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Abstract

Dengue vaccine development has made significant strides, but a better understanding of how vaccine-induced immune responses correlate with vaccine efficacy can greatly accelerate development, testing, and deployment as well as ameliorate potential risks and safety concerns. Advances in basic immunology knowledge and techniques have already improved our understanding of cell-mediated immunity of natural dengue virus infection and vaccination. We conclude that the evidence base is adequate to argue for inclusion of assessments of cell-mediated immunity as part of clinical trials of dengue vaccines, although further research to identify useful correlates of protective immunity is needed.

Introduction

The immunological basis of the efficacy of many of the most well-established vaccines is poorly understood, and, where studies to better understand vaccine efficacy have been done, they have almost always relied on tests of pathogen-specific antibodies rather than on measures of cell-mediated immunity (CMI) [1]. Several reasons likely explain this bias; serum is more easily obtained than viable lymphocytes, antibodies can be studied in isolation, and assays of antibody concentration and function are technically more straightforward and reproducible than cellular assays. Fortunately, in many cases detection of antibodies at or above a defined concentration using specific assays has proven to serve as a useful correlate of protective immunity. However, there has been ample evidence in the case of established vaccines that the information provided by assays of antibody responses is often incomplete, and that protective immunity (sometimes only partially protective) was present in some individuals without protective antibody levels.

A consultation was organized by the WHO in 2007 to "review the state of the art of dengue CMI and to discuss the potential role of CMI in advancing dengue vaccine candidates towards licensure" [2]. The participants concluded that "precise function of CMI in protection or disease pathology remains ill-defined and, at present, there is no evidence to suggest that CMI can be utilized as a correlate of protection." Recent data from dengue vaccine trials has renewed interest in addressing this issue, however. In the pivotal phase III trials of the Sanofi Pasteur chimeric dengue virus (DENV) – yellow fever virus (YFV) vaccine, plaque reduction neutralization titers (PRNT) only weakly correlated with protection, and breakthrough infections occurred in some individuals with high PRNT values [3, 4]. While efforts continue to refine assays of DENV-specific antibodies in order to discriminate effective/protective from ineffective/non-protective antibodies (assuming that this is possible), these findings re-emphasize the need to consider the role of DENV-specific T lymphocyte responses in vaccine efficacy. This review seeks

to summarize the current state of knowledge regarding DENV-specific CMI and propose potential contributions of CMI measurements to dengue vaccine development and testing.

An appraisal of the literature on DENV-specific T cell responses merits a brief review of current paradigms in T cell biology and relevant technologies. One area highlighted by recent work is the complexity of effector T cell subsets. Extending the paradigm of Th1 versus Th2 responses among CD4 T cells, at least 7 different phenotypes have now been described [5, 6]. Table 1 summarizes key proteins expressed by each subset. Cytokines and other signals produced by antigen-presenting cells during the initial T cell activation (not listed in the table) determine which pathway is taken by an individual T cell through the induction of the transcription factors listed, and this in turn controls the profile of chemokine receptors and cytokines produced. The characteristic cytokines produced by each subset are the major determinant of its role in immunity and also tend to reinforce cell polarization. The profile of chemokine receptors expressed by each cell subset determines that subset's predominant anatomical distribution, such as peripheral versus mucosal versus secondary lymphatic sites, which also contributes to its function in the response to different pathogens. Cytolytic activity, not traditionally considered an important effector function of CD4 T cells, has been increasingly recognized, mainly among cells expressing Th1 cytokines [7]. In contrast, while cytolysis has long been seen as the main function of CD8 T cells, there has been a growing recognition of more diverse subsets within this population. CD8 T cell subsets with cytokine profiles similar to several of the CD4 subsets listed in Table 1 have been described, although there is comparably less known about them. Based on studies in mice, T cell polarization has often appeared to be a fixed characteristic of the cell determined during its initial activation. However, studies in humans suggest more plasticity in T cell phenotype [8].

Another area of active research in T cell biology is the developmental relationships between naïve, effector, and memory T cells [9-11]. This topic entails significant debate, as, unlike the case with B lymphocytes, there are no universally accepted standards for defining a memory T cell; several different

schemas have been proposed to define the phenotypes of effector versus memory T cells, but it is clear that these are imperfect. From a functional standpoint, it is recognized that, among antigen-experienced T cells, there is a subset of short-lived effector cells that are destined to undergo apoptosis whereas other cells demonstrate the capacity for long-term persistence and even self-renewal. Within the long-lived memory cell population, heterogeneity in function and protein expression led to a distinction of central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). Recent data have revealed further complexity, and led to the classification of several additional subsets such as tissue-resident memory T cells (T_{RM}) and stem memory T cells. Rather than fixed cell fates, however, there is evidence that these phenotypes retain some degree of plasticity. The timing and determinants of the transitions between states are not fully understood, and remain an important area of investigation. Several markers have been clearly identified as strongly associated with a cell's capacity for long-term survival, such as high expression of IL-7R and low expression of KLRG1.

Assay methods

Persisting antibody following vaccination is recognized as the first line of defense against subsequent infection and is regarded as a distinguishing characteristic of an effective vaccine [12]. All currently licensed anti-viral vaccines elicit a robust antibody response that correlates with the level of protection provided by the vaccine [13]. If the same should prove to be true for dengue, then the search for a CMI "correlate of protection" for dengue would be unnecessary. However, dengue is one of several globally important infectious diseases, along with HIV, malaria, and tuberculosis, for which a vaccine is highly desirable yet no validated animal model or correlate of immune protection is known. While empirical testing of candidate vaccines has been successful in the past, the era of molecular biology has led to an explosion of tools and methodologies for creating new vaccine antigens and vector delivery systems. The contribution of CMI, particularly T cells, to a successful dengue vaccine is highly likely

whether it be as direct effector cells, provision of help for antibody development or creating a generalized anti-viral environment. Together with the antigenic complexity of candidate dengue vaccines (Table 2), assessing T cell responses presents a logistical problem for both vaccine developers and clinical testing laboratories – how to test or screen for all possible T cell functions when the most relevant function(s) are unknown.

Fortunately, T cell-based immunoassay development has also proceeded at a remarkable rate [14, 15]. A list of assays together with their advantages and disadvantages is presented in Table 3. Recently the focus of immune-monitoring has been upon assays that provide "minimal manipulation." Relatively high-throughput assays such as ELISPOT and intracellular cytokine staining (ICS), which utilize *in vitro* stimulation times of less than 24 hours (or no stimulation in the case of direct *ex vivo* flow cytometry), are the assays of choice as a screening tool. When well qualified, both platforms are quantitative and specific for the antigen. While validation of ELISPOT and ICS assays is not trivial, it is possible, and if a T cell-based correlate of protection for dengue is defined one of these platforms would most likely be the basis of such an assay [16, 17]. The general disadvantage of ELISPOT assays is that some *a priori* knowledge of the relevant functions is required. IFN-γ has been used extensively in vaccine development as a marker of vaccine take and as a function that is necessary, but perhaps not sufficient, for protection. ICS expands upon the functional profile of ELISPOT assays, bringing the concept of polyfunctionality of T cells to the fore. Again, some *a priori* knowledge of the relevant functional profile is required to fully interpret the results of this assay. Furthermore, ELISPOT and ICS assays are best suited for measuring and quantifying the direct effector capacity of T cells (IFN-γ, TNFα, and cytolytic potential), but are significantly less sensitive at measuring T cell helper capacity. Mass cytometry and advanced polychromatic flow cytometry are technologies that permit the analysis of as many as 36 parameters simultaneously on a single cell. These parameters may include both phenotypic and functional markers. While these methods will facilitate high-dimensional, quantitative analysis of

biomolecules on cell populations at single-cell resolution, their application to dengue research has so far been limited [18, 19].

The most sensitive assays are generally those that involve proliferation of a small number of antigen-specific precursor cells. Dye-dilution based T cell proliferation, when appropriately calibrated, can identify the phenotype of proliferating T cells as well as quantify the precursor frequency [20]. In addition, cytokines associated with helper (e.g., IL-4, IL-5, IL-13, IL-21) or regulatory (e.g., IL-10, TGF-β) capacity can be studied in supernatants collected from proliferation assays. This approach does however digress from the minimal manipulation concept, is less reproducible and is prone to *in vitro* variation artifact.

Microfluidics-based technologies have led to the possibility of extensive transcriptional profiling of T cells at the single-cell level and a description of the population dynamics of T cell responses. While better suited to a research-based environment, these methodologies provide a discovery platform that will deliver the best opportunity to uncover a correlate of protection [21, 22]. Ultimately a thorough profiling of the entire "immune space" that is occupied by a dengue vaccine will be required to compare and contrast different vaccine modalities and vaccination strategies [23]. Describing the quality, quantity and durability of immune responses elicited will involve a standardized approach incorporating many of assay procedures listed above and probably new technologies as they become deployable.

Should a CMI correlate of protection from dengue infection be identified, a significant effort will be required to qualify and validate assays platforms that will reliably detect and/or measure the correlate or function. As described earlier, validation of ELISpot or ICS format assays has proved possible; however, the further challenge will be applying these assays to meet the needs of the global dengue vaccine research community. The field would benefit from the establishment of centralized laboratory(s) that implement External Quality Assurance (EQA) Programs for overseeing the development of external proficiency testing programs for flow cytometry, ELISpot and other CMI-based

assays [24-27]. EQA programs serve three purposes and are run according to Good Clinical Laboratory Practice (GCLP) guidance: 1) provide a means for laboratories to ensure that the data generated are accurate, timely and clinically relevant; 2) provide assurance to sponsors that the data is reliable and high quality; and 3) ensure the appropriate and accurate use of human specimens obtained from clinical trials. In addition to EQA programs, the establishment of biorepositories of standardized qualified reagents and antigens (e.g. PBMCs, peptide sets, viral isolates) for use in helping laboratories validate assays would be invaluable [28-30]. Such programs have proved successful for the field of HIV vaccine testing, with the EQAPOL program run by the NIH Division of AIDS, and the field of cancer T cell therapy, with the immunomonitoring program run by the Cancer Immunotherapy Consortium (http://www.cancerresearch.org/cic) [24, 31, 32].

T cell responses to DENV

Human T cell responses to DENV were first characterized over 30 years ago, and many of the general principles originally described have remained consistent [33, 34]. Infection with one DENV induces both CD4 and CD8 memory T cells specific for DENV epitopes, with a small number of epitopes dominating the response in each individual. Epitopes are located throughout the DENV polyprotein, although several regions, especially the nonstructural protein 3 (NS3), appear to have a concentration of immunodominant epitopes. The amino acid homology across the four DENV serotypes varies for each epitope; however, most epitopes are well conserved among strains within the same serotype and differ at relatively few positions (1 to 3 of 9 residues) from the corresponding epitopes of other DENV serotypes (and other flaviviruses). The overall T cell response induced by a primary DENV infection is strongest to the serotype to which the subject had been exposed, but variable degrees of cross-reactivity are usually observed to one or more of the other serotypes.

Notwithstanding the confirmation of the above paradigms, the greater understanding of T cell biology and advancements in techniques for analysis of T cell responses described above have provided a more detailed and complex picture, particularly with regard to the different characteristics of the memory T cell response and their potential functions during the recall response to a subsequent DENV infection. Inasmuch as vaccination is intended to induce an immune response that will protect against infection or disease during a subsequent DENV exposure, these findings are highly relevant to evaluating the immunogenicity of different vaccine regimens. However, extrapolating observations from natural DENV infection to current vaccines is confounded by several important differences, as will be discussed further below.

Survey of recent literature

The pace of scientific publications describing the T lymphocyte response to DENV has greatly accelerated in recent years. A review of PubMed entries showed at least 38 papers published since 2005 that analyzed human DENV-specific T cell responses based either on functional responses to stimulation by DENV antigens or staining by HLA-peptide tetramers containing DENV peptides, 26 of which have been published since 2010 [35-75]; papers that measured serum levels of cytokines or frequencies of lymphocyte subsets during acute DENV infection were not counted if the methods could not relate the findings with antigen specificity. Taking advantage of newer techniques, these papers have greatly expanded the number of individuals whose immune responses have been characterized- tens to hundreds of subjects in each study, in comparison to fewer than 10 in most of the earlier studies. The knowledge base of DENV-specific immune responses is thus more representative of the global population, particularly among populations in dengue-endemic areas.

Several methodological trends are evident in the recent literature. ELISPOT and flow cytometry have become preferred assays; relatively few of the results from these assays- usually only for dominant

responses- have been validated by analysis of epitope-specific T cell lines. All ELISPOT and cytokine flow cytometry studies have examined the production of IFN-γ. Studies using cytokine flow cytometry have in addition measured several other effector functions, in particular TNFα, MIP-1β, or IL-2 production or release of cytotoxic granules (measured by capture of CD107a at the cell surface).

In vitro stimulation for detection of DENV-specific T cells was accomplished with synthetic peptides in nearly all of the recent studies. In comparison with crude antigen preparations used in earlier studies, such as DENV-infected cell lysates, synthetic peptides provide greater standardization and reproducibility, and also directly provide detailed epitope localization. The large number of peptides needed to provide a comprehensive analysis of all potential DENV epitopes presents a major technical challenge, however. None of the studies reviewed included overlapping peptides from the full proteomes of all four DENV serotypes. Weiskopf et al conducted the most comprehensive analysis [60]; however, although a total of 8,000 peptides were used in the study, each subject was only tested for recognition of a subset of peptides selected based on predictions of peptide binding to autologous HLA class I alleles. Epitope prediction algorithms were used in 8 other studies, but many fewer candidate epitopes were tested. Fourteen studies tested sets of overlapping peptides; of these, 4 studies tested peptides covering the full proteome of DENV-2, whereas the remaining studies tested overlapping peptides covering only a portion of the proteome, most often the NS3 protein.

At least 10 studies have used HLA-peptide tetramers to analyze DENV-specific T cells either directly ex vivo or after in vitro expansion [36, 38, 42, 47-49, 52, 59, 66, 73]. However, six of these studied the same HLA-A*1101-restricted “GTS” epitope on the NS3 protein; in total, the remaining 4 studies investigated 5 other CD8 T cell epitopes and 2 CD4 T cell epitopes. Thus, conclusions based on this body of data still are subject to considerable potential for bias.

Contributions from animal models

Differences between study populations in host genetics as well as prior DENV exposures continue to complicate the comparison of findings across studies. Given the difficulty in documenting or controlling these factors, there continues to be substantial interest in experimental animal models, particularly small, genetically defined animals such as mice. Several "humanized" mouse models have been studied. In several studies of transgenic mice expressing single HLA alleles, investigators demonstrated recognition of candidate epitopes that were selected for predicted HLA binding; subsequent testing of DENV-immune humans confirmed responses to some but not all of these epitopes [64, 69, 76-78]. Studies of immunodeficient mice in which human immune cells were reconstituted by transfusion of human hematopoietic stem cells detected T cell responses to a limited number of known human T cell epitopes [79, 80]. These studies provide preliminary evidence that these models might supplement human studies. Limited testing of heterologous secondary DENV infections was done in HLA-transgenic mice [78], but no comprehensive analysis of the different possible sequences of DENV infection has been conducted in these models to date.

Epitope distribution and cross-reactivity

Recent studies have greatly expanded the database of T cell epitopes identified on DENV proteins [81]. This reflects the combined effects of studying a larger number of humans with more diverse HLA alleles and prior DENV infection history as well as the application of single-cell assays such as ELISPOT with large numbers of synthetic peptides. It is difficult to directly compare the results from different studies, however, because of the confounding effects of differences in the numbers and characteristics of the peptides used. Overlapping peptides covering over 70% of the proteome of representative strains of all four DENV serotypes have been made available to the research community through an NIAID-funded reagent repository (www.beiresources.org), but these were not used in most

of the published studies. Additionally, there remains a lack of consensus on the optimal criteria for defining epitopes. Immunodominant epitopes- those that induce responses of high magnitude in the majority (often nearly all) of subjects with the appropriate HLA allele- have generally shown similar results across studies, but these represent a minority of the epitopes identified and the generalizability of the observations regarding these epitopes needs to be verified.

As mentioned above, the distribution of T cell epitopes across all DENV proteins, albeit with a predominance of epitopes on nonstructural proteins, has been reinforced by the expanded literature. A need to test for responses to the entire proteome of all four DENV serotypes presents challenges for performing large-scale testing of T cell responses, such as in the context of a phase II or III vaccine trial. In contrast, data pointing to the immunodominance of responses to particular regions of the polyprotein provide some support for more targeted testing. For example, Weiskopf et al have estimated that a pool of 268 peptides would include 90% or more of CD8 T cell epitopes in any study population [72]. However, this conclusion is based on their approach of HLA class I epitope prediction. It is reasonable to hypothesize that other immunologically important epitopes, especially HLA class II-restricted epitopes, have yet to be defined. Studies have yielded conflicting data on whether the distribution of CD4 T cell epitopes is similar or different from that of CD8 T cell epitopes [48, 57], with one study reporting that CD4 T cells more often recognized epitopes on structural proteins [57].

The use of single-cell assays such as ELISPOT has complicated the interpretation of serotype-cross-reactivity of T cell responses, as these assays do not assess serotype-cross-reactivity at the level of individual cells. This is a particular problem in individuals who have been exposed to more than one DENV serotype, either through sequential exposure or multivalent immunization. Although one study concluded that serotype-specific epitopes could be defined based on sequence conservation alone [78], other experimental data are directly contradictory [36, 37, 41]. Another study described a panel of CD4 T cell epitopes predicted to be serotype-specific based on high sequence divergence across serotypes

[55]. Among participants in a cohort study, individuals who experienced an interval DENV infection acquired responses to peptides of one additional serotype [74]; however, only 7 subjects were studied and the DENV serotype causing the interval infection was not identified.

Several recent findings underscore the importance of clinical, virologic, and epidemiologic data on individual subjects for the interpretation of T cell responses to DENV. Although measures of T cell responses at the population level consistently show stronger responses to the infecting DENV serotype after a primary DENV infection, exceptions to this pattern have been observed at the level of individual epitopes [37, 49, 52], and the patterns of cross-reactivity have been even more difficult to predict after secondary DENV infections. Several studies have also found sufficient sequence divergence within one or more DENV serotype(s) to affect the T cell response [67, 82], but the clinical significance of these observations is unknown.

T cell subsets and their effector functions

Recent studies using multiparameter flow cytometry have provided a more detailed picture of the effector T cell response to DENV. As noted above, most studies have focused on type 1 cytokine-producing T cells (Th1/Tc1); these studies have revealed a high degree of heterogeneity in cytokine production at the individual cell level. While polyfunctional T cells expressing 3 or more effector functions have been observed, there are also substantial populations of cells expressing 1 or 2 of the functions measured, including cells expressing only cytokines with pro-inflammatory effects (TNFα and/or β-chemokines) [37, 49, 60, 67]. Stimulation with the corresponding epitopes of different DENV serotypes has been shown to alter the profile of cytokines produced, suggesting that variant epitopes act as altered peptide ligands for some DENV-specific T cells [36, 37].

Comparably less is known regarding effector responses other than Th1/Tc1. Of the few studies that reported data on the production of type 2 cytokines, most reported little or no production of IL-4

except one study of very young children (mean age 7.7 months) [61]. Single studies have described production of IL-17 [61] or IL-21 [57] by T cells in response to stimulation, or have observed the expression of markers associated with follicular helper CD4 T cells [57] or T cells capable of homing to skin [73].

Primary vs. secondary infection

Models of sequential infection with different DENV serotypes postulate that the immune response to secondary infection will differ in several important ways from that to the primary infection: a) the memory T cell response will be induced more rapidly and achieve higher levels, b) the memory response will preferentially activate T cells directed at epitopes that are more highly conserved between the different DENV serotypes, mainly on non-structural proteins, and c) the memory T cell response will have an altered effector profile reflecting differential activation by peptides from the second DENV serotype [83]. Although testing these postulates is highly relevant to understanding both protective and detrimental immune responses in dengue, only a few studies have compared immune responses during or after primary versus secondary DENV infections. Consistent with the predictions, differences have been reported in the expression of some phenotypic markers [71], in the dominant epitopes targeted [78], and in the profile of serotype cross-reactivity [52, 82]. Surprisingly, no significant differences were observed in the kinetics of the response or in the peak T cell frequencies during the acute infection [48, 52]. These studies involved only symptomatic DENV infections, however, and the intrinsic incubation period prior to the onset of symptoms could not be determined. Also, the clearance of viremia may be more rapid in secondary infections, as suggested by some data [84]. These significant differences could have masked differences in the kinetics and magnitude of the immune response in primary versus secondary infections.

Vaccines vs. natural infection

With the expanding pipeline of vaccines in clinical testing and the wider availability of the requisite expertise and technology, there has been a growing body of literature describing the T cell response to dengue vaccines. All of the recently published studies have involved candidate live attenuated vaccines. These studies have shown that DENV-specific memory T cells, including polyfunctional Th1/Tc1 cells, are induced within 21 days after vaccination of flavivirus-naïve subjects [56]. In comparison to vaccination with its individual components, vaccination with the tetravalent formulation of the NIH/Butantan vaccine (Table 2) preferentially induced T cell responses to peptides from the more conserved non-structural proteins [70]. Interestingly, vaccination with the Sanofi Pasteur chimeric DENV-YFV vaccine induced T cell responses to epitopes on DENV NS3 protein in DENV-immune subjects but not in DENV-naïve subjects, suggesting that the heterologous YFV epitopes could reactivate pre-existing memory CD8 T cells but not antigen-inexperienced T cells [62]. Comparison of the T cell responses induced by the different dengue vaccines listed in Table 2 is not possible, however, because of significant differences in study and assay design.

<u>Potential contributions of T cell assays to dengue vaccine development</u>

The area where assessment of T cell responses to dengue vaccines would clearly have greatest impact is in identifying correlates of vaccine efficacy. A reliable immunological correlate of vaccine-induced protective immunity would accelerate vaccine testing in different populations, regimens, or epidemiological contexts. The limitations of current neutralizing antibody assays reinforce the need for a better understanding of correlates of protective immunity, although the poor discriminant ability of neutralizing antibody titers may point either to deficiencies in the assay or to non-antibody protective mechanisms. Human cohort studies and animal experiments have found associations between T cell IFN-γ production and protective immunity [51, 60, 85, 86], supporting the potential to identify T cell

responses associated with protective immunity induced by vaccination. However, the published data are quite limited. Only two studies correlated T cell responses in blood samples collected prior to exposure with clinical outcomes in individual subjects [51, 87]; both studies relied on the same prospective cohort and the sample sizes were small. Also, given the difficulty in defining individuals who are fully protected from infection, all subjects in these studies experienced DENV infections and comparisons were based on severity of illness (hospitalized dengue versus non-hospitalized dengue in one study and subclinical versus symptomatic infection in the other). Other studies measured T cell responses only during or after DENV infection, a significant confounding factor for any conclusions regarding causality. This concern is somewhat lessened in the case of experimental infection, where protective immunity was associated with early IFN-γ responses [88]. In light of the limitations of published data, however, it will be essential to validate immunological correlates against clinical endpoints in vaccine trials.

It will be important to validate any immunological correlates independently for several different vaccines, because the associations between immunological readouts and vaccine efficacy may or may not be equivalent. In addition to the differences in immune response pathways that might be stimulated by live versus inactivated or subunit vaccines, there are significant differences in antigenic content among the dengue vaccines currently in clinical development (Table 2). This is most pronounced with regard to the repertoire of flavivirus non-structural (NS) proteins, with some vaccines containing no NS proteins (subunit and inactivated vaccines, although the latter may include some NS1 protein), some containing NS proteins of one flavivirus, either DENV2 or the heterologous YFV, and one containing NS proteins of 3 of 4 DENV serotypes. Since non-structural proteins contain the majority of T cell epitopes, the repertoire of T cell responses induced by each vaccine will likely differ as well, although the resulting immunological profile is difficult to predict at this stage.

A second area where measurement of T cell responses could make an important contribution is in evaluating the durability of vaccine-induced protective immunity. This is likely to be of particular

importance for dengue vaccines given the evidence that partial immunity increases the risk for more severe illness. Substantial insight has been gained into how the initial activation of T cells contributes to the establishment of both long-lasting T cell and B cell memory, and this process has been successfully manipulated with pharmaceuticals such as rapamycin in experimental models [89, 90]. Licensed vaccines against other diseases differ significantly in the durability of pathogen-specific antibodies and T cells [91]; through comprehensive "systems vaccinology" approaches, early indicators of antibody and T cell responses have been identified for several of these vaccines [92, 93], although further studies are needed to establish their ability to predict longer-term durability of the response.

The single-cell resolution and potential to evaluate multiple T cell effector functions of newer assays offer the capacity to reveal extraordinary detail on the relationships between these responses. This capacity will likely be of special interest in the case of dengue vaccines, given the multivalent nature of dengue vaccines, the need to provide protective immunity against all four DENV serotypes, and the evidence that more severe dengue disease is associated with an inflammatory immune response. Data from several studies showing the induction of polyfunctional T cells by different tetravalent dengue vaccines are encouraging [56, 70, 75]. However, it is unclear whether the degree of 'polyfunctionality' described is optimal; similar frequencies of polyfunctional T cells are seen after natural DENV infection, a setting that does not reflect fully (i.e., tetravalent) protective immunity. Partial immunity to DENV present prior to vaccination, as was seen in the majority of subjects in phase III vaccine trials in endemic areas [3, 4], could also modify the pattern of T cell effector functions.

Conclusions and recommendations

Although assessments of pathogen-specific T cell responses have not been a priority in most vaccine development efforts, we argue that dengue is a special case and that planning and preparation for such assessments should be given greater emphasis. The example of natural infection illustrates the

potential for both positive (protective) and negative (pathological) effects of partial immunity to DENV, and potential concerns for long-term safety will likely remain a major impediment to licensure and widespread uptake of dengue vaccines. The current understanding of T cell responses to DENV indicates the potential for evaluations of T cell responses to accelerate vaccine design and testing by helping to identify correlates of vaccine efficacy and also to reduce the risk to vaccine developers by helping to understand negative outcomes of vaccine trials, should they occur [94]. Implementing analyses of T cell responses in the context of upcoming dengue vaccine trials will present a number of significant logistical challenges (Table 4). Based on current knowledge, it is not possible to define the assay or assays that would reliably serve all of the pertinent objectives. The experience from prospective dengue cohort studies [51, 87] and trials of other vaccines [95] does provide guidance to vaccine developers as to how T cell studies can be incorporated into dengue vaccine trials. There continues to be a need for studies of natural DENV infection as well as efforts to develop new technologies for assessment of T cell responses to DENV. Implementation of these efforts will require ongoing support from government, industry, and charitable foundations, as well as creative solutions from the scientific community.

Disclaimer

The opinions or assertions contained herein are the private views of the authors and are not to be construed as reflecting the official views of the United States Army or the United States Department of Defense.

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661 Table 1. Characteristics defining different subsets of effector CD4 T cells.

Subset	Cytokine(s) produced	Chemokine receptor(s)	Transcription factor(s)	Comment
Th1	IFN-γ	CXCR3	T-Bet	Cellular immunity
Th2	IL-4, IL-5, IL-13	CCR3, CCR4, CCR8	GATA-3	Humoral immunity
Th17	IL-17	CCR2, CCR4, CCR6	RORγt	Inflammation
Th9	IL-9	CCR3, CCR6, CXCR3	PU.1	Mucosal immunity
Th22	IL-22	CCR4, CCR10	AhR	Parasites
Tfh	IL-21	CXCR5	Bcl-6	B cell help
iTreg	IL-10, TGF-β	CCR6	FoxP3	Immunosuppression, tolerance

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664 Table 2. T cell antigenic content of dengue vaccine candidates in clinical development.

Vaccine developer	Structural proteins	Non-structural proteins
Live, attenuated (chimeric flaviviruses)		
Sanofi Pasteur	C: YFV; pre-M, E: DENV1-4	NS1-5: YFV
Takeda	C: DENV2; pre-M, E: DENV1-4	NS1-5: DENV2
NIH/Butantan	C: DENV1/3/4; pre-M, E: DENV1-4	NS1-5: DENV1/3/4
Purified inactivated		
WRAIR/GSK	C, pre-M, E: DENV1-4	None (? NS1)
Subunit		
Merck	E (80%): DENV1-4	None

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667 Table 3. Advantages and disadvantages of different methodologies for evaluation of pathogen-specific T

668 cell responses.

Method	Functions measured	Advantages	Disadvantages
Ex vivo (no stimulation)			
Flow cytometry (HLA-peptide tetramer staining)	Antigen specificity Phenotype	Quantitative readout of cell frequency Independent of cell responsiveness	Limited to one or few epitopes Not reflective of cell function Costly
Short-term in vitro (≤1 day)			
Flow cytometry/mass cytometry (intracellular staining)	Cytokine production Degranulation (cytolysis) Phenotype	Quantitative readout of cell frequency Multiple functions assessed	Costly Specimen requirement high
ELISPOT	Cytokine secretion Granzyme release	Quantitative readout of cell frequency Technical ease Reproducibility Specimen requirement low/modest	One (or two) functions assessed per cell

Single-cell transcriptional profiling	Any function (based on gene expression) Gene networks controlling cell fate	Provides complete profiling at the single-cell and population level	Technically complex Low throughput Expensive Data analysis requires bioinformatics expertise
Extended in vitro (5+ days)			
ELISPOT	Cytokine secretion Granzyme release	High sensitivity Technical ease Specimen requirement low/modest	One (or two) functions assessed per cell Cell frequency altered by stimulation
Flow cytometry (marker dilution)	Proliferation	High sensitivity Technical ease	Less reproducible
^{3}H-Thymidine incorporation	Proliferation	High sensitivity Low cost Technical ease	Radioisotope Less reproducible
Immunoassay	Cytokine secretion Granzyme release	Technical ease Can be multiplexed	Low sensitivity for rare cells

Cloning (characterize with other assays)	Multiple	Multiple functions measured Evaluates antigen crossreactivity	Low throughput (few cells evaluated) Costly Technical complexity

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671 Table 4. Logistical issues and recommendations for assessment of T cell responses to dengue vaccines.

Issues	Recommendations
Technical expertise and infrastructure needed for collection of viable PBMC	Study site development and staff training and supervision
Need to measure responses to all four DENV serotypes (and separately for structural and non-structural antigens)	Collect adequate volumes of blood for assessment of T cell responses
Immune correlates of vaccine efficacy have not yet been defined	Apply a diverse suite of assays of T cell function and specificity
Variation in HLA alleles and prior DENV exposure history in vaccine recipients	Enroll adequate numbers and diversity of subjects in assessments of T cell responses to vaccination Collect blood samples before and after vaccination for T cell assays
Lack of high-throughput assays to measure cross-reactivity at single-cell level	Development of new assay technologies

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