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Review

Trials and tribulations on the path to developing a dengue vaccine

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ABSTRACT

Dengue is a rapidly expanding global health problem. Development of a safe and efficacious tetravalent vaccine along with strategic application of vector control activities represents a promising approach to reducing the global disease burden. Although many vaccine development challenges exist, numerous candidates are in clinical development and one has been tested in three clinical endpoint studies. The results of these studies have raised numerous questions about how we measure vaccine immunogenicity and how these readouts are associated with clinical outcomes in vaccine recipients who experience natural infection. In this review the authors discuss the dengue vaccine pipeline, development challenges, the dengue vaccine-immunologic profiling intersection, and research gaps.

1. Introduction

Development of vaccines against dengue has been identified as a priority by the World Health Organization, the US Health and Human Services, the US Department of Defense, the Bill and Melinda Gates Foundation, and Ministries of Health from dengue endemic nations. Dengue is the acute febrile disease caused by the flaviviruses classified as dengue virus serotypes 1-4 (DENV-1, DENV-2, DENV-3, and DENV-4). Human infection with DENVs occurs almost exclusively through a transmission cycle between humans and mosquitoes of the genus Aedes, with *Aedes aegypti* serving as the principal vector. Trends in the growth and distribution of human and mosquito populations over the past 50 years have been highly favorable to DENV transmission. As a result, both *Aedes aegypti* and DENVs are widely distributed in tropical and subtropical areas of the world. It has been estimated that over 3 billion people are at risk for infection and that 390 million infections occur annually, of which approximately 96 million result in clinically apparent disease [1].

Dengue's unique clinical and epidemiologic characteristics contribute to its global health impact and challenge disease control efforts. As is true for many other viruses, many infections pass without recognized symptoms or signs [2]. Nevertheless, recent evidence confirms that clinically inapparent infections are likely to contribute to overall DENV transmission and thus are important to overall efforts to control dengue [3]. Among those infections that are clinically apparent, the spectrum of disease severity is very broad, extending from an uncomplicated febrile illness lasting several days to a life-threatening plasma leakage syndrome. At one end of this spectrum, school or work absences and disruption of normal activities due to mild dengue add significantly to overall morbidity due to the potentially overwhelming number of cases [4]. At the other end, although supportive care is very effective at keeping the case-fatality rate low, dengue is a significant concern to parents and clinicians in endemic areas and the high number of hospitalizations places a major strain on the public health infrastructure that is disproportionally borne by low and middle income countries (LMIC).

Infection with the DENV generates protective immunity, providing a theoretical basis for vaccination. In the case of natural infection, protective immunity develops in a step-wise fashion. However, the balance of humoral and cellular immune responses which constitutes an immuno-protective profile remains incompletely understood. Immune responses to a first (primary) DENV infection are mainly serotype-specific, and appear to provide long-lasting, perhaps life-long, resistance to re-infection with the same DENV serotype. Immune responses to the other (heterologous) DENV serotypes are detectable at low levels after a primary infection, and individuals become resistant to infection with heterologous serotypes, but this cross-reactive protective immunity lasts for only a few months [5]. Following this short period, secondary infection with another DENV serotype can occur, with important differences from primary infection.

Although the spectrum of dengue disease severity is equally wide in primary and secondary infection, the distribution of cases following secondary infection differs substantially; a smaller

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percentage of infections are inapparent while the likelihood of plasma leakage and hospitalization is markedly increased [6]. Immune responses in secondary infection display an anamnestic profile. Antibody levels reach high titer and show broad serotype-cross-reactivity, and T cell responses are predominantly directed at serotype-cross-reactive epitopes. These immune responses appear to provide protection against infection with the remaining serotypes for a year or longer [7]. Hospital-based seroepidemiology studies suggest that the risk of severe disease and hospitalization is lower during post-secondary infections; however, firm data are lacking because antibody profiles do not permit a clear distinction of secondary and subsequent infections [8]. In contrast to natural infection, when an individual receives a dengue vaccine he or she is exposed to attenuated or fragments of all four DENVs simultaneously. It is unclear what single dengue vaccine antigen or combination of antigens is required to sufficiently mimic natural immunity and protect its recipient from disease following infection.

2. Dengue vaccine development pipeline

2.1. General

There are numerous dengue vaccine candidates in the pipeline spanning the spectrum from pre-clinical to advanced clinical development. Vaccine candidates are diverse, representing classic live attenuated and inactivated constructs along with approaches utilizing technologies to produce recombinant antigens, DNA constructs, vector-based expression, and virus like particles (VLPs). Each approach attempts to induce immune responses to individual or combined structural and non-structural protein targets encoded by the dengue genome believed to play an essential role in DENV infection or replication once infection occurs.

The DENV envelope (E) protein attaches to host cell receptors, facilitates endosomal membrane fusion, and displays sites mediating viral neutralization [9]. Because of this, all dengue vaccine candidates in clinical development contain at least a portion of each DENV serotype's E protein (DENV-1-4). Where the candidates differ is their inclusion of DENV non-structural (NS) proteins. Certain candidates contain no NS proteins, some contain NS proteins from each serotype, others contain the NS proteins from a single serotype with different pre-Membrane (preM) and E proteins representing all serotypes, and one candidate uses the NS proteins from Yellow fever 17D vaccine with the preM and E proteins from each DENV serotype [10].

2.2. Pre-clinical development tools

Pre-clinical vaccine development activities are intended to recreate the human clinical and immunologic response to infection, immunization, and immunization followed by infection (challenge studies). The intent is to utilize small animal and non-human primate models of infection and disease to inform vaccine development decisions regarding the selection of viable antigen platforms, dose selection and formulation, and administration methods, routes, and schedules. Most importantly, animal models provide developers with an early indication of the safety profile of any particular candidate, its ability to induce a relevant immunologic response, and whether there is the potential the immunologic response could confer some clinical benefit to the recipient in the form of preventing infection, preventing disease, or significantly attenuating disease following infection. Models are intended to de-risk development programs.

Mouse models of DENV infection and pathogenesis have improved significantly and experienced increased use for early investigations of candidate vaccines or anti-dengue antivirals. In addition to increased availability and lower cost relative to non-human primates, they have improved their ability to mimic humanized immunologic responses to infection. There are numerous mouse models, each with strengths and weaknesses.

Wild type mouse models have demonstrated central nervous system findings following DENV infection but lack many classic dengue clinical features [11]. The propensity to experience neurologic abnormalities may become increasingly important as central nervous system insults are being reported more often in the dengue clinical literature [12,13]. AG129 mice lack IFN-α/β and–γ receptors and readily support DENV infection and viral replication [14]. However, like wild type mice, AG129 mice fail to demonstrate a complete spectrum of human disease phenotypes. Mice lacking only IFN-α/β receptors (IFNAR^{-}/$^{-}$) retain IFN- γ and the neuroprotective action of CD8^{+} T cells. Similar to AG129 mice, IFNAR^{-}/$^{-}$ mice support DENV replication resulting in select, but not comprehensive, human disease features [15]. Disease phenotypes of both AG129 and IFNAR^{-}/$^{-}$ mice have been made more severe by infecting mice in the presence of sub-neutralizing or enhancing antibody preparations [16].

"Humanized" mice have been developed by engrafting various human cell lines into severely immunodeficient mice. Severe combined immunodeficient (SCID) mice lacking B and T cells, irradiated non-obese diabetic (NOD)/SCID, NOD/SCID/IL-2Rγ-null, and RAG2$^{-/-}$/γc$^{-/-}$ mice have been engrafted with human hepatocarcinoma or leukemia cells (HepG2 or K562, respectively), human CD4^{+} hematopoietic stem cells, cord blood hematopoietic stem cells, and fetal thymus and liver tissue [17–23]. Fever, rash, viremia, cytokine production, and development of anti-dengue IgM following experimental infection have all been reported in these models. Although encouraging, there is inter-mouse performance variability and, similar to the "non-humanized" models, the full complement of the human immune system is not represented and may impact disease expression and/or vaccine efficacy. Practical considerations of cost and availability of sufficient numbers are also of concern.

Despite sharing a close genetic relationship to humans, non-human primates are disappointing models of dengue disease. Non-human primate species are permissive to DENV infection but typically do not display clinical pathology [24–26]. A recent literature review provides a comprehensive summary of primate species' virologic and clinical responses to infection with a variety of DENV strains [27].

The general observation is that non-human primates, following viral inoculation, experience infection, develop measurable peripheral viremia, and a neutralizing antibody and cellular immune response [28,29]. A recent literature review explored estimates of the time to detectable viremia following DENV inoculation and the duration of viremia among a number of Old and New World non-human primate species. The time to viremia in rhesus macaques ranged from 2.63 to 3.32 days for DENV-2 and -1 and the duration from 3.13 to 5.13 days for DENV-4 and -2. Differences in time to viremia and viremia duration were not significantly different between non-human primate species [30]. Quantitative measures of non-human primate viremia reveal peripheral RNAemia values to be lower than what is found in humans experiencing dengue disease, offering one explanation of why non-human primates do not develop clinical disease consistent with human dengue illness. To overcome this limitation investigators have explored intravenously inoculating non-human primates with DENV and claim the method produced "classic" hemorrhagic manifestations. This model has not been widely reproduced [31].

It is possible that additional alterations in study methodology may improve the non-human primate dengue model. Exploring new infecting viral strains, incorporating mosquitoes into the process of virus inoculation, increasing the viral concentration of the inoculation, or trying to create an immune environment conducive

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to enhancing infection (i.e. antibody dependent enhancement) may all improve model performance [26,32].

Until the non-human primate infection model becomes a disease model, vaccine developers will interpret non-human primate data with caution. If a vaccine candidate is able to induce an immune response in a non-human primate which prevents viremia following challenge there is the potential it could do the same in a human. Failure to prevent or significantly reduce viremia in non-human primates portends a low likelihood the vaccine candidate will protect a human from dengue disease.

It should be noted that investigators may assess the presence or absence of viremia using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Although PCR is informative it may not accurately quantitate the presence of infectious viral particles, a requirement to propagate infection and disease, in a previously vaccinated animal. More than one developer has reported post-challenge RT-PCR data which is not consistent with viral isolation data [33,34]. How to interpret the presence of peripheral RNAemia but failure to isolate virus from an immunized monkey following challenge is difficult and may introduce doubt into the value of the model.

In summary, neither mice nor non-human primates recreate the immunologic or clinical parameters observed following natural DENV infection. Possessing some but not all of the human immunologic components alters the sequence, magnitude, kinetics, and overall coordination of the responses which occur in humans following wild type infection and resulting in one of various potential clinical outcomes. Absence of these outcomes in animal models limits their prognostic usefulness for vaccine and drug developers.

2.3. Clinical development

The most advanced dengue vaccine candidate is Sanofi Pasteur's live attenuated Yellow fever (YF)-dengue chimeric vaccine (CYD-TDV) created by inserting the DENV pre-M and E genes into the cDNA backbone of the YF 17D vaccine, replacing the YF pre-M and E [35–39]. The CYD-TDV vaccine is administered subcutaneously (SQ) at months 0, 6, and 12 and is the first candidate tested in clinical end-point efficacy trials. Results of a phase 2b study in Thailand and two phase 3 trials in Asia and Latin America yielded overall vaccine efficacy (VE) rates between 30.2% and 60.8% [40–43]. Perplexing is the discordance between the robust and balanced neutralizing antibody profiles measured in vaccine recipients but the apparent lack of clinical efficacy across all serotypes. Encouraging is the safety profile reported during the acute vaccination period and up to one year following completion of the vaccination series.

Takeda Pharmaceuticals is developing a tetravalent chimeric dengue vaccine candidate by introducing DENV-1, -3, and -4 prM and E genes into cDNA derived from a live attenuated DENV-2 component [44–52]. A Phase 1 dose-escalation trial in DENV-non-immune subjects, involving a single dose of vaccine formulations with low or high DENV viral concentrations administered SQ or intradermally (ID), demonstrated acceptable safety. Immunogenicity is predominated by a DENV-2 neutralizing antibody response but the overall response supports additional clinical testing [53].

Whitehead et al. constructed cDNA clones of a DENV-4 candidate attenuated by constructing a 30-nucleotide deletion in the 3′ untranslated region (rDEN4Δ30) [54–67]. A tetravalent vaccine construct was created by constructing similar deletions in DENV-1 and DENV-3 while a DENV-2/-4 chimeric was created by replacing the DENV-4 preM and E genes from the DENV-4 backbone with those from DENV-2. The vaccine appears safe, inducing an asymptomatic rash and transiently lowering absolute neutrophil counts in a subset of recipients. Balanced neutralizing antibody profiles and failure to boost immune responses or measure vaccine viremia following a second vaccine dose up to six months after dose 1 raises the potential this may be a one dose vaccine [68]. Phase 2 trials in endemic populations across a diverse age range are underway in Thailand and Brazil.

The Walter Reed Army Institute of Research (WRAIR) developed multivalent live attenuated candidates (LAV) by serial PDK passage of DENV strains and final passage in fetal rhesus lung cells (FRhL) [69–79]. Newly derived vaccine lots produced in collaboration with GlaxoSmithKline Vaccines (GSK) were tested in adults in the United States (U.S.), Thailand and in Puerto Rico across a broad age range (12 months to 50 years) using two doses of vaccine delivered SQ at days 0 and 180 [80,81]. Long term follow up (up to 5 years) and administration of a 3rd dose of vaccine to children in Thailand vaccinated with a first generation tetravalent formulation has been completed. Despite early indicators of promise, the development of the WRAIR/GSK LAV candidate as a stand-alone vaccine has been halted.

The WRAIR also developed a purified inactivated virus (PIV) dengue vaccine candidate by inactivating with formalin each of the DENV serotypes [33,82–84]. Together with GSK and the Oswaldo Cruz Foundation (FioCruz), Brazil, a tetravalent PIV formulation (DPIV) is being tested in combination with GSK's proprietary Adjuvant Systems (AS). Phase 1 trials in the U.S. and Puerto Rico remain underway but preliminary data indicate an acceptable safety and immunogenicity profile [85]. As with all dengue and inactivated vaccines, durability of the immune response and associations with clinical efficacy will need to be defined.

Merck & Co. is developing a recombinant E protein candidate produced in a Drosophila S2 cell expression system [33,86,87]. A dose ranging study of the candidate (V180) alone and in combination with ISCOMATRIX™ adjuvant is being evaluated in a phase 1 human trial (clinicaltrials.gov study NCT01477580). Of interest, Merck is pursuing an exclusive license of the NIH vaccine construct.

The U.S. Navy Medical Research Center conducted a DENV-1 monovalent DNA dengue vaccine phase 1 trial with disappointing neutralizing antibody results [88]. Human clinical testing of a tetravalent DNA based formulation adjuvanted with Vaxfectin® is nearing completion [89].

A number of dengue vaccine candidates are in early clinical development establishing safety and immunogenicity profiles across diverse age, genetic, and flavivirus primed and naïve populations. It is likely more than one candidate will enter clinical endpoint studies (phase 2b or 3) in the next 2 to 3 years. Sanofi Pasteur will likely pursue licensure in highly endemic countries based on its efficacy data detailed above. If a license is granted and the vaccine is widely available to pediatric populations in dengue endemic countries it is unclear what impact this will have on applications to conduct efficacy trials in those same countries with different candidates. Vaccine safety profiles remain acceptable in populations who are both DENV primed and unprimed prior to vaccination.

3. Ongoing challenges for dengue vaccine development

Notwithstanding the significance of the first demonstration of clinical efficacy of a potentially licensable tetravalent dengue vaccine, the complex epidemiology and immunology of dengue present many ongoing challenges that will need to be addressed for a full evaluation of the benefits and risks of widespread vaccine use.

3.1. Defining protective efficacy

The interpretation of dengue vaccine efficacy and its real-world applicability are critically dependent on the criteria used for the

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primary clinical trial endpoint. Key considerations are the threshold of illness severity and whether efficacy needs to be demonstrated for each individual DENV serotype. World Health Organization guidelines proposed that virologically-confirmed symptomatic dengue (VCD) of any severity caused by any DENV serotype be used as a primary trial endpoint [90], representing a balance of public health impact and trial feasibility.

As noted earlier, a large proportion of incident DENV infections are clinically inapparent, and only a small percentage of cases of dengue illness require significant medical attention. Mild dengue that does not lead to utilization of health care resources is a lower priority for prevention than more severe disease phenotypes requiring one or multiple visits to a health care provider, missed days of school or work, and requirement to purchase medicine, particularly in LMICs. Mild cases make a greater contribution to morbidity for potential users of a vaccine in non-endemic countries such as travelers and the military.

Field studies demonstrate that immune status drastically influences the distribution of disease manifestations, but not necessarily in a consistent direction; that is, the fraction of symptomatic cases can increase with or without a proportional increase in severe cases. Therefore, the theoretical potential exists for a vaccine to reduce the overall incidence of VCD without reducing either the overall incidence of infection or the incidence of severe disease, or, more problematically, to reduce the overall incidence of infection or the incidence of severe disease (beneficial) with no reduction (or even an increase) in the incidence of all symptomatic infections (failing to meet the study endpoint). In the phase III trials of CYD-TDV, there was a trend of VE against hospitalized dengue or severe dengue being higher than VE against all VCD and was statistically significant even in several subgroup analyses where VE against VCD was not statistically significant [41,91]. Collection of data on VE against severe dengue will be very important for other vaccine candidates.

Field studies highlight the strong influence of study design on the detection of milder cases of dengue. The incidence of dengue illness in cohort studies has been significantly higher when active surveillance was used for case finding as compared to passive surveillance [2]. For school-aged children, a target population for dengue vaccination in Asia, for example, monitoring of school absences has been an efficient strategy. This approach can be criticized as a departure from standard clinical practice. However, this concern needs to be balanced against the importance of the more complete characterization of the distribution of cases.

Although all four DENV serotypes produce a similar spectrum of disease, field studies have suggested that the frequency of individual disease manifestations can differ across serotypes [92]. Data suggest that these effects may even vary by viral genotype and the DENV serotype(s) previously encountered. Although the phase III trials of CYD-TDV were not specifically powered for analysis of serotype-specific VE, it was found that VE differed across serotypes [41,91]. More problematically, serotype-specific VE could not be predicted based on post-vaccination antibody titers. This problem may be further complicated by the differences in antigenic content and immune responses engendered by the different vaccine candidates. It will therefore be important for the design of future vaccine trials to obtain robust estimates of VE for each DENV serotype. Alternatively, dengue human infection model (DHIM) studies provide a pathway to evaluate VE for serotypes that are not adequately represented during the course of the trial.

3.2. Durability of dengue vaccine efficacy under different conditions of exposure

The evolution over time of immune responses elicited by DENV vaccines is likely to have a major impact on VE, given the time-dependent effects of natural DENV infection on susceptibility or resistance to heterologous infection and disease described above. For travelers or military personnel deployed from non-endemic areas to dengue-endemic areas, exposure to DENV will usually be time limited. For these individuals, VE will initially need to be assessed over a time frame that is meaningful for the period of risk. However, many such individuals will travel to dengue-endemic areas on multiple occasions. Therefore, the durability of VE over longer periods of time, and the efficacy of booster immunizations will need to be evaluated.

In comparison to the considerations in travelers, the assessment of VE over time will be much more complicated for the population residing in dengue-endemic areas. Cohort studies have shown a high level of exposure to DENV, with annual DENV infection rates of 8% or more in some locations [2]. However, exposure varies over time and space within dengue-endemic areas [93,94]. Furthermore, the intensity of DENV transmission at the village level during one transmission season influences the likelihood of clinical illness for at least one additional transmission season [95]. It has been assumed, on the basis of experimental human infection studies done in the 1940s, that each sequential DENV infection induces solid and long-lasting immunity to reinfection with the same serotype and that therefore a maximum of four DENV infections can occur in an individual's lifetime. Evidence of significant immunological variation within each DENV serotype [96] and the recognition of DENV infections in elderly lifelong residents of endemic regions challenge this paradigm. The durability of protective immunity following inapparent infections is difficult to assess, since the serotype of infection is usually unknown. It is possible that periodic inapparent infections, by "boosting" DENV-specific memory immune responses from earlier infections, contribute to the accumulation of protective immunity among the population in dengue-endemic areas, but not all such infections may induce solid serotype-specific immunity. Attenuated or subunit vaccines cannot be assumed to generate protective immunity of the same duration as natural infection. Boosting of antibody titers after the second and third doses of CYD-TDV suggests some in vivo replication of one or more vaccine strains, implying an absence of sterile immunity.

Given the vast number of possible permutations of sequential DENV exposures, with different intervals between infections, it is unlikely that all issues regarding longevity of DENV vaccines can or should be resolved prior to their introduction for general use. Guidelines for testing of DENV vaccines have suggested a follow-up period of at least 3–5 years [90]. This represents a balance of benefits and risks; longer delays prior to licensure or introduction into general use of a truly effective vaccine expose additional people to dengue-related morbidity and mortality, but natural DENV infection shows that the risk of more severe disease can extend for decades. It will be critical to collect sufficient information to provide some level of assurance that future issues, should they arise, can be addressed, for example, by showing that waning protection can be boosted by one or more repeat immunizations. New questions are also likely to arise in the future if widespread vaccination reduces the intensity of DENV transmission and the frequency of natural boosting.

3.3. Evaluating the immune response to dengue vaccines

Guidelines for testing of dengue vaccines have recommended plaque reduction neutralizing antibody titers (PRNT) against each DENV serotype as the primary immune response measure, while noting that consideration be given to newer assays of DENV-specific antibodies as well as assays of DENV-specific T lymphocyte responses [90]. PRNT has shown a general correlation with resistance to infection and illness in some studies of natural DENV

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infection [97,98]. However, mean PRNT levels also correlate with increasing age, probably reflecting accumulating DENV infections, and studies have not validated a PRNT level that reliably indicates protective immunity. Furthermore, PRNT values are greatly affected by assay conditions and show significant interassay variation [99,100]. In the pivotal phase III trials of CYD-TDV, PRNT levels increased in response to vaccination. However, PRNT responses to the vaccine were higher for DENV-2 despite the finding of lower VE against DENV-2. This observation raises a number of questions regarding neutralizing antibodies potential to be validated as a mechanistic or non-mechanistic correlate of protection or whether each DENV serotype may have a different protective titer threshold.

The prevailing interpretation of the finding that DENV infection can occur in the presence of high pre-existing PRNT levels to the same serotype is that these in vitro neutralizing antibodies reflect serotype-cross-reactive antibody responses from prior infection with a heterologous serotype. Various antibody assays have been developed that discriminate between serotype-cross-reactive heterologous antibodies and serotype-specific antibodies such as those induced by primary infection [101]. Heterologous PRNT antibodies that fail to protect against infection in vivo have also been found to be unable to inhibit infection of some Fc receptor-positive cell lines in vitro [102,103]. These assays are promising as potential outcome measures for vaccine trials and enthusiasm for their inclusion is growing. However, their correlations with protective immunity have not yet been established.

The ability of antibodies directed at pM and E proteins to enhance DENV infection of Fc receptor-positive cells in vitro (antibody-dependent enhancement, ADE) has been thought to contribute to the increased severity of secondary DENV infections. As a result, ADE assays have been considered as a possible addition to PRNT to better evaluate VE. However, published data from studies of natural DENV infection and recipients of CYD-TDV have not shown measurement of ADE to provide additional insights [102]. These assays might be of particular interest if the situation arises where a DENV vaccine trial shows an increased incidence of severe disease.

The ability of T cells to both express antiviral effector functions and produce pro-inflammatory cytokines has led to the somewhat contradictory hypotheses that DENV-specific T cell responses contribute to protective immunity and to the pathogenesis of severe dengue [104]. Relatively few studies have examined the association of DENV-specific T lymphocyte responses with clinical outcomes, and most of these have used blood samples collected either during viremia or after recovery from DENV infection. Two papers from the Kamphaeng Phet cohort study have evaluated the correlation of DENV-specific T cell responses measured in blood samples collected before DENV infection with the outcome of infection [105,106]. These two papers found significant correlations for different assays in opposite directions- a correlation of in vitro TNFα production during 7 day incubation with hospitalized dengue in one study and a correlation of frequencies of IFNγ-producing cells at 18 h after stimulation with subclinical DENV infection. In view of the failure of PRNT to explain VE, interest in use of T cell assays has grown. The varying antigenic content of the different DENV vaccines has contributed to this interest, since non-structural DENV proteins (other than NS1) contribute little to antibody responses but include immunodominant regions for T cell responses. These studies create additional logistical hurdles for vaccine trials, given the greater complexity of specimen collection, isolation, and cryopreservation and the higher cost of T cell assays. Newer technologies have made such studies more feasible, and these assays have been successfully incorporated into trials of vaccines against HIV and malaria [107,108].

3.4. Effect of pre-existing flavivirus immunity on the response to vaccination (and vice versa)

Higher titer and breadth of antibody responses to immunization in individuals with pre-existing flavivirus-specific antibodies have been a consistent observation in clinical trials of dengue vaccines. This suggests that the anamnestic effect of memory T and/or B lymphocytes has a stronger influence than any immune-mediated restriction of replication of live attenuated vaccine viruses under typical conditions, as is the case in second and subsequent natural DENV infections. Extending this analogy, it will be important to determine if immune responses to vaccination are qualitatively different in individuals with pre-existing flavivirus-specific immune responses. In natural secondary DENV infections, both antibody and T lymphocyte responses are directed away from serotype-specific epitopes and toward conserved epitopes [109]. These heterologous serotype-cross-reactive responses are typically of lower avidity than homologous serotype-specific responses, and may be less effective in vivo as well, although longitudinal data adequate to determine the history of prior flavivirus exposure has been available in only a very small number of cases. In the phase III trials of CYD-TDV, VE was higher among subjects who were positive for DENV-specific antibody at baseline compared to DENV naïve vaccine recipients [41,91]; however, this result does not establish that antibody and T cell responses were optimal in quality.

The specific sequence of infection with different flaviviruses has been reported to affect the clinical outcome and characteristics of the immune response. However, these effects have not been uniform across studies [110,111]. The contributions of individual factors, such as genetics and immune repertoire, are difficult to dissect from differences among circulating virus strains and the timing of sequential infections. Even in large cohort studies of natural DENV infection, very few subjects represent any single sequence when applying stringent criteria. Similarly, sampling to determine the kinetics of the immune response was conducted in a minority of subjects in the pivotal phase III CYD-TDV trials. Differences in VE across study sites could be a consequence of the specific infection history in the local population impacting response to vaccination and subsequent immune response when naturally exposed. Given the diverse flavivirus exposure history in the target populations for immunization, there will be greater potential for variation in VE when a vaccine is available for widespread use; further evaluation will require prospective collection of data and specimens.

4. Research needs

The results of the first phase III dengue vaccine efficacy studies have highlighted gaps in current knowledge. With the anticipation of large-scale efficacy trials of other candidate dengue vaccines, ongoing follow-up of participants from the CYD-TDV trials, and the (hoped-for) eventual licensure and widespread use of an effective vaccine, additional questions can be anticipated and should be priority areas for public and industry-sponsored research.

4.1. Natural history/field studies

Until the population of vaccine recipients reaches a sufficient size to provide robust estimates of VE under diverse conditions of exposure, studies of natural DENV infection will remain the best source of information on acquired immunity to infection. Extended observation of cohorts under active surveillance, particularly those incorporating geographic mapping, have facilitated the definition of DENV transmission risk in spatial and temporal terms and thereby the tentative identification of individuals with

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protective immunity [98]. Blood samples from these cases will be invaluable for validation of putative immunological correlates.

4.2. Pre-clinical models

The use of animal models for testing the safety of vaccine candidates prior to clinical trials will undoubtedly continue, despite their limitations. The newer mouse models that develop dengue viremia and/or disease may improve the assessment of the safety of live DENV vaccines [22,112]. Calibration of animal data against phase I and II clinical trial results may help derisk development programs and allow informed down selection of candidates while comparison of animal data to clinical endpoint vaccine trials is necessary to determine the ability of these experimental models to predict potential for clinical benefit. More useful animal models for immunogenicity testing of vaccine candidates will require the development of even more "human-like" immune systems and/or a better understanding of the basis for the differences from humans in DENV antigen recognition. New in vitro models in which de novo antigen-specific responses can be generated [113] represent an alternative strategy, but further work is needed to establish their suitability.

4.3. Dengue human infection model (DHIM)

Vaccine and drug development efforts for malaria, diarrheal diseases, and influenza have benefitted from the availability of safe and consistently performing human infection models [114–118]. The ability to produce human infection and well-characterized disease phenotypes with a standardized and controlled pathogen exposure is a powerful tool for evaluating drug and vaccine candidates. Human infection (challenge) models complement small animal and non-human primate studies by providing developers the most comprehensive view of their drug or vaccine candidate's safety, immunogenicity profile, and its potential for clinical benefit in the field. There are numerous ethical considerations supporting and detracting from the concept of intentionally infecting healthy individuals [119–121].

Experimental infection of humans with dengue viruses is documented as early as 1902 [5,122–132]. These experiments established foundational knowledge about DENV infection and transmission of and between mosquitoes and humans. Some of the earliest descriptions of immune and clinical responses following infection were generated using experimental human infection. The limitations of animal models and the numerous other challenges facing dengue vaccine development efforts support exploring the re-introduction of the dengue human infection model (DHIM) into drug and vaccine development, assessing immune correlates of protection, and transmission studies. A comprehensive review of the topic was recently published [118,133–137].

4.4. Vaccine trials

Clinical endpoints must remain the focus for phase III dengue vaccine trials for the foreseeable future, reflecting both the lack of validated immunological correlates of protection as well as the different antigenic content of the vaccines currently in advanced development. Collection of specimens and clinical data to support the future identification of immune correlates should be a high priority and the financial resources required to conduct these studies made available. This presents logistical challenges and added costs, especially for collection of viable PBMC. There are not strong financial incentives for vaccine developers to conduct these research studies, which could be of great value to the broad field of dengue vaccine development but may provide little added value to the specific trial or development of an individual vaccine. Creative research collaborations, including public-private partnerships, are a potential solution.

5. Summary and conclusions

The global dengue burden is worsening. Widespread deployment of a safe and efficacious dengue vaccine along with more informed application of vector control programs currently represents the best strategy for reducing the burden. Dengue immunology and clinical pathology are very complex and have challenged vaccine and drug developers. Expanding in number and scope prospective field studies, improving small animal models, developing and applying advanced humoral and cellular immunology assays, and the re-exploration of dengue human infection models offer the means of appreciably increasing our understanding of dengue disease and supporting drug and vaccine development. Although serotype specific and overall efficacy of the CYD-TDV candidate did not achieve the levels making widespread licensure and large scale deployment a forgone conclusion, especially in the non-DENV primed population, the authors find the results encouraging. Proof of concept for producing a protective dengue vaccine and the potential to impact disease severity was achieved. The diversity of antigenic approach for a number of other vaccine candidates in clinical development assures the field is embarking on a very interesting time in the study of dengue and how to best reduce its global impact.

Conflict of interest statement

None declared.

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