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Improve Dengue Vaccine Design through a Comprehensive Understanding of Anti-Dengue Immunity

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Abstract

Dengue virus is one of the most prevalent arboviruses that emerges fast around the world and imposes a substantial disease burden globally. The co-circulation of four serotypes of dengue viruses causes a large number of dengue fever and dengue hemorrhagic fever/dengue shock syndrome cases. As there is no specific antiviral treatment, an effective dengue vaccine is one of the most important tools to control the global spread of dengue disease. The development of dengue vaccine, however, is complicated by the interference among four dengue serotypes and an incomplete understanding of host immune responses. This review provides an overview of current work on host immunity against dengue viral infection. The knowledge will help to modify the existing vaccine design strategies and the development of novel dengue vaccine modalities in the future.

Overview

Dengue virus (DENV) is one of the most important flaviviruses transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes in tropical and subtropical regions. Approximately one third of the world population are at risk of dengue virus infection. Dengue disease is endemic in over 100 countries, mainly in South-east Asia, Western Pacific, South America, and Africa [1-4]. It causes 50-100 million infections, and 500,000 hospitalizations each year [5,6]. Its rapid global expansion may have been accelerated by climate change, increased urbanization, globalization, and international travels [3,4].

DENVs are comprised of four serotypes, DENV-1, DENV-2, DENV-3, and DENV-4, based on their differences in neutralization and complement fixation tests. These viruses co-circulate in endemic regions. DENV has an approximately 10 kb single positive stranded RNA genome, from which one polyprotein is translated and processed into 10 viral proteins: Core (C), pr-membrane (prM), envelope (E), nonstructural protein 1 (NS1), NS2a, NS2b, NS3, NS4a, NS4b, and NS5 [7]. While the vast majority of dengue infections are asymptomatic, some show febrile illness named dengue fever (DF), among which a small proportion can also develop into life-threatening severe disease as manifested by vascular leakage, thrombocytopenia and shock, collectively called dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [8]. The risk of severe dengue disease increases in secondary infections [9,10].

The outcome of human dengue viral infection is determined by viral replication fitness and host immune responses. Its pathogenesis is closely associated with a complex network of interactions among various arms of host defense. Despite decades of study, with much scientific knowledge gained, no effective antiviral drugs or licensed vaccines are available. Here, we summarized the current status of research on immune responses to dengue viral infection during natural infection and in experimental vaccination. The knowledge will help to improve the design and testing of dengue vaccines.

Immune responses elicited by dengue virus infection

Once dengue viruses invade human bodies, innate immune responses, B cell responses, and T cell responses are triggered. Much effort had been placed on describing antibody responses to dengue virus in animal model, or in human subjects either during or after recovery from dengue viral infection. In contrast, innate and cellular immunity have not been as intensively studied.

Innate immunity

Innate immune response is the first line of host defense against infections. As new adjuvant and delivery systems are explored for vaccine development, it is useful to understand innate immune responses elicited by dengue viral infection and multiple strategies that dengue virus has evolved to evade host antiviral defense.

IFN pathway

Dengue virus is sensitive to type I interferon (IFNα and IFNβ) mediated antiviral activities. Treating cells with interferon α/β before or immediately after infection inhibits dengue virus replication, while delayed addition of IFN is less effective [11,12]. *In vivo*, type I interferon provides resistance to primary infection in a murine model of dengue viral infection [13]. To propagate its own species, dengue virus has evolved several strategies to circumvent type I interferon responses.

During viral replication, dengue ssRNA is sensed by Toll-like receptor 7 (TLR7), while dengue viral dsRNA is recognized by TLR3, retinoic acid-inducible gene I (RIG-I), and melanoma differentiation associated gene 5 (MDA5) [14-18]. The activation of such pathogen recognition receptors (PRRs) triggers downstream signaling pathways and stimulates the production of interferon α/β. Once produced, soluble interferons bind to IFN-α receptor (IFNAR), stimulates interferon-stimulated gene (ISG) transcription through Janus kinase/signal transducer and activators of transcription

(JAK/STAT) signaling. ISG proteins such as 2',5'-oligoadenylate synthetase (2',5' OAS), and interferon-inducible trans-membrane protein (IFITMs) are capable of inhibiting dengue virus infection [19,20]. *In vitro* studies indicate that dengue virus can inhibit type I IFN production by cleaving stimulator of interferon genes (STING) [21], which is an adaptor molecule of several PRRs in the cytoplasmic nucleic acid sensing pathway, activating downstream transcription pathway to induce type I IFN production [22,23]. The expression of dengue viral proteins NS2A, NS4A, NS4B or NS5, impairs JAK/STAT signaling by reducing the amount of phosphorylated STAT1 [24], or inducing degradation of STAT2 [25,26]. Dengue virus may also circumvent type I interferon response through an idiosyncratic Fc gamma receptor (FcγR) signaling pathway. When immune complexes ligate FcγR, two suppressive pathways are activated to interrupt IFN production: (1) deoxyadenosine kinase (DAK) and the autophagy-related genes 5 and 12 (Atg5-Atg12) complex inhibit MDA5 and RIG-I pathways, respectively. And, (2) activation of sterile alpha- and armadillo-motif- containing protein (SARM) and TNF receptor associated factor (TRAF) family member associated NF-κB activator (TANK) blocks the expression of TLR-3, -4,-7 [27,28].

Nevertheless, dengue virus fails to subvert host defensive mechanisms in immune competent mice, where total viral clearance is observed. Although using high titer viral infection and artificial infection route such as intracerebral injection help dengue virus to infect wild-type mice, symptoms of human dengue disease cannot be fully replicated [29-31]. Similarly, DHF/DSS are difficult to be reproduced in non-human primates or humanized mice, despite detectable viral replication in peripheral tissue and some signs of DF [32,33]. Other alternative models are to use mouse adapted dengue viral strains or immune deficient mice such as AG129, and $STAT1^{-/-}$ mice that are devoid of type I/II IFN receptors (AG129) or STAT1 ($STAT1^{-/-}$) function. Infection with mouse adapted DENV-2 strains in AG129 or $STAT1^{-/-}$ mice replicate antibody dependent enhancement and to some extent the disease profiles observed in human DF and DHF/DSS [34-36]. Such *in vivo* evidence also suggests that IFN pathway is an essential component of host immunity to control dengue infection.

NK cell

Natural killer (NK) cells are part of innate immunity. It can recognize 'self' or 'non-self' patterns through the major histocompatibility complex I molecules expressed on cell surface. They lyse target cells by secreting cytotoxic granules that contain perforin and granzyme. Alternatively, they can stimulate the apoptotic receptor on target cell surface. However, these processes are normally inhibited, when the self MHC I molecules on target cell are detected by an inhibitory receptor on NK cells [37]. Additionally, NK cells mediate antibody dependent cell mediated cytotoxicity (ADCC) through FcγRIII on their surface [38]. Early activation of NK cells is important in the defense against primary dengue infection [30]. Conversely, dengue infection has been reported to up-regulate the cell surface expression of MHC I molecules and thereby inhibits NK mediated cell lysis [39]. Moreover, studies in a murine model of dengue viral infection showed that infiltrating NK cells are associated with the liver cell death [40]. Thus, the definitive role of NK cells in dengue viral infection requires further investigation.

Complement

In a broad sense, complement response is also part of innate immunity. Complement pathways may contribute to dengue pathogenesis through interaction with dengue NS1 protein [41-44], which is being examined as a component of dengue vaccine.

Complement activation has three cascades, classical, alternative, and lectin pathways. All the processes trigger subsequent cleavage of pro-proteins and generate a series of active factors to form a membrane attack complex (MAC), phagocytes are then recruited to lyse target cells [45]. Only the classical pathway requires specific antibodies to initiate the cascade. During dengue infection, soluble NS1 protein circulating in blood plays a vital role to modulate the complement pathways. DENV NS1 was found to interact with clusterin, a regulator that inhibits MAC formation [46]. NS1 antibody was necessary in the activation of complement pathway [42], possibly by blocking the interaction of soluble NS1 with other regulators or factors in the pathways besides the classical one. In fact, studies have demonstrated that NS1 inhibits classical and lectin pathway by binding to C4 directly [47]. Some complement splitting products, such as C3a and C5a, have activities of anaphylatoxin to increase vascular permeability [48-50]. Whether the modulation of complement pathways by NS1 and the possible aggregation of splitting products are responsible for the vascular leakage developed in DHF cases is yet to be clarified by direct evidence.

In summary, innate immunity plays different roles during dengue virus infection. It can be protective if it is stimulated at the early stage of infection, but pathogenic if it is activated too late.

Humoral immunity

Previous studies on antibody responses to dengue virus have formed the basis of current vaccine development strategies. Many aspects of humoral immunity have been evaluated, including the kinetics and magnitude of antibody responses, antibody dependent enhancement, neutralizing and enhancing antibodies, and specific epitopes targeted by anti-dengue antibodies. Each of these aspects is discussed next.

Epitopes targeted by neutralizing antibody in humans and mice

Various antibodies against dengue viral proteins have been found in sera of infected patients either during acute phase of infection or convalescence. Those against prM or E could be protective and neutralizing, and thereby enhancing as well, because these antigens are exposed on virion surface [7]. E protein mediates virus binding, entry, fusion and uncoating. Its ectodomain is divided into three beta-barrel domains, domain I (EDI), domain II (EDII) and domain III (EDIII) [7]. EDII and EDIII are functional domains where fusion loop and receptor binding sites are located, respectively.

The distribution of epitopes targeted by neutralizing, cross-reactive or serotype specific antibodies is different in humans and mice. Early studies of dengue specific antibodies in mice showed that most of the strongly neutralizing monoclonal antibodies (MAb) are serotype specific and EDIII specific [51,52]. The main target regions include the lateral ridge and A strand that are partially overlapping within domain III [51-53]. Interestingly, studies on human MAb (hMAb) isolated from patient sera during dengue infection revealed only a few serotype specific neutralizing antibody, and the conserved EDII fusion loop is the major target of the serum antibodies [54]. EDIII antibody is not abundant in human sera, but it has the highest neutralizing potency among all other antibodies in sera [55]. These

data have drawn question on whether anti-EDIII antibody is essential for the neutralizing ability of human sera. A study uses recombinant EDIII protein to adsorb EDIII antibodies has demonstrated that the depletion of EDIII hardly affected the neutralizing ability of human sera, indicating other vital neutralizing epitopes exist outside the domain III region [56,57]. In fact, several newly identified epitopes of human neutralizing antibodies are mapped to the EDII fusion loop, and antibody binding to which blocks virus fusion post entry [54,58]. Recent studies also showed that some flavivirus neutralizing antibodies can only be induced in humans with whole virion but not the ectodomain of E protein [59,60]. Two neutralizing antibodies had been found to recognize the complex structure of adjacent E dimers, including residues in the hinge region of EDI and EDII, which is only presented on an intact dengue virion surface, thus may have been missed in previous studies using purified protein to determine the antibody affinity or using linear epitopes [60].

Besides antibodies specific for E proteins, high levels of antibodies against prM and NS1 had been detected in human sera. During dengue virus life cycle, assembled viral particles bud into ER lumen, in which processed prM and E protein are recruited to form the envelope of immature viruses. prM shields the E trimers during virion release, protecting it from premature release and fusing within acidic intracellular vesicles [7]. Before viruses are released from cell membrane, pr peptides are cleaved from M proteins by furin, unveiling domain II of E proteins. Meanwhile, the protruding E trimer becomes a dimeric form which lays flat on the viral surface over M proteins. Finally, E proteins are stretched and displayed on the mature virus surface [7].

However, a recent study found that a portion of the released viruses are immature or partial immature in cell cultures. With the help of antibodies targeting prM, immature non-infectious virus enters target cells, becomes mature virus and then uncoats viral RNA and finishes its life cycle inside the cells, similar to a mature virus [61,62]. Of note, prM antibodies are of higher concentrations in the secondary than primary infection [54,62]. Such antibodies provide an aid to a non-infectious virion to become an infectious one, and thus may facilitate secondary infection.

Anti-NS1 antibodies are abundant in sera of infected patients. This is of some interest because NS1 is the only non-structural protein secreted from infected cells. Its appearance in bloodstream coincides with the clinical phase of disease [63]. Furthermore, *In vitro* studies indicated that NS1 could potentially cause vascular leakage through modulation of complement pathways [42]. Consistent with this, antibodies against NS1 inhibit the development of pathology and elicit partial protection against disease in a murine model of dengue virus infection [64-67]. In contrast, antibody against NS1 has been shown to recognize self-antigen on endothelial cells, and thus implicated in causing damage to the vasculature [68,69]. Nonetheless, there is evidence that indicates soluble NS1 being unrelated to pathogenesis [70]. Therefore, further investigations are needed to determine the role of NS1 and anti-NS1 antibodies conclusively, and thereby resolve these controversial observations and provide guidance to future vaccine design.

In summary, to obtain an effective dengue vaccine, one needs to fully understand the characteristics of dengue specific antibody response. Although studies in mice with murine MAb have provided much knowledge on humoral immune responses induced by dengue infection in animals, not all information gained in mouse studies are relevant to human dengue infections. Therefore, hMAb are biologically more relevant, which must be obtained from cases with natural dengue viral infection, or those received candidate dengue vaccines. Careful study of these hMAb will be of tremendous value to rationale design of dengue vaccines.

Antibody response in primary and secondary infection

In a primary infection, dengue specific IgG antibody appears one week after the onset of fever, reaches the peak level within weeks, and then declines to a lower level that persists for decades. In contrast, in a secondary heterologous infection, IgG antibodies are detectable earlier (< Day 3 of fever) and at higher levels [71,72]. Although higher levels of antibodies are elicited earlier in a secondary infection, the majority of which are cross-reactive against the dengue serotype causing primary infection [73]. Such cross-reactive antibodies produced in a secondary infection may react with conserved epitopes, but generally bind to heterotypic virus inefficiently. In addition, the presence of such antibodies may also sequestrate new antigens that are needed for stimulation of naïve B cells. Consequently, B cell responses specific to the virus serotype in secondary infection are weaken and delayed. This phenomenon is called "original antigenic sin" [74-76]. Indeed, the highest neutralizing antibody titers in acute and convalescent sera from children experiencing secondary dengue infection are to the primary infection virus serotype [76]. A recent study of anti-EDIII antibodies in a cohort of patients during primary or secondary dengue infections also revealed stronger binding and enhancement activities against the primary infection DENV serotypes during secondary infections [77], thus providing support to the theory of "original antigenic sin".

As most cross-reactive antibodies generated in primary infection do not effectively neutralize heterotypic dengue viruses in secondary infection, such "original antigenic sin" could partially contribute to increased risk of severe dengue disease in a secondary infection. Nonetheless, a recent study has reported that flavivirus group cross-reactive antibodies isolated from patients with primary dengue infection are of low avidity and weak neutralization activity, whereas group cross-reactive antibodies from secondary infection have higher neutralization potencies compared to cross-reactive antibodies produced during primary infection [78]. Further clinical studies are needed to clearly define the similarity and difference between antibody responses during primary and secondary infections.

Antibody dependent enhancement

In the presence of preexisting antibodies, viral infection could be augmented through a progress called antibody dependent enhancement (ADE), rather than being neutralized [79,80]. Antibodies with low affinity or those with high affinity at a sub-neutralizing concentration may form immune complexes with viruses. These immune complexes interact with Fcγ receptor expressed on the surface of certain cell types through the antibody Fc domain [9,79]. Although not fully elucidated yet, there are two hypotheses explaining the mechanisms of ADE. According to the "extrinsic ADE" theory, binding of virus/antibody immune complexes to the Fcγ receptors facilitates virus entry into target cells that are either non-permissive or weakly permissive to dengue virus in the absence of antibodies, and thus increase the number of cells infected by DENV. Whereas the "intrinsic ADE" postulate involves the role of host immunity. Specifically, the internalization of immune

complexes stimulates downstream Fcγ receptor signal pathways, and blocks activation of antiviral response, such as type I interferon production and signaling. It has been shown that infection through Fcγ receptor suppresses transcription of genes of inflammatory cytokines including interleukin-6 (IL-6), IL-12, interferon–γ (IFN-γ), and tumor necrosis factor–α (TNF-α); it also inhibits an innate anti-DENV mediator, nitric oxide; and blocks the activation of STAT-1 signaling [81]. The outcome of "intrinsic ADE" is the release of more viruses from each infected cell [27].

ADE has been demonstrated *in vitro* with dengue specific antibodies when they are diluted to sub-neutralizing concentrations [27,82,83]. However, not all antibodies can reach this range of concentrations *in vivo*. For a given viral strain, key factors influencing the outcome of virus-antibody interactions are cross-reactivity, affinity, neutralizing capacity and abundance of antibodies. Thus, dengue specific antibodies may be functionally classified into neutralizing antibodies and enhancing antibodies. But there are exceptions. One recent study demonstrated that a murine MAb targeting E protein domain II has only enhancement properties *in vitro*, while another has only neutralizing activities towards homologous infection *in vitro* [84]. IgG subclass and activation of specific complement pathways also affect the function of these antibodies [84].

Not only ADE phenomenon has been consistently reproduced in primary cells or cell lines, it has also been demonstrated *in vivo* in humans or animal models. Administering antibodies at subneutralizing concentrations to non-human primate enhanced the dengue viremia following experimental infection [32,85]. Pretreatment of IFN receptor deficient AG129 mice with anti-dengue sera at a subneutralizing concentration leads to vascular leakage, and cytokine storm after being infected with D2S10 strain of DENV [35,86]. Although there is no direct evidence of ADE in secondary dengue infection in humans, the observation that infants born to dengue experienced mother are associated with higher risk of severe dengue during 6-9 months after birth, but not earlier or later, a time frame coincides with the decaying of maternal antibodies to a threshold level at around 9 months after birth, suggest a vital role for dengue specific antibodies in causing severe dengue disease in secondary infection [87].

Cell immunity

In the course of infection, dengue viruses elicit high levels of T cells that are cross-reactive among four dengue serotypes. Whether these T cells cause pathogenesis or confer protection remains controversial.

Cytokines released by T cells

T cell responses induced by dengue viral infection had been associated with pathogenesis, because of the appearance of cytokine storm during disease. Massive secretion of proinflammatory T cell cytokines such as IL-2, IFN-γ, and TNF-α were detected in patients' sera in the acute phase of dengue disease, and TNF-α could cause increased permeability of endothelial cells in the lining of blood vessels [88-90]. Elevation of these cytokines occurred slightly before or at the time of defeverscence and coincides with plasma leakage, suggesting immunopathogenic roles of these cytokines produced by T cells [91]. However, conflict results from cohort studies have confounded the interpretation of the role of T cell responses. In some studies, higher levels of cytokines were detected in patients developing DHF, comparing to DF cases [92,93]. Whereas in other studies, the elevation of IL-6, IL-10, MIF (macrophage migration inhibitory factor) levels was only observed in fatal DHF cases [94], and no differences of IFN-α and IFN-γ levels were found between DHF and DF cases [94,95]. In fact, cytokine levels changed rapidly over the course of illness, and some were detected in patient sera represent an overall production of these cytokines by many cell types. Thus the timing of measurement and type of samples from which the cytokines were gauged should be considered carefully. Also, the patients' genetic background, such as HLA types should be noted [96,97]. In sum, more studies on specific branches of cell immunity will help to elucidate the definitive role of T cell immunity during dengue disease.

CD4+ T cell response

CD4+ T cells activated during dengue primary and secondary infection mostly recognize structural proteins but also NS1, both of which are the main targets of dengue specific B cells. A portion of activated CD4+ T cells is follicular helper T cells that can interact with B cells to modulate antibody production *in vivo* [98]. However, a study in mice demonstrated that the depletion of CD4+ T cells did not affect CD8+ T cell and B cell activities in primary infection, yet dengue specific CD4+ T cells contributed to the viral clearance after vaccination with peptides derived from non-structural proteins [99].

CD4+ T cells can exert their function through the production of various cytokines. Higher levels of IL-10, mainly secreted by CD4+Th2 cells, had been detected in patients with DHF, compared to those with DF [100]. Under the modulation of IL-10, bystander T cells undergo apoptosis, vacating immunological space for memory T cells to expand during secondary dengue infections [101].

CD8+ T cell response

In contrast to CD4+ T cells, a majority of CD8+ T cells target non-structural proteins, while a smaller proportion of them targets structural proteins [98]. CD8+ T cells, recognizing structural proteins, are generally serotype specific, while those that recognize non-structural proteins (predominantly NS3) are highly cross-reactive [102]. Many studies have speculated that broadly cross-reactive T cell responses contribute to the pathogenesis of dengue disease by triggering inflammatory cytokine storm or lysing virus infected cells directly. Indeed, a recent cohort study showed that a lower magnitude of HLA restricted CD8+ T-cell response is associated with an increased disease susceptibility [97], suggesting a protective role of CD8+ T cell response against dengue viral infection [97,103]. Furthermore, immunization with immunodominant CD8+ T cell epitopes enhanced viral clearance in experimental dengue viral infection in mice [104]. However, in another murine study, the infiltration of CTL was associated with liver damage during dengue viral infection [40].

Similar to what has been observed for B cells, T cell response induced by dengue infection is also thought to be affected by the "original antigenic sin" [76,89]. Some data have showed that T cell responses induced during secondary infection are dominated by an expansion of T cells specific to the primary infection serotype [89,90,105], this may lead to diminished response to the virus causing the secondary infection and resulting in a less effective viremia control. However, in a recent study that screened human dengue specific T cell epitopes using overlapping peptides covering the full

length of DENV genome, no differences were found between CD8+ T cell responses targeting serotype specific and conserved epitopes in terms of magnitude, phenotype, multifunctionality, or avidity, thus questioning whether an original antigenic sin exists in the context of dengue-specific T cell response [97,103].

In sum, for aiding rationale dengue vaccine design, both CD4+ and CD8+ T cell immunity need further in-depth analysis. Additionally, other factors such as host (human or mouse), primary/secondary infection, host HLA type, and sampling time must be taken into consideration.

Current dengue vaccine development

To contain the spread of dengue virus around the world, researchers have tried to develop an effective dengue vaccine for years. From inactivated vaccines, live attenuated vaccines, recombinant vaccines to subunit vaccines and DNA vaccines, various candidates have been examined, and several general concepts of dengue vaccine design have been developed. First, it must be tetravalent to cover all four dengue serotypes. Second, it should elicit potent and balanced neutralizing antibody responses to all four dengue serotypes. And third, it must not induce enhancing antibodies. However, many unanticipated problems have been revealed in the process of dengue vaccine development. How to overcome these problems have become current goals of vaccine research.

Live attenuated vaccines and recombinant vaccines

Most traditional vaccines are made by inactivated pathogens. As inactivated dengue vaccines only trigger limited immune response, plus the low yield in cell culture method for preparing viral stock, its development has been largely discontinued. Much work now focuses on live attenuated vaccines and recombinant vaccines. Until the 1980s, live attenuated vaccines were still produced through sequential passage of viruses in different host or cells. More recently, molecular biology tools enabled the development of vaccines by introducing mutations to particular sites on viral genome or making chimerization with other flavivirus or viral vectors to form a genetically more defined attenuated virus. One dengue candidate vaccine with four live viruses made by attenuation through serial passages in primary dog kidney (PDK) cells and fetal rhesus lung (FRhL) cells has completed Phase II clinical trial [106]. Another type of live attenuated vaccines were developed by deleting 30nt at 3'UTR, or replacing prME of 30nt-deleted DENV4 with prME from other serotype viruses [107]. Several such admixtures of live attenuated tetravalent vaccines (LATV) consisted of selected attenuated viruses were shown to be safe and capable of inducing trivalent or better neutralizing antibodies in vaccine recipients [108].

The CYD23 recombinant vaccine had been made by swapping dengue virus prME genes with that of Yellow Fever (YF) viral genes on YF17D backbone. This vaccine has finished Phase IIb clinical trial and produced partial protection against 3 serotypes of dengue viruses [109]. The major problem of CYD23 is the lack of protection against DENV-2 in the endemic region where this serotype has the highest prevalence. One explanation offered by the trial investigators is the interference among four dengue viral serotypes *in vivo* when the four vaccine viruses were inoculated within one admixture. Another possibility is that antibodies elicited by the DENV-2 viral strain used in the vaccine could not neutralize the contemporaneous DENV-2 viral strain in Thailand [109,110]. Additionally, compare to live attenuated viruses or chimeric DENVs, CYD23 only incorporated dengue viral structural antigens, most of which stimulate B cell and CD4+ T cell responses. As the protective role of dengue specific CD8+ T cells has been implicated in recent studies [97], CD8+ T cell epitopes may have to be added to a dengue vaccine.

Structural proteins including prM from all four dengue serotypes were included in all aforementioned live attenuated or recombinant vaccines, because they are the main antigens recognized by neutralizing antibodies. However, more recently, evidences support prM protein elicits cross-reactive antibodies and facilitates the infection of immature virus were reported [61,106-108]. A new E85 vaccine that has removed prM and retained ectodomain of E protein and expressed in alphavirus vector, has been shown to induce robust protective immunity against dengue virus infection in a macaque model of dengue viral infection [111]. Direct comparison between vaccines with or without prM with respect to immunogenicity and protective efficacy will help to determine whether prM is a necessary component in a dengue vaccine.

Subunit vaccine and DNA vaccine

Subunit protein vaccines and DNA vaccines have been explored more recently. Different from attenuated vaccines, subunit vaccines and DNA vaccines avoid the potential risk of virulence caused by reversion virus, have fewer adverse effects, and can be produced outside a biosafety level III laboratory.

In mice, tetravalent EDIII proteins have induced balanced neutralizing antibody responses to all four serotype of viruses, with only minor enhancement activity observed in anti-EDIII sera [112]. Whether tetravalent EDIII vaccine elicits protective immunity *in vivo* and whether EDIII elicits neutralizing antibodies to all four serotype of viruses in humans as it did in mice are yet to be determined. A subunit vaccine that has been advanced to clinical trials is based on the expression of E protein ectodomain (80%E). It theoretically can induce anti-E antibodies that target both enhancing and neutralizing epitopes outside the EDIII region. Such tetravalent 80%E elicited neutralizing antibodies against four dengue serotypes, and conferred protection against DENV-2 in mice and monkeys [113]. Recently, neutralizing hMAbs targeting EDI/II have been found in patients recovered from dengue viral infection, thus, a direct comparison between EDIII and the whole E ectodomain with respect to their immunogenicity and protective efficacy is needed.

Most dengue DNA vaccines deliver prME nucleotide fragment as antigens and form virus like particles (VLP) in host [114-116]. The VLP is structurally similar to the naturally processed and assembled virus, and has the capacity to stimulate both B and T cell responses [116,117]. One obstacle of DNA vaccine development is that DNA has weaker immunogenicity in comparison to protein or viral particle vaccines. To solve this problem, dengue DNA vaccines are often inoculated with adjuvant or used in combination with subunit vaccines [117-119]. Although DNA vaccine has the advantage of low cost and ease of production, the optimal method of delivering a large amount of concentrated DNA into human body needs to be explored.

Obstacles in Dengue Vaccine Development

Interference among the viruses in a tetravalent Vaccine

Interference or interaction among live viruses has been observed when multivalent attenuated or inactivated viruses are mixed and

administered in a single inoculation. As the replication of each attenuated virus and immune dominance towards various viral antigens could not be predicted with precision before the inoculation, empirical modulation and adjustment of vaccine formulation and the dose of each component are often necessary. This approach, albeit worked in the trivalent polio virus vaccine [120], is cumbersome. Easier and more rationally methods to avoid the interferences remain to be discovered.

In-vitro neutralizing ability does not predict protective immunity *in vivo*

Although sera collected from subjects of CYD23 trials have an apparently balanced neutralizing activity to autologous viruses representing all four dengue serotypes in the plaque reduction neutralization test (PRNT) in Vero cells, protection against DENV-2 was not observed in the vaccinated children, despite variable degrees of protection were demonstrated against DENV-1, -3 and -4. Although PRNT titer is widely accepted as a gold standard to evaluate immunogenicity and a benchmark for the prediction of *in vivo* protective efficacy, it has not served these purposes in the large scale dengue vaccine clinical trial. The inconsistency between *in vitro* assay and *in vivo* activity of antibodies may be partially attributed to the enhancing activity of non-neutralizing antibodies elicited *in vivo*, which could not be detected in fibroblast-like Vero cells *in vitro*. Since the principal target cells of DENV are dendritic cells (DC), monocytes, and macrophages, it is necessary to perform neutralization test and enhancement assay in human primary cells to adequately evaluate antibody activities in clinical trials. At a minimum, cells that mimic the biological properties of monocytic cells should be used to more convincingly demonstrate neutralization and enhancement profiles of vaccine induced antibodies, before conclusions are made on vaccine efficacy and a decision is made on licensure.

Intra-serotype variation

Current tetravalent dengue vaccines have been designed based on the assumption that neutralizing antibodies induced by one strain of virus can protect people from infection by any viral strains within the same serotype. Although from cohort studies, it is observed that infection by one serotype can protect patient from re-infection by the same serotype of virus for decades or a lifetime [121], different neutralization activity was observed for different viral genotypes. It is common to find that one monoclonal antibody cannot neutralize a large panel of viruses from the same serotype [53,122], and the result of neutralization test also depends on the panel of viruses selected. To obtain a tetravalent vaccine with broad coverage, representative strain for each of the four components needs be selected at an early stage of vaccine development.

Future Perspective of Rationally Designed Dengue Vaccines

Genetically engineered subunit vaccine

As discussed above, subunit vaccines have advantages over live attenuated vaccines with respect to safety and ease of preparation. In addition, shorter intervals between boosts, as is often the case for subunit vaccines, also decrease the risk of enhanced infection by endemic viruses before the vaccination schedule is completed, and when antibody titers in vaccines may still be at sub-neutralizing levels. Although subunit vaccines may not be able to induce neutralizing antibodies targeting complex epitopes which only exist on virion, they have the potential to be genetically modified by selecting neutralizing epitopes and removing enhancing ones. Moreover, through a similar strategy, "good" epitopes from different viral serotypes could be selected and integrated into one genetic backbone, which could be chosen from either one dengue serotype or a consensus sequence of all serotypes. By expanding the reference genomes from representative strains of four dengue serotypes to all epidemic strains, consensus sequence will have the potential to solve the intra-serotype variation problem.

T cell vaccine

Before recent studies indicating a protective role of CD8+ T cells against dengue viral infection, T cell immunity had been to a large extent neglected as it was thought to be associated with viral pathogenesis. In view of the failure of chimeric vaccine CYD23, which was designed principally to stimulate neutralization antibodies and did not include nonstructural proteins that can stimulate T cells, a new focus on T cell immunity in dengue vaccine development may be needed. Inclusion of antigens capable of eliciting T cell response in a vaccine (besides live attenuated dengue viruses), or selection of optimal T cell epitopes for a T cell vaccine, may help to create a better dengue vaccine.

The roles of antigen presenting cells (APC) in dengue vaccine design

When dengue virus is transmitted to humans through mosquito bite, the first infected cells are thought to be Langerhans cells, which are specialized skin dendritic cells (DCs) [123]. In experimental models, dengue viruses enter immature DCs via dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). Upon maturation, DC-SIGN is down regulated and Fcγ receptors are upregulated. Through Fcγ receptor, mature DCs facilitate ADE in a secondary infection [124]. Dengue infection of monocyte derived DC (mDC) and plasmacytoid DC (pDC) lead to a diminished upregulation of MHC and co-stimulatory molecules [125], and render them incapable of priming CD4+ Th1 cells [126]. In an attempt to initiate stronger cellular and humoral protective immunity against dengue virus, some have started to test a new vaccine strategy by delivering dengue antigens to dendritic cells directly [127]. Whether such a manipulation can improve the balance and potency of dengue specific immune responses is yet to be tested experimentally.

Summary

After a half century's basic research and clinical trials, the mechanisms underlying dengue protective immunity have been gradually revealed, these knowledge provide foundation for an improvement in the design of future dengue vaccines. Besides traditional human pathogen based vaccine and antiviral drugs, expanded knowledge of flavivirus infection in mosquitoes [128-132], modulation of dengue infection in mosquitoes through infection by *Wolbachia* bacterium or genetic modification [133-135], will also provide additional novel strategies to control dengue virus transmission. The more we know, the further we can move forward in the direction of making an ideal dengue vaccine. Based on current knowledge of DENV specific immunity, either induced by natural viral infection or elicited by candidate vaccines, new dengue vaccine design and modification of current vaccine candidates can be made to

eventually obtain an efficacious human dengue vaccine.

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