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1 Analysis of cell-mediated immune responses in support of dengue vaccine development efforts

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16

17 Abstract

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

26

27 Introduction

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

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

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

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

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

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

86

87 Assay methods

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

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

103 Fortunately, T cell-based immunoassay development has also proceeded at a remarkable rate
104 [14, 15]. A list of assays together with their advantages and disadvantages is presented in Table 3.
105 Recently the focus of immune-monitoring has been upon assays that provide “minimal manipulation.”
106 Relatively high-throughput assays such as ELISPOT and intracellular cytokine staining (ICS), which utilize
107 *in vitro* stimulation times of less than 24 hours (or no stimulation in the case of direct *ex vivo* flow
108 cytometry), are the assays of choice as a screening tool. When well qualified, both platforms are
109 quantitative and specific for the antigen. While validation of ELISPOT and ICS assays is not trivial, it is
110 possible, and if a T cell-based correlate of protection for dengue is defined one of these platforms would
111 most likely be the basis of such an assay [16, 17]. The general disadvantage of ELISPOT assays is that
112 some *a priori* knowledge of the relevant functions is required. IFN- γ has been used extensively in vaccine
113 development as a marker of vaccine take and as a function that is necessary, but perhaps not sufficient,
114 for protection. ICS expands upon the functional profile of ELISPOT assays, bringing the concept of
115 polyfunctionality of T cells to the fore. Again, some *a priori* knowledge of the relevant functional profile
116 is required to fully interpret the results of this assay. Furthermore, ELISPOT and ICS assays are best
117 suited for measuring and quantifying the direct effector capacity of T cells (IFN- γ , TNF α , and cytolytic
118 potential), but are significantly less sensitive at measuring T cell helper capacity. Mass cytometry and
119 advanced polychromatic flow cytometry are technologies that permit the analysis of as many as 36
120 parameters simultaneously on a single cell. These parameters may include both phenotypic and
121 functional markers. While these methods will facilitate high-dimensional, quantitative analysis of

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

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

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

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

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

156

157 T cell responses to DENV

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

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

178

179 *Survey of recent literature*

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

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

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

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

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

215

216 *Contributions from animal models*

217 Differences between study populations in host genetics as well as prior DENV exposures
218 continue to complicate the comparison of findings across studies. Given the difficulty in documenting or
219 controlling these factors, there continues to be substantial interest in experimental animal models,
220 particularly small, genetically defined animals such as mice. Several “humanized” mouse models have
221 been studied. In several studies of transgenic mice expressing single HLA alleles, investigators
222 demonstrated recognition of candidate epitopes that were selected for predicted HLA binding;
223 subsequent testing of DENV-immune humans confirmed responses to some but not all of these epitopes
224 [64, 69, 76-78]. Studies of immunodeficient mice in which human immune cells were reconstituted by
225 transfusion of human hematopoietic stem cells detected T cell responses to a limited number of known
226 human T cell epitopes [79, 80]. These studies provide preliminary evidence that these models might
227 supplement human studies. Limited testing of heterologous secondary DENV infections was done in
228 HLA-transgenic mice [78], but no comprehensive analysis of the different possible sequences of DENV
229 infection has been conducted in these models to date.

230

231 *Epitope distribution and cross-reactivity*

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

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

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

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

264 [55]. Among participants in a cohort study, individuals who experienced an interval DENV infection
265 acquired responses to peptides of one additional serotype [74]; however, only 7 subjects were studied
266 and the DENV serotype causing the interval infection was not identified.

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

275

276 *T cell subsets and their effector functions*

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

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

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

292

293 *Primary vs. secondary infection*

294 Models of sequential infection with different DENV serotypes postulate that the immune
295 response to secondary infection will differ in several important ways from that to the primary infection:
296 a) the memory T cell response will be induced more rapidly and achieve higher levels, b) the memory
297 response will preferentially activate T cells directed at epitopes that are more highly conserved between
298 the different DENV serotypes, mainly on non-structural proteins, and c) the memory T cell response will
299 have an altered effector profile reflecting differential activation by peptides from the second DENV
300 serotype [83]. Although testing these postulates is highly relevant to understanding both protective and
301 detrimental immune responses in dengue, only a few studies have compared immune responses during
302 or after primary versus secondary DENV infections. Consistent with the predictions, differences have
303 been reported in the expression of some phenotypic markers [71], in the dominant epitopes targeted
304 [78], and in the profile of serotype cross-reactivity [52, 82]. Surprisingly, no significant differences were
305 observed in the kinetics of the response or in the peak T cell frequencies during the acute infection [48,
306 52]. These studies involved only symptomatic DENV infections, however, and the intrinsic incubation
307 period prior to the onset of symptoms could not be determined. Also, the clearance of viremia may be
308 more rapid in secondary infections, as suggested by some data [84]. These significant differences could
309 have masked differences in the kinetics and magnitude of the immune response in primary versus
310 secondary infections.

311

312 *Vaccines vs. natural infection*

313 With the expanding pipeline of vaccines in clinical testing and the wider availability of the
314 requisite expertise and technology, there has been a growing body of literature describing the T cell
315 response to dengue vaccines. All of the recently published studies have involved candidate live
316 attenuated vaccines. These studies have shown that DENV-specific memory T cells, including
317 polyfunctional Th1/Tc1 cells, are induced within 21 days after vaccination of flavivirus-naïve subjects
318 [56]. In comparison to vaccination with its individual components, vaccination with the tetravalent
319 formulation of the NIH/Butantan vaccine (Table 2) preferentially induced T cell responses to peptides
320 from the more conserved non-structural proteins [70]. Interestingly, vaccination with the Sanofi Pasteur
321 chimeric DENV-YFV vaccine induced T cell responses to epitopes on DENV NS3 protein in DENV-immune
322 subjects but not in DENV-naïve subjects, suggesting that the heterologous YFV epitopes could reactivate
323 pre-existing memory CD8 T cells but not antigen-inexperienced T cells [62]. Comparison of the T cell
324 responses induced by the different dengue vaccines listed in Table 2 is not possible, however, because
325 of significant differences in study and assay design.

326

327 Potential contributions of T cell assays to dengue vaccine development

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

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

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

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

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

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

379

380 Conclusions and recommendations

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

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

398

399 Disclaimer

400 The opinions or assertions contained herein are the private views of the authors and are not to
401 be construed as reflecting the official views of the United States Army or the United States Department
402 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	ROR γ t	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
<i>Live, attenuated (chimeric flaviviruses)</i>		
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
<i>Purified inactivated</i>		
WRAIR/GSK	C, pre-M, E: DENV1-4	None (? NS1)
<i>Subunit</i>		
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
<i>Ex vivo (no stimulation)</i>			
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
<i>Short-term in vitro (≤ 1 day)</i>			
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
<i>Extended in vitro (5+ days)</i>			
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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