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Immune correlates of protection for dengue: State of the art and research agenda

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Conflicts of Interest

The Partnership for Dengue Control has multiple operating partners, including Fondation Mérieux, Dengue Vaccine Initiative (DVI), National Institutes of Health (NIH), and Sabin Vaccine Institute, as well as funding partners, including Bill & Melinda Gates Foundation, Sanofi Pasteur, Takeda, Bayer, GSK, and bioMérieux. Ralph Baric has financial relationships with several dengue tetravalent virus vaccine manufacturers. Beth-Ann Coller is an employee, shareholder, and patent inventor for Merck & Co., Inc., Kenilworth, NJ, USA. James E. Crowe, Jr. is a consultant for Sanofi; is on the Scientific Advisory Boards of PaxVax, CompuVax, GigaGen, Meissa Vaccines, and Rensavir; and is a recipient of research grants from Moderna and Sanofi. Derek Cummings, Neil Ferguson, and Isabel Rodriguez-Barraquer advised Sanofi Pasteur Ltd., without payment, on modeling the potential impact of their dengue vaccine. Hansi Dean an employee of Takeda Pharmaceuticals Vaccine Business Unit. Aravinda de Silva has consulted for Takeda Vaccines, GSK and Merck Pharmaceuticals and is an inventor on patents related to dengue vaccines that have been filed by the University of North Carolina. Michael S. Diamond is a consultant for Inbios, Visterra, and Takeda Pharmaceuticals and on the Scientific Advisory Boards of Moderna and OvaGene. Peter B. Gilbert received a contract from Sanofi Pasteur to analyze data from its clinical trials. Duane J. Gubler is a patent holder on the Takeda dengue vaccine, has Investment in Takeda Pharmaceuticals, and has consulting activities to Sanofi, Takeda, and Merck on dengue. M. Elizabeth Halloran is in the Modelling Consortium with Sanofi, but did not receive payment and is involved in the development of the Phase IV evaluation of Dengvaxia in the Yucatan. Eva Harris served on the Scientific Advisory Board of Sanofi Pasteur during the Phase 3 vaccine trials, and her laboratory received research funds from Takeda Vaccines, Inc. to analyze samples from vaccine recipients. Nicholas Jackson, Bruno Guy, and Athanasios Papadopoulos are Sanofi Pasteur employees. Stanley Plotkin is a consultant to many manufacturers, including Sanofi Pasteur, manufacturer of a dengue vaccine. Alexander R. Precioso is the Director of the Clinical Trials and Pharmacovigilance Division and coordinator of the phase III Butantan dengue vaccine clinical trial for Instituto Butantan, a public owned Institution of the Government of Sao Paulo State, Brazil, which has a development and manufacturing program for an attenuated tetravalent dengue vaccine. Alan Rothman has served as a Consultant for Sanofi and Takeda. Alexander Schmidt is an employee of the GSK group of companies and holds shares in GSK. Gavin Screaton and Felix A. Rey are named inventors on a patent application by Imperial College and Institut Pasteur for EDE antibodies and epitope. Cameron Simmons is a principal investigator in the Eliminate Dengue program – this entity is evaluating a technology for arbovirus disease control that competes with dengue vaccines. Joseph Torresi has had speaker honoraria and consultancies with Sanofi Pasteur, GSK, and Bristol Myers Squibb and an unrestricted research grant with SanofiPasteur. Kirsten Vannice is a staff member of the World Health Organization. Leah Katzelnick, Josefina Coloma, Anna Durbin, Aubree Gordon, Scott Halstead, Richard Jarman, Shee-mei Lok, Nelson Michael, Robert Reiner, Alessandro Sette, Ashley St. John, Wellington Sun, Stephen Thomas, John Tsang, Stephen Whitehead, Annelies Wilder-Smith, and In Kyu Yoon do not report conflicts of interest.

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Abstract

Dengue viruses (DENV1-4) are mosquito-borne flaviviruses estimated to cause up to ~400 million infections and ~100 million dengue cases each year. Factors that contribute to protection from and risk of dengue and severe dengue disease have been studied extensively but are still not fully understood. Results from Phase 3 vaccine efficacy trials have recently become available for one vaccine candidate, now licensed for use in several countries, and more Phase 2 and 3 studies of additional vaccine candidates are ongoing, making these issues all the more urgent and timely. At the "*Summit on Dengue Immune Correlates of Protection*", held in Annecy, France, on March 8-9, 2016, dengue experts from diverse fields came together to discuss the current understanding of the immune response to and protection from DENV infection and disease, identify key unanswered questions, discuss data on immune correlates and plans for comparison of results across assays/consortia, and propose a research agenda for investigation of dengue immune correlates, all in the context of both natural infection studies and vaccine trials.

Keywords

Dengue virus; immune correlates of protection; immune correlates of risk; natural infection; vaccine

1. Introduction

Dengue is the most prevalent arthropod-borne viral disease globally. The four serotypes of dengue virus (DENV1-4) cause approximately 400 million infections annually [1] ranging from asymptomatic infection to severe disease manifested by vascular leak, hemorrhagic manifestations, and shock [2]. A major goal of dengue research is to identify and understand immune correlates of protection and risk (Box 1) of DENV infection, dengue illness, and severe disease, particularly in the context of vaccines (Box 2).

Here we summarize insights from the "*Summit on Dengue Immune Correlates of Protection*", sponsored by the Partnership for Dengue Control. This summit focused on research on dengue immunology and pathogenesis in relation to correlates of protection and risk. The goal of the summit was to review the state-of-the-art regarding immunity to DENV natural infections and vaccines (Box 3) and to generate a research agenda of key unanswered questions (Box 4). We also addressed methods for measuring dengue immune correlates,

measurement of safety and efficacy in vaccine trials, and a framework for comparing results across consortia, vaccines, and research sites (Box 5).

2. What have we learned from natural DENV infections?

Dynamics of immunity following natural infection

The dynamics of immunity derived from natural DENV infections provide important insights into the appropriate time-point(s) to measure immune correlates of protection for vaccines. After natural primary DENV infection, the antibody response against the homologous infecting serotype is potently neutralizing and thought to be life-long; however, individuals also generate a large quantity of less-neutralizing cross-reactive antibodies that initially protect against but may later enhance infection and/or disease with heterologous serotypes [3–5]. After primary infection, neutralizing antibody (nAb) titers against all four serotypes are highest during the first six months, when individuals are thought to be protected from disease caused by DENV infection of any serotype [6]. NAb titers wane over this time period and possibly thereafter, depending on the level of dengue endemicity/DENV exposure [7–10]. On the population level, the average period of cross-serotype protection is ~1.5-2 years against symptomatic disease and ~2.5 years against severe disease [11–13]. Recent studies of children in Thailand and Nicaragua have demonstrated that natural infections can induce cross-protective immunity over longer periods of time in individuals with high nAb titers [9,14]. However, multiple cohort studies have documented differences in the ratio of symptomatic to inapparent (S:I) infections year-to-year [15–18]; when the S:I ratio is high, perhaps the epidemic “force” is greater and there is less protection against disease.

Additionally, maternal antibodies either protect against or increase risk of severe disease in infants in a time-dependent manner. The greatest number of symptomatic and hospitalized dengue cases in infants is observed approximately six months after birth [19–21]: maternal nAb titers decay to <1:10 serum dilution by six months of age on average, whereas non-neutralizing antibodies persist for up to one year [22]. On average, sera collected at six months of age can enhance infection of monocytes more than antibodies collected at other time-points [21,23].

Homotypic reinfection

The recent observation of RT-PCR-confirmed homotypic re-infections in Nicaragua [24] and an epidemic of severe DENV2 in a population with pre-existing anti-DENV2 nAbs in Iquitos, Peru [25,26], led to a discussion about the clinical and epidemiological importance of homotypic reinfections. Homotypic immunity might not be fully sterilizing or protective and may occasionally lead to clinical disease, especially with a large viral inoculum, high strain-specific virulence/infectiousness, or poor quantity and quality of host immunity [27]. Overall, however, evidence exists for long-term protection against disease due to homotypic re-infection [6]. Thus, the clinical relevance of homotypic re-infections is likely limited, but the immunological consequences may be important.

Boosting

Homologous and mild heterologous infections may lead to boosts in immunity (<4-fold rise in antibody titers) that modify subsequent disease risk. Homologous challenge with distinct genotypes in non-human primate studies one year post-infection resulted in a persistent rise in antibody titers [28], and natural boosts have been observed in both Thailand and in Nicaragua and are consistent with case-based estimates of DENV transmission [9,29]. Determining whether and how boosting affects the durability of dengue immunity in endemic areas is important for evaluating the duration of natural and vaccine-induced immunity and the potential need for vaccine booster doses.

Post-second infection immunity

Following second heterologous DENV infection, individuals can develop potent type-specific responses to the second infecting DENV serotype in addition to potent cross-neutralizing/multitypic antibodies, which are thought to be more protective than heterotypic antibodies generated following primary DENV infection [30,31]. Although symptomatic dengue cases continue to occur, the risk of severe disease and hospitalization is low during third and fourth DENV infections [6,12,32,33]. The epidemiology of dengue is consistent with this observation, as the peak age of medically-attended dengue depends on the local force of infection [34,35]. However, some argued that more research is required on whether second infections truly induce durable cross-protective immunity. Sites with discrete introductions of DENV may aid in these studies [36]. Human challenge studies are another important approach to test whether cross-protective immunity following sequential DENV infection differs from tetravalent vaccination [37,38].

3. Immune responses in the context of dengue vaccines

Antibodies induced by vaccination

The strategy for dengue vaccination has been to immunize simultaneously with antigens from DENV1-4 in an effort to induce balanced type-specific neutralizing responses to each serotype [39] (Box 2). There are currently multiple dengue vaccine candidates, including live-attenuated vaccines (LAV), whole virus inactivated vaccines, protein-based vaccines, and more recently mRNA-based vaccines; all are tetravalent [40]. The magnitude of the vaccine-mediated immune response is generally lower than in natural infection. Several key questions remain with respect to how the antibody response elicited during vaccination (simultaneous exposure) differs from natural infection (sequential exposure). In theory, vaccines may stimulate expansion and affinity maturation of type-specific memory B cells to all four DENV serotypes simultaneously, generating a mature antibody response that may be different from natural infection. A major challenge of current vaccination strategies is achieving balanced tetravalent immunity when administering all four antigens at once [41]. The licensed tetravalent LAV studied to date appears to induce substantial cross-reactive nAbs in addition to more limited type-specific nAbs, with unbalanced immunity to DENV1-4 [42]. Rebalancing the doses of each component in the tetravalent formulation may improve replication and immunogenicity. The question of whether tetravalent immunity is needed to reduce the public health impact of dengue remains, although unpublished data of a

study from trivalent vaccination followed by heterologous challenge suggest that tetravalent formulations may be more protective against viremia (A. Durbin, unpublished).

Antibody-mediated immunity and efficacy following vaccination in relation to serostatus

Multiple candidate vaccines show differences in nAb profiles to DENV1-4 and nAb decay following vaccination in flavivirus- and/or DENV-seropositive versus -seronegative recipients. In the Dengvaxia trials, seropositive individuals ($PRNT_{50}$ titer ≥1:10 to DENV, due to prior DENV or other flavivirus infection) on average had $PRNT_{50}$ titers >1:100 following two vaccine doses without increasing after the third dose, whereas seronegative individuals had lower nAb titers following the first two doses that increased upon subsequent immunization [43]. However, for both seronegative and seropositive individuals, the proportion that seroconverted to all four serotypes increased with each vaccine dose [44]. Recent studies show that Dengvaxia induces nAbs against DENV1-4 in seronegative individuals, but most type-specific nAbs against DENV4 [42]. Unpublished exploratory analyses of vaccinated cases from the Dengvaxia Phase 2b trials suggest a relationship between nAb titers and probability of disease [45]. Further, the Phase 3 clinical trials showed an overall positive efficacy (protection against having a dengue case) of the vaccine during the initial 25 months of the trial, but evidence of reduced protective immunity over the follow-up period, with more hospitalized dengue cases in vaccinees than placebos >1 year after the final vaccine dose in children <9 years old [46,47].

With the NIH LAV vaccine, post-dose-1 peak titers declined by day 180, although most individuals, who were flavivirus-seronegative, did not show boosted responses following repeat vaccination at one year, suggesting sustained protection and sterilizing immunity [48,49]. The GlaxoSmithKline whole virus-inactivated vaccine induces good peak nAb titers against all four serotypes after the second dose, but nAb titers wane over time in non-human primates [50] and in seronegative adults [51]. In the Takeda LAV vaccine, more asymmetry in nAb titers to the four serotypes, with stronger responses to DENV2 and weaker responses to DENV4, is seen in seronegative individuals than in seropositive individuals, even following multiple doses [52]. These observations suggest that differential immune responses according to serostatus as viral and host genetic factors may be a challenge to achieving balanced, durable nAb responses for multiple dengue LAVs.

It was suggested that vaccine trial data be presented to display individual nAb variation to enable study of correlations among nAb responses to different serotypes following vaccination; this might also allow the establishment of type-specific correlates of protection. The initial target duration for vaccine efficacy of life-long protection may not be feasible for all vaccine candidates. Some dengue vaccines may require periodic booster immunizations over time, especially in non-endemic areas or areas with a low force of infection, in a model more like tetanus. Further, the kinetics of antibody waning is such that the time-point for valid measurement of a long-term antibody correlate of protection is likely to be later than 30 days post-vaccination.

Vaccines and cell-mediated immunity

The role of cell-mediated immunity (CMI) studies in the context of dengue vaccines was discussed at a WHO-led consultation in 2008, and it was recommended that exploratory studies be conducted [53]. Initially, the primary concern with respect to T cells was to ensure that vaccines did not induce a harmful T cell profile that could contribute to the immunopathogenesis seen with secondary DENV infections [54–56]. However, current data suggest that DENV-specific T cells are associated with a protective response and thus may be beneficial [57–59]. Furthermore, findings from the Dengvaxia Phase 3 trial are not entirely explained by nAb responses as measured by existing assays, making a case for further dissecting the specificity of the humoral immune response, as well as exploring other aspects of immunity, such as T cell responses, that may be immune correlates or co-correlates of protection alongside nAbs.

Differences among vaccine candidates and implications for immune correlates

Due to fundamental differences between vaccines, there will likely be differences in what can be used as a correlate of protection for each vaccine. For instance, inactivated vaccines may induce fundamentally different antibody responses than live vaccines, and while individuals may achieve the same nAb titers with two different vaccines, as judged by current *in vitro* assays, the quality and protective activity of the antibody responses may differ *in vivo*. Another key difference is that some vaccines lack DENV nonstructural genes or capsid proteins [39]. As $CD8^+$ T cells primarily target nonstructural proteins, with the strongest responses to NS3, NS4B, and NS5, and $CD4^+$ T cells target both structural (capsid and envelope) and non-structural (NS3) proteins [60,61], LAVs without these proteins differ in the quality and nature of their T cell responses [55]. In contrast, a LAV containing all DENV proteins has been shown to induce $CD8^+$ T cells comparable to natural infection responses [62]. Antibodies to NS1, which can protect against lethal vascular leak in mouse models [63], may also play an important role in vaccine-mediated protection and disease modification. Significance of these differences *vis-à-vis* protection against dengue remains to be demonstrated.

4. Considerations for defining immune correlates

The virus

Dengue virions display on their surface a lattice of pre-membrane/membrane (prM/M) and envelope (E) proteins. DENV strains vary in temperature sensitivity and maturation state, in part due to laboratory-adapted mutations in prototype and some vaccine strains, which may not be representative of circulating strains [64]. Such amino acid differences can also lead to differences in virion “breathing”, which allows cryptic epitopes to be revealed during temperature- and time-dependent changes in the virion structure [65,66]. Further evidence for antigenic variation within serotypes was presented, including amino acid variation in type-specific quaternary epitopes in highly laboratory-adapted, prototype strains [67,68]. It was suggested that natural strain variation may play a role in vaccine performance and should be further studied. The maturation state of vaccine strains could affect immunogenicity, and as prM antibodies strongly induce antibody-dependent enhancement *in vitro* [69], anti-prM antibody responses should be studied in the context of vaccines. It was

suggested that researchers perform pre-clinical immunogenicity studies on diverse DENV strains to better determine which viruses should be used for next-generation dengue vaccines.

Antibodies and neutralization assays

Serum nAb titers above a certain threshold are correlated with protection from symptomatic infection [9,14,70], but more research is required to establish specific nAb titers as correlates of protection, which will vary by vaccine, serotype, immune status, and assay [71]. Inconsistencies across laboratories exist in the neutralization assay due to differences in reagents, methods, and statistical analyses [72–74]. However, it was recommended that standard neutralization assays should still be performed, despite the challenges with interpretation. Availability of a reference panel of low-passage strains of multiple genotypes in addition to reference sera would be helpful for standardizing neutralization assays across laboratories (some reference materials are already available through NIBSC). Antigenic differences between prototype strains remain problematic; one solution is for dengue researchers to work with infectious clones to control for laboratory adaptation [75]. It was recognized that the maturation state of dengue viruses circulating in humans is not known: primary human dendritic cell-derived viruses are currently the closest approximation of the maturation state of DENV in people [76]. Resolving the maturation state of virions in humans was emphasized as critical, as maturation state affects nAb titers differentially depending on assay characteristics.

NAb titers were generally recognized as a crude measure that may not fully capture the protective component of the immune response, which is likely due to antibodies that bind type-specific or cross-reactive quaternary epitopes [64,76–79]. Combining antibody depletion methods with neutralization assays enables dissection of the cross-reactive versus type-specific neutralizing antibody response after natural infection and vaccination [80,81]. Following natural DENV infection, the majority of human anti-DENV antibodies target prM and the fusion loop in E, most of which are poorly neutralizing. Potently neutralizing type-specific and cross-reactive quaternary antibodies are generated, but in lower amounts [64,76–80]. Antibodies generated following tetravalent vaccination will likely differ depending on each vaccine. Information on the only vaccine published to date reveals that type-specific antibodies targeting quaternary epitopes were not generated against all four serotypes [81]. In experiments where blood from viremic adults was mixed with different human monoclonal antibodies (MAbs) and fed to mosquitoes, quaternary epitope-binding MAbs prevented mosquito infection most effectively, whereas cross-reactive (e.g., fusion-loop targeting) and other type-specific MAbs did not. At least one type-specific quaternary epitope has now been identified for each DENV serotype [68,82,83]. Chimeric viruses with these “transplanted” epitopes have been generated as tools for dissection of polyclonal antibody repertoire and for epitope-specific diagnostics [68,83]. However, overlap of type-specific epitopes sometimes makes it impossible to create recombinant viruses modifying only one epitope at a time. Further, individuals develop many antibodies that bind a dominant epitope in slightly different ways; additional human MAbs will help to accurately define boundaries of major antigenic sites and epitopes and develop new reagents for tracking polyclonal epitope-specific responses. New approaches that combine proteomics

and functional methods to study the antibody repertoire are promising, but require further investigation [84].

Memory B cells, plasmablasts, and long-lived plasma cells

Protection may be mediated by antibodies in plasma from long-lived plasma cells (LLPCs), antibodies encoded by memory B cells (MBCs) with the potential to differentiate following antigen exposure into plasmablasts, or from naïve plasmablasts; characterizing differences in specificity between these populations is a research priority. The plasmablast response is extensive and convenient to study, but the profile of these antibodies does not necessarily correlate with the specificity or function of memory responses. Only a small proportion of the breadth of the MBC repertoire may be found in the blood, and currently, there are numerous challenges to MBC profiling, including how to properly identify and sort DENV-specific MBCs [85]. LLPCs cannot easily be studied in human populations, limiting research to plasma antibodies. New methods are available for comparing MBC specificity and plasma antibody repertoire in humans, which may be applied to studying dengue [86].

T cells

The discussion of the role of T cells in dengue disease has shifted over the last five years from a focus on pathogenesis to a potential beneficial role based on new evidence suggesting that robust $CD8^+$ T cell responses may be protective [54–59]. More work is required to characterize the $CD4^+$ T cell response and its role in protection [57], although some evidence exists for particularly important subsets, such as follicular T helper (T_{fh}) cells in the blood. Follow-up research should be performed on the observed association between certain HLA types and more severe forms of dengue [87]. The importance of large CMI studies was discussed: if each HLA type is present in only a fraction of the population, correlations cannot be determined unless large studies are performed. Phase 1 and Phase 2 vaccine studies, which include adults who can provide larger quantities of blood, should be used to identify the most critical assays to use in efficacy trials, such as the induction of multifunctional $CD8^+$/$CD4^+$ T cells. There was consensus that it is important to measure the frequency, function, specificity, and kinetics of T cell responses in dengue patients and vaccinees.

Systems immunology

A next step for dengue research is use of comprehensive immune profiling and computational approaches to measure multiple immune parameters simultaneously via a systems immunology approach to identify correlates of protection and risk, drawing on the experience with, for example, HIV and influenza viruses [88–91]. A systems immunology approach can improve prediction of vaccine efficacy and protection at the individual level, encompassing multiple potential contributors to immune state and response variability in the human population. For example, ‘intrinsic’ variation in immunity at baseline and in response to a vaccine can be driven by age, sex, infection history, microbiome, co-morbidities, and host genetics, and systems immunology provides ways to explore these and other variables alongside multiple immune and gene expression parameters to identify correlates. There is value to this more holistic and broad approach, although it can be more expensive and complicated, and thus requires careful planning and coordination.

5. Efficacy and safety of dengue vaccines and lessons learned from recent vaccine clinical trials

Age, serostatus, and disease severity in vaccine trials

Given the differences observed in all vaccine trials to date between flavivirus- and/or DENV-seropositive and -seronegative vaccine recipients, vaccine efficacy must be assessed separately according to prior baseline serostatus; whether studies should be powered to evaluate vaccine efficacy by baseline serostatus was also discussed. An exploratory analysis of a recent Phase 3 trial showed a significant increase in risk of hospitalization with dengue disease in vaccinated 2-5 year olds 1-2 years after the third vaccine dose as well as an elevated risk, though not significant, 2-4 years after the third vaccine dose [46]. A proposed explanation is that vaccination stimulated the immune system as a natural primary DENV infection would: vaccine recipients without a moderate-to-strong month 13 nAb titer in response to vaccination have waning immunity over a year beyond vaccination/infection and are at increased risk of severe disease upon their next DENV exposure [45]. In contrast, flavivivrus-seropositive vaccine recipients had high vaccine efficacy, approximating immune responses more analogous to natural secondary DENV immunity and suggesting that seropositive children may require fewer than 3 doses for protection. Modeling results demonstrate that this hypothesis is consistent with the vaccine efficacy observations [92,93]. As pre-vaccine samples were only collected for a subset of trial subjects, efforts to estimate protection stratified by immune status, rather than just age, for all children in the trial were recommended, including measuring antibody response in post-vaccine samples to either the yellow fever chimeric backbone of the vaccine constructs or DENV nonstructural proteins to determine baseline DENV serostatus (e.g., whether immunity was vaccine-derived, from prior DENV infection, or other flavivirus infection).

The WHO Strategic Advisory Group of Experts (SAGE) on Immunization recommended that countries consider Dengvaxia vaccination only in areas where seroprevalence of anti-DENV antibodies is ≥70% in the population targeted for vaccination, namely individuals age ≥9 years [92,94,95]. A vaccine that protects seropositive individuals but not seronegative individuals provides more protection than risk if the majority of vaccinees are seropositive, but in areas with low DENV transmission intensity, vaccination may place some seronegative recipients (especially young children) at higher risk of hospitalization than if they had never been vaccinated [92,93]. Some argued that available evidence is not sufficient to prove a safety issue related to serostatus, and further research is required to evaluate the safety signal in 2-5 year-olds and whether it is a transient or a long-lasting problem. Others argued that the scale of the Phase 3 trial in Southeast Asia was sufficient to be confident that the observed risk in young vaccinated individuals demonstrates a safety problem associated with serostatus. There was debate regarding whether sufficient data are [96] or are not [44] available to determine whether negative serostatus is associated with risk of hospitalized dengue regardless of age, including children age ≥9 years. It remains to be seen whether similar safety problems will be detected for other tetravalent vaccines.

Communicating to the public risk-benefit assessments for dengue vaccines

The importance of considering the effect of vaccination at both the population level and the individual level, including effects on those who are not vaccinated, was emphasized, as well as the importance of communication and discussion of risks and benefits of any dengue vaccine to aid country-level and individual decision-making about vaccination in dengue-endemic countries. For instance, it may be difficult for non-experts to place the observed increase in the risk of severe disease in young individuals >1 year after vaccination in the context of concerns about vaccine-enhanced DENV disease in seronegatives [44,97]. Communicating potential risks as well as benefits of dengue vaccination is particularly important because the political fall-out from vaccine-induced severe dengue disease could impact future vaccination efforts. For comparison, an RSV vaccine that caused severe complications slowed development of new vaccine candidates for 30 years due to risk perception and a resulting lack of private and public investment [98]. For dengue, a major scientific and communication problem is to distinguish between vaccine-associated disease and simple breakthrough infections. Some argued that good risk management approaches are currently in place, and vaccine rollout can safely continue in settings with high background seroprevalence [99].

Mitigating individual risk of severe disease in vaccinated individuals with evaluation of individual baseline serostatus by companion diagnostics or available dengue antibody tests

Modeling work based on available data suggests that Dengvaxia can place seronegative individuals at increased risk of hospitalized dengue, regardless of transmission setting, but is highly effective in seropositive individuals in all settings [93]. Thus, modeling suggests that a companion diagnostic or available rapid serostatus diagnostic test could be used to determine seropositivity prior to vaccination to better target the population that most benefits from vaccination [93]. Such diagnostics are in development for other vaccines and treatments with efficacy concerns, and are even more important if there is a safety concern. The choice of test and feasibility of such an approach was acknowledged to be challenging, as current IgG tests are not fully reliable, and the current Zika epidemic, as well as Japanese encephalitis and yellow fever vaccination, only further complicate matters. Some argued that, until there is a good point-of-care test, available IgG tests could be used. A regulator noted that national regulatory agencies may want to mitigate individual risk of enhanced dengue disease by requiring risk-stratification based on serostatus as measured by serostatus diagnostic tests. Others argued that population-level seropositivity data can be used to determine whether vaccination should be implemented and that a pre-vaccination test is not necessary.

Future clinical trial design

There was broad consensus that in light of dependence of vaccine performance on prior DENV and/or flavivirus exposure, vaccine trials should collect serum samples from all trial participants at multiple time-points, including baseline, the end of the vaccine series, and periodically thereafter.

It may not be necessary to process all specimens immediately, but they should be stored for later analysis if needed. PBMCs should be collected from a subset of trial participants, but the size of an immunogenicity subset would be limited by feasibility and the assays/analyses required. However, identifying cell-mediated correlates of protection would likely require large immunogenicity subsets, potentially all trial participants, in order to have sufficient numbers of cases for statistical inference. For systems immunology studies, serum and PBMC samples should also be collected soon after vaccination (day 1-7).

Vaccine trial design should account for the known period of cross-protection between serotypes, estimated to be 1-2 years after the last vaccination, and should continue active surveillance for a minimum of 3-5 years after the final vaccine dose to allow for estimation of long-term vaccine efficacy as well as risks of vaccine-enhanced disease in vaccinated individuals. WHO recommends 3-5 years of follow-up of trial participants [100]; several National Regulatory Authorities accepted primary endpoint data at 12 months for licensure, but also based decisions on long-term follow up data available at the time or registration.

Post-authorization safety studies and risk management plans

When introducing dengue vaccines into routine vaccination programs, it will be difficult to determine whether an excess of severe dengue cases is occurring in vaccinated populations compared to what would be otherwise expected unless there is a control group, e.g. non-vaccinated individuals of the same age and population experiencing primary dengue. It was agreed that Phase 4 trials need: 1) natural control groups, such as age cohorts, to allow for comparison of disease incidence; 2) good case definitions of severe disease; and 3) vaccine campaigns only in areas where vaccination history and linked individual clinical data can be monitored and severe disease can be appropriately treated [94]. Many low-income countries do not have robust pharmacovigilance/disease surveillance, and routine ‘adverse events following immunization’ monitoring systems are not able to assess potential increases in dengue associated with vaccination, which may occur potentially years after vaccination. As vaccine safety is also monitored through a developer’s/manufacturer’s post-licensure risk management plan, developers work with local governments to build infrastructure and develop better tools for tracking vaccination status and linking it to hospitalized cases, as well as supporting training programs on dengue and severe dengue case management administered by local governments. An approach adopted by Butantan for the ongoing Phase 3 DENV trial in Brazil is to work with family clinics to facilitate vaccination administration and tracking of outcomes through to Phase 4 [101].

Next-generation vaccines

Rationally designed vaccines might drive immune responses towards desired neutralizing epitopes and achieve more balanced immune responses [102]. This may require engineering of antigen complexes that create the quaternary structure of E protein rafts, which is technically challenging. Conversely, it may be possible to “mask” some targets, such as the cross-reactive fusion loop, to drive immune responses towards relevant neutralizing epitopes. Another proposed approach is to use multivalent viruses in which critical features of type-specific epitopes are contained in one or two chimeric vaccine strains. Finally, sequential (as

opposed to simultaneous) vaccination was presented as an alternative method to induce cross-reactive responses to multiple serotypes.

7. Conclusions

The *Summit on Dengue Immune Correlates of Protection* highlighted recent advances in research toward identifying DENV immune correlates in the context of natural DENV infections and vaccines, as well as remaining research questions and challenges to be addressed in the future.

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Box 1

Correlates of protection: Summary points

- An immune correlate of protection is an immune response marker that is statistically associated with protection from disease or infection and may be either mechanistic (causally related to outcome) or non-mechanistic (statistically related to outcome).
- An immune marker that is a correlate of protection is defined for a specific infectious disease endpoint and may be derived from natural or vaccine-induced immunity.
- For some diseases and vaccines, useful non-mechanistic correlates in lieu of true mechanistic correlates of protection are available.
- All currently licensed vaccines work primarily through antibodies, and most vaccines approved in the last 10 years had serological markers as immune correlates measured with validated assays.
- Different aspects of the immune system often perform redundant functions or may be synergistic protective mechanistic correlates.
- Applications and uses of immune correlates of protection and risk include:
  - helping to define important aspects of infectious disease biology;
  - identifying the optimal choice of vaccine antigen and establish criteria for the consistency and potency between vaccine lots;
  - determining susceptibility to disease at the individual and population level;
  - providing a way to inform vaccine licensure in cases where establishing efficacy directly through clinical trials is not ethical or feasible; and
  - helping with bridging from first- to second-generation vaccines [103].
- Types of adaptive immunity that may modify protection include:
  - serum antibodies and their avidity, neutralization capacity, cytotoxic functionality, and ability to promote opsonophagocytosis;
  - mucosal antibodies, including local IgA and diffusion of IgG to relevant surfaces;
  - $CD4^+$ T cells and the degree to which they help activate B and T cells, promote inflammation, release cytokines, lyse cells, and maintain steady-state immunity; and

- the avidity of $CD8^+$ T cells and their ability to lyse appropriate target cells and not cause excessive damage [104,105].

Box 2

Correlates of protection for dengue vaccine licensure

Overview of correlates of protection for vaccine licensure

- The primary goal of regulators is to establish that biological agents are safe, pure, and potent.
- The traditional method for vaccine licensure requires a randomized clinical trial with comparison between treatment and control arms using a quantitative measure, either disease or an immune correlate.
- Mechanistic and non-mechanistic correlates of protection are used, but immune markers should be measured using functional assays and be regarded by the scientific community as biologically relevant.
- Other fields, such as HIV, received central funding (NIH) to take a harmonized approach for standardization of all measures of immune correlates.
- All assays should be qualified (control for variability due to reagents, the process of conducting the assay, operators, training) so that there can be confidence in the results.
- Validation is a stringent and labor-intensive process, and is important for regulatory submissions [106].

Specific considerations for dengue correlates of protection

- For dengue, safety, efficacy, and duration of protection are highly interrelated with disease due to immune enhancement.
- Valuable assays for vaccine evaluation include:
  - second generation neutralization assays, considering different types of cell substrates;
  - B cell memory assays for inactivated vaccines;
  - cell-mediated immunity assays;
  - antibody affinity/avidity;
  - serotype-specific antibody/depletion assays;
  - systems immunology; and
  - isotype/effector function.
- Currently, vaccine developers have each developed their own assays, measuring particular endpoints relevant to their vaccines.

- Attempts to harmonize neutralization assays have been difficult, and lack of a universal correlate of protection across products makes it difficult to know which assay to harmonize.

Box 3

State of the art

What have we learned from natural infections?

- Protection and correlates of protection need to be separated into “soon-after-infection/vaccination” and those that provide long-term protection.
- Cross-protection from dengue disease after primary infection is observed in those who maintain high nAb antibody titers.
- Homologous re-infections can lead to a boost in immunity.
- Tertiary and quaternary infections are typically milder than secondary, as well as primary, infections.
- Immune correlates of protection may differ by immune status, serotype, assay, and possibly by “epidemic force”.

Immune correlates in the context of dengue vaccines

- Multiple dengue vaccine candidates show differences in immunogenicity between seropositive and seronegative recipients.
- Temporal dynamics of vaccine-mediated immunity have some analogy to natural immunity for live-attenuated vaccines, although there are differences in immunity due to sequential monotypic DENV exposure (natural infection) and tetravalent DENV exposure (vaccination).
- The magnitude of vaccine-mediated immunity is generally lower than in natural infection.
- Immune correlates will likely differ by vaccine and assay employed.

Considerations for defining immune correlates

- There is antigenic variation within serotypes, including amino acid variation in type-specific quaternary epitopes in highly lab-adapted, prototype strains; DENV strains also vary in temperature sensitivity and maturation state, which may vary by assay conditions.
- NAb titers may not fully capture the ‘protective’ component of the immune response, which is likely due to antibodies that bind type-specific and cross-reactive quaternary epitopes; further research on functionality *in vivo* is required.
- Standard neutralization assays should still be performed, despite the challenges with interpretation, but efforts to standardize/qualify assays should be made.
- Current data suggest that DENV-specific T cells may contribute to protective immune responses and thus can be beneficial.

- A systems immunology approach to identify correlates of protection and risk may be a key next step for dengue, including measurement of a large number of immunological markers combined with unbiased statistical/computational learning for inference and building and validation of predictive models.

Efficacy and safety of dengue vaccines and lessons learned from ongoing vaccine clinical trials

- Vaccine efficacy should be assessed separately for baseline DENV- and/or flavivirus-seronegative and -seropositive vaccine recipients and whether studies should be powered to do so remains an important question.
- The effect of dengue vaccination must be considered on both the population and individual level.
- There is a potential risk of dengue vaccination sensitizing seronegative individuals to hospitalized dengue upon subsequent DENV exposure.
- Clear communication and discussion of the risks and benefits of dengue vaccines can aid country level and individual decision-making about vaccination in dengue-endemic countries.
- Vaccine trials should collect serum samples from all trial participants at multiple time-points, including baseline, the end of the vaccine series and periodically thereafter. The baseline samples are critical for statistical assessment of immune correlates of protection. Ideally PBMCs should be collected for assessment of T cell correlates, although it may not be feasible to collect PBMCs from all trial participants.
- Vaccine trial design should account for the known period of cross-protection between serotypes and include active surveillance of trial participants for at least 3-5 years to enable long-term vaccine efficacy estimates.

Box 4

Research Agenda

- Whether and how boosting affects the durability of dengue immunity in endemic areas is important for understanding the duration of vaccine-induced immunity.
- Further research is needed to understand the specificity of DENV memory B cells and how they differ from long-lived plasma cells and plasmablasts.
- T cell responses, including HLA, are potential immune correlates and should be further investigated in the context of natural infections and vaccines.
- Establishing correlates of protection will likely require complementing traditional “reductionist” approaches with newer systems immunology approaches that attempt to combine multidimensional datasets in unbiased statistical/computational learning analyses, including combining measurements of antibody and T cell responses.
- As countries start vaccinating their populations, it will be difficult to determine whether an excess of severe dengue cases is occurring compared to what would be otherwise expected unless there is a unvaccinated control group: strategies and tools for measuring vaccine efficacy and risk in Phase 4 trials should be implemented.
- The role of natural strain variation in vaccine performance should be further studied.
- Lessons learned from recent advances in dengue structural biology should be applied to next-generation vaccines to optimize antigens for immunogenicity evaluation.

Box 5

Future directions: Standing working committees for specific projects

Reference panels

- Learning from the experience of the HIV field, a central reference laboratory or at least a repository for standardized reference panels of diverse, low-passage strains and infectious clones, alongside sera from primary and secondary natural DENV infections, would be of enormous benefit for standardizing neutralization assays across laboratories. However, this would require identifying a funding source.

Standardizing and qualifying neutralization assays

- It was suggested that the dengue community derive a matrix of variables that affect nAb titers, and how, to be shared among new and established dengue researchers, including parameters such as: cell substrate and receptors, virus strain, source and maturation state of virus, use of serum versus plasma (presence of EDTA), etc. The community should also work toward qualifying neutralization assays, including controlling variability due to reagents, the process of conducting the assay, operators, and training, to ensure that results are highly repeatable.

T cells

- There was a call for collaboration among researchers and vaccine developers to better define the role of cellular immunity (T cells) in DENV infection and vaccine protection, including sharing results, samples, and reagents; follow-up meetings/efforts are ongoing.

Cross-cohort comparisons

- Cross-cohort comparisons (possibly including placebo arms of vaccine trials) will enable testing of specific questions that take advantage of the epidemiological differences between locations as well as increased sample size. Such questions include the impact of DENV genotype on disease, immunological determinants of severe disease, homotypic infection, and boosting, among other questions. This would involve harmonizing measurement of specific parameters and reanalyzing existing data, and follow-up plans are in progress.