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T lymphocyte responses to flaviviruses – diverse cell populations affect tendency toward protection and disease

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1 T lymphocyte responses to flaviviruses - diverse cell populations affect tendency
2 toward protection and disease

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12 ***Abstract***

13 Dengue virus (DENV), Yellow Fever virus, West Nile virus, Japanese encephalitis virus and Zika
14 virus are medically important flaviviruses transmitted to humans by mosquitoes and circulate in
15 overlapping geographic areas. Cross-reactive immune responses have been demonstrated among
16 the flaviviruses, particularly the four DENV serotypes. The immunological imprint left by a
17 flavivirus infection can therefore have profound effects on the responses to subsequent infections.
18 In this review we summarize recent research focusing on T cell responses to DENV using clinical
19 samples from prospective cohort studies in Asia. These data suggest that durability of different T
20 cell populations after natural infection or vaccination is an important consideration for the outcome
21 of subsequent flavivirus exposures and we argue for continued investigation in the context of
22 longitudinal cohort studies.

23 **Introduction**

24 The flavivirus genus in the *Flaviviridae* family of positive-strand RNA viruses includes over 33
25 viruses known to infect humans [1]. Among these, the four dengue virus (DENV) serotypes,
26 DENV1-4, represent a major global health problem, infecting an estimated 400 million individuals
27 each year [2]. A world-wide pandemic of Zika virus (ZIKV) infections occurred from 2015-2016,
28 and there remains the potential for future large outbreaks. Since these five viruses are transmitted
29 by the same mosquito vectors, *Aedes aegypti* and *Aedes albopictus*, the population living in
30 affected areas remain at risk for repeated exposures over their lifetime. Additionally, the
31 geographic distribution of DENV overlaps substantially with that of other flaviviruses, such as
32 yellow fever virus (YFV, which shares the same mosquito vector), West Nile virus (WNV), and
33 Japanese encephalitis virus (JEV).

34 The flaviviruses vary in the degree of genetic relatedness, with the four DENV serotypes forming
35 a distinct cluster in the phylogenetic tree. ZIKV is next most closely related to the DENV group,
36 with YFV, WNV, and JEV having greater genetic divergence. The genetic relatedness among these
37 viruses, discovered through the application of high-throughput sequencing, explains the serologic
38 cross-reactivity among these viruses that was reported over 50 years earlier by Casals and others
39 [3]. Serologic studies also established the concept that one flavivirus infection significantly
40 modified the immune response to any subsequent flavivirus infection, resulting in high-titer cross-
41 reactive antibody responses. These observations then led to the insight that severe dengue illness
42 was strongly associated with secondary DENV infections [4].

43 We and other cellular immunologists recognized the potential parallels between these serologic
44 observations and T lymphocyte responses to flaviviruses, and, further, the diversity of functional
45 responses possible as a result of activation of cross-reactive memory T cells during a second

46 flavivirus infection [5–7]. Initial characterizations of flavivirus-reactive T cells confirmed
47 antigenic cross-reactivity as well as altered responses after secondary flavivirus infection. As has
48 been the case for antibodies, these studies provided only circumstantial evidence of a potential
49 important contribution of cellular immunity to flavivirus infection outcomes. Our recent studies
50 have sought to establish the clinical relevance of these responses to better understand individual
51 risk factors for severe dengue illness and correlates of vaccine efficacy and safety.

52 *Technical considerations in studies of flavivirus-specific T cell immunity*

53 A major focus of our attention in recent years has been on study design and its consequences for
54 the interpretation of results. Experimental flavivirus infection models have offered the greatest
55 level of control over important parameters, e.g., the timing of viral challenge and sampling, size
56 of challenge inoculum, and genetic background of the host. These models have enabled studies
57 demonstrating, e.g., the impact of specific infection sequences and interactions with host genetics
58 on both acute and memory phases of the T cell response [8]. However, the failure of most
59 laboratory mice to faithfully recapitulate human infection and disease has been problematic for
60 translation of these findings to humans. While a number of innovative models have been developed
61 to address this limitation, such as “humanized” mouse strains and experimental infection of
62 humans with modified viruses, studies of natural flavivirus infection remain an essential element
63 of the research agenda [9].

64 Table 1 summarizes several key considerations in the design of observational studies of cellular
65 immunity to flavivirus infections, and their implications for interpretation of study results. Several
66 factors address the selection of subjects and specimens for analysis. Many studies have used blood
67 samples collected either during or after an acute flavivirus infection, due to the ready availability
68 of suitable patient populations in flavivirus-endemic areas [6,10–14]. This study design simplifies

69 the collection of specimens for detailed immunological characterization, and, with high-quality
70 associated clinical data, identifies associations with illness and generates hypotheses regarding
71 causality. However, it is clear that key immunological events precede even studies of the early
72 phase of illness; a prospective cohort study design is therefore essential to explore the role of
73 immune responses as predictors of risk. Clinical data available for statistical analyses is dependent
74 on the sample collection strategy, and also introduces additional complexity. A common
75 comparison, subjects with mild versus severe illness, relies on clear and consistently-applied
76 clinical definitions. Mistakes in applying the criteria for dengue fever (DF) and dengue
77 hemorrhagic fever (DHF) have been cited as an issue, but the newer classifications of dengue with
78 warning signs (DWS) and severe dengue (SD) have in many ways been more problematic, as
79 illustrated by the finding that antibody titers were more strongly associated with the earlier
80 classification [15]. We have preferred the DF/DHF classification for symptomatic infections,
81 which more clearly emphasizes plasma leakage as the principal component of more severe illness.
82 We have also used continuous measures of plasma leakage, thrombocytopenia, and liver injury;
83 this has allowed us to identify immunological associations with particular disease indicators [16].
84 The functional responses measured and their cross-reactivity for different flaviviruses are
85 determined by the assay format and selection of antigens. A thorough treatment of these technical
86 issues is beyond the scope of the current review, but several examples of relevant considerations
87 are listed in Table 1. In particular, our strategy has been to measure the responses to individual
88 viruses, e.g., DENV1-4, rather than to develop a single measure, as in the megapool strategy,
89 because the distribution of different clinical outcomes is critically dependent on the specific virus
90 with which each individual is infected.

92 Table 1. Considerations in the design of observational studies of cellular immunity in flavivirus
 93 infections.

Factor	Options	Implications
Timing of sample collection	Pre-infection During acute infection Post-infection	Pre-infection responses have predictive potential; acute responses reflect pathophysiology +/- pathogenesis
Clinical phenotypes	Severe illness Mild illness Inapparent infection Exposed without infection	Severe versus mild illness reflects pathophysiology, but both involve productive infection; inapparent dependent on surveillance, difficult to define exposure
T cell functions	Activation Cytokine production- IFN γ , TNF α , others Cytolysis	Effector functions that are most protective not well defined; many T cell functions also have potential role in disease pathogenesis
Antigen stimulation	No stimulation v stimulation Peptides v virus/infected cell Whole proteome v selected Single v multiple viruses	Measures the presence or function of T cells; affects percentage of virus-specific T cell repertoire detected (based on antigen specificity and/or T cell subset)

95 *Cross-reactivity and functional diversity of flavivirus-reactive T cells*

96 T cells are highly heterogeneous in peptide sequence specificity and functional responses. These
97 are particularly important considerations for flavivirus-specific T cells given the potential
98 implications for secondary flavivirus infections. Recipients of monovalent live flavivirus vaccines
99 have been useful to characterize these responses due to their ability to donate large volumes of
100 blood and well-defined virus exposure, often without confounding exposures to other flaviviruses
101 [17,18]. Multiparameter flow cytometry of antigen-stimulated memory T cells has shown
102 heterogeneous cytokine production at the single cell level for both CD4 [19] and CD8 [20] T cells.
103 Although overall the highest frequencies of cytokine-producing T cells were observed in response
104 to the homologous DENV serotype, in the case of some epitopes higher responses were observed
105 to heterologous peptides. Additionally, the relative frequencies of T cells producing IFN γ versus
106 TNF α or MIP-1 β varied for different epitope variants. These data have demonstrated the potential
107 for flavivirus antigens to act as altered peptide ligands for memory T cells during infection in
108 individuals who have had a prior heterologous flavivirus exposure.

109 Although vaccines offer advantages as model live flavivirus infections, associations with
110 infrequent clinical phenotypes and with sequential infection have continued to require the study of
111 natural infections with wild-type viruses. In addition to a careful and thorough characterization of
112 study subjects, we have found it important to analyze sequential blood samples starting before the
113 critical phase of illness. Using flow cytometry, we have demonstrated that multiple populations of
114 CD4 and CD8 T cells are activated and expanded during acute infection, and that this expansion
115 precedes the development of plasma leakage [6,12]. In one recent study [12], we found a higher
116 number of activated pT_{FH} cells during the critical phase of illness in subjects with secondary
117 infections and those with DHF; the frequency of activated pT_{FH} cells also correlated with antibody

118 responses. While most studies from our group and others have pointed to a positive association
119 between immune activation and disease severity, the direction of causality has remained a point of
120 controversy.

121 *Associations of pre-existing flavivirus-reactive T cell responses with clinical phenotype*

122 Our efforts to address this causal relationship has directed our efforts towards the analysis of blood
123 samples collected through prospective cohort studies. These studies have encountered several
124 challenges, such as the low frequency of severe clinical phenotypes, limited blood samples from
125 young subjects, and the large number of immune responses of interest based on the studies of acute
126 infection. We have therefore applied several different laboratory methods (Table 2).

127 In our first study [19], we selected subjects with symptomatic secondary DENV infections. PBMC
128 were stimulated with inactivated DENV antigens and we compared proliferation and cytokine
129 secretion in subjects with hospitalized (more severe) versus non-hospitalized (less severe) dengue.
130 There was no significant difference in T cell proliferation, however, IFN- γ production to multiple
131 serotypes was associated with milder illness and TNF α production was associated with more
132 severe illness. In our second study [21], we used the same stimulation method but measured the
133 frequency of cytokine-producing CD4 T cells by flow cytometry. We compared responses in
134 subjects with subclinical infection to those in subjects with symptomatic infection (regardless of
135 severity of illness). This study found that subjects with subclinical infection had higher frequencies
136 of CD4 T cells producing IFN- γ or IL-2 in response to DENV antigen than subjects with
137 symptomatic infection. To extend these findings, we selected another subset of subjects with
138 subclinical and symptomatic infections, and measured both the frequencies of responding cells as
139 well as cytokine secretion in response to stimulation in vitro [22]. This study confirmed the
140 association of higher frequencies of IFN- γ -producing CD4 T cells with subclinical infection. We

141 found that the secretion of IL-6, IL-15 and MCP-1 was higher by PBMC from children who
 142 developed symptomatic DENV infection, whereas the secretion of IL-12, IL-2R, MIP-1- α ,
 143 RANTES, GM-CSF, and TNF- α was lower by PBMC from these children. These studies paint a
 144 complex picture of opposing positive/protective and negative/pathological effects of pre-existing
 145 flavivirus-specific T cell responses on the outcome of secondary DENV infection.

146

147 Table 2. Comparison of studies to identify clinical associations of dengue virus-specific immune
 148 responses measured in blood samples collected prior to secondary flavivirus infections.

Study	Clinical phenotypes	In vitro stimulation	Measurements
Mangada et al [19]	Hospitalized versus non-hospitalized dengue	Inactivated DENV-infected cell lysate (7 d)	Proliferation IFN- γ , TNF α secretion
Hatch et al [21]	Subclinical versus symptomatic DENV infection	Inactivated DENV-infected cell lysate (24 h)	% cytokine-positive CD4 T cells (IFN- γ , TNF α , IL-2)
Friberg et al [22]	Subclinical versus symptomatic DENV infection	Inactivated DENV-infected cell lysate (24 h)	% IFN- γ -positive CD4 T cells
		Infectious DENV (7 d)	Cytokine secretion

149

150 *Temporal changes in responses of flavivirus-reactive T cells*

151 Most studies of flavivirus-specific memory T cells, including those described above, have based
152 their conclusions on a single time point. However, the stability of T cell memory has not been well
153 characterized, particularly in settings where frequent re-exposures are possible. We have recently
154 leveraged the longitudinal nature of our cohort study to address this gap. We measured DENV-
155 specific T cell responses by IFN γ /IL-2 ELISPOT assays in PBMC collected over 5 consecutive
156 years from individuals who had a symptomatic, subclinical or no DENV infection [23]; subclinical
157 and no infection groups were defined serologically. DENV-specific T cells were low or absent
158 before symptomatic secondary infection. Increased responses were observed following the
159 infection, however, they fluctuated over the 3-4 years after infection. We observed some increases
160 in T cell responses in the absence of serologic evidence of infection, raising the possibility of
161 inapparent viral exposures. Furthermore, some subjects, particularly those who did not experience
162 a symptomatic DENV infection during the study, showed greater stability in immune responses.

163 The above findings have led us to hypothesize that the durability of flavivirus-specific T cell
164 responses after an infection and the cytokine responses of T cells that persist at low frequency may
165 represent critical determinants of the outcome of a subsequent infection. Recently we have been
166 studying the kinetics of T cell responses over multiple years prior to infection in subjects with mild
167 (non-hospitalized) or severe (DHF) illness from our prospective cohort study [23]. To increase the
168 sensitivity for low-frequency responses, we are using cultured ELISPOT assays [24]. Figure 1
169 shows two examples of responses over time to a structural protein peptide pool (less serotype-
170 crossreactive) and a non-structural protein peptide pool (more serotype-crossreactive) in
171 individuals who had a symptomatic DENV-2 infection in 2001. IFN γ T cell responses were low in
172 2001; in one subject, this followed a decline over several years in responses to non-structural

173 proteins. Post-infection, responses were significantly increased to both structural and non-
174 structural proteins.

175 *Implications for flavivirus vaccines*

176 Applying the lessons from natural flavivirus infections to improve the design of vaccine products
177 and dosing regimens remains a key goal for us and many others. There are currently a significant
178 number of flavivirus vaccines in clinical testing [25,26]. We and others have characterized the
179 flavivirus-specific T cell responses induced by different formulations and regimens of these
180 vaccines [27,28]. Friberg et al demonstrated the complementary information that can be obtained
181 from applying a suite of immune responses assays, including ELISPOT, cytokine flow cytometry,
182 proliferation, and cytokine secretion assays [29]. Detailed single-cell analyses have demonstrated
183 the power to identify distinct gene expression and metabolic features of T cell destined to persist
184 into the memory phase [30]. However, the cohort studies described above suggest a cautious
185 approach to translating those findings to children in flavivirus-endemic areas, where blood samples
186 volumes are more limited and both pre-existing immunity and frequent re-exposures are likely.
187 We are applying the lessons we have learned from cohort studies of natural infection to a cohort
188 of Filipino children who received DengvaxiaTM as part of the phase III efficacy trial. Figure 1
189 shows examples of apparent vaccine responses. Pre-vaccination PBMC in these cases showed low
190 flavivirus-specific T cell responses, as was observed in our Thai cohort study; post-vaccination
191 PBMC showed responses to some DENV structural proteins and YFV NS3 protein, but not to
192 DENV non-structural proteins. Follow-up of this cohort will determine whether vaccine-induced
193 immune responses are associated with protection against subsequent DENV infection.

194 ***Remaining questions***

195 Recent studies have greatly advanced the characterization of flavivirus-specific T cell responses
196 during both acute and memory phases. As illustrated in Figure 2, many functionally distinct T cell
197 subpopulations are now known to be recruited into the acute response to natural infection and
198 persist into memory, where they are capable of influencing the outcome of subsequent infections.
199 Host genetic factors influence both the magnitude and specificity of this response. Qualitatively
200 and quantitatively similar responses have also been observed in the acute and early memory phases
201 after immunization of some, but not all vaccine candidates.

202 At the same time that technological advances have enhanced the detail in our understanding of
203 components of T cell responses to flavivirus infection and vaccination, we risk losing perspective
204 on the clinical relevance of these findings, like the proverbial blind men describing the elephant.
205 Data suggest that the balance between different immune responses over the entire course of
206 infection affects the clinical outcome (Figure 2 bottom). Persistence of memory responses, and the
207 impact of sequential exposures to circulating viruses and vaccines, are important influences on the
208 immunological repertoire at the time of exposure. What factors determine the durability of
209 flavivirus-specific memory after natural infection and vaccination? Which components of memory
210 contribute most to the clinical outcome of subsequent infection? How does the lifetime history of
211 flavivirus exposures affect these associations? Given the co-circulation of multiple flaviviruses,
212 host and viral genetic differences, and variable time intervals, the number of permutations to be
213 considered is enormous. We believe that studies of carefully selected cohorts of subjects with
214 detailed clinical characterization offer critical insights into these questions and should remain a
215 high priority for further study.

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326 ** This study applied single-cell RNA sequencing and T cell receptor sequencing to
327 sequential blood samples collected from recipients of a tetravalent live attenuated dengue
328 vaccine. Significant changes in the expression of metabolic gene pathways were identified
329 in memory precursor T cells during the acute phase of the immune responses which were
330 further validated using flow cytometry.

331

332 *Figure legends*

333 Figure 1. Illustrative examples of DENV-specific T cell responses in cohort studies. A.
334 Longitudinal analysis of DENV-specific T cells in a prospective cohort of Thai children.
335 Frequencies of IFN- γ spot-forming cells (SFC) detected in cultured ELISPOT assays after
336 restimulation with peptide pools from DENV-2 structural (2prM/E) or non-structural (2NSA)
337 proteins using PBMC collected at the indicated time points; subjects experienced a secondary
338 DENV-2 infection in 2001. NT, not tested. B. Analysis of DENV-specific T cell responses to
339 vaccination in a Philippine cohort. Frequencies of IFN- γ SFC detected in ex vivo ELISPOT assays
340 after stimulation with peptide pools from structural (prM/E) or non-structural (NSA) proteins of
341 each DENV serotype or NS3 protein of YFV using PBMC collected prior to vaccination (V01), 1
342 month after the first vaccination (V02), or 1 month after the third vaccination.

343 Figure 2. Model of T cell involvement in dengue disease. A) Different subsets of T cells are
344 induced during DENV infections. In a primary DENV infection, serotype-specific T cells are
345 activated including cytotoxic T cells, helper T cells comprising the recent described T_{FH} cells &
346 Tregs. In the course of convalescent-phase, T_{EM} & T_{CM} are the long-term memory subsets. These
347 memory T cell pools are modified by a second flavivirus infection or vaccination resulting in the
348 expansion of cross-reactive and recently activated serotype-specific clones. B) Multiple factors
349 associated with disease severity. Factors listed on the left side are hypothesized to contribute to
350 enhanced disease severity; factors listed on the right are hypothesized to contribute to protection
351 from infection and/or illness.