DISSERTATION

DENGUE VIRAL PATHOGENESIS AND IMMUNE RESPONSE IN HUMANIZED RAG2$^{-/-}\gamma_c^{-/-}$ (RAG-hu) MICE

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JES G. KURUVILLA ENTITLED "DENGUE VIRAL PATHOGENESIS AND IMMUNE RESPONSE IN HUMANIZED RAG2$^{-/-}\gamma_c^{-/-}$ (RAG-hu) MICE" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

DENGUE VIRAL PATHOGENESIS AND IMMUNE RESPONSE IN HUMANIZED RAG2$^{-/-}\gamma_c^{-/-}$ (RAG-hu) MICE

Dengue viral pathogenesis and vaccine studies are hampered by the lack of an ideal animal model mimicking human disease and eliciting an adaptive immune response. We utilized a novel mouse model that permits multi-lineage human hematopoiesis and immune response following transplantation with human hematopoietic stem cells. To generate immuno-competent humanized mice, we xenografted neonatal Rag2$^{-/-}\gamma_c^{-/-}$ mice with human CD34+ hematopoietic stem cells, resulting in the de novo development of functional human adaptive immune system. To evaluate susceptibility to dengue viral infection, humanized mice were challenged with dengue virus (DENV). We observed viremia that lasted three weeks post infection with all four serotypes of dengue virus. We also demonstrated the presence of human anti-dengue antibodies. Anti-dengue IgM was first detected at 2 weeks post infection followed by IgG at 6 weeks. Our results demonstrated for the first time that humanized mice were capable of dengue viral primary immune responses, thus paving the way for further dengue immunopathogenesis studies. We challenged the mice that survived a primary infection with a secondary infection of dengue virus. We report increased viremia, severe dehydration, hemorrhage and morbidity in several mice during a secondary infection. We demonstrate the first instance of dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS) like phenomenon reported in any humanized mouse model for dengue infections. We also report the identification of cells that are actively infected with DENV in the spleen and lymph

nodes of these mice using *in situ* hybridization and immunofluoresence staining methods. We used a SCID-hu model with an engrafted human thymus to study the direct infection of T-cells by dengue virus. We demonstrate that T-cells and CD34+ hematopoietic stem cells do not support a dengue viral infection. We were also able to infect monocytes and dendritic cells that were isolated and developed from human peripheral blood *in vitro*. In order to develop a consistent assay for antibody dependent enhancement (ADE) of dengue infection we were able to infect macrophages that were differentiated *in vitro* from CD34+ hematopoietic stem cells. In summary, this model provides a new tool to further our understanding of a complex disease like dengue hemorrhagic fever and dengue shock syndrome as well as provide a new system to test new vaccines as well as therapeutics against dengue viruses in the human context.

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Chapter 1

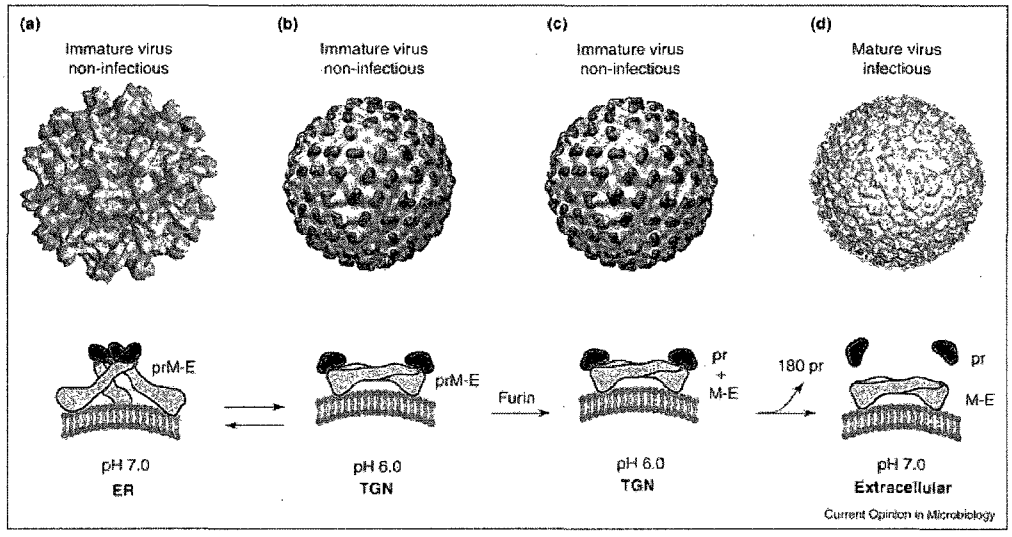
Overview of the Literature

1.1 Genomic structure and molecular organization of Dengue viruses

The dengue virus (DENV) is a single positive stranded RNA genome virus. It belongs to the Flaviviridae family which includes West Nile virus (WNV), Japanese encephalitis virus (JEV), hepatitis C. The virus is the etiological agent of dengue fever and is transmitted to humans by an infected mosquito host (Gubler, 1998a). There are four serotypes of the virus that have been isolated. Phylogenetically these serotypes are relatively distantly related yet they generate an immune response that cross reacts amongst the four serotypes, leading them to be grouped collectively as DENV 1-4 (DENV-1, 2, 3, 4) (Holmes, 2006; Rico-Hesse,1990). All four serotypes of DENV have been isolated from tropical and sub-tropical regions all over the world (Gubler, 1998a). Asiatic strains isolated from South-Asian epidemics are considered to cause a more aggressive disease as compared to those isolated from the Americas (Clyde et al., 2006; Cologna and Rico-Hesse, 2003; Rodrigo et al., 2009; Thomas et al., 2003).

DENV exists as an immature particle of 50nm in diameter or a mature particle of 60nm. The viral particle has an RNA genome which is surrounded with capsid proteins (C) to form the inner core. The inner core is covered with a lipid bilayer from the host cell and a glycoprotein shell of exactly 180 copies of envelope (E) and membrane protein (prM). The conformations of these proteins distinguish the immature particle from the mature particle (Figure 1.1). In the mature virion the E proteins lie flat against the viral surface as 90 homodimers and create a smooth protein shell. The 'pr' peptide that is part of the prM protein is cleaved and forms a protein sheath just under the envelope. In the immature virion the prM and E proteins are seen as spikes that form an ordered array of 60 trimeric spikes all around the virion. The transformation from immature (spiky) to

A


(a)
Immature virus
non-infectious
(b)
Immature virus
non-infectious
(c)
Immature virus
non-infectious
(d)
Mature virus
infectious
prM-E
prM-E
Furin
pr
+
M-E
180 pr
pr
M-E
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B

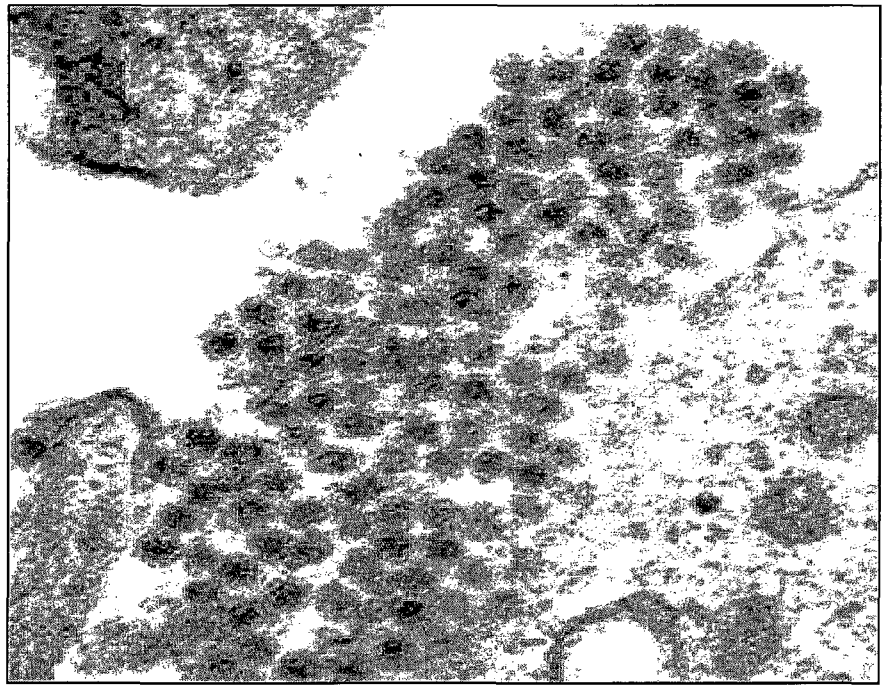


Figure 1.1 Structural representation of the dengue virus. (A) Cryo-EM representations of the dengue virus and its tranformations from an immature non-infectious virus particle to a mature infectious virion.[Figure adapted from Perera and Kuhn.,2008]. (B) Electron micrograph of dengue virus. The spiky nature of the mature virion is clearly visible [Figure obtained from CDC (http://www.cdc.gov/ncidod/dvbid/dengue/electron-micrograph.htm)]

mature (smooth) occurs in a pH-dependent manner in the trans-golgi network during the budding of the virus to the cell surface (Figure 1.1) (Pokidysheva et al., 2006; Yu et al., 2008). Low pH in the golgi triggers a conformational change from spiky to smooth followed by the final cleavage by host protease furin which cleaves the prM protein away from the envelope (E) (Perera and Kuhn, 2008).

1.1a Viral entry

Infection of susceptible cells by DENV occurs predominantly through the attachment of the virus to a cell surface receptor followed by the uptake via receptor mediated endocytosis (Figure 1.2). During a primary infection, the E protein of the virus binds to a cell surface receptor reviewed in (Clyde et al., 2006). Several putative receptors have been reported with respect to DENV infection. They include heparin sulfate (Chen et al., 1997; Germi et al.,2002), unidentified cell surface proteins ranging in 30-80kDa (Martinez-Barragan and del Angel, 2001; Salas-Benito et al., 2007; Salas-Benito and del Angel, 1997), mannose receptor on CD14+ monocytes (Miller et al., 2008) , DC-SIGN (CD209) on dendritic cells (Tassaneetrithep et al., 2003; Wu et al., 2000), GRP78/Bip on hepatic cells (Jindadamrongwech et al., 2004). Several reports have shown that using heparin prevents the attachment of DENV to glycosaminoglycans that are present on cell surface receptors (Chen et al., 1997; Lin et al., 2002; Marks et al., 2001). The consensus among the reports points to the interactions with these glycosaminoglycans as a primary non-specific binding that promotes interactions with a specific receptor or receptor complex (Pokidysheva et al., 2006). Several attempts have been made to identify specific receptor proteins and limited characterization has

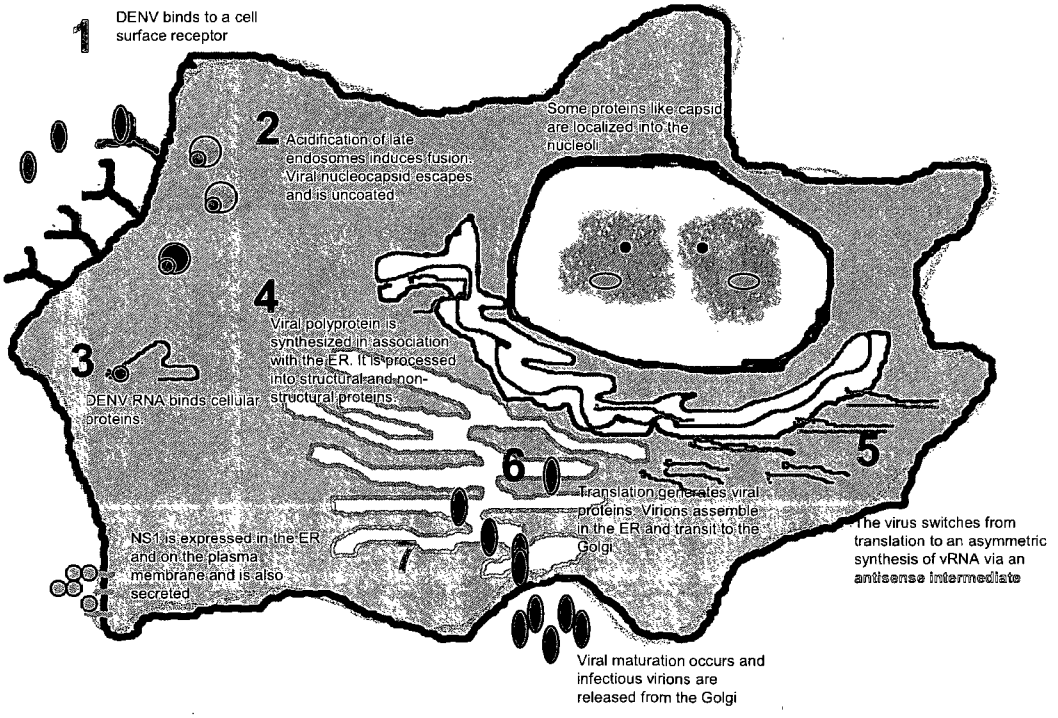


Figure 1.2 Mechanisms of entry into a susceptible cell and replication of dengue virus. DENV enters the cell through receptor-mediated endocytosis and is uncoated in the late endosome. Viral proteins are translated into a polyprotein in the ER and processed. Replication occurs in the ER through an anti-sense intermediate. Viral assembly occurs in the golgi and processed. Mature virions are secreted out from the golgi and ready to infect another susceptible cell. [Figure adapted from Clyde et al., 2006].

implicated two heat shock proteins Hsp90 and Hsp70 as putative receptor molecules (Cabrera-Hernandez et al., 2007; Reyes-Del Valle et al., 2005). Recently it was demonstrated that DC-SIGN (CD 209, a c-type lectin) is a receptor for all four strains of DENV (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). Structural studies on the virus receptor interactions have identified an interaction between the carbohydrate moiety on residue 67 of the E protein and DC-SIGN (Lozach et al., 2005; Pokidysheva et al., 2006). In the case of a severe infection with DENV, an alternative mechanism proposed implicates the presence of non-neutralizing antibodies in promoting DENV entry. The virus attachment and entry into Fc receptor bearing cells such as monocytes and macrophages is promoted by the virus bound cross-reactive antibody binding by its Fc region to its receptors (Halstead and O'Rourke, 1977b). This antibody mediated enhancement (ADE) is 5-6 fold more efficient than that of free virus binding to the monocyte-dengue receptor. This phenomenon is thought to contribute to the development of hemorrhagic symptoms of dengue (Halstead and O'Rourke, 1977a).

1.1b Post viral entry processing

After the attachment of DENV to a cell surface receptor, entry is mediated by endocytosis and pH dependent fusion with the endocytic membrane (Hase et al., 1989; Lim and Ng, 1999; Lindenbach and Rice, 2001; Lindenbach and Rice, 2003). The virion is exposed to the acidic pH in the endosomic vesicles, which also simultaneously triggers structural modifications of the envelope proteins (Gollins and Porterfield, 1985). After un-coating of the virion and digestion of the envelope by cellular lysosomal proteases the positive strand genome is released into the ER (Heinz et al., 1994a; Heinz et al., 2004;

Heinz et al., 1994b). The cellular translation machinery is initially involved in translation of a single large polypeptide that is post-translationally cleaved by cellular proteases into individual structural and non-structural components (Harris et al., 2006). The efficiency of translation is determined by the relative availability of eIF4E, which is required for eukaryotic initiation (Lindenbach and Rice, 2003). DENV has several initiation codons at the 5'end and translation initiation is also regulated by the 5' conserved hairpin structure (Chiu et al., 2005). The polypeptide is composed of structural proteins capsid (C) pre–membrane (pr-M) and envelope (E) as well as seven nonstructural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The NS2B/NS3 protease cleaves at specific sites that have dibasic amino acids (usually Lys- Arg or Arg- Arg or Arg-Lys). This protease processes the cleavage between NS2A –NS2B, NS2BNS3, NS3-NS4A and NS4B-NS5 (Falgout et al., 1993; Falgout et al., 1991). Other host proteases are responsible for the cleavage at the NS1-NS2A junction. Host signalases are responsible for the cleavage of C-prM, prM-E, E-NS1 and NS4-NS4B junctions (Figure 1.3) (Amberg et al., 1994; Beasley and Barrett, 2008; Lobigs, 1993). After cleavage specific proteins prM, E and NS1 are translocated into the lumen of the endoplasmic reticulum and can be detected in infected cells with specific antibodies directed against these proteins (Beasley and Barrett, 2008; Lambeth et al., 2005; Martin et al., 2006). The proteins are further post-translationally modified in the Golgi by the addition of carbohydrate moieties (Yamshchikov and Compans, 1993; Yamshchikov and Compans, 1995).

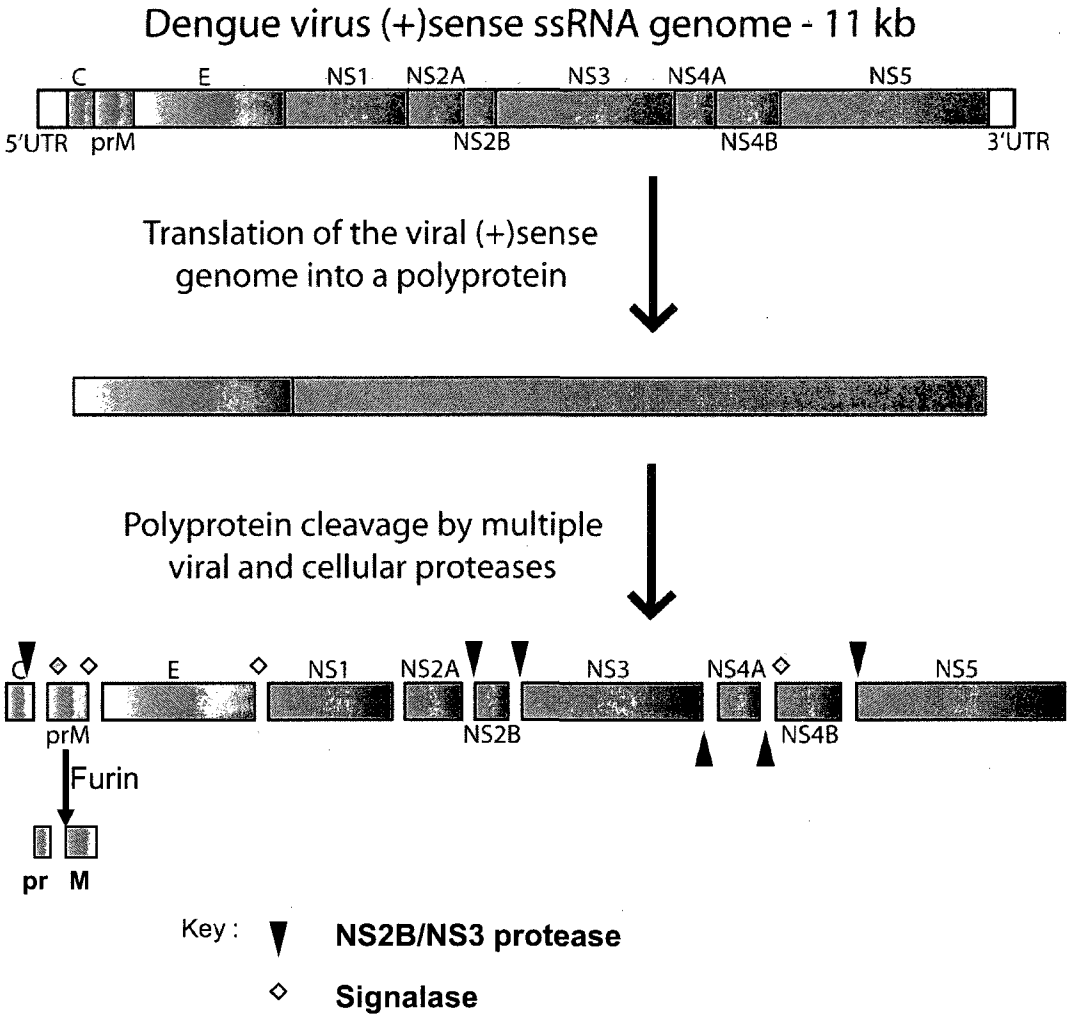


Figure 1.3 Expression and post translational processing of the dengue viral genome after entry into a susceptible cell. The virus is translated as a polyprotein and cleaved into structural and non-structural proteins both by a host signalase as well as the viral NS2B/NS3 protease. The sites of cleavage of either protein are depicted.

1.1c Viral genome replication

DENV genome is a single positive strand RNA that is 11kb long with a single open reading frame that is flanked on the 5' end as well as the 3'end with untranslated regions (UTRs)(Lindenbach and Rice, 2001). The 5' end of this RNA genome is 7-methyl guanylate capped which is required for efficient replication of the virus and conserved across all four genotypes (Figure 1.4) (Clyde et al., 2006). The 5'-UTR region is about 100-nucleotides long and contains a hairpin stem loop structure (Cahour et al., 1995). The 3'-UTR of the RNA is 450-nt long and has a conserved 3' stem-loop (3'SL) structure, which is absolutely required for efficient replication (Brinton et al., 1986; Elghonemy et al., 2005; Men et al., 1996; Proutski et al., 1997; Rauscher et al., 1997; Yu and Markoff, 2005; Zeng et al., 1998). It also lacks a polyA tail, similar to other positive strand viruses.

The 3' end also harbors a conserved sequence CS1 which is important for cyclization of the genome (Alvarez et al., 2005a; Hahn et al., 1987; Men et al., 1996). The establishment of a long-range 5'-3' RNA-RNA interaction has been proposed as a mechanism for replication of DENV (Alvarez et al., 2005a; Alvarez et al., 2005b; Khromykh et al., 2001a; Lo et al., 2003). DENV does not package any viral RNA-dependent RNA polymerases (RdRp) along with the genome. The viral RNA after release into the cytoplasm is translated using the host cell machinery to generate the viral polymerase. The viral RdRp specifically copies the positive strand genome to generate a negative strand or template strand for further amplification of the positive strand genome in the golgi apparatus. The negative strand is present in 10 to 100 fold less as compared to the positive sense strand (Westaway et al., 2003). The detection of negative strand

RNA Elements

5' UTR	Non-canonical translation; vRNA synthesis
5'/3' UAR	vRNA cyclization; viral viability
5'/3' CS	vRNA cyclization; vRNA synthesis
3' UTR	Cap dependant & non canonical translation vRNA synthesis
VR	Translation; vRNA synthesis
DB1/DB2	Translation; vRNA synthesis
3' SL	Translation; vRNA synthesis

Viral Proteins

C	vRNA packaging
prM	Prevention of premature fusion
E	Receptor binding; fusion
NS1	Signal transduction
2A/2B	NS3 serine protease cofactor
NS3	Helicase; NTPase; 5'triphosphatase; serine protease
2A/4B	Inhibition of IFN signal transduction
NS5	RdRP; methyltransferase

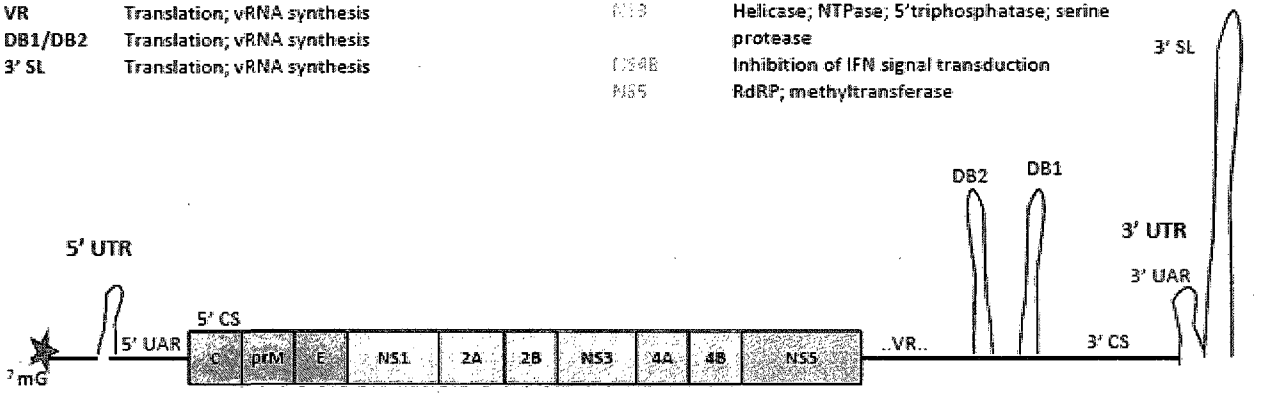


Figure 1.4 Molecular organization of dengue virus. The virus is a positive strand virus with a 5' 7-methyl guanine cap , 3'untranslated region (3'UTR) as well as several important hairpin structures in the 5' and 3' regions.[Figure adapted from Clyde et al., 2006]

using in situ hybridization methods has been critical to demonstrate cell types where active replication of DENV occurs (Kala et al., 2004). The mechanism of generation of this negative strand had been the subject of intense investigation. Recently a new RNA element was discovered that actively recruits the viral RdRp at the 5' end of the viral RNA genome and promotes synthesis at the 3'end through long-range interactions (Filomatori et al., 2006). This RNA element in the 5'end of the genome allows the RdRp to discriminate between cellular and viral RNA. The binding of the polymerase at the 5' stem-loop structure allows for interactions at the 3'end which is brought in close proximity by cyclization of the entire genome. This promotes the generation of a negative strand of the genome. It is hypothesized that genome cyclization during viral replication allows for a controlled mechanism to amplify only full length templates. Cyclization may also help to co-ordinate the signals for translation and RNA synthesis by overlapping signals at the 5' and 3'ends of the genome along with stabilizing the genome. In addition, cyclization may locate the polymerase as well as accessory proteins of the replication complex at the correct start site and also simultaneously contain a number of minus strand replication templates produced (Filomatori et al., 2006).

1.1d Viral assembly and budding

Packaging and assembly of the virus occurs in a rapid process in the endoplasmic reticulum of the infected cells within 12 hours of infection. The process is believed to occur in tandem with replication such that there is limited propagation of RNA genomes (Khromykh et al., 2001b). The capsid interacts with the RNA to form a nucleocapsid complex. The orientation of the capsid due to its interactions with the ER membrane

directs the assembly by virtue of this polarity (Markoff et al., 1997). The prM and E proteins are translocated to the lumen of the ER where they form a heterodimer complex. These proteins move through the ER and the Golgi apparatus where they are post-translationally modified by the addition of sugar residues (Courageot et al., 2000). The complete assembly of the virus occurs with the budding of the nucleocapsid complex through the ER lumen where it acquires the prM-E-lipid envelope (Wang et al., 1999). The virus then accumulates in the secretory pathway of the cell and is transported out by exocytic vesicles. Prior to exiting the cell, prM protein undergoes a furin cleavage to form a mature M protein and triggers the formation of E protein homodimers as well as other pH dependent conformational changes (Beasley and Barrett, 2008; Stadler et al., 1997).

1.2 Primary Dengue Fever

Dengue infection causes an arthropod borne viral disease in humans that ranges from a mild febrile fever to a fatal hemorrhagic disease. Primary dengue fever is a disease of children and adults. It is characterized by an onset of fever with severe joint pain, headache, nausea, retro-orbital pain, weakness and rash. Individuals suffering from dengue often report an altered taste sensation and mild sore throat (Gubler, 1998a; Hayes and Gubler, 1992; Sabin, 1952). The fever lasts up to a week with patients displaying infections of the conjunctiva, inflammation of the pharynx and lymphadenopathy. Early in the infection some patients may show a classic rash that persists for 2-3 days before clearing. Petechiae appear towards the end of the febrile period of illness. Hemorrhagic manifestations in patients with primary dengue are uncommon but may range from mild

to severe. Skin also shows the presence of petechiae and purpura. Many patients suffer from bleeding gums, epistaxis, menorrhagia and gastro-intestinal hemorrhage. Hematuria is infrequently observed but jaundice is rare (Gubler, 1998; Hayes and Gubler, 1992).

Clinical laboratory findings associated with primary dengue often include neutropenia followed by lymphocytosis with the presence of atypical lymphocytes. There is often a mild elevation in the level of liver enzymes in the serum. Patients may also show elevated levels of alanine aminotransferase as well as aspartate aminotransferase (Dietz et al., 1996; Gubler, 1998a). Some patients also demonstrate thrombocytopenia. In summary, primary dengue fever is generally a self limiting and rarely fatal disease. The illness phase typically lasts only between three to seven days with a prolonged convalescence period. The time taken for complete recovery ranges from weeks to months and is often associated with weakness and depression in adults (Gubler, 1998; Hayes and Gubler, 1992).

1.3 Secondary Dengue Infections

1.3a Dengue Hemorrhagic Fever

Dengue hemorrhagic fever (DHF) is a disease of children under the age of fifteen, although it may occur rarely in adults (Dietz et al., 1996). It is characterized by the sudden onset of fever which lasts for two to seven days and initially resembles a primary dengue infection. As the fever remits, there is a characteristic manifestation of plasma leakage, enabling an accurate diagnosis in many cases. During the stages of defervescence, signs of circulatory failure begin to show (Cohen and Halstead, 1966; Gubler, 1998a; Gubler, 1998b; Hayes and Gubler, 1992). Blood tests usually detect

thrombocytopenia and hemo-concentration that are observed with plasma leakage. Common hemorrhagic manifestations include skin hemorrhages, petechiae, purpuric lesions and ecchymoses. Epistaxis, bleeding gums, gastro-intestinal bleeding, hematuria as well as scattered petechiae on the trunk and extremities are also commonly observed. DHF is characterized by thrombocytopenia with platelet counts less than 10^5/mm3. There is significant hemo-concentration due to plasma leakage. Hepatomegaly is commonly noted with many patients. As a result, liver enzyme levels in the serum are also often elevated above normal. There is also an increased occurrence of vascular permeability that promotes hemo-concentration and decreased blood pressure (Eram et al., 1979; Gubler, 1998b; Sumarmo et al., 1986).

1.3b Dengue Shock Syndrome

During dengue shock syndrome (DSS) patients suffer from a special case of DHF. The defining feature of DSS is shock associated with plasma leakage and circulatory failure. The severity of shock may vary from mild to severe with severe shock dramatically increasing the risk of death. Without an early diagnosis, patients suffer from shock due to loss in blood volume. Characteristic symptoms of DSS may include petechia on the face, large ecchymotic lesions on the trunk as well as the extremities, and severe gastro-intestinal bleeding. Children suffering from shock often exhibit petechiae and perioral cyanosis. Capillary fragility (tourniquet – test) is often used as a diagnostic tool to identify DSS cases. During and sometimes prior to going into shock, the skin becomes cool to the touch, blotchy and congested. The pulse becomes rapid and weakens simultaneously. Although patients appear to be lethargic initially, they become restless

and rapidly pass into shock. Clinical laboratory tests show that DSS is characterized by a greater than 20% reduction in plasma volume. Pleural effusion is seen in lung x-rays from these patients. Hemo-concentration due to plasma leakage simiar to DHF patients is common. Hypoproteinemia is also observed in DSS patients. There are significant vascular changes in the patients during shock. Blood tests often reveal thrombocytopenia and coagulation disorders that are linked to lack of platelets. Patients with the mild form of shock are often able to recover after administration of electrolyte therapy. In patients with mild shock all signs and symptoms rapidly disappear shortly after fever subsides. However, patients who develop severe shock suffer from a rapid decline in health and mortality is very high in such cases (Eram et al., 1979; Gubler, 1998; Sumarmo et al., 1986).

1.4 Laboratory Diagnosis for Dengue Infections

At the onset of disease blood samples are taken to determine presence of the virus in serum or the presence of antibodies against the virus. Serological tests for dengue often include a hemagglutination–inhibition test, complement fixation, viral plaque reduction neutralization test (PRNT) and ELISA. For detection of antibodies in patient sera both sandwich ELISA as well as indirect IgG ELISA techniques can be used (Gubler and Sather, 1988; Guzman and Kouri, 1996). While the detection of antibodies against dengue is extremely useful for rapid diagnosis and treatment, the definitive detection of infection is made when the virus is isolated from an infected patient or viral RNA in serum or tissue samples has been identified.

1.4a Virus Isolation and Identification

Virus from infected patients has been routinely isolated and identified by several methods that have progressively developed over time. Baby mice were first used to routinely isolate DENV during epidemics. All four DENV serotypes have been isolated from sera of human patients that were injected into baby mice intra-cerebrally (Hotta, 1952; Sabin, 1952). This practice has been discontinued in routine use due to the lack of sensitivity and time-consuming nature of the process. Mammalian cell cultures have been routinely used to propagate DENV. Cell lines such as the LLC-MK2 and Vero are routinely used in the process of isolation and identification of DENV (Gubler and Sather, 1988; Vordnam and Kuno, 1997). However, the disadvantages to this process are similar to that of using baby mice. Several passages are required to allow the virus to adapt to the cell line. In addition a similar period of time is required for observing cytopathic effects in infected cultures (Gubler, 1998a). Direct mosquito inoculation is the most sensitive method that has been employed for DENV detection (Gubler and Sather, 1988). It has been used to successfully isolate viruses routinely from numerous fatal cases of DHF and DSS (Gubler and Sather, 1988; Gubler et al., 1979a; Sumarmo et al., 1986; Vaughn et al., 1997). Several endemic DENV strains have also been isolated using direct inoculation of mosquitoes (Gubler et al., 1978; Gubler et al., 1986). Four mosquito species have been used routinely for the purpose. They are *Aedes aegypti, Aedes albopictus, Toxorhynchities amboinensis and Toxorhynchities splendens*. Both male and female mosquitoes are equally susceptible to infection and replicate the virus efficiently to high titers (Lam et al., 1986; Thet, 1982). In a short period of time post-injection (4-5days) the virus is detected using fluorescent-labeled antibodies in the brain as well as the salivary

glands (Kuberski and Rosen, 1977). The disadvantages to the method of infecting mosquitoes include the requirement of an insectarium that can produce a large number of uninfected mosquitoes and the labor-intensive nature of the process (Gubler and Sather, 1988; Gubler et al., 1979b). There is also an additional risk of being bitten by infected female *Aedes* mosquitoes in the laboratory although this can be eliminated by using non-biting male *Aedes* as well as *Toxorhynchities* species (Rosen and Gubler, 1974). Mosquito cell cultures are the most recent additions to the methods for DENV isolation. C6/36 is a clonal *Aedes albopictus* cell line that is routinely used in culturing DENV. These cells are very useful since they are easily scaled up allowing for the rapid and economical surveillance of several serum samples at once (Gubler and Sather, 1988; Gubler et al., 1984; Kuno et al., 1985; Tesh, 1979). Another cell line AP-61 from *Aedes pseudoscutellaris* has been used due to ease of detection of cytopathic effects. The only disadvantage of the AP-61 cell line is the inability to detect any noncytopathic effect causing DENV with ease (Gubler and Sather, 1988). Since mosquito inoculation is the most sensitive for detection of DENV, it is often employed during an epidemic of DHF or DSS while mosquito cell culture is used for routine surveillance (Gubler et al., 1986).The sensitivity of the mosquito cell line towards DENV also varies by strain. Thus there are instances where the virus is not effectively isolated. This drawback is offset by the ease with which a large number of serum samples can be processed simultaneously (Gubler and Sather, 1988).

1.5 Immunity and Vaccines against Dengue

Within 3-5 days of primary infection most patients infected with DENV show detectable levels of IgM antibody. These antibodies persist for 1-2 months following infection and are usually found to be predominantly directed against E protein with few against non-structural proteins. During primary infection IgM antibodies tend to predominate over IgG antibodies (Innis et al., 1989). IgG antibodies that are detected in convalescing patients are often directed against prM E, NS1, NS3 and NS5 proteins. Antibodies generated against E protein are often neutralizing against the infecting strain of virus with the capacity to cross-react with other serotypes (Nawa et al., 2000). Neutralizing IgG antibodies produced against a particular strain confer life-long immunity to that strain (AbuBakar et al., 1997; Se-Thoe et al., 1999; Valdes et al., 2000). Thus there is a strong immunity against a homologous infecting serotype and short-lived or no protection against a heterologous serotype. After secondary infection by a different serotype, IgG antibodies are produced at very high levels against capsid, NS1, NS3 and NS5 (Halstead, 1974). These antibodies generally neutralize the original infecting strain but are non-neutralizing for the secondary infecting strain (Halstead et al., 1973). The presence of non-neutralizing antibodies in the event of a subsequent infection with a different serotype is very detrimental to the individual. The non-neutralizing antibody bound virus is taken up by a relatively large number of Fc-receptor bearing cells, resulting in the phenomenon of antibody dependent enhancement (ADE) (Halstead, 2008). This process contributes aggressively to dramatic increase in the number of cells that are infected and rapid increase in plasma virema which cumulatively contribute to the severe pathology associated with DHF and DSS (Halstead, 2008). Many of these

antibodies are cross-reactive and some are even generated against host proteins such as plasminogen. Cross-reactive antibodies against the host proteins have not been correlated with severe DHF or DSS (Chungue et al., 1994). However in mice antibodies generated against NS1 have been shown to be cross reactive against human fibrinogen (Monroy and Ruiz, 2000), platelets and endothelial cells and can cause hemorrhage in these mice (Falconar, 1997; Lin et al., 2003). These antibodies reactive against the host proteins can trigger auto-immunity related destruction of platelets and endothelial cells promoting plasma leakage, hemorrhage and shock (Chungue et al., 1994). Thus the role of antibodies during infection is profound.

In order to generate protective immunity against dengue, there is a need for four distinct vaccines to provide complete protection against all serotypes. Vaccine studies are further confounded by a lack of complete understanding of the pathogenesis of severe dengue disease and inadequate animal models. Both monkeys and mice have been used as animal models. However both mice as well as monkeys do not generate adequate viremia as compared to human patients to allow for a complete understanding of disease pathology (An et al., 1999; Halstead et al., 1973; Johnson and Roehrig, 1999; Kraiselburd et al., 1985). As described below and tabulated in Table 1.1, six different types of vaccines have been tried so far with limited success (Vaughn et al., 2008).

1.5a Attenuated live virus vaccines.

The first vaccination attempts were carried out by Sabin, who isolated and demonstrated the presence of two distinct dengue viruses DENV-1 Hawaii and DENV-2 New Guinea (strains B, C and D) for vaccination. The first vaccinations were carried out

Table 1.1 List of current Dengue vaccines.

Vaccine Type	Manufacturer	Strengths	Weakness
Attenuated live vaccine	WRAIR-Glaxo	Potent , Monovalent and Tetravalent	Reactogenic, Viral interference with booster doses
Molecularly attenuated live virus	FDA , NIH	Can be made tetravalent,less reactogenic, well tolerated,	Neutropenia
Chimeric live vaccine virus	Mahidol-CDC, Acambis	Robust antibody responses	Serotype specific T-cell memory unknown
Inactivated whole virus vaccine	WRAIR, Naval	All animals sero-positive. Vaccine does not cause an infection.	Small repertoire of proteins for immune response
DNA/RNA based vaccine	Vical, WRAIR, Naval	All four serotypes can be used	Low effectiveness of skin delivery. Risk of integration into the chromosome
Recombinant subunit vaccine	Hawaii Biotech	Cost effective, tetravalent, specific proteins for generating neutralizing response	Maintenance of post-translational modifications

by mixing convalescent serum and acute infection serum and inoculating a human volunteer intracutaneously with a known amount of each. The virus was considered neutralized if the subject did not develop symptoms of infection. Sabin confirmed that the type specific antibody provided protection against classical dengue fever for at least two months (Sabin, 1952). He attempted to attenuate the DEN-1 Hawaii strain by serial passage in mouse brain and was successful after 7 passages. Similar attempts at attenuation were made successfully with DENV-2 New Guinea and individuals were immunized without development of severe clinical symptoms. This is the first known attempt to use attenuated live vaccine in humans (Eylar and Wisseman, 1975; Sabin, 1952). In 1963 a placebo-controlled study during a DENV-3 outbreak in Puerto Rico showed partial success with the usage of mouse passaged DENV-1. These studies were later discontinued due to safety concerns with the possibility of generation of autoimmune antibodies against mouse neural proteins or inclusion of other infectious agents from mice (Wisseman et al., 1966). More recently, attenuated vaccines have been developed by Mahidol University in conjunction with Walter Reed Army Medical Instiute (WRAIR) where serial culture passaged virus was employed to develop live attenuated monovalent as well as tetravalent vaccines (Bancroft et al., 1984). For these purposes primary dog kidney (PDK), African green monkey kidney (PGMK) or fetal rhesus lung (FRhL) cells were used (Vaughn et al., 2008). These vaccines were licensed to Sanofi Pasteur and have undergone both Phase 1 and Phase 2 clinical trials recently. All four vaccines showed high seroconversion rates with DENV-1 and DENV-2 showing 100 and 92% respectively for single doses (Bhamarapravati and Sutee, 2000; Bhamarapravati et al., 1987; Vaughn et al., 1996). Although DENV-3 and DENV-4 had

lower seroconversion rates of 46 and 58%, they were the best tolerated by volunteers for a single dose. The side-effects of these inoculations at times included fever, rash, and dengue related symptoms like headache, myalgia and rash (Kanesa-Thasan et al., 2003; Mackowiak et al., 1994). The complications with booster doses involved viral interference, and reactogenicity. These experiments have still not alleviated concerns regarding vaccine-induced ADE or the possibility that a drop in concentrations of neutralizing antibodies to sub-neutralizing levels could trigger severe disease (Burton et al., 2000). An enhanced vaccine reactogenic reaction (where the vaccine induces an aggressive disease due to an ADE based phenomenon) is also possible in individuals who have pre-existing anti-flavivirus antibody (Vaughn et al., 2008).

1.5b Molecularly attenuated live virus vaccines.

DENV-4 (WRAIR 814669, Dominica 1981) was the first DENV to be cloned for vaccine research (Lai et al., 1991). After this achievement, dengue and other flaviviruses were readily altered genetically to generate attenuated variants. The first vaccine derived by cDNA technology was used to immunize Rhesus macaques (Men et al., 1996). It was found to cause vaccinemia (anemia related to vaccination) with a slightly decreased antibody response compared to other types of vaccines. Similar symptoms were seen in human volunteers who were injected with $1x10^5$ pfu in 0.5 ml subcutaneously. The vaccine was well tolerated but was seen to be vaccinemic (neutropenia) with transient rash. Though this vaccine set the precedent for future studies, several modifications will be needed prior to it being a good candidate with the ability to neutralize all four serotypes of DENV. The backbone of the virus used is now being investigated to develop

a tetravalent vaccine after insertion of genes from other DENV (Vaughn et al., 2008). In 1997, the FDA generated an infectious clone of DEN-2 that was modified at the 3'UTR to demonstrate the importance of this region in replication of DENV (Polo et al., 1997). Further studies showed that deletion in this region generated an altered virus that was incapable of replicating in insect cells but replicated in mammalian cells. These defective mutants were evaluated as vaccine candidate in Rhesus macaques and were shown to be less reactogenic than parent strains (Markoff et al., 2002; Men et al., 1996). The monkeys were protected from infection later when challenged with a homologous infectious strain of the virus. Molecular clone-based strategies thus provide an alternative where the antibody response is directed known attenuated viral strain which is an added advantage over empirically attenuated viral strains that can be used for vaccination. These mutant strains are being pursued as candidates for further live vaccine trials (Vaughn et al., 2008).

1.5c Chimeric live vaccine viruses

The generation of DENV-4 cDNA mentioned above (Men et al., 1996) paved the way for the generation of several chimeric constructs to be used for vaccine studies (Bray and Lai, 1991; Markoff et al., 2002). Several combinations of structural genes as well as non-structural genes have been combined to generate viruses that produce robust antibody responses and protection from challenge with wild-type viruses when given as monovalent or bivalent vaccines in Rhesus macaques (Bray et al., 1996). The CDC has developed a tetravalent vaccine using non-structural genes derived from the Mahidol University/Sanofi Pasteur LAV vaccine (DENV-2 16681 PDK-53) (Kinney et al., 1997).

There are proposals to insert prM-E genes from DENV-1, DENV-2 and DENV-3 attenuated viruses into this backbone to generate formulations that will hopefully generate protective antibodies against all four serotypes and allow for maintenance of attenuation (Huang et al., 2003). Chimeric viruses are being developed by Acambis Inc in conjunction with Washington University and St Louis Medical Schools using a slightly different approach in which the yellow fever 17D vaccine strain has been used as the backbone for insertion of dengue serotype specific genes. Chimeras have been produced for each of the four dengue serotypes and propagated under a controlled environment in Vero cells (Guirakhoo et al., 2001; Guirakhoo et al., 2004; Guirakhoo et al., 2000).

Injection in Rhesus macaques resulted in complete sero-conversion in all animals (Guirakhoo et al., 2002). This was seen in cases where the animals were administered 10^3 pfu of DENV-2 chimera and 10^5 pfu each of DENV-1, DENV-3 and DENV-4 chimera. A higher concentration of DENV-2 chimera demonstrated interference. The animals were completely protected from challenge with any DENV serotype (Guirakhoo et al., 2006). These vaccines may provide a better approach to generate neutralizing capability as compared to live attenuated vaccines. However, it is unknown whether these vaccines will generate enough serotype-specific memory T-cells against DENV to provide long lasting protection (Vaughn et al., 2008).

1.5d Inactivated whole virus vaccines

Inactivated whole viruses were developed first in 1929 as crude preparations to generated immunogenicity (Simmons et al., 1931). More recently the WRAIR has generated a DENV-2 S16803 vaccine preparation from Vero cells that was purified using

sucrose gradients and inactivated by formalin treatment. The virus was administered along with adjuvants to Rhesus macaques. All experimental animals, except one macaque sero-converted after the first dosage of vaccine. Upon a secondary dosage all animals showed an anamnestic response and an increase in antibody titre. Upon challenge with the parent strain the animals were protected (Putnak et al., 1996a; Putnak et al., 1996b). The advantage of an inactivated vaccine lies in the fact that the virus cannot revert to any pathogenic form. Inactivated vaccines are also less likely to interfere with other serotypes of virus used for vaccination. They have also been shown to activate cell-mediated immunity in addition to humoral immune responses (Aihara et al., 2000; Vaughn et al., 2008). The drawback however lies in the small repertoire of proteins that are processed to generate the immune response. The narrow breadth of the immune response generated is likely to be insufficient for robust protection. However, there are similar vaccines used in the case of Japanese encephalitis and tick-borne encephalitis that are used as traveler's vaccines (Hoke et al., 1988). Hence if some of the concerns regarding manufacturing and safety are addressed, inactivated virus vaccines may provide a practical alternative or may be used in combination with live attenuated vaccines (Vaughn et al., 2008).

1.5e DNA/RNA based vaccines

DNA or RNA based vaccines use specific portions of genes that elicit an immune response without the generation of an infectious virion. These vaccines are composed of plasmids containing DENV genes which are amplified in *E.coli* and injected into the recipient. After uptake in human cells the genes are transcribed and translated into their respective gene products. These proteins are later presented on the cell surface by the

host MHC system to generate a humoral and/or cellular immune response (Whalen, 1996). DNA vaccines were first evaluated by the Naval Medical Research Center where the expression plasmid contained both prM and E protein (Vical Inc, SanDiego California) from the DENV-2 New Guinea C strain under a eukaryotic promoter. Mice tested with both plasmids generated neutralizing antibody (Kochel et al., 1997). Similar plasmids were made with C-prM-E and used to immunize mice with similar results (Konishi et al., 2000).

In Rhesus macaques, usage of recombinant plasmids was able to reduce the frequency and duration of viremia on challenge with infectious virus. At the present moment usage of DNA constructs expressing antigens of all four serotypes produces only modest amounts of neutralizing antibody in non-human primates while fully protecting only a small number of animals (Raviprakash et al., 2000). The effectiveness of the vaccine may be improved if there are means to specifically deliver these genes into skin dendritic cells that are thought to be the primary targets of DENV infection. DNA vaccines in theory do provide a number of advantages over conventional vaccines in terms of ease of production, transport and stability of the vaccine (Eo et al., 2001). However it also carries the risk of integration into the host chromosome as well as generation of auto-immune disease with antibodies produced against host DNA. Unless these challenges are circumvented and more robust neutralizing responses are generated, DNA vaccines will always be viewed with skepticism (Vaughn et al., 2008).

1.5f Recombinant subunit vaccines

Several epitopes have been specifically mapped with respect to DENV proteins

that elicit a strong humoral as well as cell mediated immunity. These short regions can be cloned together as a single subunit vaccine that will hopefully be both safe and effective at a moderate cost (Trent et al., 1997). Several systems have been used to express these proteins including *E.coli*, insect cells (*S. frugiperda*), yeast, vaccinia virus as well as mammalian cells. Gene expression in Drososphila cells has been engineered by Hawaii Biotechnology Inc who have expressed and purified the E protein. Rhesus monkeys injected with this monovalent vaccine were immune against a challenge with wild-type virus of the same serotype (Simmons et al., 2006). A tetravalent formulation is also being pursued using this approach. Recently a DNA shuffling approach was used to generate chimeric tetravalent clones of the E protein to generate a recombinant antigen. It is capable of inducing production of neutralizing antibodies in mice against all four DENV serotypes (Apt et al., 2006). These and other novel approaches offer an advantage over live attenuated vaccines in terms of efficacy and cost. It is however important that the post-translational modifications such as glycosylation remain unchanged. This can be circumvented by producing the protein in mammalian cells (Konishi and Fujii, 2002). Like all other vaccines, the risk of enhanced disease when challenged with wild type viruses should also be assessed prior to approval for usage. Vaccines that generate a cytotoxic T-cell response would most likely lower such risk by inducing a robust and quick neutralizing response (Vaughn et al., 2008).

1.6 Animal Models for Dengue Fever Studies

Dengue as a severe disease has been described in medical as well as historical literature since the early 1800's. Epidemiological studies have shown that the virus was

introduced to human populations from non-human primates both in Africa and Asia. Detection of anti-dengue antibodies in these animals found in sylvatic or rural settings in these regions implies that they may be involved in virus transmission via mosquitoes (Wang et al., 2000).

1.6a Large animal models to study dengue infection

The first experiments with *Macaca mullata* (Rhesus macaques) were done in 1914 using infected patient sera injected subcutaneously to demonstrate that non-human primates can become infected with the virus. The re-isolated virus was able to produce classical dengue fever in humans but the monkeys remained asymptomatic. Later other experiments were performed using infected mosquitoes which were allowed to feed on rhesus as well as cynomolgus macaques (*Macaca fascicularis*). Both species of animals developed an infection without showing any symptoms. Mosquitoes fed on these monkeys were neither infected nor able to transmit any disease to human volunteers. Thus primates have been traditionally used in dengue research with mixed results (Bente and Rico-Hesse, 2006). Several other species of non-human primates have been used to study dengue infections with limited success. They include *Cercopithecinae* family (Rhesus macaques, Cynomolgus macaques), Japanese macaques), green monkeys (*Cercopithecus aethiops*), patas monkeys (*Erythrocebus patas*), yellow baboons (*Papio cynocephalus*) and mangabeys (*Cerocebus* spp) (Halstead et al., 1973; Rosen, 1958). The results from experiments on these animals have shown that they become infected at a very low level and do not show any overt signs of disease. Experiments with New world monkeys like night monekys (*Aotus* spp), squirrel monkeys (*Saimiri sciureus*), cotton-top

marmosets (*Saguinus oedipus*), white face monkeys (*Cebus capucinus*), black spider monkeys (*Ateles fusciceps*), Saimiri monkeys (*Saimiri örstedii*), marmosets (*Marikini geoffroyi*), howler monkeys (*Alouatta palliata*) and red spider monkeys (*Ateles geoffroyi*) have shown that they can harbor the virus due to the detection of viremia and antibody production, but also do not show any overt clinical symptoms (Kochel et al., 2005; Scherer et al., 1972; Schiavetta et al., 2003). Apes such as chimpanzees (*Pan troglodytes*) and white-handed gibbons (*Hylobates lar*) are not susceptible to dengue infection (Paul et al., 1948; Scherer et al., 1978; Whitehead et al., 1970). Thus, a lack of symptomatic infection is a major disadvantage to using non-human primates in dengue research in addition to the high cost of conducting experiments with these animals. More attention is now being focused onto small animals as dengue infection models.

1.6b Small animal models and dengue infections

Among small animal models like rats, rabbits, guinea pigs and mice, dengue research has primarily focused on mice as a suitable host to study dengue fever. The other small animals have not shown any infection when challenged. Mouse models for dengue research are divided into three categories:

1.6b[i] Immunocompetent mouse models

Initial studies with normal immunocompetent mice demonstrated that mice were permissive to dengue virus infection and replication occurred at a low level. These studies often used very high viral inoculum ($1x10^8$ pfu) injected through an intravenous route or intracranial route, both of which are not the normal modes of infection.

Moreover when the virus was passaged in mice it lost its virulence and ability to infect human volunteers (Sabin and Schlesinger, 1945). Some strains of mice like A/J are more sensitive to infection as compared to the BALB/c or C57BL/6 strains of mice. Infection in A/J mice resulted in signs of paralysis and thrombocytopenia upon infection with a very high dose of virus (Huang et al., 2000b). However, in general immunocompetent mice do not exhibit sustained DENV replication or symptoms consistent with dengue fever in humans.

1.6b[ii] Immune compromised mice

In order to circumvent the drawbacks of immunocompetent mice, attention was given to mice which can not produce interferon (AG129 strain) or the SCID mouse which does not produce functional B or T cells. Experiments with AG129 interferon knockout mice show that the infection is lethal in mice that lack receptors for interferon alpha, beta and gamma. The mice suffer from paralaysis and blindness and die by 12 days post infection. Viremia is observed up to 9 days post infection with the peak at 3 days. The lack of interferon in these mice results in death pointing to the role played by interferon in protection from dengue pathogenesis. Because these mice exhibit a severe disease outcome, they can be useful as models for testing pre-clinical vaccines. However, the severe immunodeficiency of these mice makes them a relatively unrealistic model of dengue infection and pathogenesis (Johnson and Roehrig, 1999; Shresta et al., 2006).

1.6b[iii] Humanized mice

Severe combined immunodeficiency (SCID) mice have been transplanted with primary human cells, cell lines and tumors. These mice lack humoral and cellular immunity due to the lack of mature host T and B cells. The mice engrafted with human cells have been used to study the replication of several viruses including DENV for which small animal models are not available. Several types of mice have been engrafted with human PBMCs to generate the human-PBL-SCID model. These mice were rarely infected when injected with DENV. The mice were not able to sustain a virus infection effectively since the number of target cells achieved in animal tissue was only nominal (Wu et al., 1995). In another model, SCID mice were injected with human erythroleukemic cells (K562) (Lin et al., 1998). After challenge with DENV virus intra-peritonealy the mice demonstrated the presence of virus in the blood as well as in the tumor and brain. In this model, viremia was observed 6 days post infection in the blood and 3 day post infection in the brain. The mice showed paralysis within 2 weeks of infection died by 4 weeks post infection.

SCID mice have also been engrafted with human liver cells. When engrafted with a hepatocarcinoma cell line (HepG2) (An et al., 1999) and challenged with DENV virus, the mice became infected. Viremia was detected in the brain, serum and liver but not in spleen. The mice demonstrated elevated TNF-alpha levels, along with thrombocytopenia and an elevated hematocrit, with exhibition of paralysis 2-3 weeks post infection. The increased level of TNF-alpha is a response seen similar to that in DHF/DSS cases. In another model involving hepatic cells, SCID mice were engrafted with a human hepatoma cell line (HuH-7). Intra tumor injection of these mice with DENV showed a

productive infection with DENV-4. The virus was detected in serum, liver and brain. This model has also been used to test vaccines (Blaney et al., 2005). The major drawback with all the SCID models described so far is the limited time for which the engraftment persists in these mice as well as the limited repertoire of normal human lymphocytes available for infection. SCID mice as described above are also amenable to engraftement with human cells.

To increase the stability of xeno-engraftment in these mice they were crossed with non-obese diabetic (NOD) mice to generate the NOD-SCID model which lacks natural killer cells (NK) in addition to T and B-cells (Serreze et al., 1988). The additional knockout promotes engraftment of foreign tissue, especially human hematopoietic CD34+ stem cells. The mice are engrafted a few days post birth intra-hepatically with cells sourced from human umbilical cord blood. The human CD34+ cells are progenitors for cells of both myeloid and lymphoid origin which are able to engraft into the organs as well as being present in peripheral blood. The engrafted NOD-SCID mice were challenged with DENV-2 K0049 strain through a subcutaneous injection. The engrafted mice developed clinical symptoms of rash, fever and thrombocytopenia. The results from viremia estimated from plasma and organs by q-RT-PCR suggest that the virus infected the spleen, liver and is detected in peripheral blood (Bente et al., 2005). The authors were however unable to demonstrate an adaptive human immune response from these mice to date. Another drawback with NOD-SCID mice in general is the short lifespan of these mice due to the spontaneous formation of tumors. Although it is a good model to study dengue fever with respect to primary infections, the mice do not reliably survive over a longer period of time.

Recently a novel humanized mouse model was generated by combining two separate gene knockouts. The $Rag^{-/-}\gamma_c^{-/-}$ is generated by knocking out the recombination activating gene (Rag) as well as common Il-2 receptor gamma chain (IL-2γ_c). The recombination activating genes (RAG-1 and RAG-2) plays an important role in the rearrangement of immunoglobulin genes in T and B lymphocytes. Disruption of either of these genes leads to an immunodeficient mouse which lacks an adaptive immune response. The IL-2 common gamma chain is a common receptor that is used by the Il-2 family of cytokines. The gene knockout directly affects the propagation of natural killer cells in the mice thus enabling efficient xenoengraftment in $Rag1^{-/-}\gamma_c^{-/-}$ or $Rag2^{-/-}\gamma_c^{-/-}$ mice.

The combined effect of these two mutations allows the generation of a mouse that is efficiently reconstituted with human hematopoietic progenitor CD34 cells (Traggiai et al., 2004). The neo-natal mice are first irradiated with a sub-lethal dose of gamma irradiation followed by an intra-hepatic injection of CD34+ human hematopoietic stem cells that are isolated from fetal liver tissue. The engrafted human cells migrate to every hematopoietic organ and promote development of a functional thymus and lymph nodes in addition to the propagation of human lymphocytes of myeloid as well as lymphoid origin. The human cell engrafted $Rag^{-/-}\gamma_c^{-/-}$ mice are referred to as "RAG-hu" mice. A major improvement exhibited by the engrafted RAG-hu mouse is that these mice can produce a functional human humoral immune response. We have established both $Rag1^{-/-}\gamma_c^{-/-}$ and $Rag2^{-/-}\gamma_c^{-/-}$ lines of mice and found them to be similar in their ability to engraft human cells in mouse organs. In this dissertation, I have examined the ability of RAG-hu mice to serve as models of DENV infection and immune response.

1.7 Specific aims of the dissertation

Dengue affects over 2.5 billion people worldwide each year either through a direct infection or time spent helping an infected individual recover from a debilitating infection. It affects both children and adults and does not discriminate between the sexes. Both tourists and locals at exotic destinations are equally susceptible, making it an important disease. This disease directly affects the quality of life of a substantial number of people and thus affects the GDP of many countries both in the developed as well as developing world.

Although DENV has been studied extensively for many years, there remains an absence of animal models that faithfully replicate human pathology and immune response seen in dengue infection. The lack of suitable animal models is a problem with respect to rapid testing of new drug molecules as well as testing vaccines before they are used in a susceptible human population. In addition, research on the exact nature of cells that serve as targets for DENV infection and replication has been hampered by the lack of a good small animal model. We sought to examine the potential of a mouse model which has been engrafted with human hematopoietic progenitor cells to serve as a model for DENV infection, pathology and adaptive immune response. Since secondary dengue pathogenesis in human patients is directly linked to their adaptive immune response in susceptible individuals, this model would provide us with a novel opportunity to identify and dissect observations reported in human populations.

The primary goal of this research was to identify cells that are susceptible to DENV infection. During these investigations we also sought to investigate the potential of RAG-hu mice as a model to study both primary and secondary dengue pathology. This

thesis is the result of collaborations between several individuals and laboratories and each of them in their own way has provided a significant input to putting together pieces of this puzzle. We first began with investigating the ability of DENV to directly infect T cells *in vitro* as well as *in vivo* using the SCID-hu mouse model using both flow cytometry based methods as well as immunohistochemistry staining methods. We then proceeded to analyze the primary infection of RAG-hu mice with DENV. Here we estimated the levels of plasma viremia in the infected mice and re-isolated the virus from the mice after establishment of infection. We also investigated the occurrence of fever, weight gain or loss, changes in blood cell counts. Analyzing the presence of human antibodies generated as the result of an adaptive immune response was another set of experiments that were performed. We identified the presence of and quantified human antibodies present in some RAG-hu mice. We also demonstrated the generation of neutralizing antibodies and the nature of antibodies generated in the mice, especially the presence of antibodies directed against E and prM proteins.

After analysis of the primary infections we obtained several mice who had recovered from a primary infection with production of human anti-dengue antibodies. These mice were challenged with a secondary infection after antibody levels decreased to a threshold below ELISA detection limits. During this process we investigated the levels of plasma viremia in the blood along with changes in body weight and temperature. We also investigated the changes in blood counts and identified the occurrence of dehydration, hemorrhage and shock. We analyzed the generation of protective antibodies post secondary infection and investigated the cross-reactivity of these antibodies across DENV serotypes.

In an attempt to identify specific cells in humanized mouse organs that were infected, we have identified the presence of DENV infected cells in the spleen as well as lymph nodes using *in situ* hybridization. I have also tried to characterize DENV infected human cells using immunofluorescence staining methods. We derived macrophages and dendritic cells from human peripheral mononuclear cells isolated from whole blood and investigated their ability to be infected directly by DENV. Finally we also investigated the possibility of hematopoietic stem cells being infected with DENV. We derived macrophages, dendritic cells as well as megakaryocytes from CD34+ hematopoietic progenitor cells and investigated their susceptibility to DENV.

Chapter 2

Dengue Infection of Thymic Grafts in SCID-hu Mice

2.1 Overview of thymic infections in humans with DENV

Dengue fever is characterized by high fever and prolonged recovery. Dengue hemorrhagic fever patients during a secondary infection display very high levels of inflammatory cytokines produced by T-cells in response to antigen presented by dendritic cells (Gubler, 1998a). The increase in levels of cytokines in the blood stream leads to a storm of intracellular signaling in cells that have receptors for these inflammatory cytokines. This process leads to a decrease in platelet count and vascular leakage. The effects on the patient are seen as hemorrhage and culmination in shock and death in some cases (reviewed by Mathew and Rothman, 2008). Due to the direct involvement of T-cells in precipitating DHF, several investigations have been undertaken with respect to the role of the thymus and direct T-cell infection in human patients with dengue fever (Killen and O'Sullivan, 1993). In patient autopsies performed in Burma, several histopathological changes were noted in bone marrow, thymus, spleen and medial lymph nodes of DHF cases. The investigators reported severe hypoplasia of the bone marrow, acute atrophy and wasting of the thymus, atrophy and depletion of cells in the T-cell areas of the spleen and similar effects in the lymph nodes. The investigators also hinted at a direct infection of the cells in the spleen, lymph node, thymus and thymus dependant areas of the spleen (Aung-Khin et al., 1975). Subsequent reports from other studies have been skeptical with respect to the direct involvement of T-cells in DENV infection. In order to address these discrepancies we carried out a series of experiments with direct infection of a human thymus tissue engrafted under the kidney capsule of SCID-hu mice with both primary strains of DENV as well as laboratory adapted strain DENV-2 16681. We also utilized DENV infected Vero cells as well as DENV infected dendritic cells as a

source of infectious virions. We did not observe any significant infection of T-cells in the human tissue by flow cytometry or immunohistochemistry.

2.2 Introduction

During a viral infection, the immune surveillance mechanism captures the virus and localizes it to the closest lymph node. In the case of infections like HIV-1, dendritic cells play an important role in being among the first cells to propagate the infection. These dendritic cells are thought to be responsible for the transport of the virus and its presentation to a source of T-cells in the lymph nodes and the thymus. During this process, viral antigens are processed internally and presented by antigen presenting cells (APCs) to T-cells. If an APC such as a dendritic cell is also infected, it acts as a source of new viral progeny that can expand an infection. DENV is injected into a susceptible individual by an infected mosquito. The Langerhan cells (a type of dendritic cell) in the skin are often the first recipients of the virus (Wu et al., 2000). Their migration out of the skin and into the blood stream allows for the migration of the virus to the lymph nodes. Dendritic cells, monocytes and macrophages have been shown to be easily susceptible to a DENV infection. During a severe dengue infection, viral titers often surpass $1x10^7$ to $1x10^{8.6}$ infectious particles per ml of human blood (Vaughn et al., 2000). Although viremia subsides very quickly, high viremia often triggers a quick and robust cytokine response. In an attempt to control the viremia, the immune response brings about a cytokine cascade that result in fever, hemorrhage, plasma-leakage and hypovolemic shock (Halstead, 2008). During the infection, T-cells of the immune system are constantly presented with the virus. Some autopsy results in the literature suggest that the

T-cells may be directly infected or play an important role in the enhancement of disease. In an isolated report using *in situ* hybridization, the authors demonstrated the presence of DENV transcripts in thymic autopsy tissue of patients who died due to DSS (Killen and O'Sullivan, 1993). Other reports have ruled out the possibility of DENV infecting T-cells (Jessie et al., 2004). The SCID-hu mouse model has been used previously to study DENV infections (Lin et al., 1998) (Wu et al., 1995). In these studies, the mice were engrafted with human peripheral blood monocytes. Virus was recovered from mice tissue including the thymus following infection with DENV-1. Another modification of the SCID-hu model has been the engraftment of a human thymus on the kidney capsule of the mice. This SCID-hu Thy/-liv model has been used in our laboratory to study HIV-1 infection of the human thymus as well as usage of lentiviral delivered anti-HIV genes (Akkina et al., 1994; Anderson et al., 2007; Banerjea et al., 2003). Thus these SCID-hu mice available in-house are a good source of human thymocytes as well as an efficient system to directly address the question of infection of a human thymus by DENV.

2.3 Materials and Methods

2.3a Infection of thymic grafts with DENV and DENV infected Vero cells

SCID-hu mice were implanted with fragments of human fetal thymus and liver tissue by a micro-surgical procedure under the capsule of the left kidney. The mice were sutured up post surgery and allowed to support graft growth for at least 3 months. All invasive manipulations on the mice were carried out under anesthesia.

In order to generate DENV infected Vero cells, cells were grown in 10% FBS-DMEM to 90% confluency in 6-well plates and infected at an MOI of 1 with DENV-2

16681 for 48 hours. Cells were made permeable and intracellularly stained for the dengue antigen and analyzed by flow cytometry to determine the level of infection of the cells (Lambeth et al., 2005). The infected cells were scraped from the dish and washed with plain media. They were injected along with fresh DENV-2 16681 virus into thymic grafts of SCID-hu mice.

Thymic grafts were held in place using a grabbing scissors and injected at various points with 50ul of DENV-2 16681 containing $1x10^6$ infectious particles. Controls were injected with 10% FBS-DMEM media without any virus. The grafts were harvested at regular intervals by excising the tissue from the kidney capsule under anesthesia. The tissue was sectioned into three parts. One was used to isolate individual cells and stained for DENV infection.

Cells were isolated by gently teasing a portion of the excised tissue separated for the purpose with a pair of forceps and scalpel. The single cell suspension thus generated contained several million T-cells that were analyzed for DENV infection. The cells were subjected to a permeabilization treatment with BD Perm Buffer and subjected to intracellular staining with a fluorescently labeled monoclonal antibody 4G2-Alexa 488. This method was previously described by Lambert et al 2005 and used to determine the infection in other cell types such as Vero and dendritic cells. The infected cells were subjected to a flow cytometry assay along with appropriate antibody isotype controls.

The other sections were frozen in OCT compound or fixed in 1% formaldehyde overnight. The fixed section was embedded in paraffin. The slides generated were stained to detect DENV infected cells as well as determine the distribution of cells in the thymus.

The paraffin sections were processed and stained with anti-dengue 2H2 antibody purified in-house, at Premier Laboratories Boulder CO.

2.3b Infection of thymic grafts with DENV infected dendritic cells

Dendritic cells were isolated from human blood for infection with DENV. PBMC's were isolated from whole human blood using a Ficoll density gradient centrifugation. The cells were allowed to adhere on 6-well plates for 2 hrs. Non-adherent cells were removed by washing gently with 10% FBS-RPMI. The adhered cells were then grown in the presence of cytokines GM-CSF (50ng/ml) and IL-4 (10ng/ml). The dendritic cells that mature from the population were infected with DENV-2 16681 virus on day 6 post isolation. Infected cells were collected by scrapping them off the plates using a sterile rubber policeman. The cells were washed and mixed with $1.35x10^6$ IU of DENV-2 16681 virus. The thymic tissues in the mice were injected with human dendritic cells as well as DENV-2 virus. The grafts were harvested on days 0, 1, 3, 5, 7 and 11 post-injection by excising the tissue from the kidney capsule and processed as mentioned above. In addition mice were injected with $1.35x10^6$ infectious particles of DENV-2 16681 virus alone and the tissues were harvested on day 3, 5, and 7 post-infection and processed similarly alongside other tissues.

2.3c Infection of thymic grafts with low-passage isolates of DENV-2

As mentioned above with respect to the infection with laboratory passage strain DENV-2 16681, thymic grafts were held in place using a grabbing scissors and injected at various points with 50ul of DENV-2 low passage virus. For these studies, low passage

DENV-2 strains D9590 SriLanka, AHF-100 Thailand and 10649 Philippines, a mixture of an equal amount of all viruses to obtain $3x10^6$ infectious particles were used. The graft tissues were harvested 1, 3, 5, 7, 9, 11 and 14 days post infection and processed as previously described.

2.4 Results

2.4a Infection of thymic grafts with DENV and Vero cells

Previous studies have established that SCID mice engrafted with human fetal liver-thymus-liver combination of tissues under their kidney capsule are efficiently engrafted with a human thymic tissue within 12 weeks of engraftment (McCune et al., 1988; Namikawa et al., 1990). In the first set of experiments human thymic grafts engrafted on the kidney capsule of SCID-hu mice as shown in Figure 2.1 were injected with DENV infected Vero cells or DENV-2 16681 virus. Table 2.1 shows the distribution of DENV infected Vero cells as well as grafts that were injected solely with 1.36×10^6 DENV-2 16681. The grafts were harvested day 2, day 5, day 8 and day 12 post injection. Injection of DENV directly into thymic grafts did not show any direct infection of T-cells in the thymic grafts as seen from results in Figure 2.2 panels C, D, E and F. On staining for DENV infected cells with fluorescent labeled antibody 4G2, we observed a background of 0.1% infected cells in the case of Mouse 1A as depicted in panel A. Mouse 1B shows the presence of 0.7% infected cells (panel B). This spike in infected cells is attributed to the presence of DENV infected Vero cells that were injected into the mouse thymus. Mouse 1C shows a 0.1% increase in the number of infected cells over background (panel C). This is a negligible number of cells that may be infected and the

Figure 2.1 Human thymus engrafted under the kidney capsule of a SCID-hu mice. A human thymus was developed from a fragment of human fetal thymus sandwiched between human fetal liver by a micro-surgical procedure under the left kidney capsule . The grafts were allowed to mature into a thymus which was later injected with DENV or DENV infected cells.

Table 2.1 Infection of SCID-hu thymii with DENV-2 and DENV-2 infected Vero Cells

Expt	DOC**	Mouse Code	Type of Challenge	Strains	Dose of viral inoculum	Cells Used	Histo-pathology	FACS Data
1	Day 2	1A	Control	-------	-------	-------	Neg	Neg
	Day 2	1B	Virus+ Cells	DENV-2 16681	1.35×10^6	Vero*	Neg	Low
	Day 2	1C	Virus	DENV-2 16681	1.35×10^6	-------	Neg	Neg
	Day 5	1D	Virus	DENV-2 16681	1.35×10^6	-------	Neg	Neg
	Day 8	1E	Virus	DENV-2 16681	1.35×10^6	-------	Neg	Neg
	Day 12	1F	Virus	DENV-2 16681	1.35×10^6	-------	Neg	Neg

*Infection was carried out using $1x10^6$ infected Vero cells in 50ul of DEN-2 16681.
** DOC: Day of collection of thymii post-infection.

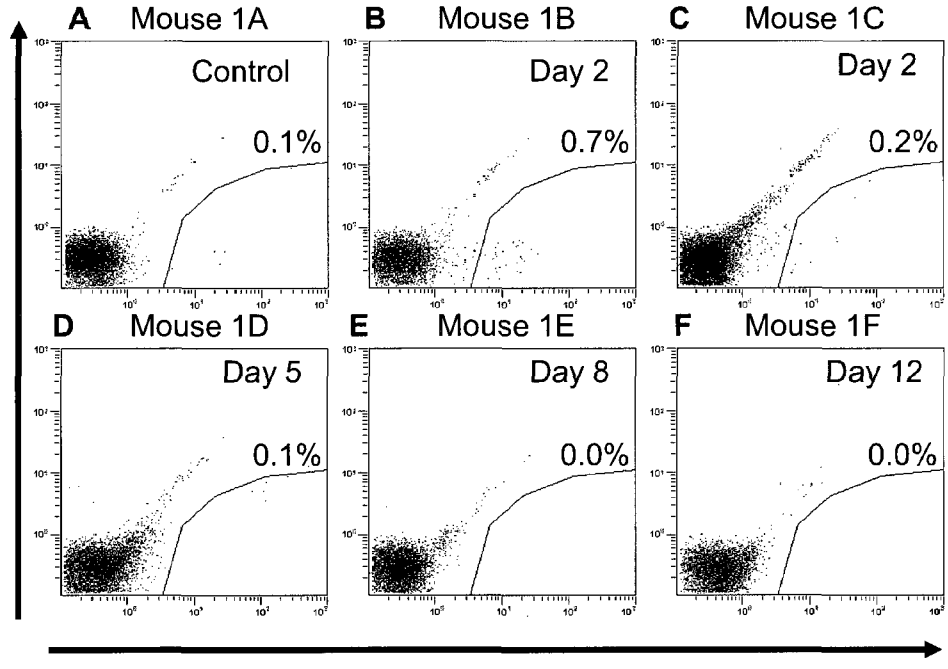


Figure 2.2 Flow cytometry analysis of dengue infection of SCID-hu thymus. SCID-hu mice with human thymic grafts were injected with DENV-2 16681 (panels C,D,E,F) or with DENV-2 16681 as well as DENV-2 16681 infected Vero cells (panel B). An uninfected thymic graft used as a control for the experiment is shown in panel A. The grafts were dissected , processed and analyzed by flow cytometry using an intracellular staining protocol for dengue antigens to identify dengue infected cells.

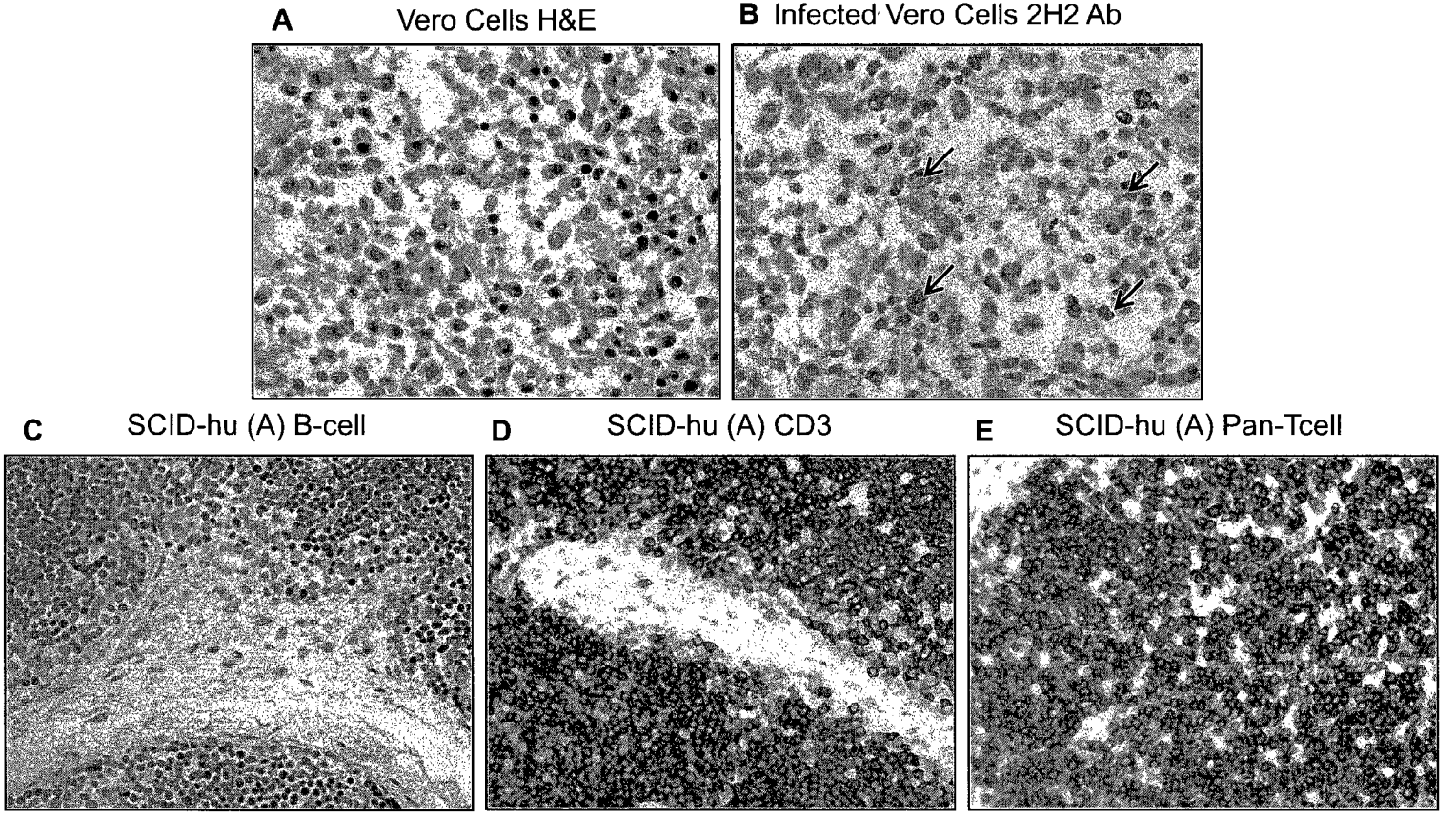

A
Vero Cells H&E
B Infected Vero Cells 2H2 Ab
C
SCID-hu (A) B-cell
D
SCID-hu (A) CD3
E
SCID-hu (A) Pan-Tcell

Figure 2.3 Immunohistochemistry analysis of Vero and thymic tissue harvested from SCID-hu mice. H&E staining of Vero cells is shown in panel A. Infected cells were detected using anti-dengue 2H2 monoclonal antibody and are denoted by black arrows (panel B). Tissue sections from SCID-hu mouse were stained for B cells (panel C) and T-cells using anti-human CD3 (panel D) as well as a Pan T-cell marker (panel E). Positive cells were identified by the presence of brown staining of the cytoplasm and on the periphery. Thymii from SCID-hu mice did not show the presence of any B-cells and are primarily composed of T-cells.

value is too close to background to be considered as a true infection. On days 5, 8 and 12 post infection we do not observe any increase in the number of infected T-cells over background (panels D, E and F). Since our results are contrary to the persistence of an infection, we conclude that thymic T-cells in the graft are not infected when directly injected with DENV-2 16681 or Vero cells infected with DENV-2 16681.

In order to directly observe the presence of infected cells in the thymic tissue a portion every thymic graft was subjected to immunohistochemistry using monoclonal antibody 2H2 directed against DENV prM protein. Prior to testing the antibody on thymic tissue, the antibody was used to stain sections of DENV-2 16681 infected Vero cells. In Figure 2.3, panel-A shows an H&E staining of infected Vero cells processed for immuno-histochemistry. The-morphology of the cells is intact with the cytoplasm and nucleus distinctly visible. When stained with 2H2 antibody by immunohistochemistry (panel B), Vero cells that are infected with DENV are made prominent by the presence of a brown cytoplasm. Uninfected cells have a clear cytoplasm. The nuclei of the cells were counter stained blue in order to be seen clearly. During this process we also stained the thymus section from Mouse-A (uninfected mouse) for the presence of human B cells using human CD20 (panel C), human CD3, a marker for T-cells (panel D) and a pan-human T-cell CD45 marker (panel E). We observe that there are no B-cells in the human thymus, and it is completely full of CD3+ human Tcells. On staining the thymic section infected with DENV-2 16681 or DENV-2 16681 infected Vero cells we did not observe the presence of any DENV infected cells (Figure 2.4 panels B, C and D). Thus the immunohistochemistry staining corroborated the finding of experiments done by flow cytometry in demonstrating that T-cells in the thymus are not infected by DENV.

2.4b Infection of thymic grafts with DENV infected dendritic cells.

During a mosquito bite, the Langerhan cells in the skin are the first cells to encounter a DENV. The dendritic cells are easily infected and enable the virus to propagate with ease when brought to a regional lymph node. The primary function of dendritic cells is to present antigen to T-lymphocytes in the thymus. It has been postulated that this is the predominant process by which HIV-1 is transmitted by dendritic cells to CD4 T-lymphocytes in a lymph node environment. Several reports in the literature have demonstrated that these cells are very easily infected *in vitro*. We isolated lymphocytes from human blood by a routine Ficoll-Hypaque density gradient method. The adherent cells were propagated in the presence of GM-CSF and IL-4, two cytokines which propagate the generation of dendritic cells from the monocyte population. These dendritic cells generated after 3 and 6 days in culture *in vitro* were analyzed for a dendritic cell marker (CD 209/ DC-SIGN) by flow cytometry. We analyzed the level of infection of these cells 1-48 hrs post infection and found that 34.1% of cells were infected on day 3 while 45.8% of cells were infected on day 6 as shown in Figure 2.5 panels B and D respectively. We used the cells infected on day 6 for our experiment to infect SCID-hu human thymus. The cells were processed and injected into the thymii as described above. In addition to the above, 3 mice were also infected with seed virus DENV-2 16681 (Table 2.2). The thymii were harvested on days 0, 1, 3, 5, 7 and 11 post infections. If the dendritic cells were able to maintain an infection in the thymus and pass on the virus whilst presenting antigen in the thymus, we expected a progressive increase in the number of infected cells visible by flow cytometry as well as

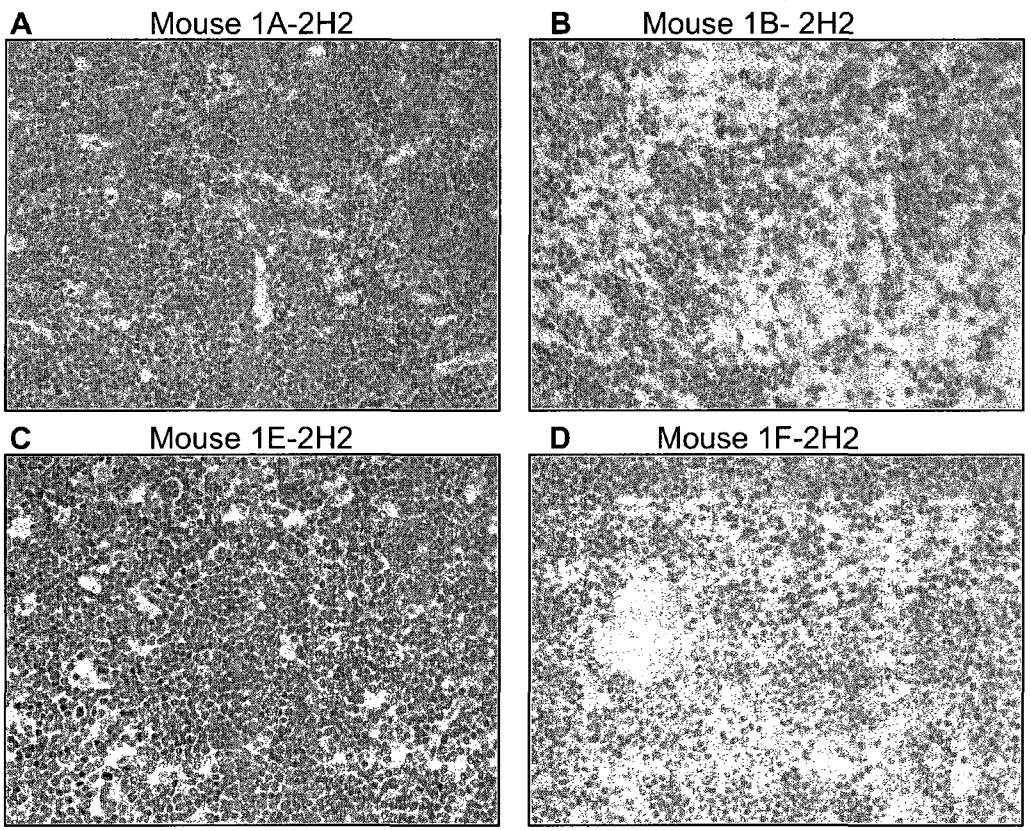


Figure 2.4 Immunohistochemistry analysis of thymic tissue harvested from SCID-hu mice. Tissue sections from thymii infected with DENV-2 16681 infected Vero cells (panel B) as well as DENV-2 16681 infected thymic grafts (panel C and D) were immunostained with anti-dengue 2H2 monoclonal antibody to detect infected cells. Thymic graft from a SCID-hu mouse that was injected with buffer (panel A) was used as control for staining. Nuclei were counter stained with Toluidene blue. No postive cells were observed in any of the sections examined.

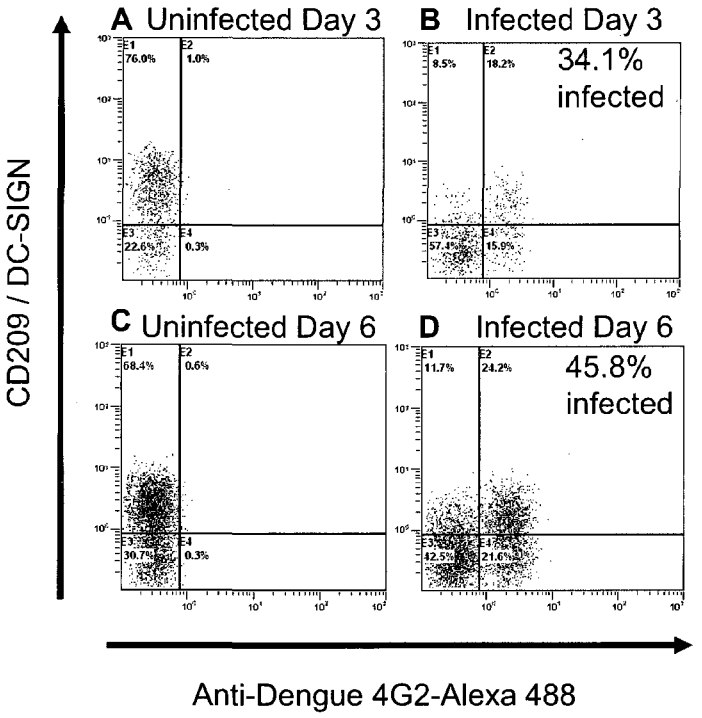


Figure 2.5 Flow cytometry analysis of dengue infection of human blood-derived dendritic cells. Dendritic cells were differentiated from human PBMC isolated from human blood using a cytokine cocktail containing IL-4 and GM-CSF. Differentiating cells were monitored for cell surface expression of CD209 (DC-SGN) marker on day 3 (panel A) and day 6 (panel C) post-differentiation. Cells were simultaneously infected with DENV-2 16681 and analyzed after 48 hours by intracellular staining.Infected cells on day 3 (panel B) and day 6 (panel) were detected by flow cytometry. A higher rate of DC infection on day 6 (45.6%) was observed as compared to day 3 (34.1%).

immunohistochemistry. On the contrary from the analysis of cells harvested from the thymii, we did not observe any infected T-lymphocytes. As shown in figures 2.6 and 2.7, the number of infected cells in the thymii did not exceed the background values observed in comparison to uninfected control cells. We analyzed the results progressively from day 0 to day 7 post infection (Figure 2.6 panels B-F) we observed a small progressive increase in the number of infected cells. This increase did not surpass 1.0% of the total number of cells on any given day, indicating that the minor increase seen was a product of the day to day were variations generated in the permeabilization based staining assay used for flow cytometry. This analysis was confirmed by the results observed for direct infection of thymic cells depicted in Figure 2.7 panels B-D, as well as from Figure 2.7 panel A.

2.4c Infection of thymic grafts with low-passage DENV strains

Following the availability of highly infectious, low-passaged DENV-2 strains D9590 SriLanka, AHF-100 Thailand and 10649 Philippines we hypothesized that the virulent nature of infecting strain may determine its ability to infect T-lymphocytes. Perhaps DENV-2 16681, which has been propagated several times *in vitro* in C6/36 cells, may have become benign and unable to actively infect mammalian cells. As tabulated in Table 2.3, SCID-hu mice were infected with a mix of all 3 DEN2 viruses. The thymii were harvested at days 1,3,5,7,9,11 and 14 post infection. The thymii were subjected to similar treatment as mentioned above. The tissue was treated exactly similar to the previous experiments. To our disappointment, we were unable to detect any aggressive infection of T-cells by these low-passaged field isolated of DENV-2 (figure 2.8 and

figure 2.9). The results obtained from the immunohistochemistry analysis of the sections corroborated the flow cytometry results.

Table 2.2 Infection of SCID-hu thymii with DENV-2 and DENV-2 infected dendritic Cells

Expt	DOC**	Mouse Code	Type of Challenge	Strains	Dose of viral inoculum	Cells* Used	Histo-pathology	FACS Data
1	Day 0	1G	Control	-------	-------	-------	Neg	Neg
	Day 5	1T	Control	-------	-------	-------	Neg	Neg
	Day 7	1U	Control	-------	--------	-------	Neg	Neg
	Day 0	1H	Virus + Cells	DENV-2 16681	1.35×10^6	DC	Neg	Low
	Day 1	1I	Virus + Cells	DENV-2 16681	1.35×10^6	DC	Neg	Neg
	Day 3	1J	Virus +Cells	DENV-2 16681	1.35×10^6	DC	Neg	Neg
	Day 3	1P	Virus	DENV-2 16681	1.35×10^6	-------	Neg	Neg
	Day 5	1K	Virus +Cells	DENV-2 16681	1.35×10^6	DC	Neg	Neg
	Day 5	1Q	Virus	DENV-2 16681	1.35×10^6	-------	Neg	Neg
	Day 7	1L	Virus +Cells	DENV-2 16681	1.35×10^6	DC	Neg	Neg
	Day 7	1R	Virus	DENV-2 16681	1.35×10^6	-------	Neg	Neg
	Day 11	1M	Virus +Cells	DENV-2 16681	1.35×10^6	DC	Neg	Neg

*Infection was carried out using 31300 infected Dendritic cells in 50ul of DENV-2 16681.
** DOC: Day of collection of thymii post-infection.

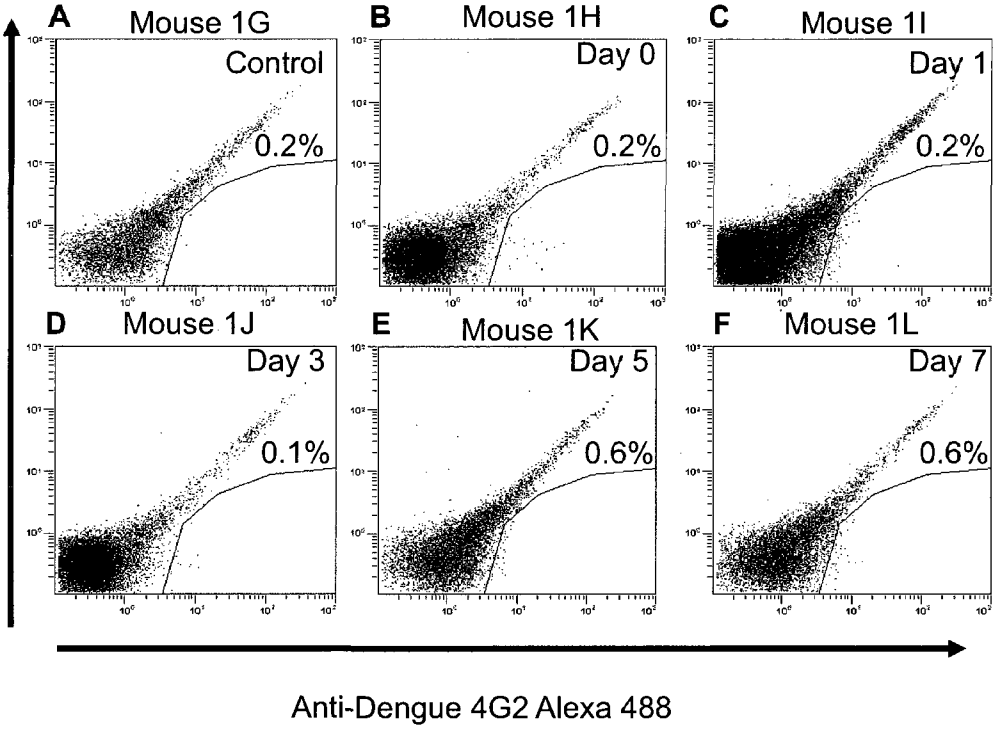


Figure 2.6 Flow cytometry analysis of infected SCID-hu thymic cells. Thymic grafts in SCID-hu mice were injected with DENV-2 16681 as well as dengue infected dendritic cells (panels B,C ,D,E and F). The grafts were excised of the mice under anesthesia and analyzed by intracellular flow cytometry staining for the presence of dengue antigen on specific days post injection. An uninfected graft from Mouse 1G (panel A) was used as a control for staining. We observed a few infected cells in the tissue harvested immediately after infection (0.2%). Subsequently at day 1 and day 3 we observed no change in the number of infected cells 0.2% and 0.1% respectively. An apparent increase in the number of infected cells was observed on day 5 and day 7 at 0.6% and 0.6% respectively attributed to background interference.

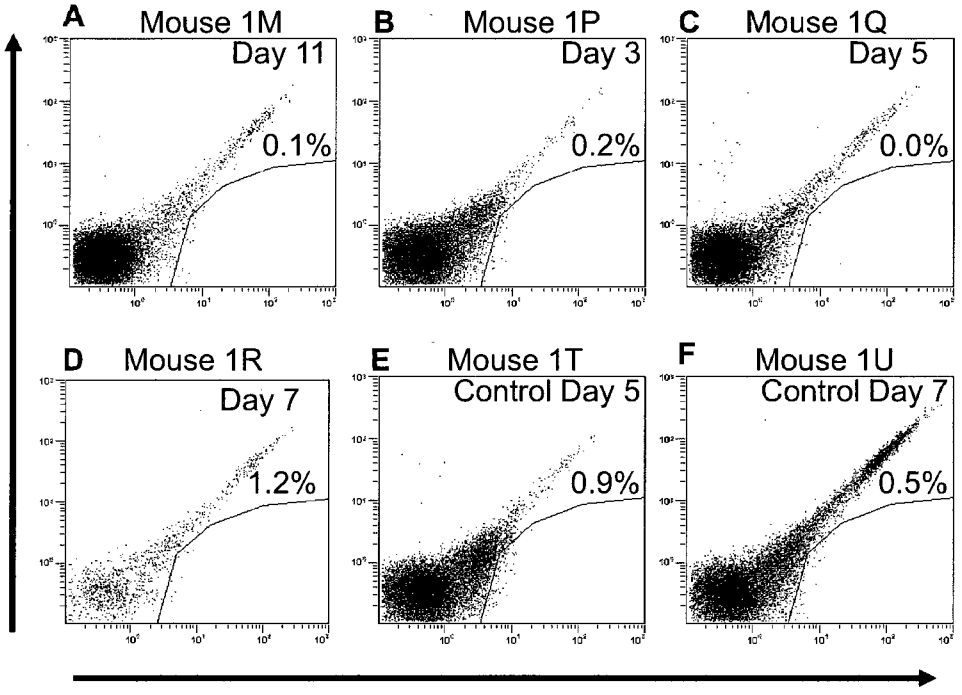


Figure 2.7 Flow cytometry analysis of infected SCID-hu thymic cells. Thymic grafts in SCID-hu mice were injected with DENV-2 16681(panels B,C,D) as well as dengue infected dendritic cells (panel A). The grafts were excised under anesthesia and analyzed by intracellular flow cytometry staining for the presence of dengue antigen on specific days post injection. An uninfected graft from Mouse 1T (panel E) and Mouse 1U (panel F) were used as a control for staining on day 5 and day 7 respectively. We did not observe an increase in the number of infected cells on day 11(0.1%). The grafts in mice which were injected with DENV-2 16681 alone showed 0.2%, 0.0% and 1.2% on infected cells on day 3,day 5 and day 7 respectively. We did not observe a significant increase in the number of infected cells over background cross reactivity.

Table 2.3 Infection of SCID-hu thymii with DENV-2 primary low-passage strains.

Expt	DOC**	Mouse Code	Type of Challenge	Strains*	Dose of viral inoculum	Cells Used	Histo-pathology	FACS Data
2	Day 1	2A	Control	-------	-------	-------	Neg	Neg
	Day 7	2G	Control	-------	-------	-------	Neg	N.A
	Day 1	2B	Virus	DENV-2 Primary	3×10^6	-------	Neg	Neg
	Day 3	2C	Virus	DENV-2 Primary	3×10^6	-------	Neg	Neg
	Day 5	2D	Virus	DENV-2 Primary	3×10^6	-------	Neg	Neg
	Day 7	2F	Virus	DENV-2 Primary	3×10^6	-------	Neg	Neg
	Day 9	2H	Virus	DENV-2 Primary	3×10^6	-------	Neg	Neg
	Day 11	2I	Virus	DENV-2 Primary	3×10^6	-------	Neg	Neg
	Day 14	2J	Virus	DENV-2 Primary	3×10^6	-------	Neg	Neg

*Infection was carried out using primary DENV-2 virus. The virus used was DENV-2 D9590 Sri Lanka, DENV-2 AHF-100 Thailand and DENV-2 10649 Philippines.

** DOC: Day of collection of thymii post-infection.

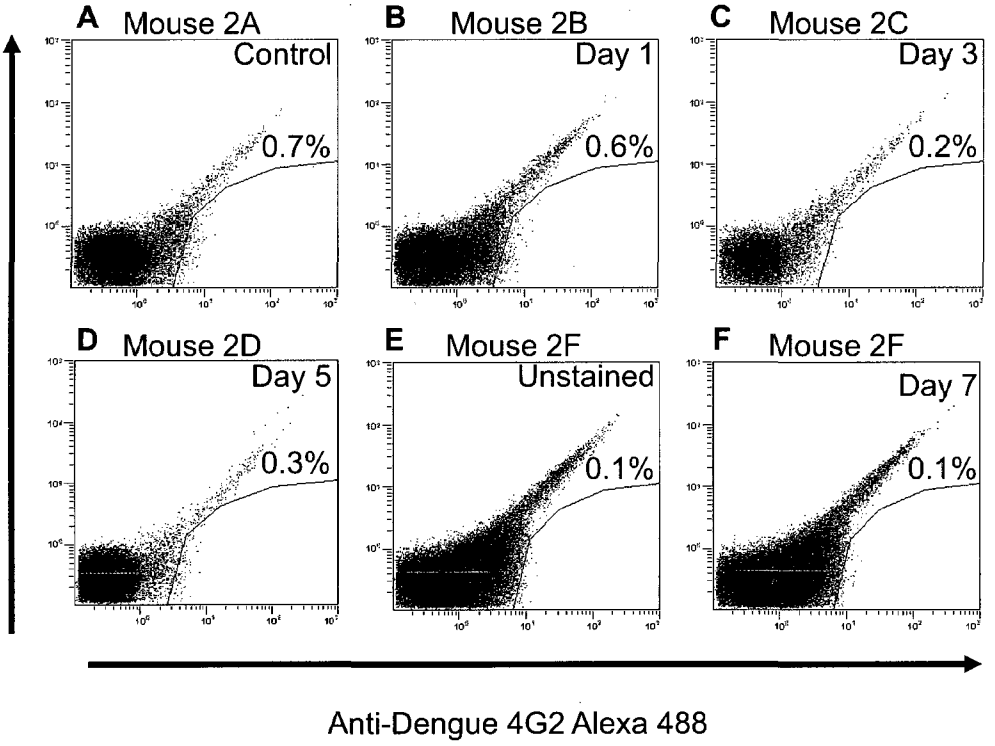


Figure 2.8 Flow cytometry analysis of infected SCID-hu thymic cells. Thymic grafts in SCID-hu mice were injected with DENV-2 primary strains (panels B,C,D, and F).The grafts were excised under anesthesia and analyzed by intracellular flow cytometry staining for the presence of dengue antigen on specific days post injection of virus and infected cells. An uninfected graft from Mouse 2A (panel A) was used as a control for staining on day 0. We observed 0.6% infection on day 1 post infection followed by 0.2% and 0.5% on days 3 and 5 respectively which are below the background level of staining in the control mouse. The level of dengue infected cells (0.1%) on day 7 (panel F) was found to be similar to an unstained sample of cells from the same day (panel E).

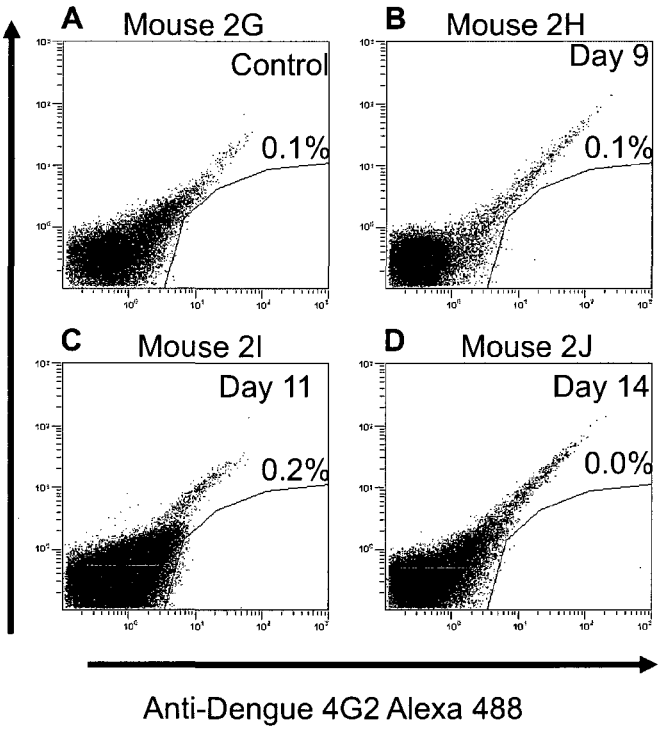


Figure 2.9 Flow cytometry analysis of infected SCID-hu thymic cells. Thymic grafts in SCID-hu mice were injected with DENV-2 primary strains (panels B,C and D).The grafts were excised out of the mice under anesthesia and analyzed by intracellular flow cytometry staining for the presence of dengue antigen on specific days post injection of virus and infected cells. An uninfected graft from Mouse 2G (panel A) was used as a control for staining on day 7. We observed 0.1%, 0.2% and 0.0% infection of cells on days 9, 11 and 14 respectively which were similar to the background levels on day 7 at 0.1%.

2.5 Discussion

Our intial hypothesis was to determine whether human thymocytes are directly infected by DENV. In our first set of experiments with the infection of SCID-hu thymus directly with DENV, we did not observe the presence of any infected thymocytes. We circumvented the process of virus clearance by the mouse innate immunity by providing actively infected Vero cells in the thymic environment. We hypothesized that the presence of cells producing active viral progeny would facilitate a more aggressive infection of the thymus. Our results suggest that the thymocytes were not infected by this process either. In order to simulate the environment of an active DENV infection as seen in human patients, we injected infected human dendritic cells directly into the human thymus in the SCID-hu mice. We hypothesized that these cells would persist and provide actively infectious virus in the thymus while presenting the antigen. Our results from this set of experiments also suggest that the human thymocytes were not permissive to DENV infection. Finally, we infected the human grafts with low passage DENV-2 that was isolated from patients during an active epidemic. We hypothesized that the virulent nature of the virus would provide a selective advantage to infect human thymocytes. Our observations from this set of experiments also indicate that human thymocytes are not infected by low passaged DENV-2 strains.

The role of T-cells in precipitating severe pathology during secondary DENV infections still remains an important area with several unanswered questions. T-cells are responsible for a cytokine cascade that leads to hemorrhagic manifestations as well as plasma leakage and subsequent onset of shock. The results obtained from all of the above experiments have shown that T-cells are not directly infected by DENV. Although

DENV may use several distinct receptors to infect various types of mammalian cells like glycosaminoglycans (Pokidysheva et al., 2006) or DC-SIGN (Navarros-Sanchez et al., 2003) or mannose receptor (Miller et al., 2008), T-cells do not seem to harbor a specific receptor or co-receptor that can be exploited by DENV. We would like to speculate that the observations on human thymus infections reported in the literature may be due to artifacts of the methods used to detect DENV. They may also have been contaminated during transportation and storage leading to false positives.

The SCID-hu mice engrafted with a human thymus in our hands, is limited in its ability to support a DENV infection and thus unsuitable to study pathology of primary and secondary DENV infections.

Chapter 3

Primary Infection of RAG-hu mice with Dengue Virus

Kuruvilla, J. G., Troyer, R. M., Devi, S., and Akkina, R. (2007). Dengue virus infection and immune response in humanized RAG2(-/-)gamma(c)(-/-) (RAG-hu) mice. Virology ***369***, 143-152.

This chapter contains results from my experiments that were included in the publication cited above. Other parts of this chapter that include results from primary infections with dengue serotypes 1, 3 and 4 were not included in the above publication

3.1 Abstract

Dengue viral pathogenesis and vaccine studies are hampered by the lack of an ideal animal model mimicking human disease and eliciting an adaptive immune response. Although current available models have been very useful in dissecting some key aspects of disease pathogenesis, a major limitation with these is the lack of a human immune response. In this study, we sought to overcome this difficulty by utilizing a novel mouse model that permits multi-lineage human hematopoiesis and immune response following transplantation with human hematopoietic stem cells. To generate immuno-competent humanized mice, neonatal $Rag2^{-/-}\gamma_c^{-/-}$ mice were xenografted with human CD34+ hematopoietic stem cells, resulting in the de novo development of major functional cells of the human adaptive immune system.

To evaluate susceptibility to dengue viral infection, humanized mice were challenged with DENV-2 serotype. Viremia lasting up to 3 weeks was detected in infected mice with viral titers reaching upto $10^{6.3}$ RNA copies/ml. Fever characteristics of dengue was also noted in infected mice. Presence of human anti-dengue antibodies was evaluated using an antibody capture ELISA. Anti-dengue IgM was first detected at 2 weeks post infection followed by IgG at 6 weeks. Sera from some of the infected mice were also found to be capable of reactivity with the viral envelope and capsid proteins in immunoprecipitation assay. These results demonstrate for the first time that humanized mice are capable of dengue viral primary immune responses thus paving way for new dengue immunopathogenesis and vaccine studies.

3.2 Overview of human cell engraftment in Rag2 $^{-/-}\gamma_c^{-/-}$ mice

The goal of investigators over a number of years has been to generate small animal models with the capacity to accept and duplicate human hematopoiesis from a xenograft with the capacity for denovo regeneration. These efforts were enabled by successes in the development of several gene-knockout immunodeficient mice. Several of these models include development in generation of mice in the severe combined immunodeficiency (SCID) as well as null mutations in genes like recombination activating genes (Rag1 and Rag 2), beta2microglobulin (B2m) and perforin (Prf1). The Rag gene mutations prevented development of mature lymphocytes. The B2M and Prf1 mutations prevent the development of mouse NK cell activity that is responsible for xenograft rejection. In a recent breakthrough, a new knockout that increases the engraftment capability was generated by combining the Rag2$^{-/-}$ knockout with a secondary IL-2r$\gamma^{-/-}$ mutation to the BALB/c background. The IL-2r$\gamma^{-/-}$ mutation in these mice leads to the lack of functional receptors for cytokines like IL-2, Il-7 etc which prevents the early expansion of NK cells and promoted xenoengraftment and longer lifespan.

The first successful xenoengraftment and hematopoiesis in BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ was described by Traggai et al in 2003. CD34+ hematopoietic stem cells that were sourced from human umbilical cord blood were used to engraft neonatal mice through an intra-hepatic injection after a mild non-lethal irradiation 3-5 days after birth. We have replicated the entire model with some modifications in our laboratory. Our first modification was the usage of CD34+ human hematopoietic stem cells isolated from human fetal liver. The disadvantage of using CD34+ cells isolated from human cord

blood is the low yield of cells from the source. The fetal livers provides a significantly larger number of CD34+ cells and under appropriate conditions maintains their totipotent nature to allow for novel manipulations. The second modification was to generate $Rag1^{-/-}$ mice similar to the Rag $2^{-/-}$ mice. We have observed that these mice have a similar constitution and equally amenable to CD34+ engraftment. Animals are allowed to grow for 12 weeks post injection of human hematopoietic stem cells in the liver at which point they are bled to estimate level of engraftment. Engraftment results suggest that there is development of both human myeloid and lymphoid lineage cells as well as re-population of lymphatic organs in the engrafted mice. The irradiation creates several niches in the bone marrow which are subsequently re-populated by the hematopoietic stem cells that migrate from the liver leading to development of B-cells, T-cells, monocytes, neutrophils, eosinophils and basophils as well as macrophages and dendritic cells of myeloid and lymphoid origin. The level of engraftment in each mouse was estimated by flow cytometry using human CD45, a pan-leukocyte marker. Therefore mice generated from the engraftment with fetal liver derived CD34+ cells are vulnerable to dengue infection due to the presence of human macrophages and dendritic cells.

3.3 Materials and Methods

3.3a Engraftment of Rag mice with CD34+ human hematopoietic stem cells

Newborn $Rag2^{-/-}\gamma_c^{-/-}$ mice were irradiated with a single dose of 3.5 Gy generated from a Cs-137 source within 3-5days of birth. At 1–24 h post irradiation, mice were injected intra-hepatically with $4x10^5$ to $1x10^6$ human CD34+ hematopoietic progenitor cells to generate the RAG-hu mice. Transplanted mice were screened for human cell

engraftment at 12+ weeks post-reconstitution with human progenitor cells. Peripheral blood was collected by a tail bleed into heparinized capillary tubes. RBCs were lysed using the Whole Blood Erythrocyte Lysing Kit (R&D Systems) according to the manufacturer's protocol. The lymphocyte fraction was stained with antibodies against the human pan-leukocyte marker CD45 and analyzed by FACS to verify engraftment. In order to detect the engraftment in the organs of RAG-hu mice, several mice were sacrificed and compared to un-engrafted animals as well as parent BALB/c mice.

3.3b Infection of RAG-hu mice with DENV

For the first primary infection with DENV 16 mice were injected with three strains of DENV-2. The viruses included in this study included DENV-2 16681 that has been highly laboratory passaged as well as primary low-passage clinical DENV-2 isolates: D9590 from Sri Lanka, 10649 from Philippines, and AHF-100 from Thailand. DENV-2 stocks were generated by amplification in C6/36 cells and titrated on Vero cells by flow cytometry FACS-based assay using 4G2-Alexa488. The mouse monoclonal antibody 4G2 was purified in house from culture supernatants and labeled with Alexa-488 fluorophore. Mice were injected with 200ul of DENV virus in four distinct places. Two injections were placed s/c on the left and right sides just above the inguinal lymph nodes. This was determined by the formation of a bolus under the skin. Two injections were placed i/p on the left and right sides respectively. All mice were challenged with $1x10^6$ IU of virus. Mice were also injected with other serotypes of DENV which included DENV-1 16007, DENV-3 SriLanka Isolate, DENV-4 241 in the same manner as mentioned above.

3.3c Clinical monitoring of RAG-hu mice

Mouse body temperatures were monitored every two days using a RET-3 rectal thermo-probe coupled to a Digi-Sense digital thermometer (Eutech Instruments). Mouse weights were monitored using an EK-1200i balance every two days for a period of 21 days post-injection. Mouse platelet counts and hematocrit were determined at pre-infection, 1 week, 2 weeks, and 3 weeks post infection using an Advia 120 hematology flow cytometer (Bayer) and validated by manually counting blood smears.

3.3d Real time qPCR for plasma viral load determination

Total RNA isolated from mouse plasma collected in heparinized capillaries using the QIAamp viral RNA kit (Qiagen) was reverse transcribed into cDNA with Superscript III™ System First Strand Synthesis for RT-PCR (Invitrogen) using DENV antisense primer NS5R (Kong et al 2006). Real time PCR was carried out using Platinum SYBR green PCR kit (Invitrogen) in a Biorad iCycler iQ thermocycler. DENV primers NS5F and NS5R (kong et al 2006) were used to initiate PCR with 5ul of cDNA as template. Reaction conditions were as follows: 50°C for 2min; 95°C for 2 min; 45cycles of 95°C for 15sec, 58°Cfor 30sec, and 72°C for 5min. Levels of DENV RNA amplified by this two step qRT-PCR method were determined by comparison to a standard curve generated against known quantities of DENV-2 16681 NS5 RNA, which were produced by transcription and quantified previously as described (Kong et al 2006).

3.3e DENV isolation from infected RAG-hu mice

Viral isolation from the sera of DENV-infected mice was done by performing two passages on C6/36 cells. The supernatants from the second passage were used to infect Vero cells which were subjected to FACS analysis 24 hours later using a mouse monoclonal anti-body 4G2-Alexa488 generated specifically against dengue envelope (E) protein. Additionally virus from the culture supernatants was also detected by PCR. The RNA was extracted from 140µl of 2nd passage of culture supernatant using QIAamp Viral RNA kit (Qiagen). RT-PCR was conducted with sense primer NS5-5 (AATCTCCATCTTGAAGGAAAGTGTG) and antisense primer NS5-6 (GCCTCTTGGTGTTGGTCTTTGC) using the SuperScriptIII One-step RT-PCR with Platinum Taq kit (Invitrogen) according to the manufacturer's instructions and recommended thermocycling conditions.

3.3f Detection and titration of human anti-dengue antibodies from infected RAG-hu mice

Levels of human anti-dengue antibodies present in the mouse serum were determined using commercial human IgM and IgG Dengue Capture ELISAs (Panbio). Sera from DENV-infected non-humanized $Rag2^{-/-}\gamma_c^{-/-}$ mice as well as humanized uninfected RAG-hu mice were used as negative controls. Positive controls included a known anti-DENV antibody positive human serum as well as samples provided with the ELISA kits. Samples were considered positive if the OD_{450} was greater than two times the background absorbance level. Serum samples which produced a positive IgM or IgG

ELISA reading at 1:10 dilution were then serially diluted two fold up to 1:81,920 and assayed by ELISA.

3.3g Detection of neutralizing antibody producers by a FACS based assay

The ability of RAG-hu mice generated antibodies to neutralize DENV-2 virus was determined using a FACS neutralization test (FNT) modified from a previously described method (Lambeth et al 2005). Mouse serum was mixed with 0.2 MOI DENV-2 16681 (determined relative to $4x10^5$ cells) in a total volume of 200ul. The 200ul serum-virus mixture was placed on ice for 30min followed by room temperature for 15min. This mixture was used to inoculate $4x10^5$ Vero cells in a single well of a 12 well plate. At 24hrs post –infection, single cell suspensions were made and subjected to FACS analysis after staining with 4G2-Alexa488 antibody as described in (Lambeth et al 2005). Serum from DENV infected non-humanized $Rag2^{-/-}\gamma_c^{-/-}$ mice as well as uninfected humanized RAG-hu mice were used as negative controls for neutralization. The FNT50 was defined as the greatest two-fold dilution of serum which produced >50% reduction in DENV infection of Vero cells.

3.3h Immunoprecipitation of DENV proteins by antibodies produced in infected RAG-hu mice

Immunoprecipitation of dengue polypeptides E and capsid proteins were carried out using antibodies generated in the infected humanized $Rag2^{-/-}\gamma_c^{-/-}$ mice using a protocol adapted from (Akkina et al., 1987). A T-75 flask containing $18x10^6$ Vero cells was cultured overnight in RPMI containing 10% heat inactivated fetal bovine serum, 2mM L-

Glutamine. The cells were infected at an MOI of 1.0 with DENV-2 16681 and washed with RPMI without fetal bovine serum to remove all traces of methionine 1-24 hrs post infection. The infected cells were methionine starved for 45 min in RPMI containing 2mM L-Glu. The proteins were metabolically labeled using 3ml of ^{35}S-methionine at 100uCi/ml for 4 hours at 37°C. After labeling the cells were washed with ice-cold PBS thrice. The cells were resuspended in modified RIPA buffer (Tris-HCl 50mM , pH 7.4, 1mM EDTA, 150mM NaCl, 1%NP-40, 0.25% Na-deoxycholate, 1mM PMSF and 1ug/ml mammalian protease inhibitor cocktail (SIGMA) to lyse the cells. The lysate was centrifuged at 14000g for 20min. For immunoprecipitation, a combination of Protein G plus Protein A agarose beads (Calbiochem) as well as anti-Human IgM specific agarose beads were used. The lysate was pre-cleared using 10ul of bead slurry per milliliter of lysate.

Polypeptides were immunoprecipitated using 10ul of sera from human or Rag mouse per 500ul of lysate overnight at 4°C on a rotary platform. The immunoprecipitated complexes were collected onto 30ul of Protein G/A and anti-human IgM beads for 30min on a rotary shaker. The beads were collected by centrifugation at 4000xg for 30secs. The beads were washed twice with RIPA buffer followed by once with 1M salt buffer (1M NaCl, 0.1% NP-40, 10mM Tris-HCl pH7.2, 1mM PMSF). The beads were given a final wash with sterile 1X PBS and collected by centrifugation. The beads were re-suspended in an appropriate volume of 2X Laemlli SDS-PAGE buffer and boiled to release the bound complexes. The proteins were analyzed on a 10% SDS-PAGE gel. The gels were dried and exposed to autoradiography film.

3.4 Results

3.4a Reconstitution of RAG-hu mice

Previous studies have established that $Rag2^{-/-}$ mice engrafted with cord blood CD34+ cells permit multi-lineage hematopoiesis (Baenzinger et al .,2006 Berges et al 2006, Gimeno et al 2004 , Traggiai et al 2004). To thoroughly evaluate their utility for DENV infection and immunity studies, we first constructed RAG-hu mice by intrahepatic injection of human fetal liver-derived CD34+ cells into conditioned neonatal mice. Our initial experiments evaluated the transplanted mice to verify levels of human engraftment, duration of persistence, tissue distribution, and the presence of different leukocyte subsets including DENV susceptible monocytes and dendritic cells. Human cell reconstitutions in blood, bone marrow, thymus, mesenteric lymph nodes, spleen and liver were evaluated by FACS analysis of cells for the presence of pan-leukocyte CD45 marker. While engraftment levels varied between mice, human cells were consistently detected in all these locations (Figure 3.1 and Figure 3.2). Additionally, enlargement of lymph nodes, thymus and spleen were also observed in RAG-hu mice when compared to non-reconstituted controls. Multi-lineage human hematopoiesis was demonstrated by FACS to detect lineage-specific markers. CD3+ T-cells, CD14+ macrophages, CD19+CD20+ B cells and both CD11c+ CD123- and CD11c- CD123c+ dendritic cells were present in the reconstituted mice (Figure 3.1). Thus RAG-hu mice harbor the essential cellular ingredients for sustaining a DENV infection and immune response. Overall, human immune reconstitution of $Rag2^{-/-}\gamma_c^{-/-}$ mice was similar to that described previously using similar methods although the source of cells was adapted to human fetal liver (Gimeno

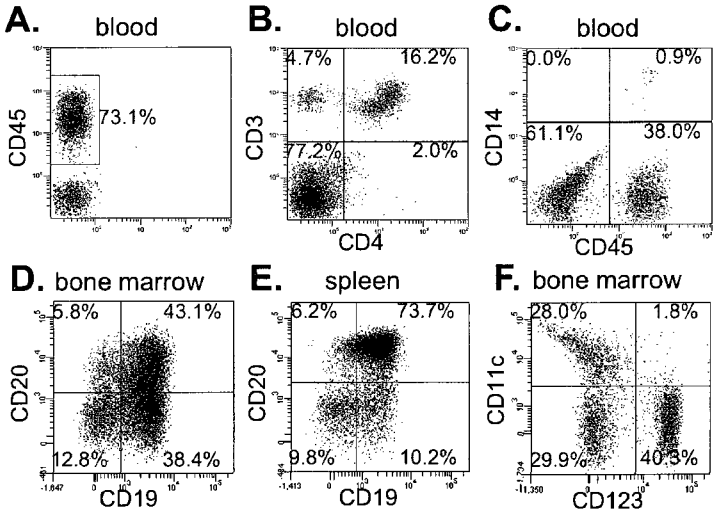


Figure 3.1 Human cell engraftment in Rag2$^{-/-}\gamma_c^{-/-}$ mice. FACS analysis was performed on cells from either peripheral blood or different lymphoid organs to detect various leukocyte subsets. CD45+ leukocytes (A) and CD3+CD4+ T cells (B) in blood were measured using a lymphocyte gate defined by staining normal human CD45+ blood leukocytes. CD14+ macrophages (C) were measured using a human monocyte/macrophage gate. CD19+ and/or CD20+ B cells in bone marrow (D) and spleen (E) were gated on CD45+ and CD3- populations. CD11c+ or CD123+ dendritic cells (F) were gated on CD45+, HLA-DR+, Lin1- populations. Dot plots are representative of at least five blood samples or organs examined from 12-20 week old mice. Cells from corresponding tissues of nonhumanized Rag2$^{-/-}\gamma_c^{-/-}$ mice were used as negative controls for all flow cytometric analyses. Appropriate fluorescently conjugated isotype matched mAbs were also used to control for background staining.

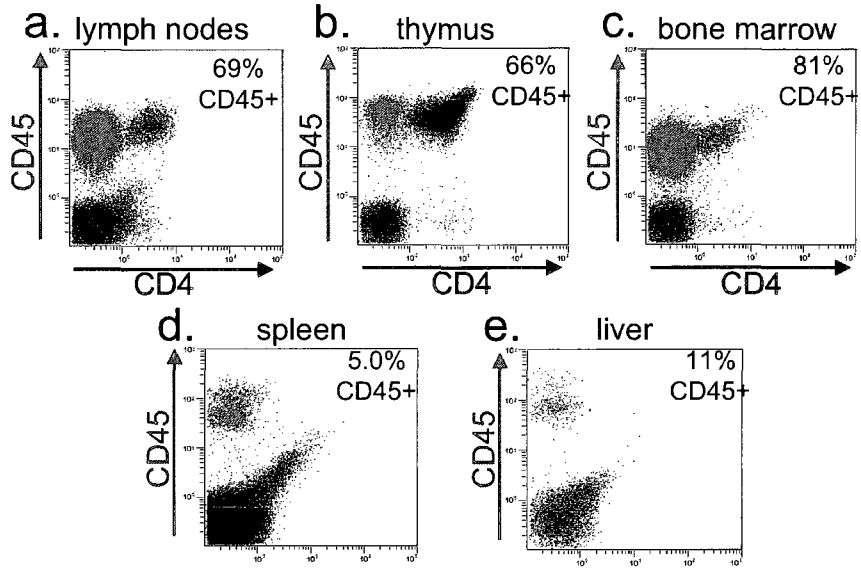


Figure 3.2 Human cell engraftment of RAG2$^{-/-}\gamma_c^{-/-}$ mice. Engraftment of CD45+ human leukocytes occurred in the lymph nodes (a), thymus (b), bone marrow (c), spleen (d) and liver (e) of humanized mice. CD4+ human T cells are indicated in red for lymph nodes, thymus and bone marrow. Dot plots are representative of at least five organs examined from 12-20 week old mice. Non-humanized RAG2$^{-/-}\gamma_c^{-/-}$ mice were used as negative controls and exhibited <0.5% CD45+ cells.

2004 Traggiai 2004). We also found that human hematopoiesis in these mice persisted for longer than one year.

3.4b RAG-hu mice support productive DENV infection

After constructing RAG-hu mice, and confirming that they generated a full complement of circulating human hematopoietic cells including monocytes and dendritic cells, we next proceeded to evaluate if these humanized mice are susceptible to DENV infection. To aid viral infection in our initial experiments, we infected mice which had >20% human leukocytes in blood. Since we were uncertain about which viral strains, ie low passage field strains versus highly adapted lab strain, would replicate efficiently in this system a pool of four isolates of DENV-2 serotype (three primary low-passage Southeast Asian DENV-2 strains, AHF-100, D9590 and 10649, and a lab DENV-2 strain 16681) was injected with half the inoculum delivered by the i/p route and half by the s/c route to each mouse. Three sets of infections were performed in which mice received different combination of viral isolates as described in methods. Non-supported productive infection leading to sustained viremia lasting up to 21 days (Figure.3.3, Table 3.1). The viral loads in plasma reached as high as $10^{6.3}$ copies/ml. Based on the quantitative PCR assay we used which has a detection sensitivity/threshold of 960 copies/ml, two distinct patterns of infection were evident: a) mice with high persistent plasma viremia lasting upto 21 days during which the virus was consistently detectable at every time point tested, b) mice with similar duration of viremia in which viral loads were below the detection threshold at some time points. Of the eight mice infected with the 4-strain DENV-2 mixture, five were positive for plasma viremia on all days whereas three had

Table 3.1 Plasma viral load (RNA copies/ml)

Mouse ID	Virus Challenge	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 21	Day 42
012	4 DENV-2*	29,676	54,714	24,957	171,435	13,571	410,866	379,665	1,031,811	-
013	4 DENV-2	81,828	263,512	668,036	76,261	185,055	230,994	24,261	41,362	-
022	4 DENV-2	77,893	21,355	6,899	7,872	96,206	296,825	7,659	8,800	-
023	4 DENV-2	83,580	46,132	106,709	98,855	32,352	382,032	14,090	186,767	-
037	4 DENV-2	16,203	22,080	177,133	73,926	157,217	101,391	338,376	530,029	-
011	4 DENV-2	106,072	-	-	-	24,694	-	-	-	-
006	4 DENV-2	20,207	-	-	-	-	-	305,141	-	-
004	4 DENV-2	-	-	-	-	-	-	37,068	-	-
020	3DENV-2†	33,667	28,589	61,165	46,989	76,507	43,162	71,995	189,234	-
027	3 DENV-2	100,508	270,023	17,836	23,281	28,392	14,041	48,484	93,605	-
032	3 DENV-2	16,297	60,214	715,155	222,741	248,028	335,043	148,799	40,077	-
034	3 DENV-2	26,903	249,574	1,041,598	513,570	133,444	2,198,366	1,295,985	243,332	-
042	3 DENV-2	-	9,120	-	-	201,120	-	-	-	-
033	3 DENV-2	-	-	19,200	-	-	-	-	-	-
041	3 DENV-2	-	-	-	-	-	-	15,560	-	-
026	3 DENV-2	-	-	-	-	-	-	10,932	-	-
031	1DENV-2 #	16,088	-	-	-	-	-	59,156	392,374	-
048	1 DENV-2	-	-	-	10,945	-	-	-	36,341	-

NH1‡	4 DENV-2	-	-	-	27,473	8,432	-	-	-	-
NH2	4 DENV-2	-	-	-	-	-	-	-	-	-
NH3	4 DENV-2	-	-	-	-	-	-	-	-	-
NH4	4 DENV-2	-	-	-	-	-	-	-	-	-
NH5	3 DENV-2	1,483	-	-	-	3,581	-	-	-	-
NH6	3 DENV-2	-	-	3,355	-	-	-	-	-	-
NH7	3 DENV-2	-	-	-	-	-	-	-	-	-
NH8	3 DENV-2	-	-	-	-	-	-	-	-	-

*4 DENV-2 = pool of four DEN-2 strains: 16681, AHF-100, D9590 and 10649
†3 DENV-2 = pool of three DEN-2 strains: AHF-100, D9590 and 10649 ; # 1 DENV-2 = DENV-2 16681 lab adapted strain
‡NH1-8 = non-humanized mice

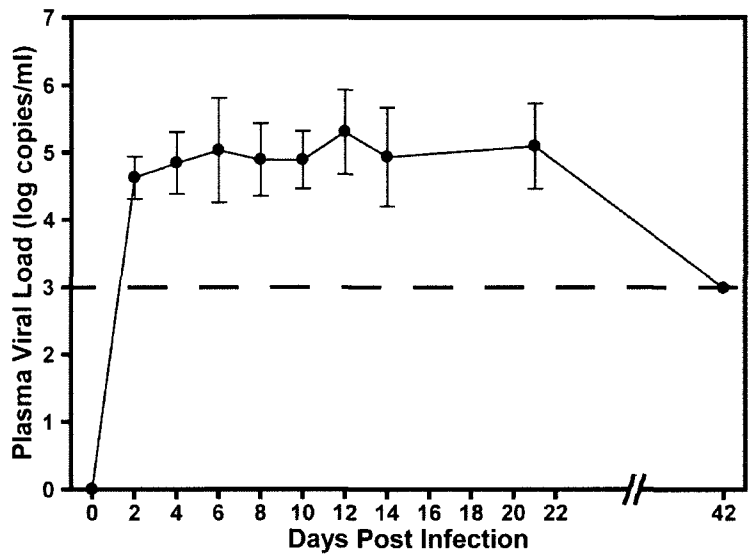


Figure 3.3 Dengue plasma viremia in infected RAG-hu mice. Plasma was collected from infected mice at different times post infection and viral RNA was isolated. Log DENV RNA copies/ml were determined by real-time qPCR. Mean viral loads (+/- one standard deviation) are shown for mice with constant detectable viremia (n=9).

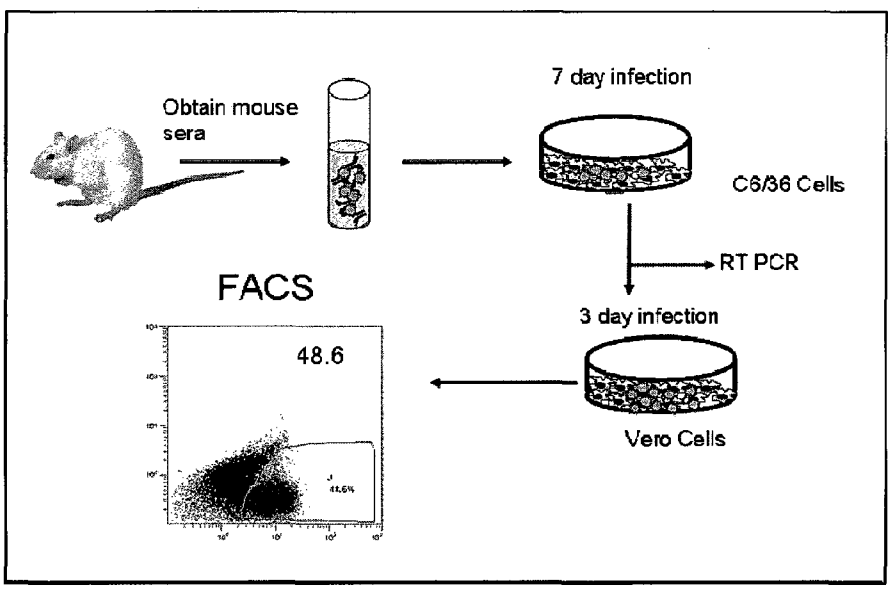


Figure 3.4 Schematic representation for viral isolation of infectious DENV from RAG-hu mouse sera. Sera from infected and uninfected mice were passaged twice on C6/36 cells then added to Vero cells, which were analyzed 48 hours post-infection by flow cytometry for DENV

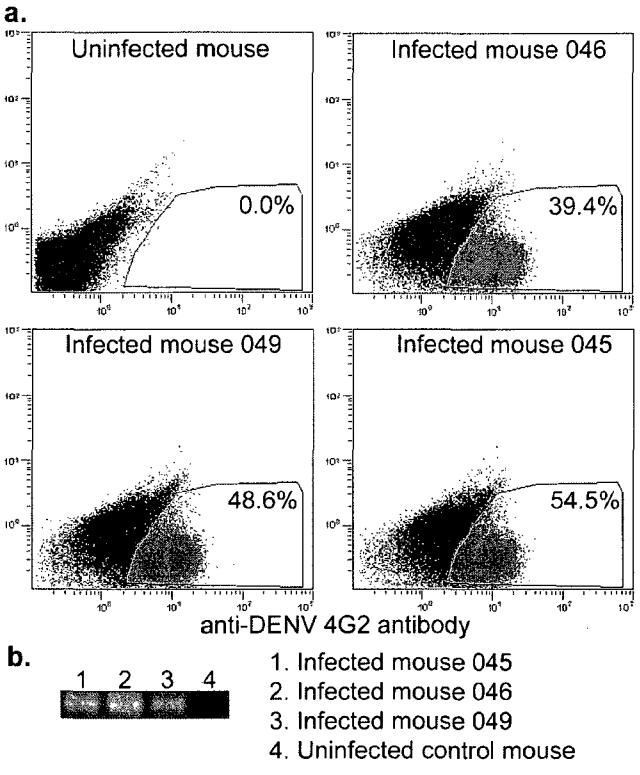


Figure 3.5 Isolation of infectious DENV from RAG-hu mouse sera. Sera from infected and uninfected mice were passaged twice on C6/36 cells then added to Vero cells, which were analyzed 48 hours post-infection by flow cytometry for DENV (a). RT-PCR on second-passage C6/36 supernatants yielded DENV-specific bands for the infected mouse sera but not the uninfected mouse sera (b).

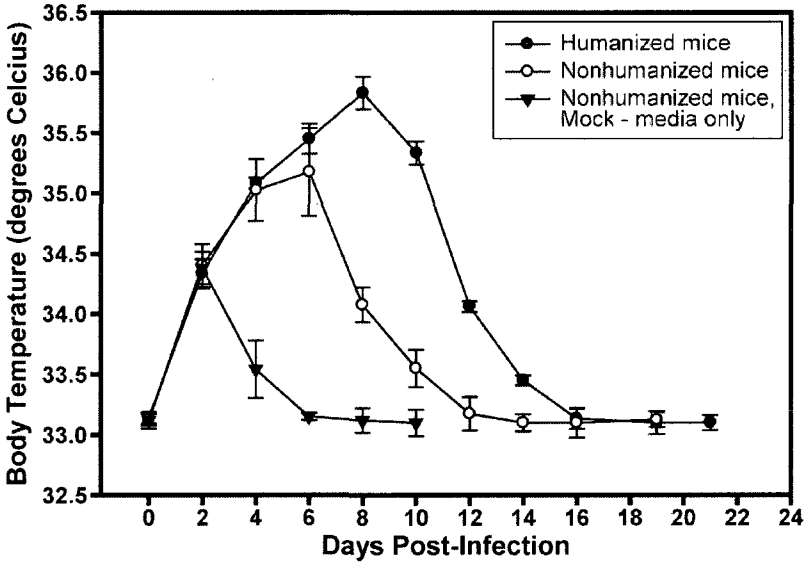


Figure 3.6 Fever in dengue virus infected mice. RAG-hu (n=14) and non-humanized RAG2-/-γc-/- (n=8) mice were challenged with DENV and rectal temperature was measured every two days. Non-humanized mice were also challenged with cell culture medium containing 10% FBS but lacking DENV (n=5).

viremia in which viral loads fell below the detection levels at some time points (Table 3.1). Similarly, of the eight mice challenged with the 3-strain DENV-2 mixture, four were positive for plasma viremia on all days tested whereas in the other four, viral detection was intermittent (Table 3.1). Both of the two mice challenged with lab strain 16681 alone also exhibited intermittent detection (Table 3.1). The plasma viral load of mice consistently detectable viremia remained at approximately 10^5 copies/ml from day 2 through day 21 (Figure 3.3). There was no difference in the plasma viral load between the mice infected with the 4-strain DENV-2 mixture and the 3-strain DENV-2 mixture. Of the eight infected non-humanized Rag $2^{-/-}$ mice, five had no detectable viremia and three had intermittent viral loads below $3x10^4$ copies/ml (Table 3.1). The latest time point at which a non-humanized mouse had detectable viremia was 10 days post infection. There is no clear correlation between the levels of human cell engraftment and the levels of viremia in infected mice (Table 3.1). Of the eight mice with >40% human cell reconstitution in the blood, four had continuous viremia and four intermittent viremia. Likewise, of the ten mice with <40% engraftment, five had continuous viremia and five had intermittent viremia. When the mice were injected with other serotypes of DENV, viremia was detected intermittently similar to DENV-2 infection. The peak viremia for each DENV strain is tabulated in Table 3.2

To confirm that viremia detected PCR was due to a viable replicating infectious virus, serum of infected animals was subjected to viral isolation by passage on C6/36 cells. Infectious virus could be isolated from all three viremic animals tested as assessed by FACS and PCR (Figure 3.4 and Figure 3.5). These results established that RAG-hu mice sustain a productive DENV infection. Since fever is a common feature in acute

Table 3.2 RAG-hu mice infection with all four serotypes of DENV

Mouse No	Primary Infection	Highest Viral Load Titer (IU/ml)*
M93	DENV-1 16007 Hawaii	1.4×10^3 Day 10
M120	DENV-1 16007 Hawaii	3.4×10^3 Day 10
M84	DENV-2 Philippines	7.6×10^5 Day 4
M85	DENV-2 Philippines	6.1×10^3 Day 2
M326	DENV-3 Sri Lanka	1.4×10^4 Day 5
M327	DENV-3 Sri Lanka	5.4×10^4 Day 7
M284	DENV-4 241	6.1×10^6 Day 17
M340	DENV-4 241	3.0×10^4 Day 9

*Plasma was collected from infected mice at different times post infection and viral RNA was isolated. Log DENV RNA copies/ml was determined by real-time qPCR. Highest viral load is reported for each viral serotype.

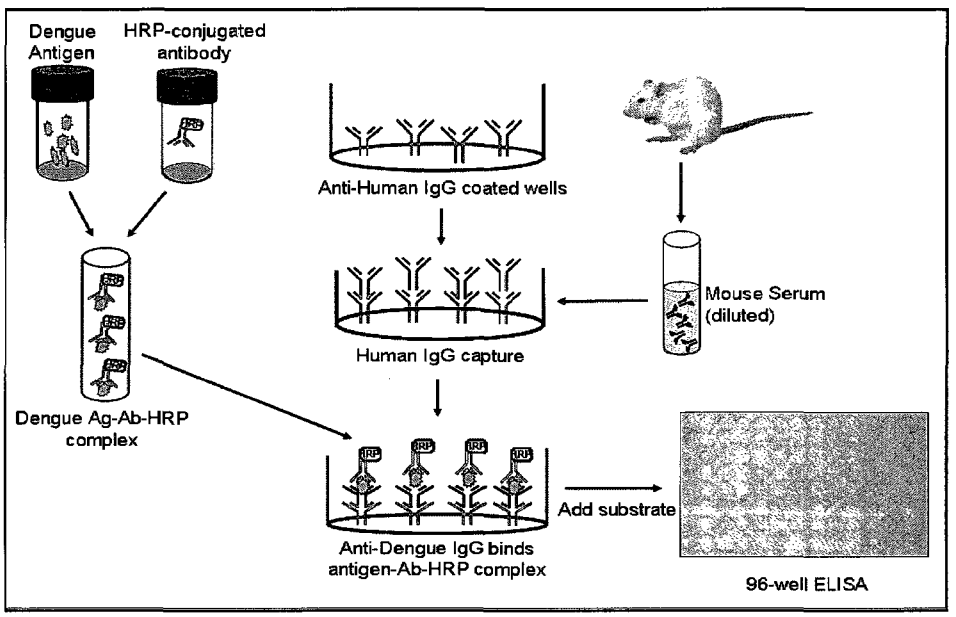


Figure 3.7 Schematic representation for PanBio sandwich ELISA to detect human anti-dengue antibodies. Sera was collected and diluted from infected as well as uninfected RAG-hu mice and bound to a human IgM/IgG capture well. An antibody sandwich is created with dengue antigen bound antibody-HRP complex. The substrate is added and the colored reaction identifies the presence of dengue specific human antibodies in RAG-hu mice sera.

Table 3.3 Detection of Human anti-dengue antibodies in RAG-hu mice sera

Mouse	1 week	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks	14 weeks
004	nd	nd	nd	nd	IgM ++	IgM IgG + +	IgM IgG + ++	IgM IgG + ++
006	nd	nd	nd	nd	IgM IgG + ++	IgM IgG + ++	IgM IgG + ++	IgM IgG + ++
012	nd	nd	NEG	NEG	IgM IgG + +	IgG +	IgG +	IgG +
020	NEG	NEG	NEG	NEG	IgM IgG +++ +	IgM IgG +++ +	IgM IgG +++ +	IgM IgG +++ +
027	NEG	NEG	IgM +	IgM IgG + +	nd	IgM IgG + +	nd	nd
034	nd	NEG	IgM +++	IgM ++	IgM +	IgM +	IgM +	nd
042	nd	IgM +	IgM +	IgM +	IgM +	IgM +	IgM +	IgM +
026	nd	NEG	NEG	NEG	IgM IgG + ++	IgM IgG + +++	IgM IgG + +++	nd
031	NEG	NEG	NEG	NEG	nd	IgM +	nd	IgM +
048	nd	NEG	NEG	NEG	IgG +	IgG +	IgG +	IgG +

+ = 2-fold greater than background fluorescence
++ = 4-fold greater than background fluorescence
+++ = 8-fold greater than background fluorescence
nd = not determined

Sera was collected from dengue infected RAG-hu mice and analyzed for the presence of anti-dengue human antibodies using a PanBio ELISA kit at various time points post infection. The IgM producers are indicated in red while IgG producers in blue. The background was established by multiplying the values obtained from uninfected RAG-hu mice sera by two.

dengue infection, we next evaluated whether the infected mice exhibited any increased body temperature. An extended fever lasting for 14 days and peaking at 35.8C on day 8 post infection was observed in infected RAG-hu mice. In contrast, non-engrafted infected and control mice receiving media alone exhibited a low grade fever lasting only a few days (Figure 3.6). The difference in mean temperature between infected humanized and non-humanized mice was statistically significant on day 8 ($p<0.001$), 10 ($p<0.001$) 12 ($p<0.001$) and 14 ($p= 0.005$, Student's t-test), indicating that the presence of human cells caused the mouse to develop a greater magnitude fever response to infection. We additionally monitored mouse platelet counts, blood hematocrit, and weight during the three weeks of infection and found no statistically significant change in any of these parameters. Mice also did not exhibit signs of rash or hemorrhage during infection.

3.4c Human immune response and DENV neutralizing antibody protection by RAG-hu mice

Since RAG-hu mice were previously shown to be capable of human immune responses to antigens as tetanus toxoid and EB virus (Traggiai et al., 2004), we wanted to evaluate if the productive DENV infection demonstrated above leads to a specific antibody response. Antibody responses were analyzed using dengue capture ELISA specific for human IgM and IgG in order to determine antibody isotype switching (Figure 3.7).

The majority of RAG-hu mice developed DENV specific human antibody responses to infection. However, the isotype, magnitude and kinetics of antibody development varied between mice (Table 3.3). DENV specific IgM was found in several

mice with its earliest detection at two weeks post infection. In most mice, antibodies were detected first at eight weeks post infection. IgG levels generally stayed constant while IgM levels waned in some mice and remained consistent in others (Table 3.3). Serum IgG titres were generally much higher than that of IgM with a median positive titre of 1:2560 and a maximum titre of 1:20,480 (Table 3.4). Eight DENV-infected non-humanized mice and three humanized uninfected mice were monitored for human DENV-specific IgM and IgG as negative controls and all were negative through 14weeks post-infection.

Neutralizing antibody responses are the most desirable since they are important in curtailing and controlling viral infections. Accordingly, we assessed the capacity of RAG-hu mouse antibodies to neutralize DENV-2 virus using a FACS based neutralization assay that has been previously demonstrated to yield equivalent results to the plaque reduction neutralization test (Figure 3.8). Our results showed that so far sera from thirty-seven ELISA antibody positive mice were able to neutralize DENV infection (Table 3.4 and Figure 3.9). In contrast, there was no evidence of neutralization by sera from all eight non-humanized infected mice, three uninfected RAG-hu mice, and all RAG-hu mice that failed to produce antibodies (Figure 3.9). We also evaluated the specificity of the infected mouse antisera by immunoprecipitation. As shown in Figure 3.10, lane C, sera from mouse M127 which showed neutralization was able to immunoprecipitate DENV–specific E and capsid polypeptides in contrast to control uninfected mouse serum.

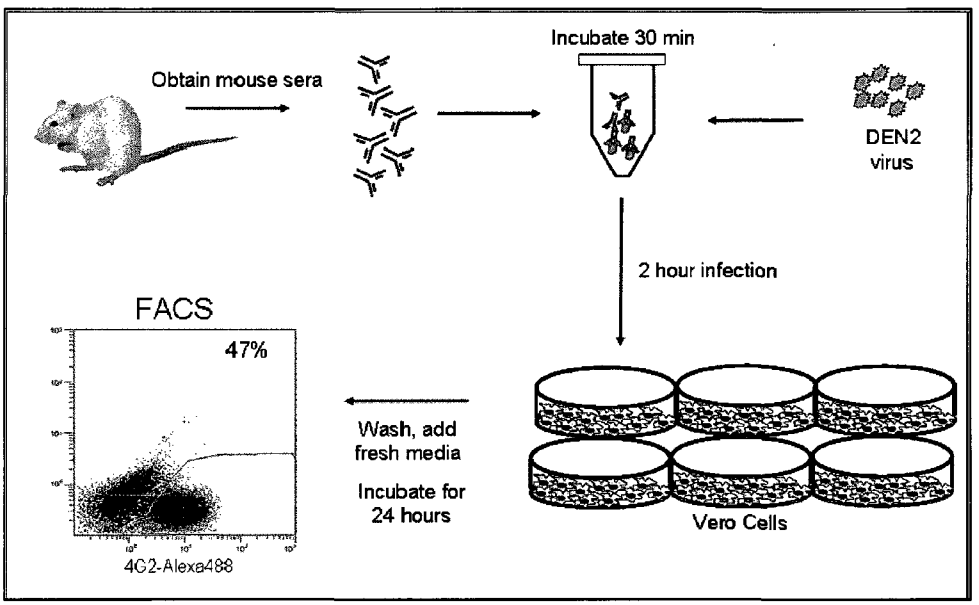


Figure 3.8 Schematic representation for a FACS based neutralization assay used to detect the presence of neutralizing antibodies in dengue infected RAG-hu mice.

Table 3.4 FACS based neutralization assay to determine neutralization titer of human anti-dengue antibodies in RAG-hu mice

Mouse	Weeks PI*	IgM titer	IgG titer	FNT_{50} titer
004	14	1:80	1:2560	1:160
006	14	1:80	1:10240	<1:40
012	14	<1:10	1:2560	<1:40
020	14	1:1280	1:2560	<1:40
048	12	<1:10	1:160	<1:40
026	10	1:40	1:20480	<1:40
034	10	1:20	<1:10	1:40
042	10	1:40	<1:10	<1:40
031	14	1:80	<1:10	<1:40
128	12	1:640	<1:10	1:1280

* post-infection

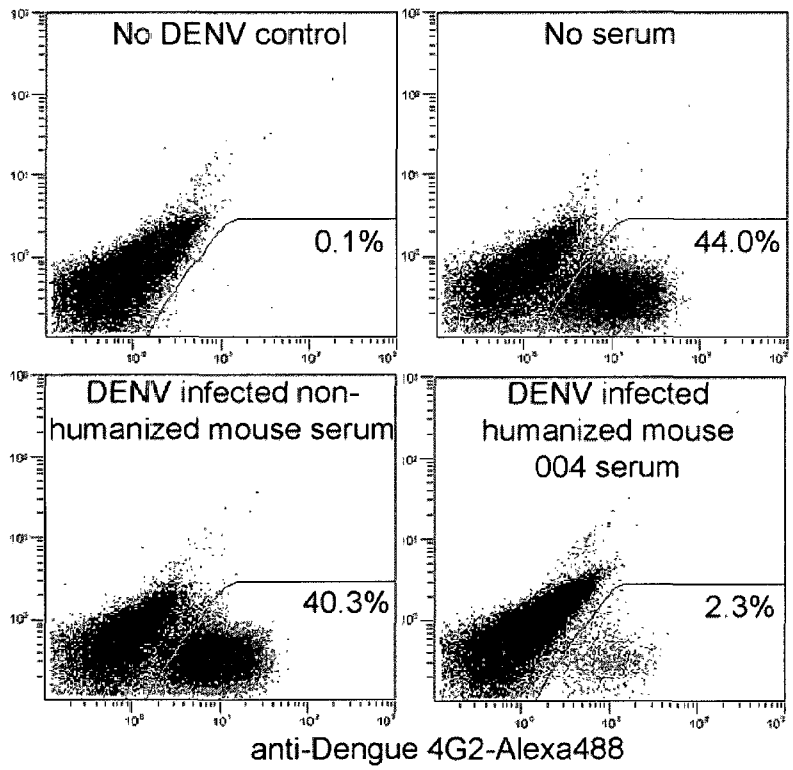


Figure 3.9 Neutralization of DEN-2 virus by infected humanized mouse serum. Controls are shown in which no dengue virus was added (flow cytometric staining background) and no mouse serum was added (level of infection without neutralization). Sera from DENV infected humanized mouse 004 neutralized DENV while sera from a DENV-infected nonhumanized mouse did not.

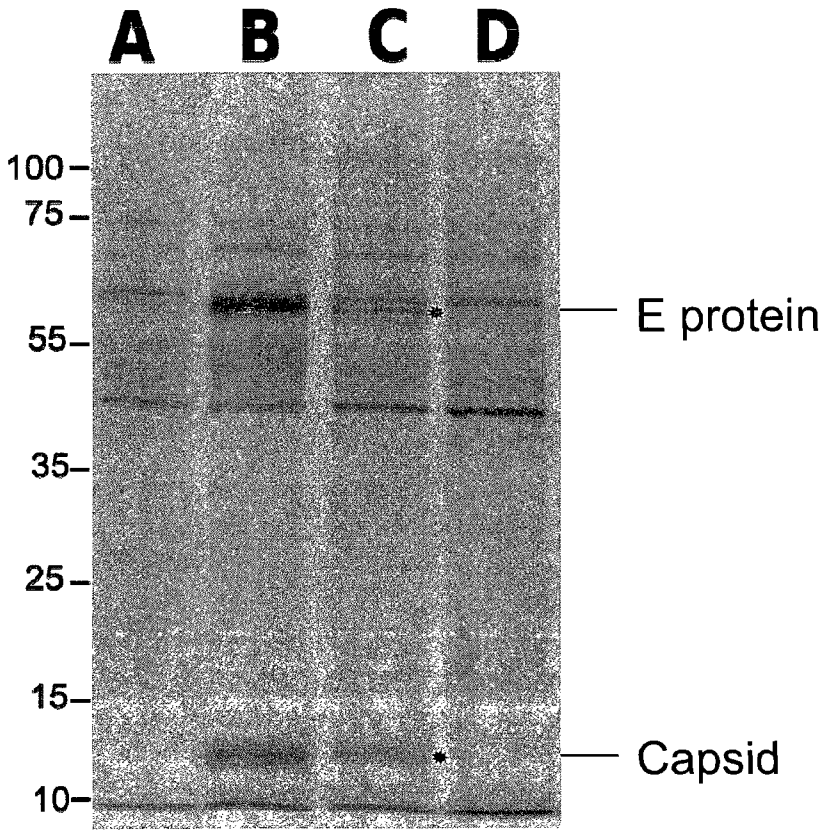


Figure 3.10 Immunoprecipitation of DENV polypeptides by infected mouse sera. DENV infected Vero cells were metabolically labeled with ^{35}S methionine and immunoprecipitated with mouse or human sera as described in methods. Viral polypeptides were resolved on a 10% SDS-PAGE gel and visualized by autoradiography. Lanes A and B : uninfected and infected cell lysates immunoprecipitated with DEN positive human sera. Lanes C and D: infected cell lysates immunoprecipitated with infected mouse antiserum and uninfected control mouse serum respectively. Immunoprecipitated DENV E (60kDa) and capsid (11kDa) polypeptides are indicated with asterics.

3.5 Discussion

Humanized mice with a full repertoire of circulating human hematopoietic cells offer novel opportunities to study DENV pathogenesis and immunity. In the present proof-of-concept experiments we showed that RAG-hu mice, capable of de novo multi-lineage human hematopoiesis, are susceptible to DENV infection and give rise to stable human antibody responses.

Small animal models such as mice have numerous advantages in general over large animal models such as primates, including ease of manipulation, lower cost and the larger number of animals that can be used for experimentation. Previous standard mouse models using mouse-adapted DENV strains have been very useful in studying mouse immune responses to DENV and some basic parameters of *in vivo* infection (Shresta 2004 Shrestra 2006). Similarly, humanized mouse models transplanted with DENV susceptible cell lines sustained limited viremia and exhibited some DENV associated pathology (An et al 1999, lin et al 1998). In a recent advancement, NOD-SCID mice engrafted with cord blood derived human hematopoietic stem cells also achieved viremia together with key symptoms of fever and thrombocytopenia (Bente et al 2005). However due to the lack of the necessary combination of essential human cells, no human immune responses could be generated in these humanized models thus restricting their potential. Furthermore, due to high incidence of lymphomas and consequent early death (Greiner, Hesselton and Shultz 1998), long-term pathogenesis studies are not possible in humanized NOD-SCID mice. In contrast, RAG-hu mice have a normal life characteristics, the present RAG-hu system overcomes most of the previous limitations and thus provides an improved *in vivo* animal model for DENV and other infectious

disease research. In fact, recent emerging studies including some from our own laboratory, showed the utility if this model for studying long-term HIV infection and virus induced CD4 helper T-cell depletion (Baenziger et al 2006 and Berges et al 2006).

Although the presence of functional human T-cells, B-cells and dendritic cells have been demonstrated previously in RAG-hu mice (Traggiai et al 2004), clear data on circulating human monocytes was lacking until recently (Baenzinger et al 2006, Berges et al 2006). Here we specifically analyzed RAG-hu mice for their presence and confirmed their existence together with T, B and dendritic cells. Thus this data has established that RAG-hu mice harbor both monocytes and dendritic cells purported to be among the primary DENV targets *in vivo*. Upon *in vivo* challenge, all the infected RAG-hu mice showed persistent viremia with viral levels reaching upto $10^{6.3}$ viral RNA copies per ml of plasma. Plasma viral loads seen here are similar to those reported in the humanized NOD-SCID mouse model (Bente et al 2005). By successful viral isolation from viremic animals, we also verified that the circulating virus as detected by RT-PCR is indeed viable infectious virus.

Interestingly, viremia persisted for 21days which is longer than that seen in humans (2-12 days) (Gubler 1998). The reason for this relatively longer viremia is not clear but may reflect qualitative differences in the innate and adaptive immune responses of these mice versus the human. We also noted that while the levels of viremia consistently remained around $1x10^5$ copies/ml in most mice, viral levels fell below the detection limit at some time points in some mice. This may have resulted from the differences in the levels of human engraftment and/or levels of monocytes and dendritic cells in different mice or due to the physiological status of each animal.

Nevertheless, all infected mice whether infected with either low passage field or highly adapted lab stains, showed sustainable viremia that lasted upto 21 days. In contrast there was no detectable viremia in the majority of non-humanized control mice although low level of intermittent viremia ($3x10^4$/ml) lasting up to 10 days was seen in some mice consistent with previous observations that various nonhumanized mice can develop a low level of infection with DENV (Huang 2000a Paes 2005). Thus, the higher level of viremia seen in RAG-hu mice is clearly attributable to more efficient viral replication in engrafted human cells. The viremic animals also showed lasting fever which is characteristic of acute DENV infection thus mimicking a key aspect of the natural infection.

A primary distinguishing feature of the current system compared to all the previous dengue humanized mouse models is the generation of human specific antibodies to DENV. Both IgM and IgG antibodies were detected in the majority of mice that showed viremia (Table 3.3). Antibodies were detected first at eight weeks post infection in most mice. Early IgM response in some mice is followed by the appearance of IgG also demonstrated antibody class switching in humanized mice. In some mice IgM levels remained stable for extended periods of time even after IgG levels increased. IgG titres were generally much higher than IgM. The level of human cell engraftment in the blood prior to infection did not correlate with the ability to produce anti-DENV antibodies. Of the ten mice which produced anti-DENV antibodies, five had >40% engraftment whereas the other five had <40%. It needs to be determined if the overall variation in antibody responses is related to the levels of individual human leukocyte subsets.

Since neutralizing antibody is an essential component of protective immunity, we analyzed the ability of infected mouse sera to neutralize DENV infectivity *in vitro*. Our results showed that three of the infected mice produced neutralizing antibody. Immunoprecipitation data confirmed that mouse neutralizing antibodies indeed recognize the DENV E protein which is the neutralization determinant. To our knowledge this constitutes the first example of human neutralizing antibody production in any humanized mouse system in response to infection with a human virus. So far we have infected several mice and 37 of 66 mice (61%) analyzed have produced a specific human adaptive response against DENV. Among these mice 18 of 37 (48%) have produced neutralizing antibodies. The present data provides the proof of concept that protective antibodies can be elicited in this system, thus paving the way for establishing more consistent neutralizing activity may be related to variability in levels of human cell engraftment and/or in the extent of functional development of specific cell types such as B-cells or CD4+ helper T-cells. As mentioned above, dengue viremia seen in RAG-hu mice is longer than that found in human infections. This could be due to delayed antibody response in RAG-hu mice. Dengue specific IgM can typically be detected 5-6 post onset of symptoms in the human, whereas in RAG-hu mice detectable DENV specific antibody appeared around 8 weeks or later. However, it is interesting to note that all viremic mice cleared the virus whether or not they produced the neutralizing antibodies. This could be due to innate immune mechanisms operating and/or due to exhaustion of virus susceptible cells that need to be investigated in future studies.

Continuous denovo human multilineage hematopoiesis together with permissiveness for acute DENV infection and specific human response make this novel RAG-hu

mouse model conducive for testing many important questions on dengue pathogenesis involving human cells. The role of cross reactive sub-neutralizing levels of antibodies in precipitating serious and often fatal DHF/DSS via ADE remains to be further evaluated in a controlled experimental system. With human antibody immune responses seen in the present model, this question can now be evaluated systematically.

In addition differences in virulence and pathogenesis between different DENV serotypes or strains can be evaluated. In the areas of the world where different viral strains coexist, simultaneous infections in the human are possible and their *in vivo* interactions are poorly understood. Thus the interplay among multiple strains infecting simultaneously can now be dissected. Since dengue is a mosquito-borne virus, the ability of infected mosquitoes to infect RAG-hu mice can now be examined with novel future experiments focusing on transgenic mosquitoes which are dengue resistant (Franz et al 2006). The involvement of different human cell types in disease pathogenesis and the specific cytokines they secrete in response to viral infection *in vivo* can also now be evaluated.

The present literature is conflicting regarding the primary cellular receptors in different cell types susceptible to DENV infection. The current model that uses hematopoietic stem cells to generate RAG-hu mice is particularly suitable to determine the roles played by putative cellular receptors. For example, gene knock-down strategies such as the use of siRNAs or ribozymes can be put to work in this model similar to that demonstrated previously with HIV and SCID-hu model systems in which viral molecules and cellular receptors were down regulated (Anderson and Akkina 2005, Bai et al 2000 Banerjea 2003). In one such experiment, CD34+ cells can be transduced with siRNA

containing viral vectors for specific receptor silencing. RAG-hu mice can be produced using these cells lacking these receptors and subsequently challenged with DENV to determine their susceptibility to infection.

In summary, the RAG-hu mouse model for DENV described in this study provides a unique research tool for addressing many important and timely questions about DENV infection and immunity.

3.6 Acknowledgements

This work reported here was supported by the Pediatric Dengue Vaccine Initative grant (PDVI-CRA TR-15) to Dr Ramesh Akkina. We would like to thank Dr Irving Weissman of Stanford University for providing the $Rag2^{-/-}$ mice, Claire Wong of CDC Fort Collins for Dengue viral strains, Dr Aravinda de Silva of UNC Chapel Hill and Dr Mary Marovich of Henry M Jackson foundation for conjugated antibodies. I would also like to thank Drs Scott Halstead and Susie Kilks for encouragement and overall support.

Chapter 4

Identification of organ tropism and dengue infected human cells post- infection in RAG-hu mice

4.1 Abstract

Dengue virus has been shown to infect several types of cells *in vitro*. These include monocytes, macrophages, dendritic cells as well as cells of hepatic, endothelial and lymphoid origin. In the case of human patients infected with DENV, monocyte-macrophage cells as well as dendritic cells are suggested to be the direct targets of infection by dengue virus. In order to determine the source of infection during primary and secondary infection of RAG-hu mice we have used two distinct methods to identify infected cells. We used an *in situ* hybridization method to detect DENV RNA in actively infected cells. We were able to identify the presence of dengue infected cells in the lymph nodes as well as spleens of infected mice. In order to determine the type of human cells that were infected we used a dual immuno-fluorescence staining method to identify the presence of dengue infected human cells. We were able to identify dengue infected dendritic cells in the lymph nodes of infected mice using immunofluoresence staining.

4.2 Introduction

DENV infects mononuclear phagocytic cells. Several reports have established that infections in patients have been identified in liver, spleen, lymph node, thymus, kidney, lung and skin by immunohistochemistry (IHC) (Migostovich et al., 1997, Hall et al., 1991, Bhoopat et al 1996., Boonpucknavig et al., 1979). A majority of the observations were obtained from post-mortem samples or fixed tissue. The presence of viral antigens only confirms the presence of viral proteins but does not confirm replication of the virus in the particular cell. In order to confirm active replication, *in situ* hybridization was performed. From our studies we have demonstrated that RAG-hu mice are successfully

infected with dengue virus by detection of viremia and re-isolation of virus from the infected mice. Further, we sought to demonstrate the presence of infected cells and determine the type of the cells that are infected during primary and secondary dengue viral infections. Due to the relatively larger number of human cells in spleen, lymph nodes and bone marrow, we focused our study on these organs in particular, especially the spleen and lymph nodes of infected mice. We have identified dengue infected cells in the spleen and lymph nodes of these mice using *in situ* hybridization. We have also identified human macrophages as well as rare dengue infected dendritic cells in infected mice tissue.

4.3 Materials and Methods

4.3a Detection of infected cells by *in situ* hybridization

DENV infected mice tissue was collected from mice undergoing primary infections as well as secondary challenges described in Chapter 3 and Chapter 5. Internal organs such as spleen, lymph nodes, liver, and bone marrow were collected and cyro-frozen from mice that developed high viremia or showed signs of severe disease. The DENV infections in mice that were used for *in situ* hybridization studies are described in Chapter 5.

In order to detect actively replicating viral RNA in dengue infected spleen and mice, we performed the *in situ* hybridization assay using Digoxigenin (DIG) labeled riboprobes directed against three distinct regions of the dengue viral RNA. cDNA against DEN-2 3'UTR region was generated by reverse transcription of viral RNA isolated from DEN2 16681 stock. Since the 3' region of the virus has consensus elements that are

conserved in all serotypes of the virus this region was particularly selected to make the 200nt probe. Primers were used to PCR amplify the desired region and the correct size was ascertained on an agarose gel. The product was purified and digested with BamHI and PstI. The digested fragment was ligated into a Roche pSPT18 plasmid at a 10:1 fragment to plasmid ratio. The ligation mixture was used to transform competent cells. Isolated colonies were picked for identification of desired clones. Mini prep DNA was generated and clones were tested with PCR primers for the generation of the desired product. After identification of the correct clones, colonies were grown for generation of a plasmid by maxi prep method. Purified plasmid was used to prepare the riboprobe in an *in vitro* transcription reaction. Using Hind III, 10ug of plasmid DNA was digested to generate a linearized fragment. *In vitro* transcription reactions were set-up using Digoxigenin labeled UTP and T7 polymerase (Promega) to generate the anti-sense probe. Similarly, Eco R1 was used to digest 10ug of plasmid DNA to generate the sense-strand probe in an *in vitro* transcription reaction using SP6 polymerase. After analysis of the probes on an agarose gel, they were used in an *in situ* hybridization reaction on tissue sections generated from dengue infected RAG-hu mice as well as control uninfected mice.

For hybridization, paraffin sections of spleen and lymph nodes from known dengue positive mice were utilized. The slides were de-paraffinized by treating them with 100% xylene solvent for 5 minutes each with 3 changes of solvent. The xylene was removed by washing in 100% ethanol for 5 minutes followed by a gradual rehydration of the tissue section in 95% ethanol, 70% ethanol, 50% ethanol and finally in 2X SSC buffer pH 7.0 (300mM NaCl, 30mM Na-Citrate) for a minute each. After rehydration into 2X

SSC, the tissue was deproteinized by digestion with Proteinase-K in 200ml of Proteinase K buffer (20mM Tris pH 7.5, 5mM EDTA pH 8.0, 250mM NaCl, 1% SDS) at 37°C for 3min. The slides were washed with DEPC water twice for 10seconds and treated in 100mM Triethanolamine buffer pH 8.0 for 3 minutes at 25°C.The slides were then given a wash with 200ml of TEA buffer containing 500ul acetic anhydride at 25°C for 10min. The slides were dehydrated after this step by dipping in 2X SSC, 50% ethanol, 70% ethanol, 95% ethanol and 100% ethanol for a minute at each step. After complete dehydration with 100% ethanol, the slides were air-dried for 60minutes at 25°C. Meanwhile the riboprobe was prepared for application onto the sections.

The riboprobe was boiled in DEPC treated water in a 15ml Falcon tube for 10 minutes and rapidly cooled on ice. To make a hybridization mix 2ug of DIG-riboprobe was mixed with 246ul of DEPC treated water, 144ul of Tris Buffered Saline, 1ml of distilled Formamide, 400ul of 40% Dextran sulfate and 200ul of a 10% blocking solution. To prepare the slides for hybridization, special hybridization coverslips were prepared by removal of the special protective backing. The 70-80ul of hybridization solution was placed on the coverslips. The coverslips were carefully rolled over onto the slides to avoid any air bubbles over the tissue sections. The slides were incubated overnight at 55-65°C and placed in a humid chamber. Post-hybridization, the coverslips are removed and the slides are washed with 2X SSC for 30 minutes at room temperature followed by a Formamide wash for 30 minutes at 65°C. The slides were washed with 2XSSC twice for 10 minutes each. The slides were then placed in RNAse Buffer to be treated with RNAse A (10mg/ml) for 30min at 37°C. Slides were then washed with RNAse buffer for 30min at 65°C to inactivate RNAses and prepped for immunohistochemical anti-DIG detection.

The slides were placed in blocking reagent with slow rocking for 2hrs at 25°C. The slides were then washed twice with 1X Maleate buffer (100mM Malic acid, 150mM NaCl pH 7.5) for 5 min with slow rocking. The DIG antibody was diluted 1:2500 with antibody dilution buffer. Slides were then rolled over carefully onto coverslips that held 210ul of the antibody solution to avoid air bubbles and placed carefully in a humid chamber. Care was taken such that the chamber was sealed with paraffin and placed at 25°C overnight.

The next day, the special coverslips were removed and the slides were washed twice with 1X Maleate buffer for 10min with gentle rocking. The slides were then washed in BCIP/NBT reaction buffer for 10min. The chromogen substrate (BCIP/NBT) was dissolved in 10ml sterile distilled water and placed onto the slides which are then covered by coverslips. The slides were placed in the humid chamber again overnight in the dark, taking necessary precautions to prevent to precipitation of the substrate on the slides.

The slides were washed in Stop buffer (10mM Tris 1mM EDTA 150mM NaCl pH 7.5) for 30 minutes at 25°C. The slides are then fixed in 4% paraformaldehyde for 20min at 25°C. The slides were then washed in stop buffer after which the slides were ready to be viewed. The sections are covered with 100% glycerol or Aqua polymount and mounted with glass coverslips and viewed under the microscope. Images were taken at 200 X and 400 X magnifications of tissue sections.

4.3b Detection of infected human cells by dual immunostaining using fluorescently labeled antibodies

Tissues, excised out of mice infected with dengue virus as well as uninfected controls, were frozen in the presence of cryoprotective OCT compound. In order obtain an infected piece of human tissue, a piece of perforated human tonsil explant, which is a valuable source of human macrophages and dendritic cells was used. The tissue was cultured in the presence of DENV-2 16681 in 10% FBS-RPMI medium for 4 days. The infected tissue was frozen in OCT compound and sectioned similar to other tissues. The slides were prepared from the frozen section using a microtome placed at -21°C at 4 micrometer thickness. The section were adsorbed onto a slide and allowed to dry for an hour at 25°C. They were then placed at -80°C until further use in the staining procedure.

Slides stored at -80°C were thawed out in the presence of Drierite to 25°C. The slides were then fixed in 1% paraformaldehyde in PBS for 20min. After fixation the tissues were demarcated with a grease pen and washed twice for 10min with 1X TBS (10mM Tris 1mM EDTA 150mM NaCl pH 7.6). The slides were then incubated with MOM (mouse on mouse Vector NTI) reagent for one hour at 25°C. The slides were then washed thrice for 10min with TBS buffer. Primary antibodies against dengue (1:100), CD68 (1:200) and CD14 (1:200) were diluted at in antibody dilution buffer (1%BSA, 0.01% NaN_3 in TBS) and applied onto the tissue sections carefully such that were no air bubbles. The slides were incubated in a humid chamber at 25°C for 30min. The slides were washed thrice in TBS with agitation for 10min. The secondary fluorescent labeled antibodies were diluted at 1:200 in antibody dilution buffer and spun at 14k rpm for 10min prior to be being carefully applied on the tissue section. Care was taken to avoid

any air bubbles and the slides were place in a humid chamber in the dark for 15min at 25°C. The slides were then washed thrice with agitation for 10min. The slides are then covered with Antifade-DAPI (Invitrogen) and placed in the dark until viewed under a fluorescent microscope.

4.4 Results and Conclusions

4.4a Detection of infected cells by *in situ* hybridization

Reports on the detection of dengue infected cells in human subjects have been very few and far between. In order to identify cells actively replicating DENV, we used an *in situ* hybridization method using digoxygenin (DIG) nucleotide labeled probes. Three distinct probes were generated against a negative strand of the virus which is routinely generated in actively infected cells as template for replicating the genome. The probes which were labeled with DIG to allow immune-detection using a colored substrate reaction were made against 5`UTR, E protein and 3'UTR regions of DENV. Infected mice as well as uninfected mouse tissues were tested. We tested several mice tissue samples for infected cells. We were unable to detect any positive cells in mice that were given a primary infection. However we found positive cells in both lymph node and spleen sections of mouse M164 that had detectable viremia day 6 post secondary infections.

The lymph nodes are made up of three distinct layers, a central medulla, a middle paracortex and outer cortex, which support distinct microenvironments. Antibody producing plasma B-cells are found in the medulla. T-cells are concentrated in the paracortex while B-cells are located primarily in the cortex, within the follicles and

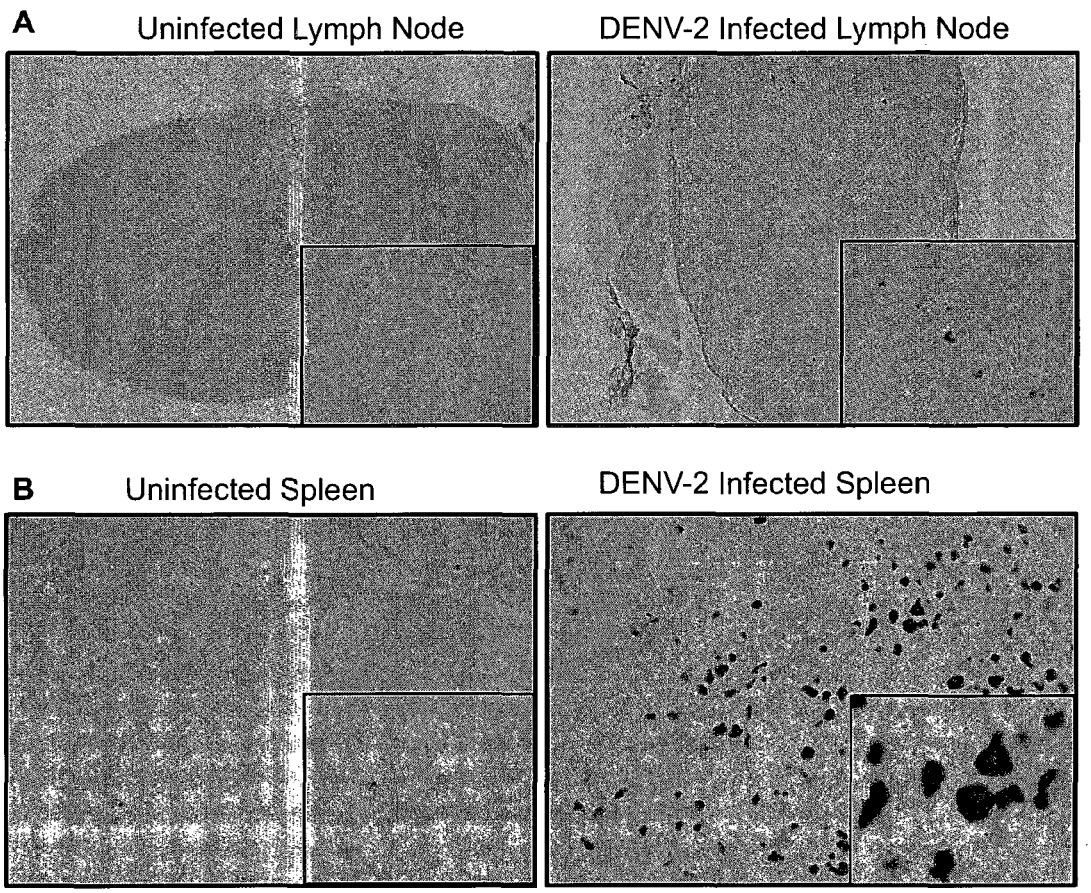


Figure 4.1 Detection of dengue infected cells in RAG-hu mice using in situ hybridization assay. Paraffin sections of infected and uninfected RAG-hu mice mesentric lymph node (A) and spleen (B) were processed and exposed to a Digoxigenin labeled anti-sense riboprobe directed against DENV-2 E protein, NS5 and 3'UTR regions. The infected cells were detected using a colored reagent that stains the cells dark blue. From the morphology of the infected cells we speculate that the infected cells are tissue macrophages in the lymph node and spleen.

germinal centers. Macrophages and dendritic cells that trap antigen are found in the cortex and paracortex. In the case of the lymph nodes, the virus was detected in cells that had an irregular shape that were distributed across the cross section of the mesenteric lymph nodes (Figure 4.1 panel A). Uninfected RAG-hu mouse lymph nodes used as controls did not show any background staining. In the infected RAG-hu mouse tissue, we speculate that the infected cells detected, based on their morphology are human macrophages or monocytes.

The mouse spleen is composed of two distinct regions namely white pulp and red pulp. The red pulp lies towards the periphery of the organ and contains a large number of erythrocytes which surround the sinusoid. The white pulp lies towards the core of the organ and forms the periateriolar lymphoid sheath that contains a large number of T-cells and B-cells. The infected cells are evenly distributed across the spleen without any specific preference for the red or white pulp of the spleen (Figure 4.1 panel B). The staining observed was seen in predominantly large cells of the spleen with irregular shape which lie close to each other. These cells may be splenic tissue macrophages that help to contain an aggressive infection.

4.4b Detection of infected human cells by dual immunostaining using fluorescently labeled antibodies.

In order to detect infected human cells in RAG-hu mice tissue we employed a dual fluorescence method of detection. The human cells were stained with mouse monoclonal antibodies directed against human cell surface antigens. We used anti-human CD14 as well as anti-human CD68 to detect human monocytes in the mice tissue. To

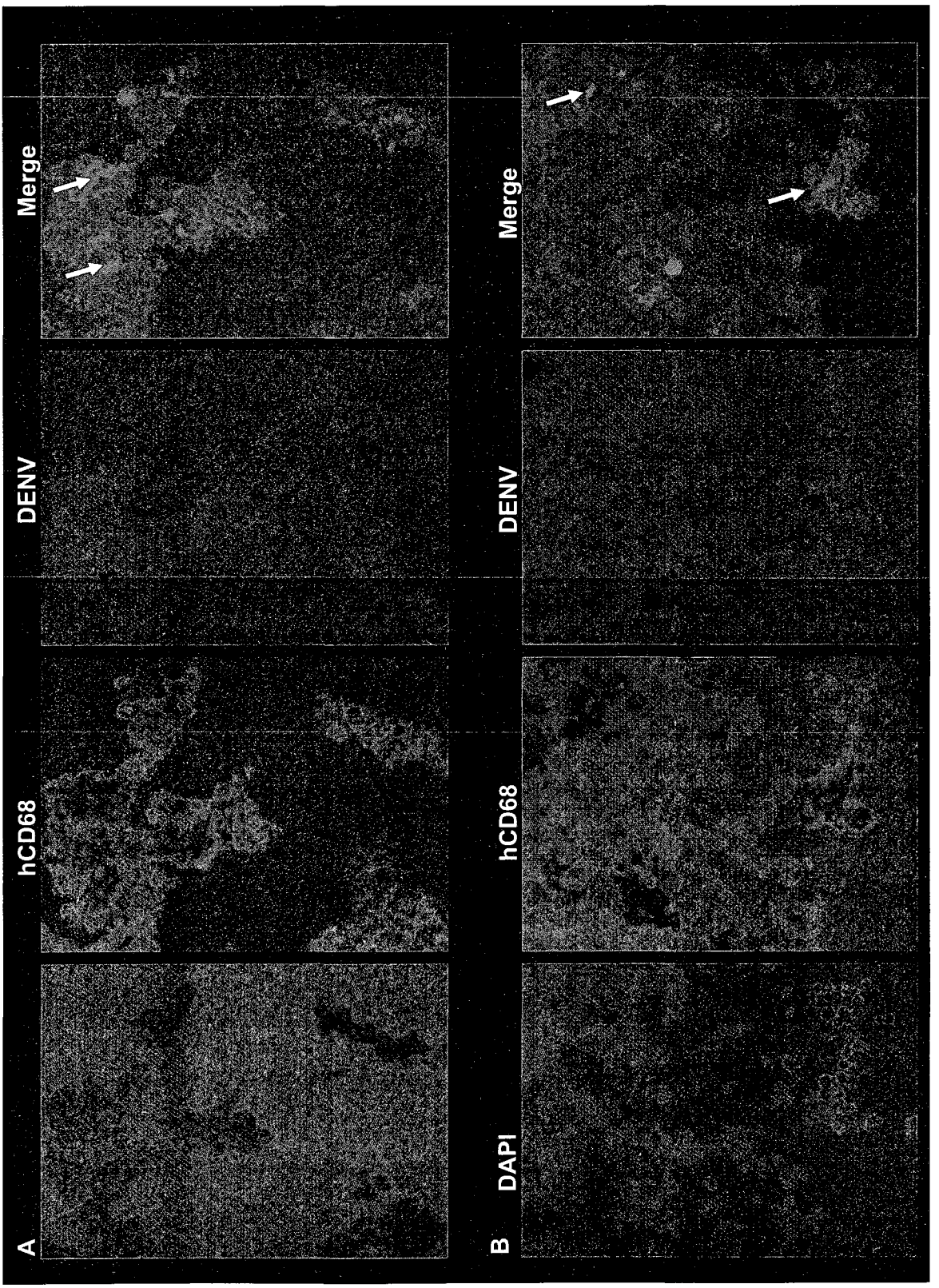

A
hCD68
DENV
Merge
B
DAPI
hCD68
DENV
Merge

Figure 4.2 Detection of dengue infected cells in human tonsil using dual immunofluroresence assay. Cryofrozen sections of DENV uninfected (A) and infected (B) human tonsil were stained with antibodies against human monocyte marker CD68 (green) and DENV (red). Dual stained human cells are depicted by arrows. Uninfected tissue does not show the presence of any dengue infected cells.

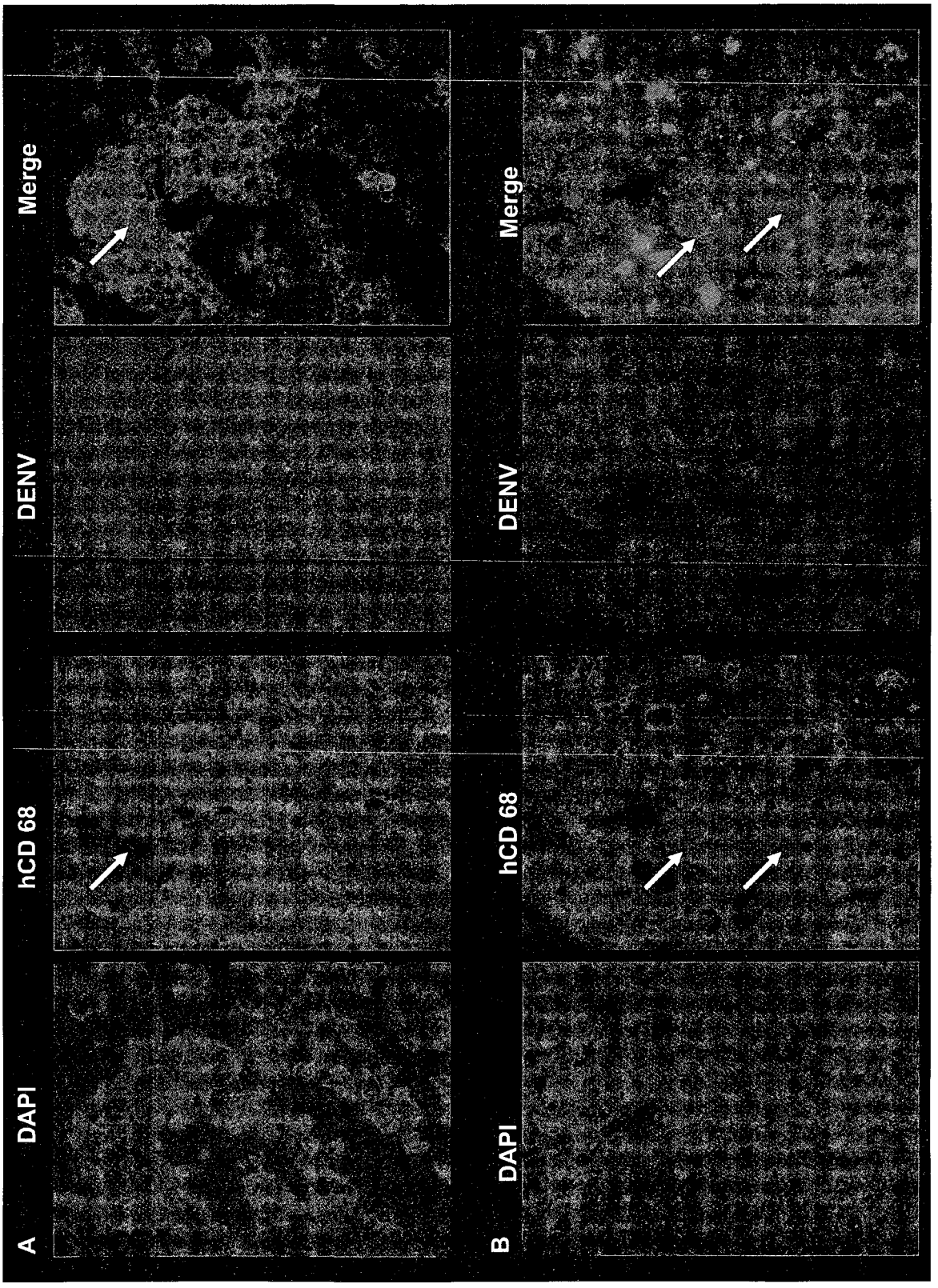

A
DAPI
hCD 68
DENV
Merge
B
DAPI
hCD 68
DENV
Merge

Figure 4.3 Detection of human cells in human tonsil and RAG-hu mice using dual immunofluroresence assay. Cryofrozen sections of uninfected human tonsil (A) and uninfected RAG-hu mouse J270 (B) were stained with antibodies against human monocyte marker CD68 (green) and DENV (red). Human monocyte cells are depicted by arrows. Uninfected tissue does not show the presence of any dengue infected cells.

A
DAPI
hCD 209
DENV
Merge
B
DAPI
hCD 209
DENV
Merge

Figure 4.4 Detection of dengue infected human dendritic cells in RAG-hu mice using a dual immunofluroresence assay. Cryofrozen sections of RAG-hu mouse J270 (panel A) and DENV infected J-15 (panel B) were stained with antibodies against human dendritic cell marker CD209 (green) and DENV (red). Human dendritic cells (green) were detected in both sections and are highlighted with arrows. An infected dendritic cell (red) was observed in lymph node of J-15 along with other infected cells. The insets in the merge of panel B show both an infected dendritic cell as well as a dengue infected cell that could probably be a macrophage.

prevent mouse on mouse cross reactive binding we use the mouse on mouse (MOM) blocking reagent from Vector as per manufacturer's recommendations. To detect the dengue antigen we utilized an anti-dengue rabbit polyclonal sera (Abcam Antibodies) raised against all four dengue serotypes.

The primary antibodies bound to their respective antigens were detected using fluorescently labeled antibodies made in an unrelated animal like chicken to prevent any non-specific staining. We used a chicken anti-mouse labeled with Alexa 488 to detect human cells and a chicken anti-rabbit antibody labeled with Alexa 594 to detect dengue infected cells for the final experiments. The primary hurdle associated with detection of rare DENV infected cells using fluorescent antibodies was the absence of a positive infected control to test the antibodies. An uninfected piece of tissue was also processed alongside and used to test the appropriate dilution of antibody to visualize the presence of human cells. As observed in figure 4.2 we were able to detect the presence of CD68 + monocytes (green) in uninfected (panel A) as well as infected (panel B) tonsil tissue. In addition, we were able to detect the presence of DENV infected cells (red) in infected tonsil tissue that merged with CD68+ cells (figure 4.2B) . From this experiment we were able to optimize the correct concentrations of antibody required for the staining procedure on infected RAG-hu mouse tissue. We were able to identify several rare human CD68+ monocytes (green) in uninfected RAG-hu mouse J270 mesentric lymph nodes (figure 4.3 B). We also identified CD209 positive cells in the lymph nodes of J270 (figure 4.4 panel A). We also observed dengue infected CD209 positive dendritic cells (green) as well as non-dendritic cells (red) in mouse J15 (figure 4.4B). The insets in figure 4.4 highlight the infected cells.

All of the cells identified in the lymph nodes of the mice were predominantly present in the outer cortex region of the lymph nodes as expected. However the total numbers of human macrophages or dendritic cells in a section are very few. Thus more optimization will be required to detect infected cells in RAG-hu mouse tissue by immunofluoresence staining methods.

Chapter 5

Secondary Infection of RAG-hu mice with Dengue Virus

5.1 Abstract

Dengue fever among human patients under clinical settings is classified into four distinct syndromes. They are categorized as a) an undifferentiated fever, b) classic dengue fever, c) dengue hemorrhagic fever (DHF) and d) dengue shock syndrome (DSS). The first two categories are relatively mild infections that affect a large population. The latter two syndromes affect a small subset of the susceptible population and are often fatal due to the large number of complications associated with them. They are hypothesized to be caused by a phenomenon referred to as antibody dependent enhancement (ADE), a condition where patients with pre-existing non-neutralizing antibodies against an infecting DENV serotype experiences a severe and aggressive form of dengue. The disease in such cases often manifests itself as hemorrhage, plasma leakage, and shock. Currently studies on DHF and DSS are hampered by the lack of an ideal animal model mimicking human disease and generation of an adaptive human immune response which is a prerequisite for DHF or DSS to occur. By using the RAG-hu mouse model system we have previously demonstrated the ability of the system to experience a primary dengue infection as well as generate an adaptive immune response against it. We utilized the mice that survived a primary dengue infection for a second challenge, either with a similar serotype of DENV as the primary infection or a heterologous serotype of DENV. We have noted an increased severity and susceptibility to the disease. Viremia with a peak titer of $7.2x10^7$ RNA copies/ml was detected in one mouse. Several mice were susceptible to severe dehydration and morbidity. Sera of many of the mice were capable of DENV neutralization with an increase in titer after the secondary infection. We also detected human cytokine production in the sera of mice

after secondary infection. We also observed a significant decrease in the platelet counts in some mice. Cumulatively, in mice which had an adaptive immune response to their primary infection, we observed several individual instances of DHF/DSS like pathological effects during the course of a subsequent infection. We believe that the RAG-hu mouse model system provides the right environment to duplicate and study the effects of ADE, DHF and DSS during a dengue infection.

5.2 Introduction

DENV infects humans causing a range of illness from subclinical infection to acute dengue fever to the severe and sometimes fatal vascular leakage conditions of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). There are over 2.5 billion people at risk for infection and it is estimated that there are 500,000 cases of DHF/DSS that occur each year (Mackenzie et al., 2004). Primary infection with any serotype of DENV confers lifelong immunity to the infecting serotype and simultaneously generates cross-reactive antibodies against other serotypes. Sometimes patients in dengue endemic areas are infected with more than one serotype during the dengue season (Araujo et al., 2006; Bharaj et al., 2008; dos Santos et al., 2003; Gubler et al., 1985). A secondary infection with another serotype of DENV is associated with the risk of DHF/DSS (Halstead, 2008; Halstead and O'Rourke, 1977a; Halstead and O'Rourke, 1977b; Kliks et al., 1989). This phenomenon has also been seen in vaccination studies using mono-valent vaccines. Antibody dependent enhancement (ADE) of infection is a phenomenon associated with sub-neutralizing levels of antibody which mediate an increased infection of Fc-bearing cells of the monocytic lineage. This

phenomenon is particularly tragic in new-born infants born to mothers who were recently exposed to a dengue infection. Antibodies passed onto the infant through the placenta, with decrease in titer post birth, put these infants at a risk of severe DHF/DSS. This observation is also supported by extensively high mortality rates observed with new-born infants in dengue endemic areas in tropical regions around the world. Thus, ADE has been proposed as a mechanism to explain the increased severity in secondary infections (Halstead, 2008; Halstead and O'Rourke, 1977a; Halstead and O'Rourke, 1977b; Kliks et al., 1989).

Several attempts have been made in large animal models like rhesus macaques to replicate the DHF/DSS pathology seen in human patients (Halstead et al., 1973). In a recently reported study, an increased viral titer was observed in animals that were administered a sub-neutralizing level of anti-dengue monoclonal antibody IgG-1A5 (Goncalvez et al., 2004). The drawback of large animal model systems has been the lack of high viremia in the animals during a primary infection. In the case of secondary infection too the viruses are quickly cleared from the animals, without any overt display of DHF/DSS. This leads to the hypothesis that the manifestations of DHF/DSS are uniquely related to pathology in humans alone. Among small animal models, primary dengue infection has been reported in several mice models. These include the SCID-hu engrafted with PBMCs, or hepatocarcinoma cells (HepG2) or erythroleukemia cells (K562) (An et al., 1999; Lin et al., 2003; Wu et al., 1995). These mice are not capable of *de novo* synthesis of human cells and thus unable to maintain the level of human cells for long periods or generate human antibodies. Interferon deficient mice which have been

used for primary dengue infection studies as well as passive transfer of antibodies studies have drawbacks of limited life-spans following an active dengue infection.

Primary dengue infections in humanized mouse system have been reported in humanized NOD-SCID mice as well as in the RAG-hu mouse model. Based on data that has been presented previously with respect to dengue infections in RAG-hu mice (Kuruvilla et al., 2007) and Chapter 3, we have observed that the infected mice can be infected with all four serotypes of DENV. We have also observed that a large majority of the infected mice are capable of mounting an immune response against the infection. In a large majority of mice that were tested, there was a lack of neutralizing antibody production despite the presence of an adaptive immune response. With the RAG-hu system we were poised to ask several important questions. A) Are mice that have generated a primary response to a certain serotype of DENV susceptible to a severe infection when challenged with a different serotype? B) Will the mice be susceptible to dengue infections and show any manifestations of disease pathology similar to that in human infections? C) Do the DHF/DSS cases show any "cytokine-storm", a condition that is seen in human patients associated with plasma-leakage? Several of the mice that were infected during primary infections were unable to generate detectable levels of primary antibodies. We also wanted to know whether re-infection with a similar serotype, would boost the capability of antibody production or does the mere presence of low non-neutralizing levels of antibody in individual mice make them susceptible to severe dengue infections? Thus the secondary challenge experiments in RAG-hu mice will help to explain several un-explored areas in dengue fever pathogenesis.

5.3 Materials and Methods

5.3a Dengue viral infection of RAG-hu mice and clinical monitoring

RAG-hu mice with human lymphoid cell reconstitution were infected with the primary challenge virus (DENV-1 16007 or DENV-2 strains D9590 SriLanka, AHF-100 Thailand and 10649 Philippines). Initial infections were performed as previously reported in Chapter 3. The mice were challenged with $1x10^6$ IU of individual virus isolates or pooled virus via intraperitoneal (100ul) and subcutaneous routes (100ul) routes. Primary infection the mice were allowed to recover for 18-20 weeks prior to being challenged with a complimentary viral strain. The secondary challenge was replicated as the primary virus challenge. Mouse body temperatures were monitored every two days using a RET-3 rectal thermo-probe coupled to a Digi-Sense digital thermometer (Eutech Instruments). Mouse weights were monitored using an EK-1200i balance every two days for a period of 21 days post-injection. Mouse platelet counts were collected pre-secondary challenge as well as during regular intervals during secondary challenge. Platelet counts were estimated after dilution of 75ul of blood in 225ul of PBS with K-EDTA and read through an automated counter.

5.3b Measurement of plasma viral load using real time qPCR

Total RNA isolated from mouse plasma collected in heparinized capillaries using the QIAamp viral RNA kit (Qiagen) was reverse transcribed into cDNA with Superscript III™ System First Strand Synthesis for RT-PCR (Invitrogen) using DENV antisense primer NS5R (Kong et al., 2006). Real time PCR was carried out using Platinum SYBR green PCR kit (Invitrogen) in a Biorad iCycler iQ thermocycler. DENV primers NS5F

and NS5R (Kong et al 2006) were used to initiate PCR with 5ul of cDNA as template. Reaction conditions were as follows: 50°C for 2min; 95°C for 2 min; 45cycles of 95°C for 15sec, 58°Cfor 30sec, and 72°C for 5min. Levels of DENV RNA amplified by this two step qRT-PCR method were determined by comparison to a standard curve generated against known quantities of DENV-2 16681 NS5 RNA, which were produced by transcription and quantified previously as described (Kong et al., 2006). These primers were also able to detect the presence of DENV-1 16007.

5.3c Detection of human anti-dengue antibodies by ELISA

Levels of human anti-dengue antibodies present in the mouse serum were determined using commercial human IgM and IgG Dengue Capture ELISAs (Panbio). Sera from DENV-infected non-humanized $Rag2^{-/-}\gamma_c^{-/-}$ mice as well as humanized infected RAG-hu mice were used as negative controls. Positive controls included a known anti-DENV antibody positive human serum as well as samples provided with the ELISA kits. Samples were considered positive if the OD_{450} was greater than two times the background absorbance level.

5.3d FACS-based estimation of dengue virus neutralizing ability

The ability of humanized $Rag2^{-/-}\gamma_c^{-/-}$ mice generated antibodies to neutralize DENV was determined using a FACS neutralization test (FNT) modified from a previously described method (Lambeth et al., 2005). Mouse serum was mixed with 1.0 MOI DENV (determined relative to $4x10^5$ cells) in a total volume of 200ul. The 200ul serum-virus mixture was placed at 37°C for 30min. This mixture was used to inoculate

$4x10^5$ Vero cells in a single well of a 12 well plate. At 1-24 hours post-infection, single cell suspensions were made and subjected to FACS analysis after staining with 4G2-Alexa488 antibody (Lambeth et al., 2005). Serum from DENV infected non-humanized $Rag2^{-/-}\gamma_c^{-/-}$ mice as well as uninfected RAG-hu mice were used as negative controls for neutralization. For cross neutralization studies, DENV-1 16007, DENV-2 16681 as well as DENV-4 H241 were used. The FNT_{50} was defined as the greatest two-fold dilution of serum which produced >50% reduction in DENV infection of Vero cells.

5.3e Analysis of human cytokine production during secondary infection

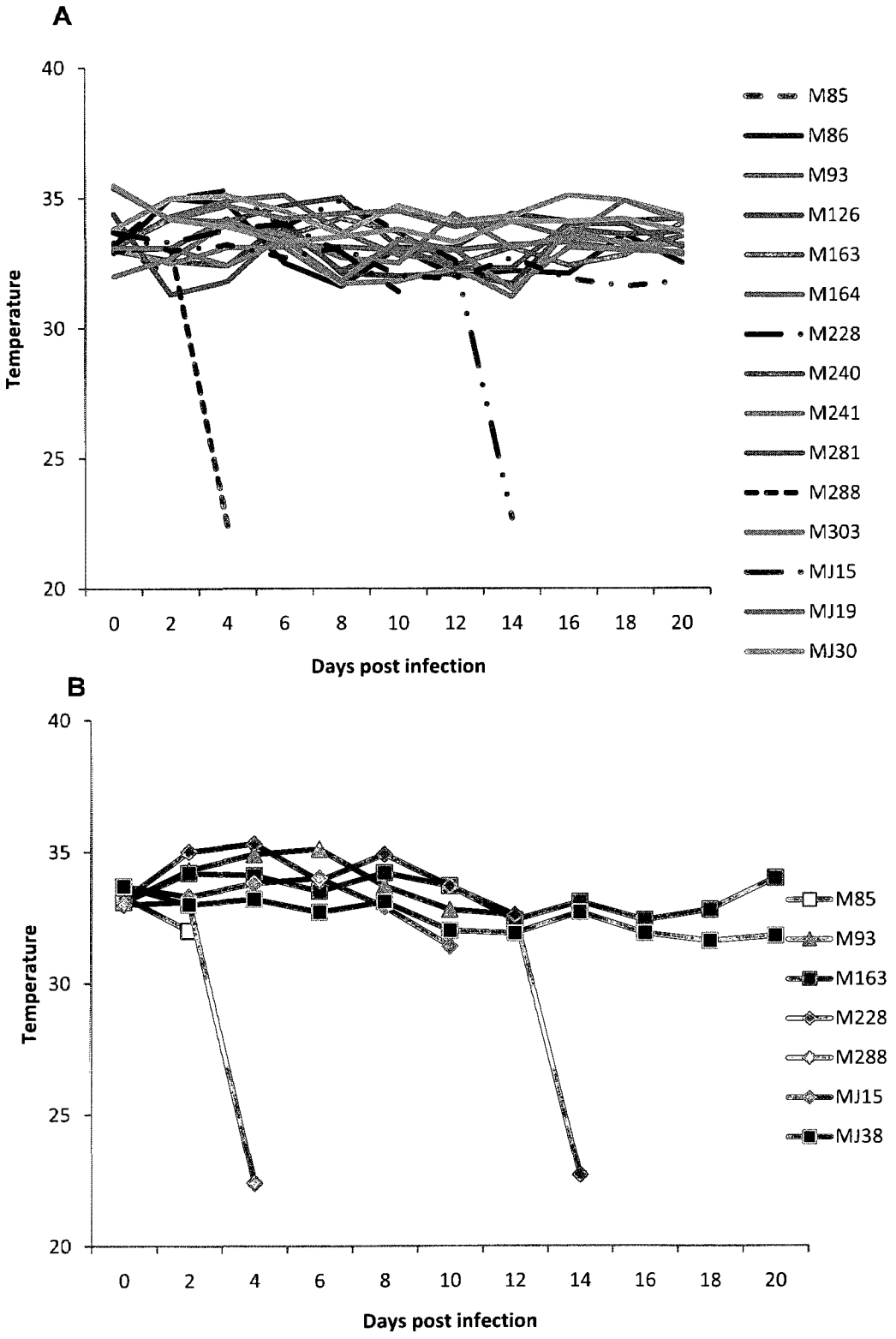
Human cytokine levels in infected RAG-hu mouse sera were determined by a FACS based assay using the Human TH1/TH2 Cytokine Kit II (BD Biosciences). The assay allows for the quantitative estimation of human IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ. The cytokine standards were serially diluted 1:2 to 1:256 and mixed with reconstitution buffer. The requisite amount of capture beads (3ul per sample) was mixed for each of the cytokines to make the requisite amounts of stock solution. To each sample or standard tube, 15ul of sample or standard was added followed by 15ul of PE-detection reagent along with 15ul of Capture-bead cocktail. The mixture was incubated at 25 °C for 2 hours. The tubes were centrifuged at 200g for 5min to collect the beads which were further washed once in 500ul of wash buffer. The beads were spun down again and collected to be solubilized in 200ul of wash buffer and analyzed on a preset and calibrated program on the flow cytometer. The standards were used to determine a curve for each individual cytokine concentration with a range of 20-5000 pg/ml. The unknown values were determined based on the standard charts generated.

5.4 Results

5.4a Dengue viral infection and clinical monitoring

Mice were monitored every two days for changes in temperature, body weight and behavior post secondary challenge with DENV. We observed significant changes in 3 out of 18 mice during the course of a secondary infection. The temperatures taken for all mice infected with a secondary infection is individually plotted in Figure 5.1. We observed that mice that suffered from severe dehydration and morbidity also suffered from a drastic drop in body temperature as identified by the curves in red. We also monitored the weights of the mice using a scale during the infection along with measuring their temperatures. We observed drastic drop in bodyweight of mice that were suffering from secondary infection related morbidity and dehydration. The changes comparing three mice suffering from severe dehydration is compared to mice that were given a primary infection (Figure 5.2).

Overall, the mice with secondary infection experienced a minimum of a 10% drop in body weight within the span of 2-3 days in the case of mice suffering from dehydration. In some cases the mouse bellies were shaved to observe the presence of any rash, hemorrhage and to document the progress of morbidity. We found several mice that were susceptible to dehydration and morbidity. Mice were euthanized as the disease burden passed beyond an acceptable limit. We observed a unique manifestation of rash and hemorrhage on the belly of M93 (Figure 5.3A) which expanded into hemorrhage in the abdominal cavity which was detected on dissection of the mouse. We also observed morbidity in mouse M228 (Figure 5.3B), as well as dehydration in mouse M288 (Figure


A
40
35
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25
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Temperature
0
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4
6
8
10
12
14
16
18
20
Days post infection
M85
M86
M93
M126
M163
M164
M228
M240
M241
M281
M288
M303
MJ15
MJ19
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Temperature
0
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16
18
20
Days post infection
M85
M93
M163
M228
M288
MJ15
MJ38

Figure 5.1 Body temperatures in RAG-hu mice during a secondary infection. Changes in body temperature in mice challenged with a secondary infection were measured using a rectal probe (panel A). The majority of the mice did not show any overt changes in temperature. Mice that suffered from severe disease and shock demonstrated a rapid decline in body temperature were grouped together and graphed in panel B to highlight the rapid decline in the health of the individual mice.

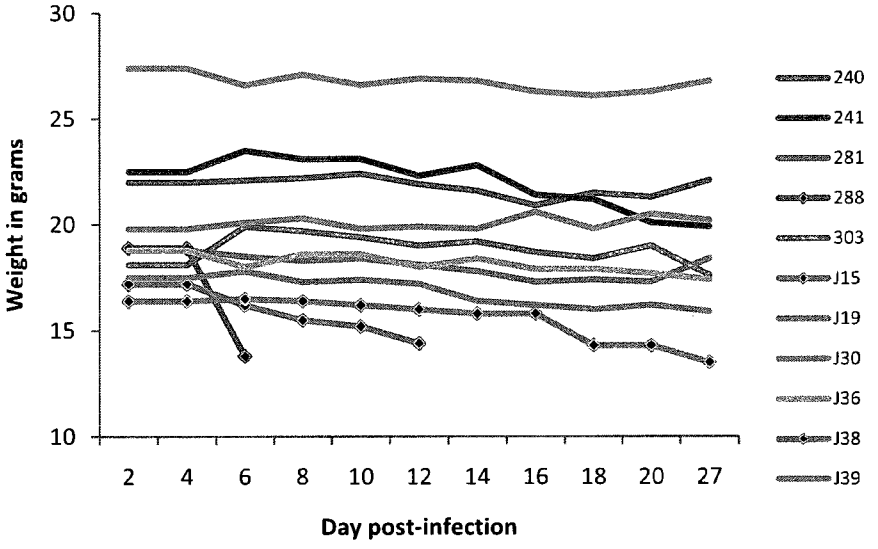

Figure 5.2 Trend in body weight changes in RAG-hu mice with secondary infection. Mice challenged with a secondary infection were weighed every two days post infection. Mice M288, MJ-15 and MJ-38 which displayed severe disease show a drastic drop in body weight over a short period of time.

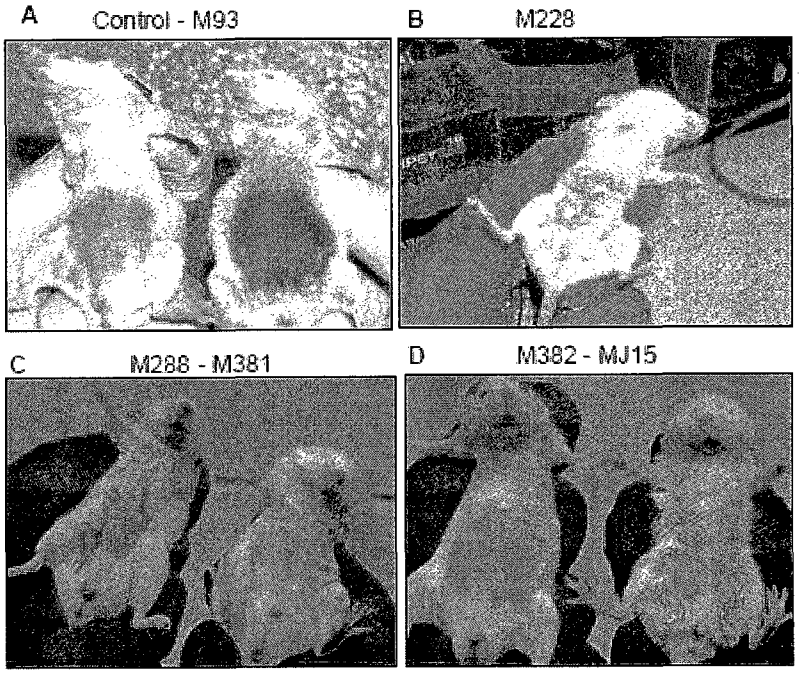

Figure 5.3 Secondary infection of RAG-hu shows hemorrhage and morbidity in several mice. Mice that were challenged with a secondary infection demonstrated various signs of morbidity including hemorrhage (panel A) and dehydration (panel B,C and D).

5.3 C) and mouse MJ-15 (Figure 5.3D). We were unable to clearly ascertain a distinct decrease in platelet counts in these RAG-hu mice.

5.4b Monitoring dengue viral infection during a secondary infection by qRT-PCR

Several RAG-hu mice were infected with different serotypes of DENV. Mice were infected with laboratory passaged strain of DENV-1 16007, as well as individual or a combination of low-passage DENV-2 D9590 SriLanka, AHF-100 Thailand and 10649 Philippines strains as shown in Table 5.1. These mice injected with $1x10^6$ IU of each virus strain. Similar to the primary infections reported earlier, we observed sporadic viremia upto 21 days post infection. The highest observed viremia is reported in Table 5.1 for each of the individual mice. We observed that one mouse M85 showed the highest viremia identified thus far in our hands ($7.2x10^7$ IU/ml). Such high titre has often been reported in human patients with severe dengue fever. Almost all of the mice suffering from a secondary dengue infection has slightly higher detectable viremia. Severity of disease was later co-related with a history of detectable human anti-dengue antibodies in most cases.

5.4c Detection of human anti-dengue antibodies by ELISA

Sera obtained from mice during the course of secondary infection were analyzed for presence of human anti-dengue antibodies using the PanBio IgM/IgG Capture ELISA kit. The assay has been previously described in Chapter 3 in detail. We observed the presence of an increase in the ability of most mice to produce antibodies after a secondary infection. As depicted in Table 5.2, we observed an increased production of

Table 5.1 Secondary infection of RAG-hu mice to determine ADE

Mouse	Primary Virus Challenge	Highest Viral Titre (IU/ml)	Secondary Virus Challenge	Highest Viral Titre (IU/ml)	Symptoms	Day of death
M85	DENV-2 Phil	ND	DENV-2 #	$7.2x10^7$ Day 4	Morbidity,	Day 4
M86	DENV-2 Phil	ND	DENV-2 #	$2.4x10^3$ Day 4	Normal	-
M93	DENV-1 16007	$1.4x10^3$ Day 10	DENV-2 #	$4.3x10^5$ Day 10	Hemorrhage	Day12
M126	DENV-1 16007	ND	DENV-2 #	$1.4x10^3$ Day 2	Normal	
M163	DENV-2 Phil	$4.2x10^3$ Day 21	DENV-2 #	ND	Morbidity	Day 40
M164	DENV-2 Phil	$3.9x10^4$ Day 21	DENV-2 #	$3x10^6$ Day 6	Normal	-
M228	DENV-2 Phil	$7.9x10^3$ Day 16	DENV-2 #	ND	Morbidity, Dehydration	Day 14
M240	DENV-1 16007	$1.5x10^5$ Day 21	DENV-2 #	$1.5x10^4$ Day 6	Normal	-
M241	DENV-1 16007	$1.1x10^4$ Day 21	DENV-2 #	$2.1x10^4$ Day 18	Normal	-
M281	DENV-2 #	$1.3x10^3$ Day 7	DENV-2 #	$1.7x10^4$ Day 18	Normal	-
M288	DENV-1 16007	$4.3x10^3$ Day 14	DENV-2 #	$4.2x10^5$ Day 4	Morbidity	Day 4
M303	DENV-1 16007	$9.3x10^3$ Day 21	DENV-2 #	$1.6x10^3$ Day 18	Normal	-
MJ-15	DENV-2 #	$2.7x10^4$ Day 2	DENV-2 #	$3.5x10^6$ Day 10	Morbidity, Dehydration	Day 10
MJ-19	DENV-2 #	$1.2x10^4$ Day 8	DENV-2 #	$2.5x10^4$ Day 4	Weak	-
MJ-30	DENV-2 #	$4.1x10^3$ Day 6	DENV-2 #	$1.0x10^4$ Day 16	Weak	-
MJ-36	DENV-2 #	$2.3x10^3$ Day 2	DENV-2 #	$1.6x10^4$ Day 18	Morbidity, Dehydration	-
MJ-38	DENV-2 #	$2.5x10^3$ Day 16	DENV-2 #	$8.6x10^5$ Day 27	Normal	Day 27
MJ-39	DENV-2 #	$4.1x10^3$ Day 6	DENV-2 #	$1.6x10^4$ Day 4	Normal	-
M381	*DENV-2 #*	*$1.4x10^4$ Day 18*	*NA*	*NA*	*Normal*	
M382	*DENV-2 #*	*$1.4x10^4$ Day 16*	*NA*	*NA*	*Normal*	
M383	*DENV-2 #*	*$1.7x10^4$ Day 2*	*NA*	*NA*	*Normal*	
MJ-80	*DENV-2 #*	*$1.0x10^4$ Day 8*	*NA*	*NA*	*Normal*	
MJ-112	*DENV-2 #*	*$6.1x10^3$ Day 8*	*NA*	*NA*	*Normal*	
MJ-113	*DENV-2 #*	*$1.1x10^4$ Day 16*	*NA*	*NA*	*Normal*	

ND : Not detected. DENV-2#: Mix of DENV-2 D9590 SriLanka, AHF-100 Thailand and 10649 Philippines strains

IgG in several mice post secondary infection, especially M93, MJ-36, MJ-38 and MJ-39. Although mice showed signs of severe disease, we did not observe a complete class switch from IgM to IgG as observed during secondary infections in human patients. This observation is unique to RAG-hu mice and is probably due to limitations associated with engrafted mice and the quality of engraftment.

5.4d Determination of neutralizing capacity of anti-sera collected from RAG-hu mice after a secondary challenge

After collection of sera from mice that were challenged for a second time with DENV, we analyzed the sera for their capacity to neutralize the challenge virus. The result from each individual mouse is tabulated in Table 5.3. We observed that the mice had an increased capability to neutralize the infecting secondary challenge. Several mice that had not previously produced any neutralizing sera were also capable of producing neutralizing sera in addition to the increase in titers of existing neutralizers.

We also investigated the ability of sera in mice that produced neutralizing antibodies to cross neutralize a heterologous viral challenge. The results from the assay are tabulated in Table 5.4. We observed that 4 out of 18 mice were able to produce neutralizing antibodies. Of these, two were able to neutralize a heterologous virus. In comparison with human patients we observe several similarities with respect to production of both homologus and cross neutralizing antibodies. However the levels of neutralization antibody were not close to the capacity generated in human subjects.

Table 5.2 Human anti-dengue antibody production in RAG-hu mice post secondary infection

Mouse	Primary Virus Challenge	Antibody PI	Secondary Virus Challenge	Antibody PI	Symptoms	Day of death
M85	DENV-2 Phil	IgM (+)	DENV-2 #		Morbidity,	Day 4
M86	DENV-2 Phil	ND	DENV-2 #		Normal	-
M93	DENV-1 16007	IgG (+)	DENV-2 #	IgG	Hemorrhage	Day12
M126	DENV-1 16007	IgM (+)	DENV-2 #	IgM	Normal	
M163	DENV-2 Phil	IgM (+)	DENV-2 #	ND	Morbidity	Day 40
M164	DENV-2 Phil	ND	DENV-2 #	ND	Normal	-
M228	DENV-2 Phil	ND	DENV-2 #	ND	Morbidity, Dehydration	Day 14
M240	DENV-1 16007	ND	DENV-2 #	IgM (+)	Normal	-
M241	DENV-1 16007	IgG (+)	DENV-2 #	IgM (+)	Normal	-
M281	DENV-2 #	IgM (+)	DENV-2 #	IgM (+)	Normal	-
M288	DENV-1 16007	IgM (+)	DENV-2 #	IgM (+)	Morbidity	Day 4
M303	DENV-1 16007	IgM (+)	DENV-2 #	IgM (+)	Normal	-
MJ-15	DENV-2 #	IgM (+) IgG (++)	DENV-2 #	IgM (+)	Morbidity, Dehydration	Day 10
MJ-19	DENV-2 #	IgG(+)	DENV-2 #	IgG (+)	Weak	-
MJ-30	DENV-2 #	ND	DENV-2 #	IgM (+)	Weak	-
MJ-36	DENV-2 #	ND	DENV-2 #	IgG (++)	Morbidity, Dehydration	-
MJ-38	DENV-2 #	IgM (+) IgG (++)	DENV-2 #	IgM (++) IgG (+++)	Normal	Day 27
MJ-39	DENV-2 #	IgM (+) IgG(++)	DENV-2 #	IgM (++) IgG (+++)	Normal	-

(+): 2 fold over background, (++): 4 fold over background, (+++): 8 fold over background
ND: Not detected
DENV-2#: Mix of DENV-2 D9590 SriLanka, AHF-100 Thailand and 10649 Philippines strains

Table 5.3 Neutralizing antibody titre RAG-hu mice post-secondary infection.

Mouse	Primary Virus Challenge	Antibody PI	FRNT Titre	Secondary Virus Challenge	Antibody PI	FRNT Titre
M85	DENV-2 Phil	IgM (+)	-	DENV-2 #	ND	-
M86	DENV-2 Phil	ND	-	DENV-2 #	ND	-
M93	DENV-1 16007	IgG (+)	-	DENV-2 #	IgG	1:40
M126	DENV-1 16007	IgM (+)	-	DENV-2 #	IgM	-
M163	DENV-2 Phil	IgM (+)	-	DENV-2 #	ND	-
M164	DENV-2 Phil	ND	-	DENV-2 #	ND	-
M228	DENV-2 Phil	ND	-	DENV-2 #	ND	-
M240	DENV-1 16007	ND	-	DENV-2 #	IgM (+)	-
M241	DENV-1 16007	IgG (+)	1:80	DENV-2 #	IgM (+)	-
M281	DENV-2 #	IgM (+)	-	DENV-2 #	IgM (+)	-
M288	DENV-1 16007	IgM (+)	1:40	DENV-2 #	IgM (+)	-
M303	DENV-1 16007	IgM (+)	1:160	DENV-2 #	IgM (+)	-
MJ-15	DENV-2 #	IgM (+) IgG (++)	1:80	DENV-2 #	IgM (+)	-
MJ-19	DENV-2 #	IgG(+)	-	DENV-2 #	IgG (+)	-
MJ-30	DENV-2 #	ND	-	DENV-2 #	IgM (+)	1:80
MJ-36	DENV-2 #	ND	-	DENV-2 #	IgG (++)	1:160
MJ-38	DENV-2 #	IgM (+) IgG (++)	1:320	DENV-2 #	IgM (++) IgG (+++)	1:640
MJ-39	DENV-2 #	IgM (+) IgG(++)	1:2560	DENV-2 #	IgM (++) IgG (+++)	1:5210

(+): 2 fold over background, (++): 4 fold over background, (+++): 8 fold over background
ND: Not detected
DENV-2#: Mix of DENV-2 D9590 SriLanka, AHF-100 Thailand and 10649 Philippines strains

Table 5.4 Neutralizing antibody titre RAG-hu mice post-secondary infection.

Mouse	Primary Virus Challenge	Antibody Post-infection	FRNT Titre	Secondary Virus Challenge	Antibody Post-infection	FRNT Titre DENV-1	FRNT Titre DENV-2	FRNT Titre DENV-4	Cross Neutralizer	Symptoms
M85	DENV-2 Phil	IgM	-	DENV-2 #	ND	-	-	-	-	Morbidity,
M86	DENV-2 Phil	ND	-	DENV-2 #	ND	-	-	-	-	Normal
M93	DENV-1 16007	IgG	-	DENV-2 #	IgG	1:40	1:40	-	Yes	Hemorrhage
M126	DENV-1 16007	IgM	-	DENV-2 #	IgM	-	-	-	-	Normal
M163	DENV-2 Phil	IgM	-	DENV-2 #	ND	-	-	-	-	Morbidity
M164	DENV-2 Phil	ND	-	DENV-2 #	ND	-	-	-	-	Normal
M228	DENV-2 Phil	ND	-	DENV-2 #	ND	-	-	-	-	Morbidity, Dehydration
M240	DENV-1 16007	ND	-	DENV-2 #	IgM	1:40	-	-	-	Normal
M241	DENV-1 16007	IgG	1:80	DENV-2 #	IgM	1:80	1:40	-	Yes	Normal
M281	DENV-2 #	IgM	-	DENV-2 #	IgM	-	1:40	-	-	Normal
M288	DENV-1 16007	IgM	1:40	DENV-2 #	IgM	-	-	-	-	Morbidity
M303	DENV-1 16007	IgM	1:160	DENV-2 #	IgM	-	-	-	-	Normal
MJ-15	DENV-2 #	IgM IgG	1:80	DENV-2 #	IgM	-	-	-	-	Morbidity, Dehydration
MJ-19	DENV-2 #	IgG	-	DENV-2 #	IgG	-	-	-	-	Weak
MJ-30	DENV-2 #	ND	-	DENV-2 #	IgM	-	1:80	1:40	Yes	Weak
MJ-36	DENV-2 #	ND	-	DENV-2 #	IgG	-	1:160	-	-	Morbidity Dehydration
MJ-38	DENV-2 #	IgM IgG	1:320	DENV-2 #	IgM IgG	-	1:640	-	-	Normal
MJ-39	DENV-2 #	IgM IgG	1:2560	DENV-2 #	IgM IgG	1:40	1:5210	1:80	Yes	Normal

ND: Not detected

DENV-2#: Mix of DENV-2 D9590 SriLanka, AHF-100 Thailand and 10649 Philippines strains

5.4e Detection of cytokine production in sera of RAG-hu mice challenged with DENV

We utilized the Human TH1/TH2 Cytokine Kit II (BD Biosciences) to estimate the levels of human cytokines produced in mice after primary and secondary DENV infections. We observed that very few mice were capable of producing large amounts of human cytokine that is detectable above the background levels in RAG-hu mice. The observations are tabulated in Table 5.5. We have observed the production of IL-2, IL-4, IL-10, TNF-α and IFN-γ in infected mice. It is interesting to note that during secondary infection, mice have been shown to produce higher amounts of IFN-γ and low levels of IL-2 and IL-4.

Table 5.5 Cytokine production during secondaryinfection of RAG-hu mice

Mouse	IL-2	IL-4	IL-6	IL-10	TNF	IFN
M163	+	+	-	-	-	++
M303	-	-	-	-	-	-
MJ-30	-	-	-	-	-	-
MJ-38	-	-	-	-	-	-
M135	+	+	-	-	-	-
M201	+	+	-	-	-	-
M202	+	+	-	-	-	-
M208	+	+	-	-	-	-
M223	+	+	-	-	-	-
M225	+	+	-	+	-	+
M226	-	-	-	-	-	+
M383	-	-	-	-	-	+
MJ-113	-	-	-	-	+	++

- : Below detectable limits
+ : 2-fold higher over background
++ : 4 fold higher over background
RAG-hu mice under secondary infection are in bold italics
RAG-hu mice under primary infectio are in italics

5.5 Discussion

The goals of these experiments were to establish an animal model that can replicate pathology observed in human dengue patients faithfully in a mouse model. Compared to all previous animal models published, we have demonstrated the presence of higher viral titers during secondary DENV infection. We observed severe morbidity and death in case of 7 out of 18 mice. Of these mice, 3 were given a heterologous secondary DENV challenge in comparison to the primary DENV infection. We observed severe forms of morbidity in these mice like hemorrhage, rash and dehydration. 4 out of 18 mice succumbed to a homologous DENV challenge. From our analysis we observed that the majority of the mice possessing antibodies (6 out of 7 mice) from a primary infection were susceptible to a severe secondary infection. The presence of a low level of non-neutralizing antibody may have triggered a severe disease in these cases. Post secondary DENV challenge we observed an increase in antibody production and neutralization capacity in 4 out of 18 mice. This provides us with preliminary evidence that the RAG-hu mice developed some memory B cell response that was activated post secondary DENV challenge. We also detected relatively low levels of human IFN production in mice post-secondary infection. We are yet to determine the significance of the role of human cytokines in precipitating any aggressive dengue disease pathology. In the RAG-hu model, aggressive disease may be a combinatorial effect of both mice and human cytokines. This hypothesis needs to be evaluated in depth in the future before any conclusions are drawn.

5.6 Conclusion

Our ability to identify and dissect the mechanisms of ADE will be developed only with development of a method to ensure antibody production in all of the mice that are infected with DENV. This preliminary study has helped us to determine several parameters that can be identified with respect to secondary infection and analysis of ADE. In future experiments we hope to expand upon these effects with larger numbers of mice.

Chapter 6

Analysis of DENV infection of human cells derived from human hematopoietic stem cells *in vitro*.

6.1 Abstract

Dengue virus infection in human patients is primarily harbored in cells of monocytic origin. These monocytes, macrophages and dendritic cells have been demonstrated to be directly infected in human tissues. Several other cells including hepatocytes and macrophages in spleen, liver and lymph nodes have been shown to be directly infected. In secondary dengue infection, during the aggressive events of DHF and DSS, endothelial cells and platelet precursor cells like megakaryocytes and platelets are also touted to be infected. In an attempt to identify these cells, we derived and infected macrophages, dendritic cells and megakaryocytes from CD34+ human hematopoietic precursor stem cells. We show that CD34+ cells in culture that loose their CD34+ marker after prolonged maintenance in culture. These auto differentiated cells were infected at a low level by DENV. Macrophages differentiated from CD34+ hematopoietic stem cells were also permissive to DENV infection at a relatively high MOI. In the presence of diluted human anti-dengue antibodies we observed that macrophages were infected with a slightly higher efficiency, demonstrating ADE. Megakaryocytes derived from CD34+ cells are also infected at a low level after differentiation over a long period.

6.2 Introduction

Dengue infections are transmitted by a mosquito vector (*Aedes aegyptii*) through a direct bite into the dermis of the individual. The infected mosquito injects a tiny amount of virus (Gubler and Rosen, 1977) into the individual which is immediately endocytosed by

skin dendritic cells (Langerhan cells) and circulating monocytes or macrophages (Wu et al., 2000). These cells are infected as the virus evades destruction in the phagosomes. The circulating macrophages deposit the virus into the nearest lymph node where several cells are brought into close proximity of infectious virus. Other reports in the literature suggest that dendritic cells, macrophages and monocytes are easily infected by DENV (Halstead and O'Rourke, 1977a; Hase et al., 1989; Kurane, 1997; Wu et al., 2000). In addition there are reports of virus infection in B cells as well as T-cells (Clyde et al., 2006). Antibody dependent enhancement (ADE) is a phenomenon where Fc bearing cells are easily infected in the presence of non-neutralizing antibodies against DENV (Kliks et al., 1989). This leads to a severe form of dengue called DHF and DSS which is often characterized by high viremia, infection of a large number of tissues, plasma leakage, hemorrhage and shock often leading to death.

In addition to lymphocytes that are reported to be infected, DENV can infect cells in the bone marrow, liver, spleen and endothelial cells (Avirutnan et al., 1998; Bonner and O'Sullivan, 1998; Cook, 1997; Couvelard et al., 1999; Halstead et al., 1977; Kuo et al., 1992; La Russa and Innis, 1995; Lum et al., 1993; Rothwell et al., 1996; Srichaikul and Nimmannitya, 2000). There are also some neuronal manifestations of dengue that have also been reported sporadically (Halstead, 2008).

In order to determine whether hematopoietic precursor cells are infected, we directly infected human CD34+ hematopoietic stem cells that were isolated from fetal liver and maintained under culture conditions. These cells have the limited ability to remain multi-potent for a short period of time in culture. We also derived macrophages from PBMC as well as CD34+ stem cells using a cytokine cocktail based differentiation

system (Caux et al., 1997; Chomarat et al., 2000; Chomarat et al., 2003). We tested these macrophages that were derived from CD34+ cells with a DENV challenge in the presence and absence of human anti-dengue sera. Dendritic cells were also derived from human PBMC and CD34+ stem cell using a cytokine cocktail. We also challenged these cells with a DENV.

Platelets play an important role in clotting blood after an injury. In the case of secondary dengue fever, plasma leakage and hemorrhagic manifestations of the disease point to an impaired clotting mechanism (Halstead, 2008; Noisakran et al., 2009). In order to determine if platelets or platelet precursor cells are directly infected by DENV, we derived megakaryocytes from CD34+ stem cell by using a cytokine cocktail and challenged with DENV.

From these *in vitro* studies, we can directly ascertain cells that are infected during the course of a DENV infection and treatments against very specific targets. We observed that macrophages and dendritic cells that were derived from PBMC or CD34+ hematopoietic stem cells were infected by DENV. CD34+ hematopoietic stem cells were not directly infected by DENV. Megakaryocyte precursors and platelets derived from CD34+ hematopoietic stem cells were infected at a low level. Thus macrophages and dendritic cells play a significant role in severity of dengue pathogenesis while other cell types play a supporting role in precipitating severe disease.

6.3 Materials and Methods

6.3a Isolation and differentiation of dendritic cells and macrophages derived from blood

Whole blood was procured from a blood bank and distributed as 40ml aliquots after dilution in an equal amount of sterile Hanks PBS. The diluted blood was slowly layered on 8ml of Ficoll-Hypaque in a 50ml Falcon tube. The tubes were subjected to 2400rpm in a Beckman Allegra 6R swinging bucket centrifuge for 30min to allow for a density gradient dependent separation of the cells. The centrifuge was allowed to come to rest without the application of brakes. The buffy layer containing lymphocytes above the RBC layer was collected and pooled from several tubes. The Ficoll was diluted out by washing the cells with fresh Hanks PBS and the cells were collected by centrifugation with the application of brakes. After two washes with Hanks PBS, the cells were diluted in 10% FBS-RPMI to $2x10^6$ cells per well and allowed to adhere to the bottom of a six-well plate. The non-adherent cells were removed with gentle washing and collected in separate tubes after centrifugation. These were frozen down for usage in other experiments. The adherent cells were differentiated into monocytes derived macrophages or dendritic cells.

6.3b Infection of blood derived macrophages and dendritic cells

Monocytes derived macrophages were generated from PBMCs by culturing the cells in 10% FBS-RPMI in presence of a cytokine mix of Il-6 (10ng/ml) Flt3-l (50ng/ml) and M-CSF (25ng/ml). The cells were maintained for over 20 days and their phenotypic characteristics as well as cell surface marker (CD14+) level was monitored every other day. Dendritic cells were generated from isolated adherent monocytes by culturing them in

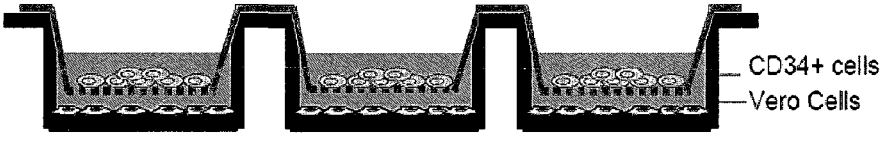

Figure 6.1 Schematic demonstration of the infection of CD34+ cells in a trans-well format. CD34+ hematopoietic stem cells were cultured in stem cell media on a porous support. The wells were suspended in the well of a 6-well plate with a mono-layer of DENV infected Vero cells as a source of DENV.

10% FBS-RPMI containing IL-4 (10ng/ml), GM-CSF (50ng/ml), TNF-α (10ng/ml), Flt3-L (50ng/ml). The cells were maintained for 20 days and their phenotypic characters as well as cell surface markers (CD1a, CD209/DC-SIGN) were monitored. The cells were grown on a well-insert which was a 1um mesh that can be moved from well to well. An adherent layer of Vero cells was infected with DEN-2 16681 virus (Figure 6.1). After two days of infection fresh media containing the requisite cytokine mix as well as the well-insert containing macrophages or dendritic cells was placed over the Vero cell layer to allow for infection of the cells with fresh virus.

6.3c Isolation and infection of fetal liver derived CD34+ hematopoietic stem cells

The fetal liver tissue was washed twice with PBS in a 150cm dish. The loose cells were aspirated away and the tissue was weighed. The tissue was then subjected to enzymatic digestion in the presence of 50ml serum free AIMS medium containing 2X antibiotic and anti-mycotic. The tissue was pre-minced using two scalpel blades. DNase, hyaluronidase and collagenase were added from 100X stocks to obtain a 1X concentration of enzyme. The tissue was pulverized by squirting it out of a 20ml syringe. The tissue was allowed to digest for 3-4 hours at 37°C with intermittent pipetting to generate a single cell suspension. The cells were then strained through a 40μm cell strainer into 50ml Falcon tubes. The single cell suspension was the subjected to a Ficoll gradient separation. The total volume of the cells was increased by adding 25ml of AIMS medium. The cells were layered on 8ml of Ficoll-Hypaque in 50ml Falcon tubes and centrifuged at 2500rpm for 30min without the application of brakes in a Beckman swinging bucket centrifuge.

The cells from the buffy layer were collected and washed by dilution with isolation buffer (PBS with 0.5%BSA, 2mM EDTA, degassed by vaccum filteration). The cells are collected by centrifugation at 1500rpm for 10min with the application of brakes. The cell pellets were solubilized in 50ml of fresh isolation buffer and enumerated using a hemocytometer. The cells were re-diluted to a density of 10^8cells/300µl. 100µl of FcR blocking reagent was added per 10^8cells to prevent nonspecific binding to magnetic beads. The cells were then labeled with CD34-Microbeads at concentration of 100µl per 10^8cells and allowed to incubate at 12°C for 30min. During this stage the magnetic separation column was set-up. The column was washed with 3ml of degassed isolation buffer. The cells were washed with isolation buffer twice and solubilized at a density of $2x10^8$ cells/ml. The cells are passed through a 30µM pre-filter before being passed over the washed magnetized column. The cells were allowed to flow through and the column was rinsed with 3ml isolation buffer thrice. The column was removed from the magnet and placed over a new sterile collection tube. The cells were removed by applying 5ml of isolation buffer and eluted from the column. This step of isolation was repeated with a fresh column that was pre-wetted with 3ml of isolation buffer. The cells were collected by centrifugation and solubilized in 10% FBS-IMDM with 2X antibiotic-anti-mycotic as well as IL-3 (10ng/ml), IL-6 (10ng/ml), SCF (10ng/ml). The cells divide rapidly and hence they are diluted to $2x10^6$ cells/well of a 6 well plate with 3-4ml medium.

6.3d Infection of CD34+ derived dendritic cells and macrophages

Dendritic cells were derived from CD34+ hematopoietic stem cells isolated from fetal liver by propagating them in a cytokine cocktail. The cells were maintained in

GMCSF (50ng/ml), FLt3-L (50ng/ml), IL-3 (10ng/ml), TNF-α (10ng/ml), IL-4 (10ng/ml), SCF (10ng/ml). SCF was removed after 3 days and the cells were propagated for 20 days. The maturation and differentiation of dendritic cells were monitored by staining for cell surface markers CD1a and CD209 (DC–SIGN). The cells were grown on a well-insert which was a 1μm mesh that can be moved from well to well. An adherent layer of Vero cells was infected with DEN-2 16681 virus. After two days of infection fresh media containing the requisite cytokine mix as well as the well-insert containing dendritic cells was placed over the Vero cell layer to allow for infection of the cells with fresh virus.

Macrophages were generated by incubation in Methocult media. CD34+ hematopoietic stem cells were placed in Methocult GF H4434 (StemCell Technologies) media containing cytokines for development of macrophages for 14days. The colonies formed by propagation in the media were solubilized in 10% FBS-DMEM containing MCSF (10ng/ml), Il-6 (10ng/ml), washed and allowed to mature for 3 days till the characteristic fried egg shaped macrophage was observed. The cells were maintained in the same media for 20 days with constant monitoring of their cell surface marker (CD14). The cells were grown on a well-insert which was a 1μm mesh that can be moved from well to well. An adherent layer of Vero cells was infected with DEN-2 16681 virus. After two days of infection fresh media containing the requisite cytokine mix as well as the well-insert containing macrophages was placed over the Vero cell layer to allow for infection of the cells with fresh virus.

6.3e Infection of CD34+ derived platelet precursors and platelets

Platelets and platelet precursors were derived from CD34+ hematopoietic stem cells by culturing the cells in megakaryocyte development media. The cells were maintained in 10%FBS-IMDM containing Thrombopoeitin (TPO) (100ng/ml) as well as SCF (10ng/ml). SCF was not added to the medium after 3 days. The cells were allowed to differentiate for 15 days and their cell surface marker characteristics were assayed to determine differentiation. For megakaryocyte development, CD41 and CD61 were used as markers of differentiation. In order to facilitate infection of megakaryocytes, the differentiating cells were maintained on an insert inside a 6 well plate. The differentiated cells were moved into a well containing infectious virus generated from a layer of Vero cells infected with DEN-2 16681 on days 6, 9, 12 and 15 days post differentiation and assayed two days post infection by flow cytometry using methods described in Chapter 2.

6.4 Results

6.4a Infection of blood derived dendritic cells and macrophages

DENV infects tissue macrophages and monocytes when injected into the dermis of an individual. These tissue macrophages have been shown to leave the tissue and enter the blood stream where they travel to local lymph node in order to present the antigen to T-cells. Differentiation of monocytes from PBMC to macrophages was monitored by the upregulation of a predominant macrophage marker CD14 in the presence of IL-4 and M-CSF. We observed a progressive upregulation of the macrophage marker over a period of 15 days. The adherent monocytes from blood as shown in figure 6.2, at day 0, have a low expression of CD14. As they mature over 15 days after isolation in the presence of

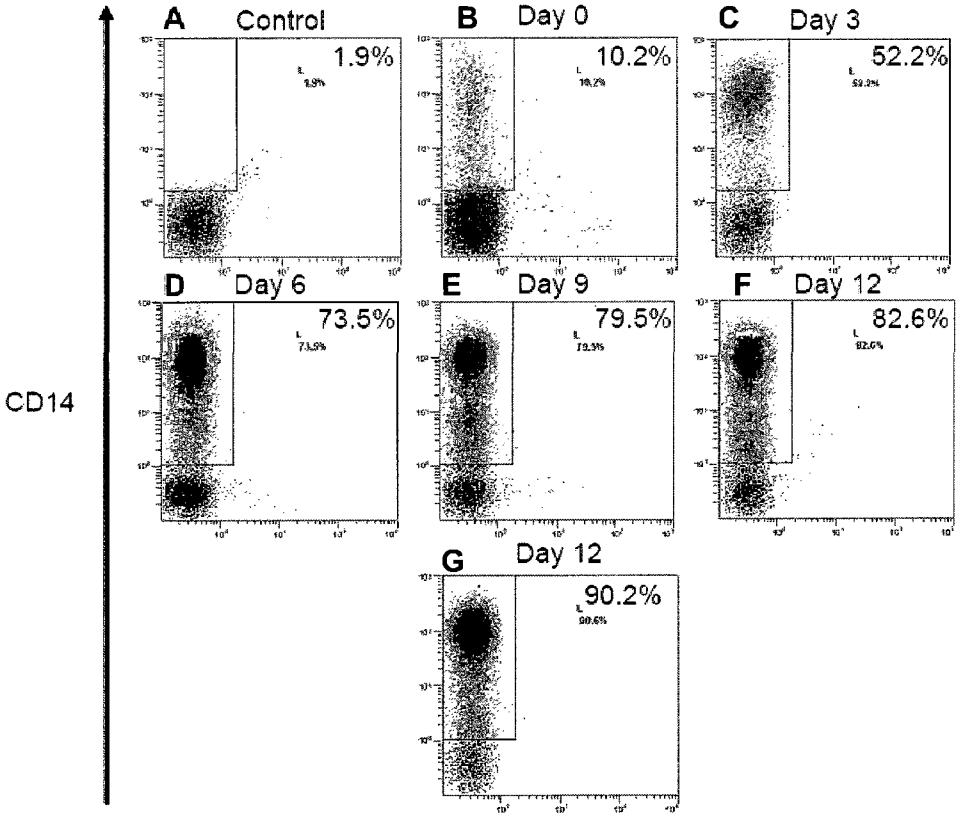

Figure 6.2 Macrophages derived from PBMC in human blood. Adherent cells isolated from human PBMC were differentiated into the macrophage lineage using a cytokine cocktail containing IL-4 and GM-CSF. The differentiation was monitored by measuring the levels of monocyte cell surface maker CD14 in a flow-cytometry based assay every third day. The highest level of differentiation was observed on day 15 .

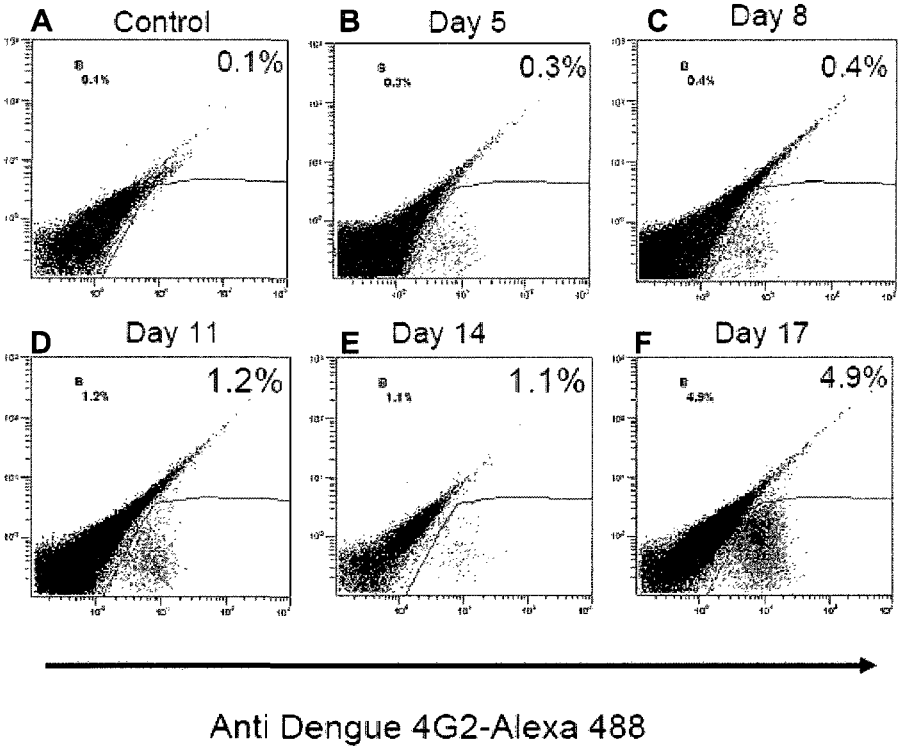

Figure 6.3 DENV infection of macrophages derived from human PBMC. Macrophages derived from PBMC were directly infected with DENV. The number of cells infected were determined 2 days post-infection by intracellularl staining with anti-dengue 4G2-Alexa488 in a flow cytometry based assay. A mouse IgG2a isotype antibody was used as the negative control against non-specific staining. The highest level of infection was observed on day 17 post-differentiation (panel F).

cytokine stimulus these cells display a higher amount of cell surface protein CD14. By day 15, 90% of the cells are mature macrophages. On infection with DENV for two days and using intracellular staining for dengue proteins as shown in Figure 6.3, we observed a distinct but progressive low level of infection amongst these macrophages. The lowest infection was observed in macrophages assayed from CD14 levels on day 3 and assayed for infection on day 5 while the highest level of infection was observed as the macrophages matured on day 15 and assayed for infection at day 17. These observations are similar to those reported in the literature where the direct infection of macrophages is always at a low level. Higher levels of infection of macrophages have been reported only in the presence of dengue positive sera or monoclonal antibodies (Kou et al., 2008).

DENV also infects dendritic cells very efficiently. We derived dendritic cells from adherent monocytes isolated from human PBMC in the presence of a cytokine cocktail that contained IL-4, Flt3-L, TNF- α, and GM-CSF. We assayed the maturation of the dendritic cells by monitoring the cell surface markers CD1a and DC-SIGN. The dendritic cells are extremely sensitive to changes in their environment and upregulation of cell surface markers is dependent on the stimulus in the surrounding medium. We observed the upregulation of CD1a and DC-SIGN over a period of 9 days with maximum upregulation on day 6 (Figure 6.4). Following infection we stained the cells for DC-SIGN as well as the dengue E protein antigen. We observed that a majority of the DC-SIGN expressing cells but not all were infected with DENV (Figure 6.5). On day 3, 34.1% of total cells were found to be infected. 18.2% of these cells were expressing DC-SIGN (Figure 6.5 panel B). The peak of infection was observed on day 6 (assayed day 8)(Figure 6.5 panel D). We show that 45.8% of total cells were infected with DENV. Amongst these cells 24.2% were

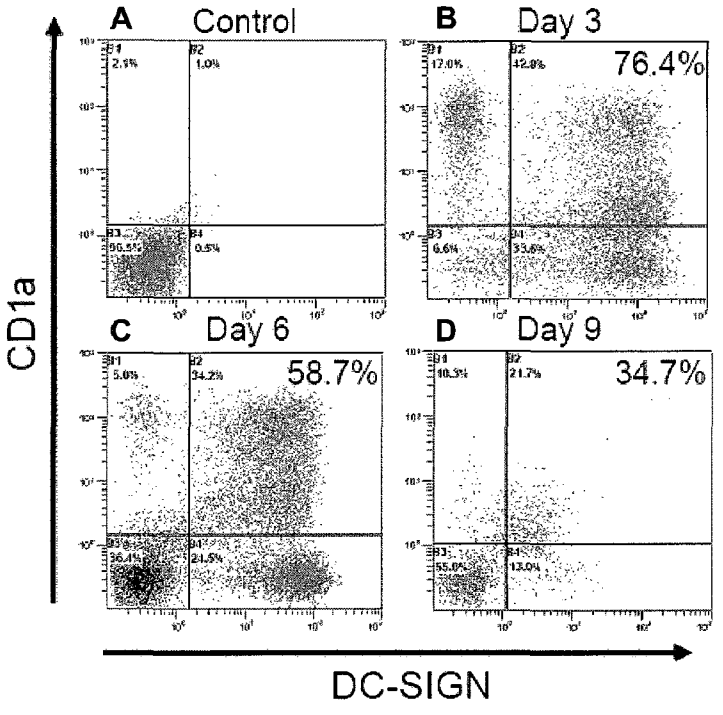

Figure 6.4 Dendritic cells derived from PBMC isolated from human blood. Adherent cells isolated from human PBMC were differentiated into a dendritic cell lineage using a cytokine cocktail containing IL-4 and GM-CSF. The differentiation was monitored by measuring the dendritic cell surface markers CD1a and DC-SIGN in a flow cytometry based assay every third day. Highest levels of dendritic cells were observed on day 3 at 76.4% (panel B) which drop over time in culture.

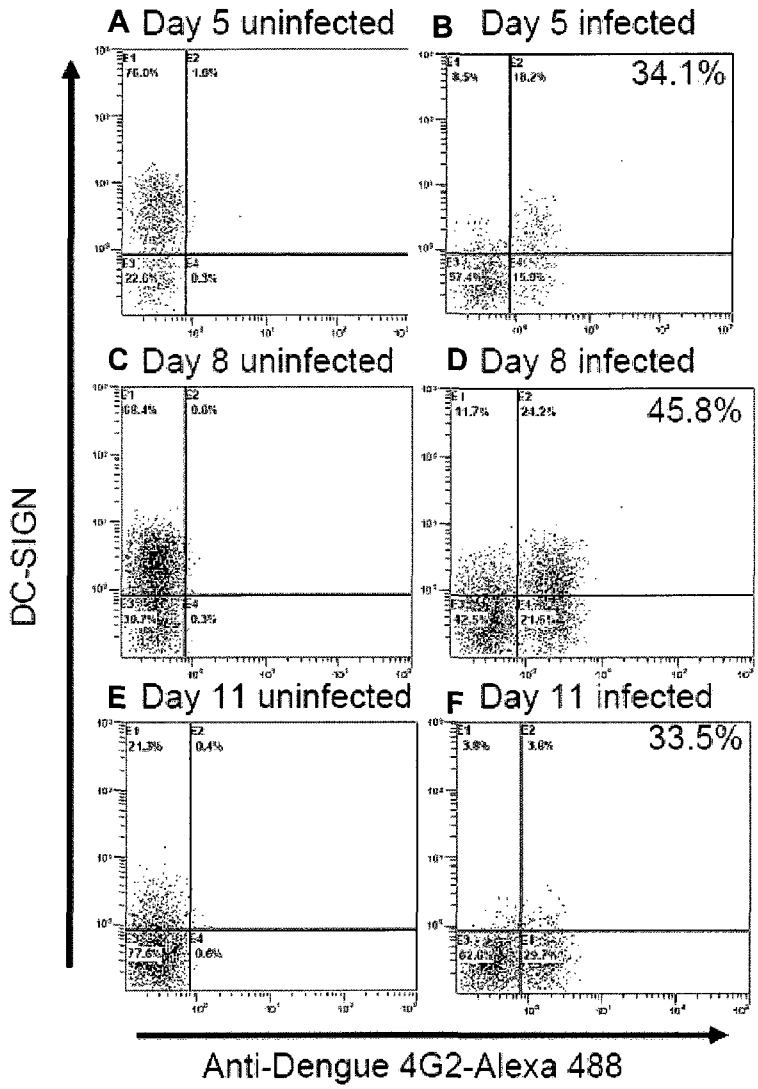

Figure 6.5 Dendritic cells derived from PBMC isolated from human blood infected with DENV. Dendritic cells derived from PBMC were directly infected with DENV. The number of cells infected we determined 2 days post-infection by intracellular staining with anti-dengue 4G2-Alexa 488 in a flow cytometry based assay. DC-SIGN cell surface marker was aslo estimated simultaneously in order to determine the number of dendritic cells being infected. Mouse IgG2a isotype antibody was used as negative control. Highest level of infection was observed in cells differentiated for 6 days and assayed on day 8 at 45.8% (panel D)

expressing the DC-SIGN marker. This observation also supports the hypothesis of that DC-SIGN is an important cell-surface receptor for DENV. As the levels of DC-SIGN expression drops during prolonged culture we observed a drop in the number of infected cells. On day 9 we observed that of the 33.5% total cells infected with DENV, only 3.8% of the cells were capable of expressing the DC-SIGN marker efficiently (figure 6.5 panel F). This observation also points to the fact that DC-SIGN may be down regulated in dendritic cells following a DENV infection.

6.4b Infection of CD34+ cells

Human CD34+ hematopoietic stem cells were isolated from human fetal liver. The fetal liver is the source of embryonic hematopoiesis. Later hematopoietic stem cells migrate to the bone marrow where they take up residence in adults in order to continually replenish the depleting reservoirs of human lymphocytes. Human CD34+ cells can be maintained in culture for a short period of time in the presence of SCF and IL-3 (1-4 days) after which they spontaneously differentiate into fibroblasts. In order to determine whether these cells can be infected with DENV, we co-cultured them in media containing IL-3 and SCF. Uninfected cells were given the same treatment as the CD34+ cells challenged with DENV, including the trans-well co-culture with uninfected Vero cells. We assayed the CD34+ cells for maintenance of CD34+ marker and observed the decrease in levels of expression of CD34 by day 5 (Figure 6.6). We also observed that CD34+ cells were not directly infected by DENV. Upon differentiation beyond day 12, only a few cells among the population were infected by DENV (Figure 6.7). Our results show that CD34+ hematopoietic stem cells were not directly infected by DENV.

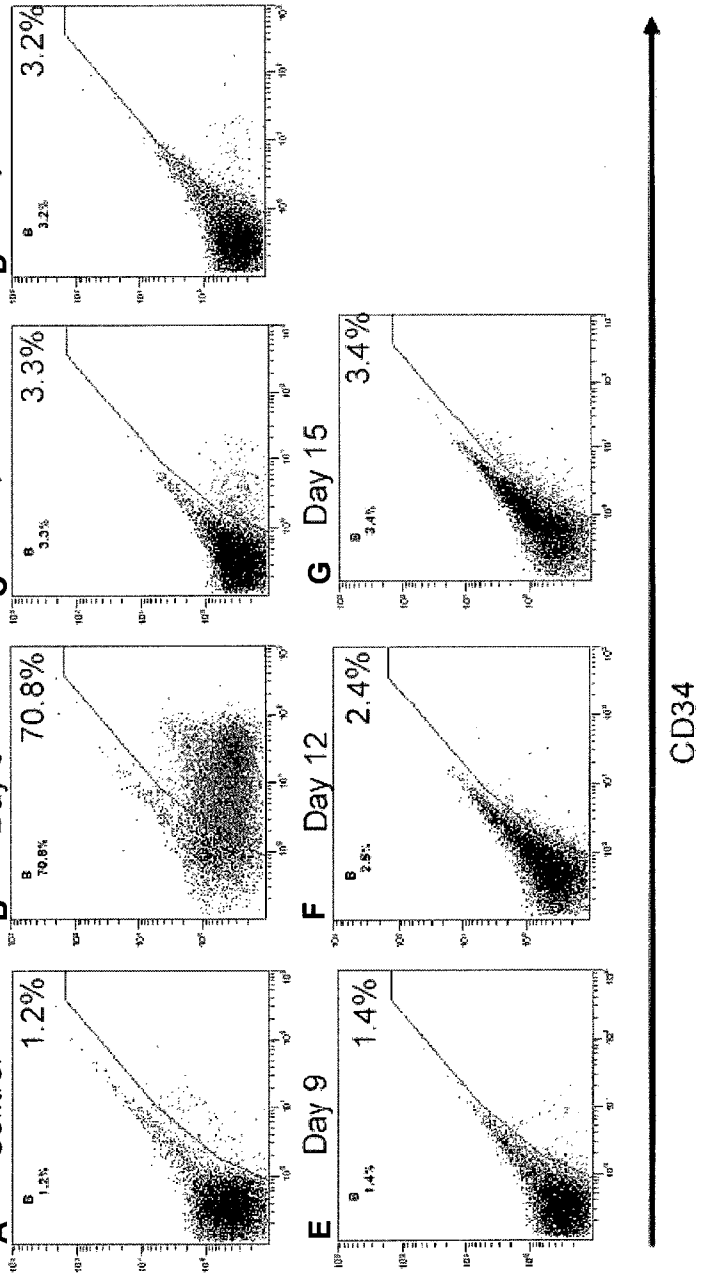
A Control 1.2%
B Day 0 70.8%
C Day 3 3.3%
D Day 6 3.2%
E Day 9 1.4%
F Day 12 2.4%
G Day 15 3.4%
CD34

Figure 6.6 Maintenance of CD34+ human hematopoietic stem cells derived from human fetal liver. CD34+ stem cells were maintained in stem cell media for 15 day. The CD34+ cell surface marker expression was monitored by a flow cytometry base assay. Decrease in the levels of CD34+ was observed over time when the cells are cultured, which points to the loss in totipotency. By day 3 (panel C) a large number of cells have lost their CD34 maker. By day 6 (panel D) the vast majority of cells have differentiated into fibroblasts and are no longer totipotent.

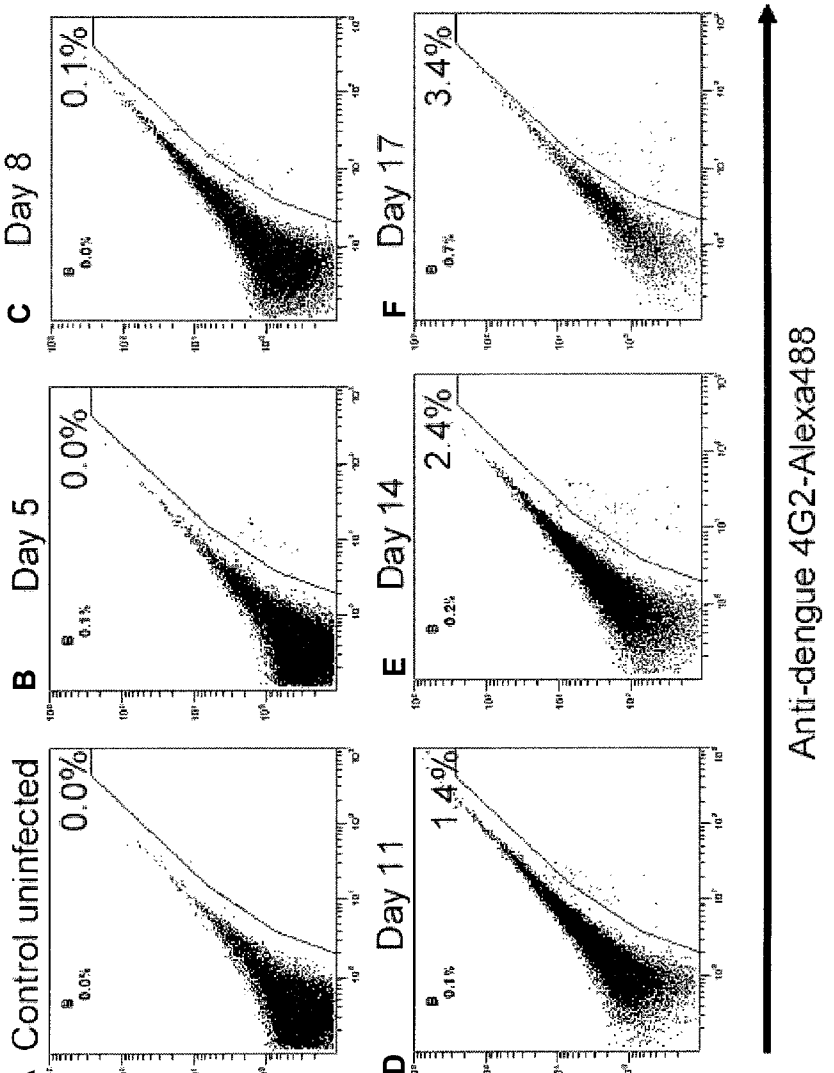
A Control uninfected
0.0%
B
0.0%
B Day 5
0.0%
B
0.1%
C Day 8
0.1%
B
0.0%
D Day 11
1.4%
B
0.1%
E Day 14
2.4%
B
0.2%
F Day 17
3.4%
B
0.7%
Anti-dengue 4G2-Alexa488

Figure 6.7 Infection of CD34+ hematopoietic stem cells maintained in culture *in vitro*. CD34+ hematopoietic stem cells were infected with DENV by using a transwell method while being maintained in stem cell media. The infected cells were identified using an intracellular staining method using anti-dengue 4G2-Alexa488 antibody by flow cytometry. CD34+ cells are not directly infected by DENV. Upon loss of CD34+ marker and differentiation, a small number of cells were found to be susceptible to dengue infection on day 8 (panel C) onwards. The highest number of cells infected was observed on day 14 in culture (panel E).

6.4c Infection of CD34+ derived dendritic cells and macrophages

Hematopoietic stem cells derived from fetal liver can be derived into human macrophages and dendritic cells using a cytokine stimulus similar to methods described above. In the case of CD34+ stem cells, the differentiation takes place over a slightly prolonged period of time. We employed a previously described protocol to differentiate CD34+ cells into dendritic cells. We observed that expression of cell surface markers like CD1a and DC-SIGN developed slowly over a period of 3-5 days, peaked between 9-11 days and were down-regulated after that period (Figure 6.8). We infected dendritic cells derived from CD34+ cells during the period of differentiation. We observed that these cells are easily infected in relatively large numbers. The number of infected cells increases with increase in the number of differentiated cells. We observed that the increase in infected cells correlated with the increase in CD1a and DC-SIGN expression on the dendritic cells (Figure 6.9). The results obtained from these experiments co-relate with results obtained from infection of dendritic cells derived from human PBMC. In order to generate macrophages from CD34+ human cells we utilized a commercially available kit (Methocult GF H4434 -StemCell Technologies). The cells were incubated at 37°C for 10 days during which period macrophage colonies are formed from CD34+ cells. These immature macrophages were removed from methocult and cultured in liquid medium containing cytokines to allow for complete maturation into adult macrophages with typical fried egg morphology. The cells were phenotypically analyzed by flow cytometry by staining for the CD14 macrophage marker (Figure 6.10). Nearly all the cells derived in methocult had differentiated into macrophages. These cells were infected with DENV. In

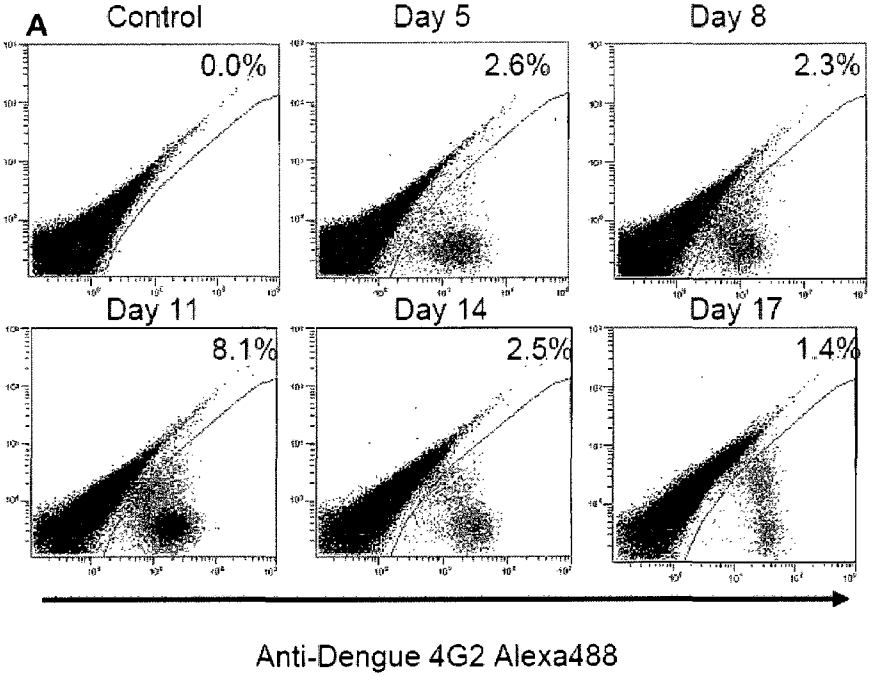

Figure 6.9 Infection of dendritic cells derived CD34+ human hematopoietic stem cells. Dendritic cells differentiated from CD34+ hematopoietic stem cells were directly infected with DENV every third day during the differentiation process. Infection of cells was assayed 2 days post infection using anti-dengue 4G2-Alexa 488 antibody in a flow cytometry based assay. Highest level of infection was observed in cells infected 9 days post-differentiation and assayed on day 11at 8.1% (panel D).

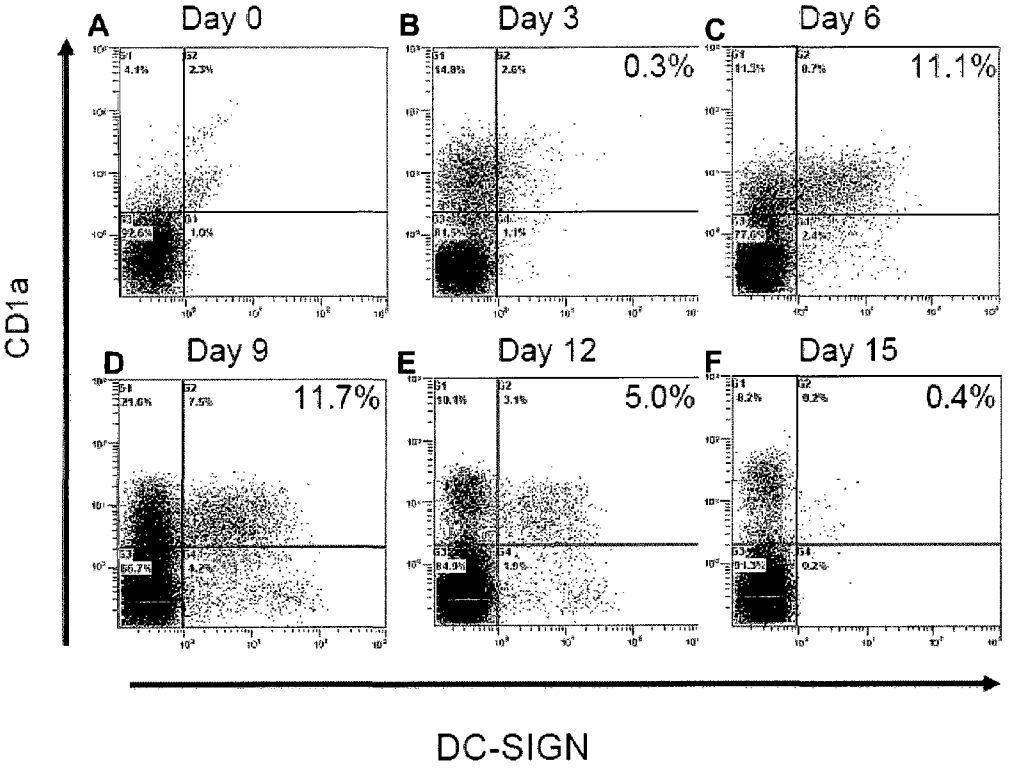

Figure 6.8 Dendritic cells derived CD34+ human hematopoietic stem cells. Dendritic cells were derived from CD34+ human hematopoietic stem cells using a cytokine cocktail containing IL-4 and GM-CSF. The differentiation was monitored by flow cytometry by measuring the levels of the dendritic cell surface marker CD1a and DC-SIGN over a period of 15 days. The highest level of differentation was observed on day 6 (panel C) and day 9 (panel D).

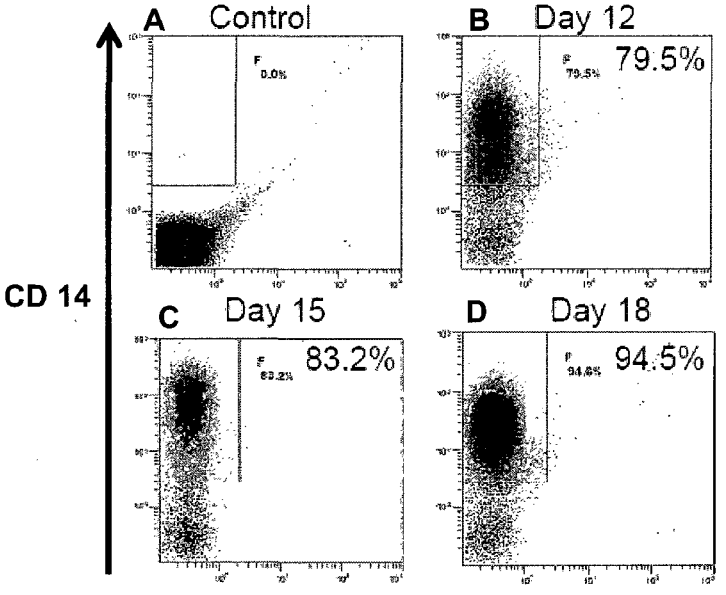

Figure 6.10 Macrophages were differentiated from CD34+ human hematopoietic stem cells. Macrophages were differentiated from CD34+ hematopoietic stem cells using a methocult based method. Macrophages obtained from the differentiation process were allowed to mature in the presence of the cytokine M-CSF. The differentiation was measure by monitoring the expression levels of the monocyte cell surface marker CD14 by a flow cytometry based assay. Highest level of macrophage maturation was observed 18 days post-differentiation (panel D).

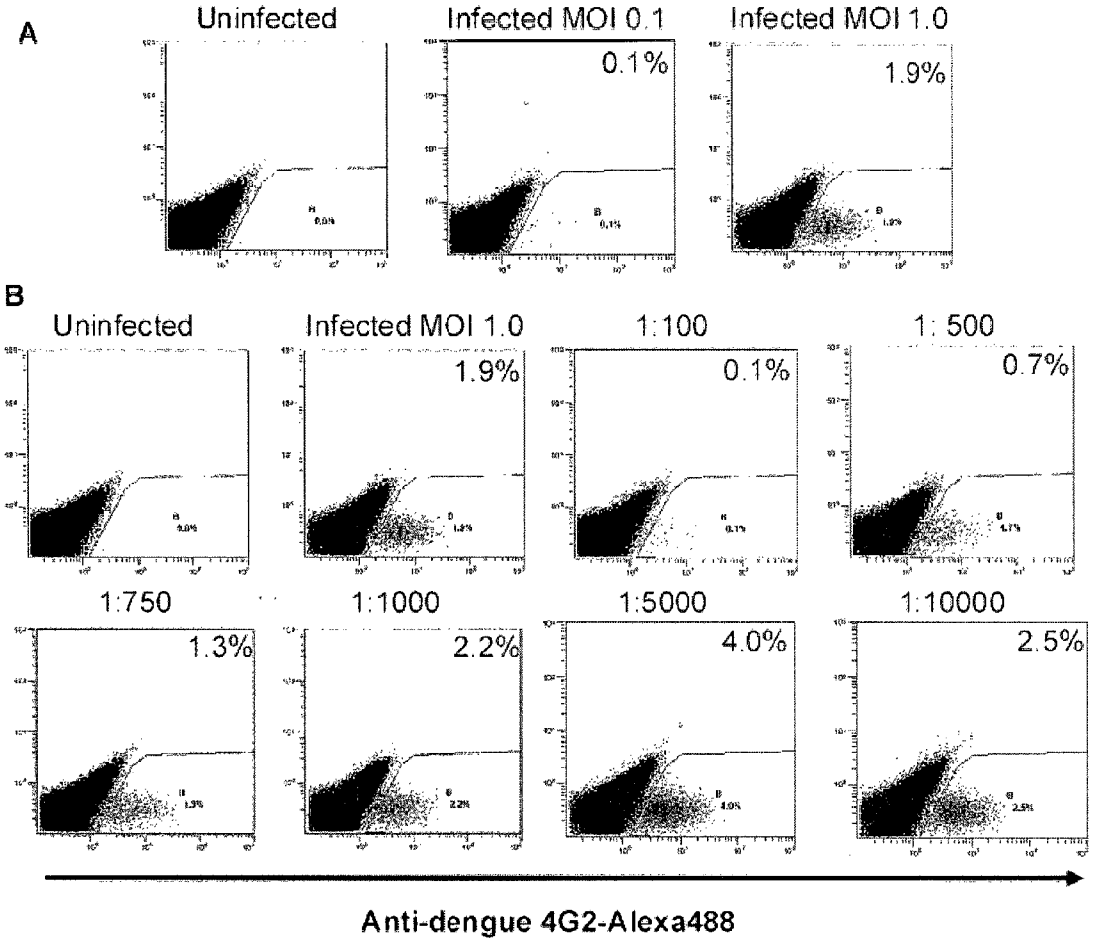
A
Uninfected
Infected MOI 0.1
0.1%
Infected MOI 1.0
1.9%
B
Uninfected
Infected MOI 1.0
1.9%
1:100
0.1%
1: 500
0.7%
1:750
1.3%
1:1000
2.2%
1:5000
4.0%
1:10000
2.5%
Anti-dengue 4G2-Alexa488

Figure 6.11 Infection of macrophages differentiated from CD34+ human hematopoietic stem cells. Macrophages differentiated from CD34+ human hematopoietic stem cells were directly infected at various MOI (panel A) or infected in the presence of dilute anti-dengue positive human sera (panel B). Infection of human macrophages occurs at a very low efficiency. Increase in MOI of direct infection does not produce a corresponding increase in the number of infected cells in these macrophages (panel A). Antibody mediated enhancement of infection of macrophages is observed in panel B. Dilution of human anti-dengue anti-sera to a concentration below neutralization titre allows for an incremental increase in the level of macrophages that were infected even at a low MOI.

absence of human anti-dengue sera, a very low number of cells are susceptible to DENV infection (Figure 6.11 panel A). Upon dilution of human anti-dengue sera (Figure 6.11 panel B), we observe that the macrophages were more susceptible to dengue infection. Macrophages in culture are not easily susceptible to DENV infection. It requires a high MOI of 1.0 to achieve a reasonable level of infection of 1.9%. Addition of diluted human anti-dengue sera allows the macrophages to take up the virus through a Fc-receptor mediated process. We observed that the addition of dilute anti-dengue sera at 1:5000 allowed for an increase in the number of infected macrophages at 4.0%. This is a relatively modest increase in comparison of infections of more DENV permissible cell lines like Vero and primary cells like dendritic cells. Further dilution of the sera does not have any significant enhancing effect due to the decrease in antibody concentrations. These observations confirm that antibody dependant enhancement of Fc bearing cells is a phenomenon that occurs in the presence of sub-neutralizing levels of anti-dengue sera.

6.4d Infection of CD34+ derived platelet precursors and platelets

During a severe DENV infection, plasma leakage along with hemorrhagic manifestations is a common occurrence. It is suspected that platelet precursors (megakaryocytes) in the bone-marrow may be infected to a certain degree thereby preventing the efficient replenishment of blood platelet levels (thrombocytopenia), which trigger the plasma leakage events. In an attempt to identify the cells that maybe infected during the course of a direct DENV challenge we decided to differentiate CD34+ hematopoietic stem cells into megakaryocytes and platelets. We derived the cells into megakaryocytes in media containing thrombopoietin as the cytokine that triggers

differentiation. We observed that CD34+ hematopoietic stem cells were derived into thrombocytes and platelet pre-cursors over 15 days incubation in the presence of thrombopoietin.

The differentiation was monitored using flow cytometry analysis of expression of CD41 as well as CD61, two cell surface markers that are predominantly expressed by thrombocytes and platelets (Figure 6.12 and 6.13). From our analysis we observed that in the presence of thromobopoietin, the cells differentiated into megakaryocytes as well as platelets that are differentiated on the basis of their forward and side scatter profiles by flow cytometry. To determine if megakaryocytes differentiated from CD34+ cells can be infected with DENV, we directly infected the differentiating cells with DENV-2 16681. We observed an extremely low-level of infection in megakaryocytes that were derived from human hematopoietic stem cells.

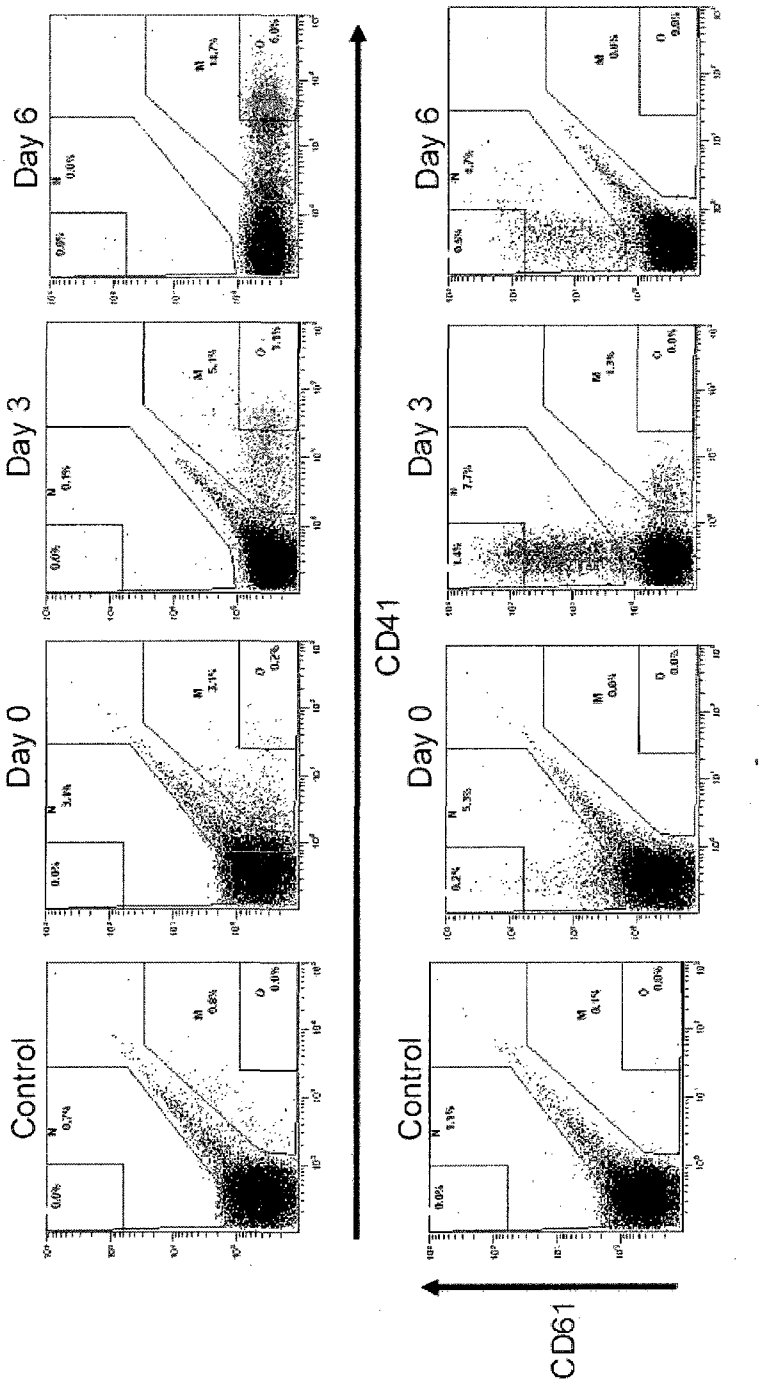
Control
Day 0
Day 3
Day 6
CD41
CD61

Figure 6.12 Differentiation of human megakaryocytes from CD34+ human hematopoietic stem cells. Megakaryocytes were differentiated from CD34+ human hematopoietic stem cells in the presence of thrombopoietin. The process of differentiation was monitored by measuring the levels of two human platelet markers CD41 and CD61 using a flow cytometry based method. Differentiation into megakryocytes was observed on day 3 and day 6 post-differentiation.

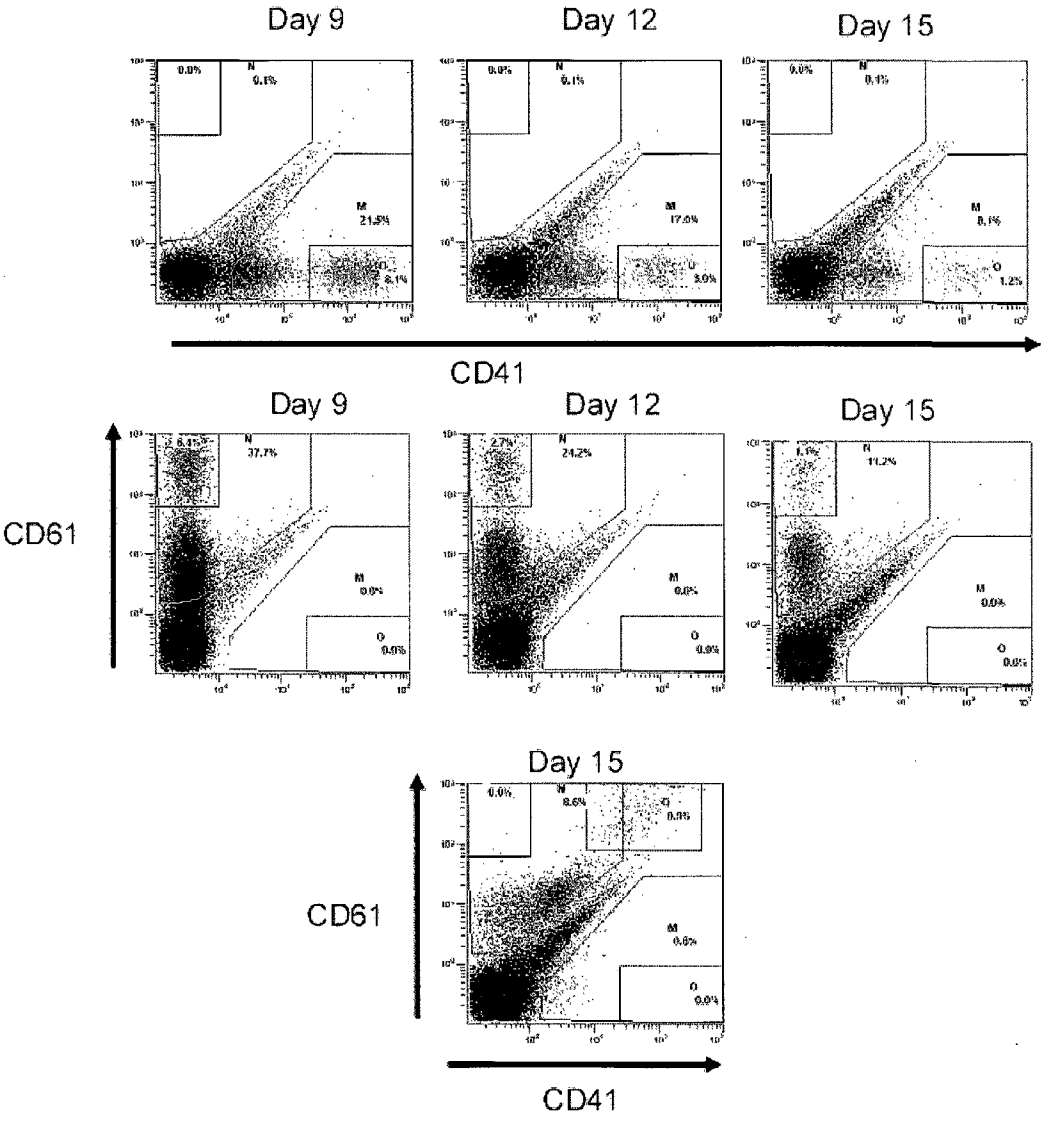
Day 9
Day 12
Day 15
CD41
CD61
Day 9
Day 12
Day 15
CD61
Day 15
CD41

Figure 6.13 Differentiation of human megakaryocytes from CD34+ human hematopoietic stem cells. Megakaryocytes were differentiated from CD34+ human hematopoietic stem cells in the presence of thrombopoietin. The process of differentiation was monitored by measuring the levels of two human platelet markers CD41 and CD61 using a flow cytometry based method. Differentiation into megakryocytes was observed on day 9,day 12 and day 15 post-differentiation. We observed the highest level of expression of CD61 on day 9 at 37.7%.

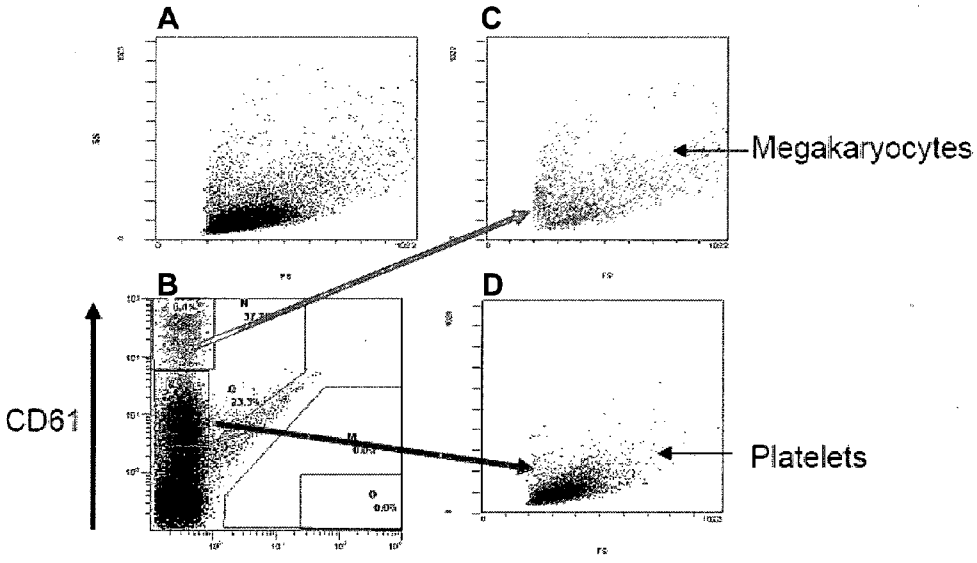

Figure 6.14 Forward and side scatter analysis of differentially staining megakaryocyte cells derived from human hematopoietic stem cells on day 9. Two distinct populations of cells were observed during the differentiation process that expressed either a high level of CD61 or low levels of CD61. Lower level CD61 expressing cells were identified as platelets (panel D) while higher CD61 expressing cells were identified as larger, megakaryocyte cells (panel C) based on their forward and side scatter patterns.

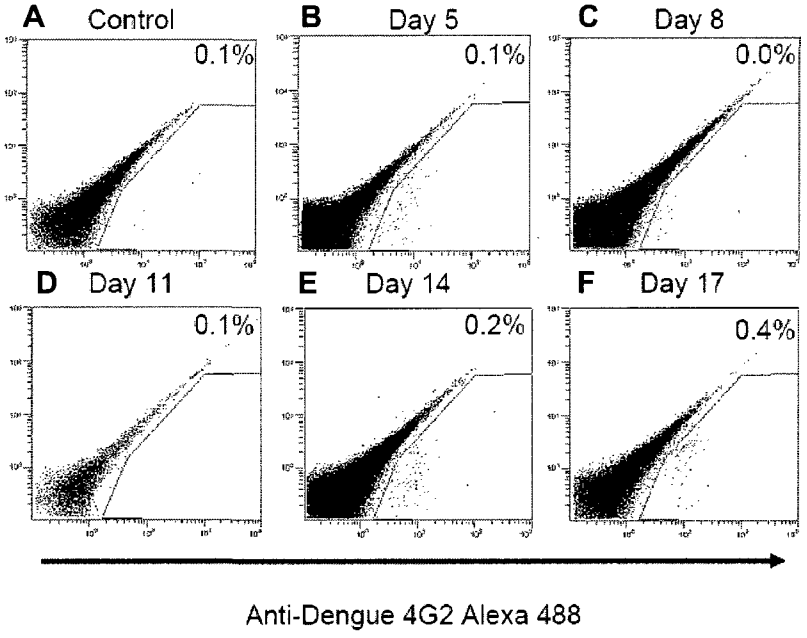

Figure 6.15 DENV infection of megakaryocytic cells derived from human hematopoietic stem cells. Megakaryocytes differentiated from human CD34+ hematopoietic stem cells were directly infected with DENV. Infected cells were identified in a flow cytometry based assay using anti-dengue 4G2-Alexa 488 antibody. Low levels of infected cells were identified day 12 (panel E) and day 15 (panel F) post-differentiation.

6.5 Discussion

During the course of these experiments we set out to identify cells that were susceptible to DENV using a flow cytometry based assay. Monocytes in blood and macrophages in tissue are the first targets that are infected with DENV in human patients. In order to ascertain that our DENV stocks were infectious we tested them on macrophages derived from PBMC. We differentiated macrophages from PBMC isolated from human blood using a cytokine cocktail. After determining the complete differentiation, the cells were infected with DENV successfully. We were also able to demonstrate the ability of dilute human anti-dengue sera to enhance the infection of macrophages at a non-neutralizing concentration.

We also differentiated dendritic cells from human blood using a cytokine cocktail of IL-4 and GM-CSF. We were able to successfully monitor the differentiation of these cells in to dendritic cells. The dendritic cells were infected with by DENV when challenged on day 3, day 6 and day 9 post-differentiation. The highest number of differentiated cells was observed on day 3 which decreased over time on day 6 and day 9.We observed that dendritic cells were efficiently infected on day 3, day 6 and day 9 post-differentiation with the peak of infection achieved on day 6.

Next, we maintained CD34+ cells isolated from human fetal thymus in culture and infected the cells in a transwell using infected Vero cells. We observed that these cells do not maintain their CD34 cell surface marker for long periods when cultured and lose their capacity to divide indefinitely. CD34+ cells are not infected by DENV. This property of CD34+ hematopoietic stem cells to be resistant to viruses plays an important role in the bone marrow where the replenishment of macrophages and monocytes are constantly

required to defend against an infection. However after spontaneous differentiation in culture of CD34+ cells into fibroblast like cells, some of them are susceptible to infection by DENV.

In order to demonstrate the direct infection of pre-cursor cells, we used cytokine cocktails to differentiate CD34+ cells into macrophages, dendritic cells and platelet precursor megakaryocytes. Dendritic cells were generated by growing the cells in a cytokine cocktail with IL-4, TNF-α and GM-CSF in order to differentiate the cells along the dendritic cell lineage. The cells were analyzed for their dendritic cell markers CD1a and DC-SIGN on day 3, day 6 and day 9. They were also infected on the same days and analyzed 48 hrs post-infection for susceptibility to DENV. We observed that dendritic cells are easily differentiated from CD34+ human hematopoietic stem cells. They are easily infected with DENV and the numbers of cells infected were directly proportional to the expression of DC-SIGN on the cell surface. The cells can easily be used as a source of DENV from human cells if required for future experiments.

In order to identify platelet precursors that are directly infected by DENV we differentiated CD34+ human cells using thrombopoietin in the differentiation medium. We observed several distinct platelet precursors develop from the mix. Some of these cells were high CD41 expressing cells while others were low expressers. We observed a distinct size difference between the low expressers and the high expressers. Although a large number of cells were differentiated in the process we observed very few cells that were directly infected when challenged with DENV. Thus we conclude that human platelet precursors are not directly infected by DENV although they may play a significant role in the pathology of the disease during a secondary infection. During our attempts to

directly infect human cells derived from CD34+ human hematopoietic stem cells we identified several cell types that are partially infected with DENV. This indicates that the disease pathology is a combinatorial effect of a cytokine storm in signaling and a hype-immune reaction to viremia.

Chapter 7

Summary and Future Considerations

During the course of my PhD dissertation we sought to understand and investigate several questions that remain unanswered in the field of dengue viral research. We have investigated the ability of DENV to infect human T-cells both *in vitro* and *in vivo*. We have shown that these cells are not infected both in the context of *in vitro* infections as well as when infected in the context of an intact thymus. Experiments with infected human dendritic cells or infected Vero cells used as a source of DENV did not yield any productive T-cell infection in a thymus. This indicates that T-cells do not have any receptors for DENV and are not susceptible to infection in the lymph node of an infected patient. We were able to infect monocytes and dendritic cells that were isolated and developed from human PBMC as well as differentiated from CD34+ human hematopoietic stem cells. We have also demonstrated a very low level of infection of human megakaryoblasts.

We have established that the RAG-hu mouse is a suitable model for studying DENV infection in context of infection of human cells in a mouse-human chimera. During primary infections of RAG-hu mice with DENV we estimated the levels of plasma viremia using a qRT-PCR assay. We successfully infected mice with all four strains of the virus. RAG-hu mice had a higher viral load as compared to unengrafted $Rag2^{-/-}\gamma_c{}^{-/-}$ mice which lasted upto 3 weeks post infection. During a primary infection we observed fever in the mice without any overt signs of serious disease. Post infection, RAG-hu mice produced an adaptive immune response against the infection. We first observed the presence of human anti-dengue IgM in the mice two weeks post infection by ELISA. We also identified mice that were able to produce IgG post infection by ELISA. Most of the mice producing IgM were able to class switch to IgG, which was the first

time it was reported in the context of RAG-hu mice. In the context of primary DENV infections, we were unable to detect any drastic elevation in production of cytokines.

As shown in human patients, production of antibodies in some RAG-hu mice predisposes them to a severe disease when challenged with secondary heterlogous or homologous serotype DENV. We have reported increased viremia, severe dehydration, hemorrhage and morbidity in several mice during a secondary infection. This is the first instance of DHF/DSS like phenomenon reported in any humanized mouse model for DENV infections. We have demonstrated cells that are actively infected with DENV in the spleen and lymph nodes of these mice.

We are in the process of identifying human cells in the infected mice that are infected with DENV using dual immunofluorescence techniques. In mice that experienced a secondary infection, we also observed the production of higher amounts of neutralizing antibody that was capable of cross neutralizing a DENV serotype that it was never exposed to. We are also in the process of developing the model to study secondary infections and ADE in RAG-hu mice. The severity of disease in mice with the presence of prior antibodies against DENV indicates that ADE is a very pertinent mechanism for describing severity of the disease. So far we have not analyzed in detail contributions made by the T-cells with respect to the aggravated disease. In the future we would like to analyze using a multi-parametric approach the critical contributions made by the thymus derived cells in primary as well as secondary DENV infections.

During secondary infections, a majority of the mice were susceptible to infection and died within 40 days post infection. Many of them suffered from spleenomegaly indicating that the chimeric spleen in RAG-hu mice is sensitive and responds to a

cytokine stimulus produced during the infection. The chimeric liver also plays an important role during secondary DENV infections with Kuffer cells that are susceptible to infection. We will analyze both of these organs in detail with respect to the distribution of human cells, DENV infection and the role played by cytokines in these vital organs. In the course of our studies, we have established a humanized mouse model that will replicate dengue pathology that is observed among human patients. We are confident that the RAG-hu mouse is a good model that will potentially be used in the future to test small anti-viral molecules as well was vaccines against DENV prior to clinical trials.

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