Interactions Between Dengue Type 3 Viruses and Human Dendritic Cells

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ABSTRACT

Cassandra Rashida Lambeth: Interactions Between Dengue type 3 Viruses and Human Dendritic Cells
(Under the direction of Dr. Aravinda de Silva)

Dengue is a mosquito-borne viral disease of global public health significance. Throughout the world more than 2.5 billion people are at risk of contracting dengue virus (DENV) infection. Each year, an estimated 100 million cases of dengue viral infection are reported worldwide with 250,000 people developing the more severe dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), which is often fatal. The pathogenesis of dengue is poorly understood due to the lack of an animal model. Both host and viral factors are believed to play key roles in dengue pathogenesis and in clinical outcome.

DENVs are difficult to study in culture because available assays are time consuming and typically used with high passage, laboratory adapted strains of the virus. I have developed flow cytometry-based assays to study DENVs. The assays were used to titer viruses and to measure virus-antibody interactions. The flow cytometry-based assays were also used to study interactions between DENVs and human dendritic cells (DCs), one of the primary targets of DENVs.

Experiments were done to analyze interactions between Dengue type 3 viruses (DENV3) and primary human DCs. DENV3 blocked activation of infected immature DCs, but not the uninfected bystander DCs in the culture. Experiments were also conducted to characterize the ability of different strains of DENV3 to infect human DCs. I identified two Sri Lankan isolates of DENV3, one from a period of mild disease and the other from a period of severe disease, which infected DCs with different efficiency. Both viruses were equally sensitive to type I IFN, indicating that host anti-viral responses were unlikely to be responsible for observed differences. Both viruses also bound to cells with similar efficiency but entered cells at different rates. The two viruses differed at a step between binding to the cell surface and viral fusion in endosomes. The significance of these results with respect to the cell biology of DENVs and virus-host interactions are discussed.

To my parents, Arlene and Douglas, my sister Cheryl and in loving memory of my brother, Bernie, who have always been there for me, provided endless love and support, and encouraged me to follow my dreams no matter where they led me. And to Adam, my best friend and love, whose never ending support has carried me through the darkest of days and the happiest moments of my life.

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Amino acid (aa)

Amino-terminus (N-terminus)

Ammonium chloride (NH_4Cl)

Antibody (Ab)

Antibody-dependent enhancement (ADE)

Antigen presenting cells (APCs)

Asparagine (Asn)

Beta (β)

Carbohydrate recognition domains (CRDs)

Capsid protein (C)

Carbon dioxide (CO_2)

Carboxyl-terminus (C-terminus)

Cluster of differentiation (CD)

Complementary deoxyribonucleic acid (cDNA)

Cytometric Bead Array (CBA)

Cytopathic effect (CPE)

Cytotoxic T lymphocytes (CTLs)

Dendritic Cell (DC)

Dengue fever (DF)

Dengue hemorrhagic fever (DHF)

Dengue shock syndrome (DSS)

Dengue virus (DENV)

Dengue virus type 1 (DENV1)

Dengue virus type 2 (DENV2)

Dengue virus type 3 (DENV3)

Dengue virus type 4 (DENV4)

Deoxyribonucleic acid (DNA)

DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)

Dulbecco's Modified Eagle's Medium (DMEM)

Eagle’s Minimum Essential Medium (EMEM)

Envelope protein (E)

Endoplasmic Reticulum (ER)

Enzyme linked immunoabsorbent assay (ELISA)

Epstein-Barr virus (EBV)

Ethylenediamine tetraacetic acid (EDTA)

Fc gamma (Fcγ)

Fc gamma receptor II (FcRII)

Fluorescein Isothiocyanate (FITC)

Fluorescence activated cell sorting (FACS)

FACS neutralization test (FNT)

Fetal bovine serum (FBS)

Glucose-regulated protein/immunoglobulin-binding protein (GRP78/BiP)

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)

Green fluorescent protein (GFP)

Heat inactivated (hi)

Heat shock proteins (Hsp)

Horseradish peroxidase (HRP)

Hour (hr)

Hours post infection (hpi)

Human foreskin fibroblast (HFF)

Human leukocyte antigen (HLA)

Human immunodeficiency virus (HIV)

Human umbilical vein endothelial cells (HUVECs)

Intercellular adhesion molecule (ICAM)

Interferon (IFN)

Interferon-alpha (IFN-α)

Interferon-beta (IFN-β)

Interferon-gamma (IFN-γ)

Interleukin (IL)

International units (IU)

Isoleucine (Ile)

Janus kinase (JAK)

Japanese encephalitis virus (JEV)

Kilobases (kb)

Kilodalton (kd)

Lipopolysaccharide (LPS)

Mature protein (M)

Major histocompatibility complex (MHC)

Mean fluorescent intensity (MFI)

Messenger ribonucleic acid (mRNA)

Minute (min)

Multiplicity of infection (MOI)

Natural killer (NK)

Neutralizing units (NU)

Noncoding region (NCR)

Nonstructural protein (NS)

Nucleoside triphosphate (NTP)

Nucleotide (nt)

Open reading frame (ORF)

Peripheral blood mononuclear cells (PBMCs)

Phosphate buffered saline (PBS)

Phycoerythrin (PE)

Plaque forming units (pfu)

Plaque reduction neutralization test (PRNT)

Polymerase chain reaction (PCR)

Premembrane (prM)

Proline (Pro)

Ribonucleic acid (RNA)

Reverse-transcriptase (RT)

Revolutions per minute (rpm)

Roswell park memorial institute (RPMI)

Seconds (secs)

Serine (Ser)

Signal transducers and activator of transcription (STAT)

Sodium azide (NaN_3)

Standard error of the mean (SEM)

Temperature (temp)

T helper type 1 (Th1)

T helper type 2 (Th2)

Threonine (Thr)

Tissue culture infectious dose 50 ($TCID_{50}$)

Transforming growth factor-beta (TGF-β)

Tumor necrosis factor-alpha (TNF-α)

Tumor necrosis factor-beta (TNF-β)

Ultraviolet (UV)

Untranslated region (UTR)

Valine (Val)

West nile virus (WNV)

Yellow fever virus (YFV)

Chapter 1

Introduction

1.1 General Introduction

Dengue is a major global health problem that affects millions of people each year. Annually, there are an estimated 100 million cases of dengue virus infection occurring worldwide, resulting in approximately 250,000 people developing dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (73). Dengue is predominately a problem in the American hemispheres, the Pacific islands and continental Asia (71). The more severe forms of the disease are most often seen in children, even though adults are susceptible to mild and severe dengue disease (31). Dengue virus infections cause a wide spectrum of disease manifestations from asymptomatic infections to dengue fever or dengue hemorrhagic fever/dengue shock syndrome. Dengue fever is a self-limiting febrile illness that is characterized by fever, headache, myalgia, joint pain, and rash (28, 55, 154). It has also been known as "breakbone fever" because the symptoms of muscle and joint pain are particularly prominent. The less common but more severe disease manifestations of dengue are DHF and DSS. DHF is marked by increased capillary permeability without morphological damage to the capillary endothelium, hemorrhagic manifestations, increased hemocrit and thrombocytopenia (19, 28, 55). DSS occurs when fluid leaks into the interstitial spaces, resulting in shock that can be fatal without proper treatment (28).

Even though dengue is an emerging virus that continues to spread throughout the tropics, it has been around for the past 1,500 years (132, 197, 202). The progenitor dengue virus (DENV) introduced into Asia likely originated from Africa. It is hypothesized that the zoonotic transfer of sylvatic strains of DENVs to human hosts occurred between 300-1,500 years ago (132, 197). DENVs can be divided into four distinct serotypes: DENV type 1 (DENV1), DENV type 2 (DENV2), DENV type 3 (DENV3), and DENV type 4 (DENV4). All four serotypes of DENV most likely evolved in the rainforest of southeast Asia (59, 86). DENV1 and DENV2 were first isolated from people during World War II in the Pacific (132, 176). DENV3 and DENV4 were later isolated in the 1950s during epidemics in the Philippines and Thailand (75, 132).

All DENVs are transmitted by the *Aedes aegypti* and *A. albopictus* mosquitoes. Migration of the *Aedes* spp. mosquito vector from rain forests into urban centers led to the switch from zoonotic transmission to the mosquito-human host transmission cycle (Figure 1.1) (58). This shift in transmission has resulted in the emergence of epidemic DF/DHF in the twentieth century (132). *A. aegypti* are domestic daytime feeding mosquitoes that prefer to feed on humans. They are usually found within close proximity to human environments and lay their eggs in artificial containers near homes.

Mosquitoes become infected after ingesting a viremic blood meal from a person experiencing an acute dengue infection. In addition, mosquitoes may also become infected by vertical transmission of DENVs from an infected female to her offspring (132). Upon infection, virus replication, or the extrinsic incubation period, begins in the mosquito. The extrinsic incubation period usually lasts 8-12 days, and after this period,

the infected mosquito can refeed and transmit the virus to a new uninfected human host. Upon infection of a new host, the intrinsic incubation period, or replication in the host, begins and continues for about 8 days. After the intrinsic incubation period, viremia is established and the onset of disease symptoms occurs (Figure 1.1) (58, 59).

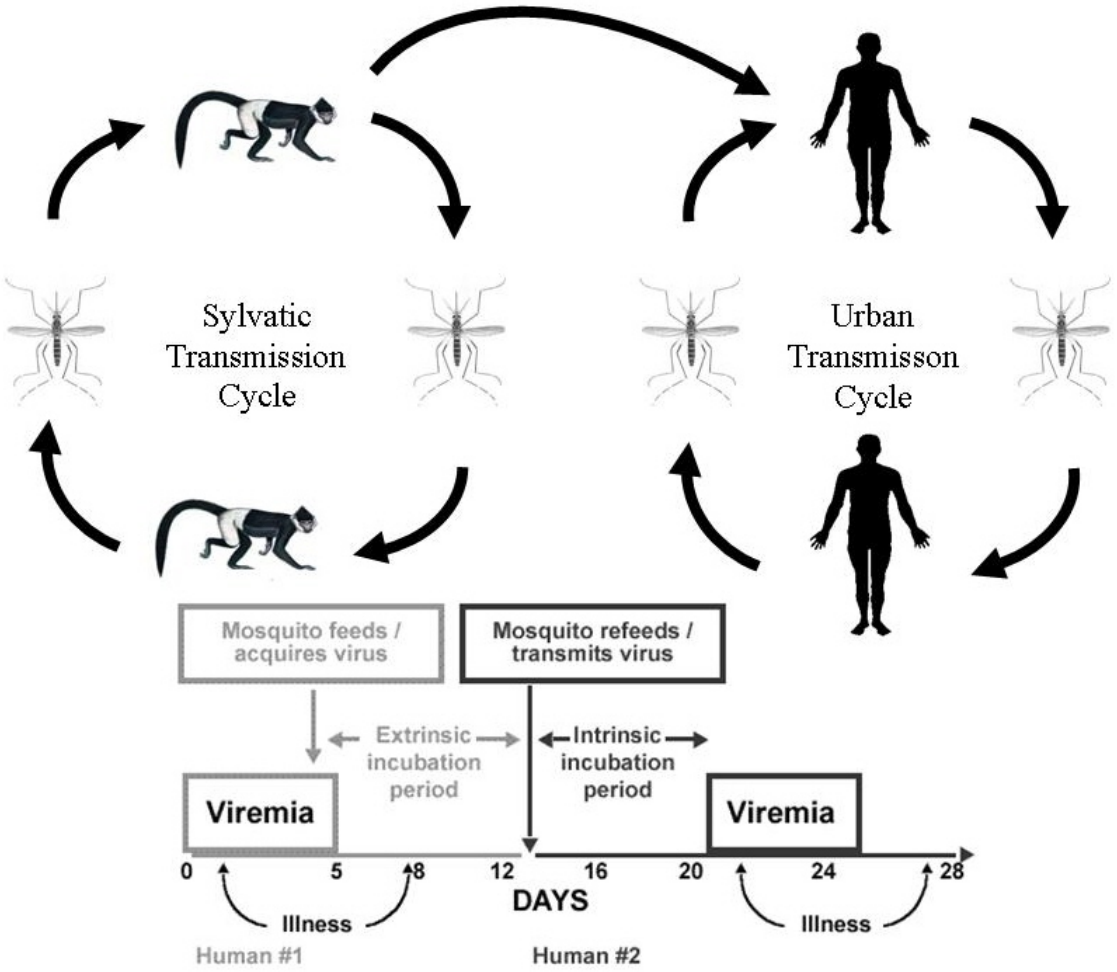

Figure 1.1. Dengue transmission cycle. Top panel: DENVs are transmitted to and from humans or lower primates from *A. aegypti* mosquitoes in either the urban transmission cycle or the sylvatic transmission cycle, respectively. Both transmission cycles co-exist as separate cycles with different DENV strains. As depicted by the arrow branching the two independent cycles, human DENVs likely emerged from DENVs transmitted in the zoonotic/sylvatic cycle. Lower panel: Transmission of DENVs begins when *A. aegypti* mosquitoes feed on an infected viremic individual, human #1. After feeding on an infected human, the extrinsic incubation period occurs in the mosquito. The infected mosquito then transmits the virus to a new uninfected host, human #2. The intrinsic incubation period begins immediately after infection of the new host and eventually results in viremia. The onset of disease symptoms occurs 1-2 days after the establishment of viremia. Lower panel figure and legend are courtesy of the CDC Division of Vector-Borne Infectious Diseases Dengue website http://www.cdc.gov/ncidod/dvbid/dengue/index.htm

1.2 Dengue viruses

DENVs are enveloped, positive single stranded RNA viruses that are members of the *Flaviviridae* family and flavivirus genus. Each serotype of DENV is an antigenically distinct virus that can be further divided into subtypes or genotypes based on genomic sequence data. The genome of DENV is approximately 11 kilobases (kb) in length and contains a 5' cap (m^7G5'ppp'A), but lacks a polyadenylated tail at the 3' end (126). The genomic ribonucleic acid (RNA) is the messenger RNA (mRNA) that is initially translated into a single polyprotein containing one open reading frame (ORF). Flanking the genome are two untranslated regions (UTRs), or noncoding regions (NCRs), located at the 5' and 3' ends. The 5' UTR is approximately 100 nucleotides (nt) in length and the 3' UTR is approximately 450 nt (84). Once the genome is translated, the large single polyprotein is processed co- and postranslationally by cellular and viral proteases into three structural and seven nonstructural proteins (28, 126). The structural proteins consist of capsid (C), premembrane (prM) and envelope (E), and the nonstructural proteins (NS) are divided into NS1, NS2A, NS3, NS4A, NS4B, and NS5 (Figure 1.2).

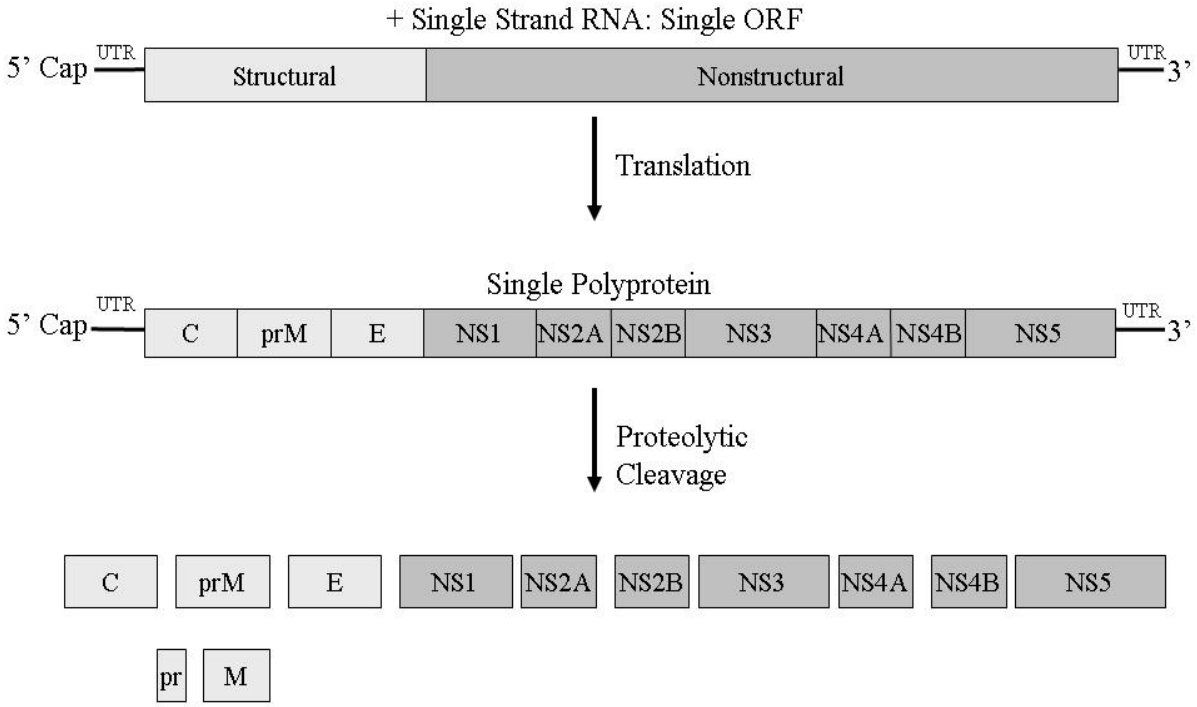

Figure 1.2. Dengue virus genome. The positive single stranded genomic RNA is translated into a single polyprotein. The single polyprotein is cleaved by cellular and viral proteases into three structural and seven nonstructural proteins. The structural proteins consist of capsid, premembrane, and envelope. The seven nonstructural proteins as divided into NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. Premembrane is further cleaved into the "pr" segment and the mature M protein.

Viral structural proteins

Capsid is an 11 kilodalton (kd) highly basic protein that is essential for specific encapsidation of the viral genome and for virus assembly (131). The mechanism of how encapsidation occurs is not fully understood, but, it is hypothesized that C protein may interact with the viral nonstructural proteins for this process (107). C protein may also interact with the viral RNA and lipid bilayer (131). The basic residues of the protein are concentrated at the amino- (N-) and carboxyl- (C-) termini and are thought to act cooperatively to specifically bind genomic RNA(126). The middle region of C contains a hydrophobic domain that interacts with cellular membranes and may be important in virus assembly (133). The C-terminal hydrophobic domain of C acts as signal sequence for the translocation of prM into the lumen of the endoplasmic reticulum (ER) (149). Once translocation is complete, the NS2B-NS3 viral protease cleaves the signal sequence, leaving the mature form of C protein (3, 131, 209).

The viral protease that cleaves C from prM is believed to regulate the processing of structural proteins and virion assembly throughout infection (126). During egression of the virion through the secretory pathway, prM is cleaved by the trans-Golgi enzyme furin (185). The protein is cleaved into two forms: the structural mature (M) protein and the N-terminal precursor "pr" segment, which is secreted from the cell. The structural M protein is located in the C-terminal portion of the prM protein and contains two transmembrane domains. The mature virion contains the structural M protein. Prior to its cleavage, prM is thought to function as a chaperone for the folding and assembly of E protein (129, 149).

The E protein is a 50 kd protein that contains two C-terminal transmembrane domains (126). This 495 amino acid (aa) membrane glycoprotein is composed of three domains: domain I is a centrally located beta (β) barrel, domain II contains a dimerization region and fusion peptide, and domain III harbors the receptor-binding activity (28, 149). The E protein forms head-to-tail homodimeric rods that lie parallel to the virion surface. In the mature virion, E exists as a homodimer, concealing the fusion peptide of domain II (28, 143). When E is exposed to low pH conditions, the E homodimers likely disassociate into monomers and then reassociate irreversibly into homotrimers (Figure 1.3) (1, 187, 188). This process occurs in the late endosome and leads to fusion of the viral membrane with the endosomal membrane (143, 149). Located between domains I and II is a flexible hinge region that is required for the structural changes that precede exposure of the fusion peptide (142, 143). Within E, two N-linked glycosylation sites are located at Asparagine (Asn)-153, which is conserved among many flaviviruses, and Asn-67, which is unique to DENVs (169). Glycosylation is important in receptor binding (28, 130, 153, 163) and endosomal fusion (28, 63, 117). Several groups have shown DENVs are differentially glycosylated depending on the virus serotype and the cell type used for virus propagation (93, 117).

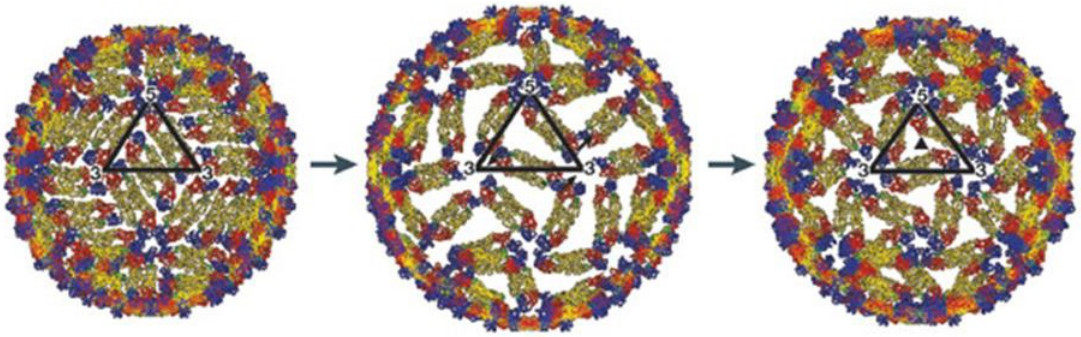

Figure1.3. Proposed rearrangement of E proteins during fusion. E protein in mature virion formation (left). When the virion is exposed to low pH conditions, E homodimers likely disassociate into monomers and then reassociate into parallel homotrimers (right). It is hypothesized that an approximate 10% radial expansion of the particle would occur to accommodate this conformational change (center). Figure and legend from Mukhopadhyay et al. (2005) (149).

Viral nonstructural proteins

There are seven nonstructural proteins of DENV: NS1, NS2A, NS3, NS4A, NS4B, and NS5. The NS1 protein exists in three forms: an intracellular form, which resides in the ER and colocalizes with the viral replication complex, a cell surface form, which is membrane anchored, and a secreted form (28, 126). NS1 contains two N-linked glycosylation sites, Asn-130 and Asn-207 (28). It has been shown previously that glycosylation of both sites is required for viral replication in mosquito cells (30). The C-terminus of E acts as a signal sequence to translocate NS1 to the ER. The C-terminus of E is removed and NS1 is then cleaved from NS2A by an unknown host protease (18, 43, 44). NS1 is a homodimer and in Kujin, another flavivirus, this dimerization appears to be required for viral replication (69, 126). During secretion in mammalian cells, a complex sugar is added to one of the N-linked glycans and three NS1 dimers come together to form a soluble hexameric form (47, 165).

NS3 is a 70 kd cytoplasmic protein that performs several roles in the viral life cycle. It is associated with membranes via its interaction with NS2B (5, 27) and contains several enzymatic activities indicating a role in polyprotein processing and RNA replication (126). The first third of the protein contains sequence homologous to trypsin-like serine proteases (9, 53). Amino acid residues 167 to 181 of NS3, along with NS2B, are sufficient for its protease activity (17, 118). The NS2B-NS3 protease cleaves in both *cis* and *trans* configurations and mediates cleavage at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 junctions (126). The C-terminal three quarters of NS3, including regions that slightly overlap with the serine protease domain, have been implicated in viral RNA replication (118). This region of NS3 contains homology to

RNA helicases, which utilize the energy of nucleoside triphosphate (NTP) hydrolysis to power RNA unwinding (53, 95). Consistent with its potential function as a helicase, DENV2 NS3 has been shown to contain an NTP-dependent RNA unwinding activity (118). In addition to its helicase activity, the C-terminal portion of NS3 has also been shown to contain RNA triphosphatase activity, which is different from the NTPase activity (205). It is likely that this enzymatic activity is involved in preparing the 5' end of the genome for the addition of the 5' cap (126, 205).

NS5, the largest of the nonstructural proteins of DENVs, is 103 kd. It is the viral RNA-dependent RNA polymerase and is homologous to other viral RNA polymerases (28, 126). NS5 is also homologous to methyltranferase enzymes that are involved in RNA cap formation and is likely to be involved in methylation of the 5' RNA cap structure (105). NS5 has also been shown to be phosphorylated by an unknown cellular serine/threonine (Ser/Thr) kinase. However, the role that this phosphorylation serves for the protein is still unknown (97, 167).

Little is known about the functions of NS2A, NS2B, NS4A and NS4B. NS2A is a 22 kd hydrophobic protein of unknown function. As mentioned above, the N-terminus of NS2A is cleaved from NS1. The C-terminus of NS2A is generated via cleavage of NS2B at the NS2A/NS2B junction by a cytoplasmic Ser protease, suggesting that this protein is membrane spanning (126). NS2B is a small 14 kd membrane-associated protein. It forms a complex with NS3 and is necessary for the serine protease activity of NS3 (5, 45, 126). NS4A is another relatively small 16 kd hydrophobic protein that is believed to function in RNA replication (126). The N-terminus of the NS4A is generated by the NS2B-NS3 serine protease, while the C-terminus acts a signal sequence to translocate

NS4B to the ER. NS4B is a 27 kd protein that is posttranslationally modified to a 2 kd smaller protein. The nature of this modification is unknown (164). NS4B, and to a lesser extent NS2A and NS4A, inhibit interferon (IFN) signaling by blocking IFN-alpha/beta (α/β)-induced phosphorylation of signal transducers and activator of transcription 1 (STAT1) (150, 151).

1.3 Dengue virus life cycle

The initial stages of the viral life cycle begin with the virus binding to one or more cellular receptors on the surface of a permissive host cell (126). It is hypothesized that carbohydrate moieties on the viral surface modulate specificity of receptor binding and that domain III of the E protein is the portion of E that interacts with the cellular receptor (28, 153). Once the virus is bound to the cell surface, it is internalized via receptor-mediated endocytosis into a clathrin-coated pit or vesicle (52). Rab 5 has recently been shown to be necessary for the transport of Dengue and West Nile Viruses (WNV) to early endosomes in Hela cells (106). The clathrin-coated pits progress into the early endosomal stage. Inside the endosome, the low pH triggers a conformational change within E. During the acidification, E homodimers disassociate into monomers and then reassociate irreversibly into homotrimers (Figure 1.3) (1, 187, 188). This conformational change leads to the exposure of the fusion peptide (13, 143). The fusion peptide then binds to the cellular membrane within the endosome and catalyzes fusion between the viral and cellular membranes, which causes the release of the nucleocapsid and viral RNA into the cytoplasm (Figure 1.4) (52, 78, 149).

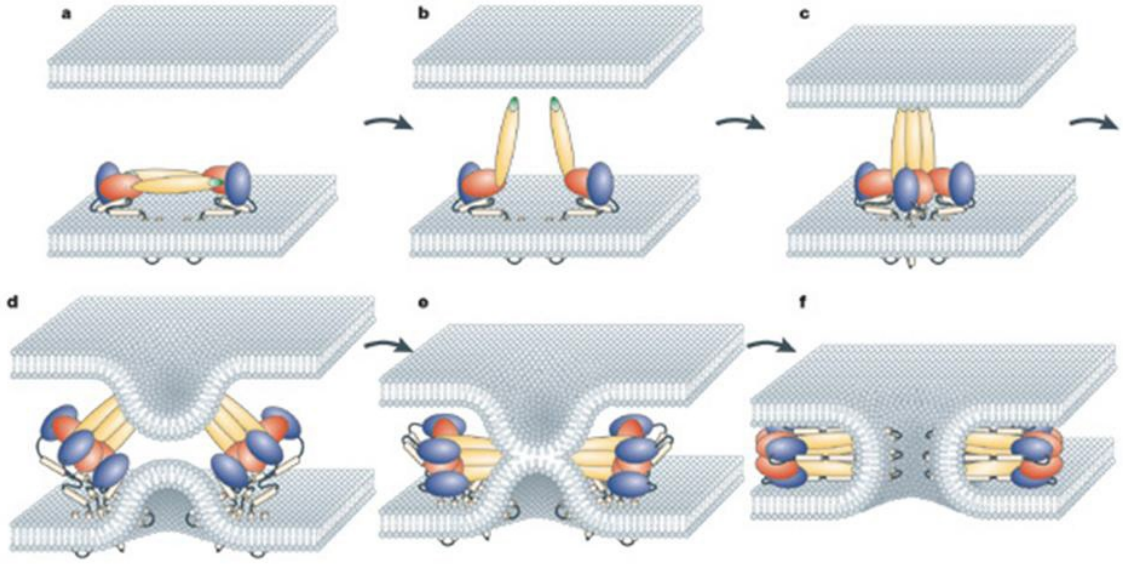

Figure 1.4. Class II fusion process. The dimeric form of E protein on the virus surface with the fusion peptide (shown in green) buried in the dimer (**a**). The virus binds the cellular receptor and is internalized into the endosome where the E protein rearranges during low pH conditions. During the rearrangement domain II swings outward towards the host cell membrane exposing the fusion peptide (**b**). The fusion peptide inserts into the host cell membrane leading to the homotrimer formation of E protein (**c**). Domain III of the E protein folds back onto itself, and in the process brings the viral membrane towards the fusion peptide and host cell membrane (**d**). As domain III moves towards domain II, hemifusion of the lipid membranes occurs (**e**), and finally formation of the trimer occurs where the transmembrane regions and the fusion peptide are in close proximity (**f**). Only the first and last steps have been observed by X-ray crystallography, the other steps have been deduced by biochemical analysis. Figure and legend from Mukhopadhyay et al. (2005) (149).

Once the viral RNA is in the cytoplasm, initiation of translation begins. The genomic RNA contains secondary structures within the 5' and 3' UTRs. The 5' and 3' UTRs are possibly capable of interacting with each other to lead to circularization of the genome (68). Conversely, mutations of the cyclization sequence domain in the 5' and 3' UTRs do not lead to translation inhibition (2, 26, 40, 85). It is unclear whether circularization of the genomic RNA is required for efficient translation of the viral genome. The exact mechanism of DENV translation is still unresolved. The lack of a polyadenylated tail in the viral genomic RNA implies that DENVs do not utilize the same mechanisms for translation as cellular mRNAs (28). In support of this theory, dengue viral proteins are still translated under inhibitory conditions for cellular protein synthesis (35, 38-40). After the large single polyprotein of dengue is synthesized, it is cleaved by cellular and viral proteases (NS2B/NS3). The viral replicase is formed by the assembly of the NS proteins with cellular membranes (126). At this time, all of the proteins present in the replication complex have not been defined. NS3 and NS5 are key components because they function as the viral helicase and RNA-dependent polymerase, respectively (126). Replication begins with the synthesis of genomic length minus strand RNA. The minus strand RNA serves as a template for the synthesis of nascent genomic RNA (Figure 1.5).

Capsid protein interacts with the nascent genomic RNA to form a nucleocapsid precursor in the cytoplasm. Based on the orientation of C, prM and E with respect to the ER, it is believed that the nucleocapsid acquires an envelope by budding into the ER lumen (126). Co-synthesis of E and prM are necessary for the proper folding of E (104). During the late stage of virion maturation, E and prM are modified by the trimming and

terminal processing of glycans (18, 136, 159). As the immature virion is transported through the exocytic pathway, prM is cleaved into M by the protease furin (149). Furin is only capable of cleaving prM once it has been exposed to a low pH, suggesting the furin cleavage site is inaccessible prior to the pH change (41, 185). Studies have shown that inhibition of prM cleavage still leads to virion release, but cleavage is required for highly infectious virus (1, 78, 166, 185). Cleavage of prM is believed to occur just prior to viral release since intracellular M-containing virions have not been detected (126). prM is thought to act as a chaperone for E functioning to prevent E from undergoing an acid catalyzed conformational change during transit of the immature virion through the acidic intracellular compartment (62, 78). The hemagglutination activity of flaviviruses is dependent on the low pH transitional step to activate the fusogenic activity of E protein (170).

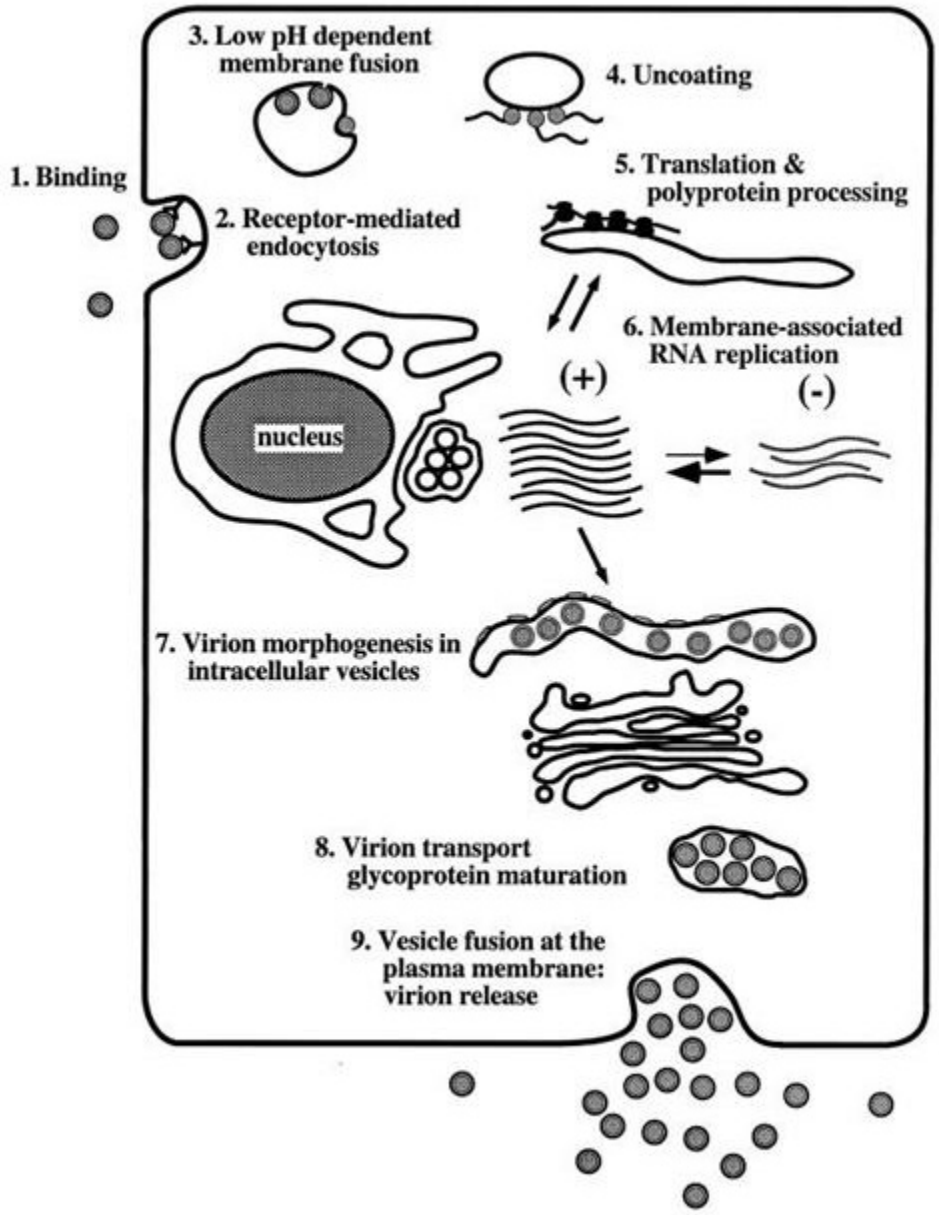

Figure 1.5. Flavivirus life cycle. Virus binds to cellular receptor(s) (1) and enters the cell via receptor-mediated endocytosis (2). In the endosome, the pH lowers triggering fusion of the viral membrane with endosomal cellular membrane (3). Viral nucleocapsid is released into the cytoplasm where the virus uncoats (4). Genomic RNA is translated into a singe polyprotein that is proteolytically cleaved by cellular and viral proteases (5). Viral proteins assist in replication of genomic RNA in negative strand RNAs that serve as templates for the synthesis of nascent positive strand genomic RNA (6). New virions are assembled and bud through the ER (7). Immature virions are proteolytically cleaved and modified by the trimming and terminal addition of glycans (8). Mature virions are transported out of the cell via an exocytic pathway (9). Figure and legend from Lindenbach et al (2001) (126).

Cellular tropism and receptors

During a natural dengue infection, dendritic cells (DCs) are probably one of the first cells to encounter DENVs. In fact, blood-derived DCs are ten-fold more permissive to dengue infection than are monocytes and macrophages (207). Using cadaveric skin explants, Wu et al. demonstrated that Langerhan DCs located in the dermis and epidermis were permissive to DENV infection (207). Conversely, *in vitro* infection data indicated that Langerhan DCs were not permissive to DENVs, but immature monocyte-derived myeloid DCs were permissive (120, 130). Even though there is still some uncertainty as to the first cell to encounter DENVs, data from clinical and autopsy studies have shown that cells of the mononuclear phagocytic lineage, such as monocytes, macrophages and DCs, are the primary *in vivo* targets (91). Other cell types that also may be important targets of DENVs are hepatocytes and endothelial cells. These cell types have been shown *in vitro* to be permissive to infection, but these findings have not been corroborated with *in vivo* data (4, 91). *In vitro* studies have also indicated human peripheral blood mononuclear cells (PBMCs) and neuronal cells are capable of dengue virus infection (98, 156, 180).

In the absence of an animal model, many *in vitro* studies have utilized mammalian and insect cell lines to study viral infection. Some of these cell lines include Vero (African green monkey kidney), BHK21 (baby hamster kidney), U937 (human monocytes), C6/36 (*A. albopictus* mosquito), and HFF (human foreskin fibroblast) cells. The glycans that are added to the progeny virions during assembly differ based on whether they were propagated in insect or mammalian cells. Studies on the glycosylation of DENVs have indicated that viruses grown in insect cells have the addition of a high

mannose sugar on Asn-67, where mammalian grown virus has the addition of a complex sugar (76, 163). These glycosylation differences potentially play an important role in receptor usage of DENVs (89, 130, 153, 163).

Investigators have attempted to define the cellular receptors for DENV. In mammalian cells, a few candidates have been identified as potential DENV receptors. These candidates include heparin sulfate (23, 51, 80, 125), heat shock protein (Hsp) 70 (171), Hsp90(171), Glucose-regulated protein/immunoglobulin-binding protein (GRP78/BiP) (92), Cluster of differentiation 14 (CD14) (24), a 37kd/67kd high affinity laminin receptor (195), DC-specific intercellular adhesion molecule-3 (ICAM)-grabbing nonintegrin (DC-SIGN) (130, 153, 194), and liver/lymph node-specific ICAM-3-grabbing nonintegrin (194). Within the past few years, the majority of studies on DENV-receptor interactions have focused specifically on the DENV receptor, DC-SIGN.

DC-SIGN is a tetrameric type II transmembrane protein in which the C-terminal carbohydrate recognition domains (CRDs) bind high-mannose oligosaccharides (46, 141). This receptor interacts with DENVs via the carbohydrate moieties on E protein (130, 153). Viral propagation in insect cells results in the addition of high-mannose glycans to residue Asn-67 (130, 153, 163). The presence of this high-mannose sugar is required for DC-SIGN-mediated entry (130, 163). High-manose carbohydrates can be added to Asn-67 of all four serotypes of DENVs, allowing all serotypes to use DC-SIGN as a cellular receptor (194) DC-SIGN mediates DENV infection of human DCs. Ectopic expression of DC-SIGN confers permissiveness to infection of normally nonpermissive cell lines (153, 194). DC-SIGN mediated viral entry may represent a common pathway used by arboviruses such as flaviviruses (i.e., DENVs) and alphaviruses (i.e., Sindbis

virus) to initiate infection of the vertebrate host via DCs by viruses transmitted by an arthropod vector (102, 153, 194).

Receptor mediated endocytosis of DENV may involve more cell surface receptors than DC-SIGN. Specifically, two or more receptors are proposed to be involved with the endocytosis of DENV. These receptors likely include a ubiquitous lower-affinity receptor such as DC-SIGN that initially captures the virus at the cellular surface, increasing the local concentration, and a less-common, high-affinity receptor that mediates internalization of the virus (28). To support this hypothesis, Lozarch et al. have shown that DENVs are capable of infecting cells in the absence of the cytoplasmic internalization signal of DC-SIGN (130). In addition, Navarro-Sanchez et al. have demonstrated that DENV infection can be drastically decreased upon blockage of DC-SIGN during viral infection (153).

1.4 Dengue pathogenesis

The pathogenesis of dengue virus infection is poorly understood due to the lack of an animal model. Currently, information concerning dengue pathogenesis is based on epidemiological and experimental data. The present theory regarding dengue pathogenesis and the generation of immune responses indicates primary infection with a given serotype confers immunity to that particular serotype. The immune response to the primary serotype is cross-protective against other serotypes only during the first several months after infection (28, 176). After the cross-protective nature of the primary infection wanes, the host is susceptible to infection by the remaining three heterologous dengue serotypes (72). Secondary dengue infection with a serotype other than the primary

infecting serotype increases the risk of developing the severe form of dengue disease, DHF/DSS. The symptoms of DHF are, initially, similar to DF with fever, headache, myalgia, joint pain, and rash (28, 55, 154). The disease then progresses, resulting in more severe symptoms including increased capillary permeability without morphological damage to the capillary endothelium, hemorrhagic manifestations, increased hemocrit and thrombocytopenia (19, 28, 55). Fluid leakage into the interstitial spaces results in shock, which can be fatal without proper treatment (28). Even though DHF is predominately seen in secondary infections, approximately 10% of DHF cases have been associated with primary infections (55). These cases have been mostly observed in very young children suggesting that waning maternal dengue antibodies may lead to these primary cases of DHF (100).

Risk factors for developing DHF

Some of the risk factors that are associated with developing DHF are: age (58, 64), viral serotype (7, 58) and genotype (140, 172), and the genetic background of the host (65, 74). The single greatest risk factor for developing DHF/DSS is secondary infection, even though a small percentage of the cases arise from primary infection. Little is known about how human genetics affect the risk of developing DHF. Some studies analyzed polymorphisms in human leukocyte antigen (HLA) alleles to determine if certain HLA types occur frequently with DHF/DSS cases. HLA genes are part of the human major histocompatibility complex (MHC) that encodes cell-surface antigen-presenting proteins. MHCs can be divided into three main classes: MHC class I, II, and III. MHC class I consists of: HLA-A, HLA-B, and HLA-C. MHC class II consists of: HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA and HLA-DRB1. The

MHC class III region of the HLA genes encodes for other immune components such as complement proteins, tumor necrosis factor-alpha (TNF-α), and -beta (TNF-β). HLAs are found on the surface of all nucleated cells and platelets and are used by the immune system to differentiate between self- and non-self antigens. Proteins that are produced by most cells are presented via MHC class I. Proteins that are presented by MHC class II are found on professional antigen presenting cells (APCs). During dengue infection, an increase in the expression levels of MHC I and II occurs. This increase in the expression levels of the MHC molecules may increase the immune response generated against virus peptides presented by HLA molecules, resulting in the more severe immunopathology seen in some DENV infections (19, 99). The studies that have been conducted to analyze HLA allele types and the severity of dengue disease have pinpointed certain HLA alleles that are more often associated with mild or severe disease, but these alleles differ based on geographic population and ethnicity (19). Based on these studies, polymorphisms in HLA class I are more often associated with DHF susceptibility in previously exposed and immunologically primed individuals (19, 186).

In addition to the potential link between HLA alleles and disease severity, polymorphisms in the DC-SIGN promoter (encoded by CD209) have also been associated with increased risk of DF compared with DHF (177). The G allele of the DC-SIGN promoter variant, DCSIGN1-336, is associated with strong protection against DF but not DHF based on results from three independent cohort studies from Thailand (177). A transition from A to G at position 336 in the promoter region of DC-SIGN alters the sequence for a SP-1 binding site and diminishes promoter activity (154, 177) .

In light of the fact that not much is known concerning how host genetics impact the potential of developing DHF, there are three main proposed mechanisms to explain DHF. The first hypothesis is that antibodies from a primary dengue infection lead to enhancement of the secondary dengue infection. This process is known as antibody-dependent enhancement (ADE). The second proposed mechanism is viral virulence, where certain viral genotypes are more often associated with severe disease. The third mechanism is based on immunopathogenesis, where viral infection triggers a heightened immune response that leads to DHF/DSS. These three proposed mechanisms are not necessarily mutually exclusive, in that it is possible that all of these hypotheses contribute to the development of severe dengue disease. Each mechanism is discussed in greater detail below.

Antibody-dependent enhancement

The ADE model postulates that DENV specific antibodies from a primary infection lead to the enhancement of the secondary infection. Under this model, the primary antibodies are thought to be cross-reactive and subneutralizing, resulting in the formation of antibody-virus complexes. These antibody-viruses complexes are then taken up by cells that express Fc gamma (Fcγ) receptors, which results in infection of these cells. This model of infection through Fcγ receptors suggests that DENVs are able to infect a different repertoire of cells that would otherwise be nonpermissive to the virus via its normal route of infection. This new route of infection would lead to increased levels of virus and result in a different population of infected cells, eventually leading to more severe disease.

In the absence of an animal model, experimental evidence supporting the ADE model has been difficult to generate. Much work has been performed *in vitro* to recreate this phenomenon of ADE. Halstead, who initially proposed this hypothesis, suggested that monocytes and macrophages were primary target cells of ADE (70, 73). A recent study reported that in addition to the enhancement of infection via Fc gamma receptor II (FcRII) by anti-E antibodies, anti-prM antibodies were able to enhance infection of both FcRII expressing cells as well as non-FcRII bearing cells (55, 88). In the context of this model, high levels of viremia in the secondary infection are believed to correlate with disease severity (42, 101, 199). Conversely, it is also believed that viremia is usually low or completely cleared before the onset of DHF/DSS, suggesting the need for additional factors that contribute to disease severity (174).

Viral virulence

Particular DENV2 and DENV3 genotypes are indicated to be associated with DHF versus DF (139, 172, 204). In South East Asia, the DENV2 strains that are endemic to this region are more often associated with DHF, whereas the DENV2 strains that are endemic to the Americas are only associated with DF (172). The introduction of the South East Asian DENV2 genotype into the Americas led to major epidemics of DHF in the Caribbean and Central America (66, 162). Studies of dengue in Indonesia pointed to differences in strains of DENV3 that have contributed to transmission and disease severity (61, 116). In 1994, DENV3 genotype III viruses, which have been associated with DHF in the Indian subcontinent, were introduced into Central America. This introduction led to the first DHF outbreaks caused by DENV3 in Latin America, suggesting the DENV3 genotype III viruses introduced were more virulent than the

native DENV3 genotype IV viruses (8, 60, 67, 158, 198). These studies imply a significant role for viral virulence in the development of DHF.

1.5 Immunopathogenesis

During the onset of DHF symptoms, viremia is usually low or completely cleared (174). The reduction in viral titers during symptomatic disease supports the hypothesis that immunopathogenesis is the major contributor to the development of DHF. DCs are believed to be an important factor in the pathogenesis of dengue since they are likely the first cells to encounter DENVs during a natural infection (207). DENV infected cells present viral antigens via MHC class I and II molecules, which leads to the stimulation of the adaptive immune response, specifically, the stimulation of memory T lymphocytes primed from the primary infection. One hypothesis of DHF suggests that the systemic activation of T 'helper' type 1 (Th1) pro-inflammatory responses mediates the progression to the severe disease (109). In DHF, the capillary leakage that is seen is not due to direct damage of endothelial cells by the virus. In studies analyzing autopsy samples from DHF patients, the endothelial cells appeared healthy and did not contain virus. These results indicate the production and secretion of host cytokines such as TNF-α, interleukin-1 (IL-1), IL-2 and IL-6 may lead to increased capillary leakage and severe disease (10).

Various clinical studies have reported that cytokine production and T lymphocyte activation are important in the pathogenesis of DHF. CD4+ T lymphocytes, also known as ‘helper’ T cells, recognize antigens that are presented via MHC class II on APCs such as B cells, macrophages and DCs (19, 25, 206). There are two main subsets of T helper

cells, Th1 and Th2. The presentation of antigen to the CD4+ T cells leads to their stimulation and subsequent cytokine production. Th1 cells produce interferon-gamma (IFN-γ), IL-2, and transforming growth factor-beta (TGF-β) cytokines and Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (20). DENV specific CD4+ T lymphocytes have been shown to produce high levels of IFN-γ, IL-2, TNF-α and TNF-β (49, 114). Chaturvedi et al., have shown that DF is most often characterized by a Th1 response, while DHF is characterized by a shift from a Th1 to a Th2 response (20).

In addition to CD4+ T lymphocytes, CD8+ T lymphocytes also play a role in dengue infection and disease. CD8+ cytotoxic T lymphocytes (CTLs) are important in controlling intracellular invading pathogens. They recognize foreign antigens that are presented via MHC class I. Since virtually every cell in the body expresses MHC class I, these CD8+ CTLs monitor all cells in the body and destroy all cells that present foreign antigens on their surface. Of the DENV proteins, NS3 was the most immunodominant of the viral proteins in the majority of T cell clones derived from a donor that received a live experimental DENV3 vaccine (210). In a study performed by Zivna et al. that evaluated T cell epitopes from Thai children during and after acute infection, a higher frequency of peptide-specific T cells was observed in patients that experienced DHF when compared to patients that experienced DF (210). The results from this study indicated that T cells specific for NS3 were more often isolated from patients with DHF, and that the NS3 epitope was an important target of CD8+ T cells in secondary infection (210).

In addition to their previously described roles in DENV infections, T cells are also hypothesized to contribute to dengue pathogenesis via “original antigenic sin”. This model proposes the induction of an inappropriate immune response by memory T cells to

a secondary infection due to clonal expansion of cross-reactive memory T cells that are specific for the previous infection rather than the current infection. This response results in delayed viral clearance and increased cytokine production. Evidence indicates that "original antigenic sin" may occur during secondary infection (145). Recent work has also identified potential epitopes, which in the context of specific HLA types, may be associated with immune enhancement (146, 184, 210).

Another immune cell type thought to be involved in pathogensis is Natural killer (NK) cells. NK cells are bone marrow derived lymphocytes that share a common progenitor with T cells. They are the main lymphoid population that is involved in innate immunity (154). NK cells are recruited to the site of infection by chemoattractant factors produced from infected cells and activated resident macrophages. These resident macrophages are capable of secreting IFN-α and IFN-β, which lead to NK cell proliferation. NK cells are one of the main producers of IFN-γ. The exact role that NK cells play in the outcome of DENV infection is still unclear. Clinical data from Green et al. imply a potential role for NK cells during early time points of DENV infection (54). Kurane et al. have reported that human blood NK cells were cytotoxic against DENV infected cells in target organs by direct cytolysis and antibody-dependent cell-mediated cytotoxicity (110). Researchers in Brazil observed increases in the percentage of NK cells, as well as more activated NK cells, associated with DF (6). Conversely, they also found lower percentages of NK cells and less activated NK cells in patients that had more severe disease (6).

Dendritic cells

DCs are important professional APCs that present antigens through MHC class I and II to stimulate differentiation of naïve T cells. Myeloid DCs, one of the three distinct subpopulations of DCs, are found in most nonlymphoid organs including the epidermis (Langerhans cells), dermis, gastrointestinal and respiratory mucosa, and the interstitial space of vascular organs (119, 168). Once immature myeloid DCs bind and internalize the virus, they release inflammatory cytokines and chemokines that initiate the recruitment of more immature myeloid DCs and other leukocytes to the site of infection. In the presence of various cytokines and chemokines, the immature DCs become activated/mature and migrate to the lymph nodes where they stimulate T cells. Myeloid DCs initially release IL-12, which drives the generation of effector cells with Th1 phenotypes. After 24 hrs, IL-12 production ceases. The cessation of IL-12 causes the generation of unpolarized memory T cells and IL-4 secreting Th2 cells that then dampen the Th1 response (127). Cytokine secretion is hypothesized to be responsible for plasma leakage. Chaturvedi et. al., have shown that DF is most often characterized by a Th1 response (secretion of IFN-γ, IL-2 and TGF-β) and DHF is characterized by a shift from a Th1 to a Th2 (secretion of IL-4, IL-5, IL-6, IL-10 and IL-13) response (20).

Various groups have shown that myeloid DCs are permissive to DENV infection (83, 119, 207). Until a few years ago, it was unclear whether or not DENV infection led to the activation of immature DCs. Libraty et al. suggested that infected DCs, as well as uninfected DCs, in *in vitro* cultures were activated by DENV infection (119). Ho et al. observed that DENV infected cultures had higher expression levels of B7-1, B7-2, HLA-DR, CD11b and produced higher amounts of TNF-α and IFN-α but not IL-6 and IL-12

(83). In a later study, Ho et al. demonstrated DENV infected DCs induced T cell proliferation and produced IL-2, IL-4, IL-10 and IFN-γ (82). These results suggest an important role for DCs in DENV pathogenesis.

Cytokines

Cytokine production is believed to be an important part of dengue pathogenesis. Type I and II IFNs, TNF-α, TGF-β, IL-4, IL-6, IL-8 and IL-10 have been implicated in the progression of severe dengue disease. Chaturvedi et al. suggest that the most significant role in the enhancement of disease severity is the cascade of cytokines, which results in a shift from a Th1 response to a Th2 response (20). Early clinical studies have shown significantly higher levels of circulating IFN-α and IFN-γ in Thai children infected with DENV and displaying symptoms of both DF and DHF/DSS over control children without dengue (111, 112). In this same study, the levels of IFN in children with DHF/DSS were higher than the levels in children with DF. Other clinical studies have shown similar findings with elevated levels of IFN-α and IFN-γ in patients infected with dengue over uninfected individuals (16, 21, 22, 157).

TNF-α, the primary cytokine implicated in the pathogenesis of DHF/DSS, acts locally to activate macrophages. The systemic release of TNF-α during infection may lead to the increase in vascular permeability seen in DHF/DSS (4). Tracey et al. have shown that TNF-α is capable of inducing plasma leakage and shock in animal models (196). Various groups have reported that IFN-γ does not directly induce plasma leakage in animals, but enhances TNF-α production by activated monocytes and interacts with TNF-α to activate endothelial cells (14, 155, 189). In addition to TNF-α, IL-10 has been

found in some, but not all, studies to be significantly elevated in patients with DHF (21, 22, 57, 157, 161). The levels of IL-10 and TNF-α have been detected as early as 2 days before the onset of plasma leakage, when shock usually occurs (56, 57). IL-10, which is an anti-inflammatory cytokine, is a potent inhibitor of pro-inflammatory cytokines and suppresses the antigen presentation capacity of APCs. One investigation correlated high levels of IL-6, IL-10, and the proinflammatory macrophage migration inhibitory factor with a fatal outcome in adult DHF patients (21, 28). Another important cytokine, IL-8 which attracts neutrophils and may activate monocytes, was shown to be transcribed at higher levels in PBMCs days before deferevescence in DHF patients (138). In this same study, IL-8 mRNA levels were also elevated in DHF patients when compared to DF patients. Medin et al. have demonstrated that *in vitro* infection of endothelial cell monolayers by DENVs resulted in IL-8 release, cytoskeletal rearrangement, and increased permeability, which was partially mitigated by treatment with antibodies against IL-8 (193). DENV infection was also shown to induce secretion of tissue plasminogen activator from endothelial cells, which was blocked in the presence of IL-6 antibodies. IL-6 is a proinflammatory cytokine that is an important mediator of fever.

1.6 Role of DENV proteins in dengue pathogenesis

Even though much is still unknown about dengue pathogenesis, the effects that some of the viral proteins have on the immune response is known. DENV proteins NS2A, NS4A and NS4B have been shown to antagonize IFN signaling. NS4B in particular has been shown to reduce STAT1 phosphorylation in response to IFN-β or IFN-γ treatment and to prevent nuclear translocation of a STAT1-green fluorescent protein (GFP) fusion protein (150, 151). DENV proteins have also been shown to block

IFN-α signaling by decreasing phosphorylation of STAT1 and expression of STAT2 (94). The antagonistic activity of DENV proteins on IFN signaling seems to be specific to type I IFN signaling since IFN-γ signaling is not affected when STAT1 and STAT3 phosphorylation was inhibited (81). Expression of DENV NS1 in DCs was reported to interact with STAT3-β, which resulted in secretion of TNF-α and IL-6, which is a cytokine that causes the phosphorylation and subsequent translocation of STAT3. The ability of DENVs to block IFN signaling supports a critical role for IFNs in controlling DENV infection. IFN-α, IFN-β and IFN-γ have been shown to inhibit DENV infection *in vitro*, but only when added prior to infection (34, 81).

Antibodies against NS1 bind to and induce apoptosis of endothelial cells, in addition to inducing the secretion of proinflammatory cytokines and chemokines. This phenomenon suggests a role for anti-NS1 antibodies in vascular permeability (121, 123). NS3 is the main antigen that stimulates DENV reactive CD4+ and CD8+ T cells to produce high levels of IFN-γ, TNF-α, TNF-β, and macrophage inhibitory protein-1β. Upon interaction with DENV infected APCs displaying NS3, CD4+ and CD8+ T cells are activated and efficiently lyse DENV infected cells *in vitro* (19, 50, 108, 128). In addition to NS3, DENV M protein also has pro-apoptotic activity (15, 122, 124, 178). Antibodies to E protein can cross-react with platelets leading to activation of both coagulation and fibrinolysis systems (122).

1.7 Tools for studying/monitoring dengue infection

In the absence of a feasible animal model, most of the work involved in studying dengue pathogenesis has been carried out using *in vitro* cell culture-based model systems. Mosquito cells are often used in the propagation of dengue viruses because most infections of this cell type develop into persistent infections with very little to no cytopathic effect (CPE) (126, 152). As a model system, mosquitoes have been used to study the biology of DENVs in the insect host as well as viral transmission from the insect vector. Since mosquitoes are useful models of DENV transmission, some progress has been made in the development of transgenic mosquitoes engineered to block transmission (48).

For evaluating the interactions of DENVs with mammalian cells, many studies have been performed using primary human cells including PBMCs (148), DCs (83, 119, 207), primary human umbilical vein endothelial cells (HUVECs) (32, 90, 203) and T cells (113, 114) as well as cell lines such as Vero, U937, and HepG2 for viral infections. Infection of mammalian cells with DENVs in many cases results in some type of CPE.

Insect and mammalian cells are widely used to study and propagate DENVs; however, the viruses often grow to low titers in these cell types when compared to other viruses. The majority of studies involving DENV infection and pathogenesis use lab-adapted strains of DENVs because they grow to higher titers than clinical isolates in cell culture. DENV titers usually range between to 10^5 and 10^7 infectious particles/ml. Very few studies utilize clinical isolates of DENV because they grow to low titers in cell culture and do not always induce a CPE, which is often used for viral titration.

Common methods that are used for titrating DENVs are plaque assays and endpoint dilutions (179, 208). Plaque assays work exceptionally well for lab-adapted DENV strains that induce a CPE, but take 6 days to titer the viruses. Another commonly used method for titrating DENVs is the endpoint dilution assay. This assay does not utilize CPE as a read out for the measurement of viral titers, but instead uses immunofluorescence to stain for infection. The viral titer is determined by the viral dilution in which 50% of the cells are infected. This method works well for both lab-adapted and clinical isolates but also takes 6 days to titer viral stocks. In order to move forward in the field of dengue pathogenesis, a shift from using lab-adapted viruses to using clinical isolates needs to occur to dissect the interactions of DENVs with host cells. However, in order to utilize clinical isolates for experiments, a new method is needed to titrate these isolates in a shorter time frame that does not require cytopathology as a read out for viral titers.

Within the past 10 years, Fluorescence Activated Cell Sorter (FACS) based methods have been developed to follow infection and titer viruses such as Human immunodeficiency virus (HIV), herpes, measles, influenza, Epstein-Barr virus (EBV) and rabies (12, 36, 137, 173, 190). FACS has also been used to detect DENV in clinical samples and to measure the ability of the virus to infect a variety of cells (33, 83, 96, 134, 192). Within the last few years, real-time polymerase chain reaction (PCR) has also been utilized to measure dengue viral RNA transcripts in infected host cells (35, 87, 182). These tools provide a basis for studying dengue pathogenesis using *in vitro* cell culture systems.

1.8 Overall focus of dissertation

In chapters 3 and 4, I discuss experiments carried out to analyze the effect of DENV infection on DCs and compare the ability of DENV3 clinical isolates to differentially infect primary human DCs. In order to complete these experiments, we needed an assay that would allow us to titrate and monitor DENV infection of cultured cells. Titration of DENVs has been difficult because most standard methods are time consuming and require the formation of clear plaques, which is not feasible for some clinical isolates of dengue. To overcome this problem, I have developed a FACS based assay to titrate DENVs which will be discussed in chapter 2. This new method is an improvement over the plaque assay because the infection period is reduced from 5-7 days to 24 hours and the assay can be used to titrate clinical isolates that frequently do not form clear plaques on cell monolayers. In addition to virus titration, FACS provides a useful tool to follow and monitor DENV infection in a variety of cells.

During a natural infection, DCs are believed to be one of the initial cells targeted by DENVs (207). Immature DCs are more permissive to DENV infection than monocytes and macrophages, with the interaction between DCs and DENV being mediated by DC-SIGN (83, 119, 153, 194, 207). These findings indicate an important role for DCs in dengue infection and disease severity. Currently, it is unclear whether DENV infection of DCs leads to activation of these cells and how this activation may or may not affect the downstream events of T cell stimulation and cytokine production (20, 83, 119, 207). Chaturvedi et al. have shown that DHF is more often associated with a switch from a Th1 to a Th2 T cell response (20). Chapter 3, describes experiments analyzing the effect of DENV3 infection on primary human DCs. In evaluating DENV3

interactions with DCs, this chapter discusses the activation state of infected and uninfected DCs. This analysis includes the characterization of two groups of clinical isolates from periods of mild and severe disease and their ability to infect primary human DCs. The scope of chapter 4 focuses on two clinical DENV3 isolates that differ in their ability to infect primary human DCs. This chapter looks further into the mechanisms behind the differences between these two clinical isolates and analyzes the effect of the host immune response and determines the stage in the viral life cycle the differences occur.

Chapter 2

A flow cytometry based assay for titrating Dengue virus

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2.1 Abstract

Plaque assays for titrating DENV are time consuming and not suitable for strains that do not plaque. FACS has been used to detect DENV infected cells. Here we describe a FACS based assay for titrating DENV. We determined that at 24 hours post infection, the number of infected cells detected by FACS represented the first round of infection, and therefore could be used as a readout of the number of infectious particles in the inoculum. When the titers of different laboratory and clinical strains of DENV, were compared using FACS, plaque and endpoint dilution assays, for most strains the FACS titers were comparable to titers obtained by plaque or end point dilution assays. The FACS assay is an improvement over the plaque assay because the infection period is reduced from 5-7 days to 24 hours and the assay can be used to titrate clinical isolates

that frequently do not form clear plaques on cell monolayers. The novel FACS based methods described here will facilitate laboratory studies of dengue.

2.2 Introduction

Dengue is a mosquito-borne viral disease of global public health significance. Throughout the world there are more than 2.5 billion people at risk of DENV infection. Each year there are an estimated 100 million cases of dengue viral infection worldwide with 250,000 people developing the more severe DHF/DSS, which is often fatal (73). DENV is a positive polarity RNA virus in the family *Flaviviridae* and the DENV complex consists of 4 distinct serotypes designated DENV1 through DENV4 (126).

Laboratory studies of dengue are difficult because the virus does not grow to high titers in cell culture and assays for titrating virus and measuring virus neutralization are time consuming. These problems are exacerbated when working with primary clinical isolates of virus. Standard methods for titrating DENV and measuring the ability of anti-sera to neutralize the virus are based on plaque assays which require 5-7 days to complete. Some isolates, especially among primary clinical isolates, do not form clear plaques on cell monolayers. Better methods for titrating the virus and measuring the ability of anti-sera to neutralize dengue need to be developed. Within the past 10 years, FACS based methods have been developed to follow infection and titer viruses such as HIV, herpes, measles, influenza, EBV and rabies (12, 36, 137, 173, 190). FACS has also been used to detect DENV in clinical samples and to measure the ability of the virus to infect a variety of cells (33, 83, 96, 134, 192). Here we report on a FACS-based assay for titrating DENVs and for characterizing the ability of antisera to neutralize the virus.

2.3 Materials and Methods

Cells, viruses and antisera

Aedes albopictus C6/36 mosquito cells were obtained from the CDC, Fort Collins. Vero 76 (African Green monkey kidney) cells were purchased from ATCC (Manassas, VA). C6/36 cells were maintained in Eagle's Minimum Essential Medium (EMEM) (Gibco BRL) supplemented with 10% heat-inactivated (hi) fetal bovine serum (FBS), penicillin G (100U/ml), streptomycin (100 μg/ml), L-glutamine and non-essential amino acids at 28°C in 5% carbon dioxide (CO_2). Vero 76 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Gibco BRL) containing 115mM HEPES buffer supplemented with 10% hi FBS, penicillin G (100U/ml), streptomycin (100 μg/ml), L-glutamine and non-essential amino acids at 37°C in 5% CO_2.

The viruses used in this study included prototype DENV2 virus (New Guinea C strain), prototype DENV3 (H87 strain) and prototype DENV4 (H241) (gifts from Dr. Robert Shope, UT at Galveston), one DENV1 high passage clinical isolate (16007, gift from Robert Putnak, Walter Reed Army Institute of Research), one DENV1 clinical isolate from Sri Lanka (UNC 1002), three DENV3 clinical isolates from Sri Lanka (UNC 3001, UNC 3009 and UNC 3011) and two DENV3 clinical isolates from Nicaragua (UNC 3017 and UNC 3018; gift from E. Harris, UC Berkeley, CA). All experiments with the clinical virus isolates, unless otherwise stated, used stocks from viruses that had been passaged in cell culture fewer than 4 times. Viral stocks were obtained by inoculating a monolayer of C6/36 cells in a 75 cm^2 tissue culture flask with 200 μl of virus diluted in 1 ml of EMEM-2% FBS. After 1 hour, 14 ml of EMEM supplemented

with 2% FBS was added and the cells were cultured for 10 days at 28°C in 5% CO_2. The supernatant was removed from the cells and centrifuged for 5 minute (min) at 2,000 g to pellet cellular debris. The supernatant was aliquoted and stored at -80°C. DENV1 (16007) was grown and passaged in Vero cells, whereas DENV4 (H241) was grown in C6/36 cells as described above and passaged one time in Vero cells.

The flavivirus group-reactive monoclonal antibody, 4G2 (79), was purchased from ATCC, Rockville, MD.

FACS titration assay

C6/36 cells were seeded in 6-well plates and incubated at 28°C in 5% CO_2 until cells were approximately 90-95% confluent (~1×10^6 cells/well). Media was removed from cells and 10-fold serial dilutions of virus diluted in EMEM with 2% FBS to a final volume of 200µl was added to the cells. The cells were incubated at 28°C in 5% CO_2 for 1 hour (hr), and plates were rocked every 15 min. The media was removed and the cells were washed in 1× phosphate buffered saline (PBS). Two ml of EMEM with 2% FBS was added to each well and the cells were incubated for 24 hours at 28°C in 5% CO_2. After 24 hours the media was removed and the cells were washed and scraped from the well and resuspended in PBS. An aliquot of cells from each well was counted using a hemocytometer. The remaining cells were centrifuged at 1,000g for 5 min and resuspended in 200 µl of the Cytofix/Cytoperm (BD Biosciences, San Jose, CA) solution and incubated on ice for 20 min in the dark. All subsequent steps were performed in Cytoperm/Cytowash solution. The cells were centrifuged at 1,000g for 5 min and washed two times before resuspending in 50 µl of fluorescein isothiocyanate (FITC)-

labeled 4G2 monoclonal antibody for 1 hr on ice. The cells were washed two times and resuspended in the Cytoperm/Cytowash solution. The samples were analyzed on a FACScan flow cytometer using Summit software. For each sample at least 100,000 events were collected. The liner range of the assay under the tested parameters was determined to be between 0.20% and 25% infected cells (between ~2000 and 250,000 infected cells per well) in a well and sample wells outside this range were not used for determining final titers. The titer of the virus was determined using the following formula: FACS Infectious Units/ml = [(% of infected cells) × (total number of cells in well) × (dilution factor)]/(volume of inoculum added to cells). Samples with a titer below 1×10^4 infectious particles/ml cannot be titered with the FACS assays as this titer is below the level of detection.

Vero plaque assay

Vero plaque assays were performed using a modified protocol from Wu et al. (208). Briefly, Vero cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and incubated overnight. Fifty µl of DENV stock was added to 450 µl of diluent containing 1% Bovine Albumin in DMEM/F12 media supplemented with penicillin and streptomycin. The virus mixture was serially diluted using 10-fold dilutions. Two hundred µl of each dilution of virus was added to each well of Vero cells in duplicates. The plates were incubated at 37°C in 5% CO_2 for 1 hour, rocking plates every 15 min. Four ml of primary nutrient agar overlay (1% Seakem LE agarose, 20% Earles's Balanced Salt Solution 10x, 7% Yeast Extract-Lactalbumin hydrolysate, 4% FBS, 0.5% Sodium Bicarbonate, 1% Gentamycin [50mg/ml] and 0.2% Fungizone (12.5µg/ml)was

added to each well and the cells were incubated at 37°C in 5% CO_2 for 5-7 days. Next, 2 ml of a secondary nutrient agar overlay (primary nutrient overlay containg 1% neutral red) was added to each well and the cells were incubated overnight at 37°C in 5% CO_2 before counting plaques and calculating viral titers. The viral titers were expressed as plaque forming units (PFU)/ml = [(number of plaques per well) × (dilution)]/ (inoculum volume).

Endpoint titration assay

Endpoint titration assays were performed using a modified protocol from Schoepp et al (179). Briefly, C6/36 cells were seeded in a 96-well plate at a density of 1.3×10^5 cells/ well in 225 µl and incubated overnight at 28°C in 5% CO_2. Twenty five µl of virus was added to the first well and the virus was serially diluted using 10-fold dilutions. The plates were incubated at 28°C in 5% CO_2 for 5 days. To fix the plates 50 µl of a 3:1 Acetone:PBS mixture was added to each well, and the plates were incubated at -20°C for 20 min. The plates were air dried and washed with 1× PBS. The plates were blocked with 5% FBS/PBS/0.05% sodium azide (NaN_3) for 15 min at room temperature. Each well was resuspended in 50 µl of FITC-labeled 4G2 monoclonal antibody diluted in blocking buffer and incubated 1 hr at 4°C. The plates were analyzed using an inverted fluorescence microscope. Wells were scored for the presence or absence of infection with either a positive (+) or a negative (–) symbol. Replicates of ten were performed for each virus. We calculated the Tissue Culture Infectious $Dose_{50}$ ($TCID_{50}$) of each virus using the Reed-Muench formula. The $TCID_{50}$ expressed as 10^x/ml = Dilution where CPE >

50% + {((% positive above 50%)-50%)/((% positive above 50%)-(% positive below 50%))} + $\log_{10}10$.

Plaque reduction neutralization test

The plaque reduction neutralization tests (PRNT) were performed using modified protocols from Russell et al., Kochel et al. and Wu et al.(103, 175, 208). Vero cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and incubated overnight. The test serum was heat inactivated at 56°C for 30 min. The serum was serially diluted 2-fold in diluent containing 1% Bovine Albumin in DMEM/F12 media supplemented with penicillin and streptomycin. DENV was diluted such that each well would be infected with 40-60 pfu. An equal volume of virus was added to the diluted serum and incubated at 37°C in 5% CO_2 for 1 hr. The virus and antibody mixtures were added to the Vero cells for 1.5 hrs. Four ml of primary nutrient agar overlay, as described above, was added to each well and the cells were incubated at 37C in 5% CO2 for 6 days. Next, 2 ml of a secondary nutrient agar overlay (primary nutrient agar overlay containing 1% neutral red) was added to each well and the cells were incubated overnight at 37°C in 5% CO_2 before counting plaques and calculating viral titers. Each antibody concentration was tested in triplicate. The numbers of plaques in each well were counted and the percent reduction was determined as follows: % reduction = ((average # of control plaques – average # of plaques for each dilution)/average # of control plaques) × 100. The plaque reduction neutralization titers of serum equaled the reciprocal of the serum dilution in which either 50% ($PRNT_{50}$) or 80% ($PRNT_{80}$) plaque reductions were observed.

FACS neutralization test

Vero cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and incubated overnight. Human serum to be tested was heat inactivated at 56°C for 30 min. The serum was serial diluted 2-fold in diluent containing 1% Bovine Albumin in DMEM/F12 media supplemented with penicillin and streptomycin. DENV was resuspended in diluent such that each well would receive a multiplicity of infection (MOI) of 0.1. An equal volume of virus was added to the diluted serum and incubated at 37°C in 5% CO_2 for 1 hr. The virus and antibody mixture was added to the Vero cells. Each antibody dilution was assayed in triplicate. The plates were incubated at 37°C in 5% CO_2 for 1 hr, rocking plates every 15 min. Two ml of DMEM/F12 containing 10% FBS was added to each well and the plates were incubated at 37°C in 5% CO_2 for 24 hours. Each well was washed with 1ml of trypsin- ethylenediamine tetraacetic acid (EDTA). Next, 0.2 ml of trypsin-EDTA was added to each well and incubated at 37°C for 5 min. One ml of chilled 1× PBS containing 10% FBS was added to each well to inactivate the trypsin. The cells were pipetted to breakup any clumps and centrifuged at 1,000g for 5 min. The PBS was removed and the cells were fixed and stained for FACS as described above. Approximately 100,000 to 200,000 cells were analyzed for each sample. The percent reduction in the number of infected cells was calculated for each serum dilution. The number of infected cells in the wells infected with virus only was used to calculate the percent reduction. The titers were expressed as the reciprocal of the serum dilution that inhibited 50% (FNT_{50}) or 80% (FNT_{80}).

2.4 Results

We conducted experiments to evaluate if FACS was a sensitive method for detecting dengue infection of C6/36 mosquito cells. Cells were infected with prototype DENV2 (New Guinea C) and DENV3 (H87) lab-adapted strains at a MOI of 0.05. Cells were harvested on days 1, 2, 3, 5 and 7 after infection and analyzed by FACS. Representative FACS scatter plots from DENV2 and DENV3 infected cells are depicted in Figure 2.1. Increasing numbers of infected cells were detected as the infection progressed indicating that the FACS assay was suitable for following kinetics of infection.

In the FACS assay DENV infected cells were detected using a monoclonal antibody (4G2) directed to the E glycoprotein of the virus. It is conceivable that some positive cells detected by FACS may represent input virus binding to cells and not productive infection of cells. Experiments were performed with normal and ultraviolet (UV) inactivated DENV2 to distinguish between infection and simple virus binding to cells. At 24 hours post infection (hpi) 5.20% of cells treated with normal virus were positive, whereas no positive cells were detected with UV inactivated virus indicating that simple binding of input virus does not result in a positive signal in the FACS assay (data not shown).

Titration of DENV by FACS

Ideally, a cell line for titering dengue by FACS should be highly permissive for infection and infected by different strains of virus with similar efficiency. Otherwise, titers obtained for different strains may reflect differences in interactions between the

viruses and cell line and may be a poor measure of the absolute number of infectious particles. We chose the C6/36 mosquito cell line because the available evidence indicates no dramatic differences in interactions between different strains or serotypes of virus with this mosquito cell line. C6/36 cells were infected with prototype DENV3 and clinical isolates of DENV3 (MOI 0.01) and the infection was monitored daily for 7 days by FACS. The different strains of virus displayed similar growth kinetics indicating that this cell line was suitable for titrating DENV (Figure 2.2). The DENV2 prototype strain NGC displayed growth kinetics similar to the DENV3 isolates in the C6/36 cell line (data not shown).

We reasoned that the number of cells infected early in infection, before cell to cell spread of virus, would be a direct measure of the number of infectious virus particles in an inoculum, and that this number could be used to estimate viral titer in a sample. Experiments were conducted to identify a time point which was late enough for the input virus to have infected cells and produced sufficient protein for detection by FACS, while being too early for release and cell to cell spread of new virus. C6/36 insect cells were infected with DENV2 (Strain NGC) at a high MOI and the cells and supernatants were collected at 9, 18, 24, 26 30 and 46 hpi. The number of infected cell increased from 9 to 24 hpi and this first round of infection plateaued between 24 and 30 hpi (Figure 2.3). A second round of infected cells were observed between 30 and 46 hpi. New virus was first detected in media at 24 hpi and the number of virus particles in the media continued to increase over time (Figure 2.3). These results demonstrate that progeny virions were first released into the media at 24 hrs and that these particles initiate a second round of cellular infection that peaked at 46 hpi. We decided to harvest cells at 24 hpi for the FACS

titration assay because the first round of infection peaks at this time point, and also because 24 hpi is too early to detect cell to cell spread of virus. Previous studies have also indicated that there is minimal cell to cell spread of DENV in C6/36 cells at 24 hpi. (96). Next we determined if the FACS assay was able to detect quantitative differences in the number of virus particles in samples. Ten-fold serial dilutions of prototype DENV2, DENV3 or clinical isolates of DENV3 were added to C6/36 cells and the number of infected cells determined after 24 hrs. In initial experiments we used the prototype DENV2 (NGC) and DENV3 (H87) strains. When cells were infected with virus diluted 1:2, 1:20 and 1:200, decreasing numbers of infected cells were detected by FACS (Figure 2.4A). Similar results were also obtained with clinical isolates of DENV3 (Figure 2.4B). Thus, a clear correlation was observed between the concentration of virus in the inoculum and the number of infected cells detected by FACS at 24 hrs after infection.

Comparing the FACS assay to other standard dengue titration assays

We estimated the FACS titer of each virus stock using the following formula: Infectious Units/ml = [(% of infected cells) × (total number of cells in well) × (dilution factor)]/ (volume of inoculum added to cells). Plaque formation on tissue culture monolayers [Vero, LLC-MK2 (Rhesus monkey kidney) and BHK (BHK 21, clone 15)] is the currently accepted method for titrating DENV. To further validate the FACS assay, we compared the titers obtained by FACS and plaque assay for different dilutions of the same preparation of virus. For these experiments, initially, we used DENV2 NGC strain, which produces clear plaques on cell monolayers. The virus stock was subjected to serial 5-fold dilutions and an equal volume from each dilution was tested by plaque and FACS assays. A liner relationship was observed between the assays over three logs of titers

ranging from 1×10^4 to 1×10^7 infectious particles or PFUs/ml (Figure 2.5) indicating that the two assays were similar measures of infectious virus in a sample. Furthermore, we observed that the effective range of the FACS assay was when between 0.2 and 25% of cells were infected (between ~2,000 and 25,000 infected cells per well). When the signal was below 0.2%, the assay was not sensitive and displayed high variability (data not shown). When more than 25% of the cells were infected, the assay was no longer linear, presumably because the C6/36 cells become limiting in the assay (data not shown). When several strains of DENV1, DENV2, DENV3 and DENV4 were titered by FACS, plaque assay and endpoint dilution assay, similar titers were obtained with the different assays for DENV1 (strains 16007 and UNC 1002), DENV2 (NGC), DENV3 (strains UNC3001, UNC3009 and UNC3011) and DENV4 (H241) (Table 1). For DENV3 strain H87 the titers obtained with the different assays varied more than the titers of the other viruses analyzed (Table 2.1).

FACS based assay to measure antibody neutralization of DENV

Interactions between antibody and DENV play roles in protective immunity and in ADE of viral infection. A current method for measuring antibody neutralization is the PRNT on Vero cells. This assay is subject to the same limitations as the plaque assay for virus titration. Experiments were conducted to determine if the FACS titration assay could be modified for measuring the ability of antisera to neutralize the virus. The DENV2 NGC strain was incubated with serial 2-fold dilutions of human dengue immune or normal serum before adding the virus to Vero cells. At each antibody dilution the number of infectious virus particles was estimated by FACS (24 hpi) and plaque assay (6

days post infection). The 50% and 80% neutralization titers obtained with each method were remarkably similar (Table 2.2).

2.5 Discussion

Any assay for titrating a virus is a relative measure of infectious virus that is defined by properties of the particular virus and the methods used. The plaque assay, which has been the standard method for titrating DENV, is time consuming and difficult to use with some primary clinical isolates. Here we describe and validate a FACS based assay for titrating DENV that overcomes the limitations of the plaque assay. Viral titers determined by FACS were similar to titers obtained by plaque assay and endpoint dilution assay for some DENV strains but not for others. We suspect that the ability of some strains to infect the cell lines used for the different assays and/or differences in plaquing efficiency may account for the discordant titers. Another explanation for the difference in viral titers may be the time at which each experimental method was evaluated for viral infection. The FACS assay conducted at 24 hrs after infection measures the ability of dengue virus particles to enter a cell and produce viral antigen. The assay does not require virus assembly or cell to cell spread. In contrast, to obtain a positive signal in the endpoint dilution method, virus particles have to enter cells and spread from cell to cell over a period of 5-7 days. The FACS assay can only be used with samples with a titer greater than 1×10^4 infectious units/ml. At titers below 1×10^4/ml, the small number of infected cells in a well were below the level of detection of the FACS assay. This is a limitation of the FACS assay compared to the plaque assay, which is more sensitive. Another limitation to the FACS assay is that it requires costly equipment and trained staff and may be difficult to establish in some settings. Even with its

limitations, FACS is still a powerful and effective tool for titrating virus. The FACS assay provides the ability to measure infectious viral titers as early as 24 hpi, where the plaque assay requires 5-7 days. Additionally, the FACS assay can be used to titrate clinical isolates that do not form clear plaques on cell monolayers.

The current method for measuring the ability of antisera to neutralize DENV is the PRNT assay. The PRNT assay is subject to the same drawback as the plaque titration assay. We report here that the FACS assay can also be adapted to measure the ability of antisera to neutralize the virus. When two human dengue immune sera were tested with the FACS and plaque neutralization assays, similar neutralization titers were obtained with both assays. Further studies are needed to validate the FACS neutralization test (FNT). Specifically, experiments need to be done with reference sera to determine if the FACS neutralization assay can be used to identify the infecting serotype of DENV and to distinguish between primary and secondary infections. A major advantage to a FNT is that it could be used to measure neutralization of DENV isolates, irrespective of their ability to form plaques. Compared to existing methods for studying dengue virus, which work best with laboratory adapted strains, we hope the FACS based methods described here will greatly facilitate studies with primary clinical isolates of dengue.

Strain	A	B	C
DENV1 (16007)*	1.5×10^7*	3.5×10^7*	Not Done
DENV1 (1002)*	2.9×10^6*	1.7×10^6*	Not Done
DENV2 (NGC)	1.8×10^6	3.8×10^6	Not Done
DENV3 (H87)	2.9×10^6	1.3×10^5	4.2×10^5
DENV3 (3001)	4.1×10^5	1.8×10^6	1.0×10^6
DENV3 (3009)	1.2×10^6	1.8×10^6	1.7×10^6
DENV3 (3011)	2.9×10^5	4.8×10^5*	1.1×10^6
DENV4 (H241)*	9.7×10^6*	1.0×10^7*	Not Done

Table 2.1. Dengue viral titers determined by FACS, plaque assay and endpoint dilution. Each virus stock titered by FACS was independently titered using plaque assay (Vero cells) and/or endpoint dilution (C6/36 cells). A = FACS titer (IU/ml), B = Plaque Assay (PFU/ml), C = Endpoint dilution assay ($TCID_{50}$/ml). Most values (exceptions are marked by an asterisks) are based on at least three independent experiments.

Serum	FNT_{50}	FNT_{80}	$PRNT_{50}$	$PRNT_{80}$
Normal Human serum	<20	<20	<20	<20
H8163	10,240	2,560	5,120	1280
H7859	<20	<20	<20	<20

Table 2.2. Antibody neutralization titers determined by FACS and PRNT assays. Vero cells were infected with DENV2 NGC that had been incubated with serial 2-fold dilutions of serum from two human patients previously infected with dengue. The FNT and PRNT were harvested analyzed at 24 hpi and 6 days post infection respectively. The 50% and 80% neutralization titers were determined based on the percent reduction observed by each method. H8163 was from a secondary infection and H7859 was from a primary infection, serotype unknown.

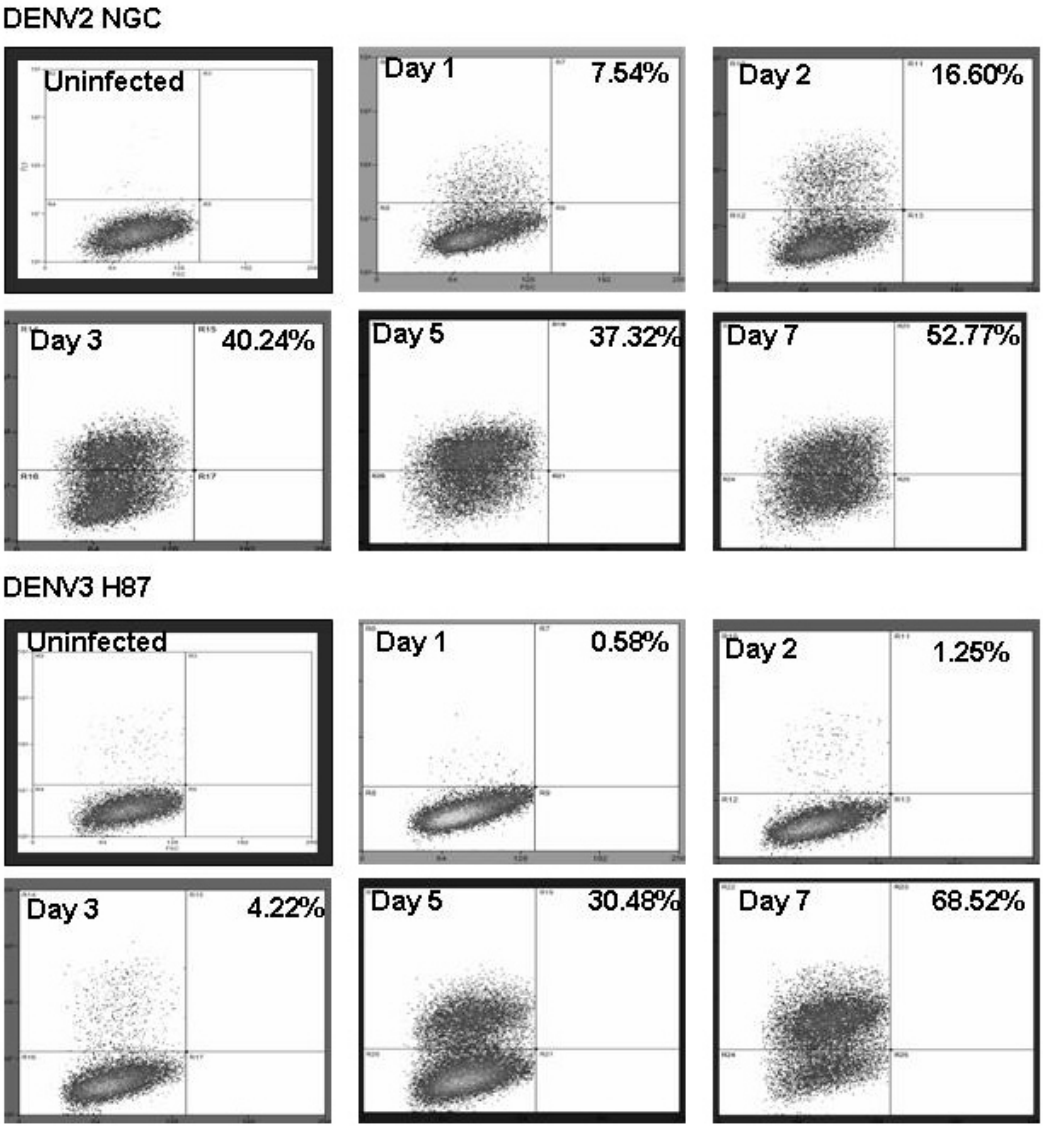


Figure 2.1. Time course of DENV2 and DENV3 infection of C6/36 insect cells. C6/36 cells grown in six-well plates were infected with DENV2 strain NGC and DENV3 strain H87 at a MOI of 0.05. One well was mock infected to serve as a negative control. Cells were harvested from duplicate wells on days 1,2,3,5 and 7 and processed for FACS. The cells were fixed and permeabilized using the Cytofix/ Cytoperm kit from BD Biosciences and stained for FACS using monoclonal antibody 4G2 that cross reacts with all 4 DENV serotypes. Cells were gated according to their size and granularity [(*x*-axis, side scattered cells (SSC) and *y*-axis, forward scattered cells (FSC)] to identify intact, single cells. The gated cells were displayed on dotplots in which the X-axis is the forward scatter and Y-axis is fluorescence (FITC) intensity. The dotplot was divided into 4 quadrants based on including 99.9% of the uninfected control cells in the lower left quadrant. The percentage of DENV infected cells was determined by counting the number of cells in the upper left quadrant and dividing this number from the total number of cells in the dotplot.

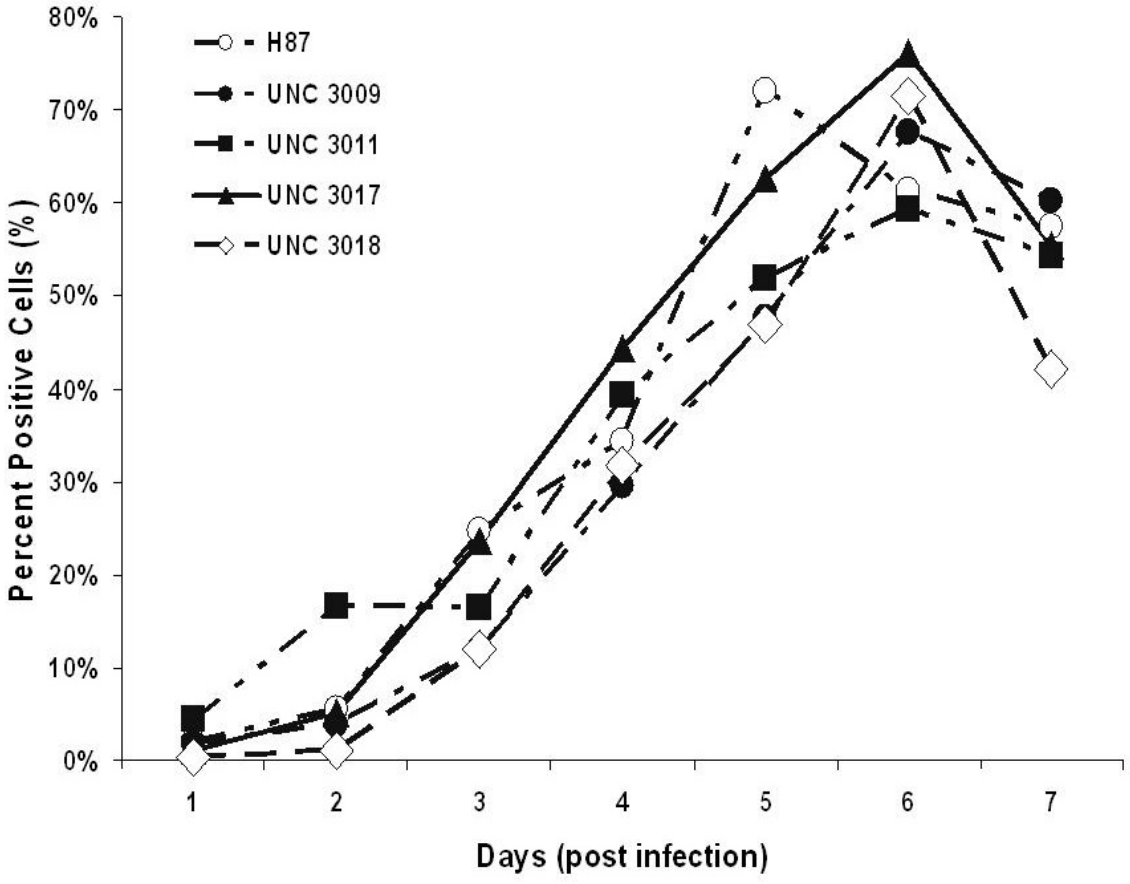


Figure 2.2. Infection of C6/36 cells by different strains of DENV. The DENV3 prototype strain H87 as well as 4 primary DENV3 clinical isolates were used to infect C6/36 cells at an MOI of 0.01. Cells were harvested at 24 hr intervals for 7 days and infected cells were detected by FACS.

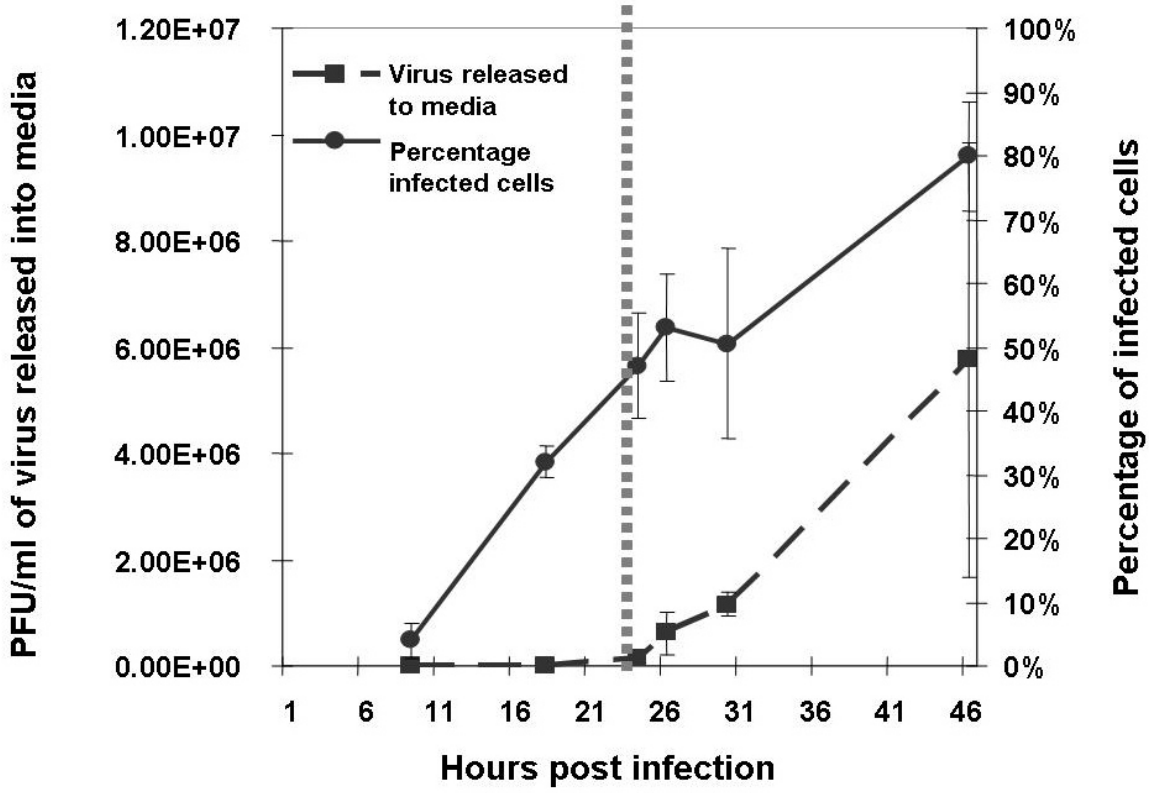
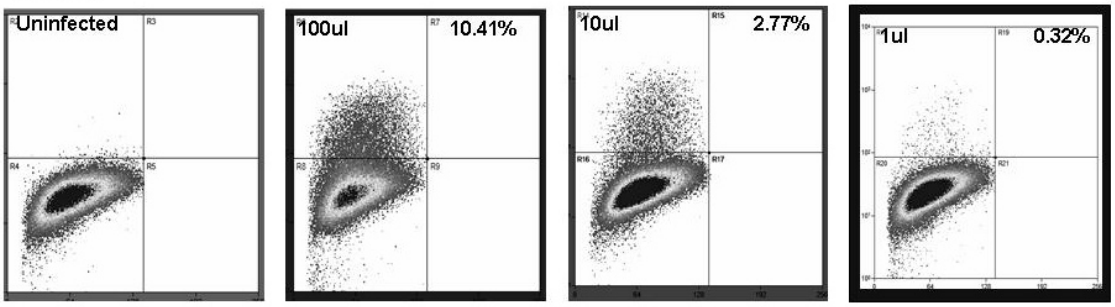


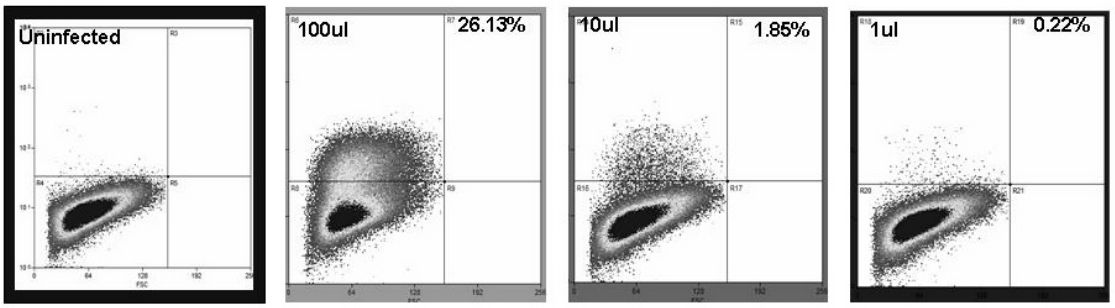
Figure 2.3. Time course of cell to cell spread of DENV2 infected C6/36 insect cells. C6/36 cells grown in six-well plates were infected with DENV2 strain NGC at an MOI of 2. Cells and supernatants were harvested from wells at 9, 18, 24, 26, 30 and 46 hours post infection. The cells were fixed and permeabilized using the Cytofix/ Cytoperm kit from BD Biosciences and stained for FACS using monoclonal antibody 4G2 to determine the percentage of infected cells. The results are expressed as a continuous line with a closed circle. The amount of virus in the supernatant at each time point was determined by plaque assay on Vero cells and expressed as a dashed line with a triangle.

A

DENV2



DENV3



B

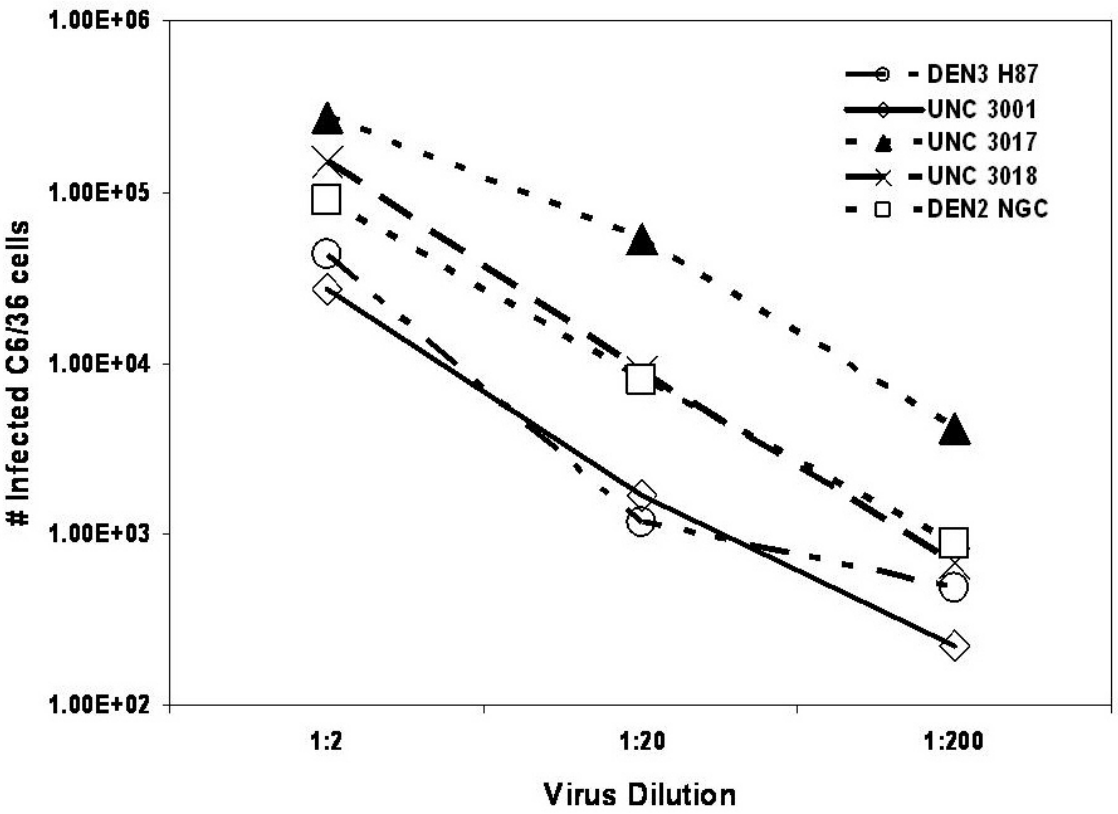


Figure 2.4. Titration of dengue virus using FACS. C6/36 cells were infected with 10-fold serial dilutions of prototype strains and clinical isolate. At 24 hpi, cells were harvested, fixed and stained for FACS analysis. (A) The contour dotplots for DEN2 and DEN3 infected cells were obtained from FACS analysis of infected C6/36 cells at 24 hours post infection. C6/36 cells grown in six-well plates were infected for 24 hours using 10-fold serial dilutions of prototype strains NGC and H87. One well served as a mock infected control. The percentage of DV infected cells was determined by counting the number of cells in the upper left quadrant and dividing this number from the total number of cells in the dotplot. At 24 hrs post infection, the cells in the upper left quadrant are likely a measure of the input virus because this time point is too early for cell to cell spread of newly assembled virus. The dotplots shown above represent one experiment out of three and one out six experiments for DENV2 and DENV3 respectively. (B) C6/36 cells grown in six-well plates were infected for 24 hours using 10-fold serial dilutions of prototype strains NGC and H87 and clinical isolates: UNC3001, UNC3017 and UNC3018.

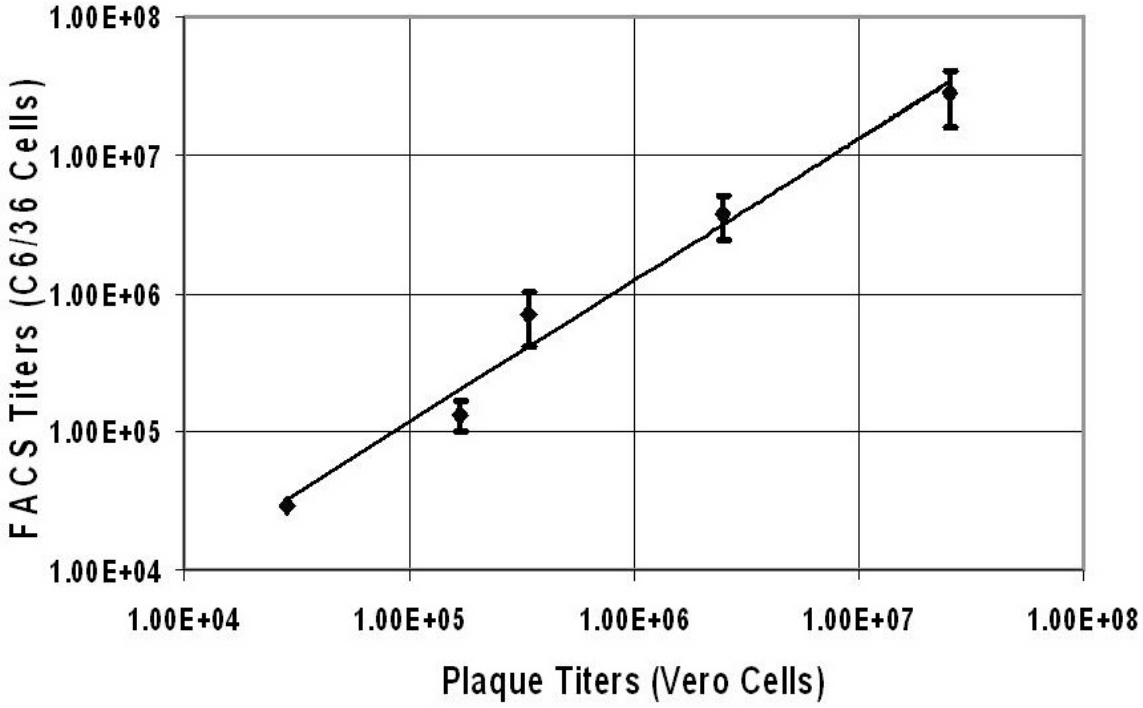


Figure 2.5. Comparison of FACS titration and plaque assay methods for estimating DENV2 titers. A stock of DENV2 (1×10^7 pfu/ml) was serially diluted 5-fold and each dilution was titrated on Vero cells by plaque assay and on C6/36 cells by FACS. The lower limit of detection by FACS is 10,000 infectious units per milliliter of inoculum. The lower limit of detection by plaque assay is 50 PFU/ml.

Chapter 3

Interaction of DENV3 with primary human dendritic cell

3.1 Abstract

DCs are one of the primary targets of DENVs during a natural infection. These APCs are responsible for stimulating innate and adaptive immune responses via their interactions with T cells, B cells and their cytokine production. The uncontrolled release of cytokines appears to be responsible for severe dengue disease, also known as dengue hemorrhagic fever. In this chapter, we present data showing that DENV3 blocks activation of infected immature DCs but not the uninfected bystander DCs in the culture. Based on these results, we next focused on the interactions between DCs and two groups of clinical DENV3 isolates from periods of mild and severe disease. In these experiments, we discovered two isolates that differ in there ability to infect DCs.

3.2 Introduction

During a natural dengue infection, DCs are believed to be one of the first cell types to encounter DENVs. Wu et al. have shown that blood-derived DCs are ten-fold more permissive to dengue infection than monocytes and macrophages (207). Langerhans DCs were initially thought to be targets of DENVs (207) until later studies demonstrated that immature monocyte-derived DCs, not Langerhans DCs, were permissive to DENV infection (120, 130). Infection of DCs is mediated by DC-SIGN, which has been demonstrated to confer permissiveness to normally nonpermissive cell lines (153, 194).

DCs are important APCs that present antigens through MHC class I and II molecules to stimulate differentiation of naïve T cells. Myeloid DCs, one of the three distinct subpopulations of DCs, are found in most nonlymphoid organs including the epidermis (Langerhans cells), dermis, gastrointestinal and respiratory mucosa, and the interstitial space of vascular organs (119, 168). Once immature myeloid DCs bind and internalize virus, they release inflammatory cytokines and chemokines that initiate the recruitment of more immature myeloid DCs and other leukocytes to the site of infection. Upon cytokine stimulation, the immature DCs become activated, and subsequently migrate to the lymph nodes where they stimulate T cells. After virus internalization myeloid DCs initially release IL-12, which drives the generation of effector cells with Th1 phenotypes. Twenty four hours after stimulation, IL-12 production from myeloid DCs ceases. The cessation of IL-12 production leads to a switch from the generation of effector cells to the generation of unpolarized memory T cells and IL-4 secreting Th2 cells, which then dampen the Th1 response (127). DC activation is measured by

increases in the surface expression levels of CD40, costimulatory molecules CD80 and CD86 (B7.1 and B7.2 respectively), CD83, and MHC class I and II molecules (HLA-ABC and HLA-DR respectively). DC activation leads to the stimulation of CD8+ CTLs, when antigens are presented via MHC class I (HLA-ABC), or to the stimulation of CD4+ T cells, when presented via MHC class II (HLA-DR). DCs also play an important role in the maintenance of B cell functions by recalling responses that give them a critical role in the establishment of immunological memory.

In the absence of an animal model to study dengue pathogenesis and DHF, the majority of studies have focused on using lab-adapted strains of DENVs to look at viral interactions with human cells *in vitro*. Previous groups have shown that low passage clinical isolates and lab-adapted viruses behave differently in cell culture systems, indicating that interactions between laboratory-adapted strains and human cells may lead to incorrect conclusions about the pathogenesis of dengue in the human host (33, 34). Here we report on studies done with low passage, clinical isolates of DENV3 and human myeloid DCs.

The DENV3 clinical isolates used in this study were isolated in Sri Lanka during periods of mild (pre-DHF) or severe (post-DHF) dengue disease. Messer et al. have shown that the pre- and post-DHF DENV3 isolates cluster within DENV3 genotype III, which is endemic to South Asia and East Africa (Figure 3.1) (139). However, within DENV3 genotype III, the pre- and post-DHF viruses form two genetically distinct groups. Thus, the isolates associated with mild and severe disease epidemics are very closely related but genetically distinct.

In this chapter, I discuss experimental data examining the interactions of primary human DCs and clinical DENV3 isolates. We demonstrated that DENV3 infection of DCs blocks activation of infected DCs but not the uninfected bystander DCs. In addition to these experiments, we also looked for differences between our pre- and post-DHF viruses in their ability to infect human DCs. This investigation led to the discovery of two clinical isolates, one from the pre-DHF group and one from the post-DHF group, which differ in their ability to infect DCs, a phenotype that appears to be specific to DCs.

3.3 Material and Methods

Cell lines, virus stocks and antibodies

Aedes albopictus C6/36 mosquito cells were obtained from CDC, Fort Collins, CO. Vero 81 (African Green monkey kidney) cells were a gift from Robert Putnak at Walter Reed Army Institute of Research, Silver Springs, MD. U937 + DC-SIGN cells were a gift from Tem Morrison in Mark Heise's lab at UNC-Chapel Hill, Chapel Hill, NC. C6/36 cells were maintained in EMEM (Gibco) (containing 10% hi-FBS, 1% pencillin-streptomycin (10,000µg/ml), and 1% L-glutamine (200mM/100x) and 1% non-essential amino acids) at 28°C in 5% CO_2. Vero 81 cells were maintained in DMEM/F12 (Gibco) (containing 10% hi-FBS, 1% pencillin-streptomycin (10,000µg/ml), and 1% L-glutamine (200mM/100x) and 1% non-essential amino acids) buffered with 15mM HEPES at 37°C in 5% CO_2. U937 + DC-SIGN cells were maintained in Roswell park memorial institute (RPMI) 1640 (Gibco) (containing 10% hi-FBS, 1% pencillin-streptomycin (10,000µg/ml), 1% L-glutamine (200mM/100x) and 0.1% 2-Mercaptoethanol (55mM/1000x)).

The viruses used in this study were six DENV3 clinical isolates from Sri Lanka (UNC 3001, UNC 3006, UNC 3009, UNC 3010, UNC 3011, and UNC 3013; gifts from Duane Gubler and Vance Vorndam, CDC) and one DENV3 clinical isolate from Nicaragua (UNC 3018; gift from E. Harris, UC Berkeley, CA). All experiments with clinical viral isolates, unless otherwise stated, used stocks of viruses that had been passaged in C6/36 cells fewer than four times. Viral stocks were obtained as described previously (115).

The flavivirus group-reactive monoclonal antibody (Ab) 4G2 (79) was purchased from ATCC. Phycoerythrin- (PE) conjugated antibodies: CD40, CD80, CD83, CD86 and HLA-DR were purchased from BD Biosciences (San Jose, CA).

Human dendritic cells (DCs)

Human DCs were generated from blood-derived monocytes as described by Moran et al. (147). PBMCs were isolated from buffy coats obtained from the American Red Cross in Durham, NC. PBMCs were isolated by diluting the buffy coats in an equal volume of PBS and centrifugation over Ficoll (GE Healthcare, Piscataway, NJ) at 2000 revolutions per minute (rpm) for 20 min. PBMCs were washed twice with PBS and resuspended in RPMI 1640 (Gibco: Invitrogen Co, Carlsbad, CA) (containing 10% hi FBS, 1% pencillin-streptomycin (10,000µg/ml), and 1% L-glutamine (200mM/100x)) and cultured at a density of 2.5×10^8 PBMCs/ 175 cm^2 tissue culture flask at 37°C in 5% CO_2 for 2 hrs. Nonadherent PBMCs were removed and the adherent PBMCs were cultured in RPMI 1640 supplemented with interleukin-4 (IL-4) (500 U/ml) and granulocyte-macrophage colony stimulating factor (GM-CSF) (800 U/ml) for 6 days at

37°C in 5% CO_2. Fresh cytokines were added to the culture at day 3 and day 6. Immature DCs were harvested on day 6.

Infection of human DCs with DENV3 clinical isolates

Immature DCs were infected with DENV3 clinical isolates in polypropylene tubes at a density of 1 x 10^5 cells/well at a specific MOI as indicated in figure legends. Viruses and DCs were maintained in RPMI 1640 supplemented with IL-4 (500 U/ml) and GM-CSF (800 U/ml) at 37°C in 5% CO_2. After 2-3 hrs of infection, cells were washed twice with PBS and resuspended in RPMI 1640 supplemented with IL-4 and GM-CSF at a density of 1 x 10^5 cells/ml. Infected cultures were seeded in 24-well low cluster plates (Corning, Lowell, MA) at a density of 1 x 10^5 cells/well. Infected cultures were maintained at 37°C in 5% CO_2 until harvested at specific time intervals indicated in figure legends.

Flow-cytometric analysis

Cells were fixed and permeablized using the Cytofix/Cytoperm Kit (BD Biosciences, San Jose, CA), washed twice with Cytoperm/Cytowash buffer and stained with FITC-labeled 4G2 monoclonal antibody for 1 hr on ice. The samples were analyzed on a FACSCan flow cytometer using Summit Software.

Activation/maturation of primary human DCs

Immature DCs from three individual donors were infected at an MOI of 1 with UNC 3009 or UNC 3018 and harvested 48 hrs post infection. The cells were initially stained with PE-conjugated CD40, CD80, CD83, CD86 or HLA-DR for 30 min on ice

and then fixed and permeablized using the Cytofix/Cytoperm Kit. The cells were then stained with FITC-labeled 4G2 monoclonal antibody for 1 hr on ice. The samples were analyzed by flow cytometry. Data measured for UNC 3009 and UNC 3018 were averaged together to represent DENV3 clinical isolates as a whole.

Pre- and post-DHF DENV3 infection

Immature DCs from two individual donors were infected at an MOI of 1 or 0.1 with three pre- and three post-DHF associated Sri Lankan DENV3 clinical isolates. The pre-DHF group contained isolates: UNC 3009 (isolated in 1989), UNC 3011 (isolated in 1985) and UNC 3013 (isolated in 1983). The post-DHF group contained isolates: UNC 3001(isolated in 1989), UNC 3006 (isolated in1997) and UNC 3010 (isolated in 1993). The DENV3 infected cultures were harvested at 48 hrs post infection and fixed and stained for flow cytometry as described above. Each sample was tested in triplicate.

IFN levels for Pre- and Post-DHF DENV3 Infection

Supernatants from the above infection experiment were collected at the time of harvest and stored at -80°C until analysis. To measure type I IFN levels, an IFN bioassay developed by the Heise lab at UNC-Chapel Hill, Chapel Hill, NC was used (181). A549 (human lung carcinoma) cells were seeded in a 96-well plate at a density of 2×10^4 cells/well for 24hrs. All samples and standards were acidified to a pH of 2 for 24 hrs and then neutralized to pH 7 prior to their addition to the 96-well plate. Each sample was serially diluted 2-fold down the plate. To quantitate the amount of IFN in each sample, an IFN standard, that was also serially diluted 2-fold, was added to one row of each plate tested. Twenty four hours after the addition of the samples to the plate, an IFN sensitive

virus, encephalomyocarditis virus, was added to each well for at least 24 hrs. Each well was scored for 50% CPE and compared to the IFN standard to determine the IFN concentration of each sample. Each sample was tested in triplicate.

Five day infection time course

Immature DCs were infected at an MOI of 0.1 or 0.01 and cells were harvested at 1, 2, 3, 4, and 5 days post infection. RPMI 1640 supplemented with IL-4 (500 U/ml) and GM-CSF (800 U/ml) was added to the samples that were collected on days 4 and 5 at day 3 post infection. Harvested cells were fixed and stained for flow cytometry as described above. Each sample was tested in triplicate.

Infection DCs, Veros and U937 + DC-SIGN cells

Immature DCs and U937 + DC-SIGN expressing cells were infected at MOIs of 1, 0.1, or 0.01 with UNC 3006 and UNC 3009 using the infection methods described earlier for DCs. Vero cells were seeded in 6-well plates at a density of 1 x 10^5 cells/well and allowed to double over night prior to infection. The Vero cells were infected at MOIs of 1, 0.1, and 0.01 with UNC 3006 and UNC 3009 and harvested at 48 hrs post infection. To harvest the Veros, the media was removed and each well was washed twice with PBS and incubated with 0.2 ml of trypsin-EDTA/well at 37°C for 5 min. One ml of chilled 1× PBS containing 10% FBS was added to each well to inactivate the trypsin. The cells were pipetted to breakup any clumps and centrifuged at 1,000g for 5 min. The PBS was removed and the cells were fixed and stained for flow cytometry as described above. The DCs and U937 + DC-SIGN expressing cells were also fixed and stained as described above. Each sample was tested in triplicate. The DC data was from eight

individual donors (n=8), and the Vero and U937 + DC-SIGN data were each from six independent experiments (n=6).

Statistics

3006 and 3009 were compared using a two-tailed unpaired Student *t* test with Welch correction using GraphPad InStat software. Differences were considered significant when $P<0.05$.

3.4 Results

DENV3 blocks activation of infected DCs but not the uninfected bystander DCs

Immature DCs from 3 individual donors were infected at an MOI of 1 with UNC 3009 (3009) and UNC 3018 (3018). The cells were harvested at 48 hrs post infection, which is a time point used by other groups studying DENV-DC interactions. The cells were first stained for surface expression of CD40, CD80, CD83, CD86, and HLA-DR and then fixed, permeabilized, stained for intracellular expression of the E glycoprotein of DENV3, and analyzed using flow cytometry. As controls, we also fixed and stained mock treated DCs, UV-inactivated 3009 infected DCs (data not shown), and DCs treated with lipopolysaccharide (LPS). To analyze the effect of DENV3 infection as a whole instead of focusing on individual isolates, we combined the results for 3009 and 3018, for which we did not observe any individual differences between to the two viruses. DENV infected DC cultures were divided into two populations: infected and uninfected, based on whether they were positive for E glycoprotein expression determined by 4G2 fluorescence indicating DENV infection. When stained for CD40, CD80, CD83, CD86,

and HLA-DR cell surface markers, we observed no difference in the mean fluorescent intensity (MFI) between mock treated cells and the DENV3 infected DCs (Figure 3.2). However, we did observe a significant difference in MFI when comparing mock treated to DENV3 uninfected bystander DCs for CD40, CD83, and CD86 expression. The positive control, LPS treated DCs, was significantly different from the negative control, mock treated DCs, for CD40 and CD86 expression. For CD80 expression, mock, uninfected, infected, and LPS treated samples showed similar MFI. These results support the idea that DENV infection results in activation of uninfected bystander DCs but not infected cells. In addition to these experiments, we also analyzed the activation of DCs at different times during infection and observed similar results (data not shown).

Pre- and Post-DHF have a similar effect on DC function

Since we initially focused on the interaction of two representative DENV3 isolates, we next wanted to test a panel of clinical isolates to measure their ability to infect the primary DC cultures. For these experiments, we focused on the Sri Lankan pre- and post-DHF groups of viruses and selected three isolates from each group (Table 3.1). For the pre-DHF group, we selected 3009 (isolated in 1989), 3011 (isolated in 1985) and 3013 (isolated in 1983). For the post-DHF group we selected 3001 (isolated in 1989), 3006 (isolated in 1997) and 3010 (isolated in 1993). Immature DCs from two individual donors were infected at an MOI of 1 and 0.1, and cells and supernatants were harvested at 48 hrs post infection. At an MOI of 1, the percent infection of both the pre- and post-DHF associated groups ranged from 32% to 60%, and at a MOI of 0.1, the percent infection ranged from 15% to 60% (Figure 3.3). From this experiment, we

concluded the pre- and post-DHF associated viruses were not different in their ability to infect primary DC cultures.

We next determined the amount of IFN that was released into the media by the infected DC cultures. The culture supernatants from the above experiments were tested for type I IFN levels using an IFN bioassay. IFN levels for the pre-DHF isolates ranged from 400 - 500 IU/ml, where as the post-DHF levels ranged from 100 - 500 IU/ml (Figure 3.4). Thus, we were unable to detect a difference in the amount of IFN secreted by cells infected with these two groups of viruses. From the pre- and post-DHF infection experiment, we did observe two Sri Lankan clinical isolates, 3006 (post-DHF) and 3009 (pre-DHF) that were different in their ability to infect DCs. At MOIs of 1 and 0.1, 3009 infected a higher percentage of the DCs than 3006 (Figure 3.3).

Clinical isolates 3006 and 3009 differ in their ability to infect primary human DCs

In the above infection experiments, at an MOI of 1, 3006 infected 35% of the DCs whereas 3009 infected 60% of the DCs (Figure 3.3). When the input virus was decreased 10-fold, 3006 infected 15% of the cells but 3009 still infected 60% of the DCs. When we compared the amount of IFN secreted by both viruses (Figure 3.4), we observed similar amounts of IFN for 3006 and 3009 (~ 400 IU/ml). To determine if the difference in ability to infect DCs was repeatable and universal between different DC donors, we infected additional DCs from a different donor at an MOI of 0.1 and 0.01 and followed the infection for 5 days. At a MOI of 0.1, 3009 infection percentages peaked with 8% of the cells infected 2 days post infection, where 3006 peaked between 2 and 3 days post infection at 3% (Figure 3.5). The infection percentage decreased over the remaining days

of the experiment. Similar to what we observed at a MOI of 0.1, a consistent difference in the percentage of infected cells existed between 3006 and 3009. Infection percentages for 3009 peaked at 3 days post infection with 4% of the cells infected. 3006 also peaked on day 3, but half the number of cells were infected when compared to 3009 (Figure 3.5).

Since we observed a difference in the infection percentages of DCs between 3009 and 3006 in DCs, we wanted to look at their ability to infect other cell types. We next infected DCs, Veros and U937 + DC-SIGN over a range of MOIs from 1 to 0.01. From earlier infection studies with U937s, we had determined this particular cell line was only permissive to DENV infection when it expressed DC-SIGN (data not shown). When we observed the ability of 3006 and 3009 to infect the different cell types, we noticed a consistent difference between our two viruses in DCs over the range of MOIs, with 3009 infecting a larger percentage of cells than 3006 (Figure 3.6). In the Veros and U937 + DC-SIGN we did not observe this difference over the range of MOIs. In these cell lines, 3006 and 3009 infected a similar percentage of cells. The differences that we observed for 3006 and 3009 in the DCs were statistically significant ($P<0.01$).

3.5 Discussion

DCs are one of the initial targets of DENV infection during a natural infection. Blood-derived myeloid DCs have been shown to be 10-fold more permissive to DENV infection than monocytes or macrophages, with this interaction being mediated by DC-SIGN. Previous work from Ho et al. and Libraty et al. have shown DENV infection of primary human DC cultures led to activation of the culture. However, it was unclear whether the infected DCs or the uninfected bystander cells in the cultures were activated.

Since the majority of studies have focused on looking at the effect of lab-adapted DENV2 on DC function and some uncertainty still existed as to whether or not DENV infection led to activation of the infected DCs, we were interested in investigating the effect of clinical DENV3 isolates on DC activation and function. From these experiments, we were able to conclude that DENV3 blocked the activation of infected DCs but not the activation of the uninfected bystander DCs in the cultures. During the course of this investigation, Palmer et al. published data for DENV2 showing similar results where the activation of infected DCs was also blocked in their system (160). Additionally, Palmer et al. showed that DENV infected DCs underwent apoptosis and had a reduced capacity to stimulate T cells (160). These findings indicate a possible immune evasion strategy by DENVs to compromise the antigen-presenting function of DCs, which is potentially important in dengue pathogenesis and the development of DHF (55).

We also used the primary DC culture system to focus specifically on two groups of viruses from periods of mild and severe disease. In our studies, we determined the ability of Sri Lankan DENV3 clinical isolates, from before (pre-) and after (post-) the emergence of DHF, to infect primary human DCs. The results from these experiments did not show differences in infection percentages between both groups of viruses at MOIs of 1 and 0.1 (Figure 3.3). We also did not observe any difference in the levels of type I IFN when we analyzed culture supernatants from DCs infected with pre- and post-DHF isolates (Figure 3.4). For these results to be statistically significant, we would have needed an n=50 (DC donors), which is not practical for our lab. So, we decided to focus our studies more specifically on two clinical isolates that did display differences in the percent infection of DCs. As mentioned previously, when we looked at the ability of our

two groups of viruses to infect the human DCs, we observed clear differences in the ability to infect DCs between 3006, a post-DHF isolate, and 3009, a pre-DHF isolate (Figure 3.3). 3009 infected a higher percentage of DCs than 3006 irrespective of MOI. Interestingly, when we determined the amount of IFN produced from DCs infected with either 3006 or 3009, we detected similar levels between the two samples (Figure 3.4).

Since we originally observed the infection difference between 3006 and 3009 at 48 hours post infection, we followed the infections of both viruses over a 5 day time course at MOIs 0.1 and 0.01. Infection percentages for both viruses peaked between 2 and 3 days post infection, with 3009 consistently infecting a larger percentage of the DCs than 3006. In these experiments, we noticed a decrease in infection percentages between days 3 and 5. This decrease may be due to apoptosis of the DC cultures, which is consistent with the findings of Palmer et al. We next looked at the ability of 3006 and 3009 to infect multiple cell types including DCs, Veros, and U937 + DC-SIGN expressing cells to determine if the previous observed differences were specific to DCs. We analyzed the percent infection of 3006 and 3009 in each cell type over a range of MOIs. In DCs, we consistently observed a difference between 3006 and 3009, with 3009 infecting a higher percentage of cells at each MOI. This difference between 3006 and 3009 was statistically significant (Figure 3.6). In Veros and U937 + DC-SIGN, we did not observe a difference in the infection percentages between the two viruses over the range of MOIs, indicating these differences were specific to DCs.

To explain the observed infection differences between the two clinical isolates, 3006 and 3009, we have developed two hypotheses: 1) the infection differences between the two viruses are due to a host immune response or 2) the infection differences between

3006 and 3009 are due to inherent differences in the ability of these viruses to infect cells. If the infection differences are due to a host immune response, we believe that type I IFN is a likely candidate for the difference, since Veros do not secrete IFN and we have been unable to detect IFN from DENV infected U937 + DC-SIGN expressing cells. Additionally, DENVs block IFN signaling through the Janus kinase (JAK)/STAT signaling pathway (94, 150, 151). Alternatively, if the differences between 3006 and 3009 are not due to a host immune response but are due to inherent differences between the viruses, we should be able to determine the stage in the viral life cycle that these differences occur. Determining the mechanism behind the infection differences between 3006 and 3009 will help us to understand how certain viruses differentially interact with primary human cells, and how this interaction may be important in the development of severe disease. The goal of the next chapter is to determine the mechanism involved in mediating the DC infection differences observed between 3006 and 3009.

Clinical Isolates	Year Isolated	Country Isolated	Source	DHF Association
UNC 3001	1989	Sri Lanka	CDC-PR	Post-DHF
UNC 3006	1997	Sri Lanka	CDC-PR	Post-DHF
UNC 3009	1989	Sri Lanka	CDC-PR	Pre-DHF
UNC 3010	1993	Sri Lanka	CDC-PR	Post-DHF
UNC 3011	1985	Sri Lanka	CDC-PR	Pre-DHF
UNC 3013	1983	Sri Lanka	CDC-PR	Pre-DHF
UNC 3018	1998	Nicaragua	Eva Harris	Post-DHF

Table 3.1. Clinical DENV3 isolates from periods of mild and severe disease.

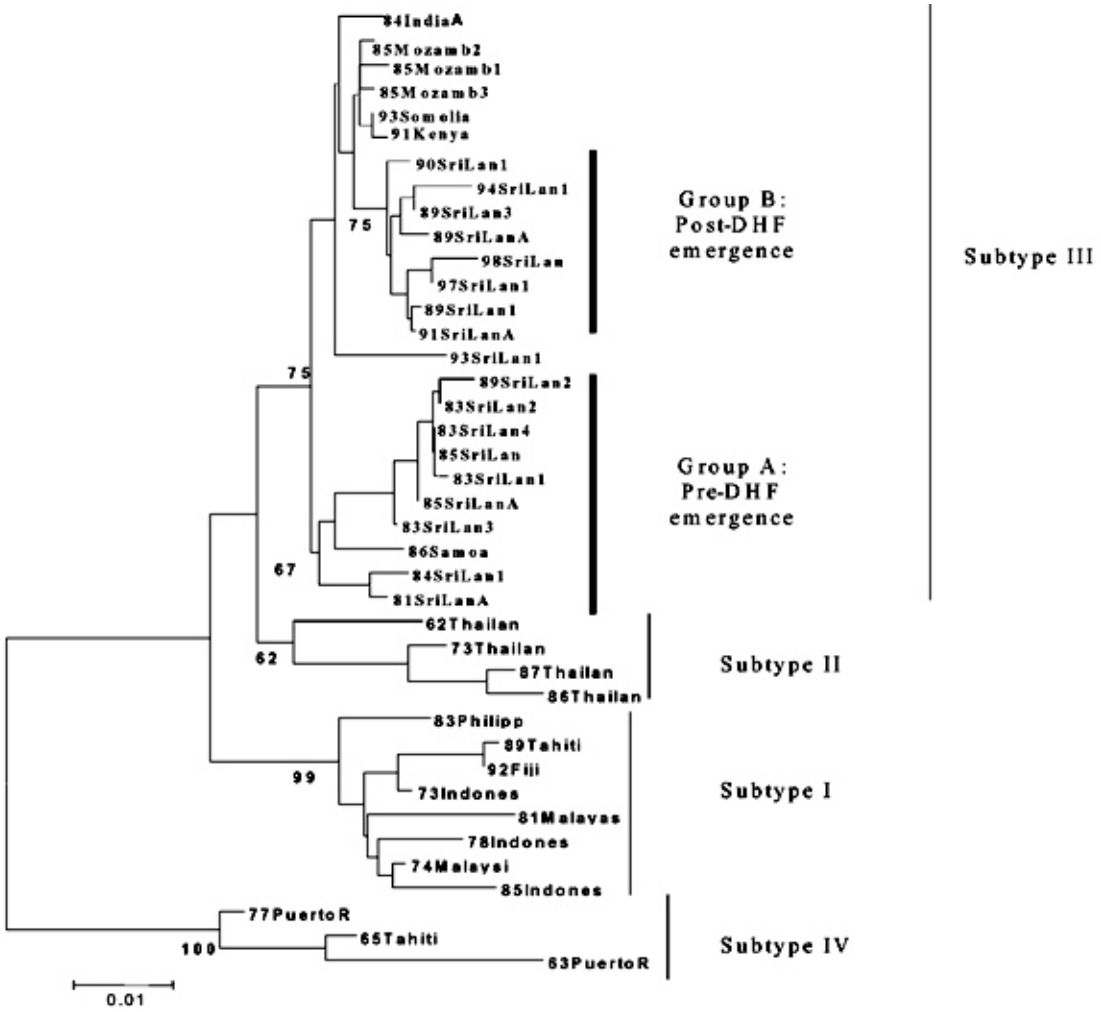


Figure 3.1. Phylogenetic tree comparing Sri Lankan DENV3 isolates to other DENV3 isolates from around the world. This tree is based on a 708 base pair sequence spanning the prM/M and partial E regions from positions 437 to 1145. Dengue serotype 3 viruses are divided into four distinct subtypes/genotypes based on the sequenced region. The scale bar indicates the number of substitutions per bases weighted by Tamura-Nei algorithm. Horizontal distances are equivalent to the distances between isolates. Numbers at nodes indicate bootstrap support values for the branch of the tree inferred at that node. Figure and legend from Messer et al. (2003) (139).

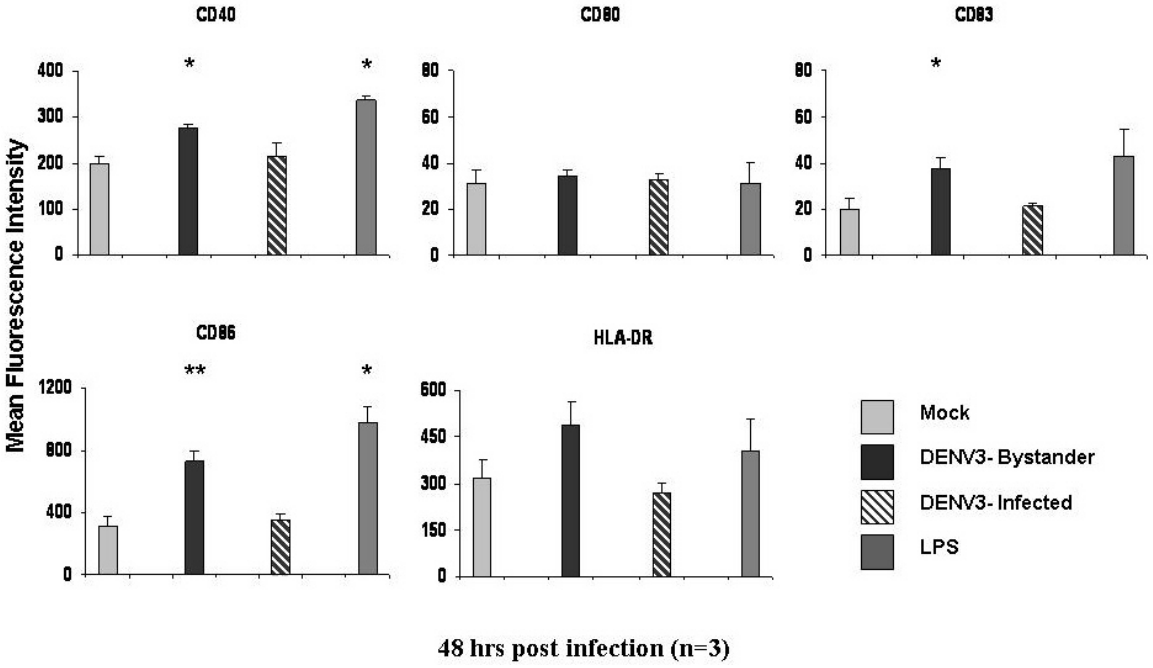


Figure 3.2. DENV3 blocks activation of infected DCs but not uninfected bystander DCs. DCs were infected at a MOI of 1 and harvested at 48 hrs post infection. DCs were first stained with PE-conjugated antibodies against CD40, CD80, CD83, CD86, and HLA-DR and then fixed, permeabilized and stained with FITC-conjugated 4G2. DENV3 infected DC cultures were divided into two groups, Infected or Bystander, based on whether they were positive or negative for DENV3 infection as determined by the mean fluorescent intensity for 4G2 (not shown) compared to mock infected samples. Displayed is the average from three individual DC donors ± SEM. * denotes $P<0.05$, ** denotes $P<0.005$ based on comparison to mock treated samples.

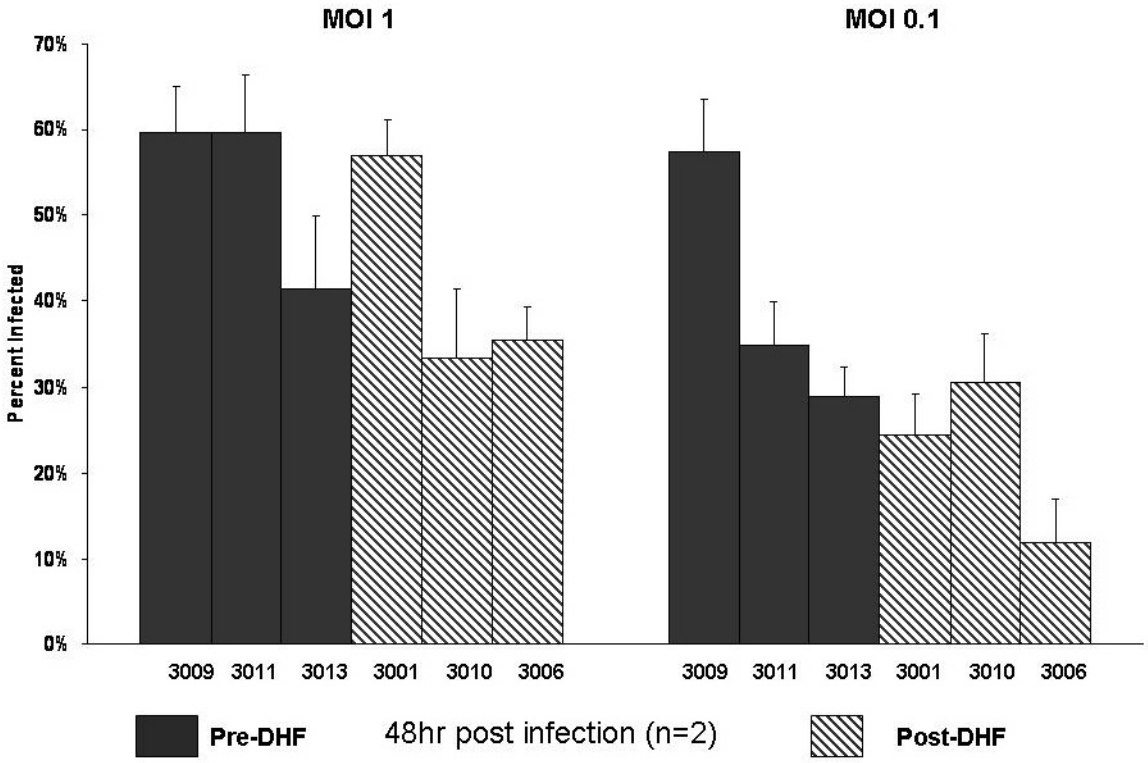


Figure 3.3. Infection of DCs with pre- and post-DHF associated clinical DENV3 isolates. DCs were infected at an MOI of 1 and 0.1 for 48 hrs. Cells were harvested, fixed, permeabilized and stained for infection using a monoclonal antibody against E protein, 4G2. Samples were analyzed using flow cytometry. Data are the average of two donors ± SEM. Each sample was tested in triplicate.

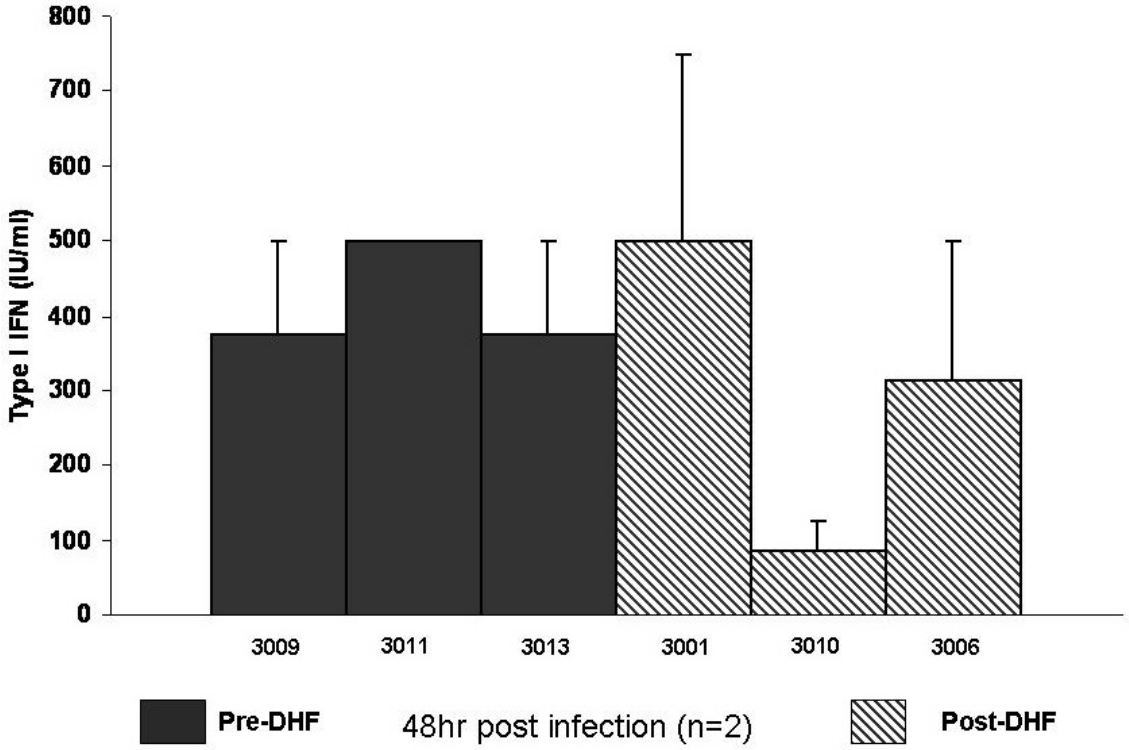


Figure 3.4. IFN production from DCs infected with pre- and post-DHF associated isolates. DCs were infected at a MOI of 1 and culture supernatants were collected at 48 hrs post infection. IFN production was measured using an IFN bioassay. Data are the average of two donors ± SEM. Each sample was tested in triplicate.

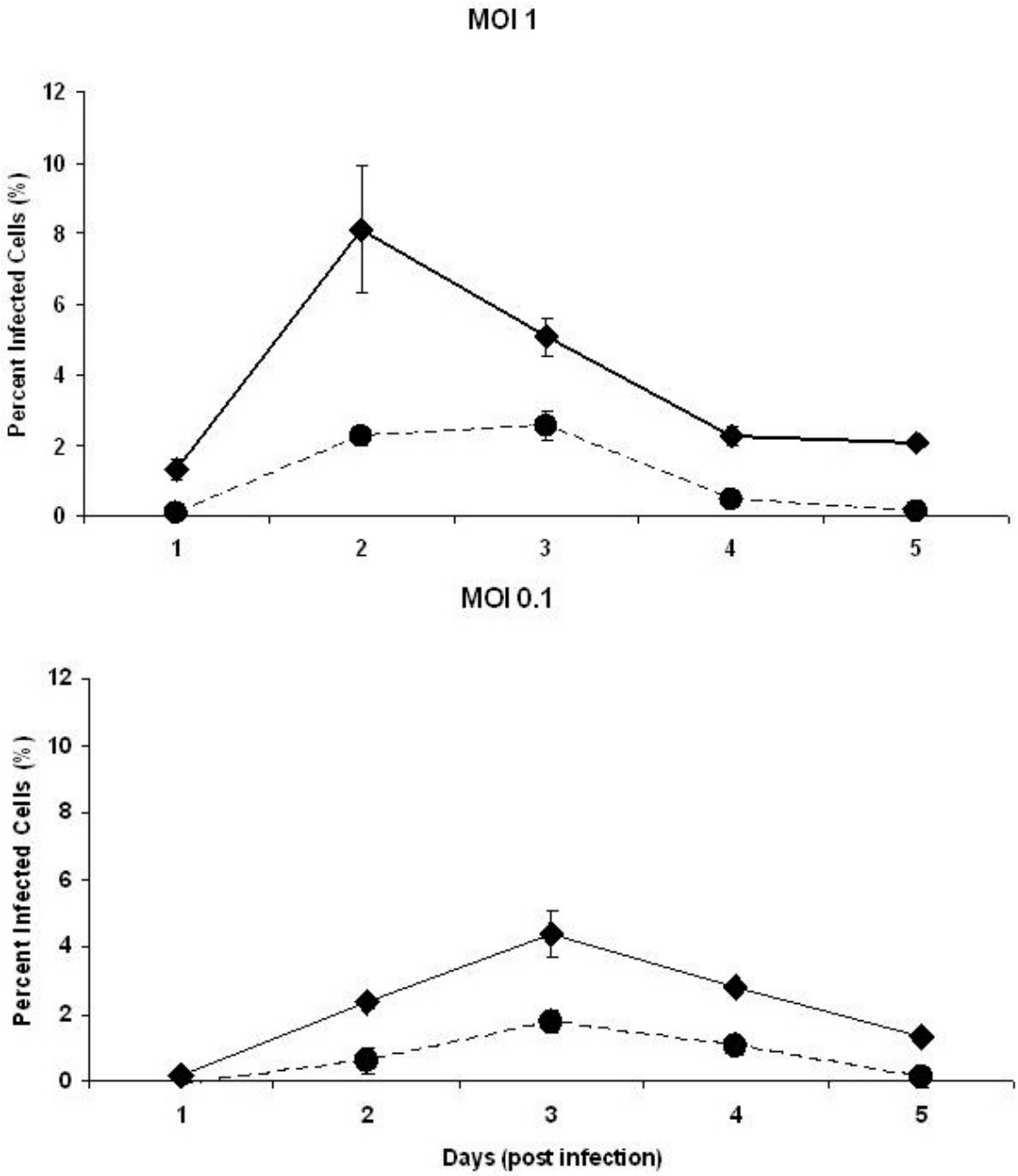


Figure 3.5. 5 day infection time course for DENV3 clinical isolates 3006 and 3009. DCs were infected at an MOI of 0.1 and 0.01 and cells were harvested at 1, 2, 3, 4, and 5 days post infection. Cells were fixed and stained as described previously and analyzed by flow cytometry. 3006 is represented by the circle and dashed line (--●--). 3009 is represented by the diamond (♦) and solid line. Data from one donor ± SEM. Each time point was performed in triplicate.

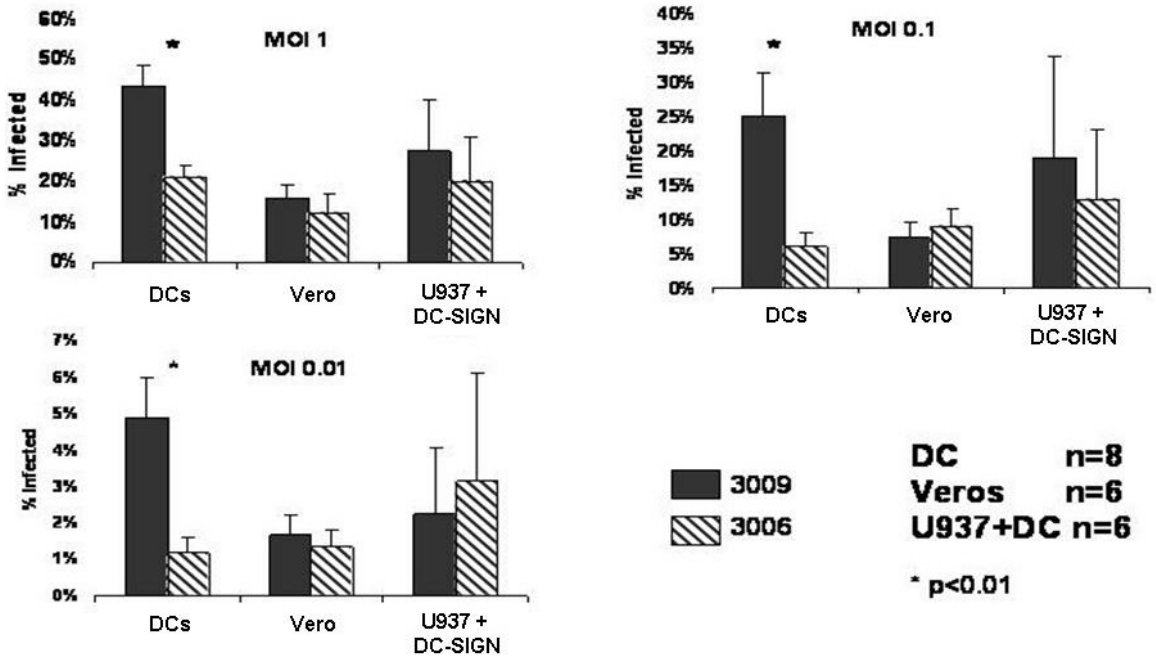


Figure 3.6. Infection of different cell types with 3006 and 3009. DCs, Veros, and U937 + DC-SIGN cells were infected at MOIs of 1, 0.1, and 0.01. Cells were harvested at 48 hrs post infection and analyzed by flow cytometry as described previously. Displayed is the average from eight individual DC donors and six separate experiments with Vero and U937 + DC-SIGN cells ± SEM. Each donor and experiment was performed in triplicate.

Chapter 4

Infection of primary human myeloid dendritic cells by clinical isolates of Dengue virus type 3

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4.1 Abstract

Much of the experimental work on dengue pathogenesis has utilized lab-adapted strains of DENVs. Very few studies have reported on the interactions of low passage clinical isolates with primary human cells. We focused on two low passage DENV3 isolates from periods of mild and severe disease in Sri Lanka. We observed differences in the ability of these two low passage isolates to infect primary human DCs. Differences in viral titers were observed early in infection but did not persist throughout the later stages of infection. IFN experiments indicated that host anti-viral responses were unlikely to be responsible for the different infectivity of these viruses to human DCs. DC-SIGN binding studies and ammonium chloride treatment experiments indicate

similarities between the DENV3 isolates in binding but suggest that differences in entry are likely responsible for the different infectivity of these two viruses to human DCs.

4.2 Introduction

Dengue is a mosquito-borne viral disease of global public health significance. DENV are enveloped, positive polarity RNA viruses in the *Flaviviridae* family. The DENV complex consists of 4 distinct serotypes designated DENV1 through DENV4 (126).

Dengue infection begins after the viral E glycoprotein binds to a cell surface receptor(s) and the virus enters cells by receptor-mediated endocytosis. E protein undergoes a conformational change in response to the low pH of the endosomal environment and fuses the viral and endosomal membranes to release the viral nucleocapsid into the cytoplasm. In the cytoplasm, the virus uncoats and the positive strand viral RNA is translated into a single polyprotein, which is then cleaved to form the three structural (C, prM, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The nonstructural viral proteins replicate the viral genome to produce negative- and then positive-strand genome-length RNA, which is used to produce more viral protein and RNA as well as new virions. New virions bud into the ER and are secreted out of the host cell.

People infected with DENV can be asymptomatic or develop symptoms that range from a mild fever to life threatening DHF and dengue shock syndrome DSS. Clinical outcome is clearly influenced by both host and viral factors. Host factors that contribute to the development of severe disease are age, genetic background and previous exposure to DENVs. DHF and DSS are often seen in children and young adults, even

though adults are also susceptible (31). Primary dengue infection confers long-term immunity to the infecting serotype but not to other serotypes. People experiencing a second dengue infection are at a greater risk of developing DHF/DSS. Polymorphisms in certain HLA types are associated with DHF susceptibility in previously exposed and immunologically primed individuals (19, 186).

Viral strains and genotypes are also important factors in disease severity. Previous studies have indicated that certain DENV2 and DENV3 genotypes are associated with DHF versus DF (139, 172, 204). In South East Asia, the endemic DENV2 strains are more frequently associated with DHF, whereas the genotype of DENV2, which is endemic to the Americas, was previously only associated with DF (172). The introduction of the South East Asian DENV2 genotype into the Americas led to major epidemics of DHF in the Caribbean and Central America (66, 162). In the 1990s, DENV3 genotype III viruses, which have been associated with DHF in the Indian subcontinent, were introduced into Central America in 1994 leading to the first DHF outbreaks caused by DENV3 in Latin America (8, 60, 67, 158, 198). Host factors as well as the genotype of the infecting virus, determine clinical outcome.

Currently there is not a feasible animal model to study dengue pathogenesis. In the absence of an animal model much experimental work is conducted using cell culture-based models. During a natural dengue infection, cells of the mononuclear phagocytic lineage, such as DCs, monocytes, and macrophages are believed to be the primary targets of DENVs *in vivo*. *In vitro* it is difficult to infect human and non-human cell lines with clinical isolates of DENV. However, primary human myeloid DCs are highly permissive to infection with clinical isolates of DENV (207). Various studies have used primary

human DC cultures as a cell culture-based system to study dengue pathogenesis in the absence of an animal model. To understand the role of DENVs in pathogenesis, here we focus on the cell biology of two low passage DENV3 strains belonging to genotypes linked to mild and severe disease epidemics. We demonstrate that these isolates differ in their ability to infect primary human DCs because one strain enters cells faster than the other strain.

4.3 Materials and Methods

Human DCs

Human DCs were generated from blood-derived monocytes as described by Moran et al. (147). PBMCs were isolated from buffy coats obtained from the American Red Cross in Durham, NC. PBMCs were isolated by diluting the buffy coats in an equal volume of PBS and centrifugation over Ficoll (GE Healthcare, Piscataway, NJ) at 2000 rpm for 20 min. PBMCs were washed twice with PBS and resuspended in RPMI 1640 (Gibco: Invitrogen Co, Carlsbad, CA) (containing 10% hi FBS, 1% pencillin-streptomycin (10,000μg/ml), and 1% L-glutamine (200mM/100x)) and cultured at a density of 2.5×10^8 PBMCs/ 175 cm^2 tissue culture flask at 37°C in 5% CO_2 for 2 hrs. Nonadherent PBMCs were removed and the adherent PBMCs were cultured in RPMI 1640 supplemented with IL-4 (500 U/ml) and GM-CSF (800 U/ml) for 6 days at 37°C in 5% CO_2. Fresh cytokines were added to the culture at day 3 and day 6. Immature DCs were harvested on day 6.

Cell lines, virus stocks and antibody

Aedes albopictus C6/36 mosquito cells were obtained from the CDC, Fort Collins, CO. Vero 81 (African Green monkey kidney) cells were a gift from Robert Putnak at Walter Reed Army Institute of Research, Silver Springs, MD. C6/36 cells were maintained in EMEM (Gibco) (containing 10% hi-FBS, 1% pencillin-streptomycin (10,000µg/ml), and 1% L-glutamine (200mM/100x) and 1% non-essential amino acids) at 28°C in 5% CO_2. Vero 81 cells were maintained in DMEM/F12 (Gibco) (containing 10% hi-FBS, 1% pencillin-streptomycin (10,000µg/ml), and 1% L-glutamine (200mM/100x) and 1% non-essential amino acids) buffered with 15mM HEPES at 37°C in 5% CO_2.

The viruses used in this study were DENV3 clinical isolates from Sri Lanka (UNC 3006 (3006) isolated in 1997 and UNC 3009 (3009) isolated in 1989; gifts from Duane Gubler and Vance Vorndam, CDC). All experiments with clinical viral isolates, unless otherwise stated, used stocks from viruses that had been passaged in C6/36 cells fewer than four times. Viral stocks were obtained as described previously(115).

The flavivirus group-reactive monoclonal antibody (Ab) 4G2 (79) was purchased from ATCC.

Infection of human DCs with DENV3 clinical isolates

Immature DCs were infected with 3006 or 3009 in polypropylene tubes at a density of 1 x 10^5 cells/well at specific MOI as indicated in figure legends. Viruses and DCs were maintained in RPMI 1640 supplemented with IL-4 (500 U/ml) and GM-CSF

(800 U/ml) at 37°C in 5% CO_2. After 2-3 hrs of infection, cells were washed twice with PBS and resuspended in RPMI 1640 supplemented with IL-4 and GM-CSF at a density of 1 x 10^5 cells/ml. Infected cultures were seeded in 24-well low cluster plates (Corning, Lowell, MA) at a density of 1 x 10^5 cells/well. Infected cultures were maintained at 37°C in 5% CO_2 until harvested at specific time intervals indicated in figure legends.

Flow-cytometric analysis

Cells were fixed and permeablized using the Cytofix/Cytoperm Kit (BD Biosciences, San Jose, CA), washed twice with Cytoperm/Cytowash buffer and stained with FITC-labeled 4G2 monoclonal antibody for 1 hr on ice. The samples were analyzed on a FACSCan flow cytometer using Summit Software.

Virus titration by immunofocus assay

Viral titers were determined using an immunofocus assay described by Durbin et al. (37). DCs were infected and harvested as described above. At time of harvest supernatants from infected cultures were collected and stored at -80°C. Vero 81 cells were seeded in 24-well plates and grown until the monolayer were 80% confluent. Viral supernatants were thawed and diluted 10-fold ranging from 10^{-1} to 10^{-8} in Opti-MEM (Gibco) (containing 2% hi- FBS, 1% pencillin-streptomycin (10,000µg/ml)). Vero 81 24-well plates were inoculated with 100 µl/well of the serially diluted virus for 2 hrs at 37°C. Monolayers were overlayed with Opti-MEM containing 1% carboxymethylcellulose and 2% hi-FBS for 5 days at 37°C and 5%CO_2. Virus plaques were visualized by immunoperoxidase staining. For the immunoperoxidase staining, cells were fixed with 80% methanol at room temperature (temp.) for 15 min, dried and

blocked with antibody buffer (5% non-fat milk in PBS) for 10 min. 200 µl of 4G2 (1.2 mg/ml) diluted 1:150 in antibody buffer was added to each well and incubated at 37°C for 1 hr. Cells were washed twice with PBS and incubated with 200 µl/well of goat anti-mouse horseradish peroxidase (HRP)-labeled antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:500 in antibody buffer for 1hr at 37°C. Cells were washed twice with PBS and 125 µl/well of TrueBlue Peroxidase Substrate (KPL, Gaithersburg, MD) was added. Visible plaques were counted. The viral titers were expressed as plaque forming units (pfu)/ml = [(number of plaques per well) × (1/dilution)]/ (inoculum volume).

Titration of viral genomes by real-time PCR

Quantification of viral stocks was performed using a modified protocol from Houng et al. (87). Briefly, virus stocks of 3006 and 3009 were thawed and serially diluted 10-fold ranging from 10^0 to 10^{-5} in RPMI 1640. RNA was extracted from 140 µl of serially diluted virus using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) and eluted in a final volume of 60 µl. In the reverse transcriptase (RT)-polymerase chain reaction (PCR) the antisense primer DV-L1 described by Houng et al. (87) was used along with Reverse Transcriptase AMV (Roche, Indianapolis IN). A total volume of 20 µl of the RT reaction mixture consisted of 10 µl extracted RNA, 4 µl 5x RT Buffer (Roche), 1 µl dNTPs (10mM), 1 µl RNasin Ribonuclease Inhibitor (Promega, Madison, WI), 0.5 µl DV-L1 primer (20 pmoles/µl), 0.5 µl AMV reverse transcriptase and 3 µl of RNAse free H_2O. The RT reaction was performed in two steps. In the first step the primer mixture; which consisted of 0.5 µl DV-L1 primer, 10 µl extracted RNA and 2 µl RNAse free H_2O, was heated to 90°C for 90 seconds (secs), and then cooled on ice. The remaining components of the RT reaction were then added to the primer mixture and the

RT-PCR was heated to 42°C for 60 min, 90°C for 2 min and cooled to 4°C. The complementary deoxyribonucleic acid cDNA obtained from the RT-PCR was used as the deoxyribonucleic acid (DNA) template for the real-time PCR amplification. The real-time PCR reaction was conducted in a final volume of 25 µl. The real-time PCR reaction consisted of 12.5 µl of SYBR Green Mix (Applied Biosystems, Foster City, CA), 2.5 µl cDNA from RT reaction, 0.5 µl DV-L1 primer (20 pmoles/µl), 0.5 µl DV3-U primer (20 pmoles/µl) as described by Houng et al. (87), and 9 µl of DNAse free H_2O. The real-time PCR amplification and data analysis were performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The real-time PCR reactions were incubated at 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 20 sec and 95°C for 15 sec. Genome amounts were determined based on standards.

Type I IFN antibody neutralization assay

Immature DCs were infected with 3006 or 3009 in polypropylene tubes at a density of 2 x 10^5 cells/well at an MOI of 0.3 in the presence or absence of IFN-neutralizing Abs (PBL Interferon Source, Piscataway, NJ). IFN-neutralizing Abs were used at concentrations of 500 neutralizing units (NU)/well of IFN-α Abs and 500 NU/well of IFN-β Abs. As a control for the neutralizing Abs, 15.61 international units (IU)/well of both IFN-α and IFN-β were added 4 hrs post infection. Cells were harvested at 48 hrs post infection and analyzed by flow cytometry.

IFN-α and IFN-β ELISAs

Supernatants were collected from DCs that were mock treated or infected with 3006 or 3009 over a range of MOIs at various time points during infection ranging from 6 hrs to 72 hrs post infection for 8 different DC donors. Mock samples were collected from 4 different donors. IFN levels were detected using human IFN-α and IFN-β enzyme linked immunoabsorbent assay (ELISA) kits (PBL Interferon Source) as directed by the manufacturer.

Viral binding

The binding experiments were performed using modified protocols from Edgil et al. and Houng et al. (38, 87). Briefly, 6 x 10^4 DCs/well were cooled in a polypropylene tube on ice in a cold room for 45 min prior to the experiment. 5 mM EDTA and 1mg/ml mannan were used as controls to block DC-SIGN binding. EDTA and Mannan were added to the cells on ice for 30 min prior to the experiment. 3006 and 3009 were cooled on ice prior to their addition to cells at an MOI of 5. The binding experiment was conducted on ice in a cold room for 3 hrs. The cells were washed twice with cold RPMI 1640 media. The cells were lysed and RNA was extracted using the RNeasy Mini Kit (Qiagen) and eluted to a final volume of 60 µl. The TaqMan One-Step RT-PCR Master Mix Reagents Kits (Applied Biosystems) was used to quantify the amount of viral RNA in each sample. The One-Step RT-PCR reaction was conducted in a final volume of 25 µl consisting of 12.5 µl 2x Master Mix, 0.625 µl 40x Multiscribe Reverse Transcriptase and RNase Inhibitor, 1 µl DV-L1 (5 µM) primer, 1 µl DV3-U (5 µM) primer, 0.25 µl DV-P1 labeled with 5'-FAM and 3'-TAMRA dyes (Integrated DNA Technologies,

Coralville, IA) probe as describe by Houng et al. (87), 5 µl RNA Template and 4.625 µl of Nuclease-free H_2O. The real-time PCR machine and software used were the same as described above. The One-Step RT-PCR reaction was incubated at 48°C for 30 min, 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and then 60°C for 1 min. Each viral experimental condition was performed in triplicate. Each triplicate was further run in triplicate during the One-Step RT-PCR analysis.

Ammonium chloride assay

Immature DCs were infected with 3006 or 3009 in polypropylene tubes at a density of 1 x 10^5 cells/well at an MOI of 5 at 37°C and 5% CO_2. After 2 hrs cells were washed twice with PBS and resuspended in RPMI 1640 supplemented with IL-4 and GM-CSF at a density of 1 x 10^5 cells/ml. Infected cultures were seeded in 24-well low cluster plates at a density of 1 x 10^5 cells/well at 37°C and 5% CO_2. Ammonium chloride (NH_4Cl) was added at a concentration of 40 mM (pH 7.4) at 0, 2, 4, 6, 8 and 10 hrs post infection and maintained until the cells were harvested at 24 hrs post infection and analyzed by flow cytometry. For the infections where NH_4Cl was added at 0 and 2 hrs post infection, these samples were washed twice with PBS containing NH_4Cl at a concentration of 40 mM and resuspended in RPMI 1640 as listed above with the addition of 40 mM NH_4Cl. Percent inhibition was calculated by: ((No NH_4Cl control – NH4Cl treat sample)/No NH_4Cl control) * 100.

Statistics

3006 and 3009 were compared using a two-tailed unpaired Student *t* test with Welch correction using GraphPad InStat software. Differences were considered significant when $P<0.05$.

4.4 Results

In previous studies we have identified distinct genotypes of DENV3 associated with outbreaks of mild and severe disease in Sri Lanka. For this study we used two DENV3 strains isolated from patients in Sri Lanka in 1989 (UNC3009) and 1997 (UNC3006). Isolate 3009 belongs to a DENV3 genotype that does not cause severe disease, whereas 3006 belongs to a genotype linked to severe disease in Asia and Latin America. To prepare stocks for infection studies, the two virus strains were grown in C6/36 insect cells and the culture supernatant with virus was harvested and stored at -80°C. The viruses were titered by plaque assay and by using real-time PCR to estimate genome copy number and to determine the genome/PFU ratio for each stock. The genome to PFU ratios for strains 3009 and 3006 were 1.04×10^4 and 7.14×10^3 respectively indicating that these virus stocks had similar ratios of defective to infectious particles. We also sequenced the nucleotides encoding the structural proteins C, prM and E of strains 3009 and 3006. When the nucleotide sequence was translated, there were only two amino acid differences (both located within E) between the two viruses. The first change was a conserved change located at aa position 6 in E, where 3006 had an isoleucine (Ile) and 3009 had a valine (Val). The second aa change was located at position 124, where 3006

had proline (Pro) and 3009 had a serine (Ser). This Ser to Pro change is located on a region of domain II of E protein that is surface exposed (144).

We compared the ability of 3006 and 3009 to infect primary human DCs from 5 individual donors. The cells were infected with similar input doses of virus and infection was assessed by flow cytometry at 4 hour intervals up to 24 hrs. With strain 3009, dengue infection was detected as early as 4 hrs after infection and the percentage of infected cells continued to increase over 24 hrs (Figure 4.1A). With strain 3006, infected cells were first observed at 16 hrs and this strain infected fewer cells than 3009 at all times tested (Figure 4.1A). We also compared 3006 and 3009 using different doses of virus to infect DCs. The difference between 3006 and 3009 in their ability to infect DCs was observed at MOIs of 1, 0.1 and 0.01 at 48 hrs post infection. Experiments were also done to compare the two viruses over a 74 hr infection period. At 4 hr intervals up to 74 hours after infection the cells and the media were collected to determine the number of infected cells and the amount of progeny virus released into the media. Over the 74 hr time span we observed a significant difference in the percentage of cells that were infected by 3006 and 3009 at most time points (*P<0.05) (Figure 4.1C). Both viruses caused peak infection between 20-30 hrs although the peak for 3009 was higher than the peak for 3006. Following peak infection, we observed a decrease in the proportion of infected cells for both viruses, most likely due to the death of infected cells. When we looked at the amount of progeny virions secreted into the media, there was a 4 to 8 hr lag in time between when we were able to detect virus for 3006 and 3009. For 3009 we were able to detect virus as early as 14 hrs post infection where 3006 did not reach this same level until 22 hrs post infection (Figure 4.1C). The differences in the amount of progeny

virus detected in the media was significantly different (*P<0.05) between the two clinical isolates until 38 hrs after infection. There was no difference in the levels of virus being secreted by either isolate throughout the remainder of the 74 hr time course. These results establish that strain 3009 is more efficient at infecting human DCs than strain 3006.

Experiments were done to determine if type I IFN was responsible for the different infectivity of DENV3 strains 3006 and 3009. Culture supernatants were assayed for IFN secretion by cultures infected with 3009 and 3006. IFN-α was induced following infection and both viruses induced similar levels (Figure 4.2A). Similarly, for IFN-β no differences were observed between cultures infected with 3006 and 3009 (Figure 4.2B).

One possibility is that levels of IFN were below the levels of detection of the ELISA or transiently induced IFN early in infection were responsible for the different infectivity of DENV3 3006 and 3009. To more clearly establish whether IFN was involved, DCs were infected with 3006 and 3009 in the presence or absence of type I IFN-neutralizing antibodies. If different levels of type I IFN induced by the viruses were responsible for the phenotype observed in DCs, both viruses should infect a similar number of cells in the presence of type I IFN neutralizing antibody. Cells were infected with DENV3 strains 3006 and 3009 using an MOI of 0.3 and the infection was allowed to proceed for 48 hrs at which point the number of infected cells were determined by flow cytometry. In the absence of exogenous IFN or IFN antibody, 3009 infected 14% of the cells whereas 3006 infected less than 1% of the cells (Figure 4.2B). When exogenous IFN was added 4 hrs after infection, both viruses were completely inhibited and this

inhibition could be overcome by treating cells with type I IFN-neutralizing antibodies (Figure 4.2B). These two controls demonstrate that both these viruses were sensitive to type I IFN and that the neutralizing antibody was effective under our experimental conditions. When infected cultures were treated with type I IFN-neutralizing antibody alone, the difference between 3006 and 3009 remained indicating that a low level of endogenous type I IFN was unlikely to be responsible for the different infectivity of DENV3 strains 3006 and 3006 to primary human DCs (Figure 4.2B). The results from these IFN experiments indicated that the difference between 3006 and 3009 in their ability to infect primary human DCs was not due to a type I IFN response. In addition to the type I IFN experiments, we have also looked at surface expression of DC maturation markers (CD40, CD80, CD 83, CD86 and HLA-DR) and levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70) and did not observe differences between cultures infected with 3006 and 3009 (data not shown).

The IFN experiments with DENV3 strains 3006 and 3009 indicated that host anti-viral responses were unlikely to be responsible for the different infectivity of these viruses to human DCs. We next explored if inherent differences in the ability of these viruses to enter and replicate in cells were responsible for the observed phenotype. DC-SIGN is a receptor or attachment factor that mediates DENV infection of primary human DCs (130, 153, 194). Experiments were done to compare the binding of these viruses to human DCs. Binding was measured by estimating the number of viral genome copies bound to cells as previously described for dengue by Edgil et al. (38). To estimate specific binding to DC-SIGN, cells were treated with mannan (1 mg/ml) or EDTA (5 mM) as these molecules inhibit the binding of ligands to DC-SIGN. When we compared

the number of genomes bound for 3006 and 3009, no difference in binding was observed for 3006 and 3009 (Figure 4.3). The binding of both viruses was inhibited by mannan or EDTA demonstrating that most of the binding to DCs involved DC-SIGN and that there was no significant difference in binding between these two viruses to DC-SIGN (Figure 4.3).

We next performed ammonium chloride experiments to determine if 3006 and 3009 entered cells at different rates. DENV binds to a cell surface receptor(s) and are taken up into endosomes, where the low pH of the endosome triggers viral fusion and delivery of the viral nucleocapsid into the cytoplasm of the host cell (135, 183). Ammonium chloride (NH_4Cl) raises the pH of intracellular compartments and blocks low pH mediated viral fusion in endosomes (191). If 3006 and 3009 enter cells and fuse with endosomes at the same rate, then both viruses should display similar kinetics of inhibition when NH_4Cl is added to cells at different times after infection. On the other hand, if 3006 and 3009 enter cells and fuse with endosomes at different rates, NH_4Cl should inhibit the two viruses to different extents depending on the time of addition. DCs from 3 individual donors were infected with 3006 or 3009 at an MOI of 5 for 24 hrs. NH_4Cl (40 mM) was added at 0, 2, 4, 6, 8 and 10 hrs post infection and maintained for the remainder of the 24 hrs. As expected both viruses were inhibited completely when NH_4Cl was added at time 0 (Figure 4.4). At all time points up to 10 hrs, NH_4Cl inhibited 3006 to a greater extent than 3009. At the earlier time points the differences between 3006 and 3009 were not significant. However, at 6, 8 and 10 hrs post infection the differences in NH_4Cl inhibition were statistically significant (6 and 8 hrs post infection, $*P<0.05$, and 10 hrs post infection, $**P<0.005$). For 3006 we observed inhibition ranging from 60-

68% when NH_4Cl was added at 6, 8 and 10 hrs post infection. Under these same conditions, inhibition of 3009 ranged from 19-28%. These results indicate that the differences we observed in the ability of 3006 and 3009 to infect primary DCs are most likely due to differences in the kinetics of entry into cells. Considering the DC-SIGN binding (Figure 4.3) and NH_4Cl data (Figure 4.4) together, we conclude that the viruses differ at a point between initial attachment to DC-SIGN and fusion with the endosome.

4.5 Discussion

In this study we have compared the ability of our two low passage clinical isolates of DENV3 to infect primary human DCs. We have demonstrated that one of these strains, UNC3009, isolated from a Sri Lankan patient in 1989, infects DCs better than the other strain isolated in 1997. Both viruses had a similar genome to PFU ratio indicating that the different infectivity was unlikely to be caused by excess genomes or infectious units in one virus stock compared to the other.

Previous studies have shown that some of the nonstructural proteins of DENVs are antagonists of type I IFN signaling (81, 94, 150, 151). We performed experiments to determine if the induction of type I IFN was responsible for the differences we observed between our two viruses. We observed no differences in the amount of type I IFN induced by the two viruses. We also did not see any effect of IFN-neutralizing Abs on the percentage of DCs infected with 3006 and 3009. If type I IFN was responsible for the differences that we observed between 3006 and 3009 then we expected to see a similar percentage of DCs infected by both viruses in the presence of neutralizing Abs. We conclude that cellular antiviral responses are unlikely to be responsible for the different

infectivity of 3006 and 3009 to human primary DCs. DENV binds to the cellular receptor which leads to viral entry/fusion with endosomes. The DC-SIGN binding studies and NH_4Cl treatment experiments point to the fact that 3006 and 3009 initially attach well to DCs but that 3009 is taken into endosomes and fuses with cellular membranes at a much faster rate than 3006. This difference in entry is, most likely, responsible for the different infectivity of these two viruses to human DCs.

Following initial attachment to DC-SIGN, DENV is likely to attach to additional co-receptors that mediate entry into endosomes and subsequent low pH induced fusion. DC-SIGN is believed to be an attachment factor and not directly responsible for viral entry because DENVs can infect cells expressing DC-SIGN without the cytoplasmic internalization signal (130). The differences between 3006 and 3009 may occur because of differences in interactions with co-receptors, differences in the efficiency of internalization or because of differences in fusion. Additional studies are needed to further delineate the specific step in entry as well as the viral and host molecules involved.

We have shown in previous studies that 3006 and 3009 are linked to two distinct groups of DENV3 that are associated with outbreaks of mild and severe disease in Sri Lanka. Even though each isolate is from a distinct genotype within DENV3 subtype 3, these viruses are very closely related but genetically distinct. The differences in DC infectivity between 3006 and 3009 are likely due to genetic differences that affect the early interactions of these viruses with the host cell. What we learn from our studies with 3006 and 3009 and their interactions with DCs will help us to understand viral determinants that may be linked with disease severity and dengue pathogenesis. Future

studies are needed to determine if the early differences between 3006 and 3009 are representative of other viruses from outbreaks of mild and severe disease.

The difference in entry between 3006 and 3009 indicate the structural regions of the viral genome for potential amino acid changes. The sequence data for C, prM and E show there are only two amino acid differences between 3006 and 3009. These amino acid changes may be responsible for the differences in DC infectivity; however, nonstructural proteins are also likely to contribute to the difference. Amino acid differences in the nonstructural region may affect processing of the viral proteins and protease resulting in a higher ratio of immature/mature viral particles. Additional studies using an infectious clone are needed to determine the effect of the structural and nonstructural proteins on DC infectivity.

In a similar study by Cologna et al., differences in primary human DC infectivity between American and Southeast Asian DENV2 genotypes were demonstrated (29). They observed higher DC infection with their DHF-associated Southeast Asian genotype than the DF-associated American genotype. A mutational change at position 390 of the E protein of the Southeast Asian virus with the American virus reduced DC infectivity. In our studies we also observed differences in DC infectivity between clinical DENV3 isolates that are associated with periods of mild and severe disease. In our studies 3009, which was isolated during a period of mild disease infected a higher percentage of DCs than 3006 which is from a period of severe disease. Additionally, we observed a Ser to Pro amino acid change at position 124 that is specific for isolates from mild and severe disease periods, respectively (139). The amino change is located at a different position than what was observed by Cologna et al. for DENV2. The differences we observed in

DC infectivity as well the differences observed by Cologna et al. indicate simple conclusions can not be made regarding disease severity and DC infectivity. Further studies need to be performed to understand the viral determinants necessary for DC infection.

Studies by Diamond et al. showed differences in productive infection between low passage Thai and Nicaraguan isolates in human foreskin fibroblast (HFF) and myeloid cells (33). Later studies by Edgil et al. demonstrated that the differences between these same two groups of viruses were not due to viral binding, entry or uncoating (38). They determined that the differences were occurring at the level of translation, where the viral RNA of low passage Thai isolates was more efficiently translated than the Nicaraguan isolates. This defect in translation was attributed to the 3' UTR of the viral genome. In our studies the difference in infectivity appears not to correlate with translation but to occur earlier at the initial step of viral entry. Similar to the findings by Edgil et al., we did not observe differences between 3006 and 3009 at the step of viral binding, but NH_4Cl treatment experiments suggest the differences occurs before uncoating of the virus. Future studies are needed to determine if there are additional differences between 3006 and 3009 at the level of translation and how this may affect DC infectivity.

4.6 Acknowledgements

We would like to thank Barbara Sherry, Jonathan Serody, and Lishan Su for their experimental suggestions and insight. We would also like to thank Karen McKinnon for her help with the DC protocols and isolations.

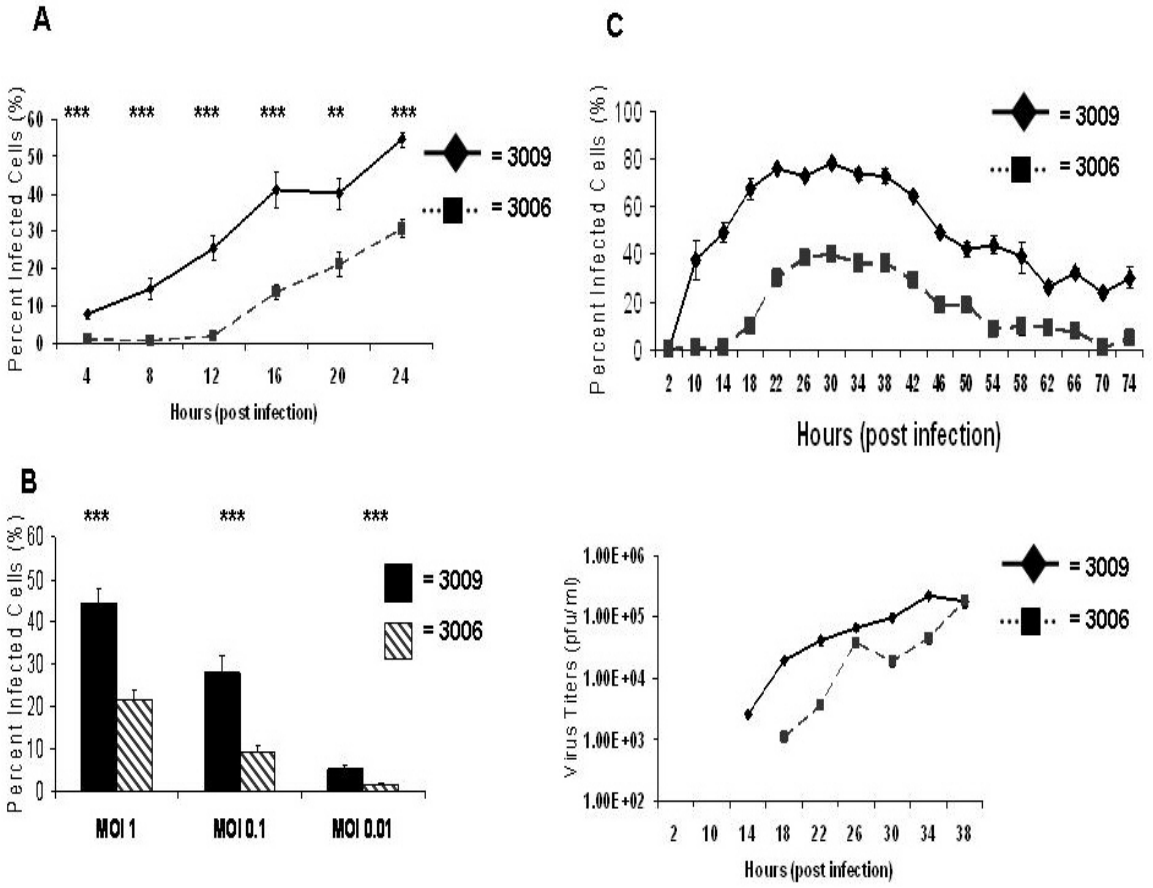

Figure 4.1. Clinical isolates 3006 and 3009 differ in their ability to infect primary human DCs. (A) 24 hr time course of infection for DCs infected at MOI of 5. Shown are the averaged data from five individual DC donors. Percent infected cells ranged between 8% and 55% for 3009 and 1% and 30% for 3006. The percentages of infected cells were statistically different for all time points (All time points except 20 hrs had ***P<0.0005, 20 hrs had **P<0.005). (B) DCs infected at MOI of 1, 0.1 or 0.01 and analyzed at 48 hrs post infection. Shown are the averaged data from eight individual DC donors. Infection percentages for 3006 ranged from 1-22% and for 3009 from 5-45%. The differences observed at each MOI were statistically significant (***P<0.0005). (C) 74 hr time course of infection for DCs infected at MOI of 5. Shown are the averaged data from one DC donor. (C) Upper panel: Percentage of infected cells. Infection percentages peaked at 30 hrs post infection with 80% and 40% infection for 3009 and 3006 respectively. All time points except 2 and 58 hrs were statistically significant with P< 0.05. (C) Lower panel: Viral titers measured by immunofocusassay. Each sample was performed in triplicate. The differences in the amount of progeny virus detected in the media was significantly different (*P<0.05) until 38 hrs after infection at which point there was no difference throughout the remainder of the 74 hr time course. All time points were statiscally significant with P< 0.05. (A-C) All experiments were analyzed by flow cytometry. Each sample from each donor was performed in triplicate. Displayed is average from all donors ± SEM.

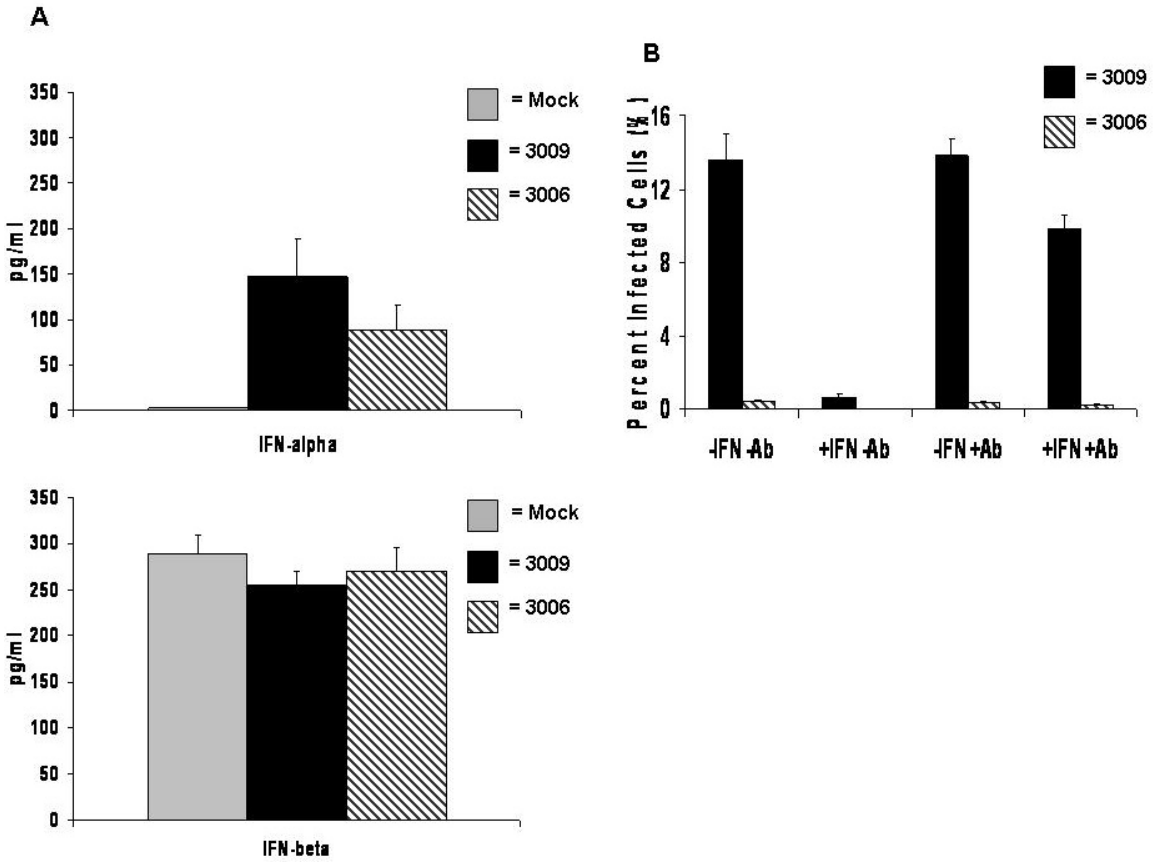

Figure 4.2. Differences between 3006 and 3009 are not due to type I IFN. (A) Levels of type I IFN measured by ELISA. (A) Upper panel: IFN-α ELISA. (A) Lower panel: IFN-β ELISA. DC samples collected from eight individual donors were infected with either 3006 or 3009 over a range of MOIs DC samples for mock infection were collected from four donors. Mean IFN-α levels for 3009 and 3006 were 146.7 ± 81.8 and 88.8 ± 52.7 pg/ml, respectively. Mean IFN-β levels for 3009 and 3006 were 256.0 ± 14.08 and 270.3 ± 25.7 pg/ml, respectively. (B) Effect of type I IFN neutralizing antibodies on DCs infected with 3006 and 3009. DCs were infected at MOI of 0.3 in the presence or absence of IFN-α and IFN-β neutralizing Abs and the infection was allowed to proceed for 48 hrs at which point the number of infected cells was determined by flow cytometry. As a control, exogenous IFN-α and IFN-β were added 4 hrs post infection. Shown are the averaged data from two DC donors ± SEM. In the absence of exogenous IFN or IFN antibodies, 3009 and 3006 infected 14% and 1% of the cells, respectively. The addition of exogenous IFN 4hrs after infection completely inhibited both viruses, this effect was overcome by treatment of type I IFN-neutralizing Abs.

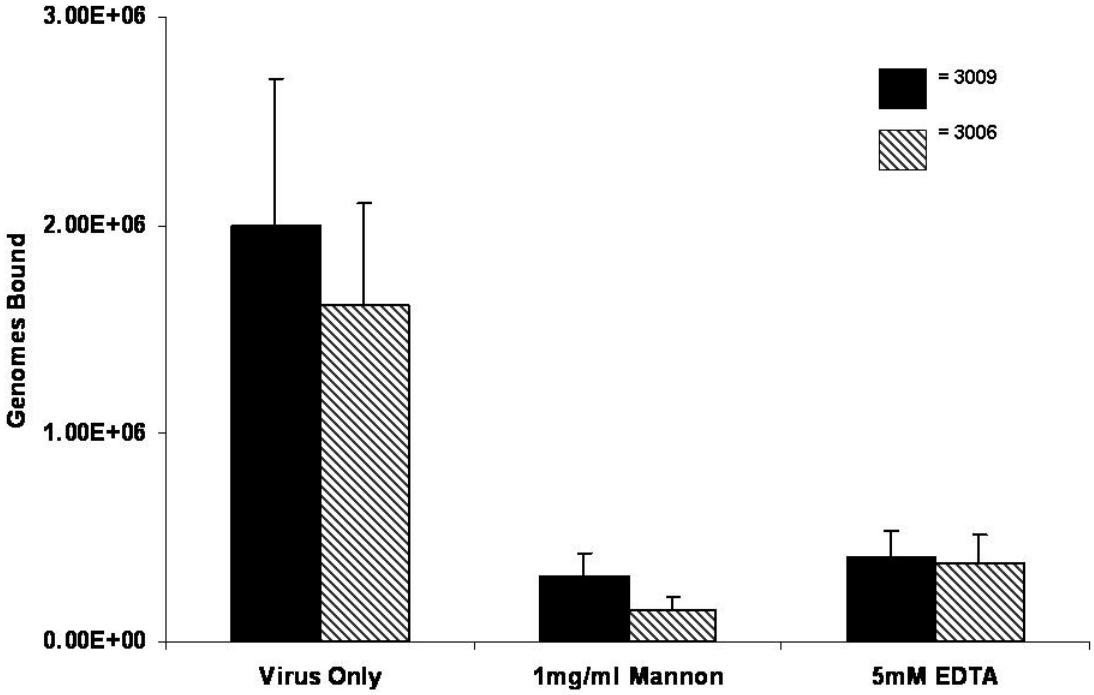

Figure 4.3. 3006 and 3009 do not differ in their ability to bind DCs via DC-SIGN. Experiments measuring binding of 3006 or 3009 to DC-SIGN were performed at 4°C for 3 hrs at an MOI of 5 and analyzed by real time PCR. Mannan (1 mg/ml) and EDTA (5mM) were used as controls. Shown are the averaged data from three individual DC donors ± SEM. The amount of genomes bound for 3006 and 3009 were 1.62×10^6 and 1.99×10^6 respectively. In the presence of mannan (1 mg/ml), binding for 3006 and 3009 was reduced to 1.56×10^5 and 3.16×10^5 genomes bound, respectively. In comparison to binding in the absence of mannan the reductions were statistically significant (*P<0.05). Binding in the presence of EDTA (5 mM) was reduced for 3006 and 3009 to 3.77×10^5 and 4.06×10^5 respectively. The reduction for 3006 in the presence of EDTA was statistically significant (*P <0.05), but the reduction for 3009 was not quite significant (P=0.0533).

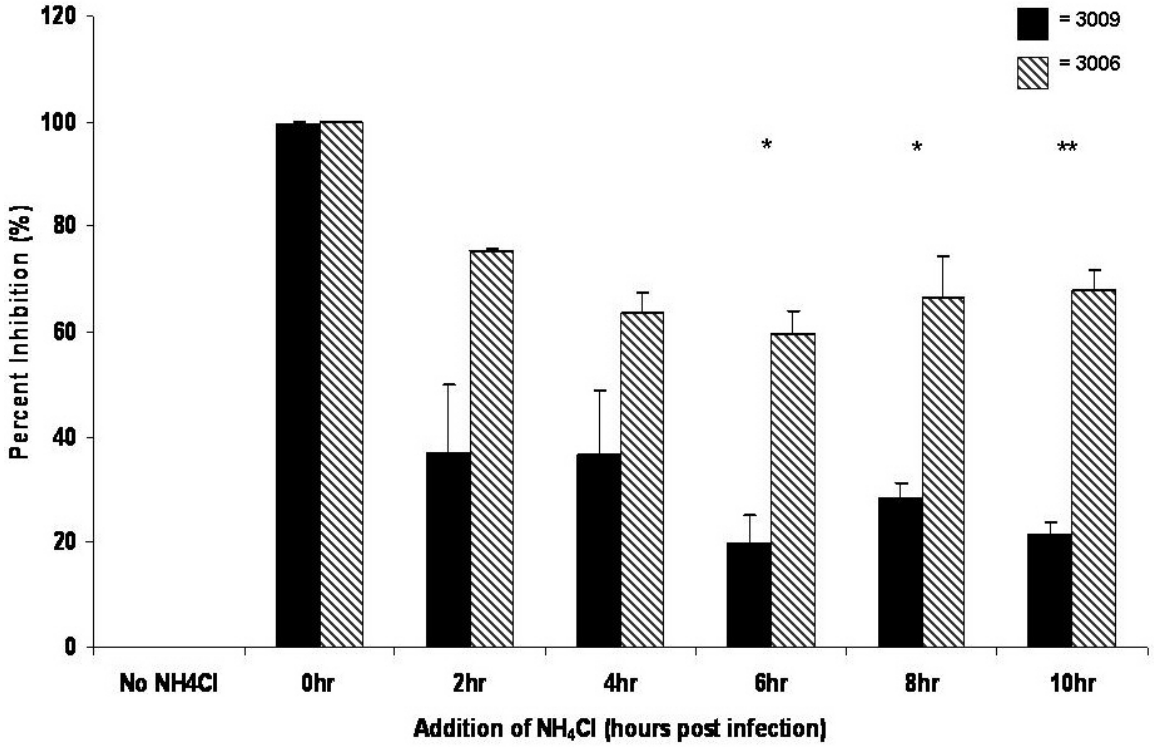

Figure 4.4. 3009 is more efficient at viral entry/fusion than 3006. DCs infected with 3006 and 3009 at an MOI of 5 were treated with 40 mM NH_4Cl at 0, 2, 4, 6, 8 or 10 hrs post infection up to 24 hrs post infection. Infection was measured by flow cytometry. Percent inhibition was calculated by: ((No NH_4Cl control – NH4Cl treat sample)/No NH_4Cl control) * 100. Shown are the averaged data from three individual DC donors ± SEM. At 2 hrs post infection inhibition induced by NH_4Cl was 75% and 37% for 3006 and 3009 respectively. For the earlier time points the differences between 3006 and 3009 were not significant. At 6, 8 and 10 hrs post infection the differences in NH_4Cl inhibition were statistically significant (6 and 8 hrs post infection, $*P<0.05$, and 10 hrs post infection, $**P<0.005$).

Chapter 5

Discussion

5.1 Summary

There are three main proposed mechanisms to explain the development of severe disease during dengue infection. The first is antibody-dependent enhancement, in which antibodies from a previous dengue infection lead to enhanced secondary dengue infection resulting in DHF (70, 73). The next is viral virulence, where certain dengue virus serotypes and genotypes are more virulent than other strains (139, 172, 204). The third mechanism, immunopathogenesis, is based on the idea that the host's immune response is responsible for the capillary leakage seen in DHF and DSS patients (10). These proposed mechanisms are not mutually exclusive and are likely to be interconnected and jointly responsible for disease outcome. The focus of my dissertation has been to develop new tools to study dengue and to use these tools to analyze interactions between DENV3 and primary human DCs.

5.2 Overview of dengue field

Cells of the mononuclear phagocytic lineage, such as monocytes, macrophages and DCs, are believed to be the primary targets of DENVs during a natural infection.

DENV infection of DCs is mediated by the attachment factor DC-SIGN (153, 194). Polymorphisms in the DC-SIGN promoter (177) and HLA alleles (19, 186) have been shown to influence dengue disease outcome in people. Cytokines are also believed to be important in the development of DHF. Investigators have observed that cytokines that favor a switch from a Th1 to Th2 T cell phenotype increase the likelihood of severe disease (20). However, without an animal model of disease, the study of dengue pathogenesis is difficult. Here we have used a primary human cell culture model to study dengue pathogenesis.

5.3 Assay development

Most investigators studying dengue have relied on lab-adapted strains of DENVs, which are likely to be quite different from low passage clinical isolates. One reason for using lab-adapted strains is that clinical isolates grow poorly in cell culture and do not efficiently form plaques. Assays such as the plaque assay and endpoint dilution work well for lab-adapted strains of DENV but they are time consuming and difficult to perform with clinical isolates. We have taken advantage of flow cytometry to follow infection of cells with clinical isolates and also to develop new assays for studying dengue.

In chapter 2 we described and validated a flow-based assay for titrating DENVs that overcame some of the limitations of the plaque assay. Our FACS-based assay allowed us to titer lab-adapted and clinical DENV isolates in 24 hrs rather than 6 days as with plaque assay and endpoint dilution. Comparison of titers measured with the FACS, plaque and endpoint dilution assays resulted in similar but not identical viral titers. We

suspect that the ability of some viral strains to infect the different cell lines used for each assay and/or differences in plaquing efficiency may account for the discordant titers. Another explanation for the difference in viral titers may be the time at which each experimental method was evaluated for viral infection. The FACS assay conducted at 24 hrs after infection measures the ability of dengue virus particles to enter a cell and produce viral antigen. The assay does not require virus assembly or cell to cell spread. In contrast, to obtain a positive signal in the plaque or endpoint dilution method, virus particles have to enter cells and spread from cell to cell over a period of 5-7 days. One of the main limitations of the FACS assay is that it can only be used for samples with a titer greater than 1×10^4 infectious units/ml. At titers below 1×10^4/ml, the small number of infected cells in a well were below the level of detection of the FACS assay. In this regard, the plaque assay is a more sensitive method that can detect smaller amounts of virus. Another limitation of the FACS assay is it requires costly equipment and trained staff to analyze the samples, which is not feasible for developing countries or laboratory field work. Even with its limitations, FACS is still a powerful and effective tool for titrating virus. One of the advantages of the FACS assay is that many more virus strains can be titered in the normal 6 day time span required for the endpoint dilution and plaque assay. Furthermore, the FACS assay can be used to titrate clinical isolates that do not form clear plaques on cell monolayers. Additionally FACS can be used to monitor infection of suspension cells.

In addition to using FACS to titrate DENVs, we report that it can be used to measure the ability of antisera to neutralize dengue infection. One of the current methods for measuring antibody neutralization, the PRNT, is a variation of the plaque assay used

for viral titration. Similar to the set backs in titrating clinical isolates using the plaque assay, the PRNT poses the same obstacles in measuring neutralization titers. When we compared the neutralization titers for two human dengue immune sera tested with the FACS (FNT) and plaque neutralization assays (PRNT), the titers measured by each assay were similar. Further studies need to be performed to validate the assay using reference sera to determine if the FACS neutralization assay can be used to identify the infecting serotype of DENV and to distinguish between primary and secondary infections. A major advantage to a FACS neutralization assay is that it could be used to measure neutralization of DENV isolates, irrespective of their ability to form plaques.

5.4 Future directions for FACS assay

The FNT has become a widely used and effectivel tool in our laboratory to study antibody neutralization. Members of our lab have proceeded to further validate the assay using reference sera from the WHO from primary and secondary infections. Using the assay they are able to distinguish between primary versus secondary dengue infections and to identify the infecting serotype. The FACS neutralization titers they measured were similar to titers determined by other labs from around the world for the same reference sera using other neutralization methods. They have further modified the assay to be used in a 96-well format and have expanded the range of cell types to include U937 + DC-SIGN expressing cells in addition to Veros (manuscript submitted, Kraus et al.).

5.5 Interactions between DENV3 and human DCs

After having developed flow-based methods, we next used these methods to study interactions between DENV and human DCs. DCs are important antigen presenting cells and likely the initial target of DENVs during infection. DCs have been shown to be permissive to DENV infection, which is mediated by DC-SIGN (153, 194). The ability of the DCs to present antigen, stimulate T cells, and secrete cytokines suggest an important role for them in the initial and downstream immune responses that are key in dengue pathogenesis.

In chapter 3 we reported that DENV3 blocks activation of primary human DCs but not the uninfected bystander DCs in the culture. This data was further supported by data from Palmer et al. showing similar results for DENV2 (160). Our results suggest that DENVs target DCs to block their activation, which leads to suppressed antigen presentation. Our hypothesis is supported by data from Palmer et al. showing that DENV infected DCs had a reduced capacity to stimulate T cells and underwent apoptosis (160). Overall, these findings indicate a possible immune evasion strategy by DENVs to compromise the antigen-presenting function of DCs, which is potentially important in dengue pathogenesis and the development of DHF.

We next focused on the interactions of clinical DENV3 isolates from periods of mild and severe disease and their interactions with DCs. In order to detect a statistically meaningful difference between the pre- and post-DHF virus groups, we would need to use a large number of strains belonging to each genotype and DCs from a large number of donors. As this is currently not feasible, we decided to focus on just two strains (3009

and 3006) belonging to the pre and post groups. Future work will address if differences observed between these two viruses apply to the larger pre- and post-DHF groups. We observed clear differences between 3006 and 3009 in their ability to infect human DCs, where 3009 infected a higher percentage of the cells. These differences were observed over a 5 day period and over a range of MOIs.

Viruses 3009 and 3006 may differ with respect to their ability to infect DCs because host cells respond differently to these viruses or because of inherent differences in the binding, entry or replication of these viruses in human cells (10). If the differences were due to a host immune response, we believed that type I IFN was a likely candidate since DENVs have been shown to block IFN signaling through the JAK/STAT signaling pathway (94, 150, 151).

To test if the differences between 3006 and 3009 were due to a host immune response, we initially looked at the levels of type I IFNs and the effect of IFN-neutralizing antibodies on infection. We did not find any evidence to indicate that type I IFN was responsible for the differences between these viruses. We then went on to test the second hypothesis which suggests that the differences between 3006 and 3009 were due to inherent differences in the viruses. We first looked at binding and did not detect differences between 3006 and 3009 in their ability to bind DC-SIGN. We then looked at the entry kinetics of 3006 and 3009 by adding NH_4Cl at different times after infection. NH_4Cl raises intracellular pH and blocks fusion. We observed that 3009 was able to fuse and exit the endosome much earlier than 3006. These results suggest that the differences between 3006 and 3009 occur early during the viral life cycle between virus binding and fusion. The differences that we observed early on may be due to structural differences

between 3006 and 3009. Structural differences could result in differential recognition by the second viral receptor, or result in improper conformational changes during fusion between the viral envelope and cellular membranes. Further studies will need to be done to pinpoint exactly when during the viral life cycle the differences between 3006 and 3009 occur and what portions of the viral genome contribute to this phenotype.

5.6 Future Directions

Since we have determined that the differences between 3006 and 3009 occur early during the viral life cycle, the phenotype we observed in DCs is likely due to inherent differences between the isolates and strongly supports that it is not due to a host immune response. With the development of an infectious clone of DENV3 by Blaney et al. we can make mutational changes to specific regions of the genome to determine the regions responsible (11). Additional experiments using microarray analysis looking at the early stages of DC infection by 3006 and 3009 could help us to understand how these early differences might affect downstream immune responses. Overall, these experiments will give us better insight into how two specific DENVs interact with human cells, which will enable us to apply what we learn to our pre- and post-DHF groups of viruses to understand how these interactions may shape the out come of disease during dengue infection.

Differences in viral entry have been shown for other flaviviruses such as Japanese encephalitis (JEV) and Yellow fever (YFV) viruses (77, 200, 201). For yellow fever, a mutation at amino acid position 360 of E protein decreased viral entry in Veros and mouse neuroblastoma cells but did not confer binding differences in Veros when

compared to the wildtype parental strain (201). This difference in entry had no effect on mouse neurovirulence and additional substitutions at positions 261 and 303 suppressed the entry defect (200). The location of amino acid position 360 in the putative receptor binding region of domain III suggests an important role for a negative charge cluster that is optimal for the function of this domain in virus-cell interactions beyond the stage of viral attachment (200). For JEV, mutations at amino acid positions 52, 364 and 367 of E protein resulted in decreased viral entry in Vero cells but had no affect on replication and production of infectious virus (77). These mutations also lead to reduced pathogenicity in mice but did not influence propagation in mice (77).

Differences in viral entry may be important in the downstream effects of the host immune response such as antibody neutralization. Viral determinants responsible for entry defects could lead to downstream effects in viral replication or alter recognition of neutralizing antibodies. Inefficient antibody neutralization could result in increased viral infection through antibody-dependent enhancement and decreased viral clearance. In the context of secondary infection, antibodies from a primary infection that inefficiently neutralize virus during a secondary infection may lead to prolonged viral infection resulting in a hyper immune response and the development of severe dengue disease.

When an animal model becomes available it would be important to look at DENVs in the context of primary and secondary infection. Viruses that differ in entry could both lead to a protective immune response during primary infection but result in the induction of very different immune responses during a secondary infection. The interaction of DCs and memory T cells, neutralizing antibody responses, and cytokine production will also be important to investigate.

To determine how differences in entry between 3006 and 3009 apply to the pre- and post-DHF viruses, viral infection within the first 24 hrs as well as the initial steps of binding and entry are important steps to examine. Additional studies are needed to look at the early time points of infection in Veros and U937 + DC-SIGN cells for 3009 and 3006. Originally we believed that the differences between 3009 and 3006 were specific to DCs, however, since the difference occurs during viral entry, these differences are likely true for other cell types as well.

What we learn from additional studies with 3006 and 3009 may aid in the development of a dengue vaccine that utilizes defects in viral entry to induce a protective immune response. However, if the differences we observes in entry affect recognition of neutralizing antibodies, this could be a problem for vaccine development and may actually enhance the development of severe disease which is a current concern in dengue vaccine research.

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