techniques for virus detection, cell culture was used for viral isolation and identification of DENV by plaque assay in LLC-MK2 cells, a rhesus cell line (Evans, Kerr et al. 1959; Hull, Cherry et al. 1962; Halstead, Sukhavachana et al. 1964; Sukhavachana, Nisalak et al. 1966). Although valid in these simple practices, these cells did not actively produce mature virus and were not able to fully recapitulate DENV susceptible cells of humans. In this regard, Vero cells, an African green monkey kidney fibroblast cell line, were one of the first cells used for DENV isolation and were easily infected (Hopps, Bernheim et al. 1963). These cells have been adapted for current titration methods using a fluorescence-activated cell sorter (FACS)-based assay (Lambeth, White et al. 2005). Additionally, due to its natural life cycle DENV replicates in high titer in female insects, illustrating the viability of a mosquito cell line for the cultivation of DENV (Rosen and Gubler 1974). The *A. albopictus* mosquito cell line, C6/36, is easy to maintain and can be used for rapid growth and isolation of DENV (Gubler, Kuno et al. 1984; Brackney, Scott et al. 2010). In order to perform studies involving DENV, high titer stock virus must be available. In this first set of experiments, all four serotypes of DENV were produced through cultivation in the C6/36 cell line for high concentration stock virus. The stock virus titers were subsequently determined using Vero cells infected with a known volume of stock virus and subjected to an Alexa-488 conjugated 4G2 antibody staining. 4G2 is an anti-flavivirus group antigen antibody and has been routinely used to detect levels of flaviviruses (Lambeth, White et al. 2005). Infected cells will convey the Alexa-488 marker and can be

analyzed by FACS analysis. Here we show the production of high titer virus for all four DENV serotypes.

2.2 Materials and Methods

2.2a Cultivation of Dengue Virus in C6/36 Mosquito Cells

C6/36 cells were cultured in either complete Dulbecco's modified Eagle's medium (DMEM) containing 10% heat inactivated fetal bovine serum (FBS) or complete L-15 medium (Leibovitz) with 3% FBS. Cells were plated and allowed to grow to 60-75% confluence before being inoculated with 20 M.O.I. dengue virus. Specific isolates cultivated included: DENV-1 16007 from Thailand, DENV-2 10649 from Philippines and 1409 from Jamaica, DENV-3 H87 from Philippines and 3009 from Sri Lanka and DENV-4 H241, D10-36 and D80-96 from Philippians, Sri Lanka and Thailand, respectively (Table 2.1). These viruses include highly laboratory passaged (DENV-2 Jamaica 1409) as well as primary low passage isolates (DENV-2 Philippines 10649). Virus was allowed to infect cells for 48 hours and supernatant was collected daily and subjected to 0.4micron filtration with aliquots stored at -80°C.

2.2b Viral Titration of Dengue Virus using Vero Cells

Vero cells were cultured in DMEM containing 3% FBS. Dengue titration was performed by first plating five-hundred thousand Vero cells in each well of a 6-well plate. Known varying volumes of virus were placed in each well and allowed to infect cells for 24 hours. Media was removed and cells were washed following staining of infected cells with a 4G2 antibody conjugated with Alexa-488 and assayed by FACS analysis as previously described (Lambeth, White et al. 2005).

2.3 Results and Conclusions

2.3a Cultivation and Titration of Dengue Virus

For studies involving dengue it is imperative to have sufficient viral stocks capable of consistent infection of cells in vitro and later for humanized mice in vivo. All four serotypes of DENV were grown in C6/36 mosquito cells, were titered by staining for anti-flavivirus group antigen using a 4G2 antibody conjugated to Alexa-488 and analyzed by FACS. As seen in table 2.1, high titers were obtained for the different isotypes of DENV with levels ranging from 7.7×10^4 to 1.5×10^8 IFU/ml. Variations in titer were consistent with varying difficulties of production of different dengue isolates and/or isotypes as previously described (Lambeth, White et al. 2005).

In conclusion, high amounts of virus were produced for each isotype including highly laboratory passaged (DENV-2 Jamaica 1409) as well as primary low passage isolates (DENV-2 Philippines 10649). These viral stocks are suitable for the studies conducted throughout this thesis and for future work.

Table 2.1 High DENV Stock Titers

Serotype	Isotype	Titer(IFU[a]/ml)
DENV-1	Thailand 16007	1.50×10^8
DENV-2	Philippines 10649	2.29×10^6
DENV-2	Jamaica 1409	1.00×10^6
DENV-3	Philippines H87	2.00×10^7
DENV-3	Sri Lanka 3009	4.67×10^7
DENV-4	Philippines H241	2.94×10^8
DENV-4	Indonesia D10-36	5.90×10^8
DENV-4	D80-96	7.70×10^4

[a]= Infectious units

CHAPTER 3

PRIMARY DENGUE INFECTION AND IMMUNE RESPONSE IN THE HUMANIZED BLT MOUSE MODEL

3.1 Introduction

Due to host specificity, until recently there has not been an ideal animal model that supports DENV infection with relevant disease progression. In this regard, the new humanized mouse models have been critical for expanding studies of dengue infection and disease. Immunodeficient mice are unable to reject foreign grafts of human immune progenitor cells. Among the commonly used humanized mouse models is the NOD/SCID mouse. NOD/SCID mice are derived through a cross between a severe BALC/c combined immunodeficient (SCID) and a non-obese diabetic (NOD) mouse. BALB/c scid mice are homozygous for the autosomal recessive mutation (scid) in the Prkdc gene that encodes for a protein kinase DNA-activated catalytic polypeptide. This results in the lack of somatic recombination, with the result of B and T cells not being able to fully mature. However, infection in these mice does not produce pathology similar to that seen in human patients infected with DENV. In this regard, RAG1 $^{-/-}$ $\gamma_c^{-/-}$ or RAG2 $^{-/-}$ $\gamma_c^{-/-}$ mice lack the common gamma chain IL2Rγ genes (γ_c), and either recombination activating genes RAG 1 or 2, respectively, and have been reported to support higher engraftment and pathogen specific antibody production (Berges, Wheat et al. 2006). When engrafted with human hematopoietic CD34+ stem cells, making them humanized (RAG-hu), these mice produced antibodies specific to dengue but only had limited neutralizing capability (Traggiai, Chicha et al. 2004; Kuruvilla, Troyer et al. 2007). In this regard, BLT mice have a human engraftment that includes implantation of <u>B</u>one marrow, <u>L</u>iver, and

Thymus, to produce a higher human cell engraftment and multi-lineage hematopoiesis. These mice are developed by engrafting human fetal liver and thymus under the kidney capsule of seven-week old RAG mice, followed by an intravenous injection of autologous bone marrow stem cells. Here we seek to improve on previous work by evaluating dengue-specific immunological responses including production of dengue-specific IgM and IgG antibodies, and DENV neutralization. The results of this study demonstrate an immune competent model for future dengue studies particularly those of severe dengue disease.

3.2 Materials and Methods

3.2a CD34 Cells, Cell Culture and Viral Stocks

As described previously, CD34+ stem cells were isolated from human fetal liver tissue and cultured for 24 hours in a medium of cytokines: IL-3; IL-6; and SCF(Akkina, Rosenblatt et al. 1994; Bai, Gorantla et al. 2000). Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 3% heat inactivated FBS. C6/36 cells were cultured in DMEM containing 10% FBS. DENV-2 Philippines 10649 stock was generated through viral amplification in infected C6/36 cells using DMEM. Dengue virus was titered in Vero cells by staining infected cells with a 4G2 antibody conjugated with Alexa-488 and assayed using FACS analysis as previously described (Lambeth, White et al. 2005).

3.2b Generation of Humanized BLT Mice

BLT mice were generated from $RAG1^{-/-}\gamma_c^{-/-}$ or $RAG2^{-/-}\gamma_c^{-/-}$mice using techniques previously described (Melkus, Estes et al. 2006). Briefly, seven-week old $RAG^{-/-}\gamma_c^{-/-}$ mice were irradiated with a single dose of 3.0 Gy generated from a Cs-137 source. At 1-3h post irradiation,

mice under anesthesia were surgically implanted with 1mm fragments of human fetal liver and thymus tissue under the right kidney capsule. Mice were allowed to recover for 24h with pain management, after which they were injected intravenously with $5x10^5$-$1x10^6$ autologous human CD34+ hematopoietic progenitor cells isolated from fetal liver. At 3-4 weeks post-reconstitution, peripheral blood was collected by tail bleeds from transplanted mice, and red blood cells were lysed using the Whole Blood Erythrocyte Lysing Kit (R&D Systems) according to manufacturer's protocol. The leukocyte fraction was stained with antibodies against the human pan-leukocyte marker CD45 and analyzed by FACS to verify human engraftment.

3.2C Engraftment Levels and Human Immune Cells in BLT Mice

To determine multi-lineage hematopoietic development, fluorescently labeled mAbs to the following cellular markers were obtained from Caltag Laboratories (Invitrogen) and used according to manufacturer's instructions: hCD45, mCD45, hCD19, hCD20, HLA-DR, hCD11c, hCD123, hCD3, hCD4, and hCD8. Antibodies were conjugated to FITC, PE, APC, PE-Cy5 or Per-CP. The leukocyte gate was defined by staining normal human blood with CD45. Cells from non-humanized $RAG^{-/-}\gamma_c^{-/-}$ mice were used in all analyses to control for non-specific staining of mouse cells. Cells were analyzed using an EPICS XL-MCL flow cytometer (Coulter) and multicolor flow cytometry was conducted using a Becton Dickinson FACSAria.

3.2d Dengue Virus Infection of BLT Mice and Clinical Monitoring

BLT mice were inoculated by a 50% intraperitoneal and 50% subcutaneous injection of $1x10^6$ IU DENV-2 Philippines. To verify infection and determine viral loads, qRT-PCR was conducted on total viral RNA isolated from mouse plasma using the QIAamp Viral RNA kit

(Qiagen) according to the manufacturer's instructions. QRT-PCR was carried out using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) in a Bio-Rad CFX96 Real-Time System C1000 Thermal Cycler. PCR was initiated on 10µl viral RNA, using NS5F and NS5R DENV primers (Kong, Thay et al. 2006). Reaction conditions were as follows: 50°C for 20 min; 95°C for 5 min; 45 cycles of 95°C for 15s, and 62°C for 30s. Levels of DENV RNA amplified by this one-step qRT-PCR method were determined though comparison to a standard curve by known quantities of DENV-2 16681 NS5 RNA, which was transcribed and quantified as previously described (Kong, Thay et al. 2006). Mouse weight was monitored using an EK-1200i balance, and mouse body temperature was monitored with a RET-3 rectal thermo-probe coupled to a Digi-Sense digital thermometer (Eutech Instruments*)*.

3.2e Detection of Human Anti-Dengue IgM and IgG Antibodies by ELISA

Levels of human anti-dengue antibodies present in humanized mouse serum were detected using the commercial PANBIO IgM and IgG capture ELISA kits. Sera collected from BLT mice were diluted in buffer provided with the kit at 1:20 dilution. Samples were considered positive if the OD_{450} was greater than two times the background absorbance level. Serum samples that produced a positive IgM or IgG reading at the 1:20 dilution were serially diluted up to 1:1280 and assayed by ELISA.

3.2f Detection of Neutralizing Antibodies by a FACS-Based Assay

The neutralizing capacity anti-DENV-2 sera obtained from humanized BLT mice were determined using a FACS neutralization test (FNT) modified from a previously described method (Lambeth, White et al. 2005). Sera from non-inoculated BLT mice were used as non-

reactive controls. Known human antibody positive anti-DENV serum was used as a reactive control for neutralization. Briefly, the sera was diluted in 2% FBS-DMEM from a 1:20 dilution up to 1:640 dilution, and mixed with DENV-2 Philippines 10649 in a total volume of 400 µl. This 400 µl serum-virus mixture was incubated for 1h at 37°C. This mixture was then added and allowed to infect a monolayer of $3x10^{5}$ Vero cells in a single well of a 12-well plate for 24h. Cells were stained using the 4G2 antibody and analyzed using an EPCIS XL-MCL flow cytometer (Coulter) as described above. FNT_{50} is the highest two-fold dilution of serum that inhibited 50% of DENV infection of Vero cells.

3.3 Results and Conclusions

3.3a Human Hematopoiesis and Immune Cell Reconstitution in BLT Mice

In this study, both RAG $1^{-/-}$ $\gamma_c^{-/-}$ and RAG $2^{-/-}$ $\gamma_c^{-/-}$ background mice were used to construct the BLT mouse model through irradiation and the surgical implantation of human fetal liver and thymus tissue under the right kidney capsule, and the next day followed by an injection with $5x10^{5}$-$1x10^{6}$ human CD34+ hematopoietic progenitor cells isolated from fetal liver. Human cell engraftment was determined by collecting peripheral blood six weeks post-engraftment and analyzing for the human pan-leukocyte marker CD45 by FACS analysis. BLT mice showed human engraftment up to 90% in peripheral blood with engraftment levels differing between mice as seen in figure 3.1A and table 3.1. Flow cytometry was also used to analyze hematopoiesis in bone marrow, thymic graft, and spleen through the detection of lineage specific markers: CD19 +, CD20+ for B cells; CD11c+CD123-, CD11c-CD123+ for dendritic cells; and CD3, CD4+, CD8+ for T cells. BLT mice exhibit a development of human immune B, T and

dendritic cells in the bone marrow, thymus graft, and spleen. Overall BLT mice showed human hematopoiesis with high engraftments in the peripheral blood.

3.3b BLT Mice Support Productive Dengue Viral Infection

After confirming hematopoiesis, RAG strain BLT mice were evaluated for susceptibility to DENV infection. Mice were inoculated by subcutaneous and intraperitoneal routes with $1x10^6$ infectious units of the primary low passage isolate DENV-2 Philippines 10649. Viremia was determined up to forty-three days post-inoculation by quantifying viral RNA from collected plasma. Viral RNA was detected as early as five days post-inoculation, and detected as late as forty-three days (Table 3.1 and Figure 3.2B). Eleven out of sixteen (70%) humanized BLT mice

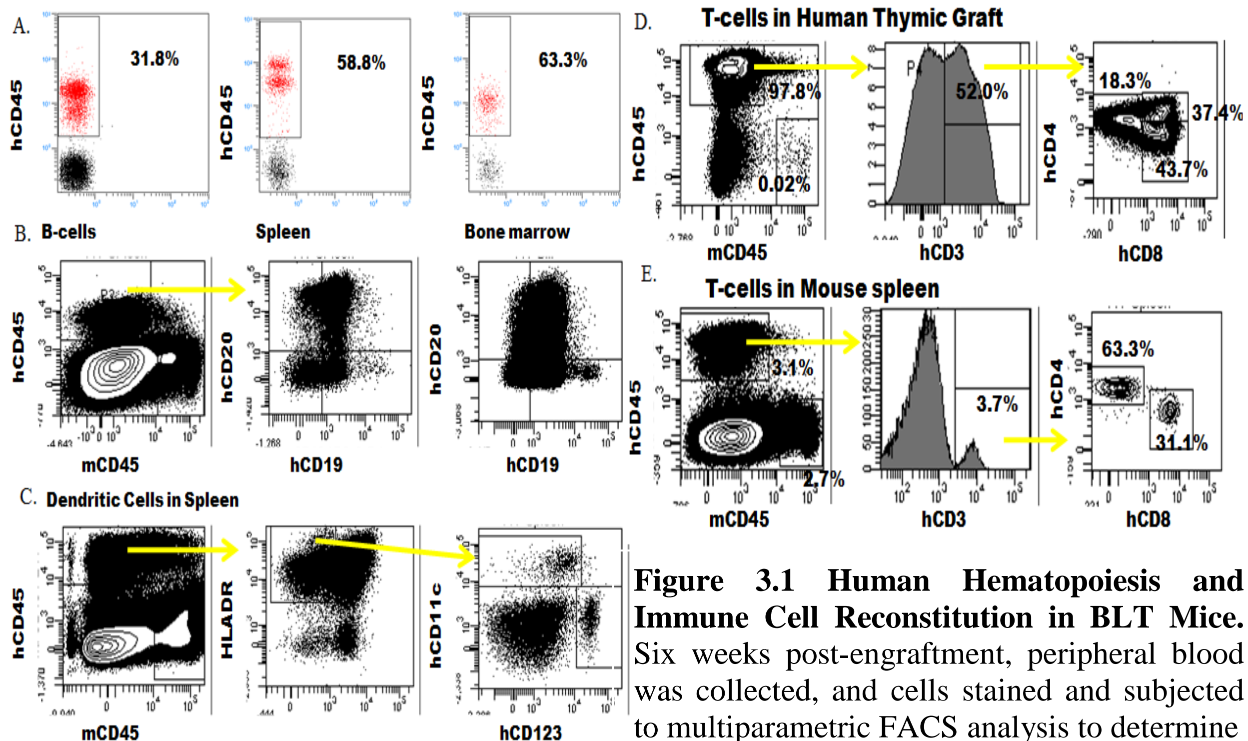

Figure 3.1 Human Hematopoiesis and Immune Cell Reconstitution in BLT Mice. Six weeks post-engraftment, peripheral blood was collected, and cells stained and subjected to multiparametric FACS analysis to determine human cell engraftment, and B cells, T cells and dendritic cells in the spleen, bone marrow and human thymus graft. **A)** Human cell engraftment in the peripheral blood. **B)** B cells in the spleen and bone marrow. **C)** Dendritic cells in the spleen. **D)** T cells in the human thymic graft. **E)** T cells in the spleen.

Table 3.1 Presence of Viremia (RNA copies/ml) as a Result of DENV Inoculation

Mouse ID	Engr[a] %	Day 2	Day 5	Day 8	Day 13	Day 15	Day 20	Day 22	Day 27	Day 29	Day 34	Day 37	Day 43
BLT 153[b]	81	<4.00E+01[d]	<4.00E+01	<4.00E+01	4.18E+02	8.19E+02	1.76E+03	5.43E+02	1.23E+02	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
BLT 154	69	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	X[e]	X
BLT 155	80	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
BLT 156	79	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
BLT 157	13	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	X
BLT 169	81	<4.00E+01	<4.00E+01	2.45E+02	9.76E+02	9.40E+02	4.07E+02	2.01E+02	1.79E+02	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
BLT 170	71	<4.00E+01	<4.00E+01	1.87E+02	9.64E+02	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
BLT 171	88	<4.00E+01	2.18E+02	6.87E+02	5.84E+01	9.87E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	X	X	X
BLT 184	90	<4.00E+01	<4.00E+01	<4.00E+01	8.93E+01	2.15E+02	3.56E+02	1.46E+04	3.78E+04	5.98E+03	1.99E+03	2.08E+03	1.10E+03
BLT 195	41	<4.00E+01	2.35E+02	5.67E+02	1.07E+02	1.02E+02	8.09E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
BLT 218	57	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	1.36E+02	8.45E+02	9.50E+02	3.52E+02	6.21E+02	8.63E+02	7.68E+03	9.49E+02
BLT 219	65	<4.00E+01	<4.00E+01	5.28E+01	4.74E+02	2.19E+02	6.55E+03	9.75E+03	3.41E+03	3.27E+03	4.57E+03	8.78E+03	9.21E+03
BLT 222	58	<4.00E+01	<4.00E+01	1.77E+02	1.63E+03	4.16E+03	9.68E+02	2.55E+02	5.92E+02	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
BLT 223	53	<4.00E+01	<4.00E+01	4.82E+01	9.31E+01	4.67E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
BLT 225	52	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
BLT 226	9	<4.00E+01	<4.00E+01	8.68E+01	1.71E+02	4.35E+02	3.31E+02	9.42E+02	4.17E+03	3.53E+02	2.03E+02	<4.00E+01	<4.00E+01
BLT 143[c]	12	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
BLT 145	61	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
BLT 193	36	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
BLT 196	45	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01

[a] Percent human engraftment in peripheral blood.

[b] BLT153-BLT226= BLT mice inoculated with DENV-2

[c] BLT143-BLT196= BLT mice not inoculatd with DENV-2

[d] <4.00E+01= Falls below the detection limit

[e] X= Mortality

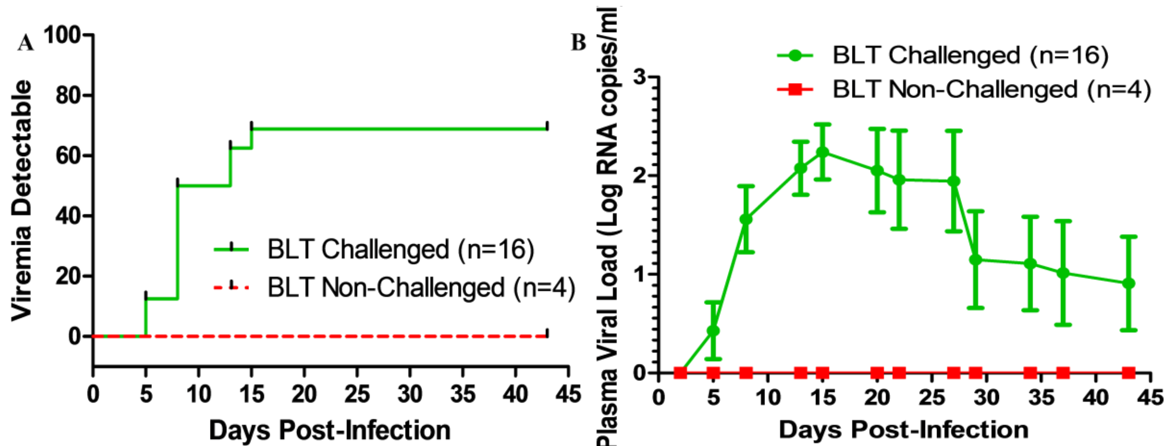

Figure 3.2. BLT Mice Infected as a Result of DENV-2 Inoculation. A) BLT mice were inoculated with DENV-2 Philippines 10649 through subcutaneous and intraperitoneal injections. Viral loads in plasma collected twice a week were evaluated by qRT-PCR. Kaplan-Meier plot of percent of mice with detectable viremia in virus inoculated and in non-inoculated control mice are shown. **B)** Viral levels increase and peak at 15 days post-inoculation, then decline and stabilize up to 43 days post-inoculation.

had detectable viral levels for at least 2 consecutive time points (Figure 3.2A). BLT mice were randomly selected and thus had variable levels (9%-90%) of engraftment (Table 3.1). However, there is no clear correlation between the levels of human cell engraftment and infection rate of DENV-inoculated mice. This is especially illustrated with mouse BLT 226 with only 9% engraftment having several time points with detectable virus compared to the high engrafted mice, BLT 154-156, that did not have a positive time point.

Additionally, the qRT-PCR assay had a detection threshold of $4x10^1$ copies/ml of plasma. Based on this, some time points had undetectable levels of DENV but were potentially still DENV positive. This limitation in the assay may have resulted in reporting less than 100% infection in these DENV susceptible mice.

3.3c Clinical Signs of Dengue Disease

Weight loss is a general sign of sickness, and can be easily measured. To further, fever is a hallmark of dengue pathogenesis. Here, weight and body temperature of experimental animals was monitored for general signs of dengue disease. On average a 40% decline in the weight of the inoculated BLT mice was observed in contrast to the stable non-inoculated mice (Figure 3.3A). This general indication illustrates that disease developed in dengue-inoculated mice. However, no significant change in average temperature of the DENV-inoculated BLT mice was seen (Figure 3.3B). Although this result leans to no developed mediated disease, it is possible that varying times of fever elicited in these mice is masked by the average temperature of the mice as a whole or the time of fever was not collected. In this regard, individual collections collected daily may or more often may not detect viral mediated fever.

3.3d Human Immune Response and Dengue Neutralizing Antibody Production in BLT Mice

Previous humanized mouse models had failed to produce active immune responses capable of virus neutralization. To address if the RAG based BLT model improves on this aspect DENV-inoculated BLT mice were monitored for human dengue IgM and IgG antibodies and collected mouse sera was challenged for dengue neutralization. Antibody levels were tested from four-to-twenty weeks post-inoculation (Table 3.2). All DENV-inoculated BLT mice developed human anti-DENV antibody responses. Human anti-DENV IgM was detected in all sixteen DENV-inoculated humanized BLT mice, which includes mice with no detectable levels of viremia. Human anti-DENV IgG was detected in three DENV-inoculated BLT mice at low levels and was detected in two BLT mice at high levels. However, even in these mice class switching was inefficient.

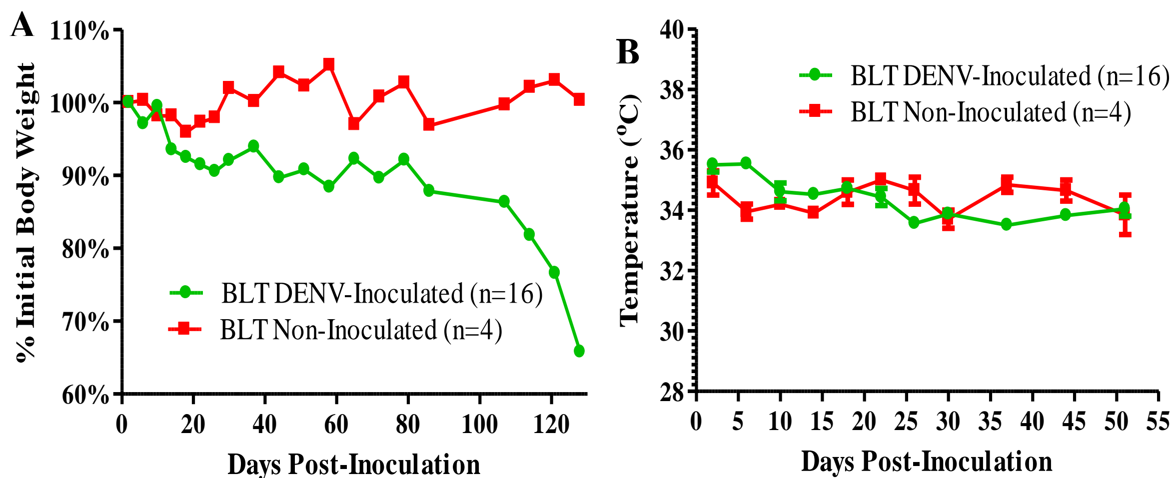

Figure 3.3 A) Weight Loss in DENV-Inoculated BLT Mice. Mice were weighed days post-inoculation as indicated, with an initial body weight prior to inoculation measured. Non-inoculated BLT control mice maintained body weight in drastic contrast to the DENV-inoculated mice which in average decreased by nearly 40% in the 127 day monitored period. **B) No Significant Increase in Temperatures.** To test for fever-like symptoms in DENV-inoculated BLT mice, a RET-3 rectal thermo-probe was utilized. No significant difference between the temperatures of DENV-inoculated BLT mice and non-inoculated mice was seen, indicating an absence of fever.

To determine if the antibodies produced by these mice have neutralizing capacity, serum of DENV-inoculated BLT mice was challenged against DENV-2. Our results showed that all sixteen DENV antibody producing BLT mice were able to neutralize DENV virus (Table 3.3). 50% neutralization ranged from a 1:20 to 1:640 dilution of the serum. Strangely, there was no significant correlation between high levels of human anti-DENV IgM or IgG and neutralizing capacity (Table 3.3). However, there is a correlation between the levels of human anti-DENV IgM and plasma viral load which indicate IgM as effectively neutralizing virus (Figure 3.4).

Table 3.2 DENV-Inoculated BLT Mice Developed Anti-DENV Antibody Responses

Mouse ID	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	9 weeks	10 weeks	11 weeks	12 weeks	13 weeks	14 weeks	15 weeks	16 weeks	17-20 weeks
BLT 153	IgM ++	IgM +++	IgM ++	IgM ++	X[a]	X	X	X	X	X	X	X	X	X
BLT 154	–[b]	–	–	–	IgM +	IgM +	–	–	–	IgM ++ IgG +	IgM ++ IgG +	IgM +++	IgM +++	IgM ++
BLT 155	–	–	–	–	IgM +	IgM +	IgM +	IgM ++	X	X	X	X	X	X
BLT 156	–	IgM +++	IgM ++	–	–	–	–	–	IgM +	IgM + IgG +	IgM +	IgM +	–	–
BLT 157	IgM +	–	–	IgM +	X	X	X	X	X	X	X	X	X	X
BLT 169	IgM ++	IgM ++++ IgG +++	IgM +++	IgM + IgG +++	IgM +++ IgG ++	IgM ++ IgG +	X	X	X	X	X	X	X	X
BLT 170	IgM ++	–	–	IgM +	IgM +	IgM + IgG +	IgM +	IgM +	–	–	X	X	X	X
BLT 171	IgM +++	IgM +	X	X	X	X	X	X	X	X	X	X	X	X
BLT 184	–	–	–	–	–	–	IgM ++++	IgM ++++	IgM ++++	IgM +++	X	X	X	X
BLT 195	–	–	–	–	–	–	–	–	IgM +	–	–	–	IgM +	IgM +
BLT 218	IgM +	IgM +	IgM +	IgM +	IgM ++	IgM ++	IgM ++	IgM ++	IgM ++	IgM +	IgM +	IgM +	IgM ++	IgM ++
BLT 219	–	–	–	IgM +	IgM +++	IgM ++++	IgM +++	X	X	X	X	X	X	X
BLT 222	–	–	IgM +	IgM +	X	X	X	X	X	X	X	X	X	X
BLT 223	–	–	–	–	–	–	IgM ++	IgM ++	IgM +	IgM +	–	–	IgM +	–
BLT 225	–	–	–	–	–	–	–	–	–	–	–	–	IgM +	–
BLT 226	IgM ++++	IgM ++++	IgM ++++	IgM ++++ IgG +	IgM ++++ IgG +	IgM ++++ IgG +	IgM ++++ IgG +	IgM ++++ IgG +	IgM +++ IgG +	IgM ++ IgG +++	IgM ++ IgG ++++	IgM ++ IgG ++++	IgM ++ IgG ++++	X
BLT 143	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BLT 145	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BLT 193	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BLT 196	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] X= Mortality

[b] –= Antibody(s) level not detectable

+=2-fold, ++=4-fold, +++=8-fold, and ++++16-fold greater than background absorbance

Overall, these RAG based BLT mice have virus specific antibody production that are capable of DENV neutralization.

Table 3.3 Sera from DENV-Inoculated BLT Mice have Neutralizing Capabilites

Mouse ID	Weeks PI[a]	IgM titer	IgG titer	FNT_{50}[b] titer
BLT 153	6-7	1:320	1:80	1:320
BLT 154	14-17	1:640	1:40	1:320
BLT 155	8-11	1:320	<1:10	1:80
BLT 156	5-8	1:120	<1:10	1:40
BLT 157	5-7	1:120	<1:10	1:40
BLT 169	6-8	1:320	1:160	1:80
BLT 170	7-10	1:160	1:80	1:320
BLT 171	5	1:80	<1:10	1:320
BLT 184	10-13	1:640	<1:10	1:40
BLT 195	16-18	1:80	<1:10	1:40
BLT 218	9-12	1:320	1:20	1:80
BLT 219	7-10	1:640	1:20	1:40
BLT 222	7	1:160	<1:10	1:40
BLT 223	13-16	1:80	<1:10	1:40
BLT 225	8-11	1:120	1:20	1:80
BLT 226	13-16	1:640	1:640	1:640
BLT 143	8-11	<1:10	<1:10	<1:10
BLT 145	8-11	<1:10	<1:10	<1:10
BLT 193	8-11	<1:10	<1:10	<1:10
BLT 196	8-11	<1:10	<1:10	<1:10

[a] PI= Post-Inoculation

[b] FNT_{50}= The serum dil. that inhibited 50% of DENV infection

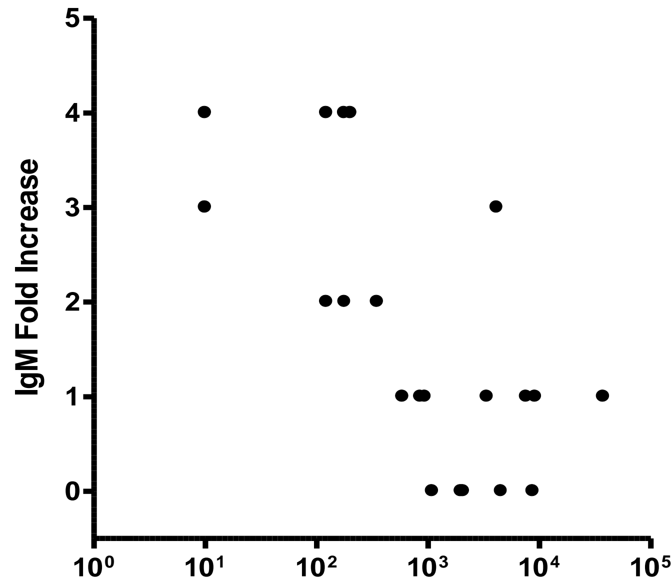

Figure 3.4. A) High IgM Levels Correlate with Low Plasma Viral Loads. IgM data was collected through a PANBIO IgM capture ELISA kits, and viral load through qRT-PCR. Low viral loads are correlated with high levels of IgM, and high viral loads are correlated to low levels of IgM.

3.4 Discussion

We have demonstrated that humanized mice, with multi-lineage human hematopoiesis, are suitable for human specific virus studies. These humanized mice provide a model to study dengue pathogenesis and immune response. Previous immunodeficient mouse models have been proven useful for DENV infection studies with viral replication but limited antibody responses (Raut, Deolankar et al. 1996; An, Kimura-Kuroda et al. 1999; Johnson and Roehrig 1999; Shresta, Kyle et al. 2004; Yen, Chen et al. 2008). The humanized NOD/SCID mouse model allowed engraftment of human hematopoietic $CD34^{+}$ stem cells, developing human immune cells in the mouse (Bente and Rico-Hesse 2006). When infected with DENV, the humanized huNOD/SCID mice showed viremia, along with clinical signs of dengue fever; including erythema and thrombocytopenia. However, these mice do not generate significant antibody production (Mota and Rico-Hesse 2009; Mota and Rico-Hesse 2011). RAG-hu mice have multi-lineage human hematopoiesis (Traggiai, Chicha et al. 2004; Berges, Wheat et al. 2006). When these mice are infected with DENV, symptoms of fever and viremia develop along with virus specific antibody production. However, viral neutralization was somewhat limited (Kuruvilla, Troyer et al. 2007). The BLT mouse model was first made using the $RAG1^{-/-}\gamma_c^{-/-}$ or $RAG2^{-/-}\gamma_c^{-/-}$ mice (BLT).

Our results demonstrate that the RAG based BLT mouse model fully supported dengue virus as evidenced by viremia lasting up to forty-three days post-inoculation. Viral loads reached as high as $3.78x10^{4}$ copies/ml, and had average viral loads from one to three logs. These plasma viral loads are similar to what was reported in the humanized BLT-NSG mouse model (Mota and Rico-Hesse 2011). Weight loss in DENV-inoculated mice was observed reaching a 40% reduction; in contrast to the sustained levels observed non-inoculated mice and in the RAG-hu

mice reported previously (Kuruvilla, Troyer et al. 2007), but was seen in the BLT-NSG mouse model (Jaiswal, Pazoles et al. 2012). However, fever that was seen in the RAG-hu mouse model was not observed. This aspect of disease may have been missed due to inappropriate timing of collections and averaging of mouse body temperatures. Additionally, the BLT-NSG mouse model has been inconsistent in showing fever in response to DENV infection (Mota and Rico-Hesse 2011; Jaiswal, Pazoles et al. 2012). DENV-inoculated BLT mice showed antibody response with production of human dengue IgM. Some mice produced IgG but were inconsistent.

We did however see a correlation between viral loads and IgM levels, with low DENV viral loads correlating with a high level of IgM. All DENV-inoculated BLT-RAG-hu mice not only presented detectable antibody levels, but also showed neutralization against DENV-2. This was first seen in the RAG-hu mouse model (Kuruvilla, Troyer et al. 2007). In conclusion, this study showed that the RAG based BLT humanized mice are an immune competent model competent for DENV studies and have accelerated the field of dengue research regarding immunity.

CHAPTER 4

MOSQUITO-MEDIATED TRANSMISSION OF DENGUE VIRUS TO THE HUMANIZED BLT AND RAG-HU MOUSE MODELS

4.1 Introduction

Naive *A. aegypti* and *A. albopictus* mosquitoes become infected when they feed on a DENV- infected human host and transmit the virus to naïve human hosts (Mackenzie, Gubler et al. 2004). To study the natural transmission of DENV, an animal model of DENV disease that mimics the natural cycle of transmission of DENV from a mosquito vector to a human host is needed. We previously established DENV infection and human immune response through DENV inoculation in the humanized BLT and RAG-hu mouse models. Here we seek to use our humanized BLT and RAG-hu mice as a model to further understand natural DENV transmission and immune response.

4.2 Materials and Methods

4.2a CD34 Cells, Cell Culture and Viral Stocks

As described previously, CD34+ stem cells were isolated from human fetal liver tissue and cultured for 24 hours in a medium of cytokines: IL-3; IL-6; and SCF (Akkina, Rosenblatt et al. 1994; Bai, Gorantla et al. 2000). Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 3% heat inactivated fetal bovine serum (FBS). C6/36 cells were cultured in DMEM containing 10% FBS. LLC-MK2 cells were cultured in DMEM containing 7% FBS. DENV-2 Jamaica 1409 stock was generated through viral amplification in infected C6/36 cells using L-15, and titrated using a plaque assay (described below) in LLC-MK2 cells.

4.2b Generation and Engraftment Levels of RAG-hu and BLT Mice

To make Rag-hu mice, 10 week old $RAG^{-/-}\gamma_c^{-/-}$ mice were irradiated with a single dose of 3.0 Gy generated from a Cs-137 source. Rag-hu mice were injected intravenously with $5x10^5$-$1x10^6$ autologous human CD34+ hematopoietic progenitor cells. BLT mice were generated from $RAG^{-/-}\gamma_c^{-/-}$mice using techniques previously described (Melkus, Estes et al. 2006). Briefly, seven-week old $RAG1^{-/-}\gamma_c^{-/-}$ or $RAG2^{-/-}\gamma_c^{-/-}$ mice were irradiated with a single dose of 3.0 Gy generated from a Cs-137 source. At 1-3h post irradiation mice under anesthesia were surgically implanted with tiny pieces of human fetal liver and thymus tissue under the right kidney capsule. Mice were allowed to recover for 24h with pain management, after which they were injected intravenously with $5x10^5$-$1x10^6$ autologous human CD34+ hematopoietic progenitor cells isolated from fetal liver. RAG-hu and BLT mice were screened for human cell engraftment at 3-4 weeks post-reconstitution. Peripheral blood was collected by tail bleeds from transplanted mice, and red blood cells were lysed using the Whole Blood Erythrocyte Lysing Kit (R&D Systems) according to manufacturer's protocol. The leukocyte fraction was stained with antibodies against the human pan-leukocyte marker CD45 and analyzed by flow cytometry to verify human engraftment.

4.2c Dengue Injections of *A. aegypti* Mosquitoes

At 5 days post-eclosion, Adult female Rex-D *Aedes aegypti* mosquitoes were intrathoracically injected with 69 nL 0f DENV-2 Jamaica 1409 viral stock (100 pfu) using a Nanojet II (Drummond Scientific Company, Broomall, PA). All injections were performed under a dissecting microscope using glass needles prepared with a vertical pipette puller (P-30, Sutter

Instrument Co., Novato, CA). Mosquitoes were then incubated for 7 days at 28°C, 80% relative humidity and fed with sucrose and water.

4.2d Transmission of Dengue Virus from *A. aegypti* Mosquitoes to Naive Humanized Mice

Dengue-exposed humanized BLT and RAG-hu mice were anesthetized by an intraperitoneal injection of ketamine/xylazine and placed into small individual cages, each containing 40 individual 7 dpi i.t. inoculated mosquitoes (described above). Mosquitoes were allowed to feed freely for up to 30 minutes. For most animals, 100% of the mosquitoes became engorged within 20 minutes.

4.2eTransmission of Dengue Virus from Humanized Mice to Naive *A. aegypti* Mosquitoes

Dengue exposed humanized BLT and RAG-hu mice were anesthetized by an intraperitoneal injection of ketamine/xylazine and placed into small individual cages, each containing 40 individual naive mosquitoes. Mosquitoes were allowed to feed freely for up to 30 minutes. For most animals, 100% of the mosquitoes became engorged within 20 minutes.

4.2f Dengue Viral Isolation from Mosquitoes

Mosquitoes were individually collected and the whole mosquito was triturated in 1.0 ml of DMEM medium containing 7% FBS. Suspensions were centrifuged for 3 min at 8000 rpm and the supernatant was syringe filtered, using Acrodisc Syringe filters with 0.2 µm HT Tuffryn membrane. Resulting samples were stored at -80°C until assayed.

4.2g Plaque Titrations of Mosquito Viral Loads

LLC-MK2 cells were grown to confluent monolayers in 12-well plates. Ten-fold serial dilutions of whole-mosquito filtered supernatants were added for 1h. These cells were then overlaid with nutrient agar. After 11 days of incubation at 37°C and 5% CO_2, cells were stained with a solution of 3 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) added directly to the plate and incubated for 4 h (Takeuchi, Baba et al. 1991; Sladowski, Steer et al. 1993). Viral titers were determined by plaque counting.

4.2h QRT-PCR Analysis for Viral Load Determination

To verify infection and determine viral loads, qRT-PCR was conducted on total viral RNA isolated from mouse plasma using the QIAamp Viral RNA kit (Qiagen), according to the manufacturer's instructions. QRT-PCR was carried out using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) in a Bio-Rad CFX96 Real-Time System C1000 Thermal Cycler. PCR was initiated on 10µl viral RNA, using NS5F and NS5R DENV primers (Kong, Thay et al. 2006). Reaction conditions were as follows: 50°C for 20 min; 95°C for 5 min; 45 cycles of 95°C for 15 s, and 62°C for 30s. Levels of DENV RNA amplified by this one-step qRT-PCR method were determined though comparison to a standard curve by known quantities of DENV-2 16681 NS5 RNA, which was transcribed and quantified as previously described (Kong, Thay et al. 2006).

4.2i Detection of Human Anti-Dengue IgM and IgG Antibodies by ELISA

Levels of human anti-dengue antibodies present in humanized mouse serum were detected using the commercial PANBIO IgM and IgG capture ELISA kits. Sera collected from

BLT and RAG-hu mice were diluted in buffer provided with the kit at 1:20 dilution. Samples were considered positive if the OD_{450} was greater than two times the background absorbance level. Serum samples that produced a positive IgM or IgG reading at the 1:20 dilution were serially diluted further up to 1:1280 and assayed by ELISA.

4.2j Detection of Neutralizing Antibodies by a FACS-Based Assay

Anti-DENV-2 neutralizing capacity of sera obtained from humanized BLT and RAG-hu mice were determined using a FACS neutralization test (FNT) modified from a previously described method (Lambeth, White et al. 2005). Sera from a non-humanized $RAG^{-/-}\gamma_c^{-/-}$ mouse exposed to DENV-infected mosquitoes, a RAG-hu mouse exposed to naïve mosquitoes, and Vero cells exposed only to DENV were used as the negative controls for neutralization. Known human antibody positive, anti-DENV serum was used as a reactive control for neutralization. Briefly, the sera was diluted in 2% FBS-DMEM from a 1:20 dilution up to 1:640 dilution, and mixed with DENV-2 Philippines 10649 in a total volume of 400 µl. This 400 µl serum-virus mixture was incubated for 1 h at 37°C. This mixture was then added and allowed to infect a monolayer of $3x10^5$ Vero cells in a single well of a 12-well plate for 24h. Cells were stained using the 4G2 antibody and analyzed using an EPCIS XL-MCL flow cytometer (Coulter) as described above. FNT_{50} is the highest two-fold dilution of serum that inhibited 50% of DENV infection of Vero cells.

4.3 Results and Conclusions

4.3a Engraftment Levels in BLT and RAG-hu Mice

In this study both humanized BLT and RAG-hu mouse models were used. RAG-hu and BLT mice have been established for permitting multi-lineage human hematopoiesis in previous studies and earlier in this report (Traggiai, Chicha et al. 2004; Berges, Wheat et al. 2006). Human cell engraftment was determined by collecting peripheral blood six weeks post-engraftment and analyzed for the human pan-leukocyte marker CD45 by FACS analysis. BLT mice show human engraftment up to 93%, and RAG-hu mice show engraftment up to 92% (Table 4.1).

4.3b *A. aegypti* Mosquitoes Support Productive Dengue Viral Infection

Adult female Rex-D *A. aegypti* mosquitoes were intrathoracically injected with highly laboratory passaged DENV-2 Jamaica 1409. Naïve BLT and RAG-hu mice were anesthetized and exposed to the infected *A. aegypti* mosquitoes. These blood fed mosquitoes were titered by plaque assays. The infected mosquitoes ranged in titer from 3.08 to 4.64 Log10 plaque forming units (PFU) with the mean of about 4 PFU per mosquito as seen in figure 4.1 and table 4.2.

4.3c Dengue Viral Infection through Mosquito Transmission into Humanized Mice

To evaluate DENV viral transmission, BLT and RAG-hu mice were exposed to DENV-infected mosquitoes. Viremia was determined up to forty-two days post-exposure by qRT-PCR. Viral loads in plasma were detected as early as one week post-exposure, and were seen as late as forty-two days (Table 4.1 and Figure 4.2B). DENV infection was successfully detected in ten out eleven BLT mice, and ten out of twelve RAG-hu mice (Figure 4.2A). The viral loads in the

Table 4.1 Presence of Viremia (RNA copies/ml) as a Result of DENV Exposure

Mouse ID	Engr[a] %	Day 6	Day 9	Day 12	Day 18	Day 21	Day 24	Day 27	Day 30	Day 35	Day 42
BLT 147[b]	50	2.33E+02	<2.00E+02	6.97E+02	1.19E+03	<2.00E+02	9.22E+02	<2.00E+02	4.42E+03	1.15E+04	<2.00E+02
BLT 149	93	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	3.72E+02	1.34E+04	X[f]	X
BLT 150	86	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	2.53E+02	3.30E+02	8.26E+02	1.24E+04	X	X
BLT 188	63	4.62E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02
BLT 189	68	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	2.53E+02	<2.00E+02
BLT 242	85	<2.00E+02	2.84E+02	<2.00E+02	<2.00E+02	2.15E+02	X	X	X	X	X
BLT 243	83	2.65E+02	6.58E+02	5.08E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	2.80E+02	2.82E+02	2.74E+02
BLT 245	84	2.04E+02	3.94E+03	1.26E+03	2.23E+02	<2.00E+02	<2.00E+02	2.44E+02	2.28E+02	2.35E+02	5.93E+02
BLT 246	82	2.35E+02	2.64E+02	<2.00E+02	4.99E+02	9.92E+02	4.35E+02	4.07E+03	2.76E+03	<2.00E+02	<2.00E+02
BLT 252	81	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	X	X	X	X	X
BLT 254	72	2.39E+02	2.11E+02	3.13E+02	2.12E+03	<2.00E+02	<2.00E+02	2.31E+02	2.46E+02	2.30E+02	2.60E+02
J912[c]	85	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02
J913	68	<2.00E+02	<2.00E+02	<2.00E+02	2.48E+02	<2.00E+02	2.12E+02	2.25E+02	<2.00E+02	<2.00E+02	<2.00E+02
J920	92	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02
J1005	44	4.11E+02	4.39E+02	<2.00E+02	4.07E+02	<2.00E+02	<2.00E+02	<2.00E+02	2.15E+04	<2.00E+02	<2.00E+02
J1006	86	2.43E+02	<2.00E+02	<2.00E+02	2.83E+02	<2.00E+02	<2.00E+02	2.50E+02	2.22E+02	2.48E+02	4.27E+02
J1007	69	2.88E+02	<2.00E+02	1.32E+03	2.91E+02	<2.00E+02	2.22E+02	2.56E+02	<2.00E+02	2.97E+02	2.54E+02
J1054	85	2.72E+02	7.61E+02	2.73E+02	<2.00E+02	2.09E+02	2.45E+02	<2.00E+02	2.43E+02	<2.00E+02	<2.00E+02
J1055	90	3.84E+02	<2.00E+02	<2.00E+02	<2.00E+02	1.46E+02	<2.00E+02	2.14E+02	<2.00E+02	<2.00E+02	2.81E+02
J1057	75	2.48E+02	2.35E+02	2.16E+02	X	X	X	X	X	X	X
M931	87	2.26E+02	4.52E+02	3.91E+02	2.39E+02	<2.00E+02	<2.00E+02	<2.00E+02	2.47E+02	1.26E+03	2.44E+02
M1012	35	<2.00E+02	3.42E+02	2.78E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02
M1013	46	2.59E+02	<2.00E+02	<2.00E+02	<2.00E+02	2.93E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02
Mos Ug[d]	0	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02
M1011[e]	7	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02	<2.00E+02

[a] Percent human engraftment in peripheral blood.

[b] BLT147-254= BLT mice exposed to DENV2 infected mosquitoes

[c] J912-J1057, M931-M1013= RAG-hu mice exposed to DENV2 infected mosquitoes

[d] Mos UG= Unengrafted mouse exposed to DENV2 infected mosquitoes

[e] M1011= Rag-hu mouse exposed to naive mosquitoes

[f] X= Mortality

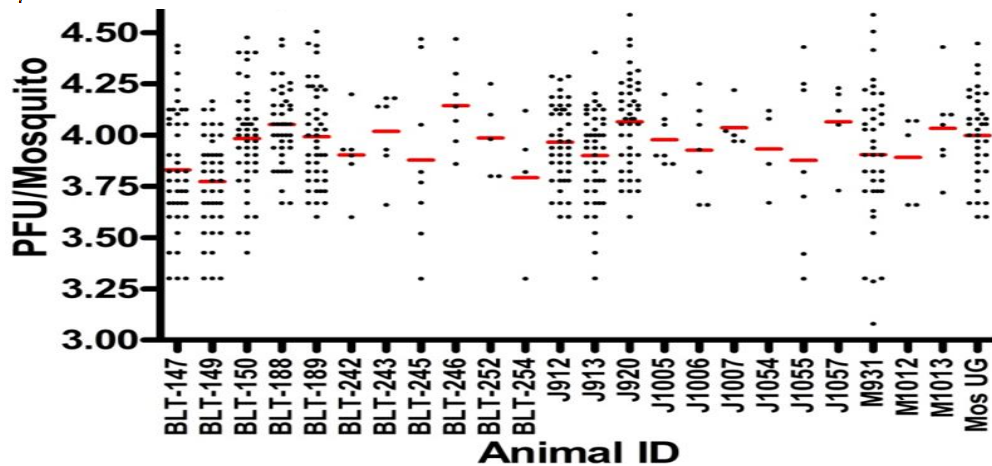

Figure 4.1 Titer of Mouse Fed DENV-Infected- Mosquitoes . Inoculated mosquitoes were used to transmit virus to mouse models. Titer of the individual mosquitoes per mouse recorded at values of Log10 PFU/mosquito.

Table 4.2 Titer of DENV-Infected Mosquitoes. Log10 PFU

Animal ID	Mean Titer	Max. Titer	Min. Titer
BLT-147[a]	3.83	4.44	3.30
BLT-149	3.77	4.17	3.30
BLT-150	3.98	4.48	3.43
BLT-188	4.05	4.64	3.67
BLT-189	3.99	4.51	3.60
BLT-242	3.90	4.20	3.60
BLT-243	4.02	4.18	3.66
BLT-245	3.88	4.47	3.30
BLT-246	4.14	4.47	3.86
BLT-252	3.99	4.25	3.80
BLT-254	3.79	4.12	3.30
J912[b]	3.97	4.29	3.60
J913	3.90	4.40	3.30
J920	4.07	4.59	3.60
J1005	3.98	4.20	3.86
J1006	3.93	4.25	3.66
J1007	4.04	4.22	3.97
J1054	3.93	4.12	3.67
J1055	3.88	4.43	3.30
J1057	4.07	4.23	3.73
M931	3.91	4.59	3.08
M1012	3.89	4.07	3.66
M1013	4.03	4.43	3.72
Mos UG[c]	4.00	4.45	3.60

[a]BLT147-254=BLT DENV-2 exposed mice
[b]J912-M1013=Rag-hu DENV-2 exposed mice
[c]Mos UG= Unengrafted DENV-2 exposed mouse
Log10 PFU

plasma reached as high as 1.34×10^4 copies/ml in BLT mice and 2.15×10^4 copies/ml in RAG-hu mice (Table 4.1). DENV-exposed BLT and RAG-hu mice are shown in log value as seen in figure 4.2B. Plasma viral load of DENV-exposed BLT mice increased and peaked at thirty days post-exposure at an average log value of 2, and declines up to forty-two days post-exposure around a value of 1 log. Plasma viral load of DENV exposed RAG-hu mice fairly remained consistent at an average value of 1 log. Infection rate in these mice may be variable due to specific characteristics caused by creating mice from tissues from different hosts. These results

establish that DENV-2 Jamaica 1409 is transmitted to BLT and RAG-hu mice through the bite of an infected mosquito, with the majority of the mice becoming infected.

4.3d Antibody Production and Dengue Neutralization in Humanized Mice

As discussed before, since both the BLT and RAG-hu mouse models have the ability to mount an immune response against DENV inoculation, we evaluated if mosquito-mediated transmission of DENV leads to a similar response. Sera collected from DENV-exposed mice was subjected to capture ELISA to detect for human anti-DENV IgM and IgG antibody levels. Antibody levels were tested from six to twenty-one weeks post-exposure, or at mortem (Table 4.3). Due to mortality, some mice died prior to antibody testing. All DENV-exposed humanized

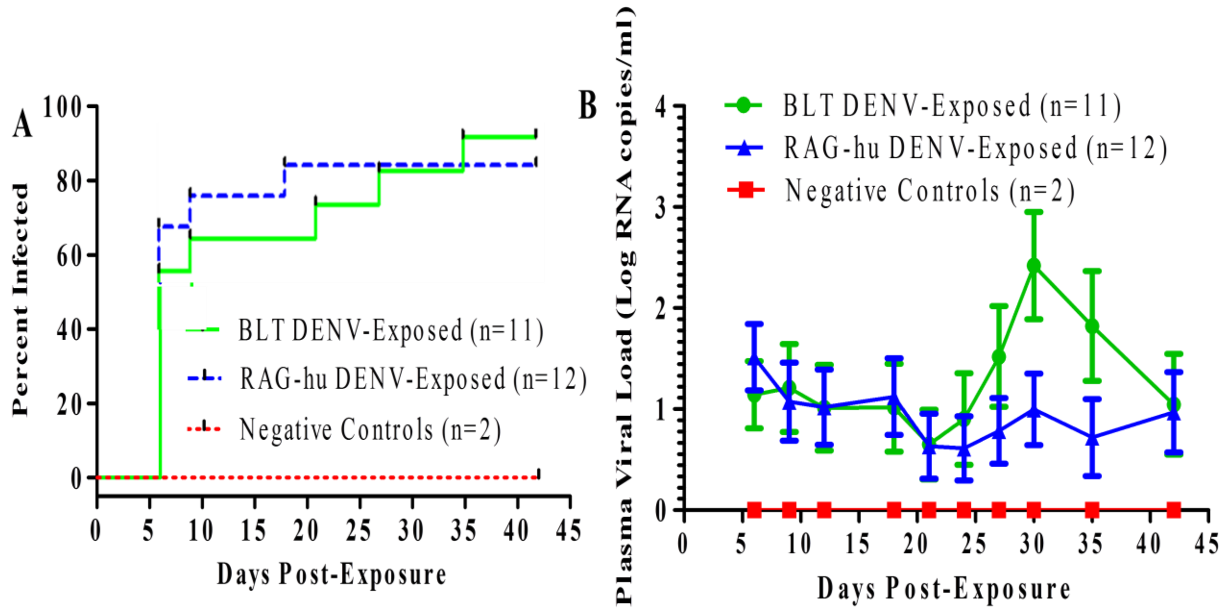

Figure 4.2. Majority of Mice Infected as a Result of Exposure to DENV-2 Infected Mosquitoes A) BLT and RAG-hu mice were exposed with DENV-2 Jamaica through infected *A. egyptii* mosquitoes. Plasma was collected from infected mice and infection determined by qRT-PCR. Kaplan-Meier plots of time course of appearance of viremia in exposed vs. non-exposed mice. **B)**Dengue viral load was monitored days post-exposure as indicated by qRT-PCR. In DENV-exposed BLT mice, levels peak at 30 days post exposure and last until 42 days post-exposure. Viral levels in DENV-exposed RAG-hu mice remain fairly constant and last until 42 days post-exposure.

mice tested for antibody production developed human anti-DENV antibody responses. Human anti-DENV IgM was detected in all BLT and RAG-hu DENV-exposed mice. IgM levels were detected up to twenty-one weeks post-exposure in BLT mice, and nineteen weeks post-exposure in RAG-hu mice. Human anti-DENV IgG was detected in only two BLT and three RAG-hu DENV-exposed mice. IgG levels were only seen at one time point per mouse, demonstrating

Table 4.3 DENV-Exposed Humanized Mice Developed Anti-DENV Antibody Responses

Mouse ID	Mortem	6 weeks	7weeks	8 weeks	9 weeks	10 weeks	11 weeks	12 weeks	13 weeks	14 weeks	15 weeks	16 weeks	17 weeks	18 weeks	19 weeks	20 weeks	21 weeks
BLT 147[a]	Na[e]	IgM + Neg[f]	IgM + Neg	IgM ++ Neg	IgM + Neg	Neg	IgM + Neg	IgM ++ Neg	IgM ++ IgG +	IgM + Neg	Neg	Neg	NA	NA	NA	NA	NA
BLT 149	5 wpi IgM +	X[g]	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
BLT 150	5 wpi IgM +	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
BLT 188	NA	Neg	Neg	Neg	Neg	IgM + Neg	X	X	X	X	X	X	X	X	X	X	X
BLT 189	NA	Neg	Neg	Neg	Neg	Neg	Neg	Neg	IgM + Neg	IgM + Neg	Neg	Neg	NA	NA	NA	NA	NA
BLT 242	NA	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
BLT 243	NA	NA	NA	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM ++ Neg	IgM ++ Neg	IgM + Neg
BLT 245	NA	NA	NA	IgM + Neg	X	X	X	X	X	X	X	X	X	X	X	X	X
BLT 246	NA	NA	NA	IgM +++ Neg	IgM +++ Neg	IgM +++ Neg	IgM ++ Neg	X	X	X	X	X	X	X	X	X	X
BLT 252	3 wpi IgM +	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
BLT 254	NA	NA	IgM + Neg	Neg	IgM ++ Neg	IgM + Neg	IgM + Neg	X	X	X	X	X	X	X	X	X	X
J912[b]	3 wpi Neg	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
J913	NA	IgM + Neg	Neg	IgM + Neg	IgM + Neg	Neg	IgM + IgG +	IgM + Neg	IgM + Neg	IgM + Neg	Neg	Neg	NA	NA	NA	NA	NA
J920	NA	NA	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + IgG +	Neg	IgM + Neg	Neg	Neg	NA	NA	NA	NA	NA
J1005	NA	NA	NA	IgM ++ Neg	IgM ++ Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	Neg	Neg
J1006	NA	NA	NA	Neg	Neg	Neg	Neg	Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	Neg	Neg	Neg
J1007	NA	NA	NA	Neg	Neg	Neg	Neg	Neg	Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM + Neg	Neg	Neg	Neg	Neg
J1054	NA	NA	IgM ++++ Neg	IgM ++++ Neg	IgM + Neg	IgM ++ Neg	IgM ++ Neg	IgM ++ Neg	IgM ++ Neg	IgM +++ Neg	IgM ++ Neg	IgM ++ Neg	IgM ++ Neg	IgM + Neg	Neg	Neg	Neg
J1055	NA	NA	IgM + Neg	Neg	Neg	IgM + Neg	IgM + Neg	IgM + Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
J1057	NA	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
M931	NA	Neg	Neg	IgM ++ Neg	IgM + Neg	IgM + Neg	IgM + Neg	IgM ++ Neg	Neg	IgM ++ Neg	IgM +++ Neg	Neg	NA	NA	NA	NA	NA
M1012	NA	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
M1013	5 wpi Neg	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Mos Ug[c]	NA	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
M1011[d]	NA	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

[a] Percent human engraftment in peripheral blood.
[a] BLT147-254= BLT mice exposed to DENV2 infected mosquitoes
[b] J912-J1057, M931-M1013= RAG-hu mice exposed to DENV2 infected mosquitoes
[c] Mos UG= Unengrafted mouse exposed to DENV2 infected mosquitoes
[d] M1011= Rag-hu mouse exposed to naive mosquitoes
[e] NA= Not applicable/ not tested
[f] Neg= Antibody(s) level not detectable
[g] X= Mortality
+=2-fold, ++=4-fold, +++=8-fold, and ++++16-fold greater than background absorbance

limited IgG production. The non-humanized exposed and RAG-hu non-exposed control mice did not produce any human anti-DENV IgM or IgG antibodies.

To determine neutralizing capability of serum from DENV-exposed BLT and RAG-hu mice, a flow cytometry based assay was used to measure 50% inhibition of DENV-2 Philippines 10649. Our results showed that all BLT and RAG-hu antibody producing mice were able to neutralize DENV infection in cell culture (Table 4.4). 50% neutralization of DENV-2 in both

Table 4.4 Sera from DENV-Exposed Mice have Neutralizing Capabilites

Mouse ID	Weeks PE[a]	IgM titer	IgG titer	FNT_{50}[g] titer
BLT 147[b]	11-14	1:160	1:20	1:320
BLT 149	5	1:160	<1:10	1:40
BLT 150	5	1:80	<1:10	1:40
BLT 188	10	1:80	<1:10	1:40
BLT 189	11,13-14	1:80	1:30	1:160
BLT 242	x[f]	x	x	x
BLT 243	16-19	1:160	<1:10	1:320
BLT 245	8	1:80	<1:10	1:40
BLT 246	8-11	1:640	<1:10	1:160
BLT 252	3	1:80	<1:10	1:80
BLT 254	8-11	1:80	<1:10	1:40
J912[c]	x	x	x	x
J913	11-14	1:80	1:20	1:320
J920	9-12	1:80	1:20	1:40
J1005	8-11	1:160	<1:10	1:160
J1006	14-15	1:80	<1:10	1:40
J1007	9-10	1:80	<1:10	1:40
J1054	13-16	1:160	<1:10	1:160
J1055	11-12	1:80	<1:10	1:160
J1057	x	x	x	x
M931	11-15	1:160	1:20	1:160
M1012	x	x	x	x
M1013	5	1:640	<1:10	1:40
Mos Ug[d]	8-11	<1:10	<1:10	<1:10
M1011[e]	8-11	<1:10	<1:10	<1:10

[a] PE= Post-Exposure

[b] BLT147-254= BLT mice exposed to DENV2 infected mosquitoes

[c] J912-J1057, M931-M1013= RAG-hu mice exposed to DENV2 infected mosquitoes

[d] Mos UG= Unengrafted mouse exposed to DENV2 infected mosquitoes

[e] M1011= Rag-hu mouse exposed to naive mosquitoes

[f] X= Mortality

[g] FNT_{50}= The serum dilution that inhibited 50% of DENV infection

DENV-exposed BLT and RAG-hu mice ranged from a 1:40 to 1:320 dilution of the serum. However, there was no significant correlation between high levels of human anti-DENV IgM or IgG correlating with an increased capacity to neutralize. Overall, antibody production and neutralization was seen in all DENV-exposed BLT and RAG-hu mice.

4.3e Infection of Naïve A. aegypti Mosquitoes through Reverse Transmission from Dengue Infected BLT and RAG-hu Mice

To evaluate if the complete natural transmission cycle of DENV can be recapitulated using this model, we tested for reverse transmission of DENV from infected mice to naïve mosquitoes. Naïve *A. aegypti* mosquitoes were allowed to feed on BLT and RAG-hu mice two weeks after exposure to infected mosquitoes. These blood fed mosquitoes were titered for DENV by plaque assays to validate reverse transmission. Reverse transmission was seen in a naïve mosquito that fed on a DENV-exposed BLT mouse (BLT 188) with a titer of 4.27 Log10 PFU and a mosquito that fed on one DENV exposed RAG-hu mouse (m931) with a titer of 4.19 Log10 PFU as seen in table 4.5. In those two cases, we saw the ability for reverse transmission through the infection of two naïve mosquitoes that fed on DENV-2 exposed humanized mice. This illustrates the complete cycle for the natural transmission of DENV, solidifying the BLT and RAG-hu mice as complete natural transmission models.

Table 4.5 DENV Titer in Mosquitoes Infected by Reverse Transmission. Log10 PFU

Animal ID	Titer
BLT 188	4.27
M931	4.19

4.4 Discussion

The natural transmission cycle of DENV includes naive *A. aegypti* and *A. albopictus* mosquitoes transmitting virus to naïve human hosts (Mackenzie, Gubler et al. 2004). To address DENV transmission, the humanized NSG mouse has been used and was shown to produce viremia, erythema, and cytokine production, but had limited antibody production and virus neutralization (Cox J, Mota J. 2012). Here we used the humanized BLT-RAG-hu and RAG-hu mice to mimic natural dengue viral transmission, pathogenesis and immune response.

Our results demonstrate transmission of DENV from infected *A. aegypti* mosquitoes to humanized BLT-RAG-hu and RAG-hu mice. The infected mosquitoes ranged in titer from 3.08 to 4.64 Log10 PFU with the mean of about 4 Log10 PFU per mosquito. Viral loads in BLT-RAG-hu and RAG-hu mice reached as high as 2.15×10^4 copies /ml. These plasma viral loads are similar to what was reported in the humanized BLT-NSG mouse model (Mota and Rico-Hesse 2011). The RAG-hu mice showed a peak in viremia at thirty days post-exposure, which is different than the typical peak in humans at the onset of illness (Eram, Setyabudi et al. 1979), but is similar to the peak at thirty days post-infection from the mosquito transmission to the NSG mice (Mota and Rico-Hesse 2011). Viremia also lasted up to forty-two days post-exposure, which is longer that the three-to-fourteen days post-infection period of viremia in humans (Eram, Setyabudi et al. 1979). However, this is similar to what was seen in DENV-inoculated BLT mice reported here and what was seen in NSG mice (Mota and Rico-Hesse 2011). Some DENV-exposed BLT-RAG-hu and RAG-hu mice showed an antibody response, but no detectable levels of viremia. This may be due to viremia below the detection limit.

Both human DENV IgM and IgG were observed in DENV-exposed BLT-RAG-hu and RAG-hu mice, with a high IgM production but low IgG. This is similar to but more consistent than what was seen in the DENV-inoculated RAG-hu mouse model (Kuruvilla, Troyer et al. 2007), and similar to what we previously showed in the IP/SC DENV-inoculated BLT mouse model. IgG production was only seen in one BLT and two RAG-hu DENV-exposed mice with limited IgG production, which is similar to reported results with other models (Jaiswal, Pazoles et al. 2012). All DENV-exposed BLT and RAG-hu mice also showed neutralization against DENV-2, which was seen in the RAG-hu mouse model to a certain extent (Kuruvilla, Troyer et al. 2007). Lastly, in two cases we saw reverse transmission with two naïve mosquitoes testing dengue positive after feeding on DENV-2 infected humanized mice. In conclusion, this study shows that BLT and RAG-hu mice are immune competent models that are capable of modeling natural transmission of dengue virus. This exciting aspect opens the door to future model studies regarding vector competence and intervention.

CHAPTER 5

DENGUE MULITIPLEX qRT-PCR

5.1 Introduction

DENV consists of four serotypes that are phylogenetically related and antigenically cross reactive. This aspect makes it difficult to specifically identify the etiological serotype in active infection (Rico-Hesse 1990; Holmes 2006). However, known differences in nucleic acid sequences can be utilized to distinguish each serotype. In this regard, nested reverse transcription PCR (nested rt-PCR) and quantitative real-time PCR (qRT-PCR) are capable of specific sequence amplification, thus differentiating between viral serotypes. Nested rt-PCR is more sensitive than qRT-PCR, due to a second round of amplification. However, qRT-PCR has a lower background since the second round of amplification is not needed, and has a much lower risk for contamination. QRT-PCR is able to detect as low as 40 copies of target sequences, making this a highly efficient method for the detection of virus in low viral loads or in small sample volumes (Callahan, Wu et al. 2001). QRT-PCR can also be used for the detection of multiple targets in a single sample by the use of specific probes with different flourophores. In this regard, a fourplex qRT-PCR has been previously established to detect the four serotypes of DENV (Johnson, Russell et al. 2005).

A DENV fourplex reaction contains four sets of primer pairs, and four probes labeled with different fluorophores, each specific for a dengue serotype 1-4. In this single qRT-PCR reaction, each serotype was successfully detected and quantified. To further simplify serotype detection in a single reaction, a multiplex qRT-PCR, which utilizes a common primer pair but has serotype specific probes, has already been established (Kong, Thay et al. 2006). However,

this multiplex was unable to efficiently quantify and/or detect all laboratory strains of DENV. Here, we sought to improve the DENV multiplex qRT-PCR in order to distinguish all four serotypes of DENV, including all laboratory strains, in a single reaction.

5.2 Materials and Methods

5.2a Cell Culture and Virus Strains

C6/36 cells were cultured in either complete Dulbecco's modified Eagle's medium (DMEM) containing 10% heat inactivated fetal bovine serum (FBS) or complete L-15 medium (Leibovitz) with 3% FBS. Cells were plated and allowed to grow to 60-75% confluence before being inoculated with 20 M.O.I. of DENV. Virus was allowed to infect cells for 48 hours and supernatant was collected daily and subjected to 0.4micron filtration. Dengue virus was titered in Vero cells by staining infected cells with a 4G2 antibody conjugated with Alexa-488, and assayed using flow cytometry as previously described (Lambeth, White et al. 2005).

5.2b Probe Design

The NS5 regions of DENV-4 H241, Indonesia 1036 and D80-96 were separately cloned into plasmid vectors, grown up in E. *coli* cells, and sent for sequencing using the TOPO Cloning Kit for Sequencing, and according to the manufacturer's instructions. These sequences were put into a multiple sequence alignment with the Basic Local Alignment Search Tool (BLAST) against The National Center for Biotechnology Information (NCBI) DENV-4 H241(Accession # AY947539) full genome, to align the NS5 region of all three DENV-4 strains. Based on the commonality of the NS5 region between all three DENV-4 strains and keeping in mind to prevent cross reactivity with other serotypes, a DENV-4 probe was designed (5′-HEX-

CTTGGAGGACATAGATAAGAGGGATGGA). The other dengue serotype primer and probe sets were previous described (Kong 2006).

5.2c RNA Extraction and QRT-PCR Analysis

Total viral RNA was isolated from stock viral samples using the QIAamp Viral RNA kit (Qiagen) according to the manufacturer's instructions. In multiplex reactions, qRT-PCR was carried out using the qScript One-Step Fast qRT-PCR Kit (Quanta) in a Bio-Rad CFX96 Real-Time System C1000 Thermal Cycler. PCR was initiated on 5µl viral RNA, using NS5 forward (5′-GGAAGGAGAAGGACTGCACA) and NS5 reverse (5′-ATTCTTGTGTCCCATCCTGCT) DENV primer set, and the 4 probes (Kong, Thay et al. 2006). Sequences of primers and DENV1-3 probes have been previously reported (Kong, Thay et al. 2006). The DENV-1 probe (5′-CTCAGAGACATATCAAAGATTCCCGGG) was labeled with 6-carboxyfluorescein (FAM) at the 5' end; the DENV-2 probe (5′-TAAGAGACGTGAGCAAGAAAGAGGGAGGAG) was labeled with texas red; the DENV-3 probe (5′-ACATTTCCAAGATACCCGGAGGAG) was labeled with Cy5; and the DENV-4 probe (5′-CTTGGAGGACATAGATAAGAGGGATGGA) was labeled with HEX. Reaction conditions were as follows: 50°C for 20 min; 95°C for 5 min; 45 cycles of 95°C for 15 s, and 60°C for 30s. Levels of DENV RNA amplified by this one-step qRT-PCR method were determined though comparison to a standard curve by known quantities of DENV-1Thailand 16007, DENV-2 Philippines, DENV-3 Philippines H87, and DENV-4 D80-96 RNA, which were transcribed and quantified as previously described (Kong, Thay et al. 2006). Viral RNA standards were made for each serotype, and used to test an established primer and probe set specific for the NS5 region of the genome (Kong, Thay et al. 2006).

5.2d Mixed Dengue Virus Infection of RAG-hu Mice

A RAG-hu mouse was inoculated with all four serotypes by a 50% intraperitoneal and 50% subcutaneous injection of DENV1- R98944a; DENV2- Sri Lanka, AHF, Thai , Philippines 10649, Jamaica 1409; DENV3-SL 3006 , SL3009 ,H87; and DENV4-D80-96 THA. To verify infection and determine viral loads using qRT-PCR, viral RNA was extracted from mouse plasma using the QIAamp Viral RNA kit (Qiagen) according to the manufacturer's instructions.

5.3 Results and Conclusions

5.3a Detection of Dengue Serotypes Using Multiplex QRT-PCR

To detect all four DENV serotypes in a single reaction and to quantify viral levels, a multiplex qRT-PCR was optimized. Figure 5.1 shows the alignment of DENV 1-4 NS5 region, indicating the binding region of the forward and reverse NS5 primers and serotype-specific probes. This reaction was capable of detecting the four serotypes of DENV as illustrated in figure 5.2. However, the reaction was only able to detect the D80-96 strain of DENV-4 and not other DENV-4 strains. Importantly, the DENV-specific probes did not cross react with the other serotypes (data not shown).

Forward NS5 Primer starts at__bp		Accession #	Forward NS5 Primer / DENV 1-4 Probes	Probes:
9091	DENV-1	AF180817	ctcagtggagtggaaggagaaggactccacaaacttggatacatactcagagacatatca	DSQ1JK:FAM
9068	DENV-2	FJ906958	ttgagtggagtggaaggagaagggctgcacaagctaggttacattctaagagacgtgagc	DSQ2JK:TR
9082	DENV-3	M93130	tacagtggagtagaaggagaaggactgcacaagctgggatacatcttgagagatatttcc	DSQ3JK:CY5
9075	DENV-4	AY947539	tggagtggagtggaaggggaaggtctgcatagattgggatatatcctggaggacatagac	DSQ4JK:HEX

	Accession #	Reverse NS5 Primer
DENV-1	AF180817	aggattccagggggaacatgtatgcagatgacacagccggatgggacacaagaataaca
DENV-2	FJ906958	aagaaagagggaggagccatgtatgccgatgacacagcaggatgggacacaagaatcaca
DENV-3	M93130	aagatacccggaggagccatgtatgctgatgacacagccggttgggacacaagaataaca
DENV-4	AY947539	aagagggatggagacctgatatatgctgatgacacagctggttgggacacaagaatcact

Figure 5.1 DENV 1-4 Alignment at the NS5 Region. DENV stains DENV-1 Thailand 16007, DENV-2 Thailand AHF, DENV-3 Philippines H87, and DENV-4 241 were aligned. The same NS5 primer set for all four serotypes is seen in red, probes are seen in orange, and blue indicates a nucleotide that would not base pair to the probe.

We next proceeded to test if multiplex qRT-PCR, with the NS5 primer and probe set, could detect the viral loads and serotypes in a mixed DENV-infected mouse. A RAG-hu mouse was chosen with 90% human cell engraftment, determined by collecting peripheral blood six weeks post-engraftment and analyzed for the human pan-leukocyte marker CD45 by FACS analysis(Table 5.1). The RAG-hu mouse was inoculated with all four serotypes through an intraperitoneal and subcutaneous injection.

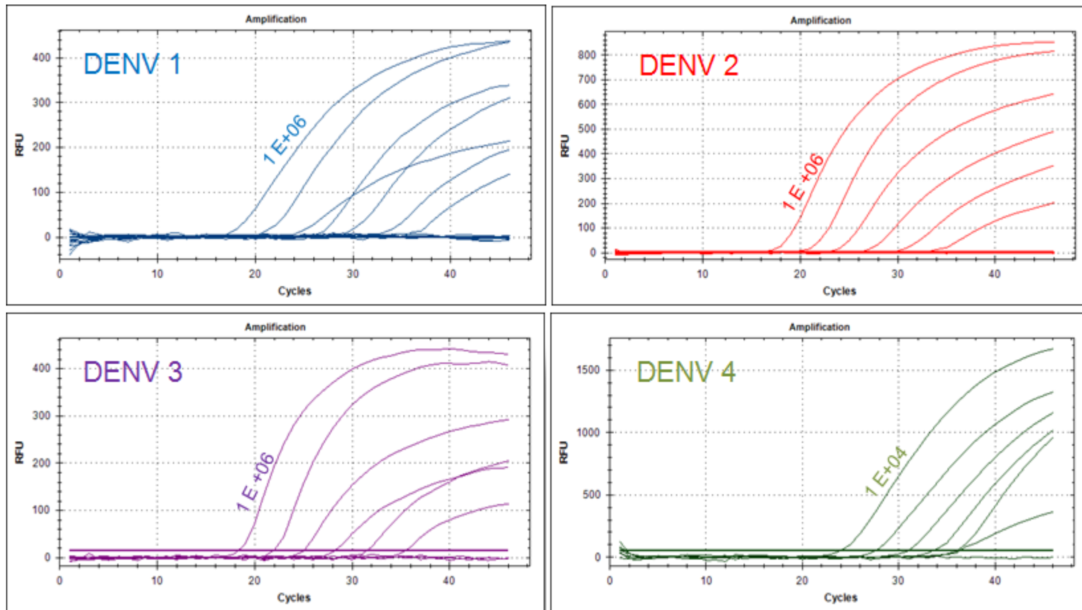

Figure 5.2 DENV 1-4 Detection and Quantification using Multiplex qRT-PCR. DENV 1-4 standards at similar titers were combined in a single reaction and viral levels were detected using multiplex.

Table 5.1 Presence of DENV1-4 Viremia (RNA copies/ml) in Mixed-Inoculated Mouse

Mouse ID	Engr[a] %	Serotype	Day 2	Day 4	Day 6	Day 8	Day 11	Day 13	Day 20	Day 22	Day 26	Day 27	Day 29	Day 35	Day 42
M1029	90	DENV-1	<4.00E+01[b]	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	3.39E+06	<4.00E+01	3.09E+04	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01
		DENV-2	5.27E+02	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	<4.00E+01	9.69E+02	6.67E+02	9.84E+02	8.34E+02	1.02E+03	<4.00E+01
		DENV-3	<4.00E+01	<4.00E+01	<4.00E+01	3.64E+02	<4.00E+01	<4.00E+01	6.15E+02	8.89E+01	<4.00E+01	<4.00E+01	7.74E+02	<4.00E+01	<4.00E+01
		DENV-4	2.87E+02	4.18E+02	3.00E+02	4.43E+02	<4.00E+01	2.30E+03	5.85E+02	1.04E+03	4.45E+02	2.71E+03	3.97E+02	4.44E+02	3.32E+02

[a] Percent human engraftment in peripheral blood.

[b] <4.00E+01= Falls below the detection limit

Viral RNA was extracted from mouse plasma at regular intervals up to forty-two days post-inoculation. Using multiplex qRT-PCR viral RNA from all four DENV serotypes was detected (Table 5.1 and Figure 5.3). However, some inconsistencies existed. Not all time points were positive for each serotype and time points were sporadic. This may be due to viremia below the detection limit of forty copies per ml. . DENV-1 was detected at only two time points, whereas DENV 2-4 showed a more frequent detection. DENV-1 was first detected at twenty days post-inoculation, DENV-2 and DENV-4 were first detected at two days post-inoculation, and DENV-3 was first detected at eight days post-inoculation. Overall, here we have illustrated the functionality of a dengue multiplex qRT-PCR for the detection of all four serotypes in a viral mixed sample. This improved assay is capable of detecting most strains of DENV; however, some isotypes of DENV-4 still need to be evaluated.

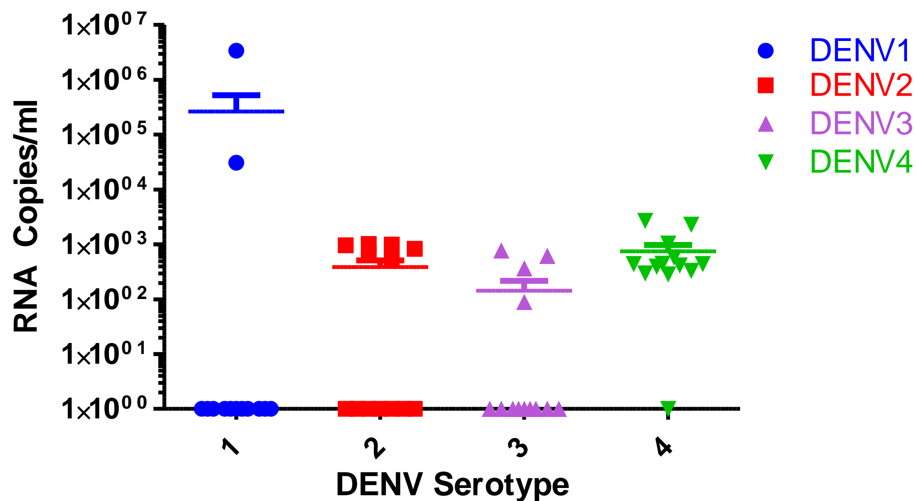

Figure 5.3 DENV 1-4 Detected by Multiplex qRT-PCR in a RAG-hu Mixed Infected Mouse. DENV 1-4 levels were detected using a multiplex qRT-PCR from viral RNA extracted from a RAG-hu mouse injected with all four DENV serotypes.

CHAPTER 6

SUMMARY AND FUTURE CONSIDERATIONS

In the studies described herein, we sought to advance DENV research through humanized mouse models. These models closely mimic human DENV infection, the natural transmission cycle of DENV, and can be used to simulate mixed infections for the development of a multiplex assay. Before utilizing the mice, high titer stocks of virus were made for each isotype including highly laboratory passaged (DENV-2 Jamaica 1409), and primary low passage isolates (DENV-2 Philippines 10649) for the studies conducted in this thesis and for future work.

We established the humanized BLT-RAG-hu (BLT) mouse model as suitable for studying DENV infection with production of human anti-DENV IgM and IgG antibodies capable of virus neutralization. BLT mice have human hematopoiesis with high engraftments in peripheral blood, including B and T cells, as well as dendritic cells in the bone marrow, human thymus tissue, and the spleen. BLT mice are susceptible to infection by DENV as evidenced by detectable levels of viral RNA in plasma. Viremia levels were detected within fifteen days post-inoculation in eleven out of sixteen or 70% of humanized BLT mice. The viral loads in the plasma reached as high as 3.78×10^4 copies/ml with levels peaking at fifteen days post-inoculation, and lasting forty-three days post-inoculation. We observed weight loss in DENV-inoculated BLT mice, but no symptoms of pathology indicative of DENV specific disease. We observed anti-DENV human IgM antibodies in all DENV-inoculated BLT mice from four-to-twenty weeks post-inoculation. Anti-DENV IgG production was detected in five out of sixteen mice. We observed low DENV viral loads correlating with a high level of IgM. All DENV-inoculated BLT mice not only presented detectable antibody levels, but also showed

neutralization against DENV-2. In conclusion, this study showed that BLT mice are an immune competent model viable for DENV studies.

We next determined if Rex-D *A. aegypti* mosquitoes are capable of DENV transmission to humanized BLT and RAG-hu mice. Rex-D *A. aegypti* mosquitoes were infected through intrathoracic injections, resulting in a mean titer of about 4 Log10 PFU per mosquito. After exposure to infected mosquitoes, DENV levels were detectable in the majority of BLT and RAG-hu mice. Viral loads in the plasma reached as high as 2.15×10^4 copies/ml, was first detected at six days post-exposure, and lasted up to forty-two days post-exposure. We observed anti-DENV human IgM antibodies in all DENV-exposed BLT and RAG-hu mice, detected as early as three weeks and up to twenty-one weeks post-exposure. IgG levels were detected in only one BLT and two RAG-hu DENV-exposed mice. Neutralization against DENV-2 was seen in all DENV-exposed BLT and RAG-hu mice. Lastly, in two cases we saw the ability for reverse transmission through the infection of two naïve mosquitoes that fed on DENV-2 exposed humanized mice, which complete the cycle for the natural transmission of DENV. In conclusion, this study showed that BLT and RAG-hu mice are immune competent models viable for DENV studies and with *A. aegypti* mosquitoes, are suitable to mimic the natural route of DENV transmission, solidifying their use in studies with regard to vector competence and intervention.

Lastly, we sought to use a multiplex qRT-PCR assay to detect all four DENV serotypes in a single reaction and to quantify viral levels. We were able to detect and quantify the four serotypes of DENV, with serotype specific probes not cross reacting. We were also able to detect the viral loads and serotypes in a mixed DENV-inoculated RAG-hu mouse. Overall, here we have illustrated the functionality of a dengue multiplex qRT-PCR for the detection of all four

serotypes in a multiplex reaction. This improved assay is capable of detecting most strains of DENV; however, some isotypes of DENV-4 still need to be evaluated.

Future studies may focus on evaluating inoculations of DENV 1, 3 and 4 serotypes in the humanized BLT mouse to further establish this model for DENV infection and immune response. Multiplex qRT-PCR can be further developed for detection of all strains of DENV, and can be used for quantifying viral loads in mixed infection studies. DENV 1, 3, and 4 serotypes can be established in transmission with Rex-D *A. aegypti* mosquitoes to BLT and RAG-hu mice, or to further establish a model for reverse transmission studies. Other future studies of DENV infection and immune response in these humanized mouse models may focus on testing for human cytokine and chemokine production, reduction in platelet levels, detection of DENV infected cells in tissues within the mouse model, and detection of the specific lymphocytes that become infected. Future studies with the BLT and RAG-hu mouse models may also focus on evaluating secondary infections, and even to test vaccines against DENV prior to clinical trials. Overall, our studies showed that BLT and RAG-hu mice are immune competent models suitable for future DENV studies, including DENV insect-mediated transmission and DENV insect reverse transmission. In addition, the humanized mouse model will permit studies of mixed infections.

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