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Role of Microparticles in Dengue Virus Infection and Its Impact on Medical Intervention Strategies

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Dengue virus (DV†) 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. DV 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 life cycle. DV 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 DV life cycle.

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†Abbreviations: DV, dengue virus; MPs, microparticles; EM, electron microscopy; MVBs, multivesicular bodies; VAM, virus of alternative morphology; DHF, dengue hemorrhagic fever; DF, dengue fever; DSS, dengue shock syndrome; WHO, World Health Organization; DDT, dichlordiphenyltrichloroethane; E, envelope; C, capsid; PrM, pre-membrane; M, membrane; NS1, nonstructural protein 1; DC-SIGN, Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Nonintegrin; RHA, Rapidly Sedimenting Hemagglutinin Antigen; SHA, Slowly Sedimenting Hemagglutinin Antigen; MEP, Megakaryocyte Erythrocyte Progenitor; BM, bone marrow; ADE, antibody-dependent enhancement.

Keywords: Dengue virus, microparticles, vaccine, vector-borne transmission, *Aedes* mosquitoes, alternative virion

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INTRODUCTION

Dengue virus (DV) is one of the most important vector-borne diseases today, contractible by the bite of a DV-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. DV 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 nonspecificity of the clinical symptoms. Currently, there is no preventive or therapeutic treatment available for dengue. Rehydration therapy and palliative care with close monitoring are the only approved practices known to reduce mortality and improve patient outcomes.

DV was once a clinically significant pathogen in the United States, 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 United States, 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 health care system has heightened public awareness and led 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 has remained unknown since it has never been recorded thus far. Our observations with patient plasma and megakaryocyte erythrocyte progenitor (MEP) cell lines, Meg01 and K562, support the idea that DV 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 DV 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 DV 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 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 more than 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 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, capable of infecting indicator cells such as Vero [40]. The VAMs manufactured in other cell lines display different characteristics. 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, suggesting it is SHA [19]. Additionally, the virus derived from the mosquito C6/36 cell line has a 30nm diameter and is deficient in capsid protein but yet still infectious [47].

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 DV life cycle, but it was later determined in vaccine preclinical trials that DV 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 DV 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 DV, suggesting that neutralization antibodies are present but bind other epitopes [59].

Surprisingly there are practically no published investigations on the DV 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]. Interest-

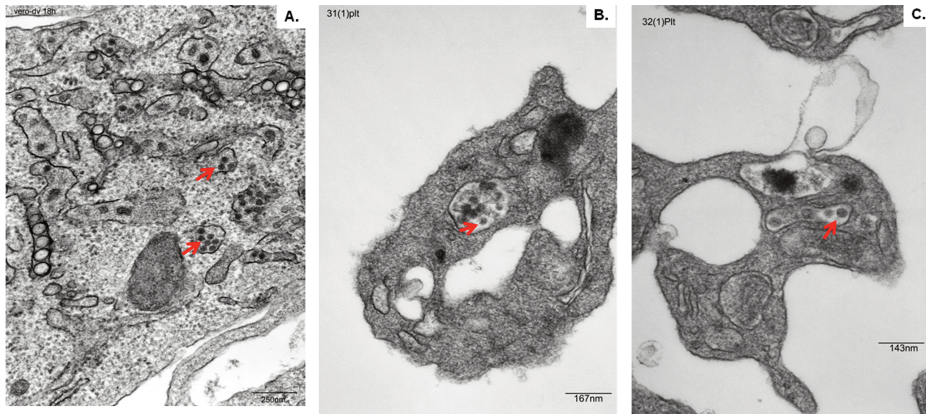

Figure 1. Transmission EM images of DV2-infected Vero cells and dengue patient platelets. Vero cells were infected with a multiplicity of infection equal to 5 for 18 hours, and samples were prepared as previously described [58]. Human platelets were isolated from acute dengue patients via Optiprep, and platelets were fixed with 4 percent glutaraldehyde in PBS. Samples were washed and fixed with 2 percent 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 arrows indicate viral- particles inside virus-induced vesicle structure.

ingly, platelets can support one round of DV replication [61]. Careful inspection of the Vero-derived and platelet-derived classical virions reveal that these particles are slightly different from each other (Figure 1). They both have diameters in the 40-50nm range, but the platelet-derived classical virus form is more heterogeneous (Figure 1B and C). Some platelet vesicles contain fuzzy debris, potentially a type of VAM (Figure 1B and C). Also there is an interesting formation blebbing off the platelet (Figure 1C). This microparticle (MP) appears to be mostly empty, containing a vesicle (a structure also seen in DV-infected Vero cells) that also could be considered a VAM (Figure 1A 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 DV 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 DV 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].

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 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 DV. Other microbes from various domains of life have been noted for their ability to alter MP content and promote their transmission [76-80]. DV, as mentioned earlier, only requires the presence of its genome to initiate an infection. If its transcripts or genome have the capacity to be

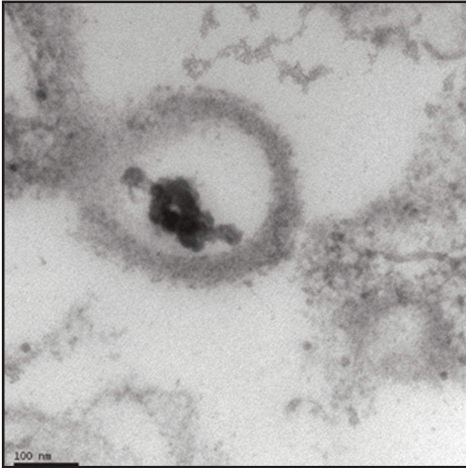

Figure 2. Transmission EM of plasma concentrate pooled from multiple patients. Plasma was spun with an ultrahigh speed at130,000xg for 30 minutes, and the pellets were prepared as described in Figure 1. Small vesicles containing virus particles were observed.

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 also have been suggested to play vital roles in shaping the immune response during infections by facilitating coagulation and delivering MHC receptors and CD40L to appropriate cell types [70,83-85]. MPs potentially may 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, DV can be found in human and monkey platelets (Figure 1B 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 are 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. All that can be found in plasma concentrates from dengue patients are small cellular vesicles, which likely contain viral components (Figure 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.

Testing this hypothesis will be difficult because the human cell population(s) that harbor and replicate DV *in vivo* have not been determined. The literature suggests that virus is likely to infect a cell frequently found in the bone marrow and capable of differentiating into megakaryocytes, shedding the DV-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 DV RNA contents, no classical virions and only host-derived MPs are readily detectable. Replication of DV 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 DV 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 DV 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 one log and make EM virion visualization more difficult. Also, this would skew the infectious virus to defective virus particle ratio. In DV2-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 quasi-species into one cell may facilitate a more robust and productive infection. These particles also may shield virus from immune system components, allowing for infection in spite of preexisting high neutralizing titers [94]. This may 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 DV 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 DV 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 gradients [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 DV 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 more than 60 years of extensive effort, little progress has been made at developing effective vaccines to prevent the occurrence of infection or disease [98]. Sev-

eral 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 four 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 DV 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 DV infection in this model have not been studied in enough detail to provide insight into dengue disease. Consequently, if they displayed symptoms 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 extensively secreted from infected cells, and viral load, 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 also has 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 DV 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 DV 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 preventive 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 putative 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 DV receptor considered for drug design is the E/DC-SIGN interaction [121]. Does blocking this interaction prevent DV infection or inhibit the protective immune response? Research has indicated the DV 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 to 5 percent) 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 DV 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 the physiological temperature of fever. 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 to 3 days of clinical illness, at which time, the viral load is either at its peak or progressing downward [128]. Thus, the severe dengue disease is 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 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] 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 DV genome may be able to be packaged into host-derived microparticles. An alternative morphology may allow DV 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 DV may complicate efforts to design safe and effective vaccines and drugs. This concept needs to be further and more carefully investigated. Successful preventive and therapeutic strategies are not possible without a more complete understanding of the DV life cycle.

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REFERENCES

1. NIAID. Dengue Fever [Internet]. 2005 April. Available from: http://www.niaid.nih.gov/factsheets/dengue.htm.
2. WHO. Dengue guidelines for diagnosis, treatment, prevention and control. World Health Organization [Internet]. 2009: Available from: http://whqlibdoc.who.int/publications/2009/9789241547871_eng.pdf.
3. Kurane I, Kontny U, Janus J, Ennis FA. Dengue-2 virus infection of human mononuclear cell lines and establishment of persistent infections. Arch Virol. 1990;110(1-2):91-101.
4. Azizan A, Fitzpatrick K, Signarovitz A, Tanner R, Hernandez H, Stark L, et al. Profile of time-dependent VEGF upregulation in human pulmonary endothelial cells, HPMEC-ST1.6R infected with DENV-1, -2, -3, and -4 viruses. Virol J. 2009;6(1):49.
5. Cabello-Gutierrez C, Manjarrez-Zavala ME, Huerta-Zepeda A, Cime-Castillo J, Monroy-Martinez V, Correa BB, et al. Modification of the cytoprotective protein C pathway during Dengue virus infection of human endothelial vascular cells. Thromb Haemost. 2009;101(5):916-28.
6. Arevalo MT, Simpson-Haidaris PJ, Kou Z, Schlesinger JJ, Jin X. Primary human endothelial cells support direct but not antibody-dependent enhancement of dengue viral infection. J Med Virol. 2009;81(3):519-28.
7. Theofilopoulos AN, Brandt WE, Russell PK, Dixon FT. Replication of dengue-2 virus in cultured human lymphoblastoid cells and subpopulations of human peripheral leukocytes. J Immunol. 1976;117(3):953-61.
8. Sriurairatna S, Bhamarapravati N, Diwan AR, Halstead SB. Ultrastructural studies on dengue virus infection of human lymphoblasts. Infect Immunol. 1978;20(1):173-9.
9. Andrews BS, Theofilopoulos AN, Peters CJ, Loskutoff DJ, Brandt WE, Dixon FJ. Replication of dengue and junin viruses in cultured rabbit and human endothelial cells. Infect Immunol. 1978;20(3):776-81.
10. Kurane I, Ennis FA. Production of interferon alpha by dengue virus-infected human monocytes. J Gen Virol. 1988;69(Pt 2):445-9.
11. Kurane I, Hebblewaite D, Brandt WE, Ennis FA. Lysis of dengue virus-infected cells by natural cell-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. J Virol. 1984;52(1):223-30.
12. Kou Z, Lim JY, Beltramello M, Quinn M, Chen H, Liu S, et al. Human antibodies against dengue enhance dengue viral infectivity without suppressing type I interferon secretion in primary human monocytes. Virology. 2011;410(1):240-7.
13. Halstead SB, O'Rourke EJ. Antibody-enhanced dengue virus infection in primate leukocytes. Nature. 1977;265(5596):739-41.
14. Blackley S, Kou Z, Chen H, Quinn M, Rose RC, Schlesinger JJ, et al. Primary human splenic macrophages, but not T or B cells, are the principal target cells for dengue virus infection in vitro. J Virol. 2007;81(24):13325-34.
15. Huang KJ, Yang YC, Lin YS, Huang JH, Liu HS, Yeh TM, et al. The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. J Immunol. 2006;176(5):2825-32.
16. Kliks SC, Nisalak A, Brandt WE, Wahl L, Burke DS. Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. Am J Trop Med Hyg. 1989;40(4):444-51.
17. Hase T, Summers PL, Eckels KH. Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. Arch Virol. 1989;104(1-2):129-43.
18. Espina LM, Valero NJ, Hernandez JM, Mosquera JA. Increased apoptosis and expression of tumor necrosis factor-alpha caused by infection of cultured human monocytes with dengue virus. Am J Trop Med Hyg. 2003;68(1):48-53.
19. Mosquera JA, Hernandez JP, Valero N, Espina LM, Anez GJ. Ultrastructural studies on

dengue virus type 2 infection of cultured human monocytes. Virol J. 2005;2:26.
20. Honda S, Saito M, Dimaano EM, Morales PA, Alonzo MT, Suarez LA, et al. Increased phagocytosis of platelets from patients with secondary Dengue virus infection by human macrophages. Am J Trop Med Hyg. 2009;80(5):841-5.
21. Marianneau P, Steffan AM, Royer C, Drouet MT, Jaeck D, Kirn A, et al. Infection of primary cultures of human Kupffer cells by Dengue virus: no viral progeny synthesis, but cytokine production is evident. J Virol. 1999;73(6):5201-6.
22. Onlamoon N, Noisakran S, Hsiao HM, Duncan A, Villinger F, Ansari AA, et al. Dengue virus-induced hemorrhage in a nonhuman primate model. Blood. 2010;115(9):1823-34.
23. Hober D, Nguyen TL, Shen L, Ha DQ, Huong VT, Benyoucef S, et al. Tumor necrosis factor alpha levels in plasma and whole-blood culture in dengue-infected patients: relationship between virus detection and pre-existing specific antibodies. J Med Virol. 1998;54(3):210-8.
24. Kuhn RJ, Zhang W, Rossmann MG, Pletnev SV, Corver J, Lenches E, et al. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell. 2002;108(5):717-25.
25. Gubler DJ, Kuno G, editors. Dengue And Dengue Hemorrhagic Fever. 1st edition. Wallingford, UK: CABI; 1997.
26. Chambers TJ, Hahn CS, Galler R, Rice CM. Flavivirus genome organization, expression, and replication. Annu Rev Microbiol. 1990;44:649-88.
27. Stadler K, Allison SL, Schalich J, Heinz FX. Proteolytic activation of tick-borne encephalitis virus by furin. J Virol. 1997;71(11):8475-81.
28. Henchal EA, Putnak JR. The dengue viruses. Clin Microbiol Rev. 1990;3(4):376-96.
29. Modis Y, Ogata S, Clements D, Harrison SC. Structure of the dengue virus envelope protein after membrane fusion. Nature. 2004;427(6972):313-9.
30. Nayak V, Dessau M, Kucera K, Anthony K, Ledizet M, Modis Y. Crystal structure of dengue virus type 1 envelope protein in the postfusion conformation and its implications for membrane fusion. J Virol. 2009;83(9):4338-44.
31. Plevka P, Battisti AJ, Junjhon J, Winkler DC, Holdaway HA, Keelapang P, et al. Maturation of flaviviruses starts from one or more icosahedrally independent nucleation centres. EMBO Rep. 2011;12(6):602-6.
32. Mondotte JA, Lozach PY, Amara A, Gamarnik AV. Essential role of dengue virus envelope protein N glycosylation at asparagine-67 during viral propagation. J Virol. 2007;81(13):7136-48.
33. Diamond MS, Edgil D, Roberts TG, Lu B, Harris E. Infection of human cells by dengue virus is modulated by different cell types and viral strains. J Virol. 2000;74(17):7814-23.
34. Lok SM, Kostyuchenko V, Nybakken GE, Holdaway HA, Battisti AJ, Sukupolvi-Petty S, et al. Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins. Nat Struct Mol Biol. 2008;15(3):312-7.
35. Hase T, Summers PL, Eckels KH, Baze WB. An electron and immunoelectron microscopic study of dengue-2 virus infection of cultured mosquito cells: maturation events. Arch Virol. 1987;92(3-4):273-91.
36. Welsch S, Miller S, Romero-Brey I, Merz A, Bleck CK, Walther P, et al. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. Cell Host Microbe. 2009;5(4):365-75.
37. Cardiff RD, Russ SB, Brandt WE, Russell PK. Cytological localization of Dengue-2 antigens: an immunological study with ultrastructural correlation. Infect Immunol. 1973;7(5):809-16.
38. Barth OM. Replication of dengue viruses in mosquito cell cultures—a model from ultrastructural observations. Mem Inst Oswaldo Cruz. 1992;87(4):565-74.
39. Barth OM, Cortes LM, Lampe E, Farias Filho JC. Ultrastructural aspects of virus replication in one fatal case and several other isolates from a dengue type 2 outbreak in Rio de Janeiro. Mem Inst Oswaldo Cruz. 1994;89(1):21-4.
40. Smith TJ, Brandt WE, Swanson JL, McCown JM, Buescher EL. Physical and biological properties of dengue-2 virus and associated antigens. J Virol. 1970;5(4):524-32.
41. Sriurairatna S, Bhamarapravati N, Phalavadhtana O. Dengue virus infection of mice: morphology and morphogenesis of dengue type-2 virus in suckling mouse neurones. Infect Immunol. 1973;8(6):1017-28.
42. Stevens TM, Schlesinger RW. Studies on the nature of dengue viruses. I. Correlation of particle density, infectivity, and RNA content of type 2 virus. Virology. 1965;27(1):103-12.
43. Brandt WE, Cardiff RD, Russell PK. Dengue virions and antigens in brain and serum of infected mice. J Virol. 1970;6(4):500-6.
44. Brandt WE, Chiewslip D, Harris DL, Russell PK. Partial purification and characterization of a dengue virus soluble complement-fixing antigen. J Immunol. 1970;105(6):1565-8.
45. Sabin AB, Schlesinger RW. Production of Immunity to Dengue with Virus Modified by Propagation in Mice. Science. 1945;101(2634):640-2.
46. Kimura R, Hotta S. Studies on dengue fever (VI). On the inoculation of dengue virus into mice (in Japanese). Nippom Igaku. 1944;(3379):629-33.
47. Junjhon J, Lausumpao M, Supasa S, Noisakran S, Songjaeng A, Saraithong P, et al. Differential modulation of prM cleavage, extracellular particle distribution, and virus infectivity by conserved residues at nonfurin

consensus positions of the dengue virus pr-M junction. J Virol. 2008;82(21):10776-91.
48. Offit PA. Vaccinated: One Man's Quest to Defeat the World's Deadliest Diseases. 1st edition. New York: HarperCollins; 2007.
49. Boriskin Yu S, Kaptsova TI, Lotte VD, Skvortsova OI, Orvell C. Laboratory markers for over-attenuation of mumps vaccine virus. Vaccine. 1988;6(6):483-8.
50. Pattnaik P, Babu JP, Verma SK, Tak V, Rao PV. Bacterially expressed and refolded envelope protein (domain III) of dengue virus type-4 binds heparan sulfate. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;846(1-2):184-94.
51. Talarico LB, Damonte EB. Interference in dengue virus adsorption and uncoating by carrageenans. Virology. 2007;363(2):473-85.
52. Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ, et al. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat Med. 1997;3(8):866-71.
53. Anez G, Men R, Eckels KH, Lai CJ. Passage of dengue virus type 4 vaccine candidates in fetal rhesus lung cells selects heparin-sensitive variants that result in loss of infectivity and immunogenicity in rhesus macaques. J Virol. 2009;83(20):10384-94.
54. Perera R, Khaliq M, Kuhn RJ. Closing the door on flaviviruses: entry as a target for antiviral drug design. Antiviral Res. 2008;80(1):11-22.
55. Wahala WM, Kraus AA, Haymore LB, Accavitti-Loper MA, de Silva AM. Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody. Virology. 2009;392(1):103-13.
56. Dejnirattisai W, Jumnainsong A, Onsirisakul N, Fitton P, Vasanawathana S, Limpitikul W, et al. Cross-reacting antibodies enhance dengue virus infection in humans. Science. 2010;328(5979):745-8.
57. Noisakran S, Onlamoon N, Hsiao HM, Clark KB, Villinger F, Ansari AA, et al. Infection of Bone Marrow Cells by Dengue Virus In Vivo. Exp Hematol. 2011 Dec 19. [Epub ahead of print]
58. Noisakran S, Gibbons RV, Songprakhon P, Jairungsri A, Ajariyakhajorn C, Nisalak A, et al. Detection of dengue virus in platelets isolated from dengue patients. Southeast Asian J Trop Med Public Health. 2009;40(2):253-62.
59. Gubler DJ. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev. 1998;11(3):480-96.
60. Sabin AB. Electron microscope studies on human dengue serum. Amer J Publ Health. 1951;41:1215.
61. Noisakran S, Chokephaibulkit K, Songprakhon P, Onlamoon N, Hsiao HM, Villinger F, et al. A re-evaluation of the mechanisms leading to dengue hemorrhagic fever. Ann NY Acad Sci. 2009;1171(Suppl 1):E24-35.
62. Lin YS, Yeh TM, Lin CF, Wan SW, Chuang YC, Hsu TK, et al. Molecular mimicry between virus and host and its implications for dengue disease pathogenesis. Exp Biol Med. 2011;236(5):515-23.
63. Aliotta JM, Pereira M, Johnson KW, de Paz N, Dooner MS, Puente N, et al. Microvesicle entry into marrow cells mediates tissue-specific changes in mRNA by direct delivery of mRNA and induction of transcription. Exp Hematol. 2010;38(3):233-45.
64. Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. Exosomes/microvesicles as a mechanism of cell-to-cell communication. Kidney Int. 2010;78(9):838-48.
65. Pilzer D, Gasser O, Moskovich O, Schifferli JA, Fishelson Z. Emission of membrane vesicles: roles in complement resistance, immunity and cancer. Springer Semin Immunopathol. 2005;27(3):375-87.
66. Jin M, Drwal G, Bourgeois T, Saltz J, Wu HM. Distinct proteome features of plasma microparticles. Proteomics. 2005;5(7):1940-52.
67. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6):654-9.
68. Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ. Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia. 2006;20(9):1487-95.
69. Horstman LL, Ahn YS. Platelet microparticles: a wide-angle perspective. Crit Rev Oncol Hematol. 1999;30(2):111-42.
70. Hugel B, Martinez MC, Kunzelmann C, Freyssinet JM. Membrane microparticles: two sides of the coin. Physiology (Bethesda). 2005;20:22-7.
71. Camussi G, Deregibus MC, Bruno S, Grange C, Fonsato V, Tetta C. Exosome/microvesicle-mediated epigenetic reprogramming of cells. Am J Cancer Res. 2011;1(1):98-110.
72. Lynch SF, Ludlam CA. Plasma microparticles and vascular disorders. Br J Haematol. 2007;137(1):36-48.
73. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol. 2009;9(8):581-93.
74. Holmgren L, Szeles A, Rajnavolgyi E, Folkman J, Klein G, Ernberg I, et al. Horizontal transfer of DNA by the uptake of apoptotic bodies. Blood. 1999;93(11):3956-63.
75. Masciopinto F, Giovani C, Campagnoli S, Galli-Stampino L, Colombatto P, Brunetto M, et al. Association of hepatitis C virus envelope proteins with exosomes. Eur J Immunol. 2004;34(10):2834-42.
76. Esser MT, Graham DR, Coren LV, Trubey CM, Bess JW Jr., Arthur LO, et al. Differential incorporation of CD45, CD80 (B7-1), CD86 (B7-2), and major histocompatibility complex class I and II molecules into human

immunodeficiency virus type 1 virions and microvesicles: implications for viral pathogenesis and immune regulation. J Virol. 2001;75(13):6173-82.
77. Soler N, Marguet E, Verbavatz JM, Forterre P. Virus-like vesicles and extracellular DNA produced by hyperthermophilic archaea of the order Thermococcales. Res Microbiol. 2008;159(5):390-9.
78. Meckes DG Jr., Shair KH, Marquitz AR, Kung CP, Edwards RH, Raab-Traub N. Human tumor virus utilizes exosomes for intercellular communication. Proc Natl Acad Sci USA. 2010;107(47):20370-5.
79. Schaar V, Nordstrom T, Morgelin M, Riesbeck K. Moraxella catarrhalis outer membrane vesicles carry beta-lactamase and promote survival of Streptococcus pneumoniae and Haemophilus influenzae by inactivating amoxicillin. Antimicrob Agents Chemother. 2011;55(8):3845-53.
80. Calistri A, Salata C, Parolin C, Palu G. Role of multivesicular bodies and their components in the egress of enveloped RNA viruses. Rev Med Virol. 2009;19(1):31-45.
81. Quesenberry PJ, Dooner MS, Aliotta JM. Stem cell plasticity revisited: the continuum marrow model and phenotypic changes mediated by microvesicles. Exp Hematol. 2010;38(7):581-92.
82. Denzer K, van Eijk M, Kleijmeer MJ, Jakobson E, de Groot C, Geuze HJ. Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface. J Immunol. 2000;165(3):1259-65.
83. Leroyer AS, Rautou PE, Silvestre JS, Castier Y, Leseche G, Devue C, et al. CD40 ligand+ microparticles from human atherosclerotic plaques stimulate endothelial proliferation and angiogenesis a potential mechanism for intraplaque neovascularization. J Am Coll Cardiol. 2008;52(16):1302-11.
84. Leroyer AS, Tedgui A, Boulanger CM. Role of microparticles in atherothrombosis. J Intern Med. 2008;263(5):528-37.
85. Gyorgy B, Szabo TG, Pasztoi M, Pal Z, Misjak P, Aradi B, et al Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci. 2011;68(16):2667-88.
86. Su EL, Snape MD. A combination recombinant protein and outer membrane vesicle vaccine against serogroup B meningococcal disease. Expert Rev Vaccines. 2011;10(5):575-88.
87. Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, et al. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. Science. 2010;327(5965):580-3.
88. Distler JH, Pisetsky DS, Huber LC, Kalden JR, Gay S, Distler O. Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases. Arthritis Rheum. 2005;52(11):3337-48.
89. Wolf P. The nature and significance of platelet products in human plasma. Br J Haematol. 1967;13(3):269-88.
90. Cecchetti L, Tolley ND, Michetti N, Bury L, Weyrich AS, Gresele P. Megakaryocytes differentially sort mRNAs for matrix metalloproteinases and their inhibitors into platelets: a mechanism for regulating synthetic events. Blood. 2011;118(7):1903-11.
91. Sadallah S, Eken C, Martin PJ, Schifferli JA. Microparticles (ectosomes) shed by stored human platelets downregulate macrophages and modify the development of dendritic cells. J Immunol. 2011;186(11):6543-52.
92. Schwertz H, Koster S, Kahr WH, Michetti N, Kraemer BF, Weitz DA, et al. Anucleate platelets generate progeny. Blood. 2010;115(18):3801-9.
93. Halstead SB, O'Rourke EJ, Allison AC. Dengue viruses and mononuclear phagocytes. II. Identity of blood and tissue leukocytes supporting in vitro infection. J Exp Med. 1977;146(1):218-29.
94. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, et al. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis. 2000;181(1):2-9.
95. Zucker JR. Changing patterns of autochthonous malaria transmission in the United States: a review of recent outbreaks. Emerg Infect Dis. 1996;2(1):37-43.
96. Janzen HG, Rhodes AJ, Doane FW. Chikungunya virus in salivary glands of Aedes aegypti (L.): an electron microscope study. Can J Microbiol. 1970;16(7):581-6.
97. Phillips A, Mossel E, Sanchez-Vargas I, Foy B, Olson K. Alphavirus transducing system: tools for visualizing infection in mosquito vectors. J Vis Exp. 2010;(45).
98. WHO. Dengue Vaccine Development: The role of the WHO South-East Asia Regional Office. 2010.
99. Murphy BR, Whitehead SS. Immune response to dengue virus and prospects for a vaccine. Annu Rev Immunol. 2011;29:587-619.
100. Thomas SJ. The necessity and quandaries of dengue vaccine development. J Infect Dis. 2011;203(3):299-303.
101. Webster DP, Farrar J, Rowland-Jones S. Progress towards a dengue vaccine. Lancet Infect Dis. 2009;9(11):678-87.
102. Thomas SJ, Endy TP. Vaccines for the prevention of dengue: Development update. Hum Vaccin. 2011;7(6):674-84.
103. Qiao M, Shaw D, Forrat R, Wartel-Tram A, Lang J. Priming effect of dengue and yellow fever vaccination on the immunogenicity, infectivity, and safety of a tetravalent dengue vaccine in humans. Am J Trop Med Hyg. 2011;85(4):724-31.
104. Thomas SJ, Endy TP. Critical issues in dengue vaccine development. Curr Opin Infect Dis. 2011;24(5):442-50.
105. Moxon ER, Siegrist CA. The next decade of vaccines: societal and scientific challenges. Lancet. 2011;378(9788):348-59.
106. Thomas SJ, Nisalak A, Anderson KB, Libraty DH, Kalayanarooj S, Vaughn DW, et al.

Dengue plaque reduction neutralization test (PRNT) in primary and secondary dengue virus infections: How alterations in assay conditions impact performance. Am J Trop Med Hyg. 2009;81(5):825-33.
107. Gusman MG. Dengue vaccines: new developments. Drugs Future. 2011;36:45-62.
108. Simmons JS, St. John JH, Reynolds FHK. Experimental studies of dengue. Philippine J Sci. 1931;44:1-251.
109. Rudnick A, Lim TW, editors. Dengue Fever Studies in Malaysia. Kuala Lumpur: The Institute for Medical Research; 1986.
110. Sabin AB. Research on dengue during World War II. Am J Trop Med Hyg. 1952;1:30-50.
111. Hotta S. Experimental studies on dengue I isolation, identification and modification of the virus. J Infect Dis. 1952;90:1-9.
112. Mota J, Rico-Hesse R. Dengue virus tropism in humanized mice recapitulates human dengue fever. PLoS One. 2011;6(6):e20762.
113. Mota J, Rico-Hesse R. Humanized mice show clinical signs of dengue fever according to infecting virus genotype. J Virol. 2009;83(17):8638-45.
114. Kuruvilla JG, Troyer RM, Devi S, Akkina R. Dengue virus infection and immune response in humanized RAG2(-/-)gamma(c)(-/-) (RAG-hu) mice. Virology. 2007;369(1):143-52.
115. Halstead SB, Shotwell H, Casals J. Studies on the pathogenesis of dengue infection in monkeys. I. Clinical laboratory responses to primary infection. J Infect Dis. 1973;128(1):7-14.
116. Guy B, Barban V, Mantel N, Aguirre M, Gulia S, Pontvianne J, et al. Evaluation of interferences between dengue vaccine serotypes in a monkey model. Am J Trop Med Hyg. 2009;80(2):302-11.
117. Simasathien S, Thomas SJ, Watanaveeradej V, Nisalak A, Barberousse C, Innis BL, et al. Safety and immunogenicity of a tetravalent live-attenuated dengue vaccine in flavivirus naive children. Am J Trop Med Hyg. 2008;78(3):426-33.
118. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, et al. Dengue in the early febrile phase: viremia and antibody responses. J Infect Dis. 1997;176(2):322-30.
119. Perng GC, Lei HY, Lin YS, Chokephaibulkit K. Dengue Vaccines: Challenge and Confrontation. World Journal of Vaccines. 2011;1(4):109-30.
120. Bierman HR, Nelson ER. Hematodepressive Virus Diseases of Thailand. Ann Intern Med. 1965;62:867-84.
121. Tassaneetrithep B, Burgess TH, Granelli-Piperno A, Trumpfheller C, Finke J, Sun W, et al. DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. J Exp Med. 2003;197(7):823-9.
122. Boonnak K, Slike BM, Burgess TH, Mason RM, Wu SJ, Sun P, et al. Role of dendritic cells in antibody-dependent enhancement of dengue virus infection. J Virol. 2008;82(8):3939-51.
123. Sun P, Fernandez S, Marovich MA, Palmer DR, Celluzzi CM, Boonnak K, et al. Functional characterization of ex vivo blood myeloid and plasmacytoid dendritic cells after infection with dengue virus. Virology. 2009;383(2):207-15.
124. Wang QY, Bushell S, Qing M, Xu HY, Bonavia A, Nunes S, et al. Inhibition of dengue virus through suppression of host pyrimidine biosynthesis. J Virol. 2011;85(13):6548-56.
125. Xie X, Wang QY, Xu HY, Qing M, Kramer L, Yuan Z, et al. Inhibition of dengue virus by targeting viral NS4B protein. J Virol. 2011;85(21):11183-95.
126. Schul W, Liu W, Xu HY, Flamand M, Vasudevan SG. A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. J Infect Dis. 2007;195(5):665-74.
127. Nakao S, Lai CJ, Young NS. Dengue virus, a flavivirus, propagates in human bone marrow progenitors and hematopoietic cell lines. Blood. 1989;74(4):1235-40.
128. Tsai J-J, Liu L-T, Chang K, Wang S-H, Hsiao H-M, Clark KB, et al. The importance of hematopoietic progenitor cells in dengue. Therapeutic Advances in Hematology. 2011 Sept 5 [Epub ahead of print].
129. Halstead SB. Dengue. Lancet. 2007;370(9599):1644-52.
130. Marchette NJ, Halstead SB, Falkler WA Jr., Stenhouse A, Nash D. Studies on the pathogenesis of dengue infection in monkeys. 3. Sequential distribution of virus in primary and heterologous infections. J Infect Dis. 1973;128(1):23-30.
131. Green S, Rothman A. Immunopathological mechanisms in dengue and dengue hemorrhagic fever. Curr Opin Infect Dis. 2006;19(5):429-36.
132. Chen JP, Cosgriff TM. Hemorrhagic fever virus-induced changes in hemostasis and vascular biology. Blood Coagul Fibrinolysis. 2000;11(5):461-83.
133. Lei HY, Yeh TM, Liu HS, Lin YS, Chen SH, Liu CC. Immunopathogenesis of dengue virus infection. J Biomed Sci. 2001;8(5):377-88.
134. Guzman MG, Kouri G, Bravo J, Valdes L, Vazquez S, Halstead SB. Effect of age on outcome of secondary dengue 2 infections. Int J Infect Dis. 2002;6(2):118-24.
135. Iyngkaran N, Yadav M, Sinniah M. Augmented inflammatory cytokines in primary dengue infection progressing to shock. Singapore Med J. 1995;36(2):218-21.
136. Green S, Vaughn DW, Kalayanarooj S, Nimmannitya S, Suntayakorn S, Nisalak A, et al. Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity. J Infect Dis. 1999;179(4):755-62.
137. Gagnon SJ, Mori M, Kurane I, Green S, Vaughn DW, Kalayanarooj S, et al. Cytokine gene expression and protein production in peripheral blood mononuclear cells of children

with acute dengue virus infections. J Med Virol. 2002;67(1):41-6.
138. Liu CC, Huang KJ, Lin YS, Yeh TM, Liu HS, Lei HY. Transient CD4/CD8 ratio inversion and aberrant immune activation during dengue virus infection. J Med Virol. 2002;68(2):241-52.
139. Suharti C, van Gorp EC, Dolmans WM, Setiati TE, Hack CE, Djokomoeljanto R, et al. Cytokine patterns during dengue shock syndrome. Eur Cytokine Netw. 2003;14(3):172-7.
140. Tsai JJ, Jen YH, Chang JS, Hsiao HM, Noisakran S, Perng GC. Frequency alterations in key innate immune cell components in the peripheral blood of dengue patients detected by FACS analysis. J Innate Immun. 2011;3(5):530-40.
141. Diacon AH, Pym A, Grobusch M, Patientia R, Rustomjee R, Page-Shipp L, et al. The diarylquinoline TMC207 for multidrug-resistant tuberculosis. N Engl J Med. 2009;360(23):2397-405.
142. Deeks SG. Protease inhibitors as immunomodulatory drugs for HIV infection. Clin Pharmacol Ther. 2007;82(3):248-50.