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Dengue vaccines: recent developments, ongoing challenges and current candidates

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Summary

Dengue is among the most prevalent and important arbovirus diseases of humans. In order to effectively control this rapidly spreading disease, control of the vector mosquito and a safe and efficacious vaccine are critical. Despite considerable efforts, the development of a successful vaccine has remained elusive. Multiple factors have complicated the creation of a successful vaccine, not the least of which are the complex, immune-mediated responses against four antigenically distinct serotypes necessitating a tetravalent vaccine providing long lasting protective immunity. Despite the multiple impediments, there are currently many promising vaccine candidates in pre-clinical and clinical development. Here we review the recent advances in dengue virus vaccine development and briefly discuss the challenges associated with the use of these vaccines as a public health tool.

Keywords

Dengue virus; vaccine; dengue fever; dengue hemorrhagic fever; antibody-dependent enhancement; immunopathogenesis

Background

Dengue virus (DENV) causes a common self-limited illness, dengue fever (DF), and a less common syndrome manifested variably by organ failure, hemorrhage, capillary leakage, shock and death (severe dengue, DHF/DSS). DENV is a globally important human pathogen. Roughly two-fifths of the world's population lives in areas that are at risk for DENV transmission [1–3]. According to the World Health Organization (WHO), "In 2012, dengue ranks as the most important mosquito-borne viral disease with an epidemic potential in the world. There has been a 30-fold increase in the global incidence of dengue during the past 50 years, and its human and economic costs are staggering." [4]. A large proportion of those affected by DENV infection are children, and it is a leading cause of serious illness and death in some Asian and Latin American countries [5]. An estimated 294 million

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asymptomatic infections and 96 million symptomatic DENV infections of any severity occurred in 2010 [6]. An enormous economic burden is associated with DENV infections [1,7,8]. While asymptomatic infections do not result in a direct burden on the health care system, these infected individuals contribute to DENV transmission.

Despite concerted efforts for the past four decades, there is no currently licensed vaccine available to protect against DENV infection. This review will focus on recent advances in the development of vaccines against DENV and the impediments facing these vaccines.

Dengue virus

The dengue viruses are a group of mosquito-borne flaviviruses composed of four antigenically distinct serotypes (DENV1–4) that co-circulate throughout Southeast Asia, Africa, and the Americas [9,10]. The *Flavivirus* genus includes many human pathogens of clinical significance, including mosquito-borne viruses such as yellow fever virus (YFV), Japanese encephalitis virus (JEV), and West Nile virus (WNV), as well as tick-borne viruses such as tick-borne encephalitis virus (TBEV). The flaviviruses are single-stranded, positive-sense RNA viruses with a genome of ~11 kilobases. The genome consists of a single open reading frame flanked by 5' and 3' untranslated regions (UTR). The open reading frame encodes a polyprotein which is processed by both virus-encoded and host proteases resulting in three structural proteins (capsid (C), membrane (M), and envelope (E)) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Figure 1 A & B) [11].

The E protein is the major surface protein of the flaviviruses and has been extensively characterized. The crystal structures for the E protein of TBEV, WNV, and DENV have been determined [12–18]. The E protein is composed of three distinct domains (DI, DII, and DIII). DIII is exposed on the virion surface and has been implicated in binding to the host cell surface receptor [19]. Furthermore, DIII is known to contain multiple type-specific neutralizing epitopes [20]. Because of its importance as an immunogen, the E protein is a major component of DENV vaccines.

Clinical Disease

Infection with any of the four serotypes of DENV may range from asymptomatic infection, classical DF or more severe clinical manifestations, including dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) [21]. Previous WHO classifications included DF and DHF/DSS, the latter being the sole representative of severe dengue [22]. DF is characterized as a febrile illness with two or more of the following: myalgia, arthralgia, headache, retro-orbital pain, rash, leukopenia, and/or hemorrhagic manifestations, plus supportive serology or occurrence at the same location and time as other confirmed cases of DF. The case definition of DHF requires the presence of fever or history of acute fever, hemorrhagic tendencies, thrombocytopenia (platelet count 100,000 cells per mm^3 or less), and evidence of plasma leakage due to increased vascular permeability, and DSS is characterized by rapid, weak pulse with narrowing of the pulse pressure or hypotension [22]. Recently, the WHO developed a new classification system that includes DF with or without warning signs for the development of severe dengue and severe dengue itself [21]. "Dengue without warning signs" is characterized by fever plus any two of the following: nausea/vomiting, rash, aches/pains, leukopenia, and/or a positive tourniquet test. Based on the WHO assessment/treatment algorithm, patients meeting the criteria for "dengue without warning signs" may safely be managed at home. "Dengue with warning signs" includes the above definition plus one or more of the following: abdominal pain/tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy/restlessness, liver enlargement >2 cm, and/or laboratory testing showing increased hematocrit (>20% above patient's baseline value or

age-specific population value if the patient's baseline is not available) with concurrent rapid decrease in platelet count. Patients meeting the "dengue with warning signs" criteria should be referred for in-hospital care. The final category, severe dengue, requires emergency treatment and includes patients with any of the following: severe plasma leakage leading to shock or fluid accumulation with respiratory distress, severe bleeding as evaluated by the clinician, or severe organ involvement [21].

Recent evaluation of the traditional and revised WHO dengue classification schemes identified increased sensitivity of the revised classification system for detecting severe disease which may be useful for clinicians in determining treatment [23]. However, because the classifications of "severe dengue" and "dengue with warning signs" are quite broad and are less precise, they may fail to adequately categorize the pathophysiology of immunopathogenesis in vaccine trials. For this reason, comparison of studies that used traditional versus revised classification systems must be evaluated with caution.

Impediments to Vaccine Development

Multiple difficulties have hindered the development of a successful DENV vaccine. These include: (1) the epidemiology of the four DENV serotypes, (2) the complex and incompletely understood immunoprotective and/or immunopathogenic responses following natural infection or vaccination, and (3) a lack of validated animal models of disease. These impediments are discussed below.

Epidemiology

DENV is arguably the most significant human arboviral disease with an excess of 2.5 billion people at risk world-wide [5,9]. Although accurately determining the number of cases is complicated by underreporting and lack of surveillance in some regions, an estimated 50–100 million DENV infections occur annually resulting in 2.3 million cases of DF reported to the WHO in 2010 [5]. Furthermore, DENV is responsible for approximately 500,000 cases of severe disease requiring hospitalization each year [9]. DENV is endemic in most tropical and subtropical regions of the world with the highest burden of disease in Asia and the Americas; however, DENV transmission has also been reported in Africa and the Eastern Mediterranean region [24,25]. Additionally, due to changes in climate, travel, and urbanization, DENV continues to spread to new areas and intensify in endemic areas.

A major factor in the re-emergence of DENV is the re-infestation of many parts of the world with *Aedes aegypti* mosquitos. DENV is transmitted primarily by the urban mosquito *Ae. aegypti*, and, less efficiently, by *Ae. albopictus* mosquitos. The geographic range of both mosquito vectors continues to expand as a consequence of environmental factors and decreased mosquito control [9,26]. Additionally, endemicity of DENV has increased as a result of rapid urbanization in regions of Asia and Latin America that provide both increased population density and an abundance of vector breeding sites [9,26].

Although there was global distribution of DENV throughout the tropics prior to World War II, most regions had only one or two serotypes co-circulating and only sporadic epidemics were reported [27]. Following the transport of troops and war materials associated with World War II, DENV spread dramatically and many countries in Asia became hyperendemic (co-circulation of all four serotypes) [27]. Severe DENV infection subsequently became a leading cause of hospitalization and death among children in Southeast Asia and the majority of deaths from severe dengue continue to occur there [25,27]. Although DENV infection has typically been considered a disease of childhood in most Asian countries, there are increasing reports of infection in adolescents and adults in Asia and the Americas [28,29].

Mosquito eradication programs in the Americas initially led to a dramatic decrease in mosquito-borne diseases. However, following the completion of these programs in the 1970s, there was re-infestation of the Americas with *Ae. aegypti* resulting in rapid re-expansion of all four DENV serotypes on this continent [30,31]. As DENV continues to spread through Latin America, changes in the epidemiological profile (increased severe disease in children and young adults) have been noted in some regions [29,31]. Expansion of DENV into new geographic regions, re-introduction of DENV serotypes following significant time lapses, and travel to endemic areas have all led to increased infections in adults [5,9].

Although all four DENV serotypes have been reported in Africa, there is no reliable prevalence or incidence data, likely due to poor surveillance/detection systems and the heavy burden of other infectious diseases like malaria [24]. Additionally, there is evidence that African ancestry is protective against DHF/DSS and DENV may therefore be circulating in Africa without causing epidemics or even sporadic cases of DHF/DSS [32,33].

DENV exhibits a complex epidemiology with co-circulation of multiple serotypes in a given geographic location and an unpredictable predominance of different serotypes at different time points. Although mathematical and epidemiological models have been developed in an attempt to predict DENV epidemics, there is no single model that takes into account the many factors that can affect DENV transmission. These include vector specific factors (including vector density, biting rates, and vector competence), as well as host factors (including population density, immune status, and travel), both of which are affected by climactic, environmental, and socioeconomic variables [34,35].

The unpredictable nature of DENV transmission and epidemics makes the design of vaccine trials difficult. Because no reliable way of accurately predicting the circulation of a specific serotype currently exists, determining protective efficacy for all serotypes requires multiple trial sites over long time periods with large numbers of volunteers. An additional concern is how genetic variation within a given serotype may impact vaccine trials. Each DENV serotype can be sub-divided into multiple genotypes. For example, the ability of sera from patients infected with DENV3 to neutralize DENV3 viruses of distinct genotypes is not equivalent *in vitro* [36]. However, this has not been confirmed in animal models, and recent studies in non-human primates indicate that antibodies generated in response to a tetravalent live-attenuated DENV vaccine are able to neutralize a broad range of DENV isolates from multiple genotypes [36]. The effect of such genetic differences on vaccine efficacy in humans remains to be determined. To summarize, co-circulation of multiple DENV serotypes in the same geographic location for many years generates the possibility of complex immune-mediated cross-protection as well as immune-mediated enhancement that must be considered in clinical trial design [9].

Immunology

Despite extensive studies, our understanding of the immune responses to DENV infection is incomplete. Following primary infection, there is initially serotype cross-reactive protective immunity; however, this wanes after a few months leaving the host susceptible to infection with heterologous serotypes [37]. Human challenge studies performed in a small number of individuals by Sabin in 1944 and published in 1952 are, to our knowledge, the only experimental evidence of the presence and duration of serotype cross-protective immunity in humans [37]. Studies of the natural history of dengue in endemic areas support these findings as primary infections are typically followed by several months of broad protection [20]. Although secondary DENV infection is a significant risk factor for severe disease, only a small percentage of individuals experiencing secondary DENV infection are severely ill,

suggesting cross-protection in some persons may persist for a considerable period of time [38].

Epidemiologically, heterologous secondary infection in children and adults is associated with higher risk of severe disease (DHF/DSS) [39]. Additionally, infants with waning maternal antibody are more susceptible to severe disease during primary infection, which may be due to the presence of heterologous maternal antibodies mimicking secondary infection [40]. It has been hypothesized that immune responses to heterologous infection play a direct role in the pathogenesis of DHF/DSS (summarized in Figure 2).

Viral factors such as peak viremia and serotype are also associated with severe disease [41]. Thus, a combination of host immunological mechanisms and viral factors likely contribute to the plasma leakage associated with severe disease. The precise interplay between host and viral factors remains to be elucidated.

The potential threat of disease enhancement due to incomplete DENV immunity necessitates the development of a tetravalent DENV vaccine (TDV) that induces long-lasting protective immunity against all four serotypes simultaneously. Furthermore, unpredictable circulation of the four serotypes also necessitates a TDV. This protective response should ideally be achieved over a short time period to prevent the theoretical possibility of disease potentiation after an infective mosquito bite during the series of primary vaccinations.

Multiple non-mutually exclusive mechanisms for enhanced disease severity due to heterologous immune responses have been proposed including antibody dependent enhancement (ADE), cell-mediated immunity (CMI), *e.g.*, the generation of cross-reactive T cells, as well as complement activation [40,42–48]. Despite extensive efforts, there is no conclusive *in vivo* data implicating the causative role of these responses in the severe manifestations (plasma leakage) of DENV infection.

Antibody Dependent Enhancement (ADE)—One proposed mechanism for the increased disease severity after a second heterotypic DENV infection is that of ADE. In addition to serotype-specific, protective antibody responses, DENV infection also induces non-neutralizing or weakly neutralizing, cross-reactive antibodies. Binding of these antibodies to a heterotypic DENV facilitates viral entrance into Fc-receptor bearing cells such as monocytes and macrophages [20,40,44]. Evidence to support a role for ADE in severe DENV infection includes observations that 1) secondary DENV infection leads to higher serum viremia and greater risk of severe disease [41], 2) the risk of severe disease is increased following primary infection in infants with waning maternal antibody titers [40], and 3) temporal associations between *in vitro* DENV enhancing activity of plasma and the epidemiology of age-related severe disease exist [49,50]. More recently, it has been suggested that in addition to increasing infection by augmenting the number of susceptible cells, Fc receptor signaling inhibits antiviral responses within the infected cells, thus contributing to increased viral replication associated with ADE [48,51]. It is important to note, however, that in a study of secondary DENV2 and 3 infection, no correlation between pre-illness enhancing activity and disease severity or viremia was identified [52]. Additionally, a recent study found no association between ADE activity and disease severity during primary DENV3 infection in infants [53]. Caution must be used in extrapolating *in vitro* mechanisms of immunopathogenesis to *in vivo* scenarios.

Cell-mediated immunity (CMI)—Another proposed mechanism of immune-mediated pathogenesis is that of a “cytokine storm” during which high levels of pro-inflammatory cytokines are released in response to heterologous DENV infection contributing to vascular leakage and severe dengue disease [42,43,54]. Increased activation and production of

cytokines/chemokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α by DENV-specific T cells have been detected [43]. Additionally, higher levels of inflammatory cytokines have been identified in sera of patients with DHF compared to DF [42,43,49,54–57]. A leading hypothesis for the "cytokine storm" is that cross-reactive memory T cells drive pathogenic responses to heterologous secondary DENV infection [54]. Prospective studies have identified T cell response patterns that may be associated with differential risk for severe disease [43,57]. Furthermore, an association between the magnitude of CD8+ T cell response during acute infection and disease severity has been identified [58]. It has also been suggested that secondary responses (to heterologous virus) may be dominated by cross-reacting memory T cells induced by primary infection that may be of lower affinity for the heterologous antigen, thus resulting in skewed cytokine responses and/or inefficient cytotoxicity [58]. Although skewed T cell responses may contribute to immunopathogenesis, the anti-viral effects of cytokines are also critical for protection. In a human challenge model of DENV infection, IFN-γ production was associated with protection from illness indicating that the quality of the T cell response may play a role in protection versus pathogenesis [56].

Complement activation—Complement activation is also thought to potentially play a role in the pathogenesis of severe DENV infection. Patients with DSS were observed to have accelerated consumption of complement, and high plasma levels of terminal complement, C5b-9, was associated with increased disease severity [46]. Anaphylotoxins, such as C5b-9, have been shown to promote plasma leakage and could thus play an important role in the pathophysiology of severe dengue [59].

Correlates of protection—There are currently no established correlates of protection against DENV infection. As already discussed above, the E protein is a major immunogen and the site of many serotype-specific neutralizing antibody epitopes. There have been extensive studies characterizing mouse monoclonal antibody epitopes of DENV, particularly to the E protein and more specifically to DIII (reviewed in [20]). The applicability of these epitopes in human immune responses remains unclear. More recent studies of human antibodies induced by natural DENV infection or by human monoclonal antibodies generated by Epstein Barr virus (EBV)-transformed B cells from DENV immune volunteers, indicate that neutralizing human antibodies recognizing epitopes on E DIII represent only a minority of the antibody spectrum. Some neutralizing human monoclonal antibodies bind to the E protein on the surface of the virion but not to recombinant E protein. These findings implicate a role for structural epitopes that may rely on the E protein configuration on the virion surface in serotype-specific neutralization [20,60,61]. In addition to serotype-specific antibodies, cross-reactive non-neutralizing or weakly neutralizing antibodies are also a significant part of the response against DENV infection. Much work remains to be done before the most relevant epitopes which correlate with protection from exposure to wild-type DENV are identified. These findings will have a great impact in accelerating vaccine development,

In general, neutralizing antibody titers are believed to be associated with protection; however, defined levels of neutralizing antibodies (*e.g.*, 50% plaque reduction, $PRNT_{50}$) have not been correlated with protection for any serotype [62]. Guidelines to standardize PRNT assays for DENV vaccine trials have been developed by the WHO [63]. A considerable drawback to traditional PRNT is the labor and time intensive nature of this assay. Microneutralization tests may provide a high throughput alternative, but remain to be validated and standardized for use in clinical DENV vaccine trials [64]. Additionally, recent studies have indicated differences in neutralization when Fcγ -receptor-transformed cells are used to perform PRNT. The use of Fcγ -receptor-transformed cells may better reflect the *in*

vivo situation in which enhancement of infectivity and neutralization presumably occurs via antibody-virus interactions with Fcγ-receptor-bearing cells [64,65].

The relationship between neutralizing antibodies and protection is not straightforward. In a recent human challenge study, 10 volunteers immunized with a live-attenuated TDV and 4 DENV naïve volunteers were subsequently challenged with either DENV1 or 3. Five of 5 vaccinated volunteers were protected against DENV1 challenge despite the fact that one of the protected volunteers had no detectable neutralizing antibody against DENV1. Three of 5 volunteers challenged with DENV3 developed clinical disease and 2 of the unprotected volunteers had low but detectable anti-DENV3 neutralizing antibodies (reciprocal $PRNT_{50}$ titers 19 and 16). All four DENV naïve volunteers developed clinical dengue [66]. Furthermore, protective titers may vary among serotypes as evidenced by recent results from a phase 2b trial in which protection against DENV2 was not obtained despite neutralizing antibody titers that were higher than those against other serotypes [67].

Identification of a reliable correlate of protection, either mechanistic or non-mechanistic [68], would allow measurement of a vaccine candidate's probable efficacy without the need to establish protective efficacy against naturally acquired infection with each of the four serotypes. Thus the sample size required for clinical trials could be reduced and/or the requirement for formal protective efficacy trials might even be eliminated. Furthermore, a well-defined correlate of protection would foster non-inferiority trials once a DENV vaccine is licensed. This would dramatically reduce trial costs and effort needed to develop additional DENV vaccines.

Animal models

Because DENV is primarily a human pathogen, identification of an animal system that accurately models the human immune response to DENV and disease pathogenesis has been elusive. Historical studies investigating intra-abdominal and/or intracerebral inoculation of infant mice, hamsters, newborn and adult guinea pigs, cotton rats, rabbits, and rhesus monkeys with serum or whole blood with proven infectivity for humans did not identify clinical signs of infection [37]. Currently available animal models possess significant limitations which necessitate careful selection of the appropriate model for specific studies and cautious interpretation of results. Models that have been used for DENV vaccine development in recent years include mice, non-human primates (NHP), rabbits, and miniature swine [69–71], as described below.

Mouse models—Immunocompetent mice do not generally develop clinical signs of DENV infection reflective of human disease (fever, rash, and thrombocytopenia). Following inoculation with DENV, most immunocompetent mice demonstrate low levels of viral replication and may present with neurotropic manifestations such as paralysis following intraperitoneal or intravenous inoculation, which is not typical of human disease [70,72,73]. While not typical, it is important to note that neurologic manifestations including coma, convulsions, and spastic paraparesis have been associated with human DENV infections [74]. There are also limited reports of histopathological liver injury and/or clinical signs of hemorrhage more consistent with human disease following inoculation of immunocompetent mice with DENV [75,76]. Additionally, adaptation of DENV strains to the mouse model by serial passage has been reported to result in clinical and histopathologic manifestations of liver injury, hemorrhage, and death [72,77]. Finally, despite the lack of clinical manifestations of disease or viremia, BALB/c mice have been shown to develop T cell responses against DENV [78]. In preclinical vaccine development, immunocompetent mice have been used to demonstrate the generation of neutralizing antibodies and in some cases protection against intracerebral challenge [79].

Due to the limitations of immunocompetent mice as a model for clinical manifestations of DENV infection, multiple immunodeficient mouse models have been developed. Severe combined immunodeficiency (SCID) mice, lacking humoral and cellular immune responses, engrafted with human tumor cells have been shown to support DENV replication [70,80–82]. Infection in these models is, however, predominantly of the engrafted tumor limiting their utility for studies of virus tropism and host immunity to DENV infection.

IFN receptor deficient mice (AG129) have also been utilized as models for DENV infection. In this model, all four DENV serotypes are capable of replication; however, in order to recapitulate clinical signs of severe DENV2 infection, virus adaptation is required [71,83,84]. This model has been extensively studied and reviewed in [84]. In AG129 mice, DENV infects the same target cells as in humans and can induce both T cell and antibody responses [71,84]; however, the lack of IFN receptors limit the spectrum of immune responses that can be studied using this model. AG129 mice are used extensively in vaccine studies to document attenuation of vaccine candidates and generation of neutralizing antibodies/protection from challenge.

Multiple strains of humanized mice have been utilized for studies of DENV pathogenesis; however, to our knowledge, there are no reports of DENV vaccine studies in humanized mice [71,85,86].

NHP models—Although NHP can be naturally infected by sylvatic strains of DENV in the wild, NHP do not develop overt clinical disease following DENV infection [71]. Viremia can be detected, albeit at lower levels than in humans, and protection from infection is shown by reduction or absence of viremia following subsequent DENV infection [70]. NHP also develop neutralizing antibody responses following DENV infection [71]. Partial protection in NHP results in reduced or undetectable viremia but the presence of an anamnestic antibody response, reflecting viral replication. Complete protection in NHP is demonstrated by both the absence of viremia and lack of an anamnestic antibody response [87,88]. Increased viremia following administration of non-neutralizing antibodies has been documented in NHP providing a partial model for ADE [89]. However, the lack of severe disease manifestations in NHP limits the utility of this model for vaccine studies and the study of effector immune responses. In spite of these limitations, prevention of viremia following DENV challenge in the NHP model has been used to estimate vaccine efficacy [70,71,90–97].

Other models—Although mice and NHP are currently the primary animal model systems being utilized for vaccine studies, other animal systems including rabbits and miniature swine are also under investigation [69,71].

Vaccine Candidates in Pre-clinical and Clinical Development

Initial efforts to develop a DENV vaccine began in the 1920s and involved attenuating DENV in blood with ox-bile or grinding DENV infected *Ae. aegypti* mosquitoes in a salt solution and chemically pure phenol and formalin [98,99]. In 1952, Sabin and Schlesinger developed an attenuated strain of DENV1 by serial passage in mouse brain. This vaccine was protective in 16 volunteers subjected to the bite of infected mosquitoes [37]. The ability to cultivate DENV in tissue culture (1970s) and more recently, recombinant DNA technology have contributed to considerable advances in DENV vaccine development.

Below we discuss many of the DENV vaccine candidates in advanced pre-clinical and clinical trials. It is important to note that for a multitude of reasons there are limitations in the comparison of vaccine candidates from one trial to another. Some of these limitations

include: differences in trial design (*e.g.* volunteer numbers, prior flavivirus experience, volunteer age, inclusion of a placebo or active control group, length of follow-up, geographic location and intensity of DENV transmission during the trial), use of different immune assays, and non-standardized reporting of adverse events and serological results. Furthermore, for vaccines in phase 1 and 2 trials, only comparisons of the degree of reactogenicity and immunogenicity can be performed. Currently, the recently completed Phase 2b trial of CYD TDV is the only clinical trial reporting vaccine efficacy data.

Live attenuated vaccines (LAV)

Safe and effective LAV vaccines for flaviviruses, including YFV and JEV, are licensed [12,100,101] indicating that LAV may also be effective for DENV. LAV candidates are the furthest along in the development pipeline to include multiple phase 1–2 trials of monovalent and tetravalent formulations and most recently, completion of a phase 2b trial of a tetravalent DENV vaccine candidate (Table 1) [67,91,95,102–108]. These vaccine candidates utilize distinct methods of attenuation including directed mutagenesis (Figure 1C) and serial passage.

Sanofi Pasteur CYD TDV—Currently, CYD TDV (chimeric YF17D- DENV tetravalent dengue vaccine) produced by Sanofi Pasteur is most advanced in clinical testing having recently completed a phase 2b clinical trial [67]. Currently, phase 3 trials of CYD TDV in more than 30,000 volunteers in 10 countries are underway with an expected completion in 2016 (Table 1: NCT01374516 and NCT01373281) [67]. This vaccine is composed of chimeric viruses which encode the prM and E regions of DENV1–4 in the backbone of YFV vaccine strain 17D [109,110]. The preclinical and early clinical development of CYD TDV has been described in detail by Guy *et al* [95]. Early clinical studies of CYD TDV indicated that CYD TDV was safe and immunogenic in children and adults, including those with previous DENV or YFV exposure [104,111,112]. Furthermore, balanced neutralizing antibody responses against all four serotypes were demonstrated in healthy adults with seroconversion rates of 70–100% after 2 doses and 100% seroconversion after 3 doses of CYD TDV [113]. In a phase 2b trial in Thailand, CYD TDV showed a disappointing overall vaccine efficacy of only 30.2%; however, differences in serotype-specific vaccine efficacy were identified [67]. Vaccine efficacy, greater than 28 days after the third immunization, was 55.6%, 9.2%, 75.3%, and 100% for DENV1, 2, 3, and 4 respectively. Neutralizing antibody responses 28 days after the third immunization were 146, 310, 405, and 155 (reciprocal geometric mean $PRNT_{50}$ antibody titers) against DENV1, 2, 3, and 4 respectively. Despite $PRNT_{50}$ antibody titers against DENV2 that were comparable to the other serotypes, no significant protection against DENV2 was observed. However, the vaccine was safe and non-reactogenic, consistent with profiles reported in prior studies [95,102,104,112,114]. The reasons for the unexpected failure against DENV2 are under intensive investigation.

Walter Reed Army Institute of Research (WRAIR) and GlaxoSmithKline (GSK) TDV—A TDV prepared from four monovalent DENV vaccines (DENV1–4) attenuated by serial passage in primary dog kidney (PDK) cells was tested in infants, children, and adults in Phase 1 and 2 clinical trials (Table 1) [105–107,115]. The vaccine was found to be safe and immunogenic in all age groups (12 months – 45 years). In adults, tetravalent neutralizing antibody responses ranged from 36–63% following 2 doses of 3 different tetravalent formulations [106]. In contrast to adults, seroconversion was seen in 100% of flavivirus naïve children following 2 doses (n=7) [105]. In the above trials, lyophilized monovalent vaccines were combined into a tetravalent preparation at the time of administration. Most recently, a phase 2 trial in adults to evaluate a re-derived tetravalent vaccine prepared from vaccine strains with three additional passages in fetal rhesus lung

(FRhL) cells was completed [116]. The monovalent bulk vaccines were formulated with a carbohydrate stabilizer rather than human serum albumin and the final product was lyophilized as a tetravalent vaccine [116]. The primary sign encountered in the vaccine groups compared to placebo was a non-specific, self-limited rash, which occurred in 13.6–31.8% of vaccine recipients depending on formulation. The febrile response was similar in vaccinees and placebo-inoculated volunteers. The tetravalent antibody response following the second dose in DENV naïve individuals was 60–66.7% and a third dose (5–12 months after the second dose) did not increase tetravalent antibody responses [116].

DENVax—A LAV DENV vaccine candidate, produced by the Center for Disease Control (CDC) and Inviragen and based on the DENV2 attenuated vaccine PDK-53, is also under investigation. DENV2 PDK-53 was attenuated by serial passage in PDK cells and has been studied in clinical trials as a monovalent vaccine candidate as well as in multivalent formulations (reviewed in [97]). A chimeric DENV2 PDK-53 based tetravalent vaccine (DENVax) has been developed by substituting the prM and E genes of DENV2 PDK-53 with those of wild-type DENV1, 3, or 4 [117]. Seed lots of DENVax have been produced under good manufacturing practices (GMP) by Shantha, Biotechnics, Ltd., Hyderabad, India and tested in AG129 mice [118]. All formulations tested were immunogenic and elicited neutralizing antibodies against all four serotypes; however, neutralizing antibody titers were not equivalent suggesting differences in immunogenicity and/or virus interference among the vaccine strains [118]. A phase 2 trial in children and adults is currently underway (Table 1) (http://www.clinicaltrials.gov).

Tetra-Vax-DV—Many LAV DENV vaccine candidates, developed by the National Institute of Allergy and Infectious Diseases (NIAID) have been produced using directed mutagenesis. Specifically, deletion of 30 nucleotides from the 3’ UTR of DENV1 and DENV4 resulted in satisfactory attenuation. In contrast, DENV2 and 3 were not sufficiently attenuated using the same methodology [119–121]. Several of these monovalent DENV vaccine candidates have been tested in phase 1 and 2 clinical trials to identify those best suited for combination into TDV formulations [91].

Following encouraging results in preclinical studies, an attenuated DENV4 candidate (rDEN4Δ30) was tested in clinical trials and found to be safe and immunogenic. The most common adverse events were an asymptomatic rash (>50% of vaccinees) and neutropenia (~20% of vaccinees). Additionally, elevated serum alanine aminotransferase (ALT) levels were identified in several volunteers [90,93]. Liver enzyme elevation appeared to be related to dose of virus administration [93]. Seroconversion (≥ 4 fold rise in neutralizing antibody titers) against DENV4 was 95–100%. Further mutations were also introduced into the rDEN4Δ30 vaccine candidate in attempts to reduce reactogenicity/hepatotoxicity while maintaining immunogenicity (rDEN4Δ30–4995 with an amino acid substitution at residue 158 of NS3 and rDEN4Δ30–200,201 with mutations at amino acid positions 200 and 201 in the NS5 polymerase gene) [94,108]. rDEN1Δ30 was also tested in clinical trials and found to be safe and immunogenic. This vaccine candidate was also associated with asymptomatic rash in 24–40% of vaccinees and neutropenia in 42–48% [92,103].

Due to insufficient attenuation of rDEN2Δ30 in preclinical trials, a chimeric DENV2/DENV4 vaccine candidate, rDEN2/4Δ30(ME), was produced and tested in volunteers [122]. It was found to be safe and immunogenic with similar adverse events as rDEN4Δ30 and rDEN1Δ30 including asymptomatic rash (45%), mild neutropenia (35%), and elevated ALT (15%) (n=20). There was a 100% seroconversion rate against DENV2 after a single dose [122].

Results of a phase 1 clinical trial of tetravalent formulations (Tetra-Vax-DV) comparing 4 admixtures of rDEN1Δ30, rDEN2/4Δ30(ME), rDEN3–3'D4Δ30 or rDEN3Δ30/31–7164, and rDEN4Δ30 or rDEN4Δ30–200,201 were recently published [123]. Tri- or tetravalent neutralizing antibody responses after a single dose were obtained in 75–90% of volunteers depending on the formulation. Additionally, the responses were generally well balanced among serotypes with the geometric mean $PRNT_{60}$ titers ranging from 32 to 154. Asymptomatic rash in 64.2% of vaccinees was the only adverse event that was significantly higher in vaccine recipients.

Interestingly, the incidence of vaccine viremia and seroconversion in Black volunteers was significantly reduced compared to non-Black volunteers. This may be in part due to protective genetic factors associated with Black race which have been previously described [32,33]. The formulation which induced the most balanced antibody responses (less than two-fold difference between the mean $PRNT_{60}$ to each serotype) induced a trivalent response in 90% of volunteers, but of these, only 45% had a complete tetravalent response. This formulation, admixture TV003 (rDEN3Δ30/31, rDEN4Δ30, rDEN1Δ30, and rDEN2/4Δ30), has been proposed for further evaluation and a phase 2 trial by the Butantan Institute in Brazil (Table 1) (http://www.clinicaltrials.gov) [123].

Purified inactivated vaccines- WRAIR/GSK

Inactivated whole virus vaccines against DENV have been shown to induce neutralizing antibody responses in animal models [124,125]. There are disadvantages and advantages of inactivated and subunit DENV vaccines compared to LAV candidates. Inactivated vaccines are frequently more expensive to manufacture than LAV. Inability to replicate within the host may be disadvantageous from the perspective of duration of immune responses, and more doses may be required. However, inactivated vaccines do not pose a risk of reversion to virulence or transmission via infected mosquitos. Furthermore, inactivated vaccines are generally safe in immunosuppressed individuals, such as those with HIV infection. Additionally, there is no interference among the four serotypes for infectivity, although immunological interference remains a potential problem. Previously, it was not possible to grow DENV to high enough titer in cell culture to make inactivated whole virus vaccines practical; however, methods have been optimized to allow replication of adequate titers of DENV in cell culture for purification and inactivation [124,125]. Preclinical studies of a formalin inactivated DENV2 vaccine candidate showed protection in a mouse model. In NHP, the vaccine induced neutralizing antibody, but only partial protection was obtained with 11 of 17 vaccinated NHP developing detectable viremia following challenge, although the duration of viremia was shorter than in control animals [124]. Currently, two phase 1 trials are underway evaluating a purified inactivated DENV1 vaccine candidate and a tetravalent purified inactivated DENV vaccine candidate (Table 1) (http://www.clinicaltrials.gov).

Recombinant subunit vaccines- Hawaii Biotech/Merck

Recombinant subunit vaccine candidates, primarily based on the E protein of DENV, are in various stages of pre-clinical and early clinical development. Advantages of recombinant subunit vaccines are similar to those for purified inactivated vaccines, as discussed above. Certainly, the possibility of an accelerated administration schedule makes these attractive candidates [126]. In attempts to identify a reliable, affordable source for production of large quantities of recombinant proteins in their native conformation, multiple expression systems for the production of recombinant E protein have been employed. Among these are *E. coli*, *Saccharomyces cerevisciae*, *Pichia pastoris*, Chinese Hamster Ovary DHFR system, Vaccinia expression in mammalian cells, Baculovirus in Sf9 or High five cells, and stably transformed *Drosophila* S2 cells (reviewed in [126]). Stably transformed *Drosophila* S2 cells

are capable of expressing high levels of truncated DENV E protein (80E) in its native conformation [127,128]. Tetravalent formulations of 80E subunits (with or without recombinant NS1) were evaluated as potential vaccine candidates in mice and NHP [127]. A balanced immune response was obtained and protection was demonstrated in a small NHP trial when tetravalent formulations were given with ISCOMATRIX® adjuvant [127]. Currently a Phase 1 trial of a tetravalent subunit vaccine candidate (V180, previously referred to as 80E) is sponsored by Merck using either alhydrogel or ISOCOMATRIX® as adjuvants compared to vaccine without adjuvant (Table 1) (http://www.clinicaltrials.gov).

Virus vectored and viral like particle (VLP)-based vaccines

Through the use of VLP and virus vectors, it is possible to more closely approximate the natural presentation of DENV surface antigens. Several vaccine candidates utilizing these platforms are currently under investigation.

VLP-based vaccines utilize carriers which self-assemble into VLP and display the antigen of interest on the surface of the particle. This has proven to be a successful strategy with the licensure of the human papillomavirus (HPV) vaccine [129]. The hepatitis B virus (HBV) core antigen is a carrier capable of forming VLP and expressing surface antigens from multiple pathogens [130]. DENV E DIII was detected on the surface of VLP using recombinant HBV core antigen, but it only induced modest DENV antibody responses in mice [131,132].

Adenoviral vectors have been extensively studied as carriers for vaccine antigens [133]. Two tetravalent DENV vaccine candidates utilizing adenovirus type 5 (AdV5) vectors have been described. The first includes AdV5 encoding tetravalent DENV E DIII, which induces both humoral and cellular immune responses against all four serotypes in mice [134]. Additionally, immune responses against DENV were not decreased by pre-existing anti-AdV5 immunity. In fact, pre-existing AdV5 antibodies appeared to facilitate entry of the vector into human monocytic cells [134]. The second vector is a combination of two bivalent AdV5/DENV constructs [135]. Immunized rhesus monkeys were protected from viremia when challenged with DENV1 or 3 and had a significant reduction in the number of viremic days when challenged with DENV2 or 4 [135].

Recently, a Venezuelan equine encephalitis (VEE) virus replicon was employed as a vector for a TDV candidate [136]. Initial studies in NHP indicate that a VEE replicon expressing a C terminally truncated segment of 85% of the E protein of DENV3 showed robust neutralizing antibody responses following 2 doses administered 6 weeks apart [136]. Additionally, a tetravalent formulation induced neutralizing antibody responses against all 4 serotypes in 16 of 16 rhesus macaques. Following challenge with wild-type virus, there was no breakthrough viremia from DENV3 or 4, and significantly reduced viremia from DENV1 and 2. Furthermore, a significant boost in neutralizing antibody responses followed challenge, indicating this platform might be useful in a prime-boost strategy with LAV as the booster.

DNA vaccines

DNA vaccines have been in development since the early 1990s. They consist of a selected gene sequence cloned into a plasmid backbone.

The plasmid is injected allowing DNA to be taken up by antigen-presenting cells, which then express the plasmid-encoded genes to generate the target antigen(s) [137]. DNA vaccines against DENV have focused on the E protein as the target antigen. Preclinical studies in mice identified a tetravalent vaccine candidate composed of four different plasmids encoding the prM/E genes of each DENV serotype [137]. Very recent evaluation

of DNA DENV vaccine candidates encoding DENV2 prM/E and NS1 show some protective efficacy in a mouse model [138]. In a phase 1 trial, a DENV1 monovalent DNA vaccine was found to be safe at low and high doses with the most commonly solicited signs/symptoms being local mild pain/tenderness, mild swelling at the vaccination site, muscle pain, and fatigue [139]. The vaccine was found to be moderately immunogenic with 5 of 12 (41.6%) high dose vaccine recipients producing low but measurable neutralizing antibody titers ranging from 1:11 to 1:135 $PRNT_{50}$ following 2–3 doses. Additionally, CMI was measured by IFN-γ ELISPOT and was positive in 83.3% of high dose recipients (n=12) and 50% of low dose recipients (n=8). Currently, a phase 1 clinical trial of a TDV is underway (Table 1) (http://www.clinicaltrials.gov).

Heterologous prime-boost strategies

Given the importance of rapidly inducing tetravalent protective immunity to avoid potential enhancement after natural infection and to rapidly protect during epidemic outbreaks, it is critical to develop strategies that improve immunogenicity and/or reduce the time-frame for DENV immunization. Heterologous prime-boost strategies, in which initial immunization with one type of vaccine is followed by a boost with a second, heterologous type of vaccine (*e.g.*, DNA vaccine followed by LAV), have shown improved immunogenicity for multiple pathogens (human immunodeficiency virus, tuberculosis, rabies, influenza, malaria) (reviewed in [140]). However, various factors such as order of administration of the prime-boost and the specific antigens used may be of critical importance in efficacy necessitating careful evaluation of these strategies in clinical trials [140,141].

Studies in NHP using non-replicating tetravalent DENV vaccines (either purified inactivated virus or DNA) to prime followed by a boost with tetravalent LAV DENV vaccines demonstrated protection from viremia for all serotypes. This strategy allowed a shorter interval between the first and second immunization while maintaining protective efficacy against all four serotypes [142]. Preliminary investigations in mice indicate that different patterns of alternating prime-boost with DNA vaccines against the E protein, adenovirus and/or vaccinia vectored vaccines (expressing the E protein) can elicit differential responses [141]. Additionally, pre-clinical studies are underway investigating the use of DNA vaccine priming as a means to “redirect” the immune response to avoid enhancing antibody responses [143].

Challenges to Vaccine Implementation

Generation and licensure of a safe and effective tetravalent DENV vaccine is only the first hurdle toward full implementation in a public health setting. Vaccine cost and stability, long-term surveillance for potential adverse events, and compatibility with current vaccine schedules will challenge implementation of the vaccine in the field.

Cost is a major determinant of the successful use of any vaccine, particularly in developing countries. A survey of policymakers in Cambodia, Indonesia, Philippines, and Vietnam indicated there would be public demand for a DENV vaccine and suggested that governments would likely be willing to pay $0.50-$1.00 per dose [144]. It is likely that the private market would tolerate vaccine costs 10–30 times higher. Given constraints in the availability of a “cold chain” in some endemic regions, a stable vaccine that does not require refrigeration would be ideal. Also, due to the potential for rare adverse events following any vaccine, DENV included, long-term post-licensure follow up will be necessary. In the case of DENV, with the theoretical possibility of potentiation of severe disease following natural infection, phase 4 studies will be critical [145]. This will add cost to vaccine development. A recent economic analysis for cost of production of a LAV TDV in Brazil suggested that with an estimated annual vaccine production of 15–60 million doses per year, production costs

would likely be $0.20-$0.65 per dose in 10-dose vials and $0.70-$1.75 for single-dose vials [146]. Even if a successful DENV vaccine is licensed, considerable efforts to improve the vaccine profile may still be required. Developing second-generation DENV vaccines with shorter intervals between immunizations or fewer required doses, more cost-effective manufacturing, and improved immunogenicity would be preferred. It is generally estimated that >80% protective efficacy will be necessary for public consideration and to confer "herd-immunity" by blocking mosquito transmission [144]. Therefore, this has been the target for vaccine developers. Consideration of the intended use (routine immunization of young children, "catch-up" campaigns, management of epidemics, or use for travellers) will affect the desired qualities of the vaccine. For example, a vaccine for travellers to endemic countries should induce rapid effective protection, but it might be costly ([147]). By contrast, a less costly vaccine intended for routine immunization in endemic areas should be compatible with existing Expanded Program on Immunization (EPI) and national dosing schedules. Finally, the possibility of immune interference or enhanced reactogenicity engendered by any DENV vaccine when given in combination with other flavivirus vaccines (i.e. yellow fever or Japanese encephalitis) must be investigated.

Strategies for the Introduction of DENV Vaccines in Resource Limited Countries

Current vaccine efforts are targeted primarily toward development of a pediatric DENV vaccine for use in endemic areas. Resource limitations in DENV endemic regions engender obstacles impeding implementation of DENV vaccines as an effective public health tool. Such countries must decide which vaccines to prioritize for public immunization programs based on consideration of the disease burden, public perception of disease impact, the safety and efficacy of the vaccine, it's cost and the economic resources available [148]. Additional considerations for most vaccines include an adequate cold chain and long term vaccine availability which must be thoroughly assessed prior to vaccine implementation. Disease surveillance following vaccine implementation is important for the introduction of all vaccines, but it is critical for DENV vaccines because of the theoretical risk of disease enhancement following incomplete or ineffective immunization.

Expert Commentary

DENV continues to be a rapidly spreading threat to global health. Re-infestation of many regions of the world with *Aedes* mosquito vectors, global travel, massive urbanization and climate change ensure this threat will only increase unless dramatic measures are taken. DENV control will require a multi-faceted approach including vector control and an effective vaccine.

Further studies of immune responses to natural DENV infection and to vaccine candidates are necessary to improve our understanding of the contributions of enhanced complement system activation, ADE, and CMI responses, which are likely to play significant roles in the immunopathogenesis of severe dengue. Racial differences in response to vaccines could affect the use of vaccines in geographic areas with distinct racial profiles. Additionally, identification of immune correlates of protection would greatly facilitate future development and licensure of second-generation DENV vaccines.

Given the limitations of current animal models to recapitulate human disease, consideration of human challenge studies for the evaluation of vaccine efficacy should be considered. Recently, volunteers previously immunized with multiple formulations of TDV [106,115] submitted to challenge with near wild-type DENV1 or 3 [66]. This study highlights the utility and safety of human challenge studies in the evaluation of DENV vaccine candidates.

There has been much debate regarding the use of human challenge studies in this context due to the potential for severe disease; however, if performed properly, these studies can be safe and provide vital information [149].

Recent advances in development of DENV vaccines are extremely promising; however, there is considerable need for further financial and political commitment to ensure the successful licensure and implementation of a DENV vaccine. Continued development of alternative vaccine platforms that may result in second-generation vaccines is also crucial. The theoretical risk of immune-mediated enhancement as a result of immunization necessitates stringent post-licensure evaluation and long-term follow-up of vaccines. The follow-up required will far exceed that possible in a clinical trial and will require surveillance systems for severe dengue in coordination with a database of vaccine recipients to enable tracking of serious adverse events remote from the immunization. It is, however, encouraging that in the completed trials summarized above (some with follow-up out to 2 years) there has been no evidence of disease enhancement or unanticipated severe or serious adverse events.

The unexpected failure of CYD TDV to protect against DENV2 in a recent phase 2b trial leaves many unanswered questions. Particularly since neutralizing antibody responses against DENV2 were within the range of what would be considered protective. It is unclear what role the heterologous nature of the non-structural proteins from YF17D may play. Although the E protein is considered to be an extremely important immunogen for the generation of neutralizing antibodies, CMI responses against NS proteins are also a major component of the immune response against DENV. It is also unclear whether or not viral genetics may affect vaccine efficacy. While NHP studies seem to indicate that CYD TDV is broadly cross-reactive against many genotypes of DENV2, it is still possible that the circulating DENV2 strain during the trial was sufficiently different to reduce vaccine efficacy. The failure of CYD TDV to protect against DENV2 presents a troublesome set-back to DENV vaccine development; however, ongoing phase 3 trials with CYD TDV may help answer some of these questions. This failure also highlights the importance of continuing to support the development of additional vaccine candidates, and to expand basic research focused on understanding mechanisms of immunopathogenesis and protection against dengue and other flaviviruses.

Five-year View

With several LAV candidates currently in advanced clinical trials, the next 5–10 years are likely to see the licensure of a tetravalent DENV vaccine. Continued efforts to confirm the vaccine profile and identify immunological correlates of protection will be critical. Certainly, the public health ramifications of continued explosion of DENV in the tropics and semi-tropics provide an enormous incentive to address these issues.

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List of abbreviations

ADE	antibody dependent enhancement
AdV5	adenovirus type 5

Ae.	*Aedes*
C	capsid
CMI	cell mediated immunity
CYD	chimeric YF17D-DENV
DIII	domain III
DENV	dengue virus
DF	dengue fever
DHF	dengue hemorrhagic fever
DSS	dengue shock syndrome
E	envelope
EBV	Epstein Barr Virus
EPI	Expanded Program on Immunization
FRhL	fetal rhesus lung
GSK	GlaxoSmithKline
HBV	hepatitis B virus
HPV	human papilloma virus
IFN	interferon
JEV	Japanese encephalitis virus
M	membrane
NHP	non-human primate
NIAID	National Institute of Allergy and Infectious Diseases
NS	non-structural
PDK	primary dog kidney
prM	pre-membrane
PRNT	plaque reduction neutralization test
TBEV	tick-borne encephalitis virus
TDV	tetravalent dengue vaccine
TNF	tumor necrosis factor
UTR	untranslated region
VEE	Venezuelan equine encephalitis
VLP	virus-like particle
WHO	World Health Organization
WNV	West Nile virus
WRAIR	Walter Reed Army Institute of Research
YFV	yellow fever virus

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Key Issues

- Dengue virus is the most significant human arboviral pathogen and poses a global public health threat which will continue without intervention.
- The phenomenon of immune-mediated enhancement and unpredictable circulation of DENV serotypes necessitate a tetravalent dengue virus vaccine that will provide long-lasting protection against all four dengue virus serotypes.
- Complex and unpredictable epidemiology as well as regional epidemiological differences will require testing of vaccine candidates in multiple geographic areas.
- Assessment of genetic differences between circulating DENV strains and vaccine strains may provide insight into vaccine efficacy and should be incorporated into clinical trials.
- The live-attenuated tetravalent vaccine candidate based on the backbone of yellow fever 17D vaccine strain is currently the most advanced having recently completed a phase 2b clinical trial demonstrating only 30% overall efficacy. Phase 3 trials are underway with an estimated completion in 2016.
- Extensive post-licensure surveillance for adverse events will be necessary to detect delayed occurrence of more frequent or severe vaccine-associated dengue, including immune-mediated enhancement.
- Identification of immune correlates of protection requires continued field trials to associate immune responses with protection against disease and will facilitate the development of second-generation vaccines.
- Development of second-generation dengue vaccine candidates should continue in pursuit of affordable, immunogenic vaccines that can be administered in one or two doses over a short period of time and provide >80% protective efficacy.
- Considerable political and financial support will be required for wide-spread adoption of a dengue vaccine, once licensed.

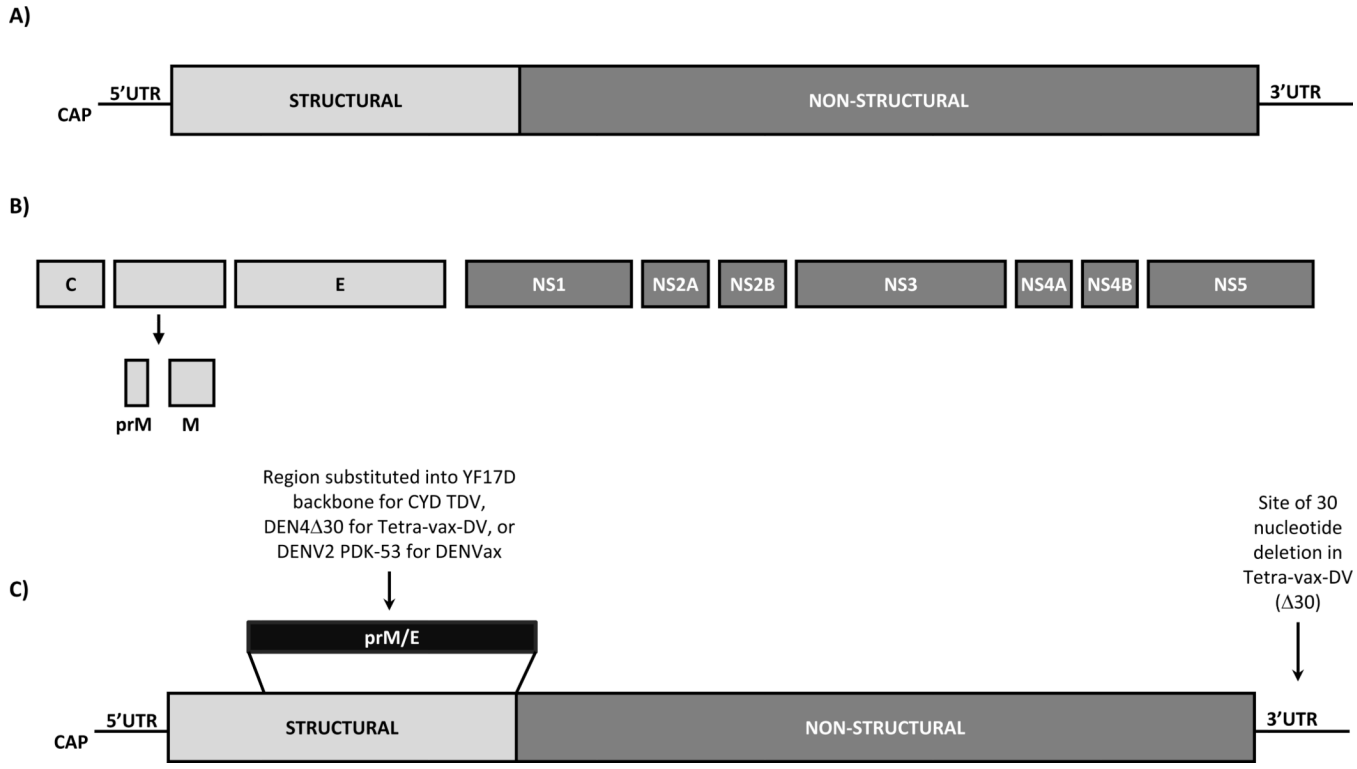

Figure 1.

Organization of the flavivirus genome. A) RNA genome including the 5' and 3' untranslated regions (UTR) and coding regions for the structural and non-structural proteins. B) Polyprotein processed by both virus-encoded and host proteases resulting in three structural proteins (capsid (C), membrane (M), and envelope (E)) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). C) Diagram indicating the alterations to the DENV genome for generation of several live attenuated virus vaccine candidates. See text for a brief description of the vaccines mentioned in this panel.

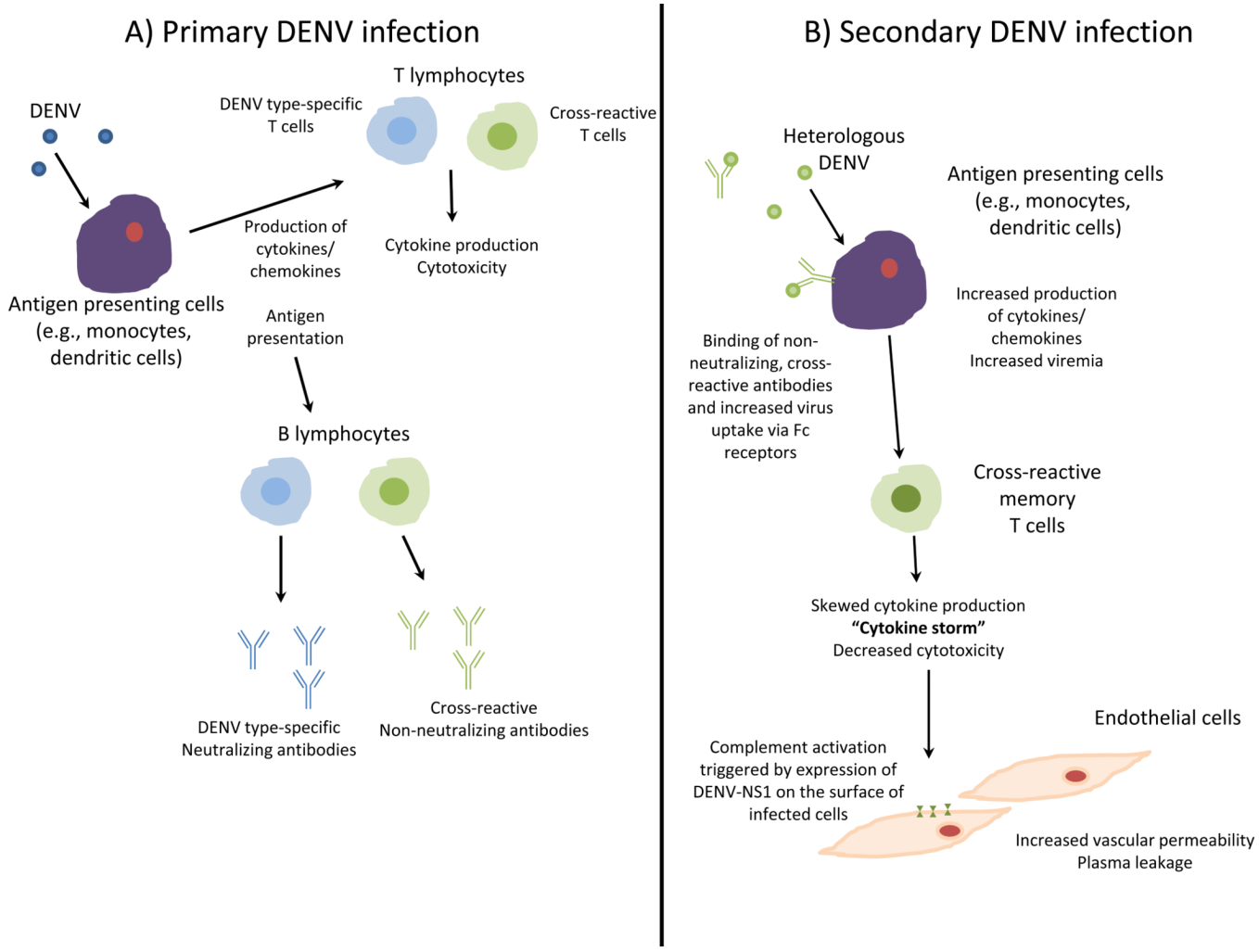

Figure 2.

Potential mechanisms for immune mediated enhancement of DENV infection. A) Immune responses following primary DENV infection include infection of monocytes and other antigen presenting cells which produce cytokines/chemokines and present antigen to lymphocytes. Type-specific and cross-reactive B and T lymphocytes are activated. B lymphocytes produce serotype-specific, neutralizing antibodies as well as cross-reactive, weakly or non-neutralizing antibodies. B) Following secondary heterologous infection, uptake of DENV is facilitated through binding of cross-reactive, non-neutralizing antibodies from a previous infection (or maternally derived antibodies in the case of primary infection in infants). Additionally, there is activation of cross-reactive T cells that produce skewed cytokine responses and demonstrate decreased cytotoxicity for DENV infected cells. Massive quantities of pro-inflammatory cytokines ("cytokine storm") result in increased vascular permeability and plasma leakage. Furthermore, expression or release of DENV-NS1 by infected cells may mediate complement activation resulting in increased vascular permeability.

Table 1

Dengue virus vaccine candidates in clinical trials. Clinical trials to evaluate DENV vaccine candidates registered on ClinicalTrials.gov are shown. We have not listed earlier studies that do not have a ClinicalTrials.gov identifier. Primary completion dates for the studies shown range from 2005-present. The primary completion/estimated primary completion date refers to the final data collection date for the primary outcome measure and is listed as provided by ClinicalTrials.gov. The enrollment is also that provided by ClinicalTrials.gov.

Vaccine type	Phase	Valent	Title	NCT Number	Vaccine name	Sponsor/Collaborators	Age Groups	Enrollment	Primary Completion/Estimated primary completion	References
Live Attenuated Virus	Phase 1	monovalent DENV1	Safety of and Immune Response to a Dengue Virus Vaccine (rDEN1delta30) in Healthy Adults	NCT00089908	rDEN1delta30	NIAID/Johns Hopkins Bloomberg School of Public Health	Adult	28	November 2005	Durbin 2006 [92]
		monovalent DENV2	Safety of and Immune Response to a Dengue Virus Vaccine (rDEN2/4delta30[ME]) in Healthy Adults	NCT00094705	rDEN2/4delta30(ME)	NIAID/Johns Hopkins Bloomberg School of Public Health	Adult	28	April 2006	
		monovalent DENV1 or DENV2	Safety of and Immune Response to Two Different Dengue Virus Vaccines in Individuals Previously Immunized Against Dengue Virus	NCT00458120	rDEN1delta30 or rDEN2/4delta30(ME)	NIAID/Johns Hopkins Bloomberg School of Public Health	Adult	36	August 2008	
		monovalent DENV3	Safety of and Immune Response to a Dengue Virus Vaccine (rDEN3/4delta30[ME]) in Healthy Adults	NCT00375726	rDEN3/4delta30(ME)	NIAID/Johns Hopkins Bloomberg School of Public Health	Adult	58	Septmeber 2008	
		monovalent DENV4	Safety of and Immune Response to a Dengue Virus Vaccine (rDEN4delta30–4995) in Healthy Adults	NCT00322946	rDEN4delta304995	NIAID/Johns Hopkins Bloomberg School of Public Health	Adult	84	August 2009	
		monovalent DENV3	Safety of and Immune Response to a Dengue Virus Vaccine (rDEN3- 3'Ddelta30) in Healthy Adults	NCT00712803	rDEN3–3'Ddelta30	NIAID	Adult	29	September 2009	
		monovalent DENV4	Safety of and Immune Response to a Dengue Virus Vaccine (rDEN4delta30–200,201) in Healthy Adults	NCT00270699	rDEN4delta30–200,201	NIAID/Johns Hopkins Bloomberg School of Public Health	Adult	56	December 2009	
		monovalent DENV2	Safety and Immune Response to Two	NCT00920517	rDEN2/4delta30(ME)	NIAID	Adult	25	January 2010	

Vaccine type	Phase	Valent	Title	NCT Number	Vaccine name	Sponsor/Collaborators	Age Groups	Enrollment	Primary Completion/Estimated primary completion	References
			Doses of rDEN2/4delta30 Dengue Vaccine							
		monovalent DENV1	Safety of and Immune Response of a 2-dose Regimen of rDEN1delta30 Dengue Virus Vaccine	NCT00473135	rDEN1delta30	NIAID/Johns Hopkins Bloomberg School of Public Health	Adult	60	January 2010	Durbin 2011 [103]
		monovalent DENV4	Safety of and Immune Response to DEN4 Vaccine Component Candidate for Dengue Virus	NCT00919178	rDEN4delta30	NIAID	Adult	70	May 2010	
		monovalent DENV2	Safety and Immune Response to an Investigational Dengue Type 2 Vaccine	NCT01073306	rDEN2/4delta30	NIAID	Adult	18	June 2010	
		monovalent DENV1	Evaluation of the Safety and Immune Response to an Investigational Dengue Type 1 Vaccine	NCT01084291	rDEN1d30	NIAID	Adult	18	June 2010	
		monovalent DENV3	Safety of and Immune Response to a Dengue Virus Vaccine (rDEN3delta30/31–7164) in Healthy Adults	NCT00831012	rDEN3delta30/31–7164	NIAID/Johns Hopkins Bloomberg School of Public Health	Adult	56	June 2011	
	Phase 2	bivalent (DENV1 & 3 or DENV2 & 4) AND tetravalent	Safety and Immunogenicity of Formulations of Dengue Vaccines in Healthy Flavivirus-Naïve Adults	NCT00740155	CYD	Sanofi	Adult	154	October 2009	
	Phase 1	tetravalent	Safety and Immunogenicity Study to Assess DENVax, a Live Attenuated Tetravalent Vaccine for Prevention of Dengue Fever	NCT01224639	DENVax	Inviragen Inc.	Adult	112	June 2011	
		tetravalent	Tetravalent Chimeric Dengue Vaccine Trial	NCT01110551	DENVax	NIAID	Adult	72	April 2012	
		tetravalent	Evaluation of the Safety and Immune Response of Five	NCT01072786	TetraVax-DV	NIAID/Johns Hopkins Bloomberg School of Public Health	Adult	141	June 2012	

Vaccine type	Phase	Valent	Title	NCT Number	Vaccine name	Sponsor/Collaborators	Age Groups	Enrollment	Primary Completion/Estimated primary completion	References
			Admixtures of a Tetravalent Dengue Virus Vaccine							
		tetravalent	A Comparison of the Safety and Immunogenicity of Various Schedules of Dengue Vaccine in Healthy Adult Volunteers	NCT01542632	DENVax	Inviragen Inc.	Adult	152	May 2013	
		tetravalent	Impact of SQ vs IM Administration of DENVax on Safety and Immunogenicity	NCT01728792	DENVax	Inviragen Inc./WRAIR	Adult	80	September 2013	
		tetravalent	Phase 1b Study Investigating Safety & Immunogenicity of DENVax Given Intradermally by Needle or Needle Free PharmaJet Injector	NCT01765426	DENVax	Inviragen Inc./NIH	Adult	96	November 2013	
		tetravalent	Evaluating the Safety and Immune Response to Two Admixtures of a Tetravalent Dengue Virus Vaccine	NCT01436422	TetraVax-DV	NIAID	Adult	112	May 2014	
		tetravalent	Evaluating the Safety and Immune Response to Two Admixtures of a Tetravalent Dengue Virus Vaccine	NCT01506570	TetraVax-DV	NIAID	Adult	112	May 2015	
	Phase 1/2	tetravalent	A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naïve Children	NCT00384670	not listed	WRAIR/United States Army Medical Materiel Development Activity/ GSK	Child	10	May 2004	Simasthien 2008 [105]
		tetravalent	Follow-Up Study of Thai Children From Dengue-003 and Evaluation of a Booster Dose of Dengue Vaccine	NCT00318916	F17	WRAIR/U.S. Army Office of the Surgeon General/GSK	Child	7	March 2006	
		tetravalent	A Phase I/II Trial of Tetravalent Live Attenuated Dengue Vaccine in Flavivirus	NCT00322049	F17	U.S. Army Medical Research and Materiel Command/GSK	Child	51	June 2009	Watanaveeradej 2011 [107]

Vaccine type	Phase	Valent	Title	NCT Number	Vaccine name	Sponsor/Collaborators	Age Groups	Enrollment	Primary Completion/Estimated primary completion	References
			Antibody Naive Infants							
	Phase 2	tetravalent	A Trial of a Walter Reed Army Institute of Research (WRAIR) Live Attenuated Virus Tetravalent Dengue Vaccine in Healthy US Adults	NCT00239577	not listed	GSK	Adult	132	June 2007	
		tetravalent	Study of ChimeriVax™ Dengue Tetravalent Vaccine in Adult Subjects	NCT00730288	Chimerivax	Sanofi	Adult	35	August 2007	
		tetravalent	A Phase II Trial of a Walter Reed Army Institute of Research (WRAIR) Live Attenuated Virus Tetravalent Dengue Vaccine in Healthy Adults in Thailand	NCT00370682	T-DEN F17 & T-DEN F19	U.S. Army Medical Research and Materiel Command/GSK	Adult	120	February 2008	
		tetravalent	A Phase II Trial of a WRAIR Live Attenuated Virus Tetravalent Dengue Vaccine in Healthy US Adults	NCT00350337	not listed	WRAIR/GSK	Adult	88	July 2008	
		tetravalent	Immunogenicity and Safety of Three Formulations of Dengue Vaccines in Healthy Adults Aged 18 to 45 Years in the US	NCT00617344	Chimerivax	Sanofi	Adult	260	December 2009	
		tetravalent	A Study of Two Doses of WRAIR Dengue Vaccine Administered Six Months Apart to Healthy Adults and Children	NCT00468858	F17 & F19	U.S. Army Medical Research and Materiel Command/GSK	Child|Adult	720	April 2010	
		tetravalent	Study of ChimeriVax™ Tetravalent Dengue Vaccine in Healthy Peruvian Children Aged 2 to 11 Years	NCT00788151	Chimerivax	Sanofi	Child	300	August 2010	Lanata 2012 [104]

Vaccine type	Phase	Valent	Title	NCT Number	Vaccine name	Sponsor/Collaborators	Age Groups	Enrollment	Primary Completion/Estimated primary completion	References
		tetravalent	Immunogenicity and Safety of Sanofi Pasteur's CYD Dengue Vaccine in Healthy Children and Adolescents in Latin America	NCT00993447	CYD	Sanofi	Child	600	September 2011	
		tetravalent	A Study of Dengue Vaccine in Healthy Toddlers Aged 12 to 15 Months in the Philippines	NCT01064141	CYD	Sanofi	Child	210	September 2012	
		tetravalent	Study of CYD Dengue Vaccine in Healthy Children and Adolescents in South America	NCT01187433	CYD	Sanofi	Child	150	December 2012	
		tetravalent	Study of a Tetravalent Dengue Vaccine in Healthy Adult Subjects Aged 18 to 45 Years in India	NCT01550289	CYD	Sanofi	Adult	189	December 2013	
		tetravalent	Efficacy and Safety of Dengue Vaccine in Healthy Children	NCT00842530	Chimerivax	Sanofi	Child	4002	December 2013	Sabchareon 2012 [67]
		tetravalent	Study to Investigate the Safety and Immunogenicity of a Tetravalent Chimeric Dengue Vaccine in Healthy Volunteers Between the Ages of 1.5 – 45 Years	NCT01511250	DENVax	Inviragen Inc.	Child|Adult	344	December 2013	
		tetravalent	Study of ChimeriVax™ Tetravalent Dengue Vaccine in Healthy Subjects	NCT00875524	Chimerivax	Sanofi	Child|Adult	180	April 2014	
		tetravalent	Phase II Trial to Evaluate Safety and Immunogenicity of a Dengue 1,2,3,4 (Attenuated) Vaccine	NCT01696422	TetraVax-DV	Butantan Institute/ Banco Nacional de Desenvolvimento Economico e Social/ Fundação de Amparo a Pesquisa do Estado de Sao Paulo/Butantan Foundation	Adult	300	May 2014	

Vaccine type	Phase	Valent	Title	NCT Number	Vaccine name	Sponsor/Collaborators	Age Groups	Enrollment	Primary Completion/Estimated primary completion	References
		tetravalent	Immune Response to Different Schedules of a Tetravalent Dengue Vaccine Given With or Without Yellow Fever Vaccine	NCT01488890	CYD	Sanofi	Adult	390	June 2014	
		tetravalent	Study of Sanofi Pasteur's CYD Dengue Vaccine in Healthy Subjects in Singapore	NCT00880893	CYD	Sanofi	Child|Adult	1200	October 2014	Sin Leo 2012 [112]
	Phase 3	tetravalent	Study of a Tetravalent Dengue Vaccine in Healthy Adults in Australia	NCT01134263	CYD	Sanofi	Adult	715	November 2012	
		tetravalent	Study of Yellow Fever Vaccine Administered With Tetravalent Dengue Vaccine in Healthy Toddlers	NCT01436396	CYD	Sanofi	Child	792	January 2013	
		tetravalent	Study of a Tetravalent Dengue Vaccine in Healthy Children Aged 2 to 11 Years in Malaysia	NCT01254422	CYD	Sanofi	Child	250	January 2013	
		tetravalent	Study of a Booster Injection of Pentaxim™ Vaccine Administered With Dengue Vaccine in Healthy Toddlers	NCT01411241	CYD	Sanofi	Child	732	April 2013	
		tetravalent	Study of a Novel Tetravalent Dengue Vaccine in Healthy Children Aged 2 to 14 Years in Asia	NCT01373281	CYD	Sanofi	Child	10278	July 2014	
		tetravalent	Study of a Novel Tetravalent Dengue Vaccine in Healthy Children and Adolescents Aged 9 to 16 Years in Latin America	NCT01374516	CYD	Sanofi	Child	20875	August 2016	
DNA	Phase 1	monovalent DENV1	Safety Study of a Dengue Virus DNA Vaccine	NCT00290147	D1ME	WRAIR/U.S. Army Office of the Surgeon General/U.S. Army Me	Adult	24	December 2006	Beckett 2011 [139]

Vaccine type	Phase	Valent	Title	NCT Number	Vaccine name	Sponsor/Collaborators	Age Groups	Enrollment	Primary Completion/Estimated primary completion	References
						dical Research and Materiel Command				
		tetravalent	Evaluation of the Safety and the Ability of a DNA Vaccine to Protect Against Dengue Disease	NCT01502358	TVDV	U.S. Army Medical Research and Materiel Command/Vical/WRAIR/Naval Medical Research Center	Adult	40	December 2012	
Purified Inactivated	Phase 1	monovalent DENV1	Safety Study of a Vaccine (DENV-1 PIV) to Prevent Dengue Disease	NCT01502735	DENV-1 PIV	U.S. Army Medical Research and Materiel Command	Adult	20	September 2012	
		tetravalent	A Two-dose Primary Vaccination Study of a Tetravalent Dengue Virus Purified Inactivated Vaccine vs. Placebo in Healthy Adults	NCT01666652	TDENV-PIV	U.S. Army Medical Research and Materiel Command/GSK/WRAIR	Adult	100	December 2012	
		tetravalent	A Two-dose Primary Vaccination Study of a Tetravalent Dengue Virus Purified Inactivated Vaccine vs. Placebo in Healthy Adults (in Puerto Rico)	NCT01702857	TDENV-PIV	U.S. Army Medical Research and Materiel Command/GSK/WRAIR	Adult	100	December 2015	
Subunit	Phase 1	monovalent DENV1	Study of HBV-001 D1 in Healthy Adults	NCT00936429	DEN1–80E	Hawaii Biotech, Inc.	Adult	16	July 2010	
		tetravalent	Study of a Dengue Vaccine (V180) in Healthy Adults (V180-001 AM1)	NCT01477580	V180	Merck	Adult	120	December 2013	