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MINI-REVIEW

Dengue viruses and promising envelope protein domain III-based vaccines

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Abstract

Dengue viruses are emerging mosquito-borne pathogens belonging to Flaviviridae family which are transmitted to humans via the bites of infected mosquitoes *Aedes aegypti* and *Aedes albopictus*. Because of the wide distribution of these mosquito vectors, more than 2.5 billion people are approximately at risk of dengue infection. Dengue viruses cause dengue fever and severe life-threatening illnesses as well as dengue hemorrhagic fever and dengue shock syndrome. All four serotypes of dengue virus can cause dengue diseases, but the manifestations are nearly different depending on type of the virus in consequent infections. Infection by any serotype creates life-long immunity against the corresponding serotype and temporary immunity to the others. This transient immunity declines after a while (6 months to 2 years) and is not protective against other serotypes, even may enhance the severity of a secondary heterotypic infection with a different serotype through a phenomenon known as antibody-depended enhancement (ADE). Although, it can be one of the possible explanations for more severe dengue diseases in individuals infected with a different serotype after primary infection. The envelope protein (E protein) of dengue virus is responsible for a wide range of biological activities, including binding to host cell receptors and fusion to and entry into host cells. The E protein, and especially its domain III (EDIII), stimulates host immunity responses by inducing protective and neutralizing antibodies. Therefore, the dengue E protein is an important antigen for vaccine development and diagnostic purposes. Here, we have provided a comprehensive review of dengue disease, vaccine design challenges, and various approaches in dengue vaccine development with emphasizing on newly developed envelope domain III-based dengue vaccine candidates.

Keywords Dengue virus · Envelope protein · Chimeric vaccine · Disease · Immunogenicity

Introduction

Dengue viruses (DVs) are emerging mosquito-borne pathogens belonging to Flaviviridae family. They are transmitted to humans by the bites of infected mosquitoes *Aedes aegypti* and *Aedes albopictus*. Because of the wide distribution of such mosquito vectors, more than 2.5 billion people (two fifth of the world's population) approximately are at risk of dengue infection. Dengue viruses are mostly prevalent in economically poor countries in the tropical and subtropical areas of the world (Amitai et al. 2009; Gubler 2002). Incidence and geographical spread of dengue have greatly increased in recent years, and it is estimated about 390 million dengue infections, of which 96 million manifest clinically (Bhatt et al. 2013) and 22,000 deaths per year (Murrell et al. 2011). It is estimated that 3.9 billion people, in 128 countries, are at risk of dengue

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infection (Brady et al. 2012). According to the report from "WHO" website, a live attenuated chimeric tetravalent dengue vaccine, Dengvaxia® (CYD-TDV) by Sanofi Pasteur, was recently licensed in Mexico and in several other countries including the Philippines, Brazil, El Salvador, Costa Rica, Paraguay, Guatemala, Peru, Indonesia, Thailand, and Singapore (http://www.sanofipasteur.com), as well. Furthermore, five additional dengue vaccine candidates are in clinical trial phases (Vannice et al. 2016). However, the efforts are underway to engineer a novel tetravalent subunit vaccine. At the present paper, we have provided a comprehensive review of dengue disease, the challenges of vaccine design, and different approaches in dengue vaccine development with emphasizing on newly developed envelope domain III-based dengue vaccine candidates.

Dengue disease

Although first written evidence of a dengue-like disease has been found from an old Chinese medical encyclopedia of 992, but in recent years incidence of dengue disease has dramatically increased, and now dengue infection is the most common vector-borne viral disease of humans. Dengue viruses cause dengue disease in different levels of severity including dengue with/without warning signs and severe dengue. Severe dengue includes hemorrhagic fever (DHF) and dengue shock syndrome (DSS) which both of them are severe life-threatening illnesses. Clinical symptoms of dengue fever (DF) are ranging from slight to mild febrile disease, which coincides with the appearance of dengue virus in blood. DF generally is a self-limiting disease, that the symptoms are included severe body ache, skin rashes, and headache. In DHF and DSS, despite the very high fever, thrombocytopenia and hemorrhagic manifestations as well as vascular leakage into interstitial spaces occur (Swaminathan and Khanna 2013).

Dengue viruses have four genetically different serotypes (DV1, DV2, DV3, and DV4), which closely related with a 65–70% nucleotide sequence homology (Rico-Hesse 1990). All four serotypes can cause the mentioned dengue diseases, but depending on the type of the virus in consequent infections, the manifestations are nearly different (Montoya et al. 2013; Murrell et al. 2011). Infection by any serotype creates life-long immunity against the corresponding serotype and temporary immunity to the others. This transient immunity declines after a while (6 months to 2 years) and is not protective against other serotypes (Montoya et al. 2013), even may enhance the severity of a secondary heterotypic infection with a different serotype through a phenomenon known as antibody-depended enhancement (ADE) (Halstead 2003). Always there is chance of infection with the other three serotypes of dengue for people who live in dengue endemic areas, which can result in disease enhancement. One of the possible mechanisms for this disease enhancement is that the induced antibodies against the primary infection are non-neutralizing for the secondary serotype and can form complexes with the viruses aiding them to entry into host cells (phagocytes and Fc receptor bearing monocytes) via Fc receptors. This phenomenon results in increased viral propagation and enhancement of infection (Kliks et al. 1988). Alternatively, reactive cytotoxic T lymphocytes against dengue antigens, with greater avidity for the primary than the secondary serotype, may accelerate the destruction of host cells and imperfect viral clearance (Mongkolsapaya et al. 2003). Furthermore, it has been shown that anti-prM/NS1 antibodies cross-react with host endothelial antigens (Falconar 2007; Huang et al. 2006) and form complex with viruses, which target them for entry into host cells via cross-recognition of host antigens (Lin et al. 2008). On the other hand, high viral titer triggers an intensive cytokine storm that damages the endothelium and results in capillary leakage and hemorrhagic manifestations (Rothman 2004).

As mentioned above, despite the potentially pathogenicity of all four serotypes, there are some correlations between dengue serotypes and the severity of infections. For example, it has been shown that primary infection with either serotype 1 or serotype 3 results in more severity disease than if serotypes 2 or 4 to be the primary infection (Vaughn et al. 2000). On the other hand, it has been found that infection by serotype 2 is associated with severe dengue diseases (Fried et al. 2010), especially DSS (Anantapreecha et al. 2005). Additionally, several researches have shown that there is a significant correlation between severity of secondary infections and host genetic backgrounds (Bravo et al. 1987; Rothman 2010). A recent study showed the impact of time intervals in severity of dengue diseases. According to this study, longer intervals ($\geq$ 2.6 years) between two infections correlate with symptomatic severe diseases, whereas shorter time intervals ($\leq$ 2 years) results in either protection or unapparent infection (Montoya et al. 2013). Hence, genetic background, age, and some specific physiological conditions of individuals could be risk factors for severity of dengue diseases. For instance, Caucasians, children, and individuals affected by bronchial asthma, diabetes mellitus, and sickle cell anemia (SCA) are more susceptible for development of severe dengue diseases (Guzman et al. 2010).

Dengue vaccine challenges and current approaches to develop dengue vaccines

Although the vaccines against other related flaviviruses, such as yellow fever (YF), Japanese encephalitis (JE), and tick-

borne encephalitis (TBE), have been developed and licensed, efforts to develop an efficient dengue vaccine have some drawbacks:

- The existence of four antigenically different but related serotypes; therefore, a dengue vaccine has to be tetravalent by the ability to induce balanced and stable immune response.
- Poor understanding of biology and pathogenesis of DVs; as the role of the immune response and the pathogenesis of DHF/DSS are not fully understood (Halstead 1988). While it has been explained a role for cell-mediated immunity in the clinical outcome of the disease, but itself cannot play a major role in protection (Edelman and Hombach 2008).
- The need for long-term protection; as it has been shown that in some cases, severe dengue diseases have been observed throughout more than 20 years after the first infection (Alvarez et al. 2006; González et al. 2005).
- Poor understanding of the mechanism of induced protective immunity; as primary infection with any of the four serotypes results in a lifelong immunity to that serotype, and interim immunity to the others (Sabin 1952). On the other hand, there is no data on the antibody titers which is required to confer complete protection against the infection. Currently, neutralizing antibody titers are measured using an in vitro plaque reduction neutralizing test (PRNT), as a standard for evaluation of induced immunity. Although, the immunological relevancy of this test should be explained (Sabin 1952; Swaminathan and Khanna 2010). A $PRNT_{50}$ titer of $\geq 1{:}10$ is concluded as indication for seroconversion (Delenda et al. 1994; Guy and Almond 2008).
- Lack of a proper animal model to study the immune response and mechanism of protection; currently, mice and monkeys are used for this purpose but they do not manifest any dengue-like diseases so that wild-type mice are resistant to dengue-induced diseases (Murrell et al. 2011). Although, there is no proper small animal model for evaluating of in vivo protective efficacy of dengue vaccines, adult mouse models (ex. BALB/c, C3H) are often used to assess protective level of dengue vaccines against DV2 (Apt et al. 2006; Wu et al. 2003c). However, these models are not sensitive to DV1, DV3, and DV4. Suckling mice which are intracranially infected with neurovirolent DV often manifest cyrtosis, hind-leg paralysis, and death. Therefore, suckling mice are currently a suitable model to assess protective efficacy of dengue vaccine candidates by observing morbidity and mortality (Mota et al. 2005). However, the main limitation of these models is that the mouse protection assays are focused on humoral responses as well as protective efficacy of cell-mediated immune response is ignored. Recently, some attempts have been performed to develop genetically engineered mice that can develop characteristics of human dengue-induced disease. For example, by knocking-out of interferon receptors, AG129 mice showed some dengue-like disease symptoms (Shresta et al. 2006). Also, a variety of different mouse models such as humanized mice, chimeric mice transplanted with human cells, immunocompromised mice, and immunocompetent mice have been developed (Balsitis et al. 2010; Clements et al. 2010; Kuruvilla et al. 2007; Zellweger et al. 2010). The induction of viral encephalitis in immunocompetent mice has been extensively used a model for the evaluation of the protective capacity of vaccine candidates against lethal challenges by dengue (Van Der Most et al. 2000). For example, a mouse encephalitis model has been used for evaluation of protective potential of a dengue vaccine candidate (Marcos et al. 2013). Alternatively, it has been reported that Asian rhesus macaques are useful animal models for evaluation of DHF (Onlamoon et al. 2010). However, humans are only real models of dengue diseases and therefore, clinical testing and long-term assessments are only means to evaluate safety, immunogenicity, and reactogenicity of vaccine candidates (Swaminathan and Khanna 2010). In this context, Gunther and colleagues have introduced "a human challenge model" for dengue infection to investigate protective effects of different cytokines in volunteers who developed illness. Their results revealed the critical role of IFN-γ in protection against dengue fever. The authors also suggested that human challenge model provides an opportunity to examine efficiency of potential vaccine candidates prior to large-scale clinical trials (Gunther et al. 2011).
- The need for vaccine trials in different geographical regions to evaluate efficacy of vaccines in different epidemiological situations (Guzman et al. 2010).
- Since the most of reported infections are from developing countries, an ideal protective vaccine should be economically inexpensive and conferring complete protection by just single inoculation.

In order to develop dengue vaccines, many different strategies have been used (Table 1). However, each of these approaches has its unique advantages and disadvantages. For example, live attenuated vaccines can provide long-lasting and robust immunity but always there is a risk for reversion to virulence forms. However, efforts of researchers over the past six decades resulted in a live attenuated dengue vaccine which is currently approved for human use (Dengvaxia®). Since natural dengue infections induce solid serotype specific immunity, live attenuated virus strains are theoretically the most promising approach for developing an effective dengue vaccine. But, live attenuated dengue vaccines have showed considerable safety problems/affectivity drawbacks. On the

Table 1 Different approaches to develop dengue vaccines

	Approaches	Method	Advantages	Disadvantages	References
1	Traditional live attenuated vaccines (LAVs) (outdated)	Passaging virus in mouse brains or semipermisive cell cultures (ex., DENV2PDK53)	Induction of humoral and cellular responses with long-lasting immunity by monovalent formulations	Different levels of attenuation among serotypes and unpredictable molecular changes Risk of reversion to the wild type Difficulties in development of balanced tetravalent formulations Interference of viral replication among the four serotypes of virus in tetravalent formulations	(Edelman et al. 2003; Innis and Eckels 2003; Sabchareon et al. 2004)
2	Recombinant live attenuated vaccines (vaccine strains)*	Introducing specific site-directed genetic mutations into the genome of the virus (ex., rDEV4Δ30)	Greater genetic stability than traditional LAVs Inducing full immune response Improved in safety	Time consuming process Difficulties in development balanced tetravalent formulations Expensive cell cultures and need to extensive purification steps to remove cellular DNA and proteins	(Blaney et al. 2008; Blaney et al. 2007; Durbin et al. 2005; Guy et al. 2017; Mcarthur et al. 2008)
3	Live chimeric vaccine viruses**	Using the backbone of a related flavivirus, and replacing the prM-E genes from dengue by infectious cDNA clone technology (ex., ChimeriVax and RepliVax)	Safe and high level of immunogenicity	Difficulties in development balanced tetravalent formulations Expensive cell cultures	(Bischof et al. 2017; Guy et al. 2008; Suzuki et al. 2009)
4	Inactivated virus vaccine	By chemically treatment	Conferring immunity without the risk of infection Reduced potential for reactogenicity	Needs high scale production of DVs and the problem of low growth titers of dengue viruses Reduction of immunogenicity during the inactivation and need for adjuvants	(Raviprakash et al. 2008; White et al. 2007)
5	DNA and virus-vectored vaccines	By inserting viral protein coding sequences in DNA/viral vectors	Inducing intracellular antigen processing for adaptive immunity Stability (without reverting to a pathogenic phenotype) Easy to manufacture and making tetravalent formulations	Low immunogenicity and short-lived protection Need for booster immunizations Some concerns about pre-existing immunity against the used basic vector	(Chen et al. 2016; Gonçalves et al. 2015; Hurtado-Melgoza et al. 2016; Mcburney et al. 2016; Zheng et al. 2017)
6	Recombinant protein vaccines	By heterologous expression of envelope E protein or derivatives (such as EDIII domain)	Inexpensive production and easy to scale-up Easy to make tetravalent formulations	Need for adjuvants and booster immunizations to induce high and long-term immunity	(Fahimi et al. 2014; Khetarpal et al. 2017; Lazo Vázquez et al. 2017; Montes-Gómez et al. 2017; Valdés et al. 2017; Versiani et al. 2017)

other hand, non-replicating vaccines such as protein subunit vaccines eliminate the risk of reversion but they are not potent immunogens and need suitable adjuvants and booster immunizations. Development of new approaches of chimeric multivalent vaccines and improvement of antigenicity of developed vaccine candidates in combination with new generation of adjuvants can overcome these potential hurdles.

Virus structure and importance of envelope protein domain III

Dengue virus particle is composed of nucleocapsid and envelope. The core nucleocapsid assembles with RNA and capsid protein (C). The viral genome is a positive-sense RNA molecule by ~10.6 kb in length, composed of an open reading frame (ORF). The single ORF is translated into a polyprotein which is cleaved by both host cell and viral proteases producing three structural (capsid C, precursor membrane prM, and envelope E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (Modis et al. 2003). Despite the structural similarities, there is a variation in the genetic sequence of different serotypes, mainly in the structural proteins that result in antigenic distinction and lack of cross protection among the serotypes. The envelope protein E is a large (495-amino acid residues), cysteine-rich (12 highly conserved cysteine residues forming six S-S bonds), and multifunctional glycoprotein. The E protein is responsible for a wide range of biological activities, including binding to host cell receptors as well as fusion to and entry into host cells. In addition, the E protein stimulates host immunity responses by inducing protective and neutralizing antibodies (Henchal and Putnak 1990; Lindenbach 2001). Therefore, the dengue E protein is an important antigen for vaccine development and diagnostic purposes. Immunomodulatory potential of dengue surface protein has been evaluated by some groups (Amitai et al. 2009; Babu et al. 2008; Fahimi et al. 2014; Kelly et al. 2000; Khanam et al. 2007; Konishi et al. 2006).

Crystallographic studies have shown that the dengue E protein consists of three structurally distinct domains: a central domain (EDI), a dimerization domain (EDII), and an immunoglobulin (Ig)-like C terminal domain (EDIII) (Modis et al. 2004, 2005) (Fig. 1). According to X-ray crystallographic structural studies, the domain I is located in the middle of the protein, including the N-terminal of protein with glycosylation sites. It is flanked by domain II and domain III (Modis et al. 2003). The EDII mediates head-to-tail dimerization of E protein and also bears the fusion loop that inserts into the target cell membrane under the pH-dependent virus fusion. There is a single disulfide bond (SS6) in DIII between Cys 302 and Cys 333, which is very critical for antigenic structure of the E protein (Lin et al. 1994; Mason et al. 1990). The folding of E protein is mainly based on beta sheet structures and disulfide bonds. The presence of many type-specific epitopes in EDIII and its relatively stable conformation suggest that it might be useful as a virus-specific antigen. It has been

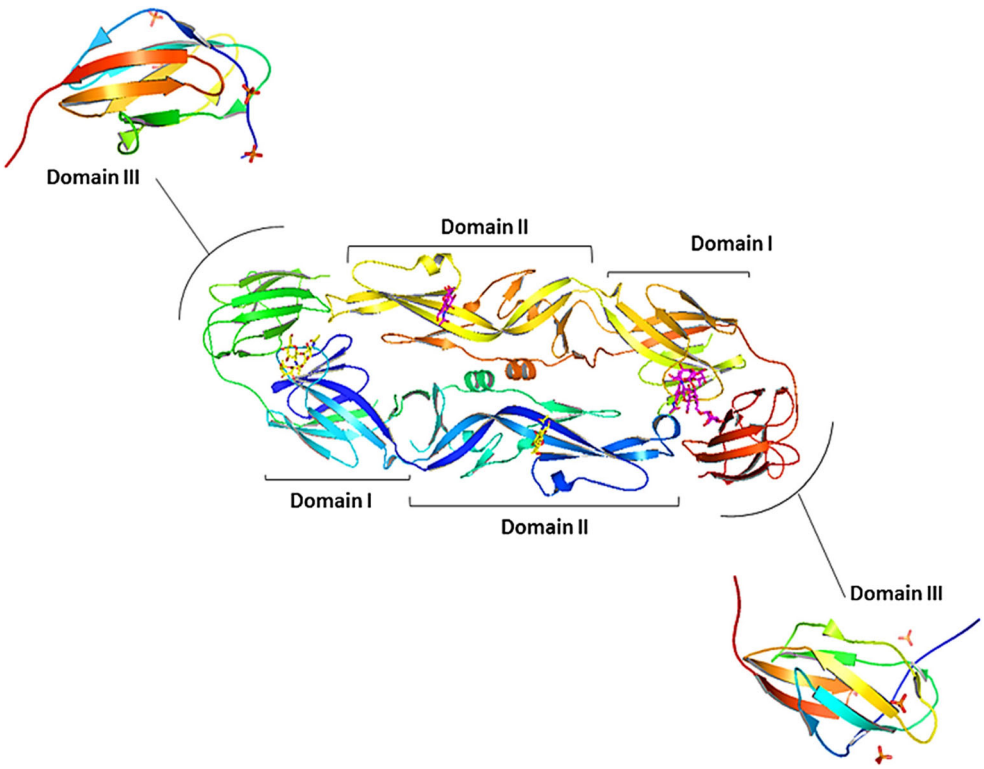

Fig. 1 Crystal structural of dimeric form of dengue envelope protein. The most important domain regarding to the vaccine research in domain III. The figure is designed based on three-dimensional structures from PDB site and PyMol software

shown that individual EDIII can be independently folded as an Ig-like structure protein (Kuhn et al. 2002) and be expressed in the form of recombinant functional protein (Etemad et al. 2008; Guzman et al. 2010). In recent years, several groups have shown that EDIII-based vaccine candidates can elicit virus-neutralizing antibodies (Chen et al. 2007; Fahimi et al. 2014; Leng et al. 2009). Envelope domain III (EDIII), which spans amino acid residues 300-400 of C-terminal of E protein, has been composed of 10 anti-parallel beta-strands and lacks glycosylation sites (Nayak et al. 2009; Rey et al. 1995; Tripathi et al. 2011). This domain, which is exposed and accessible on the virion surface and is involved in host cell surface receptor recognition, contains multiple type- and subtype-specific conformation-dependent neutralizing epitopes (Kuhn et al. 2002). Neutralizing epitopes of the dengue EDIII have been identified and included special residues 307, 333-351, and 383-389 (Modis et al. 2005). Mutations in these sites lead to escape from neutralization by conventional monoclonal neutralizing antibodies (Wu et al. 2003a). This domain appears to play a role in host cell receptor binding for viral entry (Chin et al. 2007; Hung et al. 2004), and in inducing long-lasting protective immunity against dengue virus infection (Muné et al. 2003; Zulueta et al. 2006). Anti-EDIII monoclonal antibodies are very powerful blockers of dengue infectivity (Zhang et al. 2011). A study that analyzed a large panel of anti-E monoclonal antibodies showed that those that bound to EDIII were the most potent blockers of virus (Crill and Roehrig 2001). In contrast, antibodies targeting either domains I or II are weakly neutralizing and cross neutralize several flaviviruses (Oliphant et al. 2006) that can lead to immune responses developing ADE. In addition, several reports have shown that recombinant EDIIIs can block in vitro dengue infectivity (Hermida et al. 2006; Tripathi et al. 2008). Importantly, EDIII has only a very low intrinsic potential for inducing cross-reactive antibodies (Simmons et al. 1998a, b) implicated in the pathogenesis of DHF/DSS. Choosing only domain III as a vaccine candidate instead of whole envelope protein has the advantage of reducing the risk of ADE because of the absence of other non-neutralizing and cross-reactive epitopes. There are many evidences that the most properties of the E protein, important from a vaccine perspective, are associated with domain III (Gil et al. 2017; Izquierdo et al. 2014; Mcburney et al. 2016; Poddar et al. 2016; Suzarte et al. 2014). Taken together, EDIII domain plays a crucial role in dengue pathogenesis and immunity. It has also emerged as a promising excellent vaccine candidate, so that most of recent efforts to produce a recombinant subunit dengue vaccine have focused on utilization of this domain.

In clinical context, it is suggested that EDIII peptides can have considerable potential in therapeutics for dengue infection, as it has been shown that the EDIII competes with the virus during entry by preventing viral binding to cell surface receptors. Chin et al. have shown that recombinant EDIII1 and EDIII2 inhibit the entry of dengue virus type 1 and 2 into host cells (Chin et al. 2007). Another suggestion for usage of EDIII in therapeutic approaches is the application of anti-EDIII monoclonal antibodies to prevent the dengue diseases. As it has been shown that anti-EDIII monoclonal antibodies are effective blockers of dengue entry into host cells (Crill and Roehrig 2001). It has also been reported that the murine monoclonal antibody (mAb) 4E11, which can neutralize the infectivity of all four serotypes, binds to EDIII (Thullier et al. 2001). On the other hand, Rajamanonmani et al. showed that an anti-EDIII2 mouse (Amitai et al. 2009) monoclonal antibody cross-reacts with the EDIII (corresponding to other three dengue serotypes) and WNV, blocking the first steps of viral entry (Rajamanonmani et al. 2009). The authors suggested that the viral inhibitory potential of this anti-EDIII2 antibody and its cross-reactivity have made it a candidate for humanization and therapeutic antibody-based treatment in dengue infections.

From a different view, investigation of biological properties of EDIII is very important in understanding on biology of dengue virus. Pattnaik and colleagues have showed that recombinant EDIII4 expressed in *Escherichia coli* is biologically functional and binds to soluble cell-free heparan sulfate (Pattnaik et al. 2007). In addition, Wahala et al. recently have showed that EDIII-reactive antibodies are present in primary and secondary dengue virus immune human sera. Human antibodies bound to a serotype specific epitope on EDIII after primary infection and a serotype cross-reactive epitope on EDIII after secondary infection. However, their results showed that EDIII-binding antibodies constituted only a small fraction of the total antibody in immune sera binding to dengue virus (Wahala et al. 2009). In another study, Li et al. have found that majority of cross-reactive anti-EDIII mouse mAbs do not show efficient neutralization activity (Li et al. 2013). These authors concluded to have reconsideration on efficacy of recombinant EDIII-based immunization strategies. Another study indicated that anti-EDIII antibodies contribute little to the neutralizing potency of human DENV-immune (Williams et al. 2012). It seems that the application of only rEDIII prime-boost immunization is not enough to induce a protective immune response. Taken together, it has identified that EDIII is the critical region for vaccine, therapeutic, and diagnosis developments, but it seems that integrative methods by using other parts of E protein in combination with EDIII region could be more potent approach.

EDIII-based vaccine design

As mentioned above, there are different strategies for dengue vaccine design as well as EDIII has special advantages in some of these strategies. A growing body of evidences, accumulated in recent years, have identified that envelope domain

III is the critical region from the perspective of vaccine development. Plasmids encoding EDIII and recombinant EDIII-based fusion proteins have been shown to elicit neutralizing antibodies in experimental animals (Chiang et al. 2013; Guzman et al. 2010). Similarly, the capacity of recombinant EDIII3 in the diagnosis of dengue infection and also in the induction of neutralizing antibodies has been reported previously (Reddy et al. 2012). These findings put together justify the choice of domain III for sub-unit vaccine development. An overview of different EDIII-based vaccine candidates is provided below.

EDIII-based live chimeric vaccine

To encode the domain III and/or M ectodomain of dengue virus, an EDIII-based chimeric vaccine has been produced by the engineering of live attenuated Schwarz vaccine strain of measles virus. Immunization by priming this chimera and boosting with the recombinant protein were successful in inducing of long-term specific neutralizing antibodies in mice. This approach also can be used for developing of single dose multivalent vaccines against measles virus and dengue infections, which can be very useful especially for children living in endemic areas of both diseases such as Africa and South America (Brandler et al. 2007; Brandler et al. 2010).

In a different approach, immunogenicity of a recombinant *Lactococcus lactis* strain producing the EDIII2 by oral/nasal administration has been shown (Sim et al. 2008). The authors demonstrated that this mucosal vaccine candidate induces a systemic anti-EDIII IgG antibody response in mice. They found that the immune sera from orally inoculated mice neutralize in vitro dengue infection, and also in some cases, the neutralizing antibodies were more effective than those which obtained with the intraperitoneally injection of inactivated virus.

EDIII-based subunit vaccine design

In recent years, considerable research effort has been directed towards the development of subunit dengue vaccines. Since protein subunit vaccines employ only a portion of virus, they are easier and safer in the production and use than live vaccines containing the whole genome. In terms of production costs, subunit vaccines involve low cost in compare with inactivated vaccines. In this field, *Escherichia coli* is the most commonly used host for the production of heterologous protein (Haddad Kashani et al. 2017; Hosseini et al. 2016; Kashani and Moniri 2015; Kashani et al. 2018). Firstly, given the importance of recombinant EDIIIs in development of subunit vaccines, we provide a review of recently reported investigations in this field.

Different approaches for expression of recombinant EDIIIs

Important from the perspective of recombinant protein expression, the Ig-like EDIII has been shown to be a very stable and independent folding domain (Etemad et al. 2008; Wang et al. 1999). With regard to EDIII, *E. coli* has served as the major host to date (Gromowski et al. 2008; Jaiswal et al. 2004; Saejung et al. 2006), and also there are some reports on the use of eukaryotic systems specially *Pichia pastoris* (Mani et al. 2013; Saejung et al. 2007). Some *E. coli*-expressed EDIIIs tend to be insoluble and need to be purified under denaturizing conditions, followed by renaturation (Liao and Kielian 2005; Zhang et al. 2007). The *E. coli*-based systems have the highest expression potential and cost effect scale up. To enhance the maximum potential of this system, the expressed protein should be moderate size (Goeddel 1990), as well as the coding gene should be optimized and expressed in an appropriate host. Jaiswal et al. expressed and purified recombinant EDIII2, without any carrier in *E. coli* (Jaiswal et al. 2004). Although, the N-terminal 6×-His-tagged EDIII2 protein purified during one-step purification process, but it was overexpressed in the form of insoluble inclusion bodies. Therefore, authors used the denaturation/renaturation strategy for EDIII purification. However, the yield of expression and purification was about 30 mg/L of culture and it could effectively protect cells in culture against dengue virus 2 infection by blocking the virus attachment onto host cells. Induction at lower temperatures, periplasmic expression, and fusions to partners or carriers such as GST, MBP, TrpE, and thioredoxin (Saejung et al. 2006, 2007) have been employed in attempts to increase the solubility of EDIIIs in *E. coli*. The main objective of the fusion strategy has been to ensure expression in soluble form and to provide a device for detection and purification. Soluble expression of EDIII proteins is a key point in developing a suitable strategy for both vaccine/diagnostic purposes, as previous studies have showed that the soluble forms of these proteins are more effective in production of antibody responses (Fahimi et al. 2014; Saejung et al. 2006). Furthermore, it has been shown that some special host strains have specific potential in soluble expression of disulfide bond harboring proteins. Using this strategy, Jaiswal et al. have been shown that origami strain of *E. coli* can be used as appropriate host (Jaiswal et al. 2004). They expressed soluble EDIII2 in *E. coli* using origami strain and at a low induction temperature of 18 °C. The enhanced production of the soluble protein attributed to the thioredoxin reductase (*trx*B) and glutathione reductase (*gor*) double mutations in the origami genome. This recombinant protein was reactive with human patient antiserum and could elicit the high antigen reactive antibodies in rat. In a similar way, we have showed the soluble expression of EDIII3 protein in *E. coli* by using origami strain (Fahimi et al. 2014; Prinz et al. 1997).

In order to improve the soluble expression and immunogenicity of recombinant EDIII proteins, several other different strategies have been used. One of the most successful findings was achieved by fusing of EDIII to P64k protein of *Neisseria meningitides*. There are several reports for effective immunogenicity and developing potent as well as serotype specific protective responses in immunized animal models by P64k-EDIII fusion proteins (Hermida et al. 2004; Izquierdo et al. 2014).

Recently, considering the significant importance of EDIII proteins in vaccine, diagnosis, and therapeutically related fields, large-scale recombinant production of these proteins is appeared as an interesting field in biotechnology. In this regard, large-scale production of recombinant EDIII3 in *E. coli* by using batch and fed-batch cultivation process has recently been reported (Reddy et al. 2012; Tripathi et al. 2011). Tripathi et al. have reported the production of ~ 649 mg of purified recombinant EDIII3 protein per liter of culture. By using fed-batch cultivation and immobilized metal affinity chromatography purification, they were able to produce recombinant EDIII protein in large scale, which can be used for dengue diagnosis or prophylactic studies. Furthermore, these authors have reported the large-scale production of EDIII4 (Tripathi et al. 2008). The reported production system resulted in overexpression of EDIII4 in the form of inclusion bodies in *E. coli*, which followed by affinity chromatography purification with final yield of 196 mg/L of culture.

In the field of eukaryotic expression of EDIII proteins, Batra et al. have developed a recombinant clone of the methylotrophic yeast *Pichia pastoris* capable of secreting EDIII2. They were obtained ~ 125 mg EDIII2 with ~ 94% purity after purification from a 10-L bioreactor (Batra et al. 2010; Etemad et al. 2008). In the context of yeasts as expression hosts, a new study has done to investigate the potential of *Saccharomyces cervisia* in expression of EDIII protein. *S. cerevisiae* is an attractive heterologous expression platform that offers several advantages such as post-translational modification and secretion, as well as facility of genetic manipulation and cultivation; it is also regarded as a generally recognized as safe (GRAS) organism (Nguyen et al. 2013). Nguyen et al. provided a synthetic consensus gene based on amino acid sequences of dengue envelope domain III (scEDIII) from all four serotypes, which the coding gene was optimized for expression in baker's yeast. They showed that the expressed scEDIII was secreted into the culture media, which provides a simple downstream purification process. The immunized mice with this consensus EDIII protein showed a balance immune response against all four serotypes.

Regarding to the expression of EDIII2 in a eukaryotic host, one of the most interesting reports is the production of EDIII2 in plant using tobacco mosaic virus-based vector system (Saejung et al. 2007). Saejung et al. expressed EDIII2 in *Nicotiana benthamiana* plant using a TMV-based transient expression system. The expressed recombinant EDIII2 was reactive with anti-EDIII2 polyclonal antibody and the immunized mice sera neutralized dengue type-2 virus. Furthermore, in order to achieve an edible vaccine against dengue virus, Kim et al. expressed a consensus EDIII protein in rice (Kim et al. 2012, 2013). These authors used a plant codon optimized gene encoding a consensus EDIII. The results of these studies showed the potential of transgenic rice in the expression of two EDIII-based fusion proteins: scEDIII fused with ER retention signal (scEDIII-SEKDEL) (Kim et al. 2012), and scEDIII fused with M cell-targeting peptide ligand (scEDIII-Co1). Altogether, the results of these studies showed the possibility of development of plant-based vaccines against dengue virus (Kim et al. 2012, 2013).

EDIII-based protein subunit vaccine candidates

Antigenicity of EDIII/EDIII-fusion proteins was reported by many investigators. Fonseca et al. showed that the recombinant EDIIIs of all serotypes (expressed as fusion proteins with the trpE tag) are antigenic. EDIII-trpE protein of each serotype reacted strongly with hyperimmune mouse ascetic fluid against the homologous serotype of dengue virus, with little or without cross-reactivity (Fonseca et al. 1991). Although, these trpE-fused recombinant proteins were not immunogenic in mice; however, further researches in this area were very promising. One of the earliest reports for using of domain III-containing recombinant proteins as a dengue vaccine candidate was published by Srivastara et al. in 1995. They designed a fusion protein containing domain III region of DV2 and a fragment containing 65 amino acids of N-terminal of NS1 protein with staphylococcal protein A, and expressed it in *E. coli*. Immunization of mice by this fusion protein (EDIII2-NS1-protein A) resulted in induction of virus neutralizing antibodies and protective immunity against intracranial lethal challenge of mice with DV2. A very important drawback of this vaccine candidate was high affinity of staphylococcal protein A to human IgG, which makes it unsuitable for vaccination (Srivastava et al. 1995). Another early report for using of recombinant EDIII as a dengue vaccine candidate was published in 1998. Simmons et al. cloned the EDIII2 coding gene in pMAL-c2 expression vector and expressed an EDIII2-MBP fusion protein. Immunogenicity evaluation of the purified EDIII2-MBP protein revealed the considerable potential of this protein in eliciting neutralizing and protective antibody responses. Moreover, sera from immunized mice showed no cross-neutralization activity toward any of the other dengue serotypes (Simmons et al. 1998a, b). Expression and immunogenicity of recombinant EDIII2 also have been reported by others. Yang et al. expressed N-terminally fused EDIII2 with

pelB bacterial secretion signal in *E. coli*. This protein was immunogenic and resulted in induction of high levels of neutralizing antibodies, in both BALB/c and C57BL/6 mice. They showed that both EDIII2 and its murine antibodies protect Vero cells from DV2 infection with cross protection against DV1 infection. Furthermore, the anti-EDIII2 antibodies were able to protect suckling mice from in vivo challenge (Yang et al. 2012). As it has been shown by Zhange et al., EDIII protein without any fusion tag can also be considered as a vaccine candidate (Zhang et al. 2007). They expressed EDIII2 in the form of inclusion bodies in *E. coli*, and used multistep purification and refolding methods. This recombinant protein was functional in the inhibition of plaques formation on C6/36 cells. Immunization of mice by this protein elicited neutralizing antibodies which were protective against viral challenge of mice.

One of the most studied EDIII-based fusion proteins was designed by Hermida et al. (2004). They fused a fragment of DV1 E protein (containing domain III) to P64K (a meningococcal protein) and used for mice immunization. Immunization of mice by this fusion protein elicited high levels of neutralizing antibodies and provided significant protection against lethal challenge with DV1. These investigators also produced a fusion protein containing dengue 2 E protein and P64K, so that immunization of mice with this protein resulted in eliciting high neutralizing antibodies and partially protection against lethal challenge with corresponding serotype of dengue virus (Hermida et al. 2004; Izquierdo et al. 2014). Furthermore, they investigated the immunogenicity of designed fusion protein (EDIII2-P64K) in *Macaca fascicularis* monkeys. They found that this fusion protein induces dengue 2 neutralizing antibodies with protection in vaccinated nonhuman primate model (Hermida et al. 2006).

In recent years, new strategies have been developed in the EDIII-based protein subunit vaccine context. One of these strategies is the production of EDIII-based chimeric fusion proteins in combination with other proteins of dengue virus. For instance, Marcose et al. have reported the expression, purification, and immunogenicity of a recombinant fusion protein (composed of EDIII2 and DV2 capsid protein of DVs) in mice, which has resulted in eliciting neutralizing antibodies (Marcos et al. 2013). Immunological evaluations revealed induction of neutralizing antibodies, cell-mediated immunity, and protection upon virus challenge in mice.

Special advantages of EDIIIs for designing multivalent vaccines

In the field of designing of multivalent vaccines, EDIII proteins have special advantages. EDIII proteins expressed in *E. coli* have extensively used for developing tetravalent dengue vaccine candidates. For example, EDIII proteins expressed individually and the physical mixture of all four EDIIIs formulated as a tetravalent vaccine candidate. Simmons et al. showed that these individual EDIIIs could develop high titers of serotype homologous antibody responses with high virus neutralizing capacity, and also the tetravalent formulation of four EDIII-MBP fusion proteins was very effective in eliciting high titer of virus neutralizing antibodies toward all four serotypes (Simmons et al. 2001). This study revealed the potent capacity of EDIIIs in the development of tetravalent vaccines. Although, the concerns about carrier capacity and safety of MBP in vaccine formulations should be considered.

In another report, capability of EDIII fusion in the induction of bivalent immune response has been investigated. A chimeric bivalent antigen composing of EDIIIs of serotypes 2 and 4 was expressed in *E. coli* and elicited antibody responses that efficiently blocked host cell binding and neutralized the infectivity of both DV 2 and DV4 (Khanam et al. 2006). Chen et al. developed a tetravalent bacterially expressed protein composed of four EDIIIs (Chen et al. 2007). They fused the EDIIIs of all four DVs sequentially to each other by (Gly4-Ser) 3 linkers to construct a tandem repeat of EDIII1-2-3-4. They identified specific high antibody titers in sera of the immunized mice against serotypes 1, 2, 4, and moderate antibody titer against serotype 3. In challenge experiment, 70% of mice challenged with serotypes 1, 2, 4 and 18% of mice challenged with serotype 3 were protected. The results of this study suggested that the tandem repeat of EDIIIs, forming an individual antigen, can be used as a potential tetravalent dengue vaccine. However, the unbalanced immunity that achieved in this study (weak immune response against serotype 3) attributed to conformational problems of tetravalent antigen. The linker that used in this study is widely used in antibody engineering, but the authors discussed that some special epitopes are perhaps buried inside, especially those in EDIII3. In another report for developing tetravalent vaccine, a tetravalent antigen has developed by providing a consensus EDIII sequence corresponding to all four serotypes (Leng et al. 2009). The mice immunized with this tetravalent recombinant protein developed neutralizing antibodies against all serotypes of dengue virus. The effectiveness of this protein in providing protective immunity was shown by challenge experiment on mice. The authors have suggested the possibility of developing a single tetravalent vaccine against dengue viral infections. In a newly published study, these authors also investigated the immunogenicity of this consensus EDIII in non-human primates (Chen et al. 2013). The results of this study showed the induction of immune response against all four serotypes, although the neutralizing activity was observed only for DV2. Similarly, the expression of a consensus EDIII (cEDIII) protein in *S. cervisiae* was reported by Nguyen et al. (2013). However, the results were different between

yeast- and *E. coli*-expressed cEDIIIs, in developing balanced immune responses against different serotypes.

Another EDIII-based tetravalent antigen has been produced in *Pichia pastoris*, which elicits neutralizing antibodies against all four dengue serotypes. Etemad et al. developed a tetravalent chimeric protein by fusing the EDIII of the four serotypes together, using pentaglycine peptide linkers. The designed tetravalent antigen (tandem EDIII1-2-3-4) induced neutralizing antibodies toward all four serotypes (Etemad et al. 2008). This study showed the promising potential of EDIII in developing a tetravalent dengue vaccine using *Pichia pastoris* system.

In addition to the mentioned yeast systems, in order to develop EDIII-based subunit vaccines, the capability of insect cell-based systems has also been investigated. As recently reported, Block et al. have expressed all four EDIIIs (1–4) in the insect cells (Block et al. 2010). The secreted EDIIIs used for mice immunization individually and also in a tetravalent formulation (physical mixture of four EDIIIs), which resulted in eliciting serotype specific neutralizing antibody responses. Interestingly the tetravalent formulation elicited neutralizing antibodies toward all four serotypes. To achieve balance among serotype specific neutralizing antibodies, the authors suggested dose adjustment in relative amount of each EDIII protein in tetravalent formulation.

Before the application of EDIII-based strategies, most of the efforts to develop a dengue vaccine have been based on development of monovalent (single serotype-specific) vaccines. Adjusting of balanced vaccine dose in tetravalent formulation of monovalent mixtures is a big problem. When four monovalent vaccines were mixed together into a single tetravalent formulation, immune responses were predominantly directed to one serotype with the exclusion of the others (Swaminathan and Khanna 2010). The induction of unequal levels of neutralizing antibodies against the all four serotypes can cause vaccine-induced severe dengue illnesses. EDIII-derived tetravalent fusion proteins can eliminate this problem. Taking into account the special advantages mentioned above, EDIII-based proteins have special advantages for developing of tetravalent vaccine candidates. These benefits have led to an increase in the number of reports of tetravalent dengue vaccines development by using EDIII-based recombinant/chimeric proteins (Block et al. 2010; Brandler et al. 2010; Chiang et al. 2016a, b; Izquierdo et al. 2014; Valdés et al. 2017). In this way, Bioinformatics has also been able to accelerate the design of tetravalent vaccines by using in silico methods (Fahimi et al. 2016). EDIII-based tetravalent vaccine candidates are developed in different vaccine platforms. For instance, chimeric proteins and DNA/viral vectored vaccines have developed by fusing the domain III of four serotypes (Table 2). Immunization experiments with these chimeric EDIIIs resulted in induction of neutralizing antibodies toward all four serotypes.

EDIII-based DNA and viral vectored vaccines

The DNA and vectored vaccines provide in vivo expression of target antigens. In this context, several studies have showed effectiveness of EDIII-based approaches. For instance, an EDIII-based DNA vaccine was developed by physically mixing of four individual pcDNA3 vectors containing EDIII-coding genes of four serotypes (Mota et al. 2005). Mice immunized with either individual DIII constructs or the tetravalent formulation developed antibodies against each corresponding dengue serotype. Individual EDIII plasmid/tetravalent formulations showed the neutralizing titers of 1:10 against DV2. Forty-three percent of newborn mice challenged with DV2 in combination with anti-EDIII2 serum were protected, whereas the tetravalent antiserum protected 87% of challenged mice. In this study, evaluation of possible DNA vaccine-induced cellular immune responses revealed no detectable virus-induced cell proliferation responses in immunized mice. The authors suggested that cellular responses induced by the EDIII plasmids are not stronger and to be detected in proliferation assays, in agreement with results reported by Konishi et al. (2000). In another report Ramanathan et al. developed a human codon optimized tetravalent DNA vaccine by fusing the all four consensus EDIII coding sequences together and the construction of a single ORF in a mammalian expression vector. The designed SynCon^{TM} DNA vaccine resulted in tetravalent protective immunity against all four dengue serotypes in mice. In order to separation of domains (to achieve proper protein folding) and providing proteolytic cleavage sites (for better CTL processing), they used amino acid sequence of RGRKRRS as linker between EDIII of different serotypes. The elicited tetravalent antibodies neutralized all dengue serotypes and prevented cell death induced by dengue infection (Ramanathan et al. 2009).

Khanam et al. used an adenoviral/plasmid vector expressing tandem EDIII2-4 dimer for immunization of mice. The immunization resulted in the induction of antibodies that specially bound to and neutralized the infectivity of DV2 and 4 but not DV1 and 3. Furthermore, analysis of cell-mediated immunity using in vitro re-stimulation of splenocytes from immunized mice showed a significant proliferation response accompanied by the production of high levels of cytokines (INFγ and IL-4) (Khanam et al. 2007). Another viral vectored dengue vaccine candidate was developed by using an adenovirus type 5 (AdV5) vector encoding an EDIII-based tetravalent antigen which was able to elicit immune responses against all four dengue serotypes. The expressing vector was constructed by in-frame fusion of the four EDIII-coding sequences and used for mice immunization. This rAdV5 vector induced cell-mediated immune responses and virus neutralizing antibodies specific to the all four DVs. The interesting finding of this work was that anti-AdV5 antibodies in the hyper immune sera could promote uptake of a rAdV5-

Table 2 Different strategies for development of EDIII-based dengue vaccine candidates

EDIII type-fusion tag	Vaccine strategy and host	Effectiveness in induction of protective/neutralizing immune response	References
EDIII2-NS1-protein A	Protein subunit, in *E. coli*	Neutralizing antibody and protective immunity against DV2	(Srivastava et al. 1995)
EDIII2-MBP	Protein subunit, in *E. coli*	Partial protection in mice and serotype specific virus neutralization	(Simmons et al. 1998a, b)
EDIII2-C (capsid)	Protein subunit, in *E. coli* (in the form of nucleoprotein aggregates)	Strong humoral and cellular immunity with serotype specific neutralizing antibodies and protective response in mice and monkeys against DV2	(Izquierdo et al. 2014; Suzarte et al. 2014; Valdés et al. 2017)
Consensus EDIII(1-4)*	Protein subunit, in *E. coli*	Neutralizing antibody against all serotypes in mice and against DV2 in monkeys	(Chen et al. 2013; Kim et al. 2012; Kim et al. 2013; Nguyen et al. 2013)
EDIII1-4/MBP*	Protein subunit in *E. coli*	Neutralizing antibody against all serotypes	(Simmons et al. 2001; Simmons et al. 1998a, b)
Tandem EDIII1-2-3-4*	Protein subunit, in *E. coli*	Unbalanced immunity, resulted in 70% protection against DV1, 2, and 4, and 18% against DV3	(Chen et al. 2007)
EDIII2	Protein subunit, in *Pichia pastoris*	Neutralizing antibodies against DV2	(Batra et al. 2010)
Tandem EDIII1-2-3-4*	Protein subunit, in *Pichia pastoris*	Neutralizing antibody against all serotypes	(Etemad et al. 2008)
EDIII2	Protein subunit in *E. coli*	Neutralizing antibody against DV2	(Hermida et al. 2006; Zhang et al. 2007)
tandem EDIII2-4	Protein subunit, in *E. coli*	Neutralizing antibody against DV2 and 4	(Khanam et al. 2006)
EDIII1-4*	DNA vaccine using pcDNA3	Neutralizing antibody and protection against the tested serotype (DV2)/all four serotypes	(Mota et al. 2005; Ramanathan et al. 2009)
EDIII2	Plasmid DNA and viral vaccine	Neutralizing antibody against DV2	(Khanam et al. 2007; Konishi et al. 2000; Mcburney et al. 2016; Mota et al. 2005)
EDIII1-ectoM	Chimeric live viral vaccine	Neutralizing antibodies against DV1	(Brandler et al. 2007; Brandler et al. 2010)
Tandem EDIII2-4	Adenoviral vaccine	Neutralizing antibodies and T cell responses specific to both DV2 and 4	(Khanam et al. 2007)
Tandem EDIII1-2-3-4*	Adenoviral vaccine	Neutralizing antibodies and cell-mediated immune responses to all four serotypes	(Khanam et al. 2009)
EDIII2	Protein subunit, in plant	Neutralizing antibodies against DV2	(Saejung et al. 2007)
EDIII1-4*	Protein subunit, in insect cell	Neutralizing antibodies against all four serotypes	(Block et al. 2010)
Tandem EDIII1-2-3-4*	DNA vaccine using pVAX1 plasmid (SynCon^{TM})	Neutralizing antibodies against all four serotypes and preventing cell death induced by dengue infection	(Ramanathan et al. 2009)
EDIII2-scFv	Protein subunit, in plant	Strong humoral response and neutralizing antibodies without using any adjuvant	(Coconi-Linares et al. 2013)
EDIII2-pelB	Protein subunit, in *E. coli*	Neutralizing antibodies and protection against DV2 challenge	(Yang et al. 2012)
EDIII2-P64K	Protein subunit, in *E. coli*		

Table 2 (continued)

EDIII type-fusion tag	Vaccine strategy and host	Effectiveness in induction of protective/neutralizing immune response	References
		Neutralizing antibodies and protective immunity against DV2	(Hermida et al. 2004; Izquierdo et al. 2014)
EDIII2	Recombinant *L. lactis* strain	Neutralizing antibody response	(Sim et al. 2008)
EDIII1-Ag473	Protein subunit, in *E. coli*	High levels of neutralizing antibody response	(Chen et al. 2009)

derived reporter vector into U937 cells. Authors suggested that pre-existing immunity to AdV5 may facilitate the uptake of rAdV5 vectored vaccines into antigen presenting cells and thus promote the induction of a robust antigen-specific immune response (Khanam et al. 2009). Novel EDIII-based DNA vaccines by using different strategies for immunization (DNA vaccine alone/in combination with recombinant proteins) have been recently developed (Blair et al. 2006; Lima et al. 2011; Mcburney et al. 2016; Raviprakash et al. 2006). Most of recently developed DNA vaccines resulted in induction of tetravalent immunity and neutralizing antibodies against all four serotypes.

Strategies for enhancing the immunogenicity of EDIII proteins

One of the common drawbacks of subunit protein vaccines is low immunogenicity of these antigens. In order to enhance the immunogenicity of EDIII proteins, different traditional adjuvants (e.g., Freund's adjuvant/aluminum hydroxide) and new approaches (CpG repeated DNA sequences, liposomes, and fusion tags) have been used. However, because of possible induction of strong hypersensitivity responses by complete FA, it cannot be accepted as a human vaccine component. Also safety of CpG's for human use is not clearly known yet. As an interesting alternative, liposomes are known to be safe for human use. It has reported that EDIII-based peptides could be linked to cationic liposomes as an adjuvant (Chávez et al. 2010; Wu et al. 2003b).

Combination of EDIII proteins with adjuvants is a useful approach to improve the immunogenicity of these proteins. In a study, Bernardo et al. investigated the effectiveness of two Freund's and aluminum hydroxide adjuvants on immunogenicity of a recombinant fusion protein (containing EDIII1 and P64K protein from *Neisseria meningitides*) in non-human primates (Bernardo et al. 2008). They found that vaccination by Freund's adjuvant is more immunogenic than aluminum hydroxide. Following challenge with DV1, animals which immunized by using Freund's adjuvant were protected from viremia, whereas monkeys receiving antigen in aluminum hydroxide developed a poor antibody response and were not protected from viral challenge. In another study, to enhance the immunogenicity of EDIII2-p64K fusion protein, a capsular polysaccharide from *N. meningitides* serogroup A (CPS-A), which was adsorbed on aluminum hydroxide, has been used as an effective vaccine adjuvant (Valdés et al. 2009). Babu et al. investigated the immunogenicity of a bacterially expressed EDIII4 with various adjuvants in mice (Babu et al. 2008). They showed that although EDIII4-immunized mice develop neutralizing antibody titer, but EDIII4 in combination with montanid and Freund's complete adjuvants gives highest antibody titers (1:128).

In addition to using adjuvants, application of fusion protein strategy is another different and interesting strategy to improve the immunogenicity of EDIII. For example, fusion of EDIII and bacterial A protein (Srivastava et al. 1995), MBP protein (Simmons et al. 1998a, b), lipoproteins (Chen et al. 2009), and P64K protein (Izquierdo et al. 2014; Valdés et al. 2009), have been reported previously. Lazo et al. designed a fusion protein from EDIII4 and meningococcal P64k protein carrier, by inserting two EDIII4 fragments in both sites of P64k. After immunization of mice with this fusion protein, despite the induction of low levels of antiviral antibodies, significant protection was observed against viral challenge with DV4 (Lazo et al. 2009). Furthermore, it has been shown that incorporation of the dengue capsid protein beside the EDIII can further induce cell-mediate immunity (Suzarte et al. 2014, 2015). The EDIII-based heterodimers are the other strategy in the development of fusion proteins to enhance the immunogenicity. In this field, Chiang et al. have reported a novel approach (Chiang et al. 2013). They expressed a recombinant lapidated EDIII1 (LD1EDIII) in an *E. coli* system. This new lipoprotein that contains a bacterial lipid moiety, as a potent immunomodulator, showed an inherent immunostimulation effect with activating macrophage cells. Whereas, its non-lipidated form (EDIII1) did not show similar effect. They also showed that LD1EDIII in combination with a multiphase emulsion system increased the antigen association more effective way than antigen alone. In fact, application of newly developed water-in-oil-in-water multiphase emulsion system (termed PELC) is one of the recent approaches to enhance the immunogenicity of EDIII proteins. Efficiency of PELC in potentiating the protective capacity of EDIII1 has recently reported (Chiang et al. 2012). Unlike aluminum phosphate, EDIII1 formulated with PELC plus CpG oligodeoxynucleotides induced neutralizing antibodies against DV1 and increased the INFγ secretion by splenocytes after in vitro re-stimulation (Chiang et al. 2012, 2013).

EDIII2-HBcAg is another heterodimeric fusion protein that has been developed by Arora et al. (2012). They showed that bacterially expressed EDIII2 in fusion with Hepatitis B virus core antigen (HBcAg) assembles into 35–40 nm VLPs. The produced VLPs were highly immunogenic in eliciting high titer antibodies which were able to bind and neutralized DV2. The authors suggested that EDIII-displaying nanoparticles may have potential applications in diagnostic/vaccine for dengue.

As mentioned above, Brandler et al. have used live attenuated Schwarz measles vaccine (MV) as a vector containing EDIII1 coding gene which fused to the ectodomain of the membrane protein from dengue type 1 (EDIII1-ectoM). They showed that the presence of ectoM is critical to the immunogenicity of inserted EDIII1. The remarkable adjuvant capacity of ectoM in immunogenicity of EDIII was correlated with its ability to promote the maturation of dendritic cells and the secretion of a panel of antiviral cytokines and chemokines involved in adaptive immunity (Brandler et al. 2007).

In the context of fusion of EDIIIs to lipoproteins, Chen et al. showed the effectiveness of Ag473 lipoprotein of *N. meningitidis*. According to this study, the EDIII-Ag473 fusion conferred induction of higher levels of neutralizing antibodies than EDIII alone or formulated in alum (Chen et al. 2009). Investigation on the mechanism of this enhancement revealed that EDIII-Ag473 activates antigen presenting cells (APCs) as an intrinsic adjuvant. The authors discussed that the adjuvant activity appears to be due to stimulation of innate immunity through the TLR2 signaling pathway, triggered by recombinant EDIII-lipoprotein (Leng et al. 2010).

Another alternative method to enhance immunogenicity of EDIII is to target the antigen to dendritic cells in order to induce T cells for broad antibody responses. Coconi-Linares et al. fused EDIII2 to a single chain antibody fragment (scFv), which was raised against the DEC-205 receptor. This recombinant fusion protein was expressed in *Nicotiana benthamiana* and used for mice immunization, which resulted in specific and strong humoral responses with anti-dengue neutralizing antibodies (Coconi-Linares et al. 2013). The main point in this approach was that only two immunizations were required to generate a memory response without the presence of adjuvants.

Oligonucleotides are also new adjuvants that can be used in either protein- or DNA-based approaches. The effectiveness of DNA oligonucleotides in induction of immune system has been previously reported. Marcos et al. showed the ability of the viral capsid/EDIII proteins in combination with DNA oligonucleotides (ODN) in producing nucleocapsid-like particles (NLPs) (Marcos et al. 2013). Several studies indicated the adjuvant capacity of ODNs in the induction of cell-mediated immunity against the target antigens (Gil et al. 2015; Vollmer 2006). The adjuvant capacity of NLPs was also reported by Lazo et al., where they reported that immunization of mice with a formulation containing NLPs of DV2 with chimeric EDIII4-P64k protein results in high levels of antiviral antibodies (Lazo et al. 2010). As a different strategy, the application of other carrier proteins with immune-stimulatory properties such as cholera toxin B subunit (CTB) has been suggested (Kim et al. 2016). Furthermore, in DNA vaccine context, granulocyte-macrophage colony-stimulating factor (GM-CSF) gene is one of the most attractive adjuvants (Zheng et al. 2011).

In addition to the mentioned strategies to improve the immunogenicity of dengue EDIII-based antigens, there are some other strategies that have been used for enhancement of antigenicity of other non-dengue flaviviral EDIIIs. Similarly, we suggest these strategies in the case of dengue vaccine research. For instance, a subunit vaccine against West Nile Virus (WNV) has been developed by fusing the EDIII of the WNV to STF2Delta. STF2Delta is a modified version of bacterial flagellin that has been used as an adjuvant (Huleatt et al.

2007). Immunization of mice with this fusion protein (without any routine adjuvant) elicited strong anti-WNV antibody response that neutralized viral infectivity and conferred protection against viral challenge. In another strategy by Spohn et al., a WNV vaccine candidate developed by chemically coupling of a recombinant EDIII to viral like particles (VLPs). Unlike the individual EDIII of WNV, which required three immunizations to achieve antibody response, a strong immune response was achieved after just a single injection of EDIII-VLPs. The induced antibodies were able to neutralize the virus in vitro and provide protection against viral challenge (Spohn et al. 2010).

Although there is a growing evidence of effectiveness of EDIII-based strategies in induction of protective immunity in animal models, some studies have provided special considerations about application of EDIII-based strategies for human vaccine development. As showed by Wahala et al., EDIII-binding antibodies make just a small number of the total antibody in human immune sera binding to DVs. According to their study, EDIII-binding antibodies in human sera play a minor role in virus neutralization (Wahala et al. 2009). In this context, some studies suggested the heterologous prime-boost strategy using EDIII proteins to direct immune system to specific neutralizing antibodies on EDIII protein. Recent studies done by Zlatkovic et al. revealed the low potency of WNV EDIII for priming, but excellent boosting effect was shown for this protein. They suggested a new heterologous prime-boost vaccination strategy, which primes by inactive virus/complete E protein and is followed by using EDIII as a booster antigen (Zlatkovic et al. 2011). Valdes et al. have used a similar heterologous prime-boost strategy by prime immunization of monkeys with inactive DV2 and boosting by a recombinant EDIII-Capsid fusion protein. They found that animals developed a neutralizing antibody response following primary vaccination and the immune response significantly boosted after the second immunization by recombinant EDIII (Valdés et al. 2011). The authors concluded that EDIII protein as a perfect booster antigen is able to recall the neutralizing antibody response elicited following viral infection in monkeys. Hence, it is suggested to be considered the booster effect of rEDIIIs in future vaccine strategies.

Conclusion and future perspectives

Recently, because of the spread of the mosquito vectors and worldwide trips, the occurrence of dengue infection has alarmingly increased. The vaccination could provide a promising approach for controlling dengue virus infections. Several research groups worldwide are exploring different approaches towards developing dengue vaccine on the basis of conventional live attenuated viruses, inactivated viruses, infectious clone-derived attenuated viruses, DNA/viral vaccines, and recombinant dengue viral proteins expressed in heterologous systems (Tables 1 and 2). Each approach of dengue vaccine development has its own advantages and disadvantages. Protein subunit vaccines offer several advantages over other approaches such as follows:

- No biohazard problem because of the lack of a replicating virus (which is a major problem in live attenuated and inactivated vaccines.)
- Easy to produce and scale up and can be inexpensive
- Special ability to induce a balanced tetravalent immune response compared to four replicating viruses (in the context of a tetravalent formulation) (Swaminathan and Khanna 2010).

Therefore, the recombinant subunit vaccines may provide an attractive alternative to live attenuated/killed vaccines.

Dengue virus envelope protein (E protein) is the most attractive viral protein in both vaccine and diagnostic purposes. Many attempts have been made to produce a recombinant dengue E protein in a heterologous expression system including *Escherichia coli*, yeast, insect, and plant (Table 2). Most of attempts for production of E protein in *E. coli* result in formation of an insoluble protein or an inclusion body, which further requires subsequent complicated and time-consuming denaturation/refolding for protein purification. As large proteins such as E protein tend to be expressed at lower levels, the recent attempts directed to the expressing a small and biologically critical domain (domain III) of the E protein in *E. coli*. Furthermore, chimeric proteins containing domain III have been demonstrated to be immunogenic, capable of inducing neutralizing antibodies in experimental animals. The most important benefit of EDIII-base vaccine is although EDIII represents only a fraction of the envelope protein, the removal of other epitopes that elicit non-neutralizing, cross-reactive antibodies should reduce the risk of disease progression to DHF or DSS. It is appear that a safe and effective vaccine must target neutralizing antibody responses away from less accessible and weakly neutralizing, to freely accessible and strongly neutralizing epitopes (Apt et al. 2006). Even though EDIII presents only a fraction the E protein, the absence of other epitopes which elicit non-neutralizing, cross-reactive antibodies could have advantages in reducing risk for developing ADE. In this context, the EDIII of the E protein has emerged as a very promising vaccine candidate in recent years. It is very important presenting antigens from all serotypes in a manner to elicit immune responses in equal proportion. Whenever a dengue vaccine does not provide protection against all four serotypes, a vaccinated individual will be susceptible to development of a more severe disease (DHF/DSS) if infected by untargeted serotypes. One

approach that has been suggested to minimize the possibility of antibody-dependent infection enhancement occurring after vaccination is to design a tetravalent subunit dengue vaccine. This type of vaccine contains epitopes inducing high levels of specific neutralizing antibody to all four serotypes, but contains a minimum of dengue complex cross-reactive epitopes which are presumably responsible for the induction of enhancing antibodies (Simmons et al. 1998a, b).

To achieve equal expression of antigens from all four serotypes, it should design the EDIII antigens of the four serotypes of virus as an individual protein. However, the detailed mechanism of dengue infection and also vaccine provided protection are not clearly explained, up to now. In addition, for designing a dengue vaccine which is protective against all four serotypes, without any potential risk of disease severity enhancement, the molecular mechanism of dengue pathogenesis must be considered (Miller 2010; Murrell et al. 2011). It is expected that in the coming years more data on dengue pathogenesis and protection mechanisms will be available. The new data from Phase III clinical trials of current dengue LAVs/chimeric vaccine candidates and also more evaluations of newly developed EDIII-based vaccines will help us to have a better knowledge about the mechanism of protection.

In conclusion, dengue vaccine researches especially in the field of tetravalent vaccines have increased in the last years and evaluation of these recent efforts shows that dengue domain III is the basic substance in the most of the reported studies. Now, construction of tandem repeats of consensus EDIIIs (tetravalent EDIII-based antigens) which elicit neutralizing antibodies against the all four serotypes of dengue virus is the broadly accepted approach for development of a desired vaccine. Reviewing of the findings from the recent studies has showed the effectiveness of EDIII-based approaches in tetravalent dengue vaccine design. On the other hand, new approaches by using DNA shuffling, bioinformatics, and immunoinformatics methods have enhanced the vaccine design progress (Fahimi et al. 2016; Sánchez-Burgos et al. 2010). Many promising researches have been published in the field of rational design of vaccines against dengue virus, which the most cases have been performed on the base of dengue virus E protein domain III. Despite the advent of first licensed dengue vaccine (Dengvaxia®), there are many universities, research centers, and companies which are being competition to achieve a safer and effective dengue vaccine. It seems that the current promising researches on dengue vaccines will lead us to achieve a desirable human vaccine in the near future.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

References

Alvarez M, Rodriguez-Roche R, Bernardo L, Vazquez S, Morier L, Gonzalez D, Castro O, Kouri G, Halstead SB, Guzman MG (2006) Dengue hemorrhagic fever caused by sequential dengue 1–3 virus infections over a long time interval: Havana epidemic, 2001–2002. Am J Trop Med Hyg 75(6):1113–1117

Amitai G, Callahan BP, Stanger MJ, Belfort G, Belfort M (2009) Modulation of intein activity by its neighboring extein substrates. Proc Natl Acad Sci 106(27):11005–11010

Anantapreecha S, Chanama S, A-nuegoonpipat A, Naemkhunthot S, Sa-Ngasang A, Sawanpanyalert P, Kurane I (2005) Serological and virological features of dengue fever and dengue haemorrhagic fever in Thailand from 1999 to 2002. Epidemiol Infect 133(3):503–507

Apt D, Raviprakash K, Brinkman A, Semyonov A, Yang S, Skinner C, Diehl L, Lyons R, Porter K, Punnonen J (2006) Tetravalent neutralizing antibody response against four dengue serotypes by a single chimeric dengue envelope antigen. Vaccine 24(3):335–344

Arora U, Tyagi P, Swaminathan S, Khanna N (2012) Chimeric Hepatitis B core antigen virus-like particles displaying the envelope domain III of dengue virus type 2. J Nanobiotechnol 10(1):30

Babu JP, Pattnaik P, Gupta N, Shrivastava A, Khan M, Rao PL (2008) Immunogenicity of a recombinant envelope domain III protein of dengue virus type-4 with various adjuvants in mice. Vaccine 26(36):4655–4663

Balsitis SJ, Williams KL, Lachica R, Flores D, Kyle JL, Mehlhop E, Johnson S, Diamond MS, Beatty PR, Harris E (2010) Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. PLoS Pathog 6(2):e1000790

Batra G, Gurramkonda C, Nemani SK, Jain SK, Swaminathan S, Khanna N (2010) Optimization of conditions for secretion of dengue virus type 2 envelope domain III using *Pichia pastoris*. J Biosci Bioeng 110(4):408–414

Bernardo L, Izquierdo A, Alvarez M, Rosario D, Prado I, López C, Martínez R, Castro J, Santana E, Hermida L (2008) Immunogenicity and protective efficacy of a recombinant fusion protein containing the domain III of the *dengue* 1 envelope protein in non-human primates. Antivir Res 80(2):194–199

Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O (2013) The global distribution and burden of dengue. Nature 496(7446):504

Bischof GF, Magnani DM, Ricciardi M, Shin YC, Domingues A, Bailey VK, Gonzalez-Nieto L, Rakasz EG, Watkins DI, Desrosiers RC (2017) Use of a recombinant gamma-2 herpesvirus vaccine vector against dengue virus in rhesus monkeys. J Virol 91:00525–00517

Blair PJ, Kochel TJ, Raviprakash K, Guevara C, Salazar M, Wu S-J, Olson JG, Porter KR (2006) Evaluation of immunity and protective efficacy of a dengue-3 premembrane and envelope DNA vaccine in Aotus nancymae monkeys. Vaccine 24(9):1427–1432

Blaney JE, Sathe NS, Hanson CT, Firestone CY, Murphy BR, Whitehead SS (2007) Vaccine candidates for dengue virus type 1 (DEN1) generated by replacement of the structural genes of rDEN4 and rDEN4Δ30 with those of DEN1. Virol J 4(1):23

Blaney JE, Sathe NS, Goddard L, Hanson CT, Romero TA, Hanley KA, Murphy BR, Whitehead SS (2008) Dengue virus type 3 vaccine candidates generated by introduction of deletions in the 3′ untranslated region (3′-UTR) or by exchange of the DENV-3 3′-UTR with that of DENV-4. Vaccine 26(6):817–828

Block OK, Rodrigo WSI, Quinn M, Jin X, Rose RC, Schlesinger JJ (2010) A tetravalent recombinant dengue domain III protein vaccine

stimulates neutralizing and enhancing antibodies in mice. Vaccine 28(51):8085–8094

Brady OJ, Gething PW, Bhatt S, Messina JP, Brownstein JS, Hoen AG, Moyes CL, Farlow AW, Scott TW, Hay SI (2012) Refining the global spatial limits of dengue virus transmission by evidence-based consensus. PLoS Negl Trop Dis 6(8):e1760

Brandler S, Lucas-Hourani M, Moris A, Frenkiel M-P, Combredet C, Février M, Bedouelle H, Schwartz O, Desprès P, Tangy F (2007) Pediatric measles vaccine expressing a dengue antigen induces durable serotype-specific neutralizing antibodies to dengue virus. PLoS Negl Trop Dis 1(3):e96

Brandler S, Ruffie C, Najburg V, Frenkiel M-P, Bedouelle H, Desprès P, Tangy F (2010) Pediatric measles vaccine expressing a dengue tetravalent antigen elicits neutralizing antibodies against all four dengue viruses. Vaccine 28(41):6730–6739

Bravo J, Guzman M, Kouri G (1987) Why dengue haemorrhagic fever in Cuba? I. Individual risk factors for dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). Trans R Soc Trop Med Hyg 81(5):816–820

Chávez JH, Silva JR, Amarilla AA, Figueiredo LTM (2010) Domain III peptides from flavivirus envelope protein are useful antigens for serologic diagnosis and targets for immunization. Biologicals 38(6):613–618

Chen S, Yu M, Jiang T, Deng Y, Qin C, Qin E (2007) Induction of tetravalent protective immunity against four dengue serotypes by the tandem domain III of the envelope protein. DNA Cell Biol 26(6):361–367

Chen H-W, Liu S-J, Liu H-H, Kwok Y, Lin C-L, Lin L-H, Chen M-Y, Tsai J-P, Chang L-S, Chiu F-F (2009) A novel technology for the production of a heterologous lipoprotein immunogen in high yield has implications for the field of vaccine design. Vaccine 27(9):1400–1409

Chen H-W, Liu S-J, Li Y-S, Liu H-H, Tsai J-P, Chiang C-Y, Chen M-Y, Hwang C-S, Huang C-C, Hu H-M (2013) A consensus envelope protein domain III can induce neutralizing antibody responses against serotype 2 of dengue virus in non-human primates. Arch Virol 158(7):1523–1531

Chen XY, Li DZ, Zhong XZ, Chen B, Duan ZL, Wen JS (2016) Induction of multiple cytotoxic T lymphocyte responses in mice by a multiepitope DNA vaccine against dengue virus serotype 1. Microbiol Immunol 60(12):835–845

Chiang C-Y, Huang M-H, Hsieh C-H, Chen M-Y, Liu H-H, Tsai J-P, Li Y-S, Chang C-Y, Liu S-J, Chong P (2012) Dengue-1 envelope protein domain III along with PELC and CpG oligodeoxynucleotides synergistically enhances immune responses. PLoS Negl Trop Dis 6(5):e1645

Chiang C-Y, Huang M-H, Pan C-H, Hsieh C-H, Chen M-Y, Liu H-H, Tsai J-P, Liu S-J, Chong P, Leng C-H (2013) Induction of robust immunity by the emulsification of recombinant lipidated dengue-1 envelope protein domain III. Microbes Infect 15(10):719–728

Chiang C-Y, Liu S-J, Hsieh C-H, Chen M-Y, Tsai J-P, Liu H-H, Chen I-H, Chong P, Leng C-H, Chen H-W (2016a) Recombinant lipidated dengue-3 envelope protein domain III stimulates broad immune responses in mice. Vaccine 34(8):1054–1061

Chiang C-Y, Pan C-H, Chen M-Y, Hsieh C-H, Tsai J-P, Liu H-H, Liu S-J, Chong P, Leng C-H, Chen H-W (2016b) Immunogenicity of a novel tetravalent vaccine formulation with four recombinant lipidated dengue envelope protein domain IIIs in mice. Sci Rep 6:30648

Chin J, Chu J, Ng M (2007) The envelope glycoprotein domain III of dengue virus serotypes 1 and 2 inhibit virus entry. Microbes Infect 9(1):1–6

Clements DE, Coller B-AG, Lieberman MM, Ogata S, Wang G, Harada KE, Putnak JR, Ivy JM, McDonell M, Bignami GS (2010) Development of a recombinant tetravalent dengue virus vaccine: immunogenicity and efficacy studies in mice and monkeys. Vaccine 28(15):2705–2715

Coconi-Linares N, Ortega-Dávila E, López-González M, García-Machorro J, García-Cordero J, Steinman RM, Cedillo-Barrón L, Gómez-Lim MA (2013) Targeting of envelope domain III protein of DENV type 2 to DEC-205 receptor elicits neutralizing antibodies in mice. Vaccine 31(19):2366–2371

Crill WD, Roehrig JT (2001) Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. J Virol 75(16):7769–7773

Delenda C, Staropoli I, Frenkiel M-P, Cabanié L, Deubel V (1994) Analysis of C-terminally truncated dengue 2 and dengue 3 virus envelope glycoproteins: processing in insect cells and immunogenic properties in mice. J Gen Virol 75(7):1569–1578

Durbin AP, Whitehead SS, McArthur J, Perreault JR, Blaney Jr JE, Thumar B, Murphy BR, Karron RA (2005) rDEN4Δ30, a live attenuated dengue virus type 4 vaccine candidate, is safe, immunogenic, and highly infectious in healthy adult volunteers. J Infect Dis 191(5):710–718

Edelman R, Hombach J (2008) "Guidelines for the clinical evaluation of dengue vaccines in endemic areas": summary of a World Health Organization technical consultation. Vaccine 26(33):4113–4119

Edelman R, Wasserman SS, Bodison SA, Putnak RJ, Eckels KH, Tang D, Kanesa-Thasan N, Vaughn DW, Innis BL, Sun W (2003) Phase I trial of 16 formulations of a tetravalent live-attenuated dengue vaccine. Am J Trop Med Hyg 69(6_suppl):48–60

Etemad B, Batra G, Raut R, Dahiya S, Khanam S, Swaminathan S, Khanna N (2008) An envelope domain III-based chimeric antigen produced in *Pichia pastoris* elicits neutralizing antibodies against all four dengue virus serotypes. Am J Trop Med Hyg 79(3):353–363

Fahimi H, Allahyari H, Hassan ZM, Sadeghizadeh M (2014) Dengue virus type-3 envelope protein domain III; expression and immunogenicity. Iran J Basic Med Sci 17(11):836

Fahimi H, Sadeghizadeh M, Mohammadipour M (2016) In silico analysis of an envelope domain III-based multivalent fusion protein as a potential dengue vaccine candidate. Clin Exp Vaccine Res 5(1):41–49

Falconar AK (2007) Antibody responses are generated to immunodominant ELK/KLE-type motifs on the nonstructural-1 glycoprotein during live dengue virus infections in mice and humans: implications for diagnosis, pathogenesis, and vaccine design. Clin Vaccine Immunol 14(5):493–504

Fonseca BA, Khoshnood K, Shope RE, Mason PW (1991) Flavivirus type-specific antigens produced from fusions of a portion of the E protein gene with the *Escherichia coli* trpE gene. Am J Trop Med Hyg 44(5):500–508

Fried JR, Gibbons RV, Kalayanarooj S, Thomas SJ, Srikiatkhachorn A, Yoon I-K, Jarman RG, Green S, Rothman AL, Cummings DA (2010) Serotype-specific differences in the risk of dengue hemorrhagic fever: an analysis of data collected in Bangkok, Thailand from 1994 to 2006. PLoS Negl Trop Dis 4(3):e617. https://doi.org/10.1371/journal.pntd.0000617

Gil L, Marcos E, Izquierdo A, Lazo L, Valdés I, Ambala P, Ochola L, Hitler R, Suzarte E, Álvarez M (2015) The protein DIIIC-2, aggregated with a specific oligodeoxynucleotide and adjuvanted in alum, protects mice and monkeys against DENV-2. Immunol Cell Biol 93(1):57–66

Gil L, Lazo L, Valdés I, Suzarte E, Yen P, Ramírez R, Álvarez M, Dung LT, Cobas K, Marcos E (2017) The tetravalent formulation of domain III-capsid proteins recalls memory B-and T-cell responses induced in monkeys by an experimental dengue virus infection. Clin Transl Immunol 6(6):e148. https://doi.org/10.1038/cti.2017.24

Goeddel DV (1990) [1] Systems for heterologous gene expression. Methods Enzymol 185:3–7

Gonçalves AJ, Oliveira ER, Costa SM, Paes MV, Silva JF, Azevedo AS, Mantuano-Barradas M, Nogueira ACM, Almeida CJ, Alves AM (2015) Cooperation between CD4+ T cells and humoral immunity is critical for protection against dengue using a DNA vaccine based

on the NS1 antigen. PLoS Negl Trop Dis 9(12):e0004277. https://doi.org/10.1371/journal.pntd.0004277

González D, Castro OE, Kourí G, Perez J, Martinez E, Vazquez S, Rosario D, Cancio R, Guzman MG (2005) Classical dengue hemorrhagic fever resulting from two dengue infections spaced 20 years or more apart: Havana, Dengue 3 epidemic, 2001–2002. Int J Infect Dis 9(5):280–285

Gromowski GD, Barrett ND, Barrett AD (2008) Characterization of dengue virus complex-specific neutralizing epitopes on envelope protein domain III of dengue 2 virus. J Virol 82(17):8828–8837

Gubler DJ (2002) Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol 10(2):100–103

Gunther V, Putnak R, Eckels K, Mammen M, Scherer J, Lyons A, Sztein M, Sun W (2011) A human challenge model for dengue infection reveals a possible protective role for sustained interferon gamma levels during the acute phase of illness. Vaccine 29(22):3895–3904

Guy B, Almond JW (2008) Towards a dengue vaccine: progress to date and remaining challenges. Comp Immunol Microbiol Infect Dis 31(2):239–252

Guy B, Nougarede N, Begue S, Sanchez V, Souag N, Carre M, Chambonneau L, Morrisson DN, Shaw D, Qiao M (2008) Cell-mediated immunity induced by chimeric tetravalent dengue vaccine in naive or flavivirus-primed subjects. Vaccine 26(45):5712–5721

Guy B, Noriega F, Ochiai RL, L'azou M, Delore V, Skipetrova A, Verdier F, Coudeville L, Savarino S, Jackson N (2017) A recombinant live attenuated tetravalent vaccine for the prevention of dengue. Expert Rev Vaccines 16(7):1–13

Guzman MG, Hermida L, Bernardo L, Ramirez R, Guillén G (2010) Domain III of the envelope protein as a dengue vaccine target. Expert Rev Vaccines 9(2):137–147

Haddad Kashani H, Fahimi H, Dasteh Goli Y, Moniri R (2017) A novel chimeric endolysin with antibacterial activity against methicillin-resistant Staphylococcus aureus. Front Cell Infect Microbiol 7:290

Halstead SB (1988) Pathogenesis of dengue: challenges to molecular biology. Science 239(4839):476–481

Halstead SB (2003) Neutralization and antibody-dependent enhancement of dengue viruses. Adv Virus Res 60:421–467

Henchal EA, Putnak JR (1990) The dengue viruses. Clin Microbiol Rev 3(4):376–396

Hermida L, Rodríguez R, Lazo L, Bernardo L, Silva R, Zulueta A, Lopez C, Martín J, Valdés I, Del Rosario D (2004) A fragment of the envelope protein from dengue-1 virus, fused in two different sites of the meningococcal P64k protein carrier, induces a functional immune response in mice. Biotechnol Appl Biochem 39(1):107–114

Hermida L, Bernardo L, Martín J, Alvarez M, Prado I, López C, Sierra BC, Martínez R, Rodríguez R, Zulueta A (2006) A recombinant fusion protein containing the domain III of the dengue-2 envelope protein is immunogenic and protective in nonhuman primates. Vaccine 24(16):3165–3171

Hosseini ES, Moniri R, Goli YD, Kashani HH (2016) Purification of antibacterial CHAPK protein using a self-cleaving fusion tag and its activity against methicillin-resistant *Staphylococcus aureus*. Probiotics Antimicrob Proteins 8(4):202–210

Huang K-J, Yang Y-C, Lin Y-S, Huang J-H, Liu H-S, Yeh T-M, Chen S-H, Liu C-C, Lei H-Y (2006) The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. J Immunol 176(5):2825–2832

Huleatt JW, Foellmer HG, Hewitt D, Tang J, Desai P, Price A, Jacobs A, Takahashi VN, Huang Y, Nakaar V (2007) A West Nile virus recombinant protein vaccine that coactivates innate and adaptive immunity. J Infect Dis 195(11):1607–1617

Hung J-J, Hsieh M-T, Young M-J, Kao C-L, King C-C, Chang W (2004) An external loop region of domain III of dengue virus type 2 envelope protein is involved in serotype-specific binding to mosquito but not mammalian cells. J Virol 78(1):378–388

Hurtado-Melgoza M, Ramos-Ligonio A, Álvarez-Rodríguez L, Meza-Menchaca T, López-Monteon A (2016) Differential humoral and cellular immunity induced by vaccination using plasmid DNA and protein recombinant expressing the NS3 protein of dengue virus type 3. J Biomed Sci 23(1):85

Innis BL, Eckels KH (2003) Progress in development of a live-attenuated, tetravalent dengue virus vaccine by the United States Army Medical Research and Materiel Command. Am J Trop Med Hyg 69(6 suppl): 1–4

Izquierdo A, García A, Lazo L, Gil L, Marcos E, Alvarez M, Valdés I, Hermida L, Guillén G, Guzmán MG (2014) A tetravalent dengue vaccine containing a mix of domain III-P64k and domain III-capsid proteins induces a protective response in mice. Arch Virol 159(10): 2597–2604

Jaiswal S, Khanna N, Swaminathan S (2004) High-level expression and one-step purification of recombinant dengue virus type 2 envelope domain III protein in *Escherichia coli*. Protein Expr Purif 33(1):80–91

Kashani HH, Moniri R (2015) Expression of recombinant pET22b-LysK-cysteine/histidine-dependent amidohydrolase/peptidase bacteriophage therapeutic protein in Escherichia coli BL21 (DE3). Osong Public Health Res Perspect 6(4):256–260

Kashani HH, Schmelcher M, Sabzalipoor H, Hosseini ES, Moniri R (2018) Recombinant endolysins as potential therapeutics against antibiotic-resistant *Staphylococcus aureus*: current status of research and novel delivery strategies. Clin Microbiol Rev 31(1):e00071–e00017

Kelly EP, Greene JJ, King AD, Innis BL (2000) Purified dengue 2 virus envelope glycoprotein aggregates produced by baculovirus are immunogenic in mice. Vaccine 18(23):2549–2559

Khanam S, Etemad B, Khanna N, Swaminathan S (2006) Induction of neutralizing antibodies specific to dengue virus serotypes 2 and 4 by a bivalent antigen composed of linked envelope domains III of these two serotypes. Am J Trop Med Hyg 74(2):266–277

Khanam S, Rajendra P, Khanna N, Swaminathan S (2007) An adenovirus prime/plasmid boost strategy for induction of equipotent immune responses to two dengue virus serotypes. BMC Biotechnol 7(1):10

Khanam S, Pilankatta R, Khanna N, Swaminathan S (2009) An adenovirus type 5 (AdV5) vector encoding an envelope domain III-based tetravalent antigen elicits immune responses against all four dengue viruses in the presence of prior AdV5 immunity. Vaccine 27(43): 6011–6021

Khetarpal N, Shukla R, Rajpoot RK, Poddar A, Pal M, Swaminathan S, Arora U, Khanna N (2017) Recombinant dengue virus 4 envelope glycoprotein virus-like particles derived from *Pichia pastoris* are capable of eliciting homotypic domain III-directed neutralizing antibodies. Am J Trop Med Hyg 96(1):126–134

Kim M-Y, Yang M-S, Kim T-G (2012) Expression of a consensus dengue virus envelope protein domain III in transgenic callus of rice. Plant Cell Tissue Organ Cult (PCTOC) 109(3):509–515

Kim T-G, Kim M-Y, Huy N-X, Kim S-H, Yang M-S (2013) M cell-targeting ligand and consensus dengue virus envelope protein domain III fusion protein production in transgenic rice calli. Mol Biotechnol 54(3):880–887

Kim M-Y, Kim B-Y, Oh S-M, Reljic R, Jang Y-S, Yang M-S (2016) Oral immunisation of mice with transgenic rice calli expressing cholera toxin B subunit fused to consensus dengue cEDIII antigen induces antibodies to all four dengue serotypes. Plant Mol Biol 92(3):347–356

Kliks SC, Nimmanitya S, Nisalak A, Burke DS (1988) Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am J Trop Med Hyg 38(2): 411–419

Konishi E, Yamaoka M, Kurane I, Mason PW (2000) A DNA vaccine expressing dengue type 2 virus premembrane and envelope genes induces neutralizing antibody and memory B cells in mice. Vaccine 18(11):1133–1139

Konishi E, Kosugi S, Imoto J-i (2006) Dengue tetravalent DNA vaccine inducing neutralizing antibody and anamnestic responses to four serotypes in mice. Vaccine 24(12):2200–2207

Kuhn RJ, Zhang W, Rossmann MG, Pletnev SV, Corver J, Lenches E, Jones CT, Mukhopadhyay S, Chipman PR, Strauss EG (2002) Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell 108(5):717–725

Kuruvilla JG, Troyer RM, Devi S, Akkina R (2007) Dengue virus infection and immune response in humanized RAG2−/− γ c−/−(RAG-hu) mice. Virology 369(1):143–152

Lazo Vázquez L, Gil González L, Marcos López E, Pérez Fuentes Y, Cervetto de Armas L, Brown Richards E, Valdés Prado I, Suzarte Portal E, Cobas Acosta K, Yaugel Novoa M (2017) Evaluation in mice of the immunogenicity of a tetravalent subunit vaccine candidate against dengue virus using mucosal and parenteral immunization routes. Viral Immunol 30(5):350–358

Lazo L, Zulueta A, Hermida L, Blanco A, Sánchez J, Valdés I, Gil L, López C, Romero Y, Guzmán MG (2009) Dengue-4 envelope domain III fused twice within the meningococcal P64k protein carrier induces partial protection in mice. Biotechnol Appl Biochem 52(4): 265–271

Lazo L, Gil L, Lopez C, Valdes I, Marcos E, Álvarez M, Blanco A, Romero Y, Falcon V, Guzmán MG (2010) Nucleocapsid-like particles of dengue-2 virus enhance the immune response against a recombinant protein of dengue-4 virus. Arch Virol 155(10):1587–1595

Leng C-H, Liu S-J, Tsai J-P, Li Y-S, Chen M-Y, Liu H-H, Lien S-P, Yueh A, Hsiao K-N, Lai L-W (2009) A novel dengue vaccine candidate that induces cross-neutralizing antibodies and memory immunity. Microbes Infect 11(2):288–295

Leng C-H, Chen H-W, Chang L-S, Liu H-H, Liu H-Y, Sher Y-P, Chang Y-W, Lien S-P, Huang T-Y, Chen M-Y (2010) A recombinant lipoprotein containing an unsaturated fatty acid activates NF-κB through the TLR2 signaling pathway and induces a differential gene profile from a synthetic lipopeptide. Mol Immunol 47(11):2015–2021

Li X-Q, Qiu L-W, Chen Y, Wen K, Cai J-P, Chen J, Pan Y-X, Li J, Hu D-M, Huang Y-F (2013) Dengue virus envelope domain III immunization elicits predominantly cross-reactive, poorly neutralizing antibodies localized to the AB loop: implications for dengue vaccine design. J Gen Virol 94(10):2191–2201

Liao M, Kielian M (2005) Domain III from class II fusion proteins functions as a dominant-negative inhibitor of virus membrane fusion. J Cell Biol 171(1):111–120

Lima DM, de Paula SO, de Oliveira Franca RF, Palma PV, Morais FR, Gomes-Ruiz AC, de Aquino MTP, da Fonseca BAL (2011) A DNA vaccine candidate encoding the structural prM/E proteins elicits a strong immune response and protects mice against dengue-4 virus infection. Vaccine 29(4):831–838

Lin B, Parrish CR, Murray JM, Wright PJ (1994) Localization of a neutralizing epitope on the envelope protein of dengue virus type 2. Virology 202(2):885–890

Lin C-F, Wan S-W, Chen M-C, Lin S-C, Cheng C-C, Chiu S-C, Hsiao Y-L, Lei H-Y, Liu H-S, Yeh T-M (2008) Liver injury caused by antibodies against dengue virus nonstructural protein 1 in a murine model. Lab Investig 88(10):1079

Lindenbach BD (2001) Flaviviridae. In: Fields virology. Lippincott Williams & Wilkins, Philadelphia, pp 991–1042

Mani S, Tripathi L, Raut R, Tyagi P, Arora U, Barman T, Sood R, Galav A, Wahala W, de Silva A (2013) *Pichia pastoris*-expressed dengue 2 envelope forms virus-like particles without pre-membrane protein and induces high titer neutralizing antibodies. PLoS One 8(5): e64595. https://doi.org/10.1371/journal.pone.0064595

Marcos E, Gil L, Lazo L, Izquierdo A, Brown E, Suzarte E, Valdés I, García A, Méndez L, Guzmán MG (2013) Purified and highly aggregated chimeric protein DIIIC-2 induces a functional immune response in mice against dengue 2 virus. Arch Virol 158(1):225–230

Mason PW, Zügel MU, Semproni AR, Fournier MJ, Mason TL (1990) The antigenic structure of dengue type 1 virus envelope and NS1 proteins expressed in *Escherichia coli*. J Gen Virol 71(9):2107–2114

McArthur JH, Durbin AP, Marron JA, Wanionek KA, Thumar B, Pierro DJ, Schmidt AC, Blaney JE Jr, Murphy BR, Whitehead SS (2008) Phase I clinical evaluation of rDEN4Δ30-200,201: a live attenuated dengue 4 vaccine candidate designed for decreased hepatotoxicity. Am J Trop Med Hyg 79(5):678–684

McBurney SP, Sunshine JE, Gabriel S, Huynh JP, Sutton WF, Fuller DH, Haigwood NL, Messer WB (2016) Evaluation of protection induced by a dengue virus serotype 2 envelope domain III protein scaffold/DNA vaccine in non-human primates. Vaccine 34(30):3500–3507

Miller N (2010) Recent progress in dengue vaccine research and development. Curr Opin Mol Ther 12(1):31–38

Modis Y, Ogata S, Clements D, Harrison SC (2003) A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc Natl Acad Sci 100(12):6986–6991

Modis Y, Ogata S, Clements D, Harrison SC (2004) Structure of the dengue virus envelope protein after membrane fusion. Nature 427(6972):313–319

Modis Y, Ogata S, Clements D, Harrison SC (2005) Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. J Virol 79(2):1223–1231

Mongkolsapaya J, Dejnirattisai W, Xiao-ning X, Vasanawathana S, Tangthawornchaikul N, Chairunsri A, Sawasdivorn S, Duangchinda T, Dong T, Rowland-Jones S (2003) Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med 9(7):921

Montes-Gómez AE, Vivanco-Cid H, Bustos-Arriaga J, Zaidi MB, Garcia-Machorro J, Gutierrez-Castañeda B, Cedillo-Barron L (2017) Construct and expression of recombinant domains I/II of dengue virus-2 and its efficacy to evaluate immune response in endemic area: possible use in prognosis. Acta Trop 171:233–238

Montoya M, Gresh L, Mercado JC, Williams KL, Vargas MJ, Gutierrez G, Kuan G, Gordon A, Balmaseda A, Harris E (2013) Symptomatic versus inapparent outcome in repeat dengue virus infections is influenced by the time interval between infections and study year. PLoS Negl Trop Dis 7(8):e2357. https://doi.org/10.1371/journal.pntd.0002357

Mota J, Acosta M, Argotte R, Figueroa R, Méndez A, Ramos C (2005) Induction of protective antibodies against dengue virus by tetravalent DNA immunization of mice with domain III of the envelope protein. Vaccine 23(26):3469–3476

Muné M, Rodríguez R, Ramírez R, Soto Y, Sierra B, Roche RR, Marquez G, Garcia J, Guillén G, Guzmán M (2003) Carboxy-terminally truncated Dengue 4 virus envelope glycoprotein expressed in *Pichia pastoris* induced neutralizing antibodies and resistance to Dengue 4 virus challenge in mice. Arch Virol 148(11):2267–2273

Murrell S, Wu S-C, Butler M (2011) Review of dengue virus and the development of a vaccine. Biotechnol Adv 29(2):239–247

Nayak V, Dessau M, Kucera K, Anthony K, Ledizet M, Modis Y (2009) Crystal structure of dengue virus type 1 envelope protein in the postfusion conformation and its implications for membrane fusion. J Virol 83(9):4338–4344

Nguyen N-L, Kim J-M, Park J-A, Park S-M, Jang Y-S, Yang M-S, Kim D-H (2013) Expression and purification of an immunogenic dengue virus epitope using a synthetic consensus sequence of envelope domain III and *Saccharomyces cerevisiae*. Protein Expr Purif 88(2):235–242

Oliphant T, Nybakken GE, Engle M, Xu Q, Nelson CA, Sukupolvi-Petty S, Marri A, Lachmi B-E, Olshevsky U, Fremont DH (2006)

Antibody recognition and neutralization determinants on domains I and II of West Nile Virus envelope protein. J Virol 80(24):12149–12159

Onlamoon N, Noisakran S, Hsiao H-M, Duncan A, Villinger F, Ansari AA, Perng GC (2010) Dengue virus-induced hemorrhage in a non-human primate model. Blood 115(9):1823–1834

Pattnaik P, Babu JP, Verma SK, Tak V, Rao PL (2007) Bacterially expressed and refolded envelope protein (domain III) of dengue virus type-4 binds heparan sulfate. J Chromatogr 846(1):184–194

Poddar A, Ramasamy V, Shukla R, Rajpoot RK, Arora U, Jain SK, Swaminathan S, Khanna N (2016) Virus-like particles derived from *Pichia pastoris*-expressed dengue virus type 1 glycoprotein elicit homotypic virus-neutralizing envelope domain III-directed antibodies. BMC Biotechnol 16(1):50

Prinz WA, Åslund F, Holmgren A, Beckwith J (1997) The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the *Escherichia coli* cytoplasm. J Biol Chem 272(25): 15661–15667

Rajamanonmani R, Nkenfou C, Clancy P, Yau YH, Shochat SG, Sukupolvi-Petty S, Schul W, Diamond MS, Vasudevan SG, Lescar J (2009) On a mouse monoclonal antibody that neutralizes all four dengue virus serotypes. J Gen Virol 90(4):799–809

Ramanathan MP, Kuo Y-C, Selling BH, Li Q, Sardesai NY, Kim JJ, Weiner DB (2009) Development of a novel DNA SynCon™ tetravalent dengue vaccine that elicits immune responses against four serotypes. Vaccine 27(46):6444–6453

Raviprakash K, Apt D, Brinkman A, Skinner C, Yang S, Dawes G, Ewing D, Wu S-J, Bass S, Punnonen J (2006) A chimeric tetravalent dengue DNA vaccine elicits neutralizing antibody to all four virus serotypes in rhesus macaques. Virology 353(1):166–173

Raviprakash K, Wang D, Ewing D, Holman DH, Block K, Woraratanadharm J, Chen L, Hayes C, Dong JY, Porter K (2008) A tetravalent dengue vaccine based on a complex adenovirus vector provides significant protection in rhesus monkeys against all four serotypes of dengue virus. J Virol 82(14):6927–6934

Reddy PBJ, Pattnaik P, Tripathi NK, Srivastava A, Rao L (2012) Expression, purification and evaluation of diagnostic potential and immunogenicity of dengue virus type 3 domain III protein. Protein Pept Lett 19(5):509–519

Rey FA, Heinz FX, Mandl C, Kunz C, Harrison SC (1995) The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. Nature 375(6529):291–298

Rico-Hesse R (1990) Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. Virology 174(2):479–493

Rothman AL (2004) Dengue: defining protective versus pathologic immunity. J Clin Invest 113(7):946

Rothman AL (2010) Cellular immunology of sequential dengue virus infection and its role in disease pathogenesis. Curr Top Microbiol Immunol 338:83–98. https://doi.org/10.1007/978-3-642-02215-9_7

Sabchareon A, Lang J, Chanthavanich P, Yoksan S, Forrat R, Attanath P, Sirivichayakul C, Pengsaa K, Pojjaroen-Anant C, Chambonneau L (2004) Safety and immunogenicity of a three dose regimen of two tetravalent live-attenuated dengue vaccines in five-to twelve-year-old Thai children. Pediatr Infect Dis J 23(2):99–109

Sabin AB (1952) Research on dengue during World War II1. Am J Trop Med Hyg 1(1):30–50

Saejung W, Puttikhunt C, Prommool T, Sojikul P, Tanaka R, Fujiyama K, Malasit P, Seki T (2006) Enhancement of recombinant soluble dengue virus 2 envelope domain III protein production in *Escherichia coli* trxB and gor double mutant. J Biosci Bioeng 102(4):333–339

Saejung W, Fujiyama K, Takasaki T, Ito M, Hori K, Malasit P, Watanabe Y, Kurane I, Seki T (2007) Production of dengue 2 envelope domain III in plant using TMV-based vector system. Vaccine 25(36):6646–6654

Sánchez-Burgos G, Ramos-Castañeda J, Cedillo-Rivera R, Dumonteil E (2010) Immunogenicity of novel dengue virus epitopes identified by bioinformatic analysis. Virus Res 153(1):113–120

Shresta S, Sharar KL, Prigozhin DM, Beatty PR, Harris E (2006) Murine model for dengue virus-induced lethal disease with increased vascular permeability. J Virol 80(20):10208–10217

Sim AC, Lin W, Tan GK, Sim MS, Chow VT, Alonso S (2008) Induction of neutralizing antibodies against dengue virus type 2 upon mucosal administration of a recombinant *Lactococcus lactis* strain expressing envelope domain III antigen. Vaccine 26(9):1145–1154

Simmons M, Nelson WM, Wu S, Hayes CG (1998a) Evaluation of the protective efficacy of a recombinant dengue envelope B domain fusion protein against dengue 2 virus infection in mice. Am J Trop Med Hyg 58(5):655–662

Simmons M, Porter K, Escamilla J, Graham R, Watts D, Eckels K, Hayes C (1998b) Evaluation of recombinant dengue viral envelope B domain protein antigens for the detection of dengue complex-specific antibodies. Am J Trop Med Hyg 58(2):144–151

Simmons M, Murphy GS, Hayes CG (2001) Antibody responses of mice immunized with a tetravalent dengue recombinant protein subunit vaccine. Am J Trop Med Hyg 65(2):159–161

Spohn G, Jennings GT, Martina BE, Keller I, Beck M, Pumpens P, Osterhaus AD, Bachmann MF (2010) A VLP-based vaccine targeting domain III of the West Nile virus E protein protects from lethal infection in mice. Virol J 7(1):146

Srivastava AK, Putnak JR, Warren RL, Hoke CH (1995) Mice immunized with a dengue type 2 virus E and NS1 fusion protein made in *Escherichia coli* are protected against lethal dengue virus infection. Vaccine 13(13):1251–1258

Suzarte E, Marcos E, Gil L, Valdés I, Lazo L, Ramos Y, Pérez Y, Falcón V, Romero Y, Guzmán MG (2014) Generation and characterization of potential dengue vaccine candidates based on domain III of the envelope protein and the capsid protein of the four serotypes of dengue virus. Arch Virol 159(7):1629–1640

Suzarte E, Gil L, Valdés I, Marcos E, Lazo L, Izquierdo A, García A, López L, Álvarez M, Pérez Y (2015) A novel tetravalent formulation combining the four aggregated domain III-capsid proteins from dengue viruses induces a functional immune response in mice and monkeys. Int Immunol 27(8):367–379

Suzuki R, Winkelmann ER, Mason PW (2009) Construction and characterization of a single-cycle chimeric flavivirus vaccine candidate that protects mice against lethal challenge with dengue virus type 2. J Virol 83(4):1870–1880

Swaminathan S, Khanna N (2010) Dengue vaccine-current progress and challenges. Curr Sci 98:369–378

Swaminathan S, Khanna N (2013) Experimental dengue vaccines. In: Giese M (ed) Molecular vaccines. Springer, Vienna

Thullier P, Demangel C, Bedouelle H, Mégret F, Jouan A, Deubel V, Mazié J-C, Lafaye P (2001) Mapping of a dengue virus neutralizing epitope critical for the infectivity of all serotypes: insight into the neutralization mechanism. J Gen Virol 82(8):1885–1892

Tripathi NK, Babu JP, Shrivastva A, Parida M, Jana AM, Rao PL (2008) Production and characterization of recombinant dengue virus type 4 envelope domain III protein. J Biotechnol 134(3):278–286

Tripathi NK, Shrivastava A, Biswal KC, Rao P (2011) Recombinant dengue virus type 3 envelope domain III protein from *Escherichia coli*. Biotechnol J 6(5):604–608

Valdés I, Hermida L, Martín J, Menéndez T, Gil L, Lazo L, Castro J, Niebla O, López C, Bernardo L (2009) Immunological evaluation in nonhuman primates of formulations based on the chimeric protein P64k-domain III of dengue 2 and two components of *Neisseria meningitidis*. Vaccine 27(7):995–1001

Valdés I, Gil L, Romero Y, Castro J, Puente P, Lazo L, Marcos E, Guzmán MG, Guillén G, Hermida L (2011) The chimeric protein domain III-capsid of dengue virus serotype 2 (DEN-2) successfully boosts neutralizing antibodies generated in monkeys upon infection with DEN-2. Clin Vaccine Immunol 18(3):455–459

Valdés I, Marcos E, Suzarte E, Pérez Y, Brown E, Lazo L, Cobas K, Yaugel M, Rodríguez Y, Gil L (2017) A dose-response study in mice

of a tetravalent vaccine candidate composed of domain III-capsid proteins from dengue viruses. Arch Virol 162:2247–2256

van der Most RG, Murali-Krishna K, Ahmed R, Strauss JH (2000) Chimeric yellow fever/dengue virus as a candidate dengue vaccine: quantitation of the dengue virus-specific CD8 T-cell response. J Virol 74(17):8094–8101

Vannice KS, Durbin A, Hombach J (2016) Status of vaccine research and development of vaccines for dengue. Vaccine 34(26):2934–2938

Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, Endy TP, Raengsakulrach B, Rothman AL, Ennis FA (2000) Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 181(1):2–9

Versiani AF, Astigarraga RG, Rocha ES, Barboza APM, Kroon EG, Rachid MA, Souza DG, Ladeira LO, Barbosa-Stancioli EF, Jorio A (2017) Multi-walled carbon nanotubes functionalized with recombinant dengue virus 3 envelope proteins induce significant and specific immune responses in mice. J Nanobiotechnol 15(1):26

Vollmer J (2006) CpG motifs to modulate innate and adaptive immune responses. Int Rev Immunol 25(3-4):125–134

Wahala W, Kraus AA, Haymore LB, Accavitti-Loper MA, de Silva AM (2009) Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody. Virology 392(1): 103–113

Wang S, He R, Anderson R (1999) PrM-and cell-binding domains of the dengue virus E protein. J Virol 73(3):2547–2551

White LJ, Parsons MM, Whitmore AC, Williams BM, De Silva A, Johnston RE (2007) An immunogenic and protective alphavirus replicon particle-based dengue vaccine overcomes maternal antibody interference in weanling mice. J Virol *81*(19):10329–10339

Williams KL, Wahala WM, Orozco S, De Silva AM, Harris E (2012) Antibodies targeting dengue virus envelope domain III are not required for serotype-specific protection or prevention of enhancement in vivo. Virology 429(1):12–20

Wu K-P, Wu C-W, Tsao Y-P, Kuo T-W, Lou Y-C, Lin C-W, Wu S-C, Cheng J-W (2003a) Structural basis of a flavivirus recognized by its neutralizing antibody solution structure of the domain iii of the japanese encephalitis virus envelope protein. J Biol Chem *278*(46): 46007–46013

Wu S-C, Yu C-H, Lin C-W, Chu I-M (2003b) The domain III fragment of Japanese encephalitis virus envelope protein: mouse immunogenicity and liposome adjuvanticity. Vaccine 21(19):2516–2522

Wu S-F, Liao C-L, Lin Y-L, Yeh C-T, Chen L-K, Huang Y-F, Chou H-Y, Huang J-L, Shaio M-F, Sytwu H-K (2003c) Evaluation of protective efficacy and immune mechanisms of using a non-structural protein NS1 in DNA vaccine against dengue 2 virus in mice. Vaccine 21(25):3919–3929

Yang J, Zhang J, Chen W, Hu Z, Zhu J, Fang X, Yuan W, Li M, Hu X, Tan Y (2012) Eliciting cross-neutralizing antibodies in mice challenged with a dengue virus envelope domain III expressed in *Escherichia coli*. Can J Microbiol *58*(4):369–380

Zellweger RM, Prestwood TR, Shresta S (2010) Enhanced infection of liver sinusoidal endothelial cells in a mouse model of antibody-induced severe dengue disease. Cell Host Microbe 7(2):128–139

Zhang Z-S, Yan Y-S, Weng Y-W, Huang H-L, Li S-Q, He S, Zhang J-M (2007) High-level expression of recombinant dengue virus type 2 envelope domain III protein and induction of neutralizing antibodies in BALB/C mice. J Virol Methods 143(2):125–131

Zhang D, Xia Q, Wu J, Liu D, Wang X, Niu Z (2011) Construction and immunogenicity of DNA vaccines encoding fusion protein of murine complement C3d-p28 and GP5 gene of porcine reproductive and respiratory syndrome virus. Vaccine 29(4):629–635

Zheng Q, Fan D, Gao N, Chen H, Wang J, Ming Y, Li J, An J (2011) Evaluation of a DNA vaccine candidate expressing prM-E-NS1 antigens of dengue virus serotype 1 with or without granulocyte-macrophage colony-stimulating factor (GM-CSF) in immunogenicity and protection. Vaccine 29(4):763–771

Zheng X, Chen H, Wang R, Fan D, Feng K, Gao N, An J (2017) Effective protection induced by a monovalent DNA vaccine against dengue virus (DV) serotype 1 and a bivalent DNA vaccine against DV1 and DV2 in mice. Front Cell Infect Microbiol 7:175. https://doi.org/10.3389/fcimb.2017.00175

Zlatkovic J, Stiasny K, Heinz FX (2011) Immunodominance and functional activities of antibody responses to inactivated West Nile virus and recombinant subunit vaccines in mice. J Virol *85*(5):1994–2003

Zulueta A, Martín J, Hermida L, Alvarez M, Valdés I, Prado I, Chinea G, Rosario D, Guillén G, Guzmán MG (2006) Amino acid changes in the recombinant dengue 3 envelope domain III determine its antigenicity and immunogenicity in mice. Virus Res 121(1):65–73