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Dengue Virus Targets and Efficiently Replicates in Megakaryocytes

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Abstract

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By Kristina Clark

The vectorborne pathogen dengue virus (DENV) infects millions of persons worldwide and can be lethal in the young and the old. In spite of decades of research, the primary cellular target responsible for causing high DENV viremia in humans remains elusive. Several cell lineages (dendritic cells, macrophages/monocytes, B lymphocytes, endothelial cells, and megakaryocyte-erythrocyte progenitor cells) have been implicated as targets and have been linked to different pathomechanisms. The widely recognized clinical findings of thrombocytopenia and coagulopathy observed in dengue patients directed our investigations towards the relationship between DENV and megakaryocytes, the platelet progenitors. We have examined the permissiveness of megakaryocyte-erythrocyte progenitor (MEPs) cell lines, primary rhesus macaque bone marrow cultures, and primary human bone marrow tissue and have found they are readily susceptible to DENV serotype 2 (DENV2) infection leading to productive replication. Sequential analyses of bone marrow samples from infected monkeys showed that DENV2 viral antigens were included in multinucleated cells that expressed CD61 and CD41a early post infection, at times corresponding with peak viral titer, suggesting that megakaryocytes are a target cell contributing to viremia. Of interest, after the viral peak, DENV2 antigen gradually shifted to monocyte/macrophage cells, suggesting these cells play a role later in infection not critical for initial viremia. These results have important implications for the development of dengue antivirals, the generation of effective vaccines, and the safety of blood and platelet donations in DENV endemic areas.

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CHAPTER 1

Introduction

DENGUE VIRUS GENERAL BACKGROUND

Classification and serotypes

Dengue virus (DENV) is classified in the Flaviviridae family, which includes the *Flavivirus*, *Hepacivirus*, *Pegivirus*, and *Pestivirus* genera (148, 240). Of these, DENV is a member of the *flavivirus* genus, which includes the species *Zika virus*, *yellow fever virus*, *tickborne encephalitis virus*, *Japanese encephalitis virus*, and *West Nile virus* (84). The virus' single stranded 11.5 kilobase RNA genome encodes one large polyprotein that is cleaved into individual proteins by cellular (signalase and furin) and viral (NS-2a/NS-3) proteases. DENV synthesizes three structural—envelope (env), premembrane (prM), and capsid (C)— and seven nonstructural (NS) proteins—NS-1, NS-2a, NS-2b, NS-3, NS-4a, NS-4b, and NS-5. Packaged into the host-derived lipid bilayer of the virus are structural proteins env and prM, which upon maturation are cleaved into two proteins: the pr peptide and the integral membrane (M) protein (147). Capsid protein associates with and packages the positive-sense, single-stranded RNA genome. The nonstructural proteins play roles in virus replication, assembly, and immune modulation.

DENVs can be divided into four serotypes, 1 through 4, all of which can cause severe disease (85, 264). Because of multiple factors (globalization, poor vector control strategies, insecticide

resistance, poor urban planning, lack of predators, global warming, etc.) this virus is spreading and infiltrating all corners of the globe (86, 116, 160, 175, 179, 192). All four serotypes are endemic in the countries where DENV outbreaks occur (mostly in the tropical and subtropical regions) (91), and DENV has the potential to spread further into the northern latitudes if the virus evolves to better transmit through the *Aedes albopictus* mosquito vector, which inhabits temperate environments (20).

Lifecycle

DENV is a vectorborne pathogen that is transmitted through the bite of an infected *Aedes* spp. mosquito, primarily *Aedes aegypti* and *Aedes albopictus*, although other species have been implicated (85, 87). Virus infection of the mosquito is a relatively inefficient process but occurs at a high frequency capable of perpetuating the insect-to-human transmission cycle in nature. The insect has a number of defense mechanisms to prevent infections from pathogens: salivary gland fluids, peritrophic matrix in the midgut, chitin in the trachea, hemocytes, and fat bodies (17, 99, 154, 247, 289). Bacterial coinfections can also reduce vector competence (209). When the virus successfully bypasses the insect immune system, it can infect the insect midgut epithelial cells (40). The virions then traffic to the hemocoel, where they disseminate to secondary organs, like the salivary gland. Once the salivary gland gets infected, it starts secreting virus into the saliva, making the mosquito capable of transmitting DENV to new human hosts for the rest of the it's life. Female mosquitoes can also pass down viral infections to their offspring. The initial infection of mosquitoes takes about 7–11 days to complete, but some studies suggest that infection might progress faster, depending on virus strain, mosquito genotype, and environmental parameters (e.g., temperature) (21, 151, 223, 271).

In forest environments, DENVs transmit back-and-forth between mosquitoes and nonhuman primate species through what is known as the sylvatic cycle (85). DENV sylvatic strains that dominate in rural areas are genetically distinct and are believed to cause mostly mild disease or

transmit to humans only rarely (262). However, this aspect is under investigated. In urban settings, DENV most often transmits through *A. aegypti* and, in combination with the high density of susceptible human hosts, outbreaks occur readily. In some rare areas, like Hawaii, the *A. albopictus* vector is primarily responsible for sustaining urban transmission (63). Evidence that DENV can cycle through other mammalian hosts is limited (53, 54).

VIRAL TARGETS

DENV cycles back-and-forth between humans (or nonhuman primates) and mosquitoes in the natural environment. The virus enters the mosquito proboscis through blood meals, replicates in the insect midgut, and traffics to and infects secondary organs (salivary gland and neurons). Hence, the *A. albopictus* larva-derived C6/36 cells (or whole insect larva) have been commonly used to propagate virus stocks and investigate features of the DENV lifecycle. However, several permissive cell types have been reported from analyses of human samples, and the cell lineage(s) that are capable of replicating virus and causing high viremic titers observed *in vivo* (1 X $10^{8.5}$ 50% mouse infectious dose per mL) remains a subject of debate (89, 263).

Upon blood meal, the mosquito repetitively probes the skin in search for a capillary, giving the virus many opportunities to infect cells within the skin but also within the blood vessel and in more distant tissues/cells via rapid dissemination. After experimental subcutaneous inoculation of DENV in the rhesus macaque, infectious DENV can be found distributed widely throughout the skin during and after the course of viremia (159). The skin-resident immature myeloid dendritic cells (Langerhans) (100, 169) and another dendritic cell subset (282) become infected with DENV and are likely one of the cell populations that initially acquire virus after inoculation. Immature DCs are capable of virus up-take and secreting virus to infectious titers of approximately 1 X 10^4, with low (5%–8%) levels of cells displaying antigen, although the titers and percentages can be influenced by different cytokine treatments. However, the infectiousness of this virus has been disputed. Progeny

virus obtained from infected DCs *in vitro* could not re-infect primary DCs *in vitro*, but the infectiousness could be assessed with cancerous cell lines, such as Vero and 293T (55). It has been suggested that the DCs are initially infected but primarily serve to shape the immune response against DENV and that the progeny virus from DCs then progress on to infect another target cell (141, 163).

Similarly, macrophages (161) and monocytes (48, 125) have been evaluated for their permissiveness and contribution to pathogenesis (61). Some monocytes reside in the skin and can interact with the virus there early on during infection. However, the majority of monocytes that come in contact with DENV undergo apoptosis or pyroptosis, with only around 2% becoming infected (12, 177, 251, 282). Low virus titers (1×10^4) are achievable *in vitro* (12, 251). One mechanism capable of improving the infection rates in phagocytes *in vitro* is antibody dependent enhancement (ADE), by which non-neutralizing, cross-reactive DENV antibodies facilitate virus entry into cells via the IgG Fc receptor. ADE increases the frequency of infected cells, resulting in higher virus titers, which might lead to more severe dengue disease symptoms *in vivo* (149, 176). Since virus titers stemming from infected monocytes (in the absence of antibodies) are usually low, they are not regarded as the host generally responsible for viremia at least during primary infection; however, monocytes are likely targeted by DENV and contribute to dengue disease pathology and may show higher rates of infection during secondary infections in the presence of non neutralizing cross-reactive antibodies.

Because vascular leakage occurs during severe disease, investigators have assessed the permissiveness of endothelial cells, which line the inside of blood vessel walls. When using large inocula, primary human umbilical vein endothelial cells (HUVECs) can yield high infectious titers, but more often low titers and low levels of permissiveness have been observed (2, 12, 105). Low percentages of these cells stain positive for antigen, and the numbers do not increase with the addition of antibodies in ADE experiments. Infection in these cells is restricted to the first 24 hours,

as interferon is quickly stimulated and limits DENV replication (47). Although endothelial cells can be found swollen *in vivo*, they inconsistently stain positive for DENV antigen (16, 25, 202, 224). DENV appears to also target endothelial cells, but not for the purpose of generating high titer viremia.

Because of the weakly specific and highly cross-reactive antibody response against DENV seen in dengue fever patients (49, 218), B-cell permissiveness has also been evaluated. Investigations using cancerous B-cell lines indicated they could replicate virus, but primary B-cells were a poor substrate for DENV, requiring *in vitro* culture for several days before developing replicative capacity (45, 248, 256). Increasing MOIs and adding antibodies to promote ADE and enhance B-cell permissiveness still produced only low virus titers (146), suggesting that B-cells are likely not permissive or involved in pathogenesis.

Because hepatomegaly and fulminant hepatic failure can be a component of disease in dengue patients (132, 270), the involvement of this tissue was evaluated in DENV infections (244, 257). Primary hepatocytes are poorly permissive *in vitro*, capable of infection and amplifying virus approximately 10-fold when given high MOIs (244). Patient liver biopsy tissues contain cells that stain positive for DENV antigens late in infection (111); however, these cells are predominantly the liver-localized macrophages (Kupffer cells) and endothelials, which are already known to contribute to pathology. Liver disease might more likely be attributed to the immune response rather than resulting from direct infection of liver epithelials.

Another cell type under investigation is the bone marrow resident, platelet-producing megakaryocyte because platelet abnormalities are a hallmark of dengue disease. A number of bone marrow cell populations become suppressed at early time points prior to and/or during fever onset (1, 24, 94, 119, 130, 158). The megakaryocytes and erythrocyte progenitors are two of these populations; when the bone marrow cell populations begin to repopulate and become hyperplastic, cells of the megakaryocyte lineage are replaced by younger, less-differentiated versions (24, 182, 183).

Megakaryocytes are deficient in interferon production (283), which might make them inherently susceptible to viral infections (30, 37, 284). In one study, the spleen was infiltrated by large binucleated cells that stained positive for DENV antigen (111). Earlier research suggested that the closely related erythrocyte progenitor population was the target responsible for producing DENV (180). However, dengue antigen and virus particles can be found in platelets, which are the byproducts from megakaryocyte differentiation (189-191). Also, $CD61^+$ cells isolated from DENV2-infected rhesus macaque bone marrow contained infectious virus that could readily be propagated in cell culture (190). Later chapters in this report outline further evidence for megakaryocyte lineage involvement in DENV propagation, suggesting that the megakaryocyte population is potentially an important permissive cell type that might explain the high DENV viremia, coagulopathy, and altered platelet function seen *in vivo* (24).

For *in vitro* propagation of DENV, most investigators have used either insect cells or convenient (usually interferon-deficient kidney epithelial) cell lines. Some concern has been raised about select studies that use DENV produced in kidney cell lines—which likely have different protein expression and glycosylation patterns than the true primary *in vivo* target cell type(s)—for investigations of DENV structure, host-pathogen interactions, vaccine development, and drug design. Single base pair mutations and alternative glycosylation have the ability to alter protein structure to the extent that antibodies no longer bind. A high degree of similarity *in vitro* is needed to adequately predict *in vivo* relationships.

VIRUS ASSEMBLY AND STRUCTURE

Virus structure

The proteins making up the dengue virion are capsid (C), premembrane (prM) (and/or the cleaved form—membrane M), and envelope (env) (85). Encapsulating the RNA genome is the capsid protein core, having a radius of 150 angstroms and making up 60% of the virion shell (126, 290). The

core is disordered, not forming a consistent enough structure to achieve a high clarity image by nuclear magnetic resonance imaging or crystallization (126). Its structure is somewhat blurry due to the four hydrophobic alpha helices of C that variably project out, inserting themselves into the lipid bilayer and ensuring linkage of the viral genome with the virion. Potentially these loops interact with env or prM/M within the lipid bilayer, but currently env and prM/M interaction with C during virus assembly has not been observed and thus is considered an uncoupled process. PrM is known to function as a chaperone protein for env, facilitating proper processing and folding (260). Mutations in prM have been associated with ER retention, misfolding, reduced maturation/cleavage, and decreased infectiousness (9, 113, 260). The remaining M protein, left over in the virion after prM cleavage, consists of 20 amino acids in the ectodomain above the lipid bilayer and two transmembrane alpha helices (260, 290). The short chain of M lies beneath env and is thought to act as a latch, keeping env from fusing in its mature "metastable" pre-fusion conformation (291, 293). Protonation of three histidine residues in the hydrophobic M-env pocket initiate fusion events (292).

The major protein on the surface of the virion is the env (Figure 1.1). It has an ectodomain, a stem of two alpha helices that closely interact with the host-derived lipid bilayer, and an anchor of two transmembrane alpha helices (260, 290). The ectodomain is composed mainly of beta barrels and has been divided into three domains (Ds): DI, DII (containing the fusion loop), and DIII (an Ig-like domain that projects out and interacts with the receptor). The conserved fusion loop in DII is buried between the DI and the DIII of the adjacent monomer.

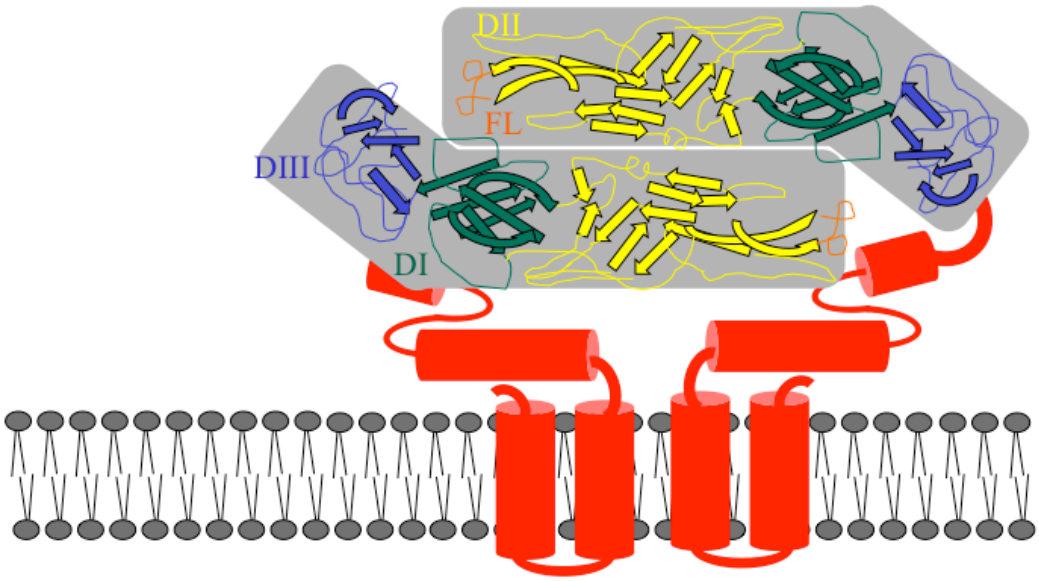

Figure 1.1. Diagram of a DENV **envelope protein dimer in the lipid bilayer.** Dimeric envelope protein is has four alpha helices and an ectodomain, which is divided into three domains (Ds) mainly constituted of beta barrels. DI (green), DII (yellow), and DIII (blue) are shown with respect to the fusion loop (FL, orange), which is buried between DI and DIII of adjacent envelope monomer.

Ultrastructural investigations of immature virus have often been performed with virions produced from insect cells treated with ammonium chloride, which prevents PrM cleavage by furin and thus inhibits virus maturation (288). The mature form can then be acquired by incubating the immature virions with furin *in vitro*. Immature virions have 90 env-prM heterodimers, oriented in trimeric spikes over the surface of the lipid bilayer, with the prM protein covering the portion of env that is fusion-capable. These structures are easily distinguishable from mature forms by EM imaging because they are wide and spikey, having an average diameter of 60 nm. DENV is unique among the flaviviruses because the immature/mature forms can fluctuate back-and-forth with changes in pH, whereas with tickborne encephalitis virus, the env dimers are more rigid and get locked into place at low pH (239, 288). However, a recent investigation suggests that this flexibility might be a unique feature of DENV2 or select strains rather than a general DENV property (124).

The mature virion averages around 50 nm in diameter, having a mostly smooth outer surface containing 90 env homodimers (126, 290). The env protein is arranged with the monomers oriented face-to-face in rafts of three homodimers. The rafts are oriented antiparallel to each other in a herringbone pattern. In this conformation, the lipid bilayer lying below the viral proteins is inaccessible. Viral protein symmetry (or the orientation of the viral proteins in relation to each other) is usually described with triangulation (T) numbers. When assessing a virus's T-symmetry, triangles are aligned over the virus structure to designate 5-fold, 3-fold, and 2-fold vertices. The herringbone orientation cannot be modeled with triangles, so the mature form of DENV is referred to as having no classic T = 3 quasi-symmetry. Interestingly, a T = 3 symmetry is predicted as one of the DENV's intermediate forms during fusion (126). DENV engages in class II fusion, so the conformation of env switches from a dimer to a trimer post fusion (178).

It was discovered that known neutralizing antibodies could not bind to the surface of DENV2 when it was purified and fixed at 4°C, so ultrastructural investigations were conducted with

DENVs incubated and fixed at 37°C (71). At the higher temperature, the conformation changed: the two alpha helix stem of env stretched out and the orientation of one env monomer in relation to the other turned, creating viruses with a more-jagged surface and a wider diameter. Other differences were noted, such as the absence of protein at the 3-fold vertices. However, when looking at structure of other DENV serotypes, these changes were not observed with changes in temperature (62, 124).

Alternative virus-induced structures

The ultrastructural investigations of DENV have mostly been performed with DENV2 propagated in insect cells (71, 83, 96, 113, 126, 185, 201, 287, 288). Fewer studies acquired images of the other DENV serotypes (122, 124, 181) or images of DENV propagated in other cell types: mammalian kidney (165, 234, 243, 275), liver (114, 275), and B-cell (248, 256). Virus structures have been captured *in vivo* rarely (189). Because of the limited diversity in DENV structure studies, DENV might be more pleomorphic than previously appreciated.

A number of additional virus-induced structures have been observed, sometimes rarely, in DENV-infected cells: filamentous forms (96), dense particles (96, 122, 165), fuzzy virions (177), satellite particles (96), subviral virions (9, 68, 113), and microparticle-associated virions (248). The filamentous form was observed with the DENV2 strain PR-159 in C6/36 cells but not with other isolates (96). Satellite particles found in experiments using C6/36 (96) varied in size but could be a number of things: escaped ribosomes, virus cores, or subviral virions (also called the slowly sedimenting hemagglutinating component) (96, 234). Dense particles potentially are subviral virions, although subviral virions are presumed to be noninfectious (96): high-density infectious particles (potentially virus cores) have been isolated from density gradients of concentrated supernatant (234). Large fuzzy virions, approximately 100 nm in width, were noted in C6/36 stock virus entering monocytes at the beginning of an infection (177). Virions have rarely appeared inside of microparticles (248). Also, DENV antigen positive vesicles have been observed blebbing off the

surface of infected C6/36 cells (96). The significance of these structures (whether they transmit messages or transmit infection to other cells) remains to be determined, underscoring the need for further exploration of parameters and determinants of DENV replication in various host cells.

For TBEV and DENV, transfection of env and prM genes alone into cells without any other viral components results in the formation of virus-like particles (9, 68). These particles, termed subviral particles or slow-sedimenting hemagglutinin components, are smaller (14–32 nm) and more heterogeneous, potentially because the core (C) protein, which usually makes up 60% of the diameter, is absent, most likely along with the viral RNA (9, 113, 234). Subviral particles are a frequent occurrence in live viral infections and have been considered noninfectious, although infectious virus can be found in the subviral virion–rich gradient fractions (113, 234). These structures do not contain C, but potentially other cell-derived RNA binding proteins could be present to package the DENV genome within the virion (113). Subviral particles are predicted to be antigenically similar (on the basis of antibody binding studies with TBEV) to classical virions, but DENV might display different protein arrangements (9). The significance of these structures is unknown; subviral particles might be an alternative way to package viral RNA, but they could also be significant for other purposes: to package and transmit mRNA messages or to block and absorb antibodies, both of which might protect the virus during the course of infection.

Virus assembly

Although the host cell of DENV is unclear, the virus has entered cells via clathrin-dependent receptor mediated endocytosis (4, 5, 107). When virus encounters acidic endocytic vesicles, fusion occurs, allowing the nucleocapsid and RNA genome to be released into the cell's cytoplasm. At early time points of infection, the endoplasmic reticulum (ER) becomes dilated and extends in size, forming stacks of ER membranes, which contain numerous membrane invaginations (108, 122). During viral infection, virus-induced structures that facilitate virus assembly and

replication form. These structures can include convoluted structures (or very dense patches of ER membranes), smooth membrane structures (SMSs), tubular structures, and virus-induced sacs (83, 114, 122, 275). The significance of convoluted structures has not been studied, but they do not appear to be required for virus assembly in C6/36 cells (114). The extensive ruffling of the ER membrane required for convoluted structure formation is thought to require cholesterol, a substance that insect cells are deficient in (83, 114). The thin long tubular structures are also poorly understood and do not consistently assemble but appear to be more prevalent in late stages of infection (114, 122).

The SMSs are circular vesicles that form inside the rough ER (83) but appear to be derived from the smooth ER because they stain positive for the marker syntaxin 17 (275). Through EM tomography studies, it was determined that SMSs are not internalized vesicles but circular concave inclusions that are continuous with the outside of the ER membrane and potentially house replication complexes (Figure 1.2) (114, 275). The thin stem region of the SMS potentially hides some of the viral components (like dsRNA) from the cellular immune system and allow for virion assembly (83, 275). The nonstructural proteins (NS-1, NS-2b, NS-3, NS-4a, NS-4b, and NS-5) can be found in close proximity, within, or touching the surface of the SMSs (114, 275), while the structural components appear to be excluded from SMSs. Structural proteins appear to be assembled by the neighboring rough ER membrane (114, 275). The high-density core complexes have always been detected on the outside of the SMS stem. The RNA genome–capsid complex associates with other lipid bilayers containing integrated prM and env proteins, which encircles the dense particle and pinches off through the rough ER into the ER lumen. Virus collects into virus-induced sacs and aggregates into crystalline structures (165, 275), although some reports have indicated that these tightly packed virion clusters form only rarely (114), suggesting a degree of potential host-specific heterogeneity in virus production/assembly during replication.

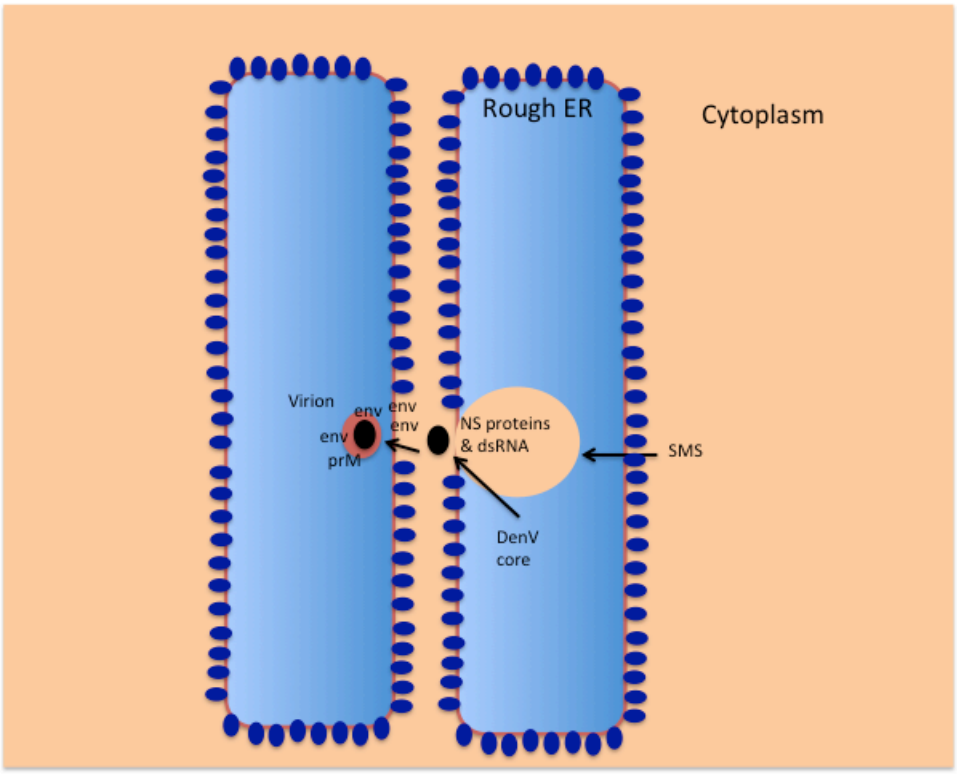

Figure 1.2. Diagram of DENV **particle assembly.** DENV genome synthesis occurs in the smooth membrane structures (SMS), or inclusions within the rough endoplasmic reticulum (ER). Nonstructural proteins can be found in close proximity and within the SMS. The RNA genome is assembled in the inclusion, but the virus core is assembled in the cytoplasmic space between neighboring rough ER structures. The structural proteins are localized on a neighboring ER surface, which serves as the site for DENV budding.

Virus maturation

Flavivirus prM cleavage into pr and M at the furin recognition site is the step responsible for virus maturation and has been studied in detail (113). After virus assembly in the Golgi apparatus, virus is trafficked to the low pH environment of the trans Golgi, where acidification exposes the prM furin cleavage site (210, 287, 288). Upon cleavage, the virus is primed for structural changes that allow for maturation at a later step. The immature virion structure and the pr peptide are retained, preventing infectious virions from fusing prematurely within the dying cell. As the virus exits and approaches the neutral pH of the extracellular environment, the virus conformation transitions to the mature form, and the pr peptides are released (201, 287). Interestingly, DENV cleavage is different from other flaviviruses; JEV and TBEV seem to have nearly complete cleavage, but DENVs have a conserved acidic residue at position 3 in the furin recognition site that inhibits cleavage (113). Some studies have indicated that as much as one-third of the prM peptides remain uncleaved, but assessing the number of mature and immature viruses is difficult (9, 108, 113). Because the DENV cleavage process is inherently inefficient, particles secreted from cells are often only partially mature, with portions of the same particle still incapable of fusion (201).

Glycosylation

Flavivirus envelope protein typically displays glycosylation at the asparagine residue at position 153/154, but in DENVs, there are two attachment sites: one at N-67 and one at N-153 (133). Glycosylation does not appear to be critical for the growth of DENV strains *in vitro*—loss of the glycosyl group on residue 154 is preferred in insect cells (112, 134). In mosquitoes, viruses with mutations at either or both residues can efficiently replicate (28). Loss of glycosylation N-153 did not considerably alter growth in insect cells or mammalian cells, but it did appear to reduce the secretion of infectious virions (28, 55, 173). Loss of N-67 glycosylation appeared more detrimental to the propagation of infectious virus in mammalian cells, also affecting the ability to enter and infect

dendritic cells (173). It has yet to be determined which DENV envelope residues are predominantly glycosylated and essential for propagation in humans.

Different glycosylation patterns were found on viruses derived from C6/36 cells, Vero cells, and monocyte-derived DCs (55). Attachment to only the GNA (Galanthus nivalis) lectin was observed with C6/36-derived virus, indicating that env protein produced in insects is mostly decorated with high- and pausi-mannose N-glycans. DC-derived virus only displayed complex patterns; their glyco groups contained α-2,6 linked sialic acids and repeating N-acetyllactosamine. Vero-derived virus is heterogeneous, displaying both the complex and high-mannose patterns seen on DC- and C6/36-derived virus, respectively. Identifying further the glycosylation patterns present on DENV will be important going forward because these structures can influence antibody interactions, immune cell recognition, and receptor-mediated entry into hosts.

DENGUE DISEASE

The distribution of DENV pathogens is expanding and is estimated to cause 390 million infections a year with 96 million experiencing clinical disease (23). DENV infections elicit a spectrum of clinical presentations, with symptoms appearing similar to other febrile illnesses (252). The geographic and vector overlap of DENV and Chikungunya viruses in particular can be problematic for rapid diagnosis in select parts of the world, and coinfection has been reported (6, 267). Dengue fever (DF) is characterized by fever, bone pain, lethargy, thrombocytopenia (low platelet count), and capillary fragility, which can be assessed by a tourniquet test. Some dengue patients experience severe forms of the disease, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Patients that display rashes and subcutaneous blood pools resulting from broken or leaky capillaries are identified as having DHF. If capillary leakage is severe and the blood circulation slows to the point of organ impairment, patients have the most severe form, DSS. These patients might also have

disseminated intravascular coagulation, caused by overactivation of the coagulation cascade, which is a dangerous condition that increases the risk of multiorgan failure and death.

A distinguishing characteristic of dengue disease is its biphasic nature (Figure 1.3) (97). The first phase is DF, during which patients experience the classical signs of dengue disease concurrent with viremia. DHF or DSS however only develop after the peak in viremia, when viremia is waning, at a time when DENV-specific antibody levels (IgM as well as IgG) are just beginning to rise after infection (200, 264), thus, DHF and DSS appear to be immune mediated, rather than a direct result of viral infection.

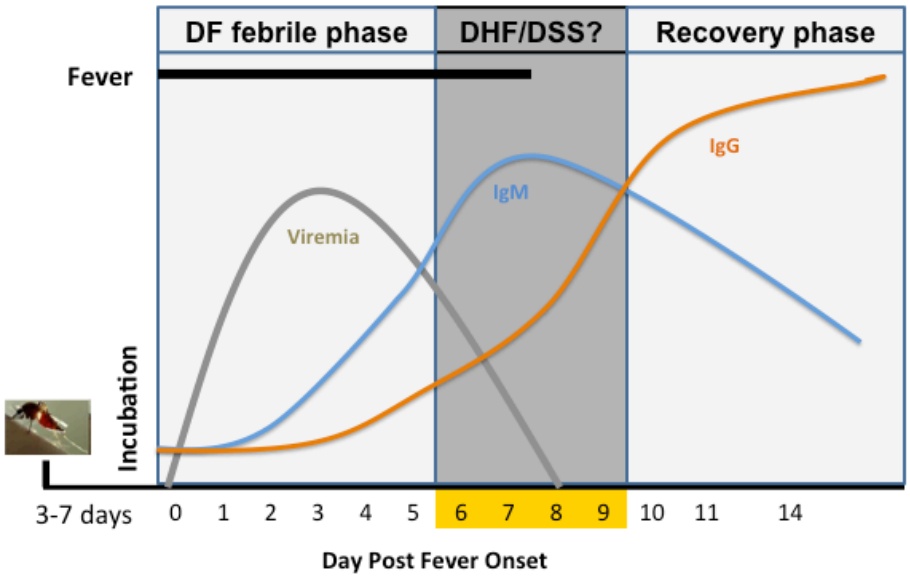

Figure 1.3. Time course of DENV **infection.** An infection begins with the bite of an infected *Aedes* spp. mosquito. An incubation period of 3–7 days precedes the onset of dengue fever (DF) febrile phase and viremia. Viremia decreases approximately 3 days post fever onset, concurrent with rise of specific antibodies, suggesting antibodies play a role in clearance. If dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) develops, it occurs after DF during viral clearance when antibody responses against DENV are most prominent. DHF/DSS is likely immune-mediated rather than a direct result of the viral infection because severe disease is not concurrent with viremia.

Dengue patients might display a number of other features: bleeding from various orifices, anorexia, hepatomegaly, vomiting, diarrhea, melena, muscle weakness, lymphadenopathy, and pleural effusion (170, 252). Because disease presentation is largely nonspecific, confirmation of the presence of DENV antigen or genomes is preferred. Rapid-test NS-1 enzyme-linked immunosorbent assay and DENV antibody ratio assessments—positive if an increase in specific-antibody is detected between acute and convalescent serum—are the most commonly performed assays, with reverse transcriptase-polymerase chain reaction assay performed, if available.

Although the ratios of asymptomatic-to-symptomatic infection vary extensively from study to study (33), a large majority of people experience asymptomatic-mild infections, and only approximately 25% or lower need to seek medical attention. Of the DF patients entering clinics and hospitals, the majority have the mild form and only a small percentage progress to DHF and fewer to DSS (255). The diagnosis rates for DHF and DSS can be particularly high in some countries with low access to healthcare. The death rates for DSS span from 1% to 2%, dependent upon the time point of illness before hospitalization, timeliness of diagnosis, age of patient, and experience of the clinical staff (208). The only approved treatment for dengue disease is rehydration therapy and palliative care, but timing is critical and the earlier the treatment is initiated, the better the outcome.

Genes associated with dengue disease

A number of studies have investigated dengue strains and mutants and their association with disease (82, 88, 117, 135). Although various mutations can alter virus fitness *in vitro*, little work has been done to confirm whether these genes are associated with disease in suitable animal models. DENV2 strains are the most dominant, and perhaps most virulent; however, all four serotypes are capable of inducing severe disease (7, 123, 167, 264).

Host factors associated with dengue disease have been extensively investigated. In the past an association of severe disease and women was noted, and a recent meta analysis confirmed a higher

risk of DSS for females (106). Interestingly, in our rhesus macaque coagulopathy disease model, hemorrhage formation was more intense in female monkeys, although this was not the only distinguishing characteristic among the tested animals (195). The females were also much older than the males and had a markedly higher body mass index, though the males were not underweight; thus it is unknown if the association in this study in nonhuman primates was due to age, sex, and/or weight (195). In endemic countries, like Thailand, dengue disease seems more prevalent in teenage and young adult cohorts (259), but young children are regarded as the most affected age group. However, the age range most susceptible to disease has been reported to vary by region and also by year (32). Of note though, persons of all age ranges can be afflicted with severe disease (259).

Some insight has been gained from studies examining the association with specific genetic traits. Comparing blood group antigens, a higher proportion of AB positive persons appear affected by severe dengue disease (115). In India, particular genotypes of human platelet antigens 1 and 2, proteins associated with alloantibody formation, are more common among DHF and DSS patients (235). Tumor necrosis factor-α (TNF-α), transporter associated with antigen presentation genes (TAPs), and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) also have been suggested to be associated with dengue disease in humans (69, 70, 222, 265). Various studies have focused on major histocompatibility complex (MHC) alleles and their contributions to disease presentation. Human leukocyte antigen (HLA)-B15, HLA-A33, and HLA-B44 are associated with protective responses, and HLA-A0207, HLA-A11, HLA-A24, HLA-B48, HLA-A02, and HLA-B07 are associated with increased susceptibility to disease, depending on the serotype and population (152, 236, 241, 265, 266). In a Sri Lankan study of healthy donors, HLAs associated with high-magnitude responses against dengue antigen were identified as protective for dengue disease; protection was also observed with alleles that could recognize specific and cross-reactive DENV epitopes, challenging the notion that "original antigenic sin" is associated with severe

disease (273). However, these types of studies are hard to reproduce across different genetic backgrounds from different countries and results at this time should not be overinterpretted.

DENGUE DISEASE MECHANISMS

Hematopoietic Dysfunction and Bone Marrow

One of the distinguishing symptoms present in DF sufferers is bone pain; thus, the disease early on earned the designation "break bone" fever. This pain is different from arthritic inflammation because it is associated with the interior of the bone and not the joints. It is widely known that the central cavity of the bone is the site of production for the majority of immune cells, which are responsible for protecting the host from invading microbial pathogens. The pain associated with the bone potentially implicates a unique phenomenon contributing to dengue pathogenesis. A number of alterations in the peripheral blood cell populations occur. Immature neutrophil cells increase in concentration—a phenomenon also known as a left shift—in DHF patients (231, 268). Many peripheral blood cell populations fluctuate: leukopenia, lymphocytosis, monocytosis, neutropenia, and the appearance of atypical lymphocytes are all phenomena well documented in dengue patients (268).

These alterations are probably reflective of bone marrow environment remodeling occurring early during infection (130). Decreases in cellularity in the bone marrow are evident at early time points of disease (24, 130, 182). However the suppression is short-lived. These cell populations often rebound, with compensatory hyperplasia occurring around the onset of severe disease. The dynamics of bone marrow cellularity were confirmed in the rhesus macaque animal model (190). Megakaryocytes, the platelet-producing cells, are one cell population that declines in dengue patient bone marrow (24)—a phenomenon that potentially contributes to increased bleeding and other platelet abnormalities prevalent in dengue patients.

Platelet Abnormalities

Platelets are the key regulators of coagulation. The activation of platelets can proceed by either the intrinsic and extrinsic coagulation pathways; activation by any of a number of different cell surface receptors causes platelets to change shape and crosslink with other biomolecules, including red blood cells, resulting in the formation of a blood clot (110). Thrombocytopenia (low platelet count) and hemoconcentration (low plasma but high red blood cell content) are not only hallmarks of dengue disease, they are also indicative of disease severity (252). The phenomenon of thrombocytopenia has been attributed to multiple mechanisms: decreased megakaryocyte differentiation and synthesis of platelets (204), decreased platelet lifespan (171), and increased consumption (102). In a limited number of studies, alternative platelet functioning was observed (171), indicating a level of reprogramming during the course of dengue disease. DENV particles have been found in patient platelets (189), suggesting they could be recognized as infected by immune cells and targeted for removal by phagocytosis. Dengue patients develop platelet-associated antibodies that contribute to disease severity (142, 215, 221); these antibodies probably contribute to platelet phagocytosis (195, 259). Alternatively, platelet-associated antibodies might bind cell surface receptors, modulating platelet activities and causing their malfunctioning. Activated partial thromboplastin time, which measures the intrinsic coagulation pathway, is the predominant coagulation pathway affected in dengue patients, indicating that the bleeding abnormalities can be associated with one or multiple coagulation factors (except factors VII and XIII) (156). Some COX-2 targeted pain relievers that work by inhibiting platelet activation can increase the bleeding risk and worsen patient outcomes (110, 276). Detailed analyses of components of the coagulation cascade in our monkey model of DENV-induced coagulopathy uncovered that a marked elevation of D-dimers along with transient increases in coagulation inhibitors (antithrombin III and protein C) corresponded with decreases in platelets and leukocytes (195). D-dimers are usually elevated but antithrombin III, protein S, and protein C are more often depressed in dengue patients (156, 277).

Platelets also are becoming increasingly recognized as components of the immune system, influencing the function and dynamics of immune responses (98, 194, 219, 228); they display activation markers, such as CD40L, contribute to complement activation, secrete chemokines and cytokines, and modulate the function of immune cells. Abnormally functioning platelets and thrombocytopenia could disrupt the immune response and contribute to pathogenesis (73).

Immune Responses

Specific antibody production has long been regarded as essential for controlling viral infections and has been studied in extensive detail with DENV. The antibodies produced during natural infection are relatively slow to initiate and thus serve poorly as diagnostic tools for determining patient treatment. Many of the antibodies are cross-reactive with other dengue virus serotypes as well as other flaviviruses (46, 49, 242, 261). Cross-reactive antibodies that are strongly neutralizing are rare, suggesting a high level of specificity of neutralizing epitopes for each serotype (232). DENV also appears to employ molecular mimicry to avoid immune recognition (145); some antibodies that develop can cross-react with host epitopes, especially those present on platelets and endothelial cells (42, 66, 143, 144, 162). Gene association studies suggest a correlation between human platelet antigen genotypes and disease (235).

Dengue-specific antibodies produced from B-cells have a long life span and are presumed to be protective for life (93, 213, 254, 264). However, some investigations of neutralizing antibody responses suggest that the presence of neutralizing antibodies does not necessarily protect from future infection or disease (64). Cross-protective antibodies that form against the other dengue serotypes are short-lived and persist for only around 3 months after infection (218). It is hypothesized that the increased levels of specific antibody coupled with antibody-mediated activities (complement, phagocytosis, antibody dependent cell-mediated cytotoxicity, and/or mast cell

activation) might actually cause pathogenesis by acting additively and inducing extensive inflammation (15, 27, 102, 157, 197).

Antibody dependent enhancement (ADE) is an important mechanism believed to be involved in the induction of dengue disease. With ADE, antibodies developed from a previous DENV infection are cross-reactive yet poorly neutralizing to a secondary infection with a DENV of another serotype. These antibodies might instead facilitate increased DENV entry into cells bearing Fc receptors, leading to increased rates of infection. *In vitro*, a greater percentage of monocytes and dendritic cells can be infected when virus is inoculated with low concentrations of DENV-specific antibodies. Higher numbers of infected monocytes and dendritic cells are believed to result in higher virus titers *in vivo* and more severe disease. This mechanism is not without controversy because while a number of studies support ADE (36, 263), other studies and observations contradict this mechanism: high virus titers in asymptomatic persons (33, 59); low specific antibody levels present during viremia (200, 264); ADE activity, preexisting antibody titers, and viremia levels not correlating with disease severity (74, 131, 140). Further studies and meta-analyses are needed to assess clinical significance. As an alternative hypothesis, subneutralizing antibodies could facilitate greater disease without increasing rates of infection.

Research characterizing the human B-cell populations and their resultant immune responses against DENV has become a recent focus to identify potential mechanisms. A polyclonal IgM response is a preferred response of B-cells exposed to dengue antigens, even in secondary infection; DENV interacts with nonspecific receptors on B-cells, and antibody development appears to work through a germinal center independent mechanism (45, 79). When evaluating the composition of the B-cell populations present in dengue patients experiencing secondary exposures to DENV, a massive plasmablast response was observed comprising primarily DENV-specific IgG secreting plasmablasts (76, 278), which are short-lived cells that require further differentiation to become long-lived plasma cells. The observed increase in plasmablast response reached 1000-fold baseline values in select

patients. Eighty percent of $CD19^+$ B-cells in dengue patients were plasmablasts, contrasting with the approximate 3% of the healthy volunteers receiving vaccines (279). Plasmablasts, which usually peak in the peripheral blood on day 7 after immunization, had a unique pattern of expression in dengue patients; they were detectable on multiple days, increasing slowly until day 7, the day of disease onset. Moreover, there was a correlation observed between magnitude of the plasmablast response and severity of disease in a Brazilian cohort suggesting an association between inflammation and B-cell responses (76).

Other immune responses have been associated with the severity of disease. $CD8^+$ T-cell responses during secondary infection are thought to be inadequate by retaining specificity to the primary infecting serotype while being poorly potent at inhibiting the virus from the secondary exposure. Some studies suggested that these cells produce the same robust cytokine response but have deficient cytolytic activity, causing less efficient removal of infected cells while contributing to inflammation (58). Proinflammatory factors TNF-α, IFN-gamma, soluble(s) CD4, sCD8, interleukin (IL)-2, and sIL-2 receptor levels are all elevated in patients with severe disease, suggesting that T-cell mediated immunity contributes to disease (52, 109, 128). It has also been reported that a switch from a Th1 to a Th2 response occurs as disease progresses from mild dengue fever to severe dengue hemorrhagic fever (34). Some gene association studies suggest that HLAs are linked with severe disease (241, 266). However, other studies argued that T-cell responses are protective. Certain HLAs correlate with reduced disease, and robust, specific T-cell responses are elicited in dengue patients (266, 274). When assessing T-cells from healthy individuals from a dengue-hyperendemic population, antigen-specific T-cells, regardless of whether they were specific for a given serotype or for a conserved epitope, were triple–cytokine-producing (273). T-cells are one of many cell lineages (iNKT, mast cells, endothelial cells, and macrophages) proposed to participate in pathogenesis; dengue disease is likely complex and could involve multiple immune system components (27, 47, 164, 281).

Factors secreted by various immune cells (monocytes, T-cells, macrophages, and endothelial cells) have been associated with disease severity: monocyte chemoattractant protein (MCP)-1, TNF-α, IL-4, IL-6, IL-8, IL-10, interferon-gamma, vascular endothelial growth factor, complement, thrombomodulin, protease activated receptor-1, tissue factor receptor, tissue factor inhibitor, and activated protein C (15, 29, 35, 174, 186, 197, 198, 238). In addition to cytokines, oxidative and nitrosative stress contribute to the environment and induce cytokine storms (31). There are also suggestions that increased gut permeability and increased peripheral blood lipopolysaccharide levels ignite an inflammatory environment and increase disease severity (258). A number of different mechanisms have been linked to dengue disease severity, but more research is needed to elucidate which common biochemical pathway(s) are responsible for pathogenesis.

TREATMENT AND PREVENTION

Treatment and Therapeutics

Although much research has focused on designing antivirals against flaviviruses (>70 in number), there are no approved therapeutic drugs for any of these viruses (77). Inhibiting the activities of NS-5 and NS-3 has been the primary strategy for DENV drug design (77, 188). Antivirals designed against the protease (encoded by NS-3/2b) (280), helicase (NS-3) (18), RNA-dependent RNA polymerase (NS-5) (187), and methyltransferase (NS-5) (250) are underway. Successful inhibition of these enzymes is dependent on understanding their functional properties, which requires crystallization and elucidating complex protein interactions, the mechanisms of which have not been fully elucidated. Nucleoside analogs are other therapeutic candidates (41): phosphorodiamidate morpholino oligomers that penetrate the cell, directly target DENV RNA, and shut off transcription are under development (120).

Another favorite target protein for drug development is env (77). Promising regions on this protein include the n-octyl-β-D-glucoside (β-OG) pocket, the conserved alpha helices on the env

stem, and the receptor binding sequences on domain III (77, 139, 172, 227). Drugs that bind these sites prevent the required conformation changes needed for the initiation of infection: viral fusion, uncoating, and RNA release. Another therapeutic drug design option involving inhibiting virus fusion is DENV-specific antibody development. Isolating naturally occurring human B-cells with the capacity to recognize and neutralize DENV is a promising strategy for developing therapeutics. Currently, the literature suggests that the naturally occurring neutralizing antibodies found in humans function by recognizing complex three-dimensional epitopes on env.

Many other antiviral drugs that were specifically designed to prevent DENV replication and maturation are undergoing testing (26). These include a drug that disrupts viral genome packaging (184), lycorine, which prevents virus assembly (295), and celgosivir, which blocks glycosylation (211). Molecules that interfere with the prM cleavage and pr peptide dissociation represent another potential strategy that can be used to treat dengue patients (77).

Therapeutic hormone drug candidates have shown little promise. Interferons, which convey antiviral properties, have been promising for HCV treatment; pegylated α-interferon 2a is one current treatment available for HCV patients. In preliminary dengue studies, type I interferons worked best (57); however in trials in rhesus monkeys, α-interferon only postponed the viral infection for 3 days, while the pegylated form of the drug showed only modest decreases in virus titers (8). Corticosteroids and dexamethasone have been tested in dengue patients; they are safe but do not change the course of disease (127, 249). Combination treatment of antivirals with immune system-modulating drugs has not been tested. One major therapeutic challenge for antiviral treatments is the late onset of disease, which often corresponds with the peak in viremia or later, and thus a time point when viremia itself is already regressing (Figure 1.3).

Hydration therapy (isotonic crystalloid or colloidal solutions) with close monitoring of hematocrit and giving blood or plasma transfusions when necessary is still the only current recommended treatment for dengue patients (252). Giving platelet transfusions to hemodynamically

stable patients is not recommended (276), although blood product transfusions are often administered to treat thrombocytopenia and other dengue symptoms. Anti-D immune globulin, a standard treatment for idiopathic thrombocytopenic purpura, was tested in a small population of dengue patients; counter to expectations, the antibodies did not increase platelet levels when given to dengue patients, but it sped platelet recovery after disease (51).

Vaccines

Vaccines are valuable public health tools that have lead to the reduction (and even the eradication) of diseases caused by viral or bacterial etiological agents. Vaccines equip a person's immune system with a protective immune response against pathogens by pre-exposing the body to components of that pathogen (e.g., proteins, glycoproteins, polysaccharides and lipoproteins). Given the wide dissemination of DENV infection, vaccines represent the only economically viable solution to curb DENV infection and disease.

Vaccine development to prevent dengue disease has been underway since the 1920's (231), yet still no vaccine is available that can prevent dengue infection or disease for everyone. While several vaccines are in various stages of human testing, recently Sanofi Pasteur has obtained licensing for Dengvaxia, a tetravalent dengue vaccine delivered by a live attenuated yellow fever vector for use among persons older than 9 years. The vaccine prevents hospitalization from all four serotypes in 65.6% of persons over the age of 9, but only 44.6% of younger children (22, 92). Even though only partially effective, roll out of this vaccine is expected to halve the global incidence of dengue severe disease. Although this vaccine is not approved for use by travelers or young children, who tend to develop more severe disease, several additional vaccine candidates are under development (discussed later), one of which might be able to serve as protective for these populations.

Designing DENV vaccines has been a challenge and might continue to be a challenge for those who are immunologically naïve. One major challenge to vaccine design has been the lack of

adequate animal models to test vaccine efficacy against disease (166). Testing usually begins with evaluations in a mouse model, and if the immune responses measured and safety observed are adequate, testing progresses to the primate animal model (90). Although DENV can replicate in both of these organisms, disease progresses quite differently; in immune compromised mouse models, neurological disease is prominent (14, 229, 294), and in primate models, disease is either absent or limited (43, 75, 226, 285). The lack of a disease model has lead to poor predictability of vaccine efficacy in humans. When using nonhuman primate models, vaccines can only be selected for on the basis of challenge virus titers, but this is not an ideal parameter to evaluate efficacy because a reduction in viremia does not necessarily correlate with protection (38). Virus can replicate in humans (sometimes to high titers) without causing disease (59, 81).

Exacerbating the lack of adequate disease animal models is the lack of reliable correlates of protection from disease. Antibody responses are typically measured in vaccine trials, despite a clear correlation between neutralization titers and protection from dengue disease (64, 166, 207, 225). In fact, protection has been observed in individuals with subneutralizing antibody responses (199, 246), while an absence of protection has been observed in those with clear neutralizing antibody responses (64, 217). Antibodies directed against env domain III was shown to be protective in mice (230, 245), but humans typically do not make many antibodies directed against this epitope (233, 269). Vaccines that promote the development of antibodies specific to env domain III are under development (13, 118), although there is as yet no proof that they will indeed correlate with protection in humans. There is more evidence suggesting that the env domain I/II hinge and similar quaternary epitopes are the targets for naturally formed neutralizing antibodies in humans (49, 168, 253). Until better correlates of protection are identified, vaccine efficacy in humans will depend on the performance of large and onerous phase III clinical trails spanning long periods of time (166).

Another challenge to designing dengue vaccines stems from the potential for eliciting antibody dependent enhancement responses, which might increase infection and disease by weakly

cross-reactive antibodies upon secondary DENV exposures involving other (and sometimes the same) DENV serotypes (3). It is believed that an antibody response that is not protective against all four serotypes will inevitably lead to incidences of more severe disease. Because of this concern, it is required that DENV vaccines elicit protection against all four serotypes simultaneously; it is not possible to obtain approval for a vaccine successful at preventing disease for only a subset of DENVs. This requirement has made vaccine design particularly difficult for live-attenuated vaccines, considering that the closely related strains have to compete for replication in the same cellular hosts. For live-attenuated vaccines presenting each serotype on separate vectors, often one or more subsets dominate, either due to better replication or greater immunogenicity in comparison to the others, leading to an unbalanced immune response and therefore an insufficient response to select serotypes. Since all four DENV serotypes tend to cohabitate in various parts of the world, the need for a tetravalent vaccine is imperative. A lot of effort has focused on the careful selection of strains that can replicate equally and stimulate similar immune response levels (60, 78, 121, 136, 150, 216).

The challenge of finding strains that can replicate similarly to each other *in vivo* has been achieved by mutating viruses so that they replicate poorly. Tetra-Vax-DV (designed by NIAID) contains genomes with 30-bp deletions on the 5' end (the portion of the genome required for circularization of the RNA genome, which facilitates transcription, translation, and replication) with additional mutations in protein coding regions (60). Inviragen/Takeda developed their live-attenuated virus (DENVax) by the classic technique of repetitively passaging them in a cell type not naturally infected by DENV (dog kidney) (196). A few vaccine developers decided to display DENV structural proteins on other virus backbones. The most successful candidate thus far is Sanofi's vaccine, which utilized the yellow fever virus backbone (136). However, the hepatitis B and AdV5 backbones are also being tested in other vaccine candidates (13, 118).

Other companies have decided to test inactivated vaccines, the benefit being that the individual serotype components do not have to compete with each other for the same cellular

reservoir, making a balanced immune response more feasible, although immune hierarchy and dominance might still exist. With GSK's inactivated whole virus vaccine (205, 206) and Merck's 80E subunit (44, 95), a balanced antibody response might be more easily acquired, but this strategy limits the development of cellular immune responses capable of attacking DENV-infected cells and generally is a more costly method considering large scale production. DNA vaccines may represent an ideal compromise as they do produce viral proteins within cells and can induce cellular immunity; one such vaccine is under development that synthesizes env, prM, and NS-1, the most prominently produced DENV proteins in natural infections (19, 153), but DNA-based vaccines have thus far only been able to show modest responses in humans unless used in combination with electroporation to amplify vector uptake and expression. More testing will be needed to tell which of these vaccine candidates progress; with so many different strategies under evaluation, soon we will know if it is possible to develop a vaccine protective for DENV naïve persons.

CHAPTER 2

Characterization of dengue virus 2 growth in megakaryocyte erythrocyte progenitor cells

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Title: Characterization of Dengue Virus 2 Growth in Megakaryocyte-Erythrocyte Progenitor Cells

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ABSTRACT

Megakaryocyte-erythrocyte progenitor (MEP) cells are potential *in vivo* targets of dengue virus (DENV); the virus has been found associated with megakaryocytes *ex vivo* and platelets during DENV-induced thrombocytopenia. We report here that DENV serotype 2 (DENV2) propagates well in human nondifferentiated MEP cell lines (Meg01 and K562). In comparison to virus propagated in Vero cells, viruses from MEP cell lines had similar structure and buoyant density. However, differences in MEP-DENV2 stability and composition were suggested by distinct protein patterns in western blot analysis. Also, antibody neutralization of envelope domain I/II on MEP-DENV2 was reduced relative to that on Vero-DENV2. Infectious DENV2 was produced at comparable kinetics and magnitude in MEP and Vero cells. However, fewer virion structures appeared in electron micrographs of MEP cells. We propose that DENV2 infects and produces virus efficiently in megakaryocytes and that megakaryocyte impairment might contribute to dengue disease pathogenesis.

Keywords: dengue virus, megakaryocyte, erythrocyte, progenitor, Meg01, K562, MEP, electron microscopy

INTRODUCTION

Dengue virus (DENV) is an increasing public health threat, largely because of its ability to transmit not only by *Aedes aegypti*, a tropical and subtropical vector, but also via *Aedes albopictus*, the more prevalent mosquito vector endemic in temperate zones (WHO, 2015). Approximately 390 million people get infected annually, although most of these infections do not progress to the point of major clinical disease (Bhatt et al., 2013). Persons of a wide range of ages can become infected and experience a variety of clinical manifestations (from mild dengue fever to more severe dengue hemorrhagic fever/dengue shock syndrome) with approximately 22,000 deaths occurring annually (Tsai et al., 2012). DENV is also a heavy public health burden because no specific therapeutics are available; one vaccine recently became available, but it was approved only for previously exposed populations. Moreover, in spite of its widespread recurrence and emphasis in the literature, a number of its basic biologic and pathologic DENV mechanisms remain to be fully elucidated.

During blood meal, mosquitoes inoculate DENV directly into the skin. But more importantly, when mosquitoes probe the skin, they can find blood vessels and deposit virus directly into the capillaries, releasing virus into circulation (O'Rourke, 1956; Styer et al., 2007) and exposing many different cell types to pathogen. Permissiveness has been investigated in various cell types: dendritic cells (Ader et al., 2004; Ho et al., 2001; Sun et al., 2009; Wu et al., 2000), monocytes/macrophages (Arevalo et al., 2009; Daughaday et al., 1981; Diamond et al., 2000; Tan and Chu, 2013; Theofilopoulos et al., 1976), endothelial cells (AbuBakar et al., 2014; Arevalo et al., 2009; Diamond et al., 2000), and B-cells (Takasaki et al., 2001; Theofilopoulos et al., 1976). A number of these cell lineages can get infected and reprogrammed, and many of these events might even contribute to disease pathology (Butthep et al., 1993; Green and Rothman, 2006; Lee et al., 2013; Libraty et al., 2001; Nielsen, 2009). But while a cell type might be permissive to DENV infection, a separate issue is whether that cell can efficiently produce high titers of infectious virus. The

infectiousness of virus released from a number of cell types has been questioned (AbuBakar et al., 2014; Marianneau et al., 1999; Mosquera et al., 2005), and thus the cellular target responsible for viremia in humans remains controversial.

DENV infection of bone marrow cell populations has been implicated in a number of previous reports. It was noted even in early studies that bone marrow resident cells change in morphology and frequency (Bierman and Nelson, 1965; Kho et al., 1972; La Russa and Innis, 1995; Nelson et al., 1964; Noisakran et al., 2012). Bone marrow-derived megakaryocyte-erythrocyte progenitor cells were permissive and yielded high DENV2 titers (1×10^5 FFU/mL and 1×10^8 genome copy number [GCN]/mL)(Basu et al., 2008; Clark et al., 2012; Nakao et al., 1989). Also, a recent publication reports a positive correlation between DENV titers in dengue fever patient plasma and circulating $CD61^+$ (megakaryocyte marker) cell count numbers (Hsu et al., 2015). While not conclusive, these observations suggest that $CD61^+$ cells might contribute to DENV replication *in vivo*, since DENV can be propagated *ex vivo* from $CD61^+$ cells isolated from bone marrow of infected animals (Noisakran et al., 2012). Studies have indicated that megakaryocytes stain positive for viral antigen and antigen positivity correlates with peak infectious titer and virus-like particle (VLP) production (Basu et al., 2008; Clark et al., 2012; Noisakran et al., 2012). However, despite an association of DENV2 with the megakaryocyte, the cell types that initially encountered and took up the virus in these experiments were uncertain because the effect could be due to infection of any of several cell types capable of differentiating into megakaryocytes. Thus, it is not known if megakaryocytes can be infected directly by DENV.

In this investigation, we sought to examine further cells of the megakaryocytic lineage as potential DENV2 hosts. Because bone marrow samples are difficult to acquire, and because of the low frequency of megakaryocytes in the bone marrow in general, our investigations were conducted with megakaryocyte-erythrocyte progenitor (MEP) cell lines: Meg01 (Ogura et al., 1985), a megakaryocytic cell line that has rarely been used in DENV research, and K562 (Lozzio et al., 1981)

a MEP cell line that has the ability to differentiate into megakaryocytes and has been used in a number of DENV studies. We characterized DENV2 replication and production in Meg01, K562, or Vero cell lines, a gold-standard tool in DENV investigations, and also studied the structure and antigenicity of viruses produced in cultures of these cells. In all cell lines examined, DENV2 propagated to similar titers with comparable kinetics and produced infectious virions of similar density and structure. However, our study also revealed that particular composition and antigenicity differences did exist. This work supports previous findings indicating that cells of the megakaryocyte-erythrocyte lineage were permissive to DENV infection and might contribute to DENV pathogenesis (Clark et al., 2012; Diamond et al., 2000; Nakao et al., 1989; Noisakran et al., 2012).

RESULTS

DENV2 propagates efficiently and produces virus particles in MEP cell lines

We examined virus growth kinetics with *in vitro* cell lines of the MEP lineage. Propagation of DENV2 in Meg01 or K562 cells was compared in parallel with Vero cells. All cells were inoculated with DENV2 that had been propagated previously in Vero cell monolayer cultures (Vero-DENV2) and cultured under similar conditions (Fig. 2.1A). Plaque assay analysis of passage 1 (p1) supernatants indicated that similar levels of infectious DENV2 were produced in all three cell lines, but virus growth in Meg01 and K562 cells appeared slightly delayed, reaching consistent titers of approximately 1×10^5 PFU/mL on day 4 after inoculation, at least 2 days after Vero-DENV2. To determine if slower growth was a consequence of the cell line or level of adaptation to the host, viruses Meg01-DENV2p1 and K562-DENV2p1 were passaged again in Meg01 or K562 cells, respectively, to yield suspensions designated Meg01-DENVp2 and K562-DENV2p2 (Fig. 2.1B). Meg01-DENV2p2 and K562-DENV2p2 grew with kinetics similar to those of Vero-DENV2, indicating that DENV2 can grow in these MEP cell lines equally well. Because of their similar replication kinetics, all further experiments were conducted with the p1 viral stocks.

In addition to infectious titers, RNA genome copy number (GCN) quantification suggested that virus was released into the supernatant with comparable kinetics for all three cell lines tested, with K562-DENV2p2 yielding slightly higher values on days 6 and 7 (Fig. 2.1C). Although these cell lines appeared to release infectious virus and viral RNA with similar kinetics, GCN:PFU ratios differed slightly. Meg01 and K562 cells yielded lower GCN:PFU ratios at early time points, though only day 2 differences were significant ($p=0.013$ and $p=0.012$, respectively) (Fig. 2.1D). The mean ratios at this time point were 24.4 (Meg01-DENV2), 9.2 (K562-DENV2), and 107.4 (Vero-DENV2) (Table 1). Thus, Meg01 and K562 cell lines appeared to release fewer noninfectious virions than Vero cells at early time points of infection. Meg01-DENV2 and K562-DENV2 GCN:PFU ratios appeared to increase over time, suggesting an increase in the release of noninfectious virus at later time points or an increase in virus particle degradation over time (perhaps as a consequence of cell culture proteases). Vero-DENV2 also showed an increase in GCN:PFU ratio with time, except on days 5 and 6, when they dropped and then rose again on day 7 (Fig. 2.1D). The reason for this dip in GCN:PFU ratio is unknown but might be due to a second round of virus amplification.

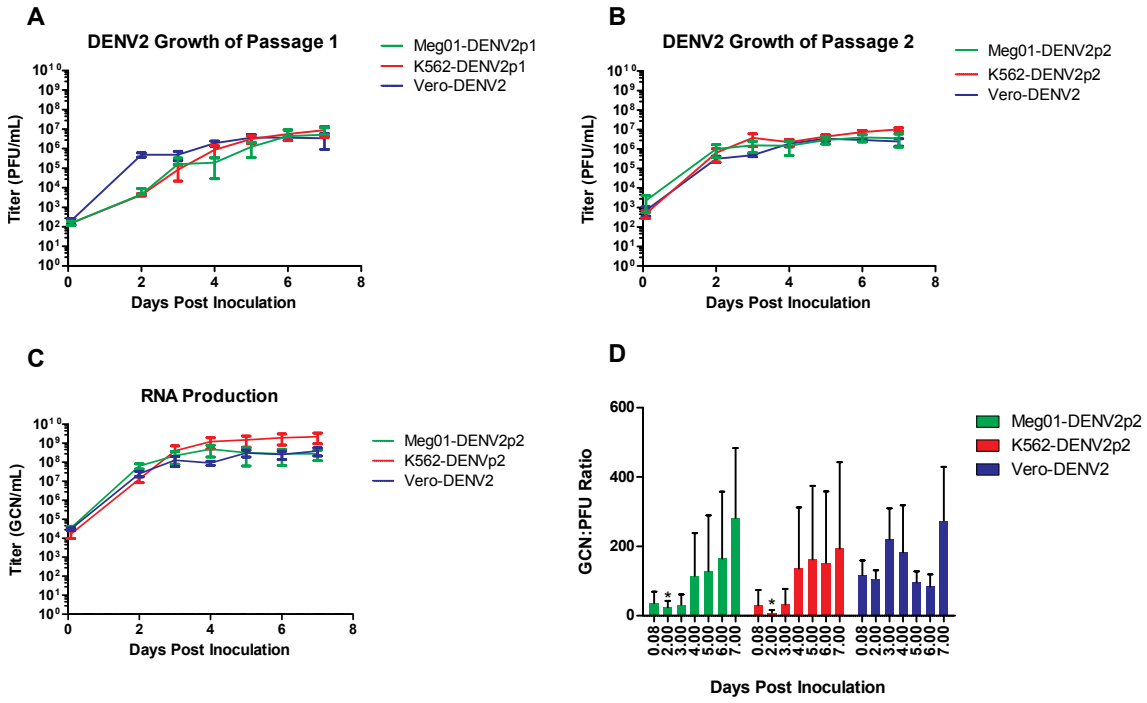


Figure 2.1. Replication kinetics of DENV2 in Meg01, K562, or Vero cells.

Cells were inoculated at an MOI = 0.1 FFU/mL. Virus from Meg01, K562, and Vero cell supernatants acquired days 2–7 were quantified by either plaque assay or RT-qPCR. Time courses were done at least in triplicate and error bars represent SD. (A) Infectious virus titer time course of Vero-DENV2 passaged in the indicated cell lines. (B) Infectious virus titer time course of virus passaged a second time in the same cell line. Vero-DENV2 data is the same as (A). (C) Quantification of passage 2 virus in (B) by RT-qPCR. (D) GCN:PFU ratios (n = 5). *$p<0.05$ when compared with corresponding value from Vero-DENV2 using student's t-test.

Abbreviations: FFU = focus forming unit; GCN = genome copy number; MOI = multiplicity of infection; PFU = plaque forming units; RT-qPCR = reverse transcription-quantitative polymerase chain reaction; SD = standard deviation.

Table 2.1. Time course of GCN:PFU ratios of Meg01-DENV2p2, K562-DENV2p2, and Vero-DENV2 from cell supernatant.

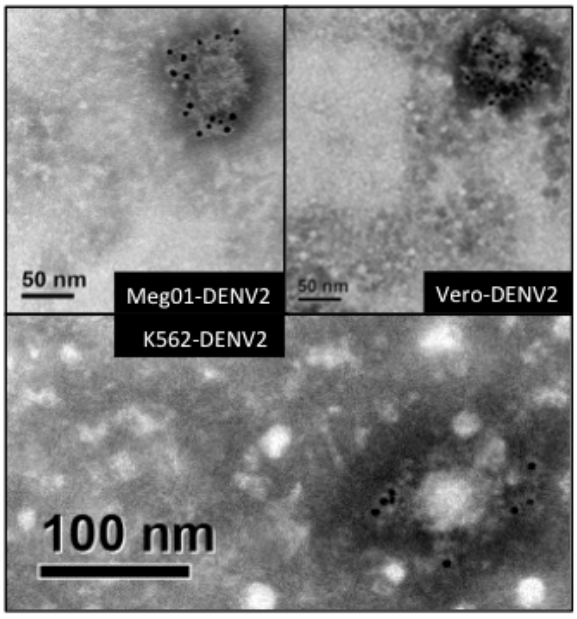
DPI	K562-DENV2p2		Meg01-DENVp2		Vero-DENV2	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
0.083	22.7	44.5	36.1	33.3	117	94.9
2.000	9.21*	6.88	24.4*	18.3	107	53.1
3.000	32.0	45.1	32.0	29.2	221	199
4.000	136	176	115	124	182	305
5.000	162	212	129	161	96.9	69.2
6.000	153	206	165	193	84.3	78.3
7.000	194	249	282	202	274	348

*$p<0.05$ compared with corresponding value from Vero-DENV2 using student's t-test.

Abbreviations: DPI = days post-inoculation; GCN = genome copy number; PFU = plaque-forming unit; SD = standard deviation.

Meg01-DENV2, K562-DENV2, and Vero-DENV2 were compared for their ability to replicate in cells from human bone marrow tissue specimens. These viruses were isolated through sucrose gradients, quantified by RT-qPCR, and then propagated in human bone marrow tissue specimens. Virus production then was evaluated by an enzyme-linked immunosorbent assay specific for detection of DENV nonstructural protein-1 (NS-1). In these experiments, NS-1 peaked at similarly high levels (>4,000 ng/mL) in human bone marrow supernatants, irrespective of the cell type in which the inoculated virus had been produced (data not shown).

Analysis of concentrated supernatants from day 3 revealed that Meg01 and K562 cells released virus similarly as Vero-DENV2 (Supp. Fig. 2.1). Virions, identified by staining with 3H5 (envelope-specific) monoclonal antibody, were in the 50 nm range of size and had a "hairy" appearance.



Supplemental Figure 2.1. Immuno-EM of Meg01-DENV2, K562-DENV2, or Vero-DENV2 from concentrated supernatants.

Supernatants were clarified, PEG 8,000-concentrated, and ultracentrifuged. Concentrated viruses were fixed with paraformaldehyde, labeled with envelope-specific monoclonal antibody 3H5, and processed for negative-staining immuno-EM.

Abbreviation: EM = electron micrograph; PEG 8,000 = polyethylene glycol 8,000.

DENV2-infected MEP cell lines synthesize lower numbers of virus-induced structures

We examined the morphology of DENV2-infected MEP cells. Meg01, K562, or Vero cells were inoculated at a low multiplicity of infection (MOI), harvested on days 1 or 2, thin-sectioned, and imaged by electron microscopy (EM) (Fig. 2.2). DENV2-inoculated Meg01 and K562 cells produced virus particles and replication complex structures similar to DENV2-infected Vero cells, although there was some variability in the shape of replication complex shape. Meg01 and K562 replication complexes often appeared elongated/elliptical (data not shown).

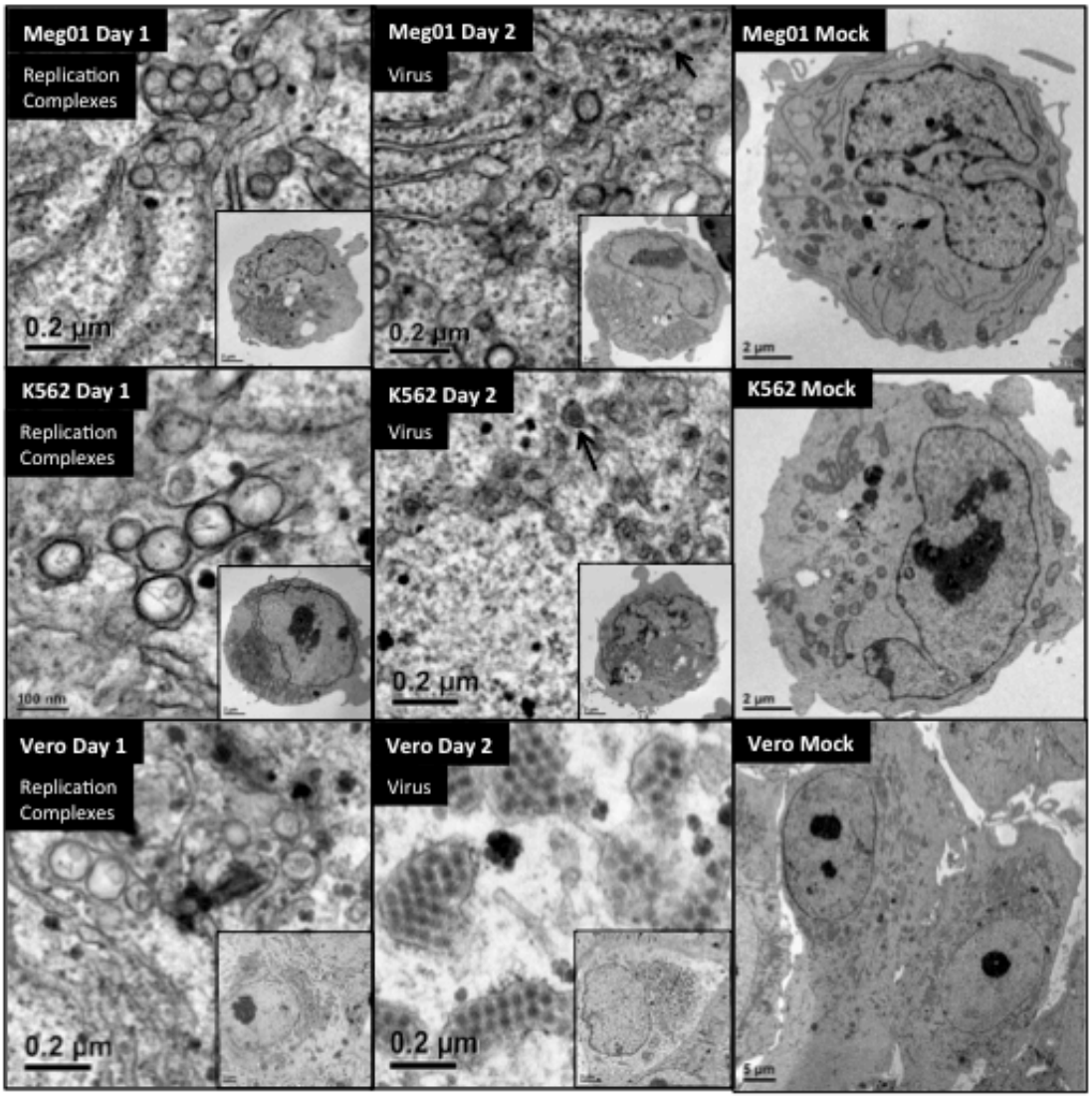


Figure 2.2. EM imaging of DENV2-infected Meg01, K562, or Vero cells.

Meg01, K562, or Vero cells were inoculated with DENV2 at low MOI or mock infected and cell pellets or monolayers were fixed with glutaraldehyde and processed for thin-sectioning EM. (Top) Meg01, (Mid) K562, and (Bottom) Vero depict the following structures (left-to-right): replication complexes from day 1 DENV2-infected cell; virus from day 2 DENV2-infected cell (arrows indicate virus); day 2 mock-infected cell. Insets show cell of origin. Abbreviations: EM = electron micrograph; MOI = multiplicity of infection.

Virus particles appeared more numerous in DENV2-infected Vero cells, so virus particles from day 2 were enumerated. The analyses were performed using 20–27 cell cross-sections from each of the infected cell lines. Significant differences were observed between the infected MEP and Vero cell lines. Meg01 and K562 cells both produced fewer numbers of virus particles per cell cross-section (averaging 140.9 and 94.9, respectively) than did Vero cells per cross-section (764.2; $p<0.0001$) (Fig. 2.3) (Table 2.2). In addition, fewer crystalloids formed in infected Meg01 and K562 cell lines ($p<0.0001$). The majority of K562 cells did not have a single virus cluster. Less variation in numbers of replication complexes was observed between MEP cells and Vero cells, although infected K562 cells had fewer complexes (average, 49.7) than Vero cells (average, 92.2; $p=0.0062$). In addition to the lower frequency of virions per cell, fewer numbers of MEP cells appeared to be infected. When evaluating cells on an entire EM grid square, the endoplasmic reticulum (ER) was distended in 17.5% of Meg01 and 19.2% of K562 cells, while most of the Vero cells (85.5%) appeared to contain virus-induced structures (Table 2).

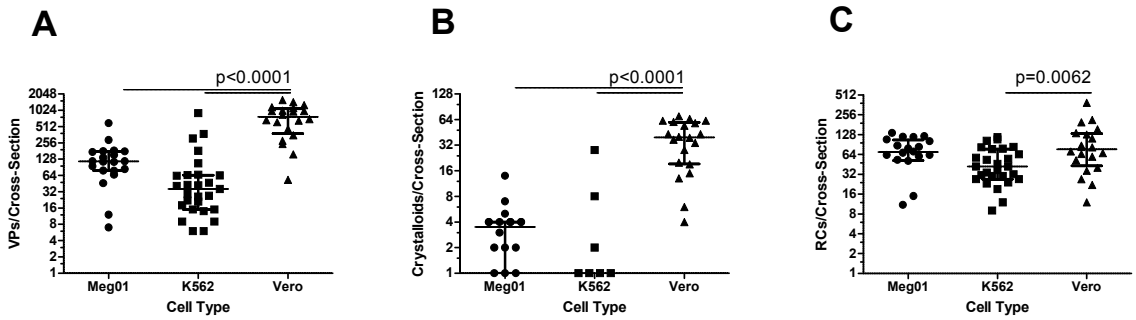
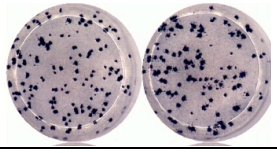
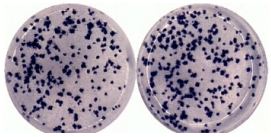
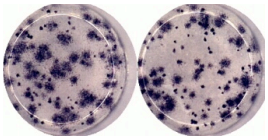


Figure 2.3. Quantitative comparison of virus-induced structures in Meg01, K562, or Vero cells.

Meg01, K562, or Vero cells were inoculated at a low MOI and cell pellets or monolayers from day 2 were fixed, thin-sectioned, stained, and analyzed. Cross-sections of 20 Meg01, 27 K562, or 20 Vero cells were evaluated for the formation of VPs, crystalloid structures, and RCs. (A) Concentration of VPs per cell cross-section. (B) Concentration of crystalloid structures per cross-section. (C) Concentration of RCs per cross-section. Bar indicates median, and whiskers show standard deviations. P values were obtained using unpaired student's t-test.

Abbreviations: MOI = multiplicity of infection; RCs = replication complexes; VPs = virus particles.

Table 2.2. Quantification of DENV2-induced structures from day 2 infected Meg01, K562, or Vero cells.

	Meg01		K562		Vero	
	Mean ± SD	**Median (Q0,1,3,4)**	**Mean ± SD**	**Median (Q0,1,3,4)**	**Mean ± SD**	**Median (Q0,1,3,4)**
VPs per cross-section	140.9 ± 125.1**	116 (7, 78.8, 174.3, 593)	94.9 ± 185**	36 (6, 16.5, 64.5, 908)	764.2 ± 443.2	769 (49, 393.8, 976.3, 1689)
Crystalloids per cross-section	3.3 ± 3.1**	3.5 (0, 1, 4, 14)	1.6 ± 5.5**	0 (0, 0, 0.5, 28)	35.5 ± 22.5	37 (0, 16.8, 54.8, 71)
VPs per crystalloid	9.2 ± 5.7*	8.8 (0, 6.4, 12.3, 23)	2.6 ± 5.7**	0 (0, 0, 0, 22)	14.5 ± 6.6	12.9 (0, 10.8, 19.4, 27.4)
Replication complexes per cross-section	70.4 ± 40.9	69.5 (0, 52.5, 103.5, 136)	49.7 ± 29.9*	42 (9, 28.5, 70.5, 117)	92.2 ± 78.9	71 (12, 38.3, 120.8, 342)
Percent infected (number of EM grids analyzed)	17.5% ± 5.8% (10)**	—	19.2% 6% (11)**	—	85.8% ± 3% (5)	—
Percent small focus size	100% 		98.8% 		65.8% 	

*p<0.01 compared with the corresponding value from Vero cells using student's t-test.

**p<0.0001 compared with the corresponding value from Vero cells using student's t-test.

Abbreviations: EM = electron micrograph; SD = standard deviation; Q = quartile; VPs = virus particles.

A link has been suggested between numbers of viruses produced by a cell and virus plaque diameter (Junjhon et al., 2008; Lee et al., 2010). DENV2 derived from Meg01 and K562 cells had more uniform sizes of small foci (Table 2) and plaques (data not shown), while DENV2 derived from Vero cells formed foci with various widths. It is important to note that other DENV strains were not examined in such a detailed manner, and thus, it is not known if reduced intracellular virion numbers correlate with reduced focus/plaque diameters for other strains grown in MEP cell lines.

Growth of a limited number of strains was examined in Meg01 and K562 cells. Production of prototypic strains from the three other DENV serotypes, DENV1 (Hawaii, gift from CDC), DENV3 (H87, gift from CDC), and DENV4 (Hawaii, gift from Dr. Duane Gubler), were tested in a limited number of experiments with Meg01 and K562 cells. Using a focus-forming unit assay (FFA) for quantification, all strains could be propagated in MEP cell lines but not reproducibly. Titers of 1 x 10^5 FFU/mL were obtained with all viruses in both MEP cell lines, except for DENV4 in Meg01, which only reached approximate 1 x 10^4 FFU/mL titers (data not shown). Reduced replication might be attributable to the strain type and the presence of defective interfering particles. The DENV4 strain gave rise to large foci when grown in Vero cells, but focus sizes varied when grown in MEP-DENV4 cells.

Minor differences in quantity and density observed with purified Vero-DENV2 and MEP-DENV2

Virus was propagated on a larger scale in Meg01, K562, or Vero cells for 3 days, and supernatants were collected for virus purification. After fractionation through 0%–35% potassium tartrate gradients and removal from gradient solutions, virus was assayed for infectivity by FFA. The infectious titers of fractions from all virus purifications performed are displayed (Fig. 2.4A–C). The data represent seven Meg01-DENV2, five K562-DENV2, and four Vero-DENV2 large-scale purifications. The highest infectious titers were found consistently in either fraction 7 or 8 at the

approximate density of 1.39 g/mL (Fig. 2.4D), which differs from the density specified using cesium chloride gradients (1.22–1.24 g/mL) (Smith et al., 1970; Stevens and Schlesinger, 1965). Virus peaked in fraction 7 with 60% of the K562-DENV2 purifications (3 of 5), 50% of the Vero-DENV2 purifications (2 of 4), and 71% of the Meg01-DENV2 purifications (5 of 7). The variation in localization in fraction 7 versus 8 might be attributable to minor differences in gradient preparation rather than differences in virus density.

Average peak infectious titers for Vero-DENV2 (6.5×10^6 FFU/mL) were generally two times lower than those from the MEP cells lines (Meg01-DENV2, 1.2×10^7 FFU/mL; K562-DENV2, 1.8×10^7 FFU/mL), even though on average about twice as many cells were used to propagate Vero-DENV2. In representative virus purifications, the Vero-DENV2 peak titer was at least 10 times lower than MEP cell line titers (Meg01-DENV2, 2.1×10^7 FFU/mL; K562-DENV2, 9.2×10^6 FFU/mL; Vero-DENV2, 6.8×10^5 FFU/mL) (Fig. 2.4E), a difference that did not correspond with the starting cell populations. Vero-DENV2 might be considered less-stable through purification processes than MEP-DENV2. However, our observations from immuno-EM imaging experiments did not agree with that notion, based on the observation that Vero-DENV2 was the easiest virion to detect.

GCN titers and GCN:FFU ratios of purified fractions also were evaluated (Fig. 2.4E). In general, the lowest GCN:FFU ratios from purified and fractionated virus were found in fractions 7 and 8, corresponding with the infectious virus peaks. Similar to the results from viral supernatants, Meg01-DENV2 peaks had the lowest ratios (73.1 GCN/FFU [fraction 7] and 22.8 GCN/FFU [fraction 8]), and Vero-DENV2 had the highest ratios (689 GCN/FFU [fraction 7] and 1640 GCN/FFU [fraction 8]), suggesting again that markedly higher numbers of noninfectious virus are produced in Vero cells relative to MEP cells.

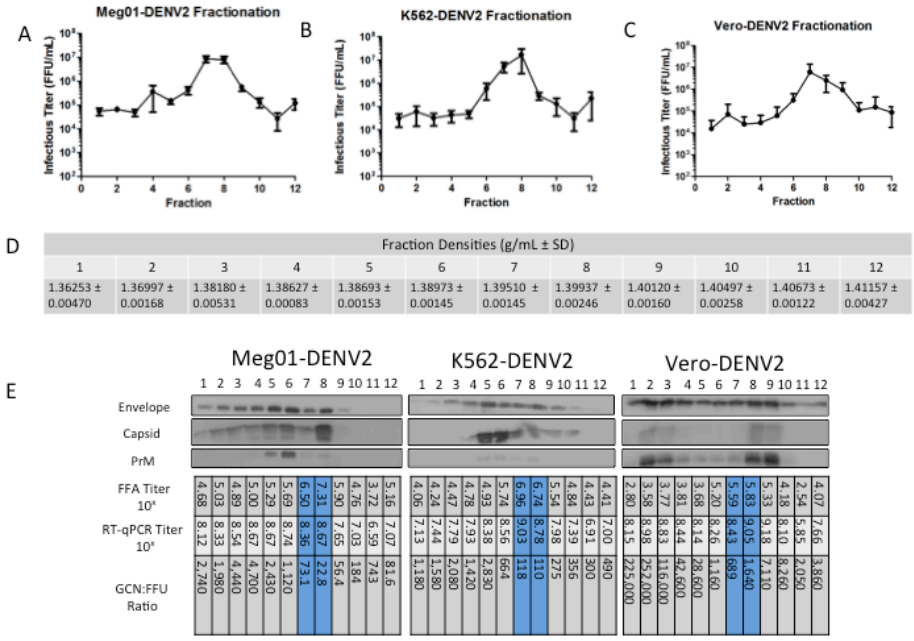


Figure 2.4. Characterization of gradient-fractionated Meg01-DENV2, K562-DENV2, or Vero-DENV2.

Large-scale batches of DENV2 were propagated in (A) Meg01, (B) K562, or (C) Vero cells and then purified through potassium tartrate-glycerol gradients and fractionated. From each fraction, virus was analyzed by FFU and western blot assays. (A–C) Graphs indicate the mean concentration and SD of infectious virus from all replicates performed (Meg01-DENV2, n = 7; K562-DENV2, n = 5; and Vero-DENV2, n = 4) per fraction. (D) Density readings for fractions 1–12 (n = 3). (E) Western blot, FFA titer (FFU/mL), RT-qPCR titer (GCN/mL), and GCN:FFU ratio comparison from a representative DENV2 purification from each cell line. Envelope, capsid, and prM proteins were detected with 4G2, 6F3-1, and GeneTex polyclonal antibody, respectively.

Abbreviations: FFA = focus-forming unit assay; FFU = focus-forming unit; GCN = genome copy number; prM = premembrane; RT-qPCR = reverse transcriptase-quantitative polymerase chain reaction; SD = standard deviation.

MEP-DENV2 structural protein fractionation patterns vary from that of Vero-DENV2

Western blots were performed with equal volumes of each fraction to compare protein content from fraction-to-fraction and to compare MEP-DENV2 with Vero-DENV2 (Fig. 2.4E). Envelope and premembrane (prM) proteins appeared more abundant in Vero-DENV2 than MEP-DENV2 samples in several fractions. On the other hand, capsid protein was more abundant in DENV2 produced in MEP cells.

In general, the amount of envelope protein correlated poorly with the titers of infectious virus, although infectious virus did correspond somewhat with the presence of capsid and prM proteins. These structural proteins from Vero-DENV2 purifications peaked in fractions 8 and 9, close to the infectious virus peaks in 7 and 8. (Note that in this purification similarly high titers [1×10^5 FFU/mL] were present in fraction 9.) A second focus of concentrated protein occurred in Vero-DENV2 fractions 2 and 3, which probably corresponds with the smaller virion structures reported in the literature (Allison et al., 2003; Ferlenghi et al., 2001; Ishikawa and Konishi, 2006; Junjhon et al., 2008). Capsid and prM proteins were present in K562-DENV2 and Meg01-DENV2 infectious virus peaks, although higher concentrations were found in adjacent fractions. Potentially, these bands (which are not present in Vero-DENV2 purifications) resulted from damaged virus particles migrating to slightly lower densities; their intensity might reflect lower MEP virus stability.

Antigenicity of MEP-DENV2 differs at domain I/II of envelope protein

We examined antigenicity because post-translational modifications of viral proteins such as glycosylation are known to vary among host cell types (Bryant et al., 2007; Dejnirattisai et al., 2011; Lee et al., 2010). Neutralizing antibody concentrations were determined by plaque reduction neutralization assay (PRNA) for Meg01-DENV2, K562-DENV2, and Vero-DENV2 with a series of monoclonal antibodies: 3H5, 4G2, 2D22, 2C7, 3F13, and VRC-01 (Fig. 2.5).

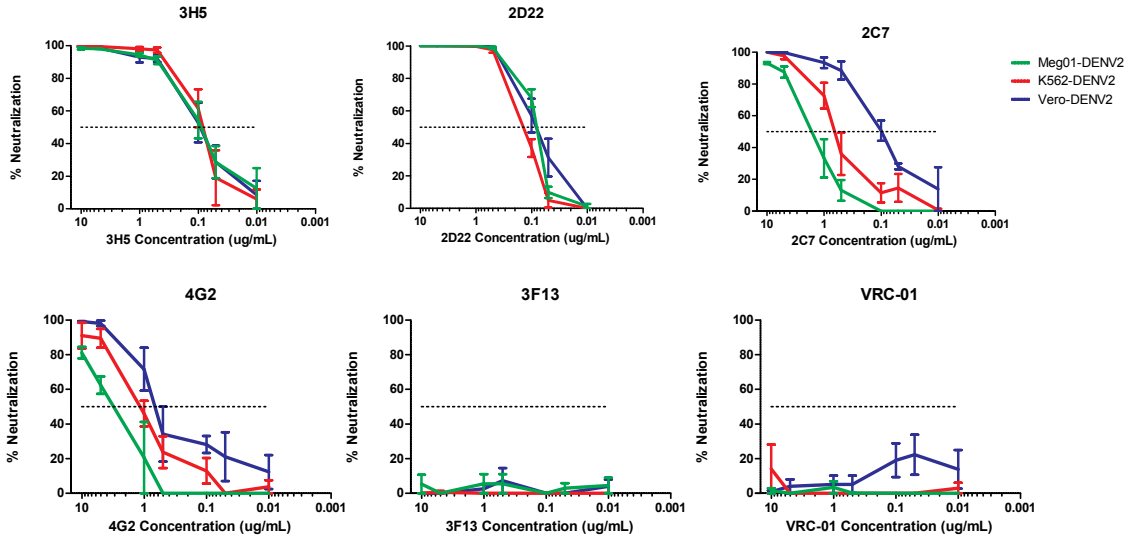


Figure 2.5. Neutralization assays of Meg01-DENV2, K562-DENV2, or Vero-DENV2 with monoclonal antibodies.

Mouse (3H5 and 4G2) or human (2D22, 2C7, and 3F13) anti-DENV2 envelope antibodies or control anti-HIV envelope antibody (VRC-01) were tested for their neutralization capacity via plaque reduction neutralization assays. Graphs indicate the average percent neutralization with decreasing concentrations of antibody (n = 3).

Anti-DIII envelope monoclonal antibodies 3H5 (mouse-derived) and 2D22 (human-derived) neutralized all three viruses similarly. However, neutralization of MEP-DENV2 with anti-envelope DI/II antibodies 4G2 (mouse-derived) or 2C7 (human-derived) required higher levels of antibody. The half maximum inhibitory concentrations (IC_{50}) of 2C7 for Meg01-DENV2 (4.1 μg/mL) and K562-DENV2 (0.45 μg/mL) were elevated markedly in comparison with the IC_{50} value for Vero-DENV2 (0.11 μg/mL, $p<0.05$) (Table 3). The IC_{50} of 4G2 for Meg01-DENV2 (5.42 μg/mL) also was much higher than that for Vero-DENV2 (0.33 μg/mL, $p=0.0011$). Most antibodies were capable of neutralizing K562-DENV2 and Vero-DENV2 completely, but 2C7, 4G2, and 3H5 did not fully neutralize Meg01-DENV2. Neutralization with human polyclonal antibodies also was performed. Serum sample DF 3457 neutralized Meg01-DENV2 to a lesser extent, but no difference was detected with endemic plasma (Supp. Fig. 2.2), or with plasma of a person from a DENV endemic country. In summary, modest differences in neutralization were observed with Meg01-DENV2, suggesting Meg01-produced DENV2 might be more resistant to neutralization, at least *in vitro*. As expected, the control non-neutralizing anti-dengue antibody 3F13 and HIV-specific monoclonal antibody VRC-01 did not neutralize dengue virus.

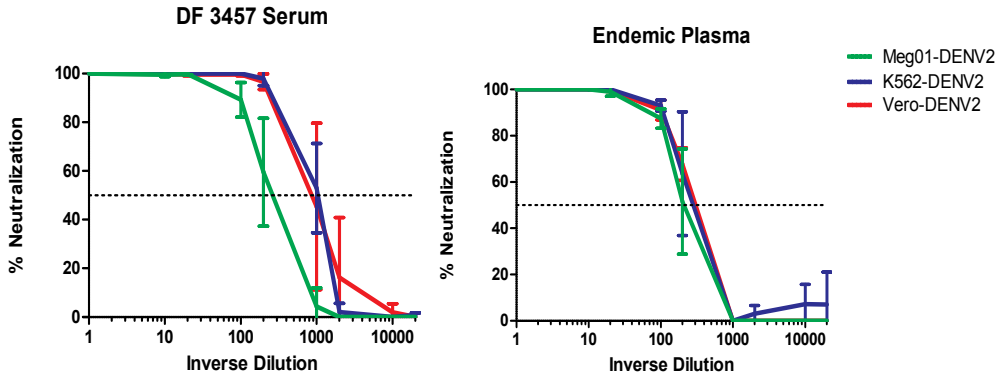
Table 2.3. Neutralization capacity (measured by IC_{50}, IC_{90}, and maximum neutralization) of antibodies against Meg01-DENV2, K562-DENV2, or Vero-DENV2.

Antibody	Meg01-DENV2			K562-DENV2			Vero-DENV2		
	IC_{50} (μg/mL)	IC_{90} (μg/mL)	Max (%)	IC_{50} (μg/mL)	IC_{90} (μg/mL)	Max (%)	IC_{50} (μg/mL)	IC_{90} (μg/mL)	Max (%)
2D22	0.12	1.54	100	0.18	1.88	100	0.1	1.48	100
2C7	4.14*	8.12	94.1	0.45*	5.27	100	0.11	1.93	100
4G2	5.42**	10.0	87.3	0.87	13.6	98.8	0.33	5.73	100
3H5	0.1	1.91	99.2	0.11	1.62	100	0.11	1.86	100
3F13	NN	NN	16.7	NN	NN	2.13	NN	NN	12.1
VRC-01	NN	NN	10.4	NN	NN	42.3	NN	NN	38.6

*$p<0.05$ compared with the corresponding value from Vero-DENV2 using student's t-test.

**$p=0.0011$ compared with the corresponding value from Vero-DENV2 using student's t-test.

Abbreviations: IC_{50} = half maximal inhibitory concentration; IC_{90} = 90% maximal inhibitory concentration; NN = non-neutralizing.



Supplemental Figure 2.2. Humoral characterization of Meg01-DENV2, K562-DENV2, or Vero-DENV2 with human polyclonal antibodies.

Neutralization capacity of DF 3457 serum or plasma from an person from a dengue endemic location were tested via plaque reduction neutralization assay. Graphs indicate the average percent neutralization with decreasing concentrations of antibody (n = 3).

DISCUSSION

Megakaryocytes and platelets are dysfunctional in dengue patients, and direct infection of megakaryocytes is one potential attributing factor that might explain this phenomenon. To examine whether cells of the megakaryocyte-erythrocyte lineage can be directly infected by DENV, we took advantage of the readily available Meg01 megakaryoblast and the related K562 erythroid cell lines to assess DENV2 viral growth and virus particle characteristics and compared them with those from the Vero epithelial cell line that is typically used to propagate DENV. Our data suggest DENV2 production in Meg01 and K562 is more efficient than that in Vero, based on their lower viral GCN:PFU ratios and reduced virus particle levels despite easily quantifiable infectious virus. Also, despite similar levels of infectious virus in day 3 cell supernatants, EM analyses of unconcentrated supernatants failed to reveal MEP-DENV2 particles, suggesting that EM particle:infectious virus ratios also might be reduced in these cell lines. This observation is not surprising. DENV particles have rarely been documented directly from patient and rhesus macaque tissues; the virions that have been imaged were found inside of platelets (Noisakran et al., 2009; Noisakran et al., 2012). Because little work has been done to characterize DENV particles directly produced in human patients, the potential that *in vivo* virus structure differs from cell culture virus remains a viable possibility. Alternative virus structures with different protein content have been suggested for DENV produced *in vivo* (Hsu et al., 2015).

Ultrastructural studies have indicated that different DENV-infected cell lines display unique features (e.g., convoluted structures are absent in the insect cell line C6/36 and crystalloid structures rarely form in cell lines) (Junjhon et al., 2014); thus analyses of the megakaryocyte-erythrocyte lineage were conducted. Our previous report evaluating mature megakaryocytes indicated that they produced DENV2, with an abundance of virus-like particles in the cytoplasm (Clark et al., 2012). However, EM analyses with Meg01 and K562 suggested a far more controlled production of classical virus particles – 50 nm electron dense structures within ER-derived vesicles. Although the differentiated

megakaryocytes contained virus and crystalloid structures, it is possible that a number of the virus-induced structures observed in that report might have been polysomes (strings of ribosomes linked together by mRNA). Polysomes (also known as dense particles) are electron dense and approximately the same size as the virus core (Hase et al., 1987; Ko et al., 1979; Sriurairatna et al., 1973); they are indicative of high levels of protein production and were numerous in the cytoplasm of DENV2-infected Vero cells in this study. In contrast, we did not observe abundant polysome-like structures in Meg01 or K562 cells, which could reflect reduced viral protein production and account for the lower levels of virus particle assembly relative to Vero cells.

This study supports the concept that abundant virion production is not required for high infectious titers. Although high levels of viral protein production can be observed in kidney epithelial (Vero) cells, these observations should be evaluated cautiously because kidney cells are not likely natural targets of DENV infection *in vivo*. Kidney cell lines have a tendency to produce noninfectious subviral virions, while western blot results of MEP-DENV2 did not indicate the presence of these types of particles. DENV2 protein production in MEP cell lines appeared to be coordinated, leading to lower amounts of excessive viral protein production, thereby reducing the likelihood of immune recognition. Many mutations associated with reduced virus production have already been identified (Junjhon et al., 2008; Lee et al., 2010; Pryor et al., 2004; Yoshii et al., 2004) and could potentially play a role in shaping virus particle production in MEP cells.

In addition to differences in virus particle production, we also found minor variations in virus composition and structure. While the three cell lines examined propagated virus of comparable morphology and density, MEP-DENV2 appeared to have less prM protein. DENV is known to be unique among the flaviviruses for its inherently inefficient prM cleavage process, which is facilitated by a mutation in the prM trypsin cleavage recognition site that inhibits cleavage (Junjhon et al., 2008). Less prM was noted in purified fractions of MEP-DENV2, which could indicate more proficient cleavage and virus maturation, potentially explaining the efficient infectious virus production

observed in this report. Also, antigenic composition presented subtle differences. In particular, we observed differences in neutralization with envelope domain I/II antibodies, which could be significant since many potent anti-DENV neutralizing antibodies produced in humans are directed against this epitope, and poorly neutralizing domain I/II antibodies do not correlate with protection (Beltramello et al., 2010; de Alwis et al., 2012; Shrestha et al., 2010; Smith et al., 2012; Wahala et al., 2009). In one example, *in vitro* neutralization of Vero-DENV2 was demonstrated clearly with serum from vaccinated volunteers; however, no protection was observed against this serotype in vaccine recipients (Sabchareon et al., 2012). Potentially, this discrepancy might be attributable to differential antigenicity of the envelope domain I/II protein epitopes displayed on Vero-DENV2 and on *in vivo*-DENV2. Data in this report suggests that vaccine recipient serum might neutralize Meg01-DENV2 differently from Vero-DENV2. The importance of these differences in antigenicity remains to be fully elucidated, but *in vivo* protection in the aforementioned vaccine study could have been predicted better potentially with neutralization assays involving virus propagated in a target cell line, such as Meg01-DENV2. Additional work examining Meg01-DENV antigenicity and the role of the megakaryocyte lineage in DENV pathogenesis is warranted. This new system for propagating infectious DENV provides a new tool for the design of dengue vaccines and for the evaluation of antiviral compounds.

MATERIALS AND METHODS

Virus and cells

The DENV strain used in these experiments was 16681 (DENV serotype 2) originally grown in Vero-E6 cells. This virus is referred to here as Vero-DENV2. Stocks of Vero-DENV2 were propagated once in Meg01 cells (Meg01-DENV2) or K562 cells (K562-DENV2). Meg01 cells were a gift from Dr. Ofori-Acquah at Emory University. Vero and K562 cells were grown in RPMI medium (Cellgro, Manassas, VA) with 10% fetal bovine serum (FBS) (Atlanta Biologicals) and

penicillin-streptomycin (PS) (Cellgro), while Meg01 was cultured in RPMI with PS and 20% FBS. (Meg01 had poor growth kinetics at low cell densities; high FBS concentrations were used to ensure continuous doubling and permissiveness). All infected cells were maintained in RPMI medium supplemented with PS 10% FBS medium, unless otherwise specified. For imaging studies, we used exosome-free FBS (prepared by centrifuging FBS at 100,000 *g* for 18 hrs and passing through a 0.2 μm cellulose acetate filter unit [Corning]).

Comparison of virus growth kinetics in different cell lines

Vero-DENV2 was propagated in 2×10^6 cells of Meg01, K562, or Vero cells by inoculation with an MOI of 0.1 FFU/cell. Cells were incubated with virus for 2 hrs in a 15 mL polypropylene conical tube in a CO_2 incubator at 37°C, washed three times with D-PBS (Lonza, Walkersville, MI), and resuspended to a final concentration of 5×10^5 cells/mL with RPMI 10% FBS (K562 and Vero cells) or RPMI 20% FBS (for Meg01 cells) in T25 flasks (Corning). Medium was added and aliquots were taken daily from day 2–7. Samples were analyzed via plaque assay and RT-quantitative PCR.

Plaque assay and plaque reduction neutralization assay

Cells were seeded into either 6- or 12-well plates (Falcon, Durham, NC) the day before the experiment. For regular plaque assays, virus was 10-fold serially diluted in medium. Medium was removed from plates, virus dilutions applied in duplicate, and incubated at 37°C for 1 hr.

For Plaque Reduction Neutralization Assays (PRNAs), cells were seeded in a similar manner. Antibodies were serially diluted in RPMI 5% FBS medium. Viruses also were diluted in RPMI 5% FBS and mixed equal-volume with the antibody dilutions. A no-antibody control (~1,000 PFU/reaction) was treated in a similar manner and used as the virus titration control. Samples were incubated in cell culture incubators at 37°C for 1 hr. After the incubation period, virus was diluted to

a final volume of 1 mL and 5% of the reaction was applied to wells. Additional medium was added to cover the cells, and plates were incubated at 37°C for 30 min.

For both plaque assays and PRNAs, cells and inocula were overlaid with 1.5% methylcellulose 1,500 cps (MP Biomedicals, Solon, OH) medium (0.5X RPMI, 5% FBS, PS, pH 8.0) and incubated at 37°C for 12 days. With PRNAs involving polyclonal human antibodies, the same medium except with a different methylcellulose (1.0% of 1,500–5,600 cps [Fisher Science Education]) was used and harvested on day 7. Plaques were visualized by staining monolayers with 0.1% crystal violet (Sigma-Aldrich) in 20% methanol before counting.

Reverse transcription-quantitative polymerase chain reaction

RNA was isolated from virus supernatants or concentrates with QIAamp viral RNA mini kit or EZ-1 virus mini kit v2.0 (Qiagen) using the manufacturer's protocol. RNA was reverse transcribed into cDNA and amplified in a one-step reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay with LightCycler 480 RNA Master Hydrolysis Probe (Roche) using primers (DENV2U and DVL1) and probe (DVP1) for 40 cycles of 95°C (15 sec) and 60°C (1 min) on LightCycler 480 II (Roche), similar to a previous publication (Houng et al., 2001).

Antibodies

Mouse monoclonal antibodies 4G2 and 3H5 (CTK Biotech, San Diego, CA) specific to DENV2 envelope proteins were used in various assays. Anti-capsid 6F3-1 hybridoma supernatant and anti-polyclonal prM antibody (Genetex) were used in western blot assays. Human monoclonal antibodies (2C7, 2D22, and 3F13) were used in PRNAs. Dr. Chokephaibulkit, Dr. Pattanapanyasat, and Patcharee Songprakhone from Siriraj Hospital in Bangkok, Thailand provided convalescent patient serum samples used in PRNAs. Endemic plasma (or plasma from a healthy donor native to a country endemic for DENV) was obtained through Emory University's blood donation program.

VRC-01 (Mapp Biopharmaceutical, San Diego, CA), a human anti-HIV envelope monoclonal antibody, was used in PRNA as a negative control.

Virus purification

Vero-DENV2, K562-DENV2, and Meg01-DENV2 were propagated in a similar manner as described for the growth kinetics experiments. Vero cells: T162 flasks (Falcon, Durham, NC) were seeded with cells days before and about 4.8×10^8 cells were inoculated at an MOI of 0.1 FFU/cell with Vero-DENV2 stock virus. Inocula were removed and replaced with 45 mL of RPMI PS 10% FBS (exosome-free) medium. Meg01 or K562: $1–4 \times 10^8$ cells were inoculated at an MOI of 0.02 FFU/cell. Cells were incubated in T162 flasks for 1–2 hrs. Cells were washed three times with RPMI PS medium and resuspended to a final volume of 5×10^5 cells/mL in RPMI PS 10% exosome-free FBS. After 3 days of propagation, supernatant was clarified at 3,000 rpms for 30 min. Supernatant was treated with polyethylene glycol (PEG) 8,000 (Fisher BioReagents, Fair Lawn, NJ) solution (final concentration: 8% PEG 8,000, 1 M NaCl, 5 mM EDTA, pH 8.5) overnight. Virus was concentrated with a Beckman Optima L-70K ultracentrifuge at 12,000 rpm in SW28Ti rotors for 25 min and resuspended in TNE buffer (50 mM Tris-HCl, 75 mM NaCl, 5 mM EDTA, pH 8.0). Concentrated virus was fixed in 2% paraformaldehyde (Sigma-Aldrich) in TNE buffer (final pH 7.0) for EM. Continuous potassium tartrate dibasic hemihydrate (Sigma-Aldrich, St. Louis, MO) (0%–35% w/w)-glycerol (30%–12.5% w/w) gradients were formed with Gradient Master IP 107 (BioComp) using glycerol program 10%–20% (v/v) in 14 x 89 mm ultraclear tubes. Concentrates were centrifuged in an SW41Ti rotor at 40K rpm for 16–18 hrs. Twelve fractions were isolated by pipette, starting from the top of the gradient. An aliquot of each fraction was taken from some gradients (Meg01-mock, K562-mock, and Vero-mock) and averaged to determine the buoyant density with a Bausch & Lomb refractometer. Fractions were diluted with TNE buffer and centrifuged in the SW28Ti rotor at 28K rpm for 1.5 hrs. Virus was resuspended in TNE buffer and aliquoted for further analyses.

Negative-staining immuno-EM and thin-sectioning EM

For immuno-EM, samples were fixed in 2% paraformaldehyde in TNE buffer and given to the Robert P. Apkarian integrated electron microscopy core service at Emory. Samples were applied to carbon-coated grids, incubated with DENV2 envelope-specific primary antibody (3H5), gold-conjugated anti-mouse secondary antibody, and tungsten stained.

For thin-sectioning EM, DENV2-infected K562, Meg01, and Vero cells at 1 and 2 days post-inoculation were washed twice with D-PBS, fixed in 4% glutaraldehyde in phosphate buffer overnight, and given to the EM core. The cells were processed for thin-sectioning EM as previously reported (Noisakran et al., 2009). Using IMOD imaging software (http://bio3d.colorado.edu/imod/), multiple images of different sections of the same cell were acquired and patched together into one continuous cell image. A total of 20 Meg01, 27 K562, and 20 Vero single cell cross-sections were examined. In cell image analyses, a virus particle was defined as a circular electron dense object in the 30–60 nm range that appeared to be enclosed within the ER or an ER-derived membrane vesicle. Replication complexes were larger, circular, mostly-empty objects that also were enclosed within ER-derived membranes. Crystalloids were defined as a cluster of at least five virions that were not aligned linearly.

Focus-forming unit assay

Flat bottom 96-well plates (Celltreat) were seeded with 2×10^4 Vero cells per well the day before titration. Medium was removed from 96-well plates and 10-fold serial dilutions of virus samples were applied in duplicate. Plates were incubated for 1–2 hrs at 37°C. Subsequently, cells and inocula were overlayed with 1.5% methylcellulose cps 1,500 medium (1X EMEM [Lonza, Walkersville, MI], 5% FBS, 2 mM L-glutamine, 10 mM HEPES, PS) and incubated for 3 days. Cells were washed three times in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4,

pH 7.5), and fixed in 3.7% formaldehyde for 1 hr at room temperature or overnight at 4°C. Cells were permeabilized for 10 min with 1% triton X-100 (Acros) in PBS and washed five times with PBS. Monolayers were blocked with 2% normal goat serum (Jackson Immuno Research) in PBS for 1 hr and then incubated with 10 μg/mL 4G2 in PBS for 1 hr at 37°C. After three washes, monolayers were incubated with goat anti-mouse IgG-HRP human absorbed antibody (Southern Biotech) in PBS-Tween 20 for 1 hr at 37°C. After three washes, foci were incubated in DAB (0.6 mg/mL 3,3'-diaminobenzidine tetrahydrochloride [Sigma-Aldrich], 0.08% $NiCl_2$, 0.01% H_2O_2 in PBS) until development was complete.

Western blot

Purified virus fractions (1–12) were diluted in 4x SDS-PAGE loading buffer (160 mM Tris, 6.4% SDS, 20% glycerol), loaded onto 10% or 12% separating SDS-polyacrylamide (Bio-Rad) gels and run in Tris-Glycine-SDS (TGS) running buffer at 90 v for 2–3 hrs with EPS 1001 power supply (General Electric). For western blots with 4G2, samples were not heated or reduced; for blots with 6F3-1, samples were heated; and for prM, samples were heated and reduced with β-mercaptoethanol. Gels were transferred to methanol-pretreated PVDF membranes (Bio-Rad) in transfer buffer (2.5 mM Tris, 19.2 mM glycine, 20% methanol) for 15–17 hrs at 30 v. Membranes were blocked for 1 hr at room temperature with blocking buffer (5% milk in PBS-Tween 20). Membranes were incubated with 4G2 (10 μg/mL, 1 hr), 6F3-1 (neat, 2 hr), prM antibody (1:1,000, 2 hr) in blocking buffer. After five washes with PBS-Tween 20, membranes were incubated 1 hr with 1:1,000 dilution of appropriate secondary anti-mouse or anti-rabbit IgG-AP conjugated antibody. After washing, a 30-min incubation with Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega, Madison, WI) allowed for visualization of viral antigens.

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CHAPTER 3

Title: Multiploid CD61$^+$ cells are the pre-dominant cell lineage infected during acute dengue virus infection in bone marrow

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Title: Multiploid CD61^{+} cells are the pre-dominant cell lineage infected during acute dengue virus infection in bone marrow

Running Title: Dengue Virus Infection in Bone Marrow

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ABSTRACT

Depression of the peripheral blood platelet count during acute infection is a hallmark of dengue. This thrombocytopenia has been attributed, in part, to an insufficient level of platelet production by megakaryocytes that reside in the bone marrow (BM). Interestingly, it was observed that dengue patients experience BM suppression at the onset of fever. However, few studies focus on the interaction between dengue virus and megakaryocytes and how this interaction can lead to a reduction in platelets. In the studies reported herein, BM cells from normal healthy rhesus monkeys (RM) and humans were utilized to identify the cell lineage(s) that were capable of supporting virus infection and replication. A number of experiments were performed: viral RNA quantification, nonstructural protein assays, infectious virus analysis, phenotypic studies utilizing immunohistochemical staining, anti-differentiation DEAB drug treatment, and electron microscopy. Cumulative results from these studies revealed that cells in the BM were indeed highly permissive for DENV infection, with human BM having higher levels of infectious virus production compared to RM. DENV-like particles were predominantly observed in $CD61^+$ cells with multiple nuclei. These data suggests that megakaryocytes are the predominant cell type infected by DENV in BM, which likely explains thrombocytopenia and the dysfunctional platelets characteristic of dengue.

INTRODUCTION

Bone marrow (BM) is one of the largest and most widely distributed organs in the body. It is the principal site for blood cell formation; the daily production of which in adults is 2.5 billion red cells and platelets each, and 1.0 billion granulocytes per kilogram of body weight. The bone marrow compartment is a highly dynamic environment; even small changes can lead to a very significant modification in the cellular constituents in the corresponding peripheral blood.

There is extensive evidence implicating the involvement of the BM in dengue virus infection. Excruciating bone pain can be a common symptom in dengue patients; hence the term "break-bone fever" was coined and has become synonymous with dengue fever (Gubler 1997). Pain localized to the BM suggests the involvement of this organ during dengue virus infection. *In vitro* studies have found that cells in the BM are highly permissive for dengue virus infection (Nakao, et al 1989) and are more so than those from the spleen, lymph node, and thymus (Halstead, et al 1977). Moreover, a report documented the transmission of dengue virus from a donor to a recipient as a result of a BM transfusion (Rigau-Perez, et al 2001). In this case study, the donor was at an early stage of infection and did not have any signs of illness. But fever was noticed 2 days after the donation, and it was later confirmed that the donor was indeed infected with dengue type 4 by serological tests.

Bone marrow suppression has long been recognized as a clinical feature contributing to dengue disease. An early investigation in Thailand and Malaysia revealed that the bone marrow mass is at its nadir at the onset of fever and at its peak 2–3 days later (the time when most patients start enrolling in the hospital) (Bierman and Nelson 1965, Kho, et al 1972, Na-Nakorn, et al 1966). The kinetics of bone marrow changes makes it very difficult to study this subject in detail for obvious practical reasons (Tsai, et al 2012). This difficulty is compounded by the bleeding tendencies of these patients making it clinically impractical to acquire BM samples. Thus, except for some earlier investigations of bone marrow during acute infection of dengue patients, the practice of bone marrow sampling is now clinically contra-indicated making it difficult to ascertain the relationship

between dengue virus infection and the role of the bone marrow during acute infection. It is important to note that despite decades of research, the primary permissive target cell lineage for dengue virus replication in vivo continues to remain unclear. The fact that acute dengue disease is accompanied with a marked disappearance of megakaryocytes and the stagnation of erythropoiesis (Bierman and Nelson 1965) in conjunction with thrombocytopenia (a hallmark feature of dengue disease) led us to postulate that dengue virus may indeed target the megakaryocytes.

Recently, dengue virus-induced loss in BM mass was substantiated in the dengue virus coagulopathy model in rhesus macaques (Noisakran, et al 2012). In these animals, the cells capable of generating infectious dengue virus displayed integrin CD61, a cell surface marker specifically expressed by platelets and their megakaryocyte precursors. In order to further understand the nature of dengue virus infection, ex vivo experiments were performed with BM samples from healthy rhesus macaques and humans. The results of these studies showed that i) human BM cells were more permissive than those from rhesus monkeys for dengue virus infection *in vitro*, as determined by RNA and NS-1 quantification assays, ii) densely packed dengue virus-like particles were visualized predominantly in the cytoplasm of multi-lobulated cells, as indicated by electron microscopy (EM), iii) the virus from human and monkey whole BMs were infectious, iv) dengue virus antigen was present in multi-lobulated cells expressing CD61 as determined using immunohistochemical techniques, and v) virus containing cellular debris were engulfed by phagocytic cells, evidenced by EM and histochemical stainings. Taken together, these data strongly indicate that the megakaryocytes are likely to serve as the major target of dengue virus infection and replication in the BM. We reason that the mechanisms of platelet dysfunction and thrombocytopenia are in part due to the targeting of megakaryocytes in the BM by dengue virus during acute infection. The marked destruction of these cells accompanied by release of pro-inflammatory cytokines such as but not limited to TNF-alpha, IL-1 and IL-6 normally associated with pain, followed by their rapid reconstitution in the BM and

probable exudation into the periphery, along with cellular signaling, may account for the extreme deep bone pain during the disease.

METHODS

Healthy rhesus monkey bone marrow procurement

BM was aspirated from the iliac crest of healthy rhesus monkeys and supplemented with heparin. BM cellularity was analyzed as previously described (Noisakran, et al 2012). All experimental protocols and procedures were conducted following approval by the Emory Institutional Animal Care and Use Committee (IACUC), and all animals were housed at the Yerkes National Primate Research Center of Emory University and cared for in conformance to the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council and the Health and Human Services (Institute of Laboratory Animal Research 1996).

Healthy human bone marrow procurement

Healthy human BM samples that would otherwise be discarded were obtained from the Stem Cell Processing Laboratory of the Emory Center for Transfusion and Cellular Therapy. The experiments were conducted following appropriate approval by the Emory IRB.

In vitro infection of the bone marrow

Results from a pilot study revealed that whole BM without any further processing was just as permissive as fractionated populations of bone marrow cells for dengue virus infection (Supplementary Figure 3.1). Thus, all experiments were performed with unfractionated bone marrow preparations. The total number of nucleated cells were determined as previously described (Noisakran, et al 2012). Dengue virus, strain 16681 (reference or describe), grown in Vero cells, was

used to infect the unfractionated bone marrow at an MOI of 0.1. The infected Vero cells following incubation for 2 hours at 37^{o}C were washed X3 with media to remove unbound virus. The infected cells were re-suspended in 2 ml of culture media and incubated in suspension without shaking and 400 μl of the cell suspension was removed at different time points as indicated in the text. BM smears were prepared by pelleting cells at low speed and applying them to slides.

Infectious virus analysis of BM supernatant in Vero cells

Cocultures were performed by adding a volume consistent with an MOI of 0.1 from monkey BM supernatant fluids collected on days 2 and 5 were added to Vero cells at 80% confluency. Subsequently supernatant fluids from these Vero cells were collected at the indicated time points and immediately stored at -80˚C until real time (RT)-PCR analysis.

Focus forming unit assays were performed by infecting a monolayer of Vero cells in 96-well plates with serial dilutions of human bone marrow supernatant in MEM media. After a two-hour absorption, the cells were overlayed with 1% methylcellulose in EMEM (with 2mM L-Glutamine, 1mM sodium pyruvate, 2% FBS, HEPES). Cells were incubated for 3 days and fixed with 3.7% paraformaldehyde. Cells were permeabilized with 1% triton-X for 10 minutes. Cells were washed 5 times with PBS and incubated with monoclonal cell line 4G2 supernatant for 1 hour at 37°C. Cells were washed 3 times and incubated with HRP-conjugated rabbit anti-mouse (Dako) for 1 hour at 37°C. Cells were washed 3 times and incubated with diaminobenzidine for 10 minutes.

FACS analysis of bone marrow aspirated from DENV infected rhesus monkey

Rhesus monkeys (Macaca mulatta) of Indian origin that were part of two separate experiments as previously described (Onlamoon, et al 2010) were the source of the samples described herein. At different time points post infection, bone marrow was aspirated from the iliac crest and supplemented with heparin. BM cells were stained with specific cell markers and

monoclonal antibody to dengue viral antigen and subjected to FACS analysis. All experimental protocols and procedures were conducted following approval by the Emory Institutional Animal Care and Use Committee (IACUC), and all animals were housed at the Yerkes National Primate Research Center of Emory University and cared for in conformance to the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council and the Health and Human Services (Institute of Laboratory Animal Research 1996).

Periodic Acid Schiff and Giemsa staining

Staining of cell smears was performed using the Periodic Acid Schiff stain with a PAS kit and Giemsa staining according to the manufacturer's suggested protocol (Polysciences, Inc., Warrington, PA).

Immunohistochemistry/immunofluorescent staining

Immunohistochemical staining for the detection of dengue viral antigen in BM smears was performed by employing the Vectastain ABC immunohistochemistry kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. Mouse anti-E monoclonal antibody (clone 4G2) or isotype-matched control (IgG2a) antibody was utilized in the primary staining step unless otherwise indicated. The stained samples were incubated with 3-amino-9-ethylcarbazole (AEC) or diaminobenzidine (DAB) as an enzyme substrate for peroxidase followed by mounting with DAPI (Invitrogen) or counterstaining with hematoxylin.

The identification of the dengue virus cell lineage consisted of dual staining of the preparation for dengue viral antigen in addition to a variety of cell lineage specific cell surface markers. Thus appropriate BM smears were fixed onto slides with 4% paraformaldehyde for 20 min

and permeabilized with 0.2% triton X-100 for 10 min at RT. The samples were then treated with 0.6% H_2O_2 for 30 min to block endogenous peroxidase followed by 30-min incubation with 10% human AB serum. After two washes with PBS, the samples were blocked according to the manufacturer's instructions and then incubated with mouse anti-E monoclonal antibody (clone 4G2) or its isotype-matched control (IgG2a) antibody at 4°C overnight. The samples were washed three times with PBS and incubated with biotinylated horse anti-mouse immunoglobulin at RT for 30 min followed by three washes with PBS. The samples were then incubated for 30 min each with Vectastain ABC reagent and AEC substrate (all reagents from Vector Laboratories, Inc., Burlingame, CA) for the development of peroxidase signal. Thereafter, the samples were washed three times with PBS, incubated with 10% normal mouse serum for 30 min, and then labeled with FITC-conjugated mouse anti-human CD41a (for megakaryocyte and platelets) or BDCA2 (for phagocytic dendritic cells) antibodies (Genway Biotec, San Diego, CA) for 1 hr. Following washing with PBS, the samples were incubated with 1: 250 dilution of rabbit anti-FITC antibody conjugated to alkaline phosphatase (Sigma Aldrich, St. Louis, MO) and the signal was developed by using Vector blue alkaline phosphatase substrate kit III in the presence of levamisole solution (Vector), an inhibitor of endogenous alkaline phosphatases. The resulting images were captured using a Zeiss microscope equipped with an Axis 5 digital camera. $CD41a^+DENV^+$, $CD41a^-DENV^+$, $BDCA^+DENV^+$, $BDCA^-DENV^+$ cells were counted by assessing the number of cell surface positive or negative cells among all $DENV^+$ cells in 3–5 slides. Numbers are expressed as a percentage of total $DENV2^+$ cells.

For immunofluorescent staining, smears of BM cells on glass slides were fixed with methanol for 5 minutes and rinsed with PBS. The slides were then incubated with 10% human AB serum in PBS at room temperature (RT) for 15 min followed by either mouse anti-NS1 monoclonal antibody (ab41616, Abcam, Cambridge, MA) or isotype-matched control (IgG1) antibody for 1 hr. The slides were washed and then incubated with PE-conjugated goat anti-mouse IgG antibody (eBioscience, San Diego, CA) at a dilution of 1:1000 for 1 hr. The slides were washed in PBS and

then incubated with 10% normal mouse serum in PBS for 30 min at RT to block the remaining binding sites followed by the addition of FITC-conjugated mouse anti-CD61 antibody (eBioscience) for 1 hr. The slides were washed three times with PBS and mounted with DAPI mount reagent (Invitrogen, Carlsbad, CA), and images were captured using a Zeiss microscope equipped with an Axis 5 digital camera.

Electron microscopy

BM cells collected at different time intervals following infection were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer overnight and then processed for electron microscopy by the Robert P. Apkarian Integrated Electron microscopy Core Facility Service at Emory University. Quantitative real-time RT-PCR (qRT-PCR) for the detection of viral RNA

RNA was extracted from 140 μl of culture supernatant fluid isolated from the BM using QIAmp Viral RNA mini kit (QIAGEN). The resultant RNA was then subjected to quantitative RT-PCR using the Taqman RT kit (Perkin Elmer Applied Biosystem) and a Bio-Rad iCycler system according to a previously described method (Onlamoon, et al 2010). An aliquot of RNA from a viral stock of DENV was used as a control. The detection limit of this assay was about 100 copies of viral RNA genome equivalents per ml.

Measurement of NS1 concentration in supernatant of infected bone marrow

Supernatant fluids of the culture were collected at the indicated days and stored at -80^{o}C until assay for NS1 as a surrogate measure of virus replication. Standard ELISA was set up to quantify the level of NS1 antigen in the collected supernatant fluid by using purified NS1 antigen (CTK Biotech. Inc, San Diego, CA) to derive a standard curve. Supernatants and various concentrations of NS-1 were incubated with coating buffer on ELISA plates (Nunc Maxisorp) overnight at 4^{o}C. After 2 washes with PBS, samples were blocked with 5% milk in PBS-Tween 20 for

30 minutes at RT. Polyclonal rabbit anti NS-1 antibody (2 μg/ml) in 5% milk was incubated for 1 hour at 37^{o}C. Plates were washed and incubated with horseradish peroxidase-conjugated donkey anti rabbit IgG (1:2500) in 5% milk for 1 hour at 37^{o}C. Tetramethylbenzidine OptEIA substrate (BD) was prepared and 50 μl was dispensed into individual wells of the microtiter plates and incubated for 5 minutes. The samples were neutralized with 25 μl 4N H_2SO_4 and read at OD 490. Time point zero or mock infected was used to subtract out background signal. Values obtained with the NS1 standard were plotted and used to calculate the amount in the experimental sample.

Colony Forming Unit Assay

Methylcellulose cultures of the bone marrow cells were used to study the capacity of these cells to produce colonies of hematopoietic origin after dengue virus infection. All necessary reagents were purchased from Stem Cells Technologies, Inc. (Vancouver, Canada), including methylcellulose medium and pre-screened FCS. A total of 1X10^{5} cells were plated in individual 35-mm Petri dishes (Costar, USA) in 1.5 ml of methylcellulose medium with 20% FCS. To promote growth of colony-forming units (CFU), 10 ng/ml SCF, 50 U/ml IL-3, 25 U/ml IL-6, and 2 U/ml erythropoietin were added to detect burst-forming units (BFU)-Erythroid, CFU-Granulocyte-myeloid (CFU-GM) and CFU-megakaryocytes (CFU-MEG). After an incubation period of 12 days at 37°C, 5% CO_2, colonies were scored using an inverted microscope. Colonies from mock dish were picked for expansion and aliquots subjected to phenotype analysis and pooled for virus infection.

Treatment with Aldehyde Dehydrogenase (ALDH) inhibitor

Diethylaminobenzaldehyde (DEAB) was used to treat unfractionated bone marrow cells at 1 mmol/l for 2 days prior to dengue virus infection or immediately after the infection. DEAB non-treated and DEAB concurrently treated cells that had been infected with dengue virus served as controls. The characteristics of DEAB pre-treated cells were examined before performing dengue

virus infection. The infected cells that were DEAB pre-treated, concurrently-treated (added after virus adsorption) and non-treated were harvested at different time points post infection and subjected to quantitative RT-PCR to determine the levels of viral RNA.

Statistical Analysis

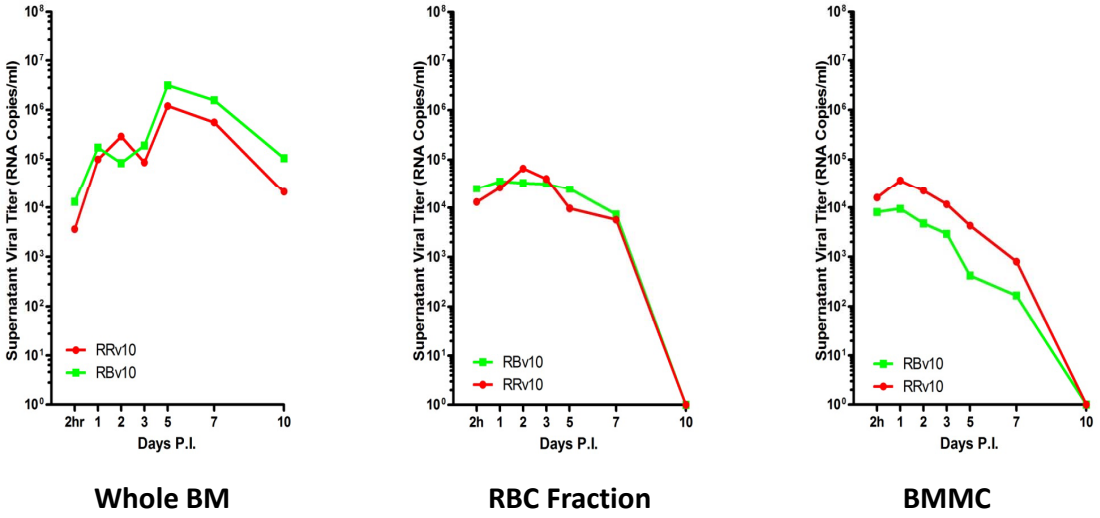
Statistical analyses were performed with GraphPad Prism V5.04, a GraphPad Software Inc. product. Results were considered statistically significant when P was <0.05.

RESULTS

Kinetics of *in vitro* viral replication in bone marrow cells

Results from an initial attempt to infect isolated mononuclear cell subsets from the BM of healthy rhesus monkeys indicated that cells optimally permissive for dengue virus infection were in fact present in unfractionated BM (Supplementary Figure 3.1). Consequently, all subsequent experiments were performed utilizing unfractionated BM cells to demonstrate the infectability of cells by dengue virus. Studies of the kinetics of virus replication in cultures of unfractionated BM cell preparations from healthy monkeys showed that whereas these cells were highly permissive for infection by dengue virus, the degree of permissiveness varied with different individual samples (Figure 3.1A). The levels of nonstructural protein 1 (NS1), a protein that should be expressed by all productively infected cells and a surrogate marker for dengue virus replication, also showed a similar trend (Figure 3.1B). Viral titers in these BM cultures peaked either on days 2 or 3 after the beginning of infection (Figure 3.1). As a whole, the trend of viral replication and levels of NS1 in cultures of BM cells from a total of 20 different monkeys was very similar (Figure 3.2A). However, an increase in the levels of viral RNA does not equate to the production of infectious viral particles. Thus, to demonstrate the infectiousness of the virus obtained in supernatants from infected BM cell cultures, aliquots of randomly selected samples of the cultures from day 2 and 5 containing similar amounts of

viral RNA were incubated with fresh Vero cells. Results indicated that virus recovered during the early phase of BM infection contained low but readily detectable levels of infectious virus (Figure 3.2B). The level of infectious virus in BM rapidly decline, consistent with an earlier report indicating that supernatants taken from cord blood mononuclear cells at day 8 and co-cultured with C6/36 cells are rarely positive for virus (Murgue, et al 1997).



Supplementary Figure 3.1. Whole bone marrow supports dengue virus replication.

Freshly obtained monkey bone marrow was infected with dengue virus at an MOI = 0.1 and supernatants were collected at the indicated times. Viral RNA was quantified as previously described (Noisakran, et al 2012). (A) Increased viral RNA levels in whole bone marrow. A portion of the same whole bone marrow specimen was subjected to Ficoll-Paque gradient fractionation; two fractions, (B) red blood cells (RBC) and (C) bone marrow mononuclear cells (BMMC), were collected and infected with dengue virus. Both fractions did not appear to support dengue virus replication.

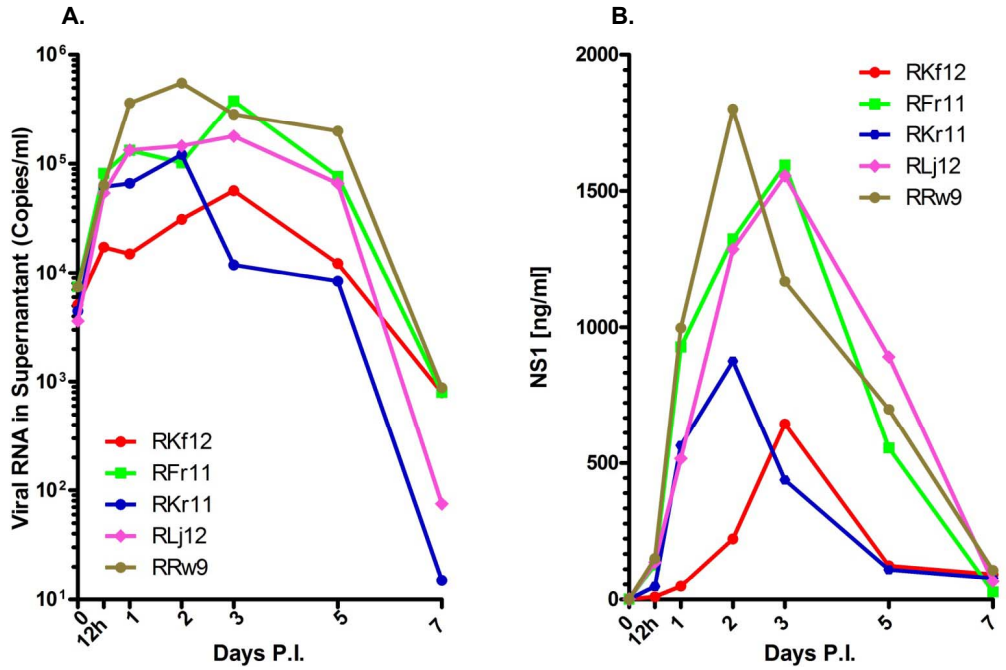


Figure 3.1. Bone marrow cells from rhesus monkeys are permissive for dengue virus infection *in vitro*.

Fresh whole BM cells were infected with dengue virus at an MOI = 0.1. Supernatant fluids were collected at the indicated times and analyzed by qRT-PCR and nonstructural protein 1 (NS1) ELISA as described in Methods. (A) Viral RNA in supernatants. (B) NS1 in supernatants. Varying degrees of susceptibility to dengue virus infection was noticed.

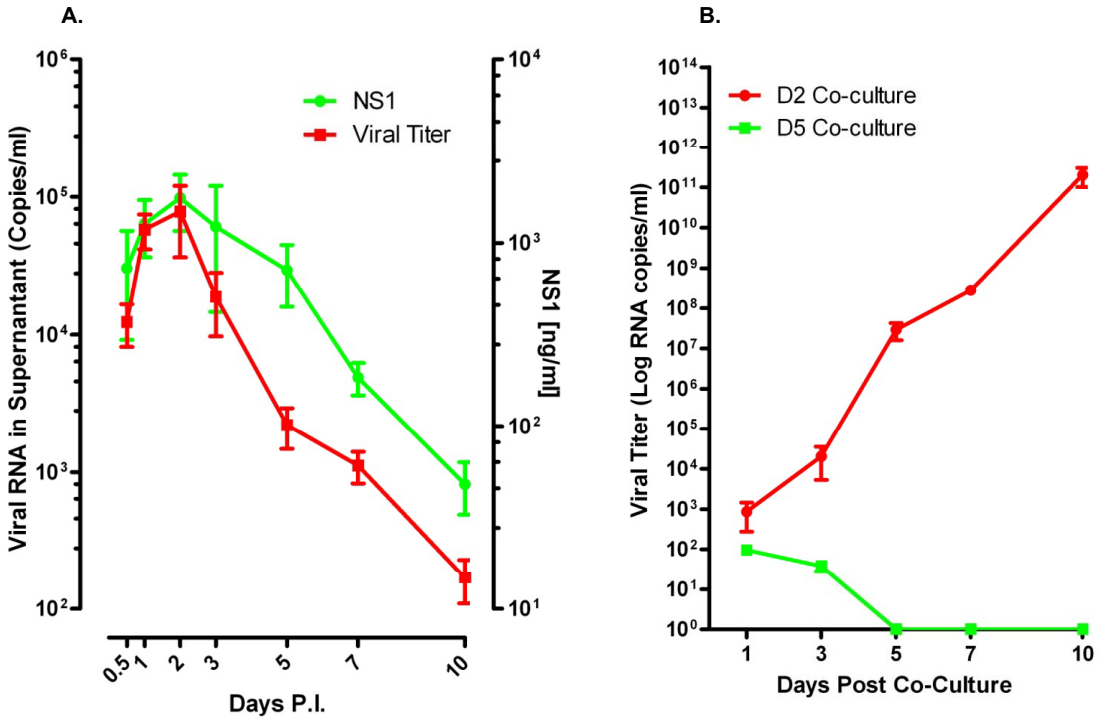


Figure 3.2. Supernatant fluids early post infection contain infectious virus.

All in all, 15 BM samples from healthy rhesus monkeys were studied as described in Figure 3.1. (A) Kinetics of viral replication in 15 bone marrow cell cultures. Red line indicates RNA titers and green line indicates NS1 protein levels. (B) Infectious virus recovery from supernatant fluids. Supernatants from days 2 (red line) and 5 (green line) were cultured with Vero cells. Approximately equal amounts of viral RNA from 5 randomly chosen monkey BM supernatant fluids were utilized in these culture experiments. Supernatant from day 2 contained infectious dengue virus.

Infectability of megakaryocytes

In an attempt to identify the lineage of BM cells that are permissive for dengue virus infection, BM cells harvested at different days after infection were smeared onto slides and stained with antibody to dengue viral antigen. Among the cells positive for dengue antigen, those with megakaryocytic characteristics, such as multiple nuclei, were specifically positive for dengue viral antigen at various days p.i. (Figure 3.3, A, B, C, and Supplementary Figure 3.2 A, B, and C), while slides stained with the isotype control (Figure 3.3F and Supplementary Figure 3.2D) were negative. The lineage of DENV positive cells was also tested using dual staining for CD41a (a marker of platelets and megakaryocytes) or BDCA2 and DENV (Table 3.1). While $CD41a^-/DENV^+$ negative cells were detected at day 1, these cells rapidly declined to undetectable levels, while $CD41a^+DENV^+$ cells increased up to day 5 p.i. and stayed above 50% for the duration of the cultures. $BDCA2^+/DENV^+$ cells were initially negative and showed a gradual and continuous increase throughout the culture (Supplementary Figure 3.3). In addition, dengue viral antigen positive vesicles shedding from apparent megakaryocytic cell were consistently observed (Figure 3.3C) and phagocytic cells engulfing dengue antigen-positive vesicles could also be detected (Figure 3.3 D and E). We interpret these results as suggestive of the megakaryocytic cell lineage as the predominant early target and the bone marrow phagocytic cells as critical for subsequent clearance of virus.

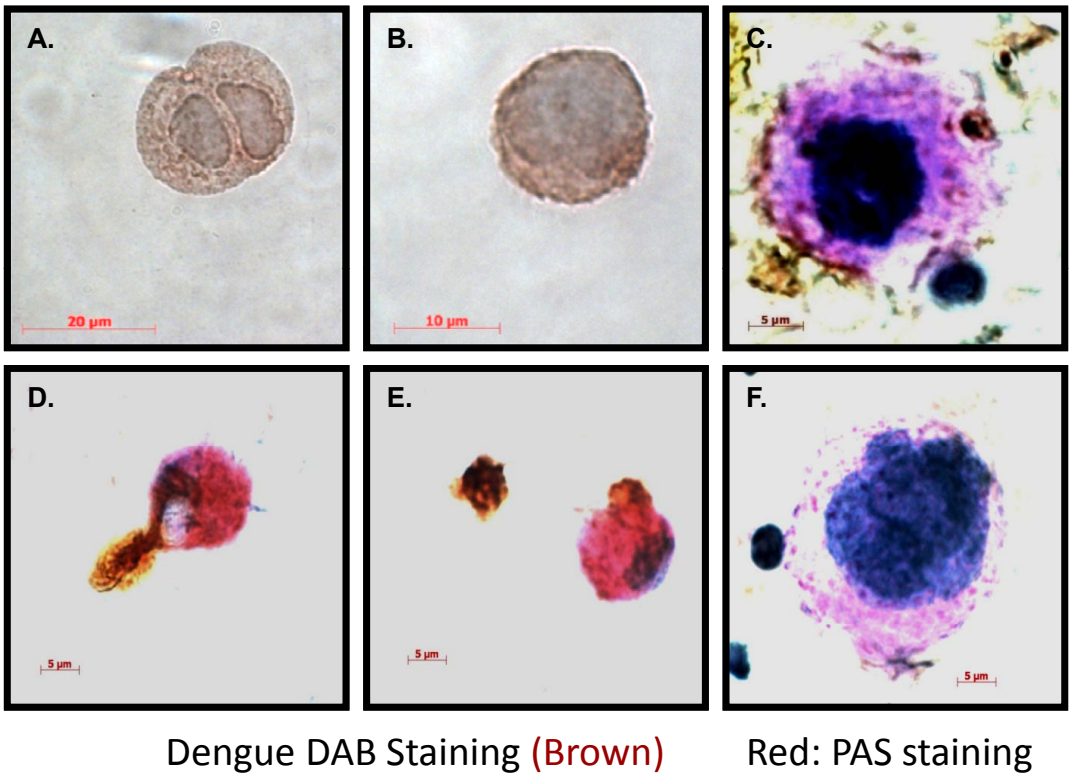
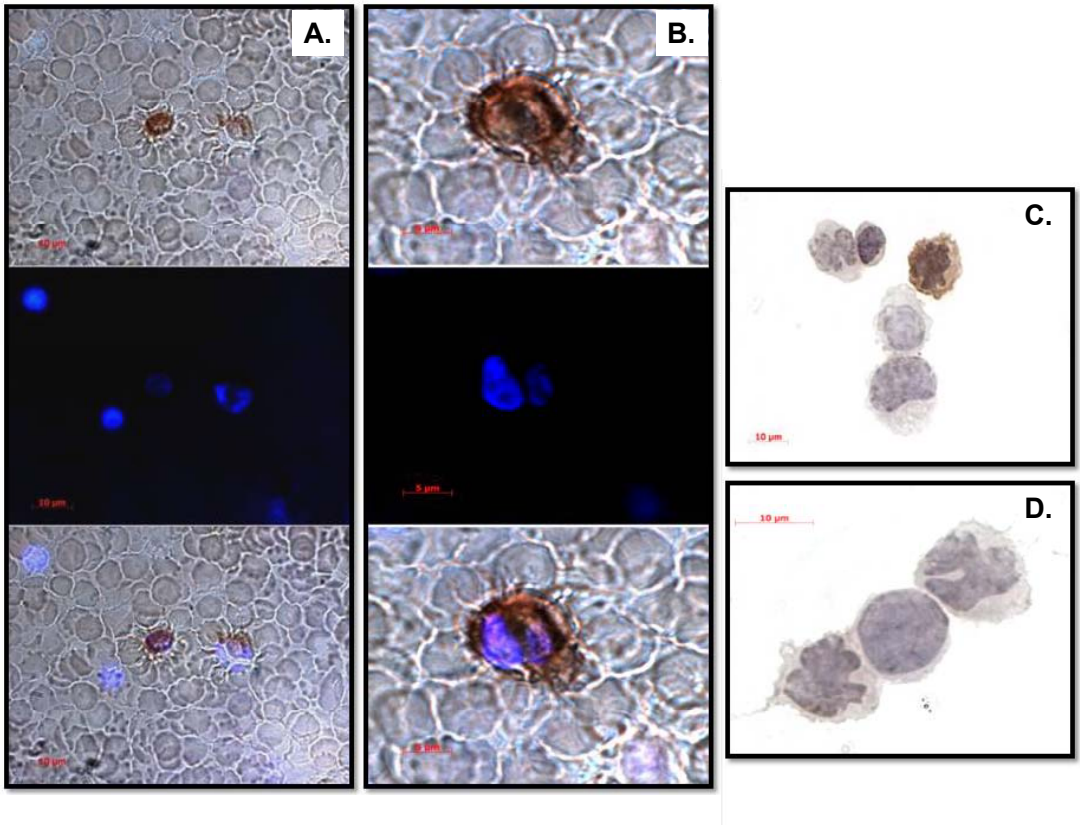


Figure 3.3. Megakaryocytes were likely the dominant dengue virus antigen positive cells in monkey bone marrow.

Smears of bone marrow cells were prepared and immunohistochemical stainings were performed as described in Methods. Dengue antigen is indicated by DAB staining (brown) (A) Dengue viral antigen in a diploid megakaryocyte. (B) Dengue antigen in a multi-lobulated megakaryocyte.. (C), Dengue antigen in cellular debris. Red, PAS staining. Blue, hematoxylin staining. (D and E) Dengue viral antigen-containing vesicles engulfed by phagocytic cells. (F) Isotype control.



Supplementary Figure 3.2. Dengue viral antigen was dominantly observed in multi-nucleated cells.

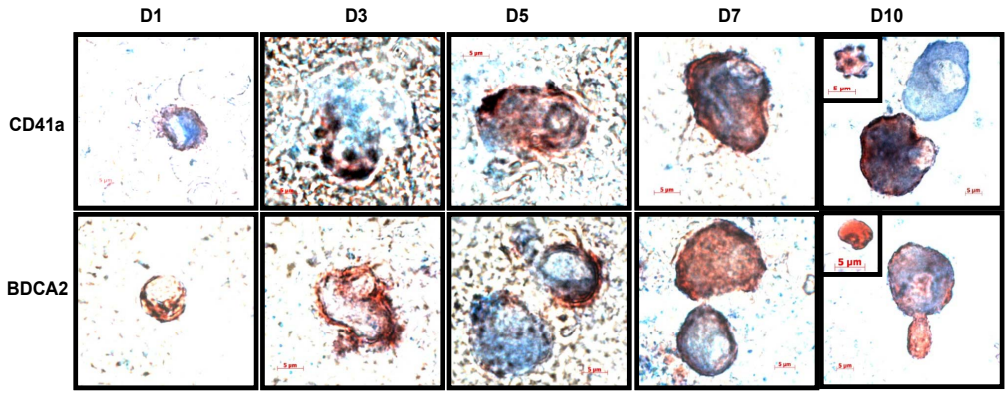
Immunohistochemical staining was performed as described in the Methods. (A) and (B) Dengue viral antigen was specifically observed in multi-nucleated cells. (C) DENV infected cells were stained with DENV antibody after lysis of red blood cells. (D) Isotype control staining.

Table 3.1. Quantification of monkey bone marrow cells positive for dengue viral antigen[a].

Days P.I.	1	3	5	7	10
$CD41a^{+}DENV^{+}$	11.3±2.3	43.4±3.6	50.7±2.9	59.2±7.0	61.4±6.5
$CD41a^{-}DENV^{+}$	17.5±19	13.6±2.2	10.0±2.4	0.0±0.0	0.0±0.0
$BDCA2^{+}DENV^{+}$	2.0±0.4	2.6±1.2	41.8±3.6	64.5±8.3	85.5±3.3
$BDCA2^{-}DENV^{+}$	15.6±2.4	12.2±3.1	4.4±1.5	0.0±0.0	0.0±0.0

[a]values represent the percentage of surface marker positive or negative among 200 dengue positive cells with 3-5 histochemical stainings.

± standard deviation, P.I., post-infection, BDCA2, plasmacytoid dendritic cell antigen 2.



Supplementary Figure 3.3. Dengue viral antigen is present in CD41a$^+$ cells and not BDCA2$^+$ cells at early time points of infection.

Monkey bone marrow smears were prepared from whole bone marrow infected with dengue virus at an MOI = 0.1. Cells were harvested at the indicated times, smeared onto slides, and stained with the indicated cell markers, CD41a (Blue), marker for platelets, and BDCA2 (Blue), maker for plasmacytoid dendritic cells, and antibody specific to dengue viral antigen (Red).

Finally, a kinetic study was performed on BM aspirated from DENV infected rhesus monkeys collected at various time points after infection and stained for CD41a, CD61, CD14 and DENV antigen (Figure 3.4), whereby viral antigen was observed early in $CD61^+$ cells with a decreasing trend, the opposite trend was evident in $CD14^+$ monocytic cells.

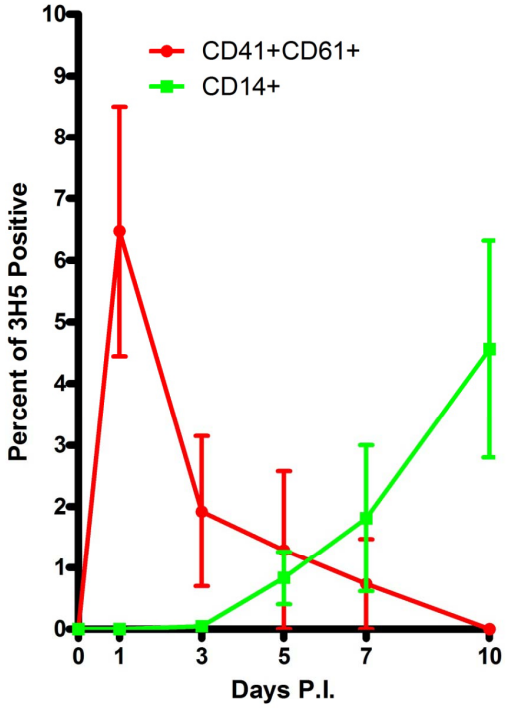


Figure 3.4. $CD61^+$ cells were the early cells infected by dengue virus bone marrow.

Freshly aspirated bone marrows at various time points from DENV infected rhesus monkeys were stained with dengue viral specific monoclonal antibody (clone 3H5) and cell lineage markers CD41, CD61, and CD14, and subjected to FACS analysis. Results revealed that viral antigen was observed early in $CD61^+$ cells with a decreasing trend while the opposite trend was evident in $CD14^+$ monocytic cells.

Infection of human bone marrow cells

It has been known for a long time that dengue virus can infect monkeys, but their levels of viremia are lower than that of human beings. Thus, it was reasoned that studies similar to the above studies should be attempted using human-derived BM cells to construct comparative data. Left over healthy human BM samples were thus obtained from the BM transfusion center at Emory University School of Medicine and infected with dengue virus *in vitro*. To our surprise, not only were human BM cells easier to infect by the virus, but, in addition, the levels of virus in the supernatant fluid could reach as high as 10^9 viral RNA copies per ml, which is similar to the level of viral load in the peripheral blood of dengue patients (Figure 3.5A). Similar results were also noted in the levels of NS1 in the same supernatants (Figure 3.5B). Importantly, the pattern of the average focus forming unit (FFU) viral titer was similar but lower than that of the viral RNA titer determined by qRT-PCR assays, peaking on day 3 after infection (Supplementary Figure 3.4). The higher viral titers and NS-1 secretion documented in human BM cultures was statistically significant (Figure 3.6). BM smears prepared from the human BM cell cultures at different times post-infection were similarly stained with monoclonal antibodies specific to dengue viral antigen and cell surface markers as described above. Results revealed that cells with the megakaryocytic characteristic/marker were positive for dengue viral antigen (Figure 3.7A and B). Viral antigen containing vesicles shedding from a megakaryocyte with a multi-lobulated nucleus were routinely observed (Figure 3.7A).

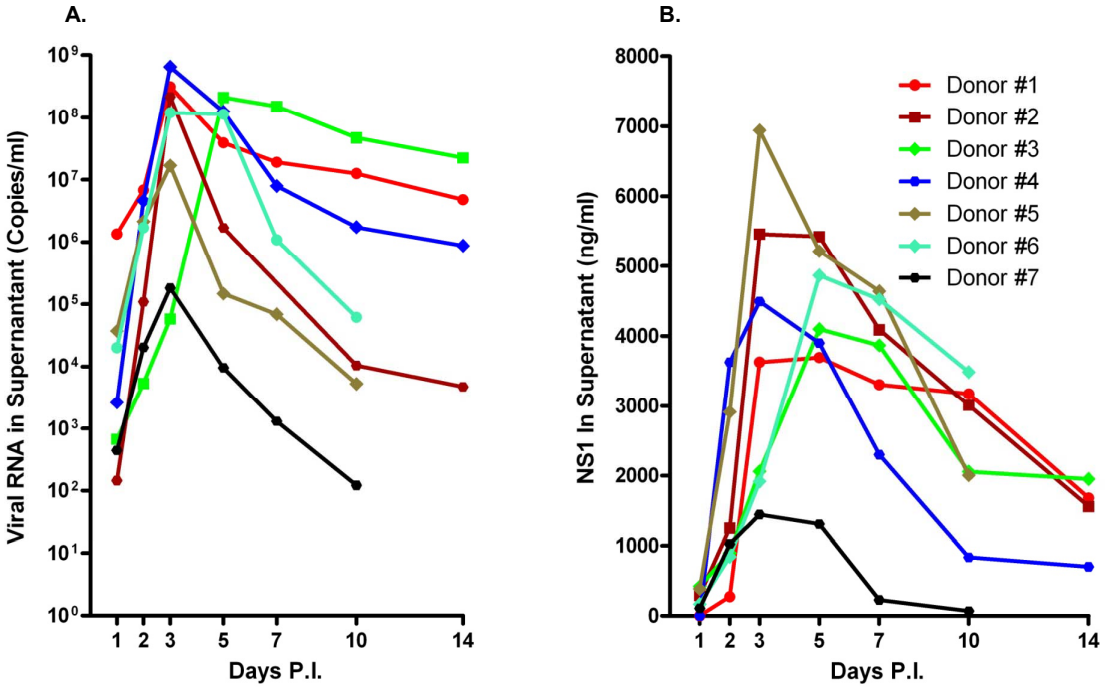
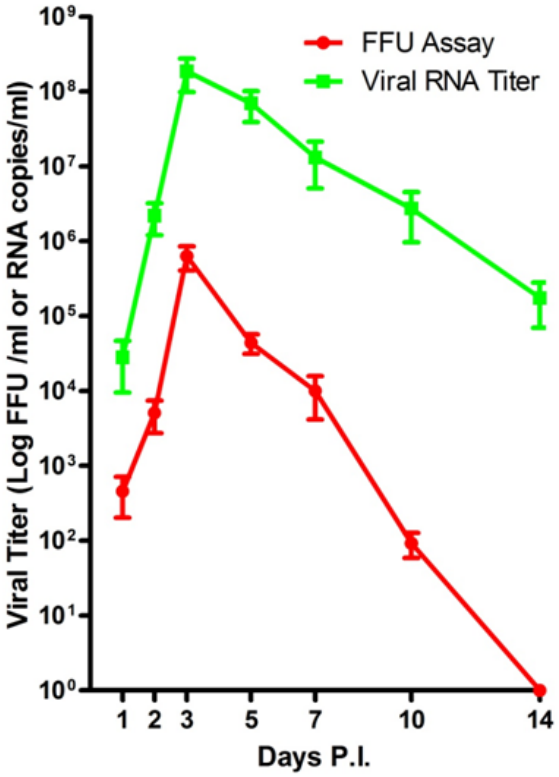


Figure 3.5. Human bone marrow is permissive for dengue virus infection *in vitro*.

Healthy human BM cells were obtained from the BM transplantation center at Emory University and infected with dengue virus as described in the Methods. Supernatant fluids were collected at the indicated times; viral RNA and NS1 were quantified as described in the Methods. (A) Viral RNA in supernatant fluids. (B) NS1 in supernatant fluids.



Supplementary Figure 3.4. Quantification of infectious viral titers with focus forming unit assays (FFA).

The viral titer and the infectivity of the virus in the collected specimens were determined using an FFA. [12]. Titers were expressed as FFU per ml. The pattern of the average viral titer was similar to that of viral RNA titer determined by qRT-PCR assays, peaking on day 3 after infection.

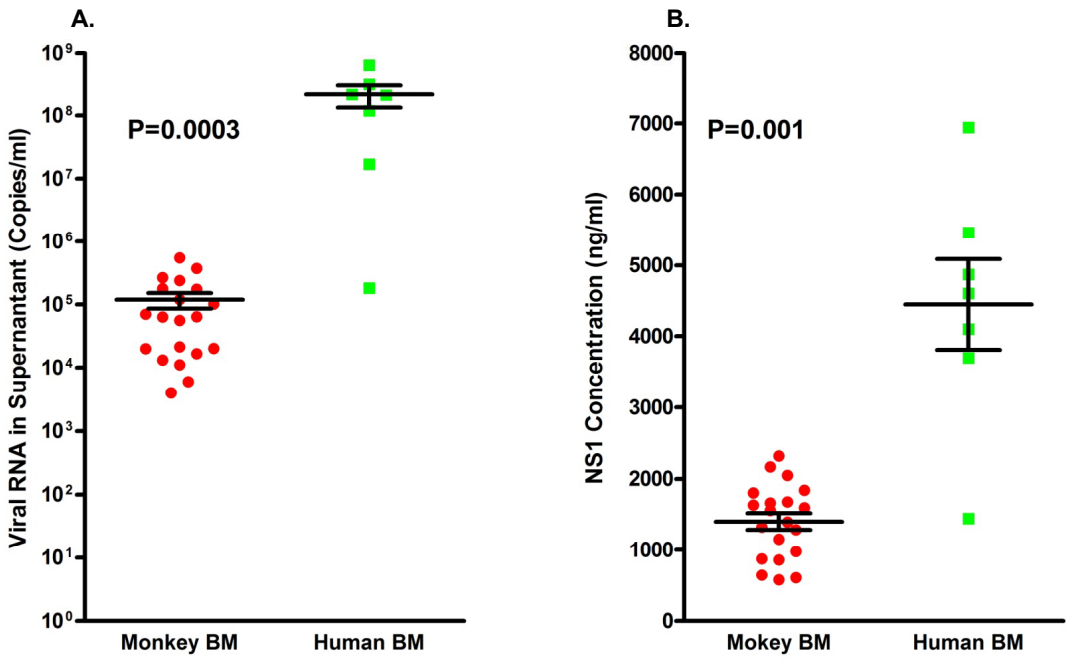


Figure 3.6. Human bone marrow is more permissive than rhesus macaque bone marrow to dengue virus infection *in vitro*.

(A) A comparison of peak virus genome copy number levels in human and monkey BM cultures. (B) Comparison of NS1 in the supernatant fluid of human and monkey BMs. The levels of viral RNA and NS1 in the supernatant fluid from infected human BM were significantly higher than that from the rhesus monkey.

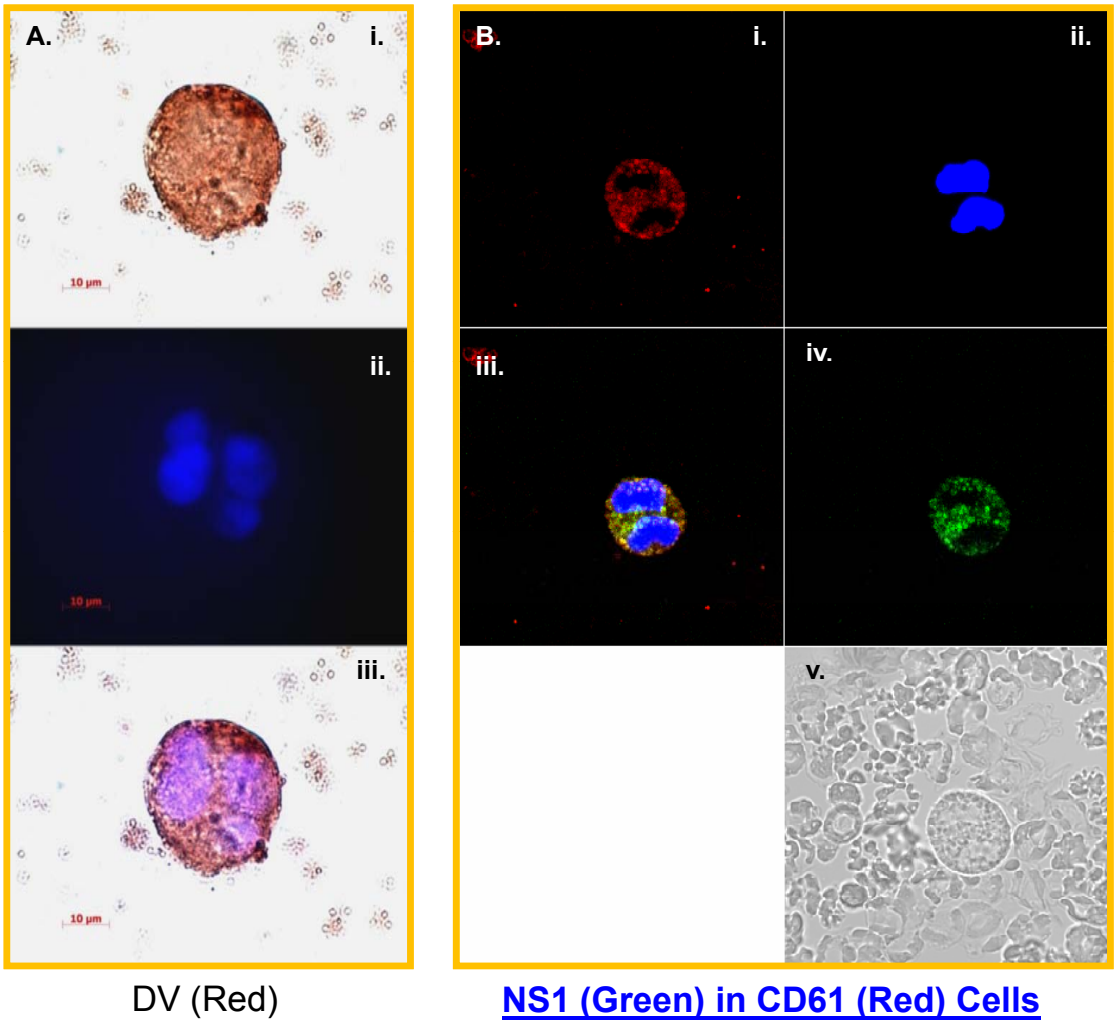


Figure 3.7. Megakaryocytes from human bone marrow contain dengue virus antigen.

Bone marrow smears were prepared and fluorescent cell stainings were performed as described in the Methods. (A) Dengue viral E antigen in tetraploid megakaryocyte in the process of shedding vesicles as evidenced by immunohistochemical staining in the presence of DAPI. Dengue viral antigen (red) and nucleus (blue) (B) Dengue NS1 antigen in a $CD61^{+}$ megakaryocytic cell depicted by immunofluorescence staining. NS1 (green), CD61 (red) and nucleus (blue). Scale bar, 10 μm.

Electron microscopy studies

Electron microscopy (EM) studies were performed on aliquots of bone marrow cell cultures collected on different days after infection. As seen in Figure 3.8, viral particles appear primarily within multi-lobulated cells (Figure 3.8), with viral replication complexes visible on day one (Figure 3.8B) and large numbers of virions present within the cytoplasm by day 3 post-infection (Figure 3.8C and D). As seen, viral particle-containing vesicles appear to be shedding from the cytoplasm (Figure 3.8D and E). We infer that phagocytic cells engulf these virus-containing vesicles at later times post-infection (Figure 3.8F). EM studies also suggest that phagocytic cells, such as monocytes, are highly activated, featuring numerous vacuoles as early as day one post infection (Supplementary Figure 3.5A and B). However, virus-like particles were not detectable at this point in these mononuclear cells (Supplementary Figure 3.5C and D). In contrast, at later times post infection, these cells appear to engulf vesicles containing viral particles (Supplementary Figure 3.6A and B), which seemed to infiltrate the phagocytic cell cytoplasm upon plasma membrane fusion (supplementary Figure 3.6C). The morphology of the viral particles is unclear in these phagocytic cells and are likely degenerated (Supplementary Figure 3.6D).

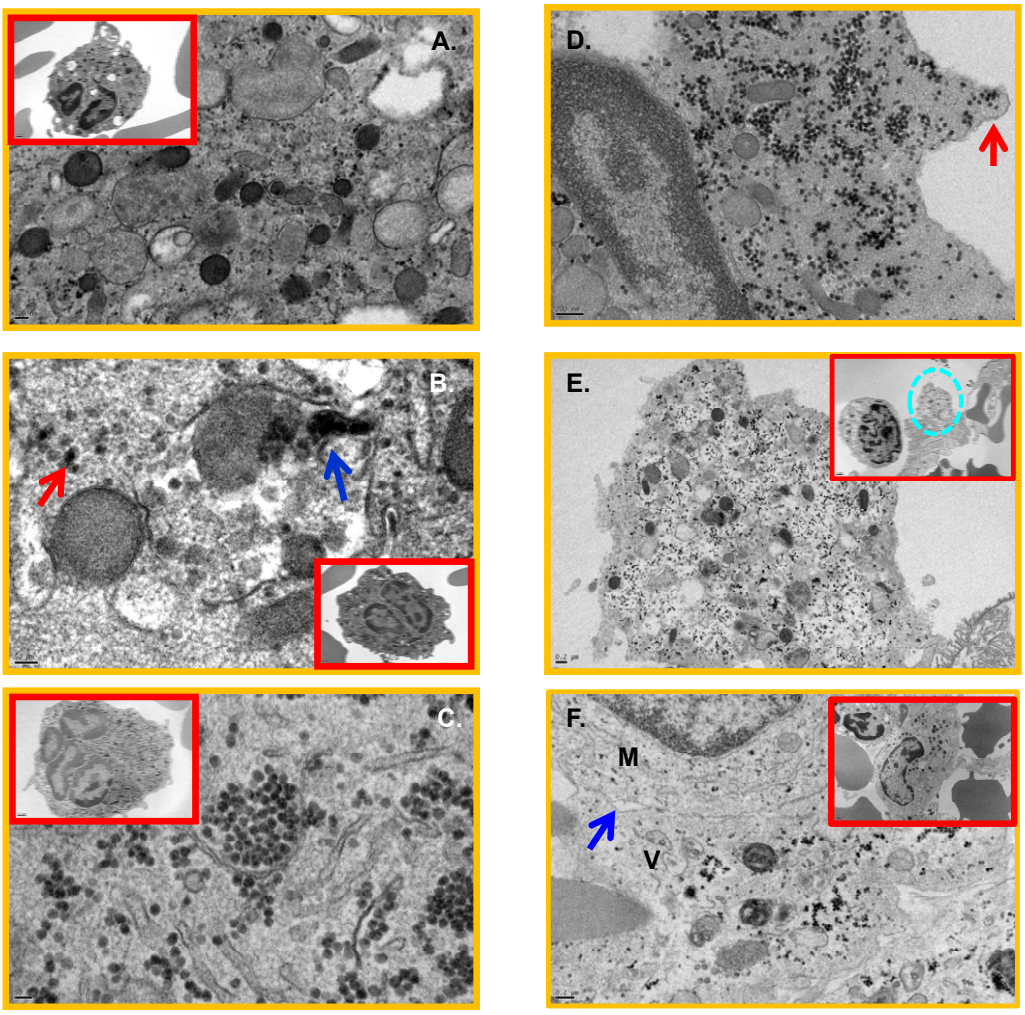
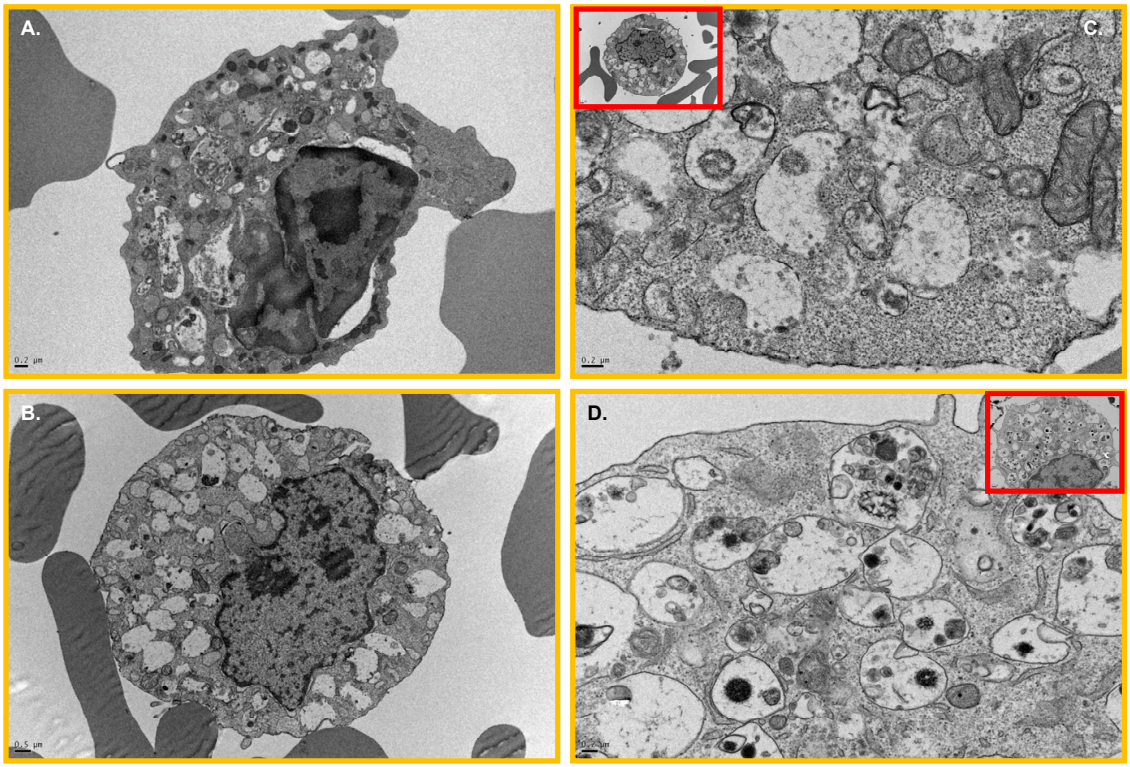


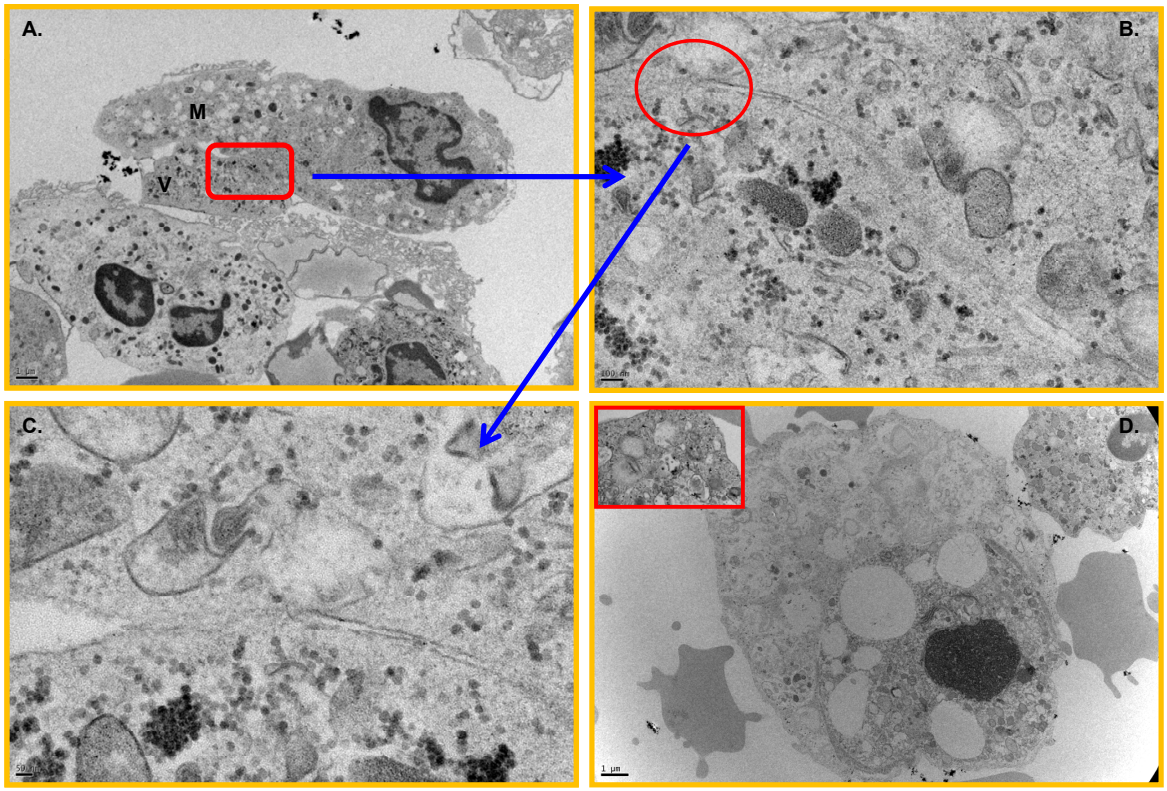
Figure 3.8. Viral particles are present in megakaryocytes from the human bone marrow.

Sample preparations for EM studies were performed as described in the Methods. (A) Uninfected control. (B) Cellular vesicle containing viral particles (single particle, red arrow; cluster of viral particles, blue arrow) inside a diploid megakaryocyte on day one post-infection. (C) Large numbers of viral particles inside the cytoplasm of a multi-lobulated megakaryocyte on day three post-infection. (D) Cytoplasm containing many virus particles shedding off in a vesicle (red arrow). (E) A virion-containing vesicle (dash circle) at the vicinity of an activated mononuclear cell. (F) Virion containing vesicle (V) fusing with a monocyte (M). A zipper junction (blue arrow) is indicated. No viral particles were observed in the monocytes. A scale bar is 0.2 μM.



Supplementary Figure 3.5. Monocytes from infected human bone marrow appear uninfected and activated.

Infected bone marrows were processed for EM investigations. (A and B) Activated and vacuole-loaded phagocytic cells, likely monocytes or macrophages. (C and D) Absence of discernible viral particles or replication complexes in vacuolated cytoplasm of activated monocytes or macrophages.

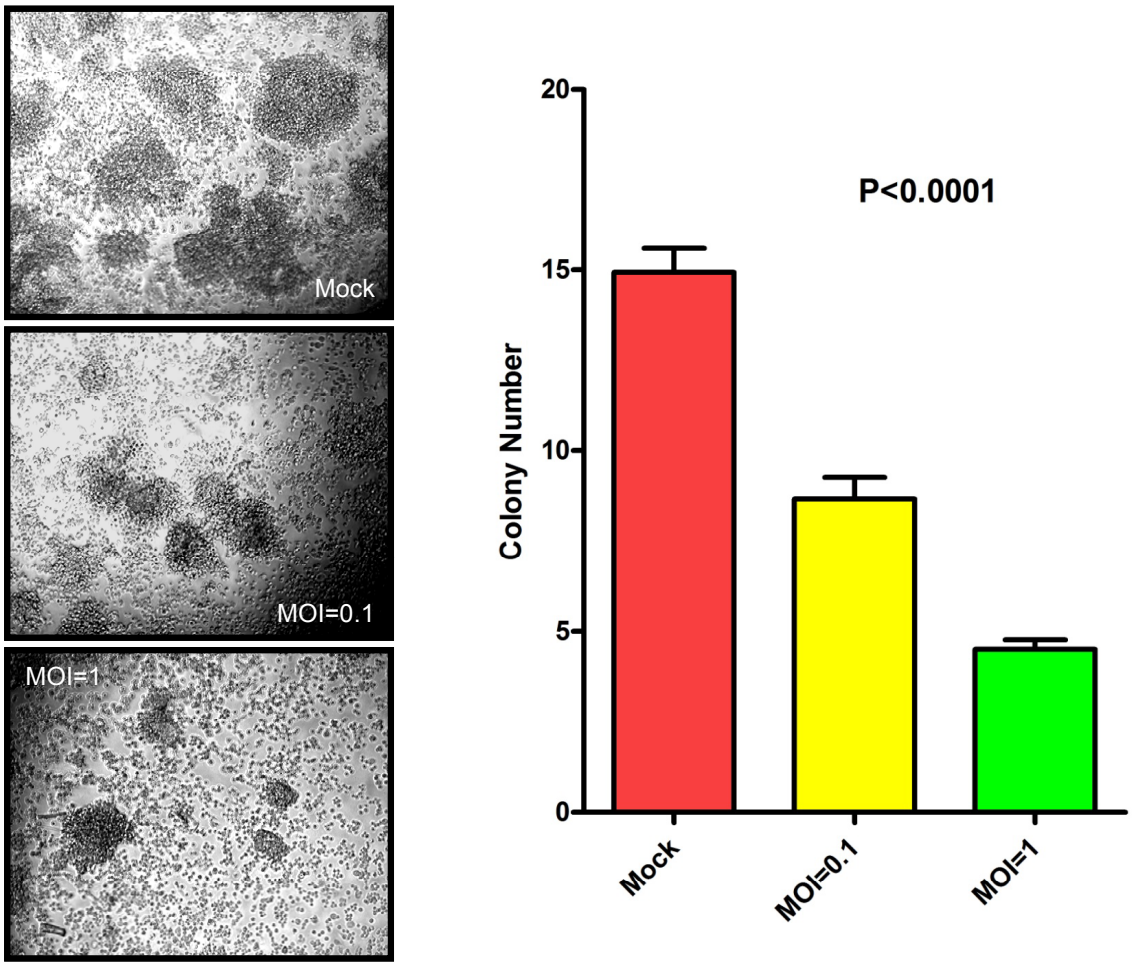


Supplementary Figure 3.6. Phagocytic cell engulfs virion-containing vesicles.

Images of human whole bone marrow were captured by EM on day 5 after infection. (A) A vesicle loaded with virus-like particles (V) fusing with a monocyte or macrophage (M). (B) Zipper junction (circle) at the fusion point. (C) Virions transfering from the vesicle to the cytoplasm of the phagocytic cell. (D) Degenerated viral particles inside the cytoplasm of phagocytic cells on day 7 after infection.

Colony forming unit (CFU) assays

Numerous heterogeneous progenitor cells are present within the bone marrow; these delicate cells are highly responsive to microenvironment alterations, which likely prompt the differentiation and proliferation of certain cell lineages. Therefore the efficiency of colony formation in the bone marrow post virus infection was evaluated. Data obtained showed that the number of CFUs were reduced post virus infection in a dose-dependent manner (Supplementary Figure 3.7). The dose-dependent inhibition results are in line with a previous report using purified cord blood mononuclear cells and cord blood $CD34^+$ cells (Basu*, et al* 2008, Murgue*, et al* 1997). Mock colonies were picked, expanded, aliquots identified by Giemsa staining and then the rest infected with dengue virus. Results indicated that cells from the colonies identified as CFU-megakaryocytes were more susceptible to dengue virus infection than colonies identified as CFU-other cells, which likely include a mixture of cell lineages. In contrast, cells from CFU-erythrocyte appeared not to support viral replication (Table 3.2). These results also suggest that hematopoietic stem cells are capable of getting infected with dengue virus. Accordingly, infections were performed with expanded stem cell cultures. Aldehyde dehydrogenase (ALDH) is a receptor on hematopoietic stem cells (HSC) and is a key regulator of HSC differentiation. Human stem cells were treated with the drug DEAB, which interferes with ALDH, down-regulating HSC differentiation and promoting short term stem cell proliferation. After 2 days of treatment with this inhibitor, the majority of cells displayed a multi-lobulated morphology, implicating an increase in the number of megakaryocytes (Supplementary Figure 3.8). These cells were much more permissive to dengue virus infection than untreated or concurrently treated BM cells (Supplementary Figure 3.9 and Supplementary Figure 3.10A). Immunohistochemical staining revealed that these cells expressed CD41a, indicating they were likely of megakaryocytic origin (Supplementary Figure 3.10B and C). Viral antigen was observed on globoid-like vesicles that were undergoing budding from the surface of the cells (Supplementary Figure 3.10B and C).



Supplementary Figure 3.7. The efficiency of colony formation in human bone marrow was inhibited by dengue virus in a dose-dependent manner.

Healthy human bone marrow was exposed to dengue virus at an MOI = 1 or 0.1 for two hours. Unbound virus was removed with three washes of media, and cells were cultured with CFU media according to the protocol suggested by the manufacture (Stem Cells Technologies Inc., Vancouver, Canada). Uninfected human bone marrow was used as control.

Table. 3.2. Infectivity of dengue virus in Colony Forming Unit cells picked from human bone marrow[a].

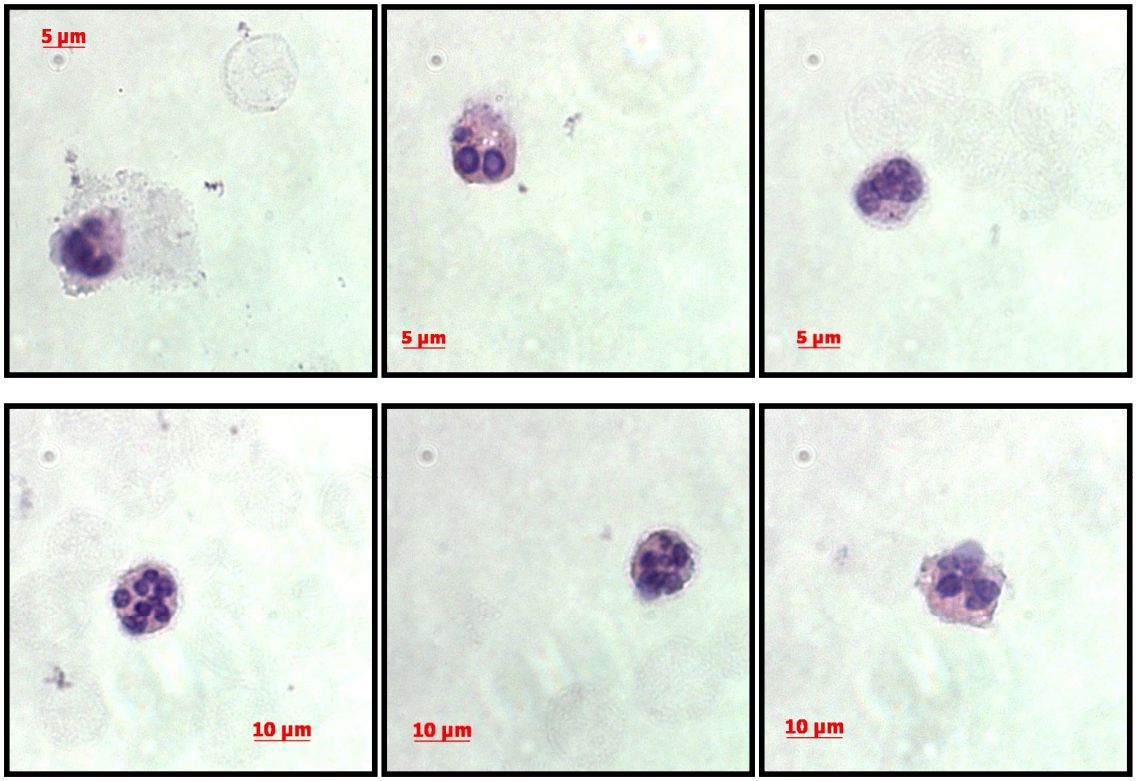
Days P.I.	0[b]	1	3	5	7	10
CFU-other cells	74[c]	215	198	136	124	103
Fold Increase[d]	0	190.5	167.6	83.3	67.6	39.2
CFU-Megakaryocytes	82	114	363	145	92	26
Fold Increase	0	39.5	344.3	77.5	12.1	0
CFU-Erythroid	3530	1000	657	433	194	0
Fold Increase	0	0	0	0	0	0

[a]Cells were characterized based-upon their morphology and giemsa staining characteristics.

[b]Day 0 means the time point 2 hours after adsorption, in which culture supernatants were extensively washed for unbound virus. The amount of residual virus in the culture supernatant was determined and used as the baseline.

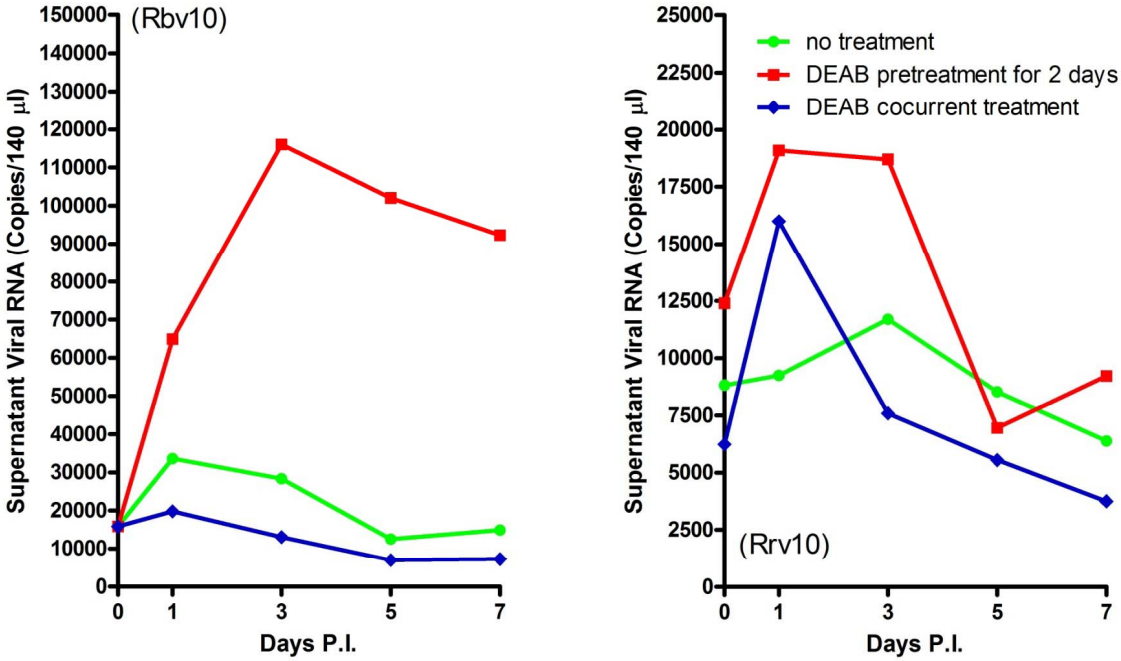
[c]Quantification determined by qRT-PCR (unadjusted copy number per 140 μl of the supernatant).

[d]The fold increase relative to the viral titer at Day 0, supernatant at 2 hours post-infection, was calculated.



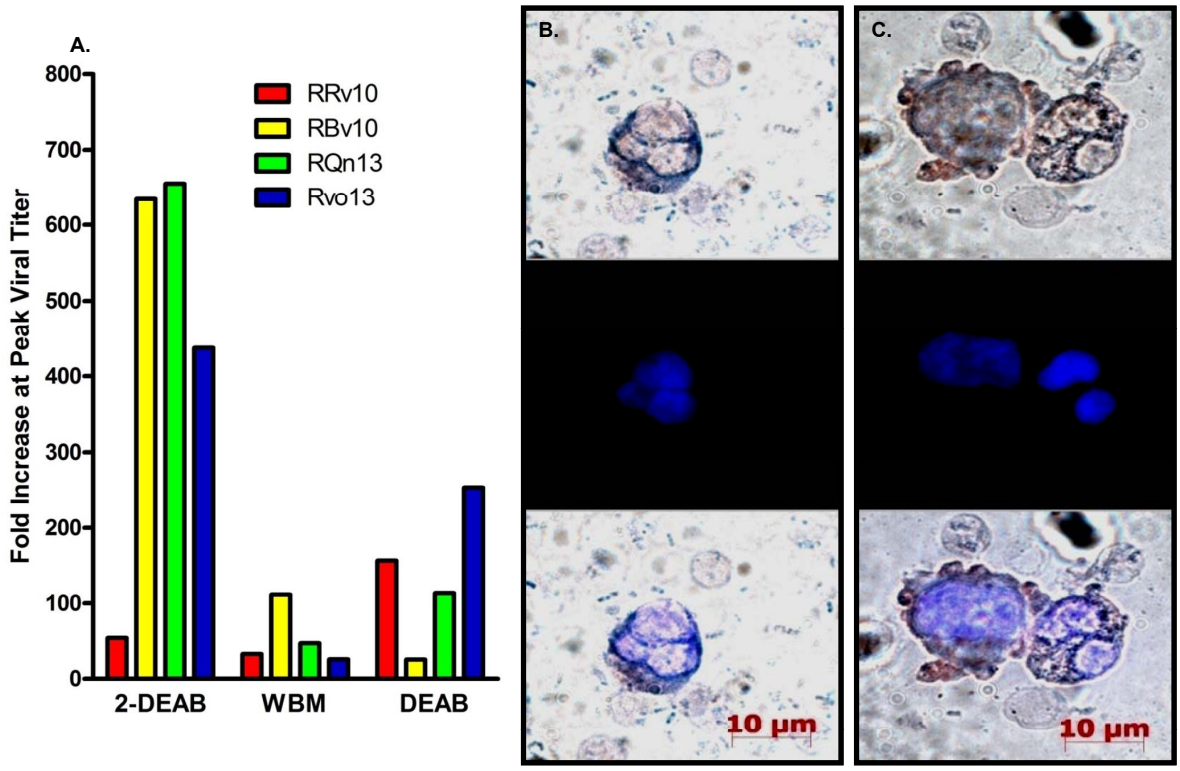
Supplementary Figure 3.8. Multi-lobulated cells were the dominant population present in monkey bone marrows treated with the drug diethylaminobenzaldehyde (DEAB).

Bone marrows were treated with DEAB, an inhibitor for aldehyde dehydrogenase (ALDH), at a concentration of 1μmol/l for two days. Cellular morphology of cells after Wright Giemsa staining was captured with a Zeiss inverted microscope.



Supplementary Figure 3.9. Bone marrow cells pre-treated with DEAB are permissive for dengue virus infection *in vitro*.

Monkey whole BM cell samples treated in three different ways (DEAB-untreated and DENV-infected, pre-treated with DEAB for 2 days prior to DENV infection and DEAB-treated post infection) were performed as described in methods. Results from two representative monkeys are shown. The peak in genome titer was at days 2 or 3 days post initiation of infection.



Supplementary Figure 3.10. Monkey bone marrows treated with DEAB for two days are highly permissive to dengue virus infection.

(A) RNA quantification was performed with three experimental groups from four monkeys: 2-DEAB, bone marrow pre-treated with DEAB for two days before virus infection; WBM, DEAB-untreated and DENV-infected whole bone marrow; DEAB, DEAB added to culture immediately after dengue virus infection. The kinetic fold-increase in viral titer compared to that at time 0, or two hours after absorption, was calculated. The peak fold increase in viral titers is presented. Cells were cytospun onto slides and immunohistochemical staining for CD41a and dengue E antigen was performed as described in the Methods. (B) Isotype control. (C) Viral antigen observed in infected megakaryocyte that was ongoing shedding vesicles. Dengue E antigen (brown), CD41 (blue), and nucleus (DAPI stained).

DISCUSSION

It is well known that the bone marrow is composed of a complex and heterogeneous mixture of cell lineages that can vary greatly in composition from individual to individual. This delicate compartment is highly sensitive to any subtle stimulation, which can dramatically change the cellular constituents and its functional capacity. The hierarchical order among the various cell lineages is critical in orchestrating the activities of the host and sustaining homeostasis. The plasticity of hematopoietic progenitor cells in BM bestows upon them the ultimate power to restore homeostasis. Infectious agents are a type of stimulation that likely disturbs the equilibrium, requiring BM progenitors to respond to re-establish order.

Dengue is one of the most important vector-borne diseases in humans. Although the disease predominantly circulates in tropical and subtropical zones, it has recently been acknowledged as a potential public health threat in several other locations around the world. The majority of those infected remain asymptomatic, but many experience dengue fever (DF) that is a self-limited illness. Only a small percentage of affected subjects progress to the very severe and life-threatening clinical form termed dengue hemorrhagic fever (DHF) accompanied with shock syndrome (DSS), which is characterized by increased vascular permeability, plasma leakage and internal bleeding. The degree of thrombocytopenia has been demonstrated to significantly correlate with the severity of the disease. Understanding the mechanisms accounting for the drop in platelet counts has been one of the central themes for several decades. The following processes, acting successively or in combination, have been demonstrated to interfere with the number of platelets in the peripheral blood of dengue patients: reduced platelet production through early transient marrow suppression with damage to megakaryocytes (Noisakran*, et al* 2012, Rothwell*, et al* 1996); platelet aggregation with endothelial cells upon dengue virus activation (Butthep*, et al* 1993, Noisakran*, et al* 2009); hemo-phagocytosis (Jacobs*, et al* 1991, Wong*, et al* 1991); and finally, immune destruction of platelets displaying dengue-antibody complexes on their membranes (Hathirat*, et al* 1993). Profound hematopoietic suppression has been

noted to occur in dengue virus infected patients early post infection occurring prior to hospital admission (Bierman and Nelson 1965, Kho*, et al* 1972, Na-Nakorn*, et al* 1966). Thus, direct suppressive action of the virus on megakaryocytes was suggested as a mechanism contributing to thrombocytopenia long ago (Nelson and Bierman 1964); however, this hypothesis was never properly evaluated and remained un-confirmed.

We first observed that unfractionated BM cultures are highly permissive for dengue virus infection relative to purified populations of BM mononuclear cells. Selective destruction of cells during the Ficoll-Paque gradient separation procedure may account for the difference. Combinations of immunohistochemical staining and electron microscopy imaging authenticate that multi-lobulated megakaryocytes are highly permissive for dengue virus infection *in vitro*. This can be inferred from previous findings indicating that hematopoietic cells other than megakaryocytes are very seldom polyploid in healthy BM (Bessman 1984, Larramendy*, et al* 1994, Levine 1980). Productive infection of these megakaryocytic cells likely plays an important part in the development of thrombocytopenia in dengue-infected patients.

Megakaryocytes are one of the most unique cells in the mammalian system, accounting for only 1% of healthy BM. They express all proteins required for cell division and yet never divide to generate daughter cells. The surface area of the cell membrane progressively expands to an enormous size, which then, via internal operational signaling, extends itself into a demarcation membrane that sheds to produce platelets, a mechanism likened to apoptosis. Correspondingly, the contents of the chromosome increase, continuously doubling the genome to numbers as large as 128N. Each megakaryocyte can produce between 3000 to 5000 platelets dependent upon the size of the membrane and thus differentiation stage of the cell (Stenberg and Levin 1989). Thrombopoiesis normally takes 4 to 7 days for completion with 2/3 of the newly produced platelets destined to the peripheral blood for circulation, while 1/3 becomes sequestered within the spleen. The multi-lobulated cells observed during dengue virus infection appeared to be smaller in size, likely classified

as micro-megakaryocyte, as opposed to a late stage megakaryocyte, population. This could be an indication that dengue virus infection may inhibit differentiation, transiently holding off the doubling of the genome and expansion of the membrane, resulting in a reduced efficiency in platelet production. Furthermore, if platelets are produced from these infected cells, they are likely dysfunctional. Perhaps, this may be one of the reasons why in some patients, the levels of platelet counts are within normal range, but hemorrhagic manifestations are still observed.

Interestingly, despite careful study, we were unable to observe viral particles in activated monocytes of the BM during the early days of infection. However, we frequently observed virus-containing vesicles becoming engulfed by monocytes and degenerated virus-like particles in the cell cytoplasm at later times post infection. The evidence is in line with a previous publication, in which the authors report that only cells from the bone marrow are capable of supporting dengue virus replication after a side-by-side comparison with cells from other monocyte rich organs (spleen, lymph node, and thymus) (Halstead and O'Rourke 1977). The activated mononuclear cells we observed could well be inflammatory monocytes that have the ability to differentiate into dendritic cells equipped with a high degree of phagocytic activity (Auffray*, et al* 2009). Interestingly, it has been suggested that the elimination of apoptotic bodies by phagocytic cells is a pathway of dengue virus clearance in infected tissues (Marianneau*, et al* 1998) and that the shedding of platelets is a mechanism operationally similar to apoptosis in megakaryocytes (Josefsson*, et al* 2011). This may perhaps explain the observation that BDCA2^{+} cells become antigen-positive late in infection, probably due to phagocytosis of dengue-containing apoptotic debris. Nevertheless, the results are in line with reports on the importance of monocytes/macrophages in the clearance of virus in the circulation (Fink*, et al* 2009, Marchette*, et al* 1973, Mosquera*, et al* 2005, Onlamoon*, et al* 2010, Tsai*, et al* 2011).

In addition, results from the DEAB inhibition assays indicated that viral yields in the supernatants were readily detectable in cells with multi-lobulated nuclei. Interestingly, it has been

reported that cells highly resistant to gamma irradiation are concentrated in DEAB-treated hematopoietic stem cells and that they are likely to be multi-lobulated megakaryocytes. Importantly, it has already been documented that dengue viral titers are not reduced in bone marrow cells treated with gamma radiation (Halstead*, et al* 1977). Therefore, utilizing a variety of approaches, our results suggest that one major target for dengue virus infection in BM is likely to be megakaryocytes. The potential mechanism at the origin of this preference may be that megakaryocytes are defective in interferon alpha/beta synthesis (Fuhrken*, et al* 2007, Kim*, et al* 2002), a critical inhibitory molecule that can limit dengue virus gene expression. Perhaps, with their defective defense machinery, megakaryocytes are an easy target for multiple pathogens. Thrombocytopenia is a common clinical feature seen in patients infected with other infectious agents; Junin virus, the causative agent of Argentinian hemorrhagic fever, (Carballal*, et al* 1981, Carballal*, et al* 1977), murine lymphoid viruses (Dalton*, et al* 1961) and HIV (Boukour*, et al* 2006, Zucker-Franklin and Cao 1989), the causative agent of AIDS have all been shown to attack the megakaryocytes as well.

AUTHOR CONTRIBUTIONS

Kristina B. Clark discussed the experimental strategy, performed EM and the immunohistochemistry staining, and prepared the manuscript; Sansanee Noisakran discussed the experimental strategy, performed the subset of the EM and partially wrote the manuscript; Hui-Mien Hsiao performed real time PCR and ELISA; John Roback assisted in IRB protocol approval and obtained human bone marrow samples for the study; Francois Villinger discussed the experimental strategy, directed the IACUC protocol and obtained monkey bone marrow samples for the experiments, and edited the manuscript; Aftab A. Ansari discussed the experimental strategy, assisted in approval of IACUC protocol, and edited the manuscript, and Guey Chuen Perng designed, defined and discussed the experiment strategy, wrote the IACUC and IRB protocol, and directed the research effort.

All authors declare no competing financial interests.

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CHAPTER 4

Title: Role of microparticles in dengue virus infection and its impact on medical intervention strategies

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Title: Role of microparticles in dengue virus infection and its impact on medical intervention strategies

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ABSTRACT

Dengue virus (DENV) is one of the most important vector-borne diseases in the world. It causes a disease that manifests as a spectrum of clinical symptoms, including dengue hemorrhagic fever. DENV is proficient at diverting the immune system to facilitate transmission through its vector host, *Aedes* spp. mosquito. Similar to other vector-borne parasites, dengue may also require a second structural form, a virus of alternative morphology (VAM), to complete its lifecycle. DENV can replicate to high copy numbers in patient plasma but no classical viral particles can be detected by ultra-structural microscopy analysis. A VAM appearing as a microparticle has been recapitulated with *in vitro* cell lines Meg01 and K562, close relatives to the cells harboring dengue virus *in vivo*. VAMs are likely to contribute to the high viremia levels observed in dengue patients. This review discusses the possible existence of a VAM in the DENV life cycle.

INTRODUCTION

Dengue virus (DENV) is one of the most important vector-borne diseases today, contractible by the bite of a DENV-infected female *Aedes* spp. mosquito [1]. It causes 500,000 hospitalizations a year and threatens to infect two-fifths of the world's population. These statistics are only likely to increase with the lack of success at controlling transmission and preventing outbreaks. DENV causes a disease that manifests as a spectrum of clinical presentations, with initial symptoms appearing similar to other common febrile illnesses such as influenza. The most common form is severe fever, myalgia and thrombocytopenia (dengue fever [DF]), and the less common forms of disease are hemorrhaging (dengue hemorrhagic fever [DHF]) or DHF with plasma leakage leading to shock and multi-organ failure (dengue shock syndrome [DSS]). Although the acute DF is a self-limiting infection, a subset of DF patients rapidly progress into a secondary phase, known as DHF/DSS. This life-threatening condition often occurs after the clearance of viremia and is generally thought to be an immune-mediated disease. Adequate and timely diagnosis is a major challenge to physicians, considering the delay in patient hospital enrollment and the variety and non-specificity of the clinical symptoms. Currently there is no preventative or therapeutic treatment available for dengue today. Rehydration therapy and palliative care with close monitoring are the only approved practices known to reduce mortality and improve patient outcomes.

DENV was once a clinically significant pathogen in the US, before the mosquito vector was nearly eradicated in the Americas by spraying with DDT. However, dengue was not eliminated in the rest of the world. With increased human travel, unplanned urban development, global warming, lack of effective vector control and the expansion of the *Aedes* spp. niche, dengue has penetrated to almost every corner of the world [2]. It is perceivable that dengue will infiltrate back into the US since effective mosquito control measures are still in their infancy and the population is immunologically naïve. The increasing incidence of dengue disease worldwide and its escalating costs to the healthcare system has heightened public awareness and lead to an augmentation in activity

developing vaccines and drugs. Medical interventions that can prevent and alleviate dengue symptoms are greatly needed but promising candidates will not be likely without a clearer understanding of dengue virus life cycle.

Much has been established in the dengue virus field, such as the clinical progression of disease in dengue patients and the virus structure and life cycle *in vitro*. However, the structure and the life cycle of the virus in human plasma or the form that enters the insect proboscis have remained unknown since it has never been recorded so far. Our observations with patient plasma and megakaryocyte erythrocyte progenitor (MEP) cell lines, Meg01 and K562, support the idea that DENV can take on a different form, residing in host-derived microparticles (MPs). In this review, we will discuss the possibility of a virus of alternative morphology (VAM) that may allow dengue to divert the immune system, comparable to other vector-borne diseases such as malaria. *This implies that antibodies to Vero-derived virus may not be a good predictor for protection against dengue or an index for virus neutralization within the human host* and an alternate method should be used to evaluate efficacy of drugs and vaccines.

DENGUE VIRUS'S PROPAGATION AND STRUCTURE *IN VIVO* AND *IN VITRO*

Various *in vitro* [3] as well as numerous primary cell lineages have been studied for their relative permissiveness for dengue virus infection, including endothelial, fibroblast, myeloid-derived and lymphocytic cells [3-11]. Due to difficulties and inconsistencies in identifying the cell lineages responsible for dengue viremia at the acute stage *in vivo* and the low infectivity of the primary phagocytic cells [12], the hypothesis of antibody-dependent enhancement (ADE) infection was postulated [13]. The ADE hypothesis attempts to explain why disease is much more severe in people upon re-infection with heterologous dengue viral serotypes. The assumption is that the antibody made during the first infection does not have a high enough affinity to neutralize the secondary

heterologous serotype; this partial cross-reactive (or sub-neutralizing) antibody may enhance the virus opsonization and uptake by Fc-bearing cells such as monocytes and macrophages, leading to increased virus production. However, conflicting reports with results obtained *in vitro* abound in the literature on the immune-mediated pathogenesis; some reports support the view [14-16], while others dismiss the theory [3, 17-23]. *It is still disputed which cells take up dengue virus in vivo, as well as the receptors required for virus entry.* Consequently much of the research on dengue virus biology has been performed with convenient *in vitro* cell lines.

The genome of DENV is a positive-sense RNA strand of about 11 kilobases [24, 25]. The viral RNA has the same polarity as mRNA and, if the viral RNA can be delivered into a cell's cytoplasm through biologically active vesicles, translation and genome synthesis can occur and induce infection without the need of virus-encoded proteins [26]. From this sequence a polyprotein is translated and becomes proteolytically cleaved into at least 10 known viral protein subunits: three structural proteins designated capsid (C), premembrane/membrane (PrM/M), envelope (E) and seven nonstructural proteins (NS) [27]. The order of the gene products encoded by the genome is C-PrM/M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 [28].

The most investigated DENV structures (intact virion and the envelope protein) were produced in Vero or insect cell lines such as C6/36 and Schneider 2 [24, 29, 30]. These classical virus particles are known to have three dominant stages: immature, mature and mature fusion-ready (or mosaic particles) [24, 31]. The immature intermediate structure has a rough surface consisting of 60 spikes of E/PrM dimers; further processing (low pH alterations in combination with cleavage by cellular furin protease) results in the mature cleaved form, which is smaller with a smooth outer surface made up of 90 E dimers. In the third classical viral form, the E protein rearranges into a homotrimer conformation, which is capable of fusion with the host lipid bilayer. It is assumed that the mature virion is the dominant form contained in insect saliva because it is the most infectious in

cell culture; however, the input virus acquired by mosquitoes after blood meal has never been imaged.

Less information is known about the dengue virus particles that are formed in mammalian cells. They are presumed to be identical to the insect cell structural form with likely variation in post-translational modifications [32]. To the best of our knowledge, crystallography has not been performed with mammalian derived virus to confirm this. Electron microscopy (EM) techniques have been the most frequently employed methods to visualize virus structures from other cell types. Dengue virus has been cultured in quite a high number of cell lines, totaling over 30 [33]. As of yet, EM pictures of progeny virions have only been obtained from a few of these, mainly insect and kidney cell lines [34-37]. Only Barth et al. has investigated the structure of virus from human serum. These low-resolution images depict "fuzzy" virions, suggesting the presence of a virus of alternative morphology (VAM) *in vivo* [38, 39].

VIRUSES OF ALTERNATIVE MORPHOLOGY (VAMs)

Heterogeneous populations of dengue virus particles have been observed for more than four decades [40-42]. The types found have been highly dependent on the cell type examined. The term "viruses of alternative morphology (VAMs)" is defined as any structures or conformations that are deviated from the classical dengue virus particle. Thus, in the old literature, VAMs are referred to as the rapidly- and slowly-sedimenting hemagglutinin antigens (RHA and SHA), which were virus forms fractionated from mouse brain [40, 43, 44]. This SHA was 9nm in diameter, increased in frequency with processing and appeared to be noninfectious [40, 41]. This particular form is likely to be an artifact from replication in an abnormal organism and may help explain why mouse-derived virus is attenuated in humans [45, 46]. *RHA is the 50nm classical virion, which is capable of infecting indicator cell, such as Vero [40]*. The VAMs manufactured in other cell lines display different characteristics. For

instance, the lighter weight virus found in C6/36 cell line has a 30nm diameter and is deficient in capsid protein but yet still infectious [47]. Also a fuzzy virus morphology has been noted in a few sources [19, 39]. *This morphology has been viewed to be an apoptotic particle in the virus infected monocytic cell and is not infectious, a suggestive of SHA [19]. While the fuzzy-coated virus-like particles in infecetd mosquito C6/36 cell is infectious and is in the category of RHA [39].*

All of these VAMs may not be relevant *in vivo*. Viruses can easily evolve to replicate in cell lines that they cannot normally infect; this has been countlessly demonstrated in the past with vaccine development. Viruses have been propagated in alternative organisms or cell types to produce an attenuated strain [48]. This strategy is thought to force the virus to evolve toward better replication in another cell type, making them less capable of infecting the appropriate host cells or diverting their ability to counter the immune system when placed back into man. Viruses can also be over-propagated through cell culture, potentially replicating too well in these cells, and fail at preventing disease in experimentation [49].

Likewise over-adaptation and good replication of viruses in these *in vitro* cell lines often leads to the development of characteristics that are irrelevant *in vivo*. In the absence of the appropriate receptor synapse, viruses can still find a way inside the cell. The virus receptor may bind weakly to abundantly expressed host proteins leading to clustering and high avidity interactions. When placed into cell culture at high concentrations for prolonged periods of time, these weak interactions eventually lead to the right conditions that favor fusion for a portion of virions. One example of a virus entry mechanism brought about by cell culture adaptation is dengue virus interactions with heparan sulfate [50-52]. Much attention was spent investigating this feature of the DENV life cycle, but it was later determined in vaccine preclinical trials that DENV with high affinity to this receptor was actually attenuated in macaques [53]. This emphasizes the importance of studying virus entry in the most appropriate cell types, the ones they naturally infect. This should improve the chances of

investigating mechanisms still relevant *in vivo*. For example, *in vitro* the domain III of the DENV E protein, a drug target, is predominantly exposed on the mature virus and can easily be bound by rodent-derived neutralizing antibodies to prevent fusion [54]. However, work with human serum has demonstrated the lack of antibodies specific to these epitopes, suggesting that this structure is specific to *in vitro* virus and is not present in humans [55, 56]. One explanation for the absence of domain III antibodies may be masking by heavy glycosylation, suggested by the fuzzy virion morphology occasionally noted in some investigations [19, 38, 39]. Another explanation may be that the structural conformation is completely different. The literature indicates that virus-like particles differing from the classical virion can be observed in dengue infected human and rhesus macaque platelets [57, 58]. Human serum also possesses the capacity to neutralize *in vitro* cultured DENV, suggesting that neutralization antibodies are present but bind other epitopes [59].

Surprisingly there are practically no published investigations on the DENV morphology *in vivo*, despite the high levels of viremia in patients. It is presumed that many researchers have tried but failed to detect classical dengue virions either in plasma, serum or peripheral blood mononuclear cells [60]. One reason for this failure may be because the investigators were looking for the structure crystallized from insect cell lines. Another reason may be isolation of the wrong blood components. Only recently were virus particles depicted in human and rhesus macaque platelets [57, 58]. Interestingly, platelets can support one round of DENV replication [61]. Inspection of the Vero-derived and platelet-derived classical virions carefully reveal that these particles are slightly different from each other (Figure 4.1, *A*–*C*). They both have diameters in the 40–50nm range, but the platelet-derived classical virus form is more heterogeneous (Figure 4.1, *B and C*). Some platelet vesicles contain fuzzy debris, potentially a type of VAM (Figure 4.1, *B and C*). Also there is an interesting formation blebbing off the platelet (*Figure 4.1C*). This microparticle (MP) appears to be mostly empty, containing a vesicle (a structure also seen in DENV-infected Vero cells) that also could be considered a VAM (Figure 4.1, *A and C*). It has the appearance of a virus-induced vesicle, which has

also been noted in other EM studies [36]. The function of these virus-induced vesicles is unknown, but we hypothesize them to be a possible alternative DENV RNA-containing virion that may allow the virus to escape aspects of the immune system. We suggest that MPs may play a role in dengue virus infection and transmission, potentially by shielding DENV from aspects of the immune system. Dengue specific antibodies often cross-react with self-proteins, suggesting that VAM can hide from the neutralizing antibody response [62].

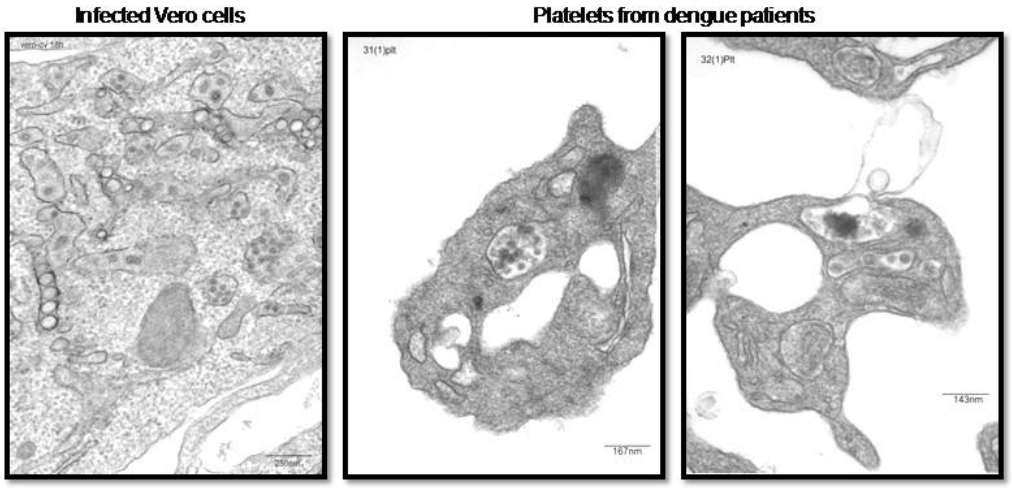

Figure 4.1. Transmission EM images of DENV2-infected Vero cells and dengue patient platelets.

Vero cells were infected with a multiplicity of infection equal to 5 for 18 hours and samples were prepared as previously described (58). Human platelets were isolated from acute dengue patients via Optiprep and platelets were fixed with 4% glutaraldehyde in PBS. Samples were washed and fixed with 2% osmium tetraoxide and stained with uranyl acetate. Stained specimens were infiltrated with propylene oxide and epoxy resin, embedded in a polypropylene capsule and visualized with a Hitachi Transmission Electron Microscope. (A) Dengue classical virions can be seen in endocytic vesicles of infected Vero cells. (B and C) Dengue viral particles inside platelet vesicles isolated from two acute dengue patients. Red arrow indicates viral-particles inside virus-induced vesicle structure.

MICROPARTICLES (MPS) AND THEIR INVOLVEMENT IN INFECTIONS

Microparticles (MPs), the vehicles of cell-cell communication, often contain mRNA, miRNA and proteases [63-67]. These vesicles can bleb off the plasma membrane or form within multivesicular body (MVB) compartments, which then fuse at the cell surface releasing their microvesicular contents. Many review articles are available that have discussed MP involvement in various biological phenomenon [68-73]. There are a few investigations that have observed transmission of virus through MPs [74, 75]. One notable example of this is Hepatitis C virus, a close relative to DENV. Other microbes from various domains of life have been noted for their ability to alter MP content and promote their transmission [76-80]. DENV, as mentioned earlier, only requires the presence of its genome to initiate an infection. If its transcripts or genome have the capacity to be packaged into microvesicles, like host mRNA and miRNAs, they may easily get distributed broadly throughout the body and taken up by a wide variety of cells. *These* MP and cell interactions as far as selectivity, attachment and fusion are poorly understood. Bone marrow (BM) progenitors are recognized as frequently accepting MPs from BM and other cell types [63, 81]. However, one study showed that B-cell exosomes bound abundantly only to follicular dendritic cells, suggesting that MPs contribute to an elaborate and selective communication system [82]. MPs have also been suggested to play vital roles in shaping the immune response during infections by facilitating coagulation and by delivering MHC receptors and CD40L to appropriate cell types [70, 83-85]. MPs may potentially serve as a biomarker for pathogenesis or vaccine effectiveness [85-88]. Investigations describing MP participation during the course of infection can offer great insight and should be studied further.

In humans, the majority of MPs are derived from platelets [69]. Interestingly DENV can be found in human and monkey platelets (Figure 4.1, B and C) [57, 58], which are shed from megakaryocytes during differentiation; these anucleated cells can fragment into many smaller vesicles, termed platelet-derived particles or "platelet dust" [89-92]. This evidence suggests that there is likely

to be platelet-derived vesicles containing dengue virus found *in vivo*. Accordingly, dengue virus can be easily cultured from human serum or plasma, which doesn't contain detectable virions or platelets but does have platelet-derived MPs (cite PMPs in plasma). All that can be found in plasma concentrates from dengue patients are small cellular vesicles, which likely contain viral components (Figure 4.2). Virus quantity may be too low in these types of samples for visualization by EM but there is an alternative hypothesis to explain these findings. Dengue virions may resemble the host's cellular vesicles.

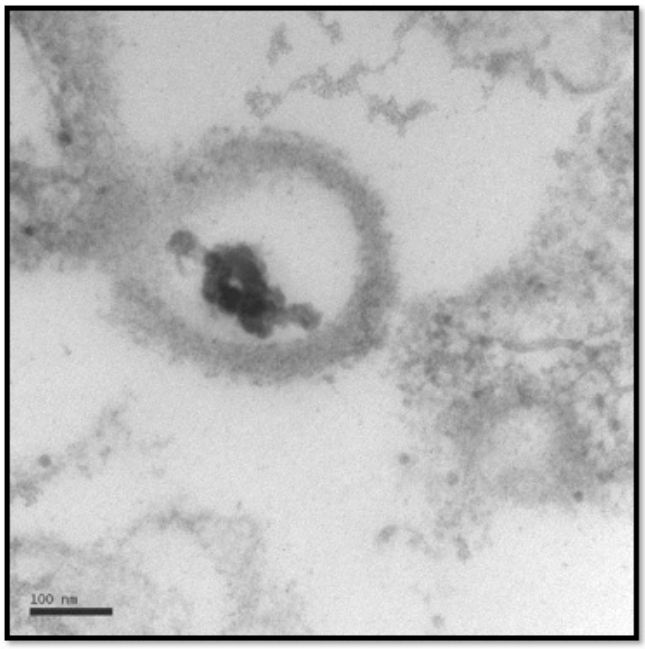

Figure 4.2. Transmission EM of plasma concentrate pooled from multiple patients.

Plasma was spun with an ultrahigh speed centrifuge at 130,000 x g for 30 minutes and the pellets were prepared as described in Figure 4.1. Small vesicles containing virus particles were observed.

Testing this hypothesis will be difficult because the human cell population(s) that harbor and replicate DENV *in vivo* have not been determined. The literature suggests that virus is likely to infect a cell that is frequently found in the bone marrow and capable of differentiating into megakaryocytes, shedding the DENV-containing platelets noted in the literature [58, 93]. Additional evidence acquired with platelet progenitor cell lines, Meg-01 and K562, demonstrates that the MEP lineage is highly permissive for dengue virus infection (unpublished results and [3]). With these cell types, even in sucrose fractions with the highest DENV RNA contents, no classical virions and only host-derived MPs are readily detectable. Replication of DENV in this lineage *in vivo* may explain the inability to find obvious virions in patient samples and would suggest that Meg01 and K562 are the most appropriate for studying human components of the DENV life cycle.

Additionally, encapsidation of multiple genomes into MPs could partially explain the difficulty of detecting virions in patient blood with high RNA copy numbers. It is well known that there is a difference between DENV quantified by real time RT-PCR and by plaque or focus forming unit assays. It is generally accepted that these assays result in different virus titers because there are higher levels of RNA than there are infectious virus. If instead a single MP packages 10 or more virus genomes, then this could account for lower infectious virus quantities. The concentration of infectious particles would decrease by at least 10-fold and make EM virion visualization more difficult. Also, this would skew the infectious virus to defective virus particle ratio. In DENV2 infected K562 and Meg01 cells, this ratio spans anywhere from the upper 100s to lower 10,000s [unpublished results]. With the MP transmission scenario, there could be many functional genomes clustered into the same microvesicle, but only one MP and cell fusion event, resulting in one infectious focus unit. Fusion of multiple genomes or a quasispecies into one cell may facilitate a more robust and productive infection. These particles may also shield virus from immune system components, allowing for infection in spite of preexisting high neutralizing titers [94]. This may also

permit the retention of virus in the blood for extensive periods of time, making possible efficient transmission to the mosquito vector.

VECTOR-BORNE DISEASE TRANSMISSION

Dengue is a vector-borne virus that is contracted through the bite of an infected female *Aedes* spp. mosquito. It is often the case with vector-borne parasites that the infectious agent takes on a different structural form to accomplish infection in divergent species. In the case of malaria transmission, the plasmodium needs to assemble into the sporozoite in the mosquito salivary gland in order to be transmitted to and infect humans [95]. However, the merozoite must be present in human blood imbibed by the *Anopheles* spp. mosquito vector to complete the cycle and be available for future transmission. Without transmission of the appropriate form the next host organism cannot acquire the infection.

To the best of our knowledge, these types of polymorphisms have not been noted with vector-borne viruses. Insect cell DENV progeny have been described with multiple morphologies: the classic, capsid-less and filamentous [35, 47]. We have observed a microparticle-associated VAM, which may be present in other mammalian cell lines. However, the physical structure of the virus in the mosquito saliva acquired during blood meal from an infected individual has not been documented. Virus morphology is usually observed in the gut or salivary glands after, rather than before, propagation in the insect vector [96]. The investigations that have visualized virus entering the proboscis have infected *Aedes* with cell culture derived virus rather than patient blood [97]. This detail may have escaped DENV investigators due to unsuccessful attempts to detect virus in this substance. It may have been assumed that virus particles were too few and below detection limits to be visualized by EM [personal communications, Dr. Duane Gubler]. Interestingly, it has been known that dynamic dengue viral particles exist *in vivo*, based upon fractionation with sucrose density

gradient [40, 42, 47]. Therefore, the lessons learned from parasitology, that infectious agents often morph into other forms at different stages of their life cycles may have been overlooked. VAMs may be present in patient blood, potentially required for productive evasion of the immune system and transmission to the vector or for specific host-pathogen interactions. It is not unreasonable to expect that the dengue E-M protein complex in the classical structure cannot fuse equally well with receptors on mammalian and insect cells. Differential glycosylation has already been attributed to variations in virus titers in insect versus mammalian cells [32]. We propose that at least two different forms of DENV could be generated to complete its life cycle in nature*: a classical and a microparticle-associated form*. Both forms would need to be considered when designing effective vaccines and drug candidates.

IMPLICATIONS AND CURRENT DENGUE VACCINE EFFORTS

Despite over 60 years of extensive effort, little progress has been made at developing effective vaccines to prevent the occurrence of infection or disease [98]. Several strategies (attenuated, intra-strain chimeras, subunit and plasmid-based DNA vaccines) have been or are currently being attempted; most have failed to elicit protective immunity in children [99-101]. Currently there are no approved vaccines but a number of candidates are under development; the clinical trials evaluating their reactogenicity and immunogenicity have not yet resolved [102]. The furthest along, beginning phase III clinical trials, is the Sanofi Pasteur dengue vaccine (chimeric yellow fever backbone-dengue attenuated vaccine, CYD), which contains 4 intra-strain chimeras that are highly attenuated in humans and noted for its capacity to elicit neutralizing antibodies [103]. Assuming one of these candidates is successful at reducing severe disease, it will still be another 5 to 10 years before one of these candidates will reach the market.

However a highly protective vaccine against dengue virus is very unlikely for a number of reasons. One of the difficulties in vaccine design has been attributed to dengue virus genetic diversity. Because there are four distinct serotypes and sequential infections with different strains may be a risk factor for severe manifestations, it is imperative to have a tetravalent vaccine that can efficiently and simultaneously prevent disease from all four viral serotypes. Clinical trial evaluations have revealed that imbalances and interference in the immune responses between the four strains in the formulation is a major concern [98, 104]. When infecting with multiple related viruses, which likely compete with each other for the same cellular hosts, there is always a tendency for one of them to dominate (or out-replicate) the others. This results in an uneven immune response, eliciting better antibody titers to a few serotypes rather than all of them. Thus development of a vaccine with the right combination is critical to achieve a balanced immune response that does not contribute to immune-mediated dengue disease (DHF/DSS) in vaccinated individuals [105]. A successful vaccine is also unlikely because there is no known correlate of protection; the neutralizing antibody response has not been proven to predict disease severity [106, 107].

Another factor that has contributed to the slow progress toward an effective vaccine is the lack of a suitable disease animal model. These model systems are integral for evaluating drug and vaccine candidates and gaining insight into the molecular mechanisms responsible for clinical presentations. Since the early 1900s, many attempts to reproduce the disease in animals have been conducted. More than 500 species of animals have been tested to date; however, none of them were capable of being infected by dengue virus and displaying the cardinal features of the disease [108, 109]. Dynamic clinical manifestations of dengue patients, ranging from dengue fever, DF with abnormal bleeding, DHF, DHF/DSS, to DSS with complications, have hindered the progress toward an animal disease model. Although certain rodent species have been implicated to display some clinical symptoms, the main phenotype of the disease is neurovirulence without bleeding diathesis or plasma leakage, which is not characteristic of human illness [45, 110, 111]. In addition,

virus propagated in rodents display altered biological properties since it is attenuated in humans [45, 110, 111]. Recently a humanized mouse model was developed to determine its suitability as a dengue disease model [112-114]. These animals are capable of becoming infected with DENV as well as displaying hemorrhages. Still they do not present with other salient human features such as thrombocytopenia, plasma leakage or shock. The immune responses to DENV infection in this model have not been studied in enough detail to provide insight into dengue disease. Consequently, if they displayed symptoms that were more similar to human disease in response to dengue virus infection, rodents would be an ideal, small animal model.

Despite the inadequacies of the rodent model to study dengue virus pathogenesis, there is another type of animal model– the non-human primate [22, 115]. It is accepted that they are a natural reservoir for this pathogen in the wild [108]. However, infections in primate species do not consistently or as extensively develop the prominent dengue clinical symptoms. Further investigations have revealed that the levels of NS-1, a nonstructural protein that is extensively secreted from infected cells, and viral load, which are both indicators of disease severity, are far lower in monkeys than in humans, potentially explaining their milder symptoms [115]. Recently a primate dengue coagulopathy model was developed by administration of a high dose of dengue virus intravenously [22]. Perhaps this model could be a useful tool to evaluate the efficacy of future candidate dengue vaccines.

A common unfortunate finding in live attenuated vaccine studies is the reoccurrence of viremia upon booster shots, regardless of the route of infection and high neutralizing antibody titers [116, 117]. High viremia in dengue patients with pre-existing neutralizing antibody has also been documented but the mechanism is poorly understood [94]. Viral strain differences, immune-mediated inhibition and individual genetic background, age and nutritional status have all been suggested to be contributing factors. However, the problem with viremia is dismissed by DENV vaccinologists, who

have relaxed their standards for sterilizing immunity [99]. It is considered acceptable to get viremia levels of 10^3 pfu per ml, because it will theoretically eliminate transmission [99]. However, this value assumes that infected individuals are bitten only once by a mosquito during the 3 to 5 day period of viremia. Unfortunately, without the elimination of blood borne virus, transmission to mosquitoes cannot be prevented and herd immunity cannot be achieved. If vaccine recipients are still getting an infection and shedding virus into their circulation, they are still capable of transmitting to the mosquito and contributing to the occurrence of outbreaks.

This inability to eliminate viremia may be due to the lack of an adequate antibody response to the VAM or alternatively, it could be explained by antibody depletion that occurs sometime after DENV infection and before hospitalization [118]. Dengue patients that come to the hospital and are diagnosed with DF, often display low levels of dengue specific antibody at admission, even in secondary infections [119]. The mechanism for this inhibition is unknown, but is likely due to the alteration in cellularity of the bone marrow and the potential death of the residing plasma cells [57, 120]. This disruption in antibody production is likely required for adequate human-to-mosquito transmission. Determining vaccine efficacy by neutralizing antibody response demonstrated *in vitro*, especially with monkey kidney cell lines like Vero, may be inefficient as an indicator for disease prevention. Another approach is needed.

Many formulations have been used in vaccine design. However, virus isolated from MEP cell lines, which assemble an alternative virus form, has not been tested for its ability to stimulate the immune response and prevent disease. Heat killed MP-associated virus may be a viable candidate to test in future trials.

IMPLICATIONS ON DRUG DESIGN

Besides preventative vaccines, other medical interventions under development are pharmaceuticals that can prevent virus entry or replication in the host. Drugs blocking virus uptake is being attempted by many groups. One difficulty facing this effort is the uncertainty over the host-pathogen interactions to inhibit. Many host proteins that mediate attachment to the virus have been suggested but the true receptor(s) responsible for triggering fusion and entry have yet to be discovered and agreed upon [54]. Therefore the design of small molecule fusion inhibitors has been focused against the virus receptor envelope protein. Molecules may be designed to fit into the binding pockets observed between envelope and some attachment receptor proteins. Although drug design can progress without knowing the true host receptor protein interactions that need to be blocked, the absence of a suitable animal model makes drug efficacy difficult to determine. One potential DENV receptor that has been considered for drug design is the E/DC-SIGN interaction [121]. Does blocking this interaction prevent DENV infection or inhibit the protective immune response? Research has indicated the DENV E protein interacts with the DC-SIGN receptor on dendritic cells; however, DC-SIGN is present on a high percentage of these cells while only a small percentage (~2%–5%) of DCs support infection [122, 123]. A drug against DC-SIGN would likely massively alter downstream signaling in a number of cells, changing the innate immune system response and contributing to toxicity in the host. Foreseeably, this candidate is more likely to contribute to immune-mediated disease. Another drug design strategy is to target the N-octyl-β-D-glucoside molecule, which should prevent confirmation changes associated with classical virus maturation and fusion [54]. Interestingly, there are also antibodies that bind to DENV particles better after hidden epitopes are exposed at higher temperatures, for instance when shifted from room temperature to normal body temperature [34], implicating that the same scenario could occur under fever physiological temperature. Lastly, there are attempts at designing dengue viral drugs that interfere with dengue virus genome replication [124, 125]. No drugs are currently available for therapeutic treatment; very few have been successful in animal models [126]. Inhibition of viral

replication is often screened in convenient cell lines and never in more relevant cells, such as the MEP cell lines or whole bone marrow, the suggested site for dengue virus replication *in vivo* [57, 93, 127]. Evaluation of *dengue virus replication in these cell types* may be a helpful strategy for screening drug candidates.

As aforementioned, dengue patients generally do not seek professional help until the late stage of fever, often after 2–3 days of clinical illness, at which time, the viral load is either at its peak or progressing downward [128]. Thus, the severity of dengue diseases are observed not at the time when the viral burden is at its highest *in vivo*, but rather when the virus is being rapidly cleared from host tissues by the innate and adaptive immune responses [129]. It is critical to bear in mind that dengue viral antigen in leukocytes are most likely seen after the cessation of viremia [130]. This suggests that the pathogenesis of clinically important complications is closely linked to the host immune response [129, 131]. However, the underlying mechanisms causing DHF/DSS are in debate. Current evidence strongly suggests that the immune response to dengue virus infection, predominantly inflammatory cytokines in the serum of patients, plays a key role in the pathophysiological cascade leading to plasma leakage and shock [132-139], which presumably results from the action of phagocytosis [22, 140]. Consequently, despite the large amount of work dedicated to dengue drug design targeted on blocking virus replication and entry, treatments with any of these candidates are unlikely to work in the clinic. Additionally, since there is no evidence suggesting that classical viral particles exist in the human, the success of this drug design approach is likely to be low.

The inability to find classical dengue virions in patient serum or plasma and the dynamic clinical presentation of illness in dengue patients [60, 111], both suggest the phenotypic structure of the virus *in vivo* is likely to be a versatile VAM. Therefore, targeting immune modulators that work by selectively blocking mechanisms involved in the inflammatory and immune response would be a way to go for therapeutic drug development. Thus, more attention should be spent on designing immune

system modulators that down-regulate the responses that contribute to vascular permeability and shock. For chronic infectious diseases, this strategy is not preferred because inhibition often leads to the unchecked amplification of the pathogen and increased risk of death in patients. On the contrary, dengue is an acute disease; by the time DHF/DSS occurs, the virus has likely run out of appropriate cellular hosts. This drug design strategy may be more safe and feasible with dengue disease than with other infectious agents that has been tested in the past [141, 142].

SUMMARY AND CONCLUSION

Dengue virus causes a challenging disease with diverse and nonspecific symptoms that are difficult to control. These problems are amplified by the tendency for patients to seek health care at late stages of infection, often during the phase of viral clearance. This review suggests that like other vector-borne pathogens, dengue virus may also be able to take on different structural forms, a classical virion and a VAM, in order to complete its life cycle in different hosts. Investigations using patient plasma and Meg01 and K562 cell lines have suggested that the DENV genome may be able to be packaged into host-derived microparticles. An alternative morphology may allow DENV a way to escape the immune system while in search for its next host and may also allow for a more robust infection in the vector. In combination with other issues such as the absence of a good animal, the dynamic biological morphology and life cycle of DENV may complicate efforts to design safe and effective vaccines and drugs. This concept needs to be further and more carefully investigated. Successful preventative and therapeutic strategies are not possible without a more complete understanding of the DENV life cycle.

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CHAPTER 5

Title: Can nonhuman primates serve as models for investigating dengue disease pathogenesis?

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Title: Can nonhuman primates serve as models for investigating dengue disease pathogenesis?

Running title: Nonhuman primate models of dengue disease

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ABSTRACT

Dengue virus infects between 50 and 100 million people globally, with public health costs totaling in the billions. It is the causative agent of dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), vector-borne diseases that initially predominated in the tropics. Due to the expansion of its mosquito vector, *Aedes* spp., DENV is increasingly becoming a global problem. Infected individuals may present with a wide spectrum of symptoms, spanning from a mild febrile to a life-threatening illness, which may include thrombocytopenia, leucopenia, hepatomegaly, hemorrhaging, plasma leakage and shock. Deciphering the underlining mechanisms responsible for these symptoms has been hindered by the limited availability of animal models that can induce classic human pathology. Currently, several permissive nonhuman primate (NHP) species and mouse breeds susceptible to adapted DENV strains are available. Though virus replication occurs in these animals, none of them recapitulate the cardinal features of human symptomatology, with disease only occasionally observed in NHPs. Recently our group established a DENV serotype 2 intravenous infection model with the Indian rhesus macaque, which reliably produced cutaneous hemorrhages after primary virus exposure. Further manipulation of experimental parameters (virus strain, immune cell expansion and depletion, etc.) can refine this model and expand its relevance to human DF. Future goals include applying this model to elucidate the role of pre-existing immunity upon secondary infection and immunopathogenesis. Of note, virus titers in primates *in vivo* and *in vitro*, even with our model, have been consistently 1000-fold lower than those found in humans. We submit that an improved model, capable of demonstrating severe pathogenesis may only be achieved with higher virus loads. Nonetheless, our DENV coagulopathy disease model is valuable for the study of select pathomechanisms and testing DENV drug and vaccine candidates.

INTRODUCTION

Dengue Virus (DENV), the causative agent of dengue fever (DF), is the most important vector-borne human pathogen, infecting between 50 and 100 million people annually (WHO, 2012). Moreover, DF is an escalating human problem that is increasingly spreading across the globe and extending in seasonality. This recent growth is attributed to the expansion in the niche of the virus-transmitting vectors, primarily *Aedes albopictus* and *Aedes aegypti* (WHO*, 2011)*. Thanks to the lack of vector control, increased human travel and global warming, DF, once considered a tropical disease, may reach a worldwide distribution.

The majority of DENV infections are asymptomatic or mild, but for about a quarter of infected people, disease may present as an illness that is indistinguishable from other febrile diseases or as DF with minor hemorrhagic abnormalities, bone pain, decreases in platelet counts and leucopenia, the most common form of disease. Rarely people present with the severe forms– dengue hemorrhagic fever (DHF) in which patients display hematomas with a marked thrombocytopenia or extremely low platelet counts and dengue shock syndrome (DSS), a disease similar to DHF but including plasma leakage/heme concentration, pleural effusion and the increased risk of multi-organ failure (TDR, 2009). Other symptoms (abnormal bleeding, melena, hepatomegaly, vomiting, etc.) have also been reported (Cobra et al., 1995). The majority of severe DHF/DSS cases in endemic countries occur in healthy adolescents 10 to 24 years of age (Tsai et al., 2012). Early identification of the causative agent and immediate hydration therapy with extensive monitoring of symptoms is important for resolving symptoms and preventing fatal outcomes (WHO and TDR, 2009). There is currently no targeted therapy to modulate disease severity of those most vulnerable.

It has been surmised that factors such as genetic susceptibility, developmental stage, environmental exposures and immune system programming induced by previous infections may predispose young adults to more severe disease (Halstead et al., 2007). Epidemiological data obtained

from endemic countries reveal that DHF/DSS most often occurs in people with a secondary antibody response, which has led many to champion the antibody-dependent enhancement (ADE) of infection hypothesis (Endy et al., 2004;Fox et al., 2011). ADE proponents believe that weakly specific, cross-reacting antibodies facilitate virus entry into permissive cells, increasing titers and thus, disease. Though some ADE proponents suggest that dengue-specific antibody increases immunopathology without necessarily enhancing virus replication (Markoff et al., 1991;Lei et al., 2001;Oishi et al., 2003). On the contrary, many reports have failed to demonstrate an association of DHF/DSS with secondary infection (Murgue et al., 1999;Murgue et al., 2000;Cordeiro et al., 2007;Guilarde et al., 2008;Libraty et al., 2009;Meltzer et al., 2012). A better association may exist between virus titers and disease severity (Murgue et al., 2000;Libraty et al., 2002). Despite the uncertainty over ADE, it is required that this potential risk factor be considered during the formulation in all vaccines under development (WHO, 2011). Standard preventative modalities incorporate representative antigens of each serotype in effort to simultaneously induce protection to all four DENV strains.

In the past, vaccines were designed without an exact understanding of the mechanism(s) responsible for disease pathogenesis; this was done by selecting for candidates that reduced viremia and elicited strong antibody responses (Cox, 1953;Togo, 1964). Unfortunately this approach has failed with DENV, a pathogen that does not elicit strong humoral immunity in natural infections. Neutralizing antibody to DENV can be elicited in a variety of primates (chimpanzees, cynomolgus macaques, African green monkeys, etc.) after primary infection, but they are often weak and short-lived (Scherer et al., 1978;Bernardo et al., 2008;Martin et al., 2009). In addition, protection from viremia has been reported in rhesus macaques that develop poor neutralizing antibody titers (Scott et al., 1980;Putnak et al., 1996) and after the response waned (Raviprakash et al., 2000). Interestingly, some evidence suggests that humans may also be protected from disease during high viremia without ever developing specific antibodies (Stramer et al., 2012;Perng and Chokephaibulkit, 2013); these

observations raise concern that neutralizing antibody quantification is not the best approach to evaluate vaccine efficacy.

A more thorough understanding of the mechanisms contributing to disease and protection in humans are clearly needed to accelerate progress toward better drug and vaccine candidates. Severe disease is known to arise after the clearance of viremia, suggesting that DHF/DSS and lethality are more likely immune than viral-mediated (WHO and TDR, 2009). In fact, immune activities elicited via antibodies (Saito et al., 2004), complement (Avirutnan et al., 2006) and T cells (Green et al., 1999) have been associated with disease in human studies. Importantly, the delay in severe disease presentation until late after infection limits our ability to interrogate early events that set the stage for immunopathogenesis. Thrombocytopenia, plasma leakage and coagulation abnormalities appear to be the critical phenomena to prevent in patients, but the events preceding these phenomena have been incompletely elucidated. Carefully controlled experiments performed in relevant animal models are needed to explore the dynamics of hematological dysfunction and other factors potentially involved in dengue disease. Unfortunately an adequate animal model that is capable of recapitulating human disease is largely unavailable.

DEVELOPMENT OF DENV INFECTION ANIMAL MODEL SYSTEMS

The search for animal model systems began in the early 1900s, far before the availability of cell culture techniques to propagate or quantify virus stocks. Pathogens had to be amplified in animals that were permissive and quantified by mortality studies. Unfortunately none of the animals tested (hamster, mouse, rat, lizard, etc.) ever displayed signs of disease, limiting the progress in studying DENV (Simmons et al., 1931). The research that was conducted often involved virus propagation in human volunteers, who suffered from typical DF (Simmons et al., 1931). Eventually, a young suckling mouse model inoculated intracranially with DENV that displayed mild disease was

developed (Sabin and Schlesinger, 1945). This model was quite limited, with paralysis observed only after 3–4 weeks in 10%–20% of the mice, but this provided a starting point for virus adaptation and lead to the first small animal infection model.

MOUSE MODEL

There are a number of mouse breeds that have been employed in DENV investigations– wildtype, engrafted-SCID, AG129, RAG-hu and the NOD/SCID/IL-2Rγ/human CD34 transplant or humanized mouse (Lin et al., 1998;Kuruvilla et al., 2007;Zhang et al., 2007;Mota and Rico-Hesse, 2011;Zompi et al., 2011). AG129 mice have been the most commonly utilized strain; they are highly susceptible to dengue, replicate virus to high titers and display vascular leakage (Shresta et al., 2006;Zompi and Harris, 2012). The NOD/SCID/IL-2Rγ mice reconstituted with human $CD34^+$ cells are infrequently used but have the greatest potential as future mouse models. These animals demonstrate several symptoms of human disease (fever, erythema, thrombocytopenia) (Mota and Rico-Hesse, 2011;Cox et al., 2012).

However the symptomatology observed with inbred, immune-compromised mice differs from that seen in humans, likely because of the susceptibility of various cell lineages and the extensive differences in immune system dynamics (Nussenblatt et al., 2009). AG129 mice predominantly display neurological symptoms and splenomegaly (Schul et al., 2007;Zompi and Harris, 2012) and engrafted-SCID mice present with paralysis (Zompi and Harris, 2012). While the humanized mouse may be the closest to replicating patient pathology, there still remain a few caveats to using this model. Challenges involved in humanized mouse preparation and data interpretation are compounded by the considerable mouse-to-mouse variation observed (Akkina et al., 2011). Additionally this mouse model, with murine stroma and endothelium, cannot completely mimic the

immune response of humans. A number of mechanisms suspected to play critical roles in dengue pathology are differentially regulated in these mice. Processes that are dependent on stromal cell interactions, such as B lymphocyte maturation and specific antibody production (Akkina, 2013), and involve endothelial microparticle signaling, such as the coagulation cascade (Mairuhu et al., 2003;Lynch, 2007), may unfold differently in these mice and lead to alternative outcomes. The human $CD34^+$ engrafted mouse model system can provide a great starting point in interpreting important biological processes involved in human DENV disease but results will still need to be confirmed in nonhuman primate species.

NONHUMAN PRIMATE (NHP) MODELS

It has been hypothesized that the close genetic relationship between primates and humans and the presence of a comparable immune responses make NHPs the best models for studying DENV. While this may be, NHPs have been particularly unreliable at modeling DENV pathology, producing mild symptoms at best (Scherer et al., 1972;Halstead et al., 1973b). Monkeys thus far appear to be incapable of succumbing to life-threatening DENV disease. However, several Old and New world primate species are in fact permissive to experimental DENV infection (Scherer et al., 1978;Schiavetta et al., 2003;Onlamoon et al., 2010;Yoshida et al., 2012). A recently published review detailed the characteristics of viremia in many of these species (Hanley et al., 2013). Table 1 summarizes the pathology and immunopathology observed thus far in approximately 20 NHP species from 15 different genera.

Table 5.1. Summary of *in vivo* DENV studies.

Primate	Route	Strain	Type	Virus stock[a]	Dose[b]	Infected	Viremia[c]	Findings (source)
Macaca mulatta	iv, sc	ND	ND	Humans	ND	ND	ND	no disease, leucopenia **(Lavinder and Francis, 1914)**
Macaca cyclopis	sc, iv, ip	ND	ND	Humans	ND	ND	ND	no disease **(Koizumi and Tonomura, 1917)**
Macaca mulatta	NI	ND	ND	Humans	ND	Yes	ND	animal chilly and morose, rash on chin & throat **(Chandler, 1923)**
Macaca fascicularis	sc	ND	ND	Humans	ND	Yes	ND	first to demonstrate unquestionably that some primates were permissive to DENV infection but that they are asymptomatic **(Blanc et al., 1929)**
Cercopithecus callitrichus		ND	ND	Humans	ND	Yes	ND	
Papio spp.		ND	ND	humans	ND	No	ND	
Cercocebus spp.		ND	ND	humans	ND	No	ND	
Macaca mulatta	sc, mi	ND	ND	humans, mosquitoes	ND	No	ND	no fever, some leukopenia and lymphocytosis, demonstrated transmission of DENV from primates to humans through mosquitoes **(Simmons et al., 1931)**
Macaca fascicularis philippinensis	sc, mi, ic	ND	ND	humans, mosquitoes	ND	Yes	ND	
*Macaca fascicularis fusca**	sc, mi	ND	ND	humans, mosquitoes	ND	Yes	ND	
Pan troglodytes	sc	Hawaiian	NI	human	ND	Yes	ND	mild fever (101°F)

*	, id							**(Paul et al., 1948)**
Homo sapiens	id	NI	NI	human	1^{d}	Yes	+	low dose gave multiple patterns of disease: 1) unmodified attack, 2) short febrile illness without rash or 3) no illness but partial immunity
	id				10^{d}	Yes	+	progression of symptoms:1) edema & erythema, 2) fever, 3) maculopapular eruptions with sparing at the site of the original skin lesion
	into scars				conc. human serum	Yes	+	unmodified dengue
	eye				$2E5^{d}$	Yes	+	typical dengue
	eye				$1E4^{d}$	No	-	no disease or immunity
	in				$1E6^{d}$	Yes	+	unmodified dengue or mild rash
	in				$1E4^{d}$	No	-	no disease or immunity **(Sabin, 1952)**
Cebus capucinus	scor ip	Hawaiian, NGC	DENV1, DENV2	human	ND	Yes	+	no overt signs of illness
Ateles geoffroyi						Yes	+	
Ateles fusciceps						Yes	+	
Alouatta palliata						Yes	+	
*Callithrix geoffroyi**						Yes	ND	
Saimiri oerstedii						Yes	ND	
Aotus trivirgatus						Yes	ND	**(Rosen, 1958)**
Hylobates lar	sc	BKM725-67	DENV1	LLC-MK2	800	Yes	+	fever and hemorrhagic manifestation occurred

								but were associated with acute
		BKM1179-67	DENV1		800			lymphomatous leukemia, no correlation between antibody titers to
		BKM1749	DENV2		1.6E3			DENV and protection from viremia
		24969	DENV3		6.6E2			
		KS168-68	DENV4		5E3			**(Whitehead et al., 1970)**
Saimiri sciureus	sc	Hawaii	DENV1	mice	1E6.4[e]	Yes	+	some fever in DENV1 infection
		16007	DENV1	LLC-MK2	1E5.7	Yes	-	no platelet, hematocrit or leukocyte count
		NGC	DENV2	mice	1E6.7[e]	Yes	+	changes.
		NGC	DENV2	mosquitoes	1E2.5	Yes	+	
		16681	DENV2	LLC-MK2	1E5.5	Yes	-	
		Pak-20	DENV3	LLC-MK2	1E3.4	Yes	50	
		16562	DENV3	LLC-MK2	1E5.7	Yes	-	
		4328S	DENV4	LLC-MK2	1E3.9	No	-	
Saguinus oedipus	sc	Hawaii	DENV1	mice	1E6.4[e]	Yes	+	brief fever in DENV1 infection
		NGC	DENV2	mice	1E6.7[e]	Yes	+	
		NGC	DENV2	mosquitoes	1E2.5	Yes	+	
		H87	DENV3	mice	1E5.8	Yes	-	
		Pak-20	DENV3	LLC-MK2	1E3.4	Yes	-	
		H241	DENV4	mice	1E6.6[e]	No	-	
Saimiri sciureus	in	Hawaii	DENV1	mice	1E6.4[e]	Yes	ND	no disease reported
		NGC	DENV2	mice	1E5[e]	No	ND	
		NGC	DENV2	mosquitoes	1E2.5	No	ND	
		Pak-20	DENV3	LLC-MK2	1E2.1	No	ND	
		H-241	DENV4	mice	1E6.6[e]	No	ND	

Saguinus oedipus	in	NGC	DENV1	mice	1E5.3[e]	Yes	+	
		H87	DENV3	mice	1E6.2[e]	Yes	ND	
		Pak-20	DENV3	LLC-MK2	1E2.7	No	ND	
		H241	DENV4	mice	1E5.6[e]	No	ND	
Aotus trivirgatus	in	Hawaii	DENV1	mice	1E5.7[e]	No	ND	
		NGC	DENV2	mice	1E6.6[e]	Yes	ND	
		Pak-20	DENV3	LLC-MK2	1E2.1	Yes	ND	**(Scherer et al., 1972)**
Macaca mulatta **(India)**	sc	16007	DENV1	LLC-MK2	5E5	Yes	1.7E3	lymphadenopathy in DENV1,2 &4, rare hemorrhaging in DENV1& 4, leucopenia
		16681	DENV2	LLC-MK2	5E5	Yes	4.8E2	in DENV2 & 4, lymphocytosis common.
		16562	DENV3	LLC-MK2	5E5	Yes	+	thrombocytopenia in 21%–33% of animals with all serotypes,
		4328S	DENV4	LLC-MK2	5E5	Yes	2.8E2	complement decreases in secondary DENV2, no change in behavior, eating or prothrombin.
Macaca fascicularis *fascicularis**	sc, id	16007	DENV1	LLC-MK2	NI	Yes	-	no disease
		16681	DENV2		NI	Yes	-	
		16562	DENV3		NI	Yes	-	
		4328S	DENV4		NI	Yes	-	
*Chlorocebus aethiops**	sc, id	16007	DENV1	LLC-MK2	1E5	Yes	+	no disease
		16681	DENV2		1E5	Yes	+	
		16562	DENV3		1E4.5	Yes	+	
Erythrocebus Patas	sc, id	16007	DENV1	LLC-MK2	NI	Yes	+	no disease
		16681	DENV2		1E5	Yes	+	
		16562	DENV3		1E4.5	Yes	-	

		4328S	DENV4		1E3.3	Yes	-	**(Halstead et al., 1973a;b)**
Macaca mulatta	sc	16007	DENV1	LLC-MK2	1.2E5	Yes	350	lymphadenopathy, virus distribution after sc injection indicated
		16681	DENV2		2E6	Yes	443	that most virus did not move far from
		16562	DENV3		1E5	Yes	40	the inoculation site, day after
		4328S	DENV4		1E6	Yes	1085	viremia virus was distributed widely throughout skin **(Marchette et al., 1973)**
Pan troglodytes	id, sc	49313	DENV1	mosquitoes	1E3.1	Yes	1E6.6g	nasal discharges and lymphadenopathy
		NC38	DENV2	humans	1E3.6	Yes	1E5.6g	symptoms found in individual animals:
		49080	DENV3	mosquitoes	1E2.7	Yes	1E5.2g	splenomegaly, leucopenia,
		17111	DENV4	mosquitoes	1E2.8	Yes	1E6g	hemorrhage, shaking chill, lethargy **(Scherer et al., 1978)**
Macaca mulatta	sc	16681	DENV2	LLC-MK2	1E5	Yes	1E5.7	cyclophosphamide treatment caused chronic infection, 3/9 died, internal hemorrhaging, enlarged kidney, severe acute proliferative glomerulonephritis, pleural effusion, passively transferred antibody aided viral clearance **(Marchette et al., 1980)**
Macaca mulatta	sc	PR-159	DENV2	FRhL	5.6	Yes	ND	no disease
		H-241	DENV4		1.44			**(Kraiselburd et al., 1985)**
Macaca mulatta & Macaca fascicularis	is, im, it	16007	DENV1	PDK	2.5E5	Yes	ND	mild neurovirulence **(Angsubhakorn et al., 1987)**
Aotus	sc	Western	DENV1	NI	2E4	Yes	+	pathology more pronounced in DENV1,

nancymae		Pacific 74						mild leucopenia, changes in attitude and appetite,
		S16803	DENV2					changes in fecal consistency,
								2/20 became lethargic,
		CH53489	DENV3					common symptoms: lymphadenopathy,
		341750	DENV4					nasal discharges and splenomegaly. **(Schiavetta et al., 2003)**
Aotus nancymae	sc	IQT6152	DENV1	NI	1E4	Yes	+	no disease
		IQT2124	DENV2				-	
		OBS8041	DENV2				+	**(Kochel et al., 2005)**
Macaca Mulatta	sc	60305	DENV1	Vero	1E5	Yes	1E1.6	no disease
		16007	DENV1	Vero	1E5	Yes	1E2.4	
		16007	DENV1	C6/36	1E5	Yes	1E1.9	
		40247	DENV2	C6/36	1E5	Yes	1E3.6	
		44/2	DENV2	Vero	1E5	Yes	1E2.9	
		H87	DENV3	Vero	1E5	Yes	1E2.7	
		16562	DENV3	Vero	1E5	No	-	
		74886	DENV3	C6/36	1E5[f]	Yes	1E2.2	**(Freire et al., 2007)**
Macaca fascicularis	sc	40514	DENV1	NI	1E6.4[f]	Yes	400[f]	no disease, characterized T-cell and neut antibody cross-reactivity, no changes in
		28128	DENV4		1E6.2[f]		20[f]	IFN-γ, TNFα, IL4, IL8, IL10 transcription during infection. **(Koraka et al., 2007)**
Macaca mulatta	sc	Western Pacific 74	DENV1	NI	1E4	Yes	ND	no disease, increases in AST, transcriptional upregulation of ISGs, OASs, Mxs, etc., no increases in cytokine gene expression **(Sariol et al., 2007)**

Chlorocebus aethiops sabaeus	sc	SB8553	DENV2	NI	1E6	Yes	+	no fever or lymphomegaly, no changes in behavior or weight, no respiratory, digestive or nervous system disturbances, lower inoculum titers gave prolonged viremia and better neut antibody responses **(Martin et al., 2009)**
Macaca mulatta **(Indian)**	iv	16681	DENV2	Vero	1E7	Yes	~8E3	consistent hemorrhaging in 9/9 animals, decline in platelet count and leucopenia, elevated thrombin-antithrombin, D-dimers, ALT and CK, no increases in hematocrit, prothrombin or activated PTT. **(Onlamoon et al., 2010)**
Callithrix jacchus	sc	02-17/1	DENV1	C6/36	3.5E7	Yes	5E5[h]	no disease
		DHF0663	DENV2		6.7E7		1.6E7[h]	found differing NK, NKT, and naive,
		DSS1403	DENV3		4.5E6		5.5E4[h]	effector memory and central T-cell kinetics during DENV infection with
		05-40/1	DENV4		1.5E6		2.5E4[h]	different strains
		Jam/77/07	DENV2		1.2E5		2.8E6[h]	
		Mal/77/08	DENV2		1.9E5		9.6E6[h]	**(Omatsu et al., 2011;Yoshida et al., 2013)**
Homo sapiens	sc	45AZ5	DENV1	FRhL	2E3	Yes	+	$CD8^+$T-cell-dervied IFN-γ associated with protection form fever and viremia, sIL-R2α correlated with disease onset and severity, PBMC-derived TNF-α, IL-2, 4, 5, 10 did not correlate with protection or

		CH53489	DENV3	FRhL	1E5			disease. **(Gunther et al., 2011;Sun et al., 2013)**
Macaca nemestrina	sc	98900645	DENV3	C6/36	1E7-1E8	Yes	62.94	inoculation route influenced virus-tissue distribution
	id						47.98	minimal hepatitis
	iv						58.62	**(Pamungkas et al., 2011)**
Saguinus midas and Saguinus labiatus	sc	DHF0663	DENV2	C6/36	6.7E7	Yes	2.7E6[h]	no disease , $CD16^+$ NK cell depletion did not alter virus replication or pathogenesis
	iv						2E7[h]	**(Yoshida et al., 2012)**
***Macaca mulatta* (Indian)**	sc	NGC	DENV2	NI	1E5	Yes	257	day 14 PI showed the highest levels in T-cell activation, Anti-NS1, 3 & 5 T-cell responses were characterized **(Mladinich et al., 2012)**
***Macaca mulatta* (Chinese)**	iv, sc	16681	DENV2	Vero	1E7	Yes	+	hemorrhaging in 50% of iv inoculated primates (unpublished)

[a]Cell type or organism in which DENV stock was propagated, [b]Highest inoculum dose is given when there were variable doses, [c]Titers given when available, [d]HID, [e]MLD50 or MLD50/ml, [f]TCID50 or TCID50/ml, [g]MID50/ml, [h]RNA/ml, +/- indicates presence or absence of viremia

Abbreviations: ic-intracardial, mi-mosquito inoculation, iv-intravenous, sc-subcutaneous, id-intradermal, ip-intraperitoneal, in-intranasal, im-intramuscular, is-intraspinal, it-intrathalmic, NI-not indicated, ND-not determined, MID50-mosquito infectious dose 50, TCID50-tissue culture infectious dose 50, MLD50-suckling mouse intracranial lethal dose 50, HID-human minimal infectious dose.

The most consistent pathological finding in these animals has been lymphadenopathy of the inguinal and auxiliary lymph nodes (Halstead et al., 1973a;Marchette et al., 1973;Scherer et al., 1978;Schiavetta et al., 2003). In one species, *Chlorocebus aethiops sabaeus*, the absence of lymphomegaly (Martin et al., 2009) and in a few reports, splenomegaly (a rare symptom in humans) were noted (Scherer et al., 1978;Schiavetta et al., 2003). Fever is a valid parameter to assess, but its recording in DENV-infected primates is logistically difficult, and is therefore rarely reported (Scherer et al., 1972). NHPs in general have higher body temperatures and greater variability than human bodies (Scherer et al., 1972;Fuller et al., 1985), so unless readings are measured on awake animals by telemetry, the anesthesia used profoundly alters the body's temperature, making accurate readings impossible (Baker et al., 1976). Another human dengue symptom, cutaneous rashes, are not commonly observed in primates but may be underreported; also tourniquet tests are never performed on primates to assess capillary fragility. Behavioral changes, like lethargy, have been documented in only a few studies (Chandler, 1923;Scherer et al., 1978;Schiavetta et al., 2003). In general, primates kept and bred in captivity rarely display overt disease.

Despite the low incidence of pathology observed in these studies, dengue infections in primates share many characteristics with human disease. The onset and duration of viremia is similar to humans, or about 3–6 days starting from the second day after inoculation (Freire et al., 2007;Koraka et al., 2007). Leucopenia has been observed (Onlamoon et al., 2010). Thrombocytopenia has never been captured in NHPs, likely because of their naturally high platelet counts, but moderate platelet decreases have been document in *M. mulatta* (Halstead et al., 1973a;Onlamoon et al., 2010). A DENV-induced reduction of dengue-specific antibodies during the early phases of secondary homologous infection, a phenomenon observed in viremic patients, has been seen in marmosets (Omatsu et al., 2011). The anti-dengue antibodies that are elicited in primates are highly cross-reactive against other closely related flaviviruses (Scherer et al., 1978). DENV infection of monkeys elicits a vigorous innate response (Sariol et al., 2007) leading to

activation and marked shifts in circulating subsets of T, NK, and NK-T cells in a marmoset model (Yoshida et al., 2013). The role of DENV specific cell-mediated responses in NHP models has received relatively less attention, although some studies reported recognition of nonstructural proteins in addition to viral components by both $CD4^+$ and $CD8^+$ T cells (Koraka et al., 2007;Mladinich et al., 2012). However, such responses have been difficult to detect in immunized monkeys, even in those that show protection from challenge (Chen et al., 2007;Porter et al., 2012).

The similarities observed in these studies imply that primates may present with more suitable symptoms than mouse models upon further manipulation. A comparison of the benefits to using the NHP and murine animal models is given (Table 2). Several strategies to improve the NHP model may be explored—for instance increasing the number of permissive cells or altering the immune environment. Here we discuss boosting viremia with different virus delivery strategies.

Table 5.2. Relative advantages in using primate and murine model systems to study DENV disease.

	Primate models	**Murine models**
Ease of use/cost	-	+
Susceptibility to human DENV strains	+	-
Mimic human viremia	(+) reduced	+
Mimic human immune responses	+	-
Model human disease		
Fever	-	CD34-engrafted humanized mouse
Hemorrhages	Indian rhesus monkey	CD34-engrafted humanized mouse, C57BL/6
Platelet count reduction	Indian rhesus monkey	CD34-engrafted humanized mouse
Hepatomegaly	-	Balb/c
Pleural effusion	-	-
CNS disease*	-	+
DHF/DSS	-	-
Lethality	-	+

+ = commonly present,- = absent, *rarely observed in human dengue infections

VIRUS DELIVERY

Only a limited number of studies have attempted determining the infectious dose delivered during natural dengue infection. One study suggests the amount of DENV transmitted by *A. aegypti* ranges between 1 x 10^4 to 1 x10^5 (Gubler and Rosen, 1976). However, there are disagreements over the best methods to conduct such studies; the controversial points include mosquito species, generation number, feeding strategy, infection method, incubation temperature and length, virus strain and technique used to quantify transmitted virus. All these variables have the potential to affect the infection dynamics and alter the conclusions of the study (Chamberlain et al., 1954;Grimstad et al., 1980;Mellink, 1982;Watts et al., 1987;Colton et al., 2005;Smith et al., 2005). Some studies have suggested levels as high as 1 x $10^{8.7}$ genome equivalents or almost 1 x 10^7 PFUs can be transmitted, though rarely (Colton et al., 2005;Styer et al., 2007). Currently we know as few as 1000 PFUs can cause viremia and disease symptoms in humans (Sun et al., 2013). Ultimately the natural inoculum dose is more suggestive of the amount of virus needed for continual DENV transmission *in vivo* and does not necessarily reflect the quantity required for disease induction. Viremia levels and disease may be less dependent on inoculum size and more contingent on host-pathogen interactions. These matters should be considered when modeling DENV infection in animals.

Virus delivery to the proper tissues is important for inducing the appropriate interactions with the host and promoting disease presentation. DENV deposition is believed to occur exclusively by direct inoculation into the subcutaneous layer by mosquitoes. However, the subcutaneous infection route does not promote adequate virus dissemination (Marchette et al., 1973;Pamungkas et al., 2011). Potentially the virus is restricted by less frequent encounters with migrating cells and immobilization by attachment to extracellular matrix proteins (Anez et al., 2009). Consider that mosquito feeding involves the probing of all layers of skin, including the cutaneous layer and capillaries, to find a blood meal. These tissues are an integral part of the arbovirus-vector lifecycle

and are frequently evaluated in transmission studies (Chamberlain et al., 1954;Styer et al., 2007). Virus injected directly into these tissues have better access to and faster dissemination throughout the body, affording the virus more opportunities to rapidly reach distant target cells (Pamungkas et al., 2011). Additionally, pathology induction is likely promoted by rapid viral dissemination and replication in distant cells and organs. This assumption led us to hypothesize that an intravenous infection strategy would favor wide dissemination and allow for rapid simultaneous replication of virus in various tissues, invoking a more pronounced innate immune response, potentially reflective of the human immune environment during high viremia. Although the kinetics of viremia did not markedly differ between subcutaneous and intravenous DENV2 infection (Onlamoon et al., 2010;Omatsu et al., 2011), it will be critical to delineate the overall kinetics of DENV dissemination to and replication in various tissues and how this relates to the induction of symptoms.

RHESUS MACAQUE MODEL OF COAGULOPATHY

Only a few NHP dengue investigations have reported rashes post-infection (Lavinder and Francis, 1914;Halstead et al., 1973b;Onlamoon et al., 2010). In most of these studies, hemorrhaging was a rare event. However, our group reported a reproducible coagulopathy disease model in the Indian rhesus macaque when 9 out of 9 monkeys inoculated intravenously with $1x10^7$ PFUs of DENV2 (16681) displayed evidence of subcutaneous hemorrhage (Onlamoon et al., 2010). The viremia noted in these animals remained at the high end of the range typically reported in other NHP studies and were reached relatively consistently at early time points post-infection.

The most prominent symptoms observed in our studies with the Indian rhesus macaque were cutaneous hemorrhages, starting at day 3 and 4 post-infection (PI) and lasting as long as 10 days (Figure 5.1A) (Onlamoon et al., 2010). In a pilot study using Chinese rhesus macaques, disease presentation with the same virus was more modest, suggesting that these NHPs may be less

susceptible to disease. Large hematomas developed in only one of the two primates infected intravenously with DENV2 (Figure 5.1B).

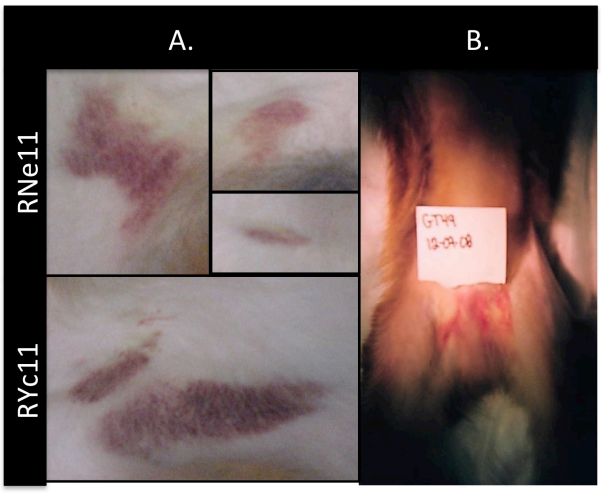

Figure 5.1. Hematomas are seen in intravenously inoculated rhesus macaques.

(A) Indian rhesus macaques were injected intravenously with 1 x 10^7 PFUs of DENV2 16681 as previously reported (Onlamoon et al., 2010). Hematomas of various degrees of severity were present on days 3 till 14 post-infection (PI). Prominent ecchymoses were visible in two young male animals, RNell and RYc11, on day 7. **(B)** Four Chinese rhesus macaques were injected intravenously (n = 2) or subcutaneously (n = 2) with 1 x 10^7 PFUs of DENV2 16681 strain. Hemorrhaging was only observed in 1 of 2 IV-injected monkeys (GT49), depicted in the picture above day 6 PI. No hematomas were observed in subcutaneously inoculated macaques.

The dynamics of various leukocyte subsets were followed longitudinally PI. Similar to human dengue, these animals experienced the typical leucopenia or a modest but consistent decrease in white blood cells that reached a nadir at day 7 PI, but returned to normal levels by day 10 (Onlamoon et al., 2010). Platelets also modestly decreased until day 3, corresponding to the time of peak DENV RNA load (Noisakran et al., 2012). While these leukocyte values did not fall out-of-range for macaques the changes were clearly noticeable and consistent. There was also a modest decrease in hematocrit, which resolved with the clearance of viremia at day 7, in spite of continuous blood and bone marrow draws (Onlamoon et al., 2010).

A longitudinal monitoring of coagulatory parameters hinted that a number of features may be important for hemorrhage formation (Onlamoon et al., 2010). Increased time to clotting was noted during blood collection of some Indian rhesus macaques, indicating an increased susceptibility towards bleeding. However, thromboplastin and prothrombin times did not indicate abnormal clotting. Protein C and anti-thrombin III levels did not vary from pre-inoculation values, but they were predominantly in the high end of the reference range. Marked elevations were noted for D-dimers, TAT complexes and protein S, with peaks most consistently present on days 5 to 10 PI, corresponding to the resolution of viremia. This data requires further confirmation with additional time points, more animals spanning various ages and other DENV isolates. However, we submit that we might for the first time have a model to investigate coagulopathy similar to DHF, which can allow for better evaluation of preventative and therapeutic strategies to prevent pathogenesis, not just infection.

Interestingly, analysis of serum chemistry parameters indicated relatively modest changes for all parameters except for creatine phosphokinase (CK), which was markedly elevated on day 7 (Onlamoon et al., 2010). CK is a component in energy metabolism (with multiple isoenzymatic forms: MM, MB, and BB) that is altered in individuals with a number of different illnesses (Roberts

and Sobel, 1973;Saks et al., 1978). Heightened levels of CK have been noted in Crimean Congo and Influenza patients (Middleton et al., 1970;Ergonul et al., 2004). Additionally, a recent report confirms elevation of this enzyme in dengue patients and suggests it is linked to muscle weakness/dysfunction during malaise (Misra et al., 2011). However, CK is a nonspecific biomarker that is elevated in various conditions, and thus its diagnostic value is limited. Since these enzymes are quite highly elevated during DENV infection, there could be a meaningful relationship between CK and disease. CK and creatine phosphates in combination are known as ADP scavengers and participate in modulating platelet activities, such as aggregation (Chignard et al., 1979;Chesney et al., 1982;Krishnamurthi et al., 1984;Jennings, 2009), which may consequently modulate immune cell activation/function and by extension, pathogenesis (Wong et al., 2013).

BONE MARROW (BM) TARGETING

The bone marrow (BM) can be involved in hemodynamic defects; alterations in the BM environment may result in altered leukocyte function and contribute to pathogenesis (Wilson and Trumpp, 2006;Duffy et al., 2012). DENV has long been known to alter hematopoiesis in human BM (Bierman and Nelson, 1965;La Russa and Innis, 1995). However, collecting BM aspirates from DENV patients is contraindicated. Additionally, infections in patients can be misleading due to the variability in disease onset and the uncertainty of sample time points. Experimentation in animal models in which the induction of infection is known allows for better analysis in real time. Our rhesus monkeys were sampled for BM repeatedly on a rotating basis resulting in the collection of at least 3 samples at each time point spanning days 1 to 14 PI. This has allowed for us to confirm that BM cellularity is indeed depressed during early acute DENV infection (Noisakran, 2012). Aspirates were also monitored for the presence of DENV in attempts to identify the initial cellular reservoirs of infection. While the general consensus is that DENV targets phagocytes, such acquisition could be secondary to amplification in other cell types. *In vitro* both human and monkey BMs are permissive

for DENV replication, and similar to *in vivo*, peak titers differ by 1000-fold (Figure 5.2) (Clark et al., 2012). Characteristics of the early host cells were also evaluated in our model both *in vivo* and *in vitro* (Clark et al., 2012;Noisakran et al., 2012). Of interest DENV antigen was primarily detected in $CD41^{+}CD61^{+}$ cells during the first 3 days, followed by a gradual shift towards $CD14^{+}$ phagocytes at later time points, coinciding with viral clearance (Clark et al., 2012). The results suggest that megakaryocytes represent the initial target of DENV in BM, rather than a member of the monocytic lineage. Direct infection of these cells may account for the altered megakaryocyte composition (Nelson et al., 1964), impaired platelet function (Srichaikul and Nimmannitya, 2000;Cheng et al., 2009) and the incidence of platelet phagocytosis observed in previous studies (Nelson et al., 1966;Honda et al., 2009;Onlamoon et al., 2010). Platelet activation and function during the course of infection has been under-investigated but may be critical for unraveling the mechanisms responsible for dengue pathology.

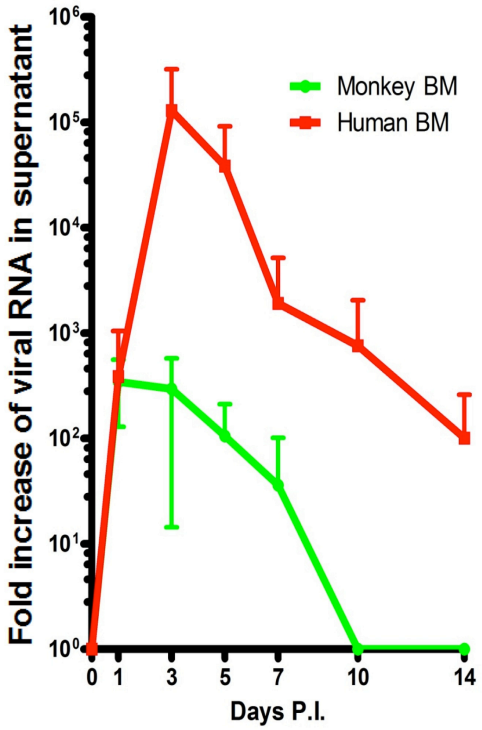

Figure 5.2. Peak DENV titers in rhesus macaque BMs is markedly lower than that of humans.

BMs were acquired and infected as previously described (Clark et al., 2012). Samples from days 1 through 14 were quantified by realtime PCR. Human (red) and monkey (green) titers are depicted in RNA copy numbers per ml. The *in vitro* experimentation of whole BM indicates that human BM is able to produce far more virus than monkey BM. Titers appear to max out on average closer to day 1 in monkey BM but reach their peak (~1000-fold higher) on day 3 PI in humans.

PLATELET ACTIVITIES

The role of platelets in the crafting of the immune response is imperfectly defined and only recently becoming recognized (Klinger and Jelkmann, 2002;Ombrello et al., 2010). These anucleated cells are able to associate with and deliver signals to other lineages and shape immune responses. Abnormal platelet behavior during dengue infection may play a significant role in modifying lymphocyte, monocyte, and granulocyte function. When platelet-leukocyte interactions were quantified *in vivo*, macrophages/monocytes appeared to be the most commonly associated cell lineage with platelets (Onlamoon et al., 2010), with a majority of these monocyte-platelet aggregates expressing activation marker CD62P (Onlamoon et al., 2010). This data is reminiscent of other reports linking activated monocytes to disease pathology in humans (Mustafa et al., 2001;Bozza et al., 2008;Durbin et al., 2008).

Platelets binding to neutrophils and lymphocytes were less frequent (Figure 5.3A,B,C) (Onlamoon et al., 2010). Only about 20%–40% of neutrophils were bound with platelets, with 30%–60% expressing CD62P. This may underestimate the extent of neutrophil-platelet aggregates, since these cells are short-lived and other markers for neutrophil (CD11b and CD66b) and platelet (CD154, cleaved PAR1, CD63) activation were not tested (Heijnen et al., 1999;Claytor et al., 2003;Kinhult et al., 2003;Sprague et al., 2008). Lymphocyte-platelet aggregation occurred the least (Figure 5.3B,C). This was examined with Indian and Chinese rhesus macaques during primary DENV2 (16681) infection and in Chinese macaques during secondary DENV3 (Hawaii) infection (Figure 5.3B,C, respectively). Since the dominant phenotype of the lymphocyte-platelet aggregate (LymPA) population was CD62P negative, this was the only population evaluated. Chinese and Indian macaques have different baseline levels of $CD41^{+}CD61^{+}CD62P^{-}$ lymphocytes, approximately 2% and 12%, respectively (Figure 5.3B and 5.3C). The average response from five Indian macaques suggests that the LymPA population is down-regulated (to about 7%) during infection but returns to

normal levels after viral clearance (Figure 5.3B). In Chinese macaques, there appeared to be higher LymPA frequencies with the IV-inoculated monkeys, ranging up to 8% but only as high as 4% in SC-inoculated primates (Figure 5.3C). There was a late phase expansion of this population after primary but not after secondary infection. The functional significance of such changes is unclear at the present, but it would be interesting to compare these findings with other viral infections, like influenza, which produce robust long-lived B cell memory responses (Ikonen et al., 2010;Li et al., 2012). It remains to be seen whether this observation represents a common immune phenomenon or a DENV specific response, which would potentially open a new line of investigation.

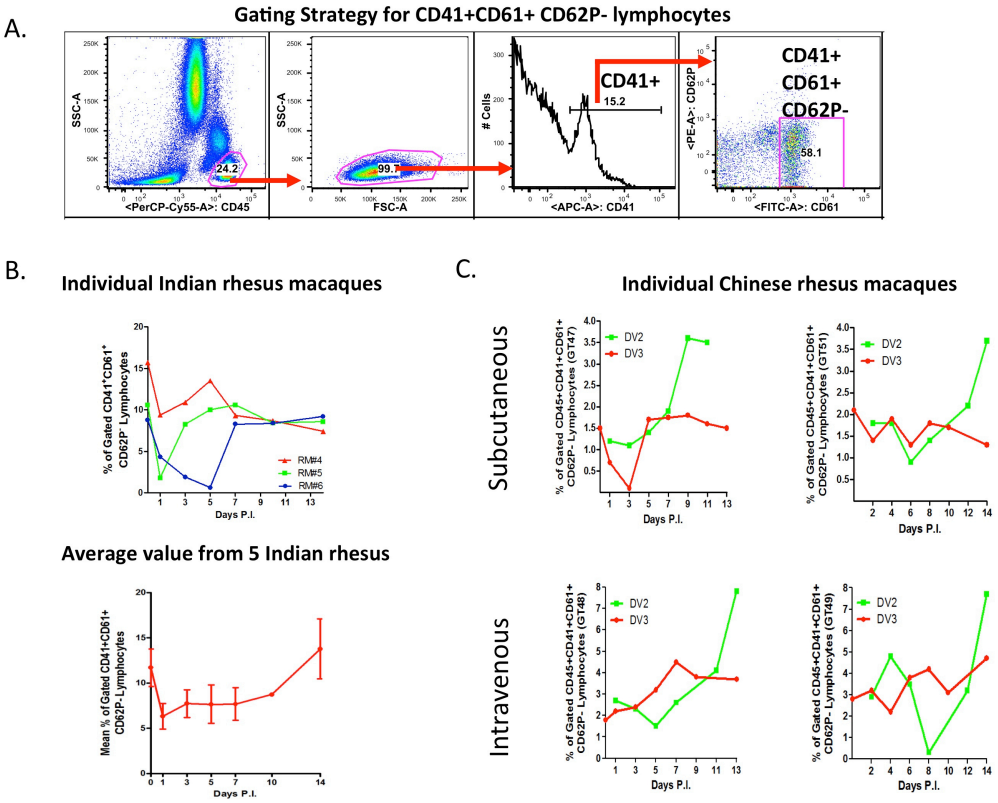

Figure 5.3. Dynamics of lymphocyte-platelet aggregates (LymPA) during DENV infection.

Indian and Chinese rhesus macaques were infected as detailed in Figure 5.1. In addition, the Chinese macaques were challenged two months later with DENV3 strain Hawaii. Peripheral blood samples obtained on days 1 through 14 were subjected to flow cytometric analysis with CD45, CD41, CD61 and CD62P fluorescent antibodies. The frequencies of $CD45^{+}CD41^{+}CD61^{+}CD62P^{-}$ cells over time is graphed. **(A)** Panels to illustrate the gating strategy employed to analyze lymphocyte-platelet aggregates (LymPA). **(B)** The kinetics of LymPA in Indian rhesus macaques. The top graph displays LymPA frequencies from 3 individual macaques and the bottom graph, the average population frequency from 5 primates. The LymPA population is down-regulated during DENV infection in Indian rhesus macaques. **(C)** LymPA kinetics in subcutaneously and intravenously infected Chinese rhesus macaques during primary DENV2 (green line) and secondary DENV3 infection (red line). The frequency of LymPA increases late after primary but not after secondary infection.

POTENTIAL REFINEMENTS TO THE COAGULOPATHY MONKEY MODEL

Virus selection

While the data obtained with our rhesus macaque model appears promising, many parameters remain to be examined and refined. Arguably, the most important factor to evaluate is different strains. The viruses we used had been propagated extensively in cell culture, and thus the next step will be to evaluate primary DENV strains, which are considered more capable of inducing pathology. Interestingly, the earliest DENV studies (pre-1940s) in primates were conducted with human-derived virus that had never been propagated through cell culture (Lavinder and Francis, 1914;Chandler, 1923;Blanc et al., 1929;Simmons, 1931), yet these investigations induced minimal overt disease. The human-derived Hawaiian and New Guinea strains from Sabin's work were pathogenic in humans (when inoculated intradermally) but demonstrated no pathology in Rosen's study when inoculated into various primate species via a subcutaneous or intraperitoneal route (Sabin, 1952;Rosen, 1958). In recent studies, a large number of the strains employed were recent clinical isolates minimally passaged *in vitro* (Freire et al., 2007;Omatsu et al., 2011;Pamungkas et al., 2011;Yoshida et al., 2012). While these viruses are often close in sequence to the original isolate, these strains are not necessarily the most virulent or capable of achieving the targeted pathology in primates (Omatsu et al., 2011) and may require further evaluation before use *in vivo*.

The major drawbacks of primate models are the logistics and cost. Ideally one would perform preliminary experiments and evaluate strain virulence through a screening tool before *in vivo* studies with NHPs. Virulence could be assessed by testing the induction of disease in the humanized mouse or potentially by growth characteristics in monkey whole bone marrow. Alternatively, passage of dengue in organisms (humanized mice or rhesus macaques) may ensure that the strain is more fit for these studies. It has been suggested that mouse-passaged viruses are more capable at causing viremia in NHPs than *in vitro*-passaged strains (Scherer et al., 1972).

Considering the viruses that have already been tested in NHPs, a select few appear promising for future studies. WP-74 (DENV1) and S16803 (DENV2) caused extreme lethargy in owl monkeys (Schiavetta et al., 2003) but not in cynomolgus (Koraka et al., 2007) or rhesus macaques (Ajariyakhajorn et al., 2005;Robert Putnak et al., 2005). Besides the 16681 DENV2 virus, strains 49313 (DENV1), 16007 (DENV1) and 43283 (DENV4) were associated with hemorrhage in previous studies (Halstead et al., 1973b;Scherer et al., 1978). Testing these strains in our Indian macaque model could lead to a more frequent presentation of coagulopathy and models for 3 of the 4 dengue serotypes. For future preclinical vaccine and drug studies, one strain of each serotype that can induce easily quantifiable disease will be needed for better vaccine evaluation.

Other parameters

A number of additional parameters may be manipulated in rhesus macaques that could amplify disease severity. Factors from infected mosquito saliva may potentiate the virus in down-modulating immune responses during the initiation of infection and help raise peak titer levels (Cox et al., 2012;Reagan et al., 2012;Surasombatpattana et al., 2012;Le Coupanec et al., 2013). Mosquito inoculation of DENV into NHPs was modeled long ago without inducing much disease (Simmons et al., 1931). However, a number of confounding factors (preexisting immunity, inoculum quality, etc.) were not accounted for in these studies, indicating that this approach in worth revisiting.

Modulation of *in vivo* cell populations with drug treatments has rarely been attempted (Marchette et al., 1980;Yoshida et al., 2012). Potential treatment of macaques with megakaryocytic growth factors, like thrombopoietin, could increase the number of early permissive targets and enhance peak viral load if indeed megakaryocytes are the primary replication site for DENV (Nakorn et al., 2003). General immunosuppression has been attempted but led to chronic viremia, which does not mimic human DENV disease (Marchette et al., 1980). Depletion of macrophages, neutrophils or other innate immune responders may enhance titers by altering the dynamics of viral clearance. One

previous attempt at $CD16^+$ natural killer cell depletion did not modulate virus titers (Yoshida et al., 2012), although such depletions are generally partial at best. Additionally, various inoculum sizes and alternative inoculation routes may be tested. The intradermal inoculation route was suggested to lead to better virus tissue distribution, but did not result in better dissemination to the BM (Pamungkas et al., 2011). Characterization of these parameters are necessary for the further refinement of the coagulopathy disease animal model.

HOST CHARACTERISTICS OR GENETIC FACTORS THAT INCREASE SUSCEPTIBILITY TO COAGULOPATHY

Epidemiological studies of dengue patient characteristics, including age, sex, and genetic polymorphisms have been frequently studied, but none of these findings have been validated in animal models (Loke et al., 2001;Stephens et al., 2002;Cordeiro et al., 2007;Kalayanarooj et al., 2007;Soundravally and Hoti, 2007;Stephens, 2010). In humans, the age of greatest susceptibility to disease is seen in young adults (Tsai et al., 2012). In our Indian rhesus macaques, we have evaluated age as a contributing factor to viremia by comparing the titers of DENV when propagated in whole BM *in vitro* (unpublished data). However, no difference was noted in virus growth kinetics or magnitude related to age of BM donors (n = 11), which spanned 2–15 years of age. *In vivo*, anecdotal observations suggested that coagulopathy appeared to be more extensive in older female macaques when compared to young males, which were the populations included in the study, although sample size was too low to be conclusive. This nevertheless raises an interesting question about the potential for host factors contributing to the severity of symptoms.

Various MHC alleles, blood group, and platelet antigens have been found to be associated with dengue disease and protection (Kalayanarooj et al., 2007;Soundravally and Hoti, 2007;Alagarasu et al., 2013;Weiskopf et al., 2013). In general these associations are weak as biomarkers of disease. One of our goals is to assess gene alleles involved with regulating platelet activation and the

coagulatory cascade e.g. *HPA1*, *HPA2* for association with disease presentation. Available techniques, such as *Macaca mulatta* typing and gene expression analyses, will need to be an integral part of future experiments with the rhesus monkey model to facilitate identification of genetic factors involved with dengue-induced abnormal coagulation.

CONCLUSION

The induction of disease symptoms upon the inoculation of DENV in primates has been an elusive objective. Recently a coagulopathy disease model was developed using the serotype 2 strain 16681 injected intravenously into Indian rhesus macaques. We submit that this approach provides a strategy for detailed investigation of the mechanisms potentially involved in DHF. Moreover, the model provides an attractive algorithm for testing the efficacy of preventative vaccines and therapeutics that not only limit virus replication but also prevent disease development *in vivo*. Various host and viral parameters can begin to be evaluated *in vivo* to help us gain a better understanding of dengue biology and disease pathogenesis. Can pathology be induced in other NHPs by switching to the intravenous route? Will different virus strains promote coagulopathy, or other symptoms? Can we alter other parameters and achieve a more severe disease model? The establishment of this new rhesus macaque infection model has proved insightful on ways to improve disease presentation in primates.

CONFLICTS OF INTEREST

This research was conducted in the absence of any commercial or financial relationships. Publication of this article cannot be construed as a conflict of interest for any of the authors.

HUMAN SUBJECT AND ANIMAL RESEARCH

Use of de-identified human BM was provided by Emory Hospital and approved by the Emory University Internal Review Board. Investigations with rhesus macaques were approved by Yerkes and Tulane IACUCs and conducted at either the Yerkes or Tulane National Primate Research Centers. Research was performed in accordance with institutional and national guidelines and regulations.

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CHAPTER 6

Discussion and Future Directions

This compendium characterizes the relationship between dengue virus (DENV) and bone marrow cell populations, particularly the megakaryocyte-erythrocyte progenitor (MEP) lineage, and emphasizes that these cells are involved and potentially play an important role in DENV replication. A number of key observations were made in this work. After absorption with virus, human bone marrow was capable of amplifying DENV to high titers reflective of those found in patients. Using electron microscopy (EM), virion assembly was seen in megakaryocyte-like cells of the bone marrow. The cell types in humans that serve as initial viral targets *in vivo* and fuel the high dengue fever viremia has remained elusive. However, this research suggests that *in vivo* at least one such host for DENV is cells of the MEP lineage. This work contributes to the understanding of basic DENV biology and provides avenues for future research in regards to dengue disease pathogenesis.

Previous studies suggested that the bone marrow and bone marrow resident cells (potentially the megakaryocytes) contributed to the development of disease pathology (24, 130, 182). Efforts to further evaluate bone marrow involvement have been thwarted by the difficulty of obtaining bone marrow and the increased mortality associated with acquiring human bone marrow from acutely

infected dengue patients. Also, the lack in availability of a model *in vitro* megakaryocytic cell line delayed the progress that could have been made using basic research. In Chapter 2, we were able to revisit the hypothesis that megakaryocytes contributed to dengue disease by using one established megakaryocyte progenitor cell line, Meg01 (193), and included for comparison the closely related erythrocyte progenitor cell line, K562, which is also capable of differentiating into megakaryocytes (10, 103, 220). Megakaryocytic lineage cell lines could be directly infected by DENV2, 16681, *in vitro* and produce high infectious titers (Figure 2.1). K562 cells have been used previously in other studies and are capable of producing high DENV titers (56, 129, 214), but their permissiveness is disputed because of lab-to-lab variation; other labs have found them poorly permissive (80). Although this cell line is predominantly composed of erythrocytic progenitors, this myelogenous leukemic cell line was later discovered to express monocyte cell markers (212) and thus has been regarded by many as a myeloid cell line. Consequently, many researchers have regarded K562 as a poorly permissive myeloid-derived cell line and have used them for studies evaluating ADE (50, 80, 149). Differences in K562 peak titers were also found among the various cellular stocks present at Emory University. To explain this variation among laboratories, three different cell populations present within the heterologous K562 cell line were isolated and examined for their capacity to replicate DENV2 (Figure 6.1). Different K562 subsets differed in their ability to propagate DENV2, type A having a lower capacity (1 X 10^3) and types B and C (MEPs) having greater capabilities (1 X 10^5 and 1 X 10^7), suggesting great variability based on cell stocks used. Thus, with our data, we resubstantiate that the MEP cell lines are highly permissive and likely closely related to the cell population infected by DENV *in vivo* (Figure 2.1).

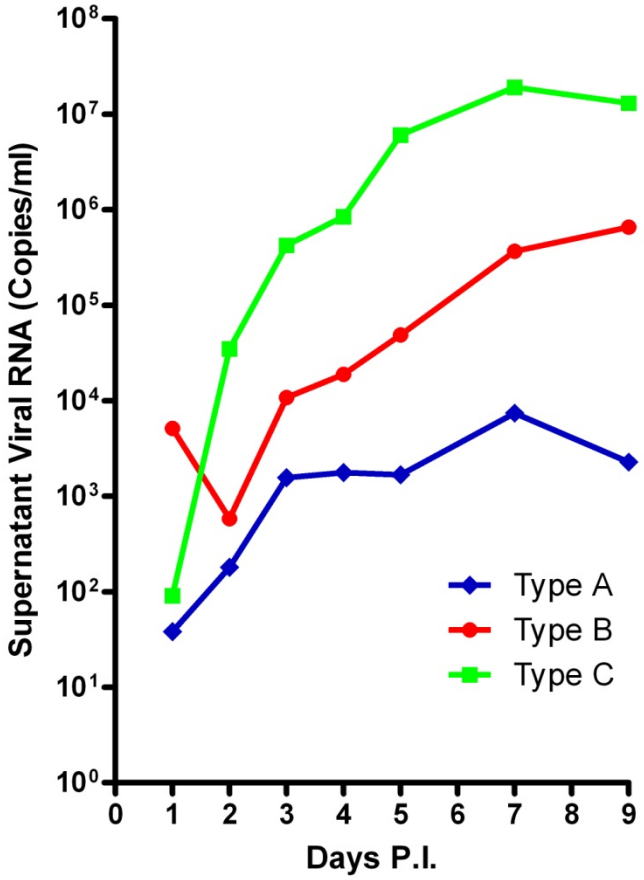

Figure 6.1. Different K562 subset populations are differentially permissive.

Cell colonies A (other, monocytes), B (erythrocytes), and C (megakaryocytes) were isolated from K562 cell cultures grown in methylcellulose media and selected based upon their burst forming unit and giemsa staining properties. Subpopulations were infected with DENV2, 16681, with an MOI of 0.1, supernatants taken daily from days 1–9, and virus genomes quantified by RT-qPCR. Data contributed by Sansanee Noisakran, Ph.D.

Megakaryocyte permissiveness was also assessed *ex vivo* by evaluating infections in whole bone marrow, the tissue having the highest concentration of megakaryocytes (or 0.01% of nucleated cells) (155). By studying human bone marrow and repeating experiments with monkey bone marrow, we not only indicated that the bone marrow was permissive to DENV infection (Figures 3.1 and 3.5 and Supplementary Figure 3.4) but that the corresponding tissue from the rhesus macaque (a commonly used animal model) could serve as a substitute for studying virus replication. Virus amplified by at least three logs in comparison with the starting time point in human bone marrow, indicating a cell population in this tissue is a target cell for DENV (Supplementary Figure 3.4). Considering that low virus titers result from infections of cells derived from the peripheral blood, the host cell type responsible for high dengue virus viremia might be predominantly located in the bone marrow.

DENV antigen has been found in many different cell types *in vivo* (202), but antigen can be found in cells for reasons other than productive infection. In our studies, DENV antigen was not only found in megakaryocytes (Figures 3.3 and 3.7 and Supplementary Figure 3.2), but they were found at times corresponding with early time points of infection when the virus was still replicative, unlike detection in phagocytes at later time points during which the virus was losing replicative capacity. Increasing the number of megakaryocytes lead to improvements in bone marrow permissiveness (Supplemental Figures 3.9 and 3.10). Additionally, crystalloid structures were uncovered in large cells with multiple nuclei (most likely megakaryocytes) (Figure 3.8), indicating that this cell type is capable of not only infection but also efficient virion assembly and production. This evidence strongly suggests interactions between DENV and the MEP cell lineage (or the closely related stem cell lineage that differentiates into MEPs) *in vivo*. Identifying the cell type that initially encounters and takes up the virus is still ongoing. Because sampling bone marrow from DENV patients is contraindicated, cells displaying megakaryocytic or stem cell CD markers are being isolated

from the peripheral blood of patients and tested for DENV antigen. When the cell type(s) is better identified, attempts will be made to isolate these cells and test permissiveness.

When examining the virus produced within *in vitro* MEP cell lines, Meg01 and K562, they produced lower numbers of virions (Figure 2.3 and Table 2.2) as compared with Vero cells, but similar or higher viral DENV titers were obtained from MEP cell line supernatants, suggesting a more efficient viral production. Previous work involving virus supernatant revealed that EM particle concentrations closely mirrored genome copy number concentrations (272), although few studies have compared both genome copy number and EM virus particle titers (113, 286). A common phenotype observed with DENV structural protein mutants is reduced virus secretion, without necessarily affecting intracellular infection rates. The phenomenon occurring in MEPs is the opposite, reduced intracellular infection rates but unaltered virus particle secretion. Previous studies have not reported this phenomenon, and determining whether such differences in production of infectious DENV are caused by differences in viral genetics or other factors is needed.

It is not known whether efficient virus production is a general characteristic of DENV production in MEP cell lines and shared across strains. We have examined the growth of DENV of other serotypes in MEPs, but under the experimental conditions used, viral replication appeared less robust overall and rigorous testing of other serotypes remains to be evaluated with the same degree of detail to fully validate our findings across all four serotypes. It is also unknown whether low production of defective virions and reduced virion assembly is reflective of *in vivo* replication of the virus. Examination of particles in the megakaryocytes *ex vivo* demonstrated many virus-induced structures in the cytoplasm but few classical virus particles in the ER-derived membranes, which was consistent with the *in vitro* findings with MEP cell lines, suggesting a mechanism that might explain the kinetics of virus production.

Acute viruses that replicate to high titer *in vivo* are thought to produce robust numbers of virus particles, many of them defective, potentially functioning to absorb antibodies, providing virus

a better opportunity to propagate in the presence of a specific immune response *in vivo*. Defective interfering (di) particles, a type of noninfectious virus, may also inhibit virus growth in *in vitro* cell culture systems by binding and blocking receptors and competing with infectious virions for access to target cells. These di particles may also have incomplete genomes that compete with infectious virus for transcriptional and translational machinery (39, 138). The Vero cell line appears to produce robust levels of DENV, though many of them noninfectious, as suggested by their genome copy number to plaque-forming unit ratios (Chapter 2 Table 2.1), while noninfectious virus formation in MEPs are less prominent. Because of Vero's high production of noninfectious virus, they likely produce higher quantities of di particles. DENV production in MEP cell lines might allow for the production of better virus stocks with lower concentrations of defective virions, which could improve the quality of assays that evaluate host-pathogen interactions, in particular protective responses.

The time point of sample collection is not only relevant for virus particle quality, it is generally important for understanding pathomechanisms. Investigators often find various cell types (e.g., monocytes, macrophages, and dendritic cells) and tissues (e.g., liver and skin) in DENV patients to be antigen positive, and these observations have been interpreted as supportive of virus replication in phagocytes (111). However, these observations generally coincide with patients seeking treatment corresponding to post peak viremia and DENV at this point is often no longer infectious, an observation consistent with removal and inactivation of DENV particles, rather than productive infection. Dengue pathology is more pronounced after the peak in viremia, when the virus is undergoing degradation and the immune response is highly activated. DENV taken from whole bone marrow at these late time points was indeed found to be associated with macrophages ($CD14^+$) and dendritic cells ($BDCA^+$) (Figure 3.4, Table 3.1, and Supplemental Figures 3.3 and 3.6), were poorly infectious in cell culture (Figure 3.2), suggesting these immune cells might be more involved with virus clearance, as suggested in other studies (72). The timing of these findings suggest that

phagocytes might actually be involved in the pathogenic mechanisms that occur post viral peak, either by contributing to the inflammatory process or the release of factors that promote vascular leakage.

Despite evidence supporting a protective role for the humoral immune responses, vaccines are predominantly evaluated for the quality of specific antibody they elicit. The lack of a correlate of protection (an immune response that correlates with protection from disease) has lead to challenges interpreting DENV vaccine efficacy in animals and humans. Consequently, vaccine evaluation requires human clinical phase III trials in which vaccine recipients have to be followed for extensive periods of time to assess the difference in dengue disease between vaccinated and nonvaccinated persons over several seasons, with considerable costs and delays. Identifying correlates of protection for DENV will make developing a preventive modality for dengue disease easier and considerably cheaper. Of note too, investigations assessing immune responses predominantly use Vero-derived DENV; however, antigenicity can vary somewhat when produced in different cell lines (Figures 2.6 and Supplemental Figure 2.1) and the presence of large amounts of di particles described above may skew the results. Vero-derived DENV structure is likely distinct from that of *in vivo*-produced DENV. Thus, immune responses might be evaluated correctly in vaccine trials, but perhaps using the wrong reagents. If testing is done using antigen that is poorly representative of the *in vivo* pathogen, the outcome of the testing might be skewed to varying levels. Including megakaryocyte-DENV and comparing results with Vero-DENV in neutralization assays to test vaccine efficacy are plans that are underway.

The megakaryocyte's primary function is to differentiate and produce platelets (155), which are integral for controlling coagulation/bleeding and contribute to immune responses. Thrombocytopenia (low platelet counts) and platelet dysfunction, e.g., altered platelet production (237), altered platelet function (104, 171), reduced platelet half-life, and increased platelet consumption (171), are hallmarks of dengue disease (171). Platelet counts correlate with disease

severity. DENV targets the platelet and is a common contaminant in the platelet and blood supply in DENV-endemic areas (11, 137). Intact DENV particles can be found inside of dengue patient platelets (Figure 4.1) (189). These virions appear to be found in vesicles not interconnected with the open canalicular system, the dominant location in the platelet for alpha-granule release and particle degradation (65), suggesting DENV might have entered the platelet at a step preceding their formation/release and had opportunity to alter platelet function. Other viruses that cause thrombocytopenia have also been captured within the megakaryocytes (37, 296). Further defining the relationship between DENV and platelets is needed to elucidate the mechanisms involved in platelet malfunction contributing to dengue pathogenesis (i.e., bleeding abnormalities, disseminated intravascular coagulopathy, and inflammation).

Infection of the megakaryocytes and inclusion of virus particles in platelets yields the virus opportunity of inclusion in platelet debris and platelet microparticles. Microparticle involvement has been implicated in other viral infections (67, 101), and platelets are the dominant contributor to microparticles in the peripheral blood (203). Because limited research has been performed on DENV particle morphology *in vivo*, little is known about its structure or how it transmits. Potentially the virus can become enclosed in platelet microparticles, which might account for another mechanism of cell-to-cell transmission or allow for escape from humoral immune response. The presence of virion assembly in EM images confirmed that DENV could be produced in cells of the MEP lineage (Figure 2.2). However, we observed the presence of an additional feature in K562 cells on day 1 only, potential virus assembly, or replication complex inclusion in microparticles (Figure 6.2), suggesting another potential route of virus transmission. This DENV-induced structure inclusion in microparticles did not persist into day 2 of culture, when virus was more prevalent intracellularly. If microparticles transmit DENV, they are shed early post-infection when virus is more difficult to detect. Analysis of cell debris or microparticles released from infected MEP cells at these early time

points (day 1 and 2) would better indicate if DENV is released within microparticles and whether these microparticles are infectious.

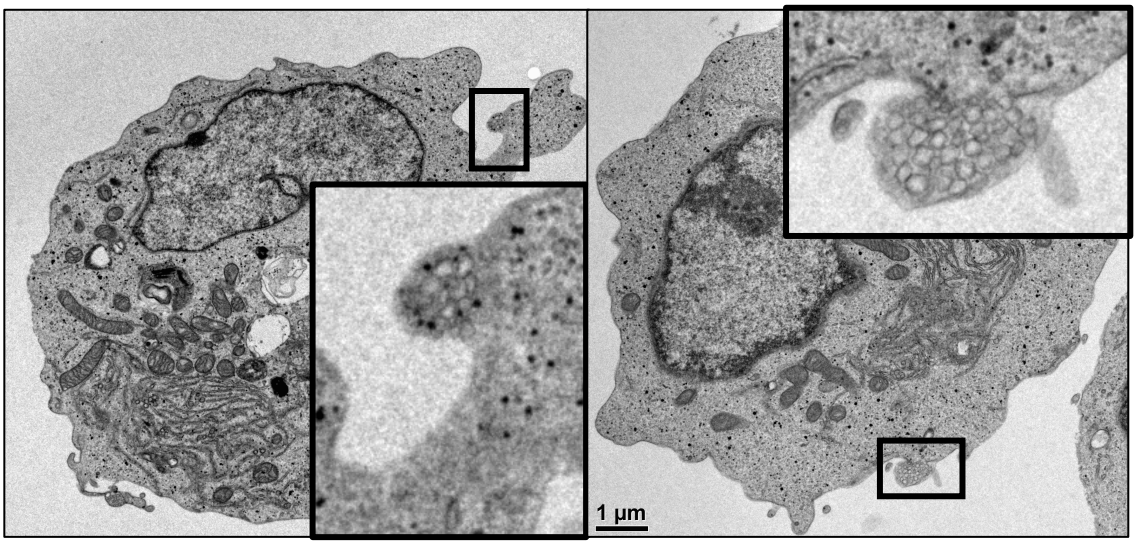

Figure 6.2. Small intracellular vesicles budding off within microparticles from DENV2-infected K562 cells.

K562 cells were infected at an MOI = 0.1 and cells harvested day 1 for thin-section EM imaging. Vesicles appear to be in the size range of DENV replication complexes and suggest viral RNA trafficking or virus assembly occur in MEP-derived microparticles. Images provided by Robert P. Apkarian Integrated Electron Microscopy Core, Emory University.

Another option could be to evaluate platelet microparticles from humans or model organisms. Total human microparticles have been evaluated for infectious DENV. These microparticles were positive for infectious virus, but half of the virus was lost with acid wash treatments, indicating a sizable proportion of microparticle-associated virus might be trafficking within host-derived vesicles (203). Typically, persons who experience severe disease arrive at the hospital after the peak in viremia, and thus dengue patient samples obtained at these times are not the most suitable for detecting infectious virus. The rhesus macaque animal model, which can be infected, followed, and sampled at specific early time points, might be a better tool to investigate *in vivo* infections, although this is not without controversy.

As of yet no animal model has been capable of recapitulating severe dengue disease; only minor symptoms (lymphadenopathy, hepatomegaly, and hematomas [Figure 5.1]) have inconsistently presented in nonhuman primate models (Table 5.1). Some researchers insist on returning to the use of the experimental human infection model to interpret dengue disease pathology (246), though such an approach comprises obvious ethical concerns. The nonhuman primate model can be infected, although most such infections were asymptomatic associated with markedly lower viral titers, approximately 1000-fold lower than that seen in humans (Figure 5.2), suggesting that high titers might not be achievable in this model. Because some studies suggest a correlation between disease severity and virus titer, severe disease might not be possible to investigate using nonhuman primates. However, considering that consistent cutaneous hematomas were observed in our Indian rhesus macaque model inoculated via the intravenous route, it is likely the nonhuman primate model has not been manipulated appropriately in most studies to allow for disease presentation. With the dissection of pathomechanisms and testing of additional infection parameters (e.g., nonhuman primate species and genetics, virus adaptation, inoculum levels, etc.), the development of an even better animal model is possible.

The data presented and summarized herein has elucidated the involvement of the bone marrow and suggested the megakaryocyte-erythrocyte lineage is playing a role in viral dynamics and pathology, likely through altering the function of platelets. Future research will determine in greater detail what cells within or closely related to the megakaryocyte-erythrocyte lineage are infected, to what extent this lineage is involved across different virus strains and serotypes, how involved the platelets and platelet microparticles in dengue disease and transmission, and whether the nonhuman primate animal model can be further improved and serve as a reliable animal model to study disease.

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