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Cross-Reactivity and Expansion of Dengue-Specific T cells During Acute Primary and Secondary Infections in Humans

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Serotype-cross-reactive memory T cells responding to secondary dengue virus (DENV) infection are thought to contribute to disease. However, epitope-specific T cell responses have not been thoroughly compared between subjects with primary versus secondary DENV infection. We studied $CD8^+$ T cells specific for the HLA-A*1101-restricted $NS3_{133}$ epitope in a cohort of $A11^+$ DENV-infected patients throughout acute illness and convalescence. We compared the expansion, serotype-cross-reactivity, and activation of these cells in PBMC from patients experiencing primary or secondary infection and mild or severe disease by flow cytometry. Our results show expansion and activation of DENV-specific $CD8^+$ T cells during acute infection, which are predominantly serotype-cross-reactive regardless of DENV infection history. These data confirm marked T cell activation and serotype-cross-reactivity during the febrile phase of dengue; however, A11-$NS3_{133}$-specific responses did not correlate with prior antigenic exposure or current disease severity.

The four dengue virus serotypes (DENV 1–4) have a significant and growing impact on global health. They are responsible for over 38 million reported dengue cases each year with ~21,000 deaths[1]. Dengue disease encompasses a wide range of symptoms, usually presenting as an uncomplicated acute febrile illness (dengue fever, DF); however, a small percentage of infections are associated with a plasma leakage syndrome (dengue hemorrhagic fever, DHF), which can be life-threatening. Plasma leakage in DHF coincides with defervescence and viral clearance[2,3] suggesting that severe disease arises from the immune response rather than a direct viral effect. In support of this, epidemiological studies indicate that severe dengue disease most often occurs during secondary heterotypic DENV infection[4–6].

Current hypotheses suggest that serotype-cross-reactive memory T cells reactivated in response to secondary DENV infection mediate a sub-optimal immune response, contributing to dengue disease pathology[7]. Various studies have explored the immunopathogenic role of cross-reactive memory T cells in DENV infection utilizing blood samples from infected patients. A high percentage of DENV-specific T cells recognize multiple DENV serotypes, as demonstrated by peptide-MHC (pMHC) tetramer binding and in vitro functional assays[8–17]. Furthermore, patients with DHF have been shown to have greater T cell activation in vivo than patients with DF, based on serum markers of activation[18–22] or cell surface CD69 expression[23]. Few studies have reported a higher frequency of DENV epitope-specific T cells in patients with DHF[13,14,24], but other studies have questioned the timing of T cell activation and its association with disease severity[25,26]. None of the previous reports have compared responses to multiple serotypes in cohorts of dengue patients, distinguishing between primary and secondary infection, in association with data on outcome of infection. In this study, we compared the expansion, serotype-cross-reactivity, and activation of DENV-specific $CD8^+$ T cells in serial blood samples during acute infection and convalescence in patients experiencing primary or secondary infection and mild or severe disease.

Table 1 | Summary of HLA-A11$^+$ cohort information.

Serotype[a]	Diagnosis[b]	Serology (no.)		Total (no.)
		Primary	*Secondary*	
DENV-1	DF	6	5	11
	DHF	1	6	7
DENV-2	DF	2	2	4
	DHF	-	3	3
DENV-3	DF	5	4	9
	DHF	3	6	9
DENV-4	DF	-	1	1
	DHF	-	-	-
				44

[a]Of current infection
[b]According to original WHO guidelines; DF = dengue fever, DHF = dengue hemorrhagic fever

Results

Dengue virus-specific CD8+ T cells expand during acute infection. We identified 44 HLA-A*1101$^+$ children experiencing primary or secondary DENV infection (Table 1 and Supplementary Table S1). We prepared pMHC tetramers using three peptide variants of the previously defined A11-restricted NS3$_{133–142}$ DENV epitope[13], corresponding to different DENV serotypes, with different fluorochromes (Table 2). The specificity of each tetramer, and conditions for simultaneous staining of cells, was demonstrated using PBMC and epitope-specific T cell lines (Supplementary Figure S1; see also [ref 10]). We stained PBMC from all time points available for each subject with all three tetramers along with antibodies to activation and phenotypic markers. Each experiment included PBMC from a healthy, A11$^+$ DENV-naïve subject as a negative control and healthy PBMC spiked with an epitope-specific T cell line as a tetramer-positive control. A positive cutoff value for tetramer frequencies was defined as any frequency greater than that measured for 11 of 12 A11$^+$ DENV-naïve subjects (>0.14% of total CD8$^+$ T cells equated to >92% specificity). Our flow cytometry gating strategy is presented in Fig. 1a.

A11-NS3$_{133}$ tetramer-positive CD8$^+$ T cells demonstrated clear expansion and contraction from acute infection into convalescence (Figure 1b and c and Supplementary Figure S2). Peak tetramer frequencies could be defined in 36 of the 44 subjects (see Supplementary Table S1). Of the remaining 8 subjects, 3 had data from one or no sample during acute infection and 3 were missing data for one of the tetramer variants; only 2 subjects with adequate data failed to show an epitope-specific T cell response. Peak frequencies ranged from 0.15% to 19% of total CD8$^+$ T cells (median = 0.75%, mean = 2.33%). Dramatic changes in tetramer-positive T cell frequencies between consecutive daily blood samples were observed in a few subjects (Supplementary Figure S2); however, sample unavailability precluded confirmation of these findings and we therefore interpret these values with caution. Contraction of epitope-specific CD8$^+$ T cells averaged 76% of the peak frequency at 1 year post-infection for 16 of 20 subjects with samples available. The remaining 4 subjects had tetramer-positive T cell frequencies at 1–3 years post-infection that were equal to or higher than the peak frequency during acute infection. These subjects showed lower frequencies at 6 months post-infection; although there was no serologic evidence of reinfection in these individuals we cannot exclude the possibility that these subjects experienced another DENV infection.

Antigen-specific CD8+ T cells are serotype-cross-reactive in both primary and secondary dengue virus infection. We compared the distribution of tetramer-positive T cells in subjects with primary versus secondary DENV infection and with mild (DF) versus severe (DHF) disease (Figure 2). The proportion of A11-NS3$_{133}$-specific T cells that bound tetramers specific for heterologous DENV serotypes was similar in subjects with primary infection compared to those with secondary infection. For example, at 1 week post-defervescence median frequencies of 77% and 65% (p=0.72) of epitope-specific T cells bound heterotypic tetramers (those specific for serotypes other than the currently infecting serotype) in primary and secondary infection, respectively. The pattern of tetramer staining showed great sample-to-sample variability, but, notably, it varied less across time points from the same individual than between individuals. Despite the lack of clear and consistent staining patterns according to the serotype of the infecting virus or clinical diagnosis, we did note some similarities among individuals within particular subject groups. For example, subjects with primary DENV-3 infections had more pD2$^+$ cells (shown in yellow) than subjects with secondary DENV-3 infections (median = 15% versus 6% of epitope-specific T cells, respectively; p=0.02) or any DENV-1 infection (median = 4%; p=0.002) at 1 week post-infection. In addition, subjects infected with DENV-1 appeared to have a greater percentage of pD3/4$^+$ cells (shown in red) than those infected with DENV-3 at fever day +1, although the difference did not reach statistical significance (median = 30% versus 13% of epitope-specific T cells; p=0.16). In general, relatively few T cells bound to two or more tetramers at once (shown in green, purple, orange and brown) and were more often found in subjects with secondary infections (median = 4% of epitope-specific T cells for primary infections versus 12% for secondary infections at 1 week, p=0.03).

We detected preferential binding to the pD1 tetramer when T cells were stained with the three A11-NS3$_{133}$ tetramer variants. We observed a similar staining pattern in peptide-stimulated short term bulk cultures and epitope-specific T cell lines[10]. To evaluate the possibility that preferential binding to the pD1 tetramer was an artifact of our staining conditions, we compared staining with all three tetramer variants at once to staining with each variant individually (Supplementary Figure S3a). Relative staining with individual tetramer variants was consistent with staining detected when all three tetramers were added together. To determine whether there was competition for tetramer binding and if the concentration of tetramer influenced the staining patterns, we stained T cell lines with decreasing concentrations of the pD1 or pD3/4 tetramer while

Table 2 | Summary of epitope sequence information.

Epitope	HLA restriction	Serotype	Sequence	Designation[a]
DENV NS3$_{133–142}$[b]	A*1101	DENV-1	GTSGSPIVNR	pD1
		DENV-2	GTSGSPIVDR	pD2
		DENV-3, -4	GTSGSPIINR	pD3/4
DENV NS3$_{222–231}$[c]	B*07	DENV-2, -3, -4	APTRVVAAEM	B7-DENV
Influenza M1$_{58–66}$[d]	A*0201	n/a	GILGFVFTL	A2-Flu

[a]As used in this paper
[b]Identified in [ref 13]
[c]Identified in [ref 41]
[d]Identified in [refs 43] and [44]

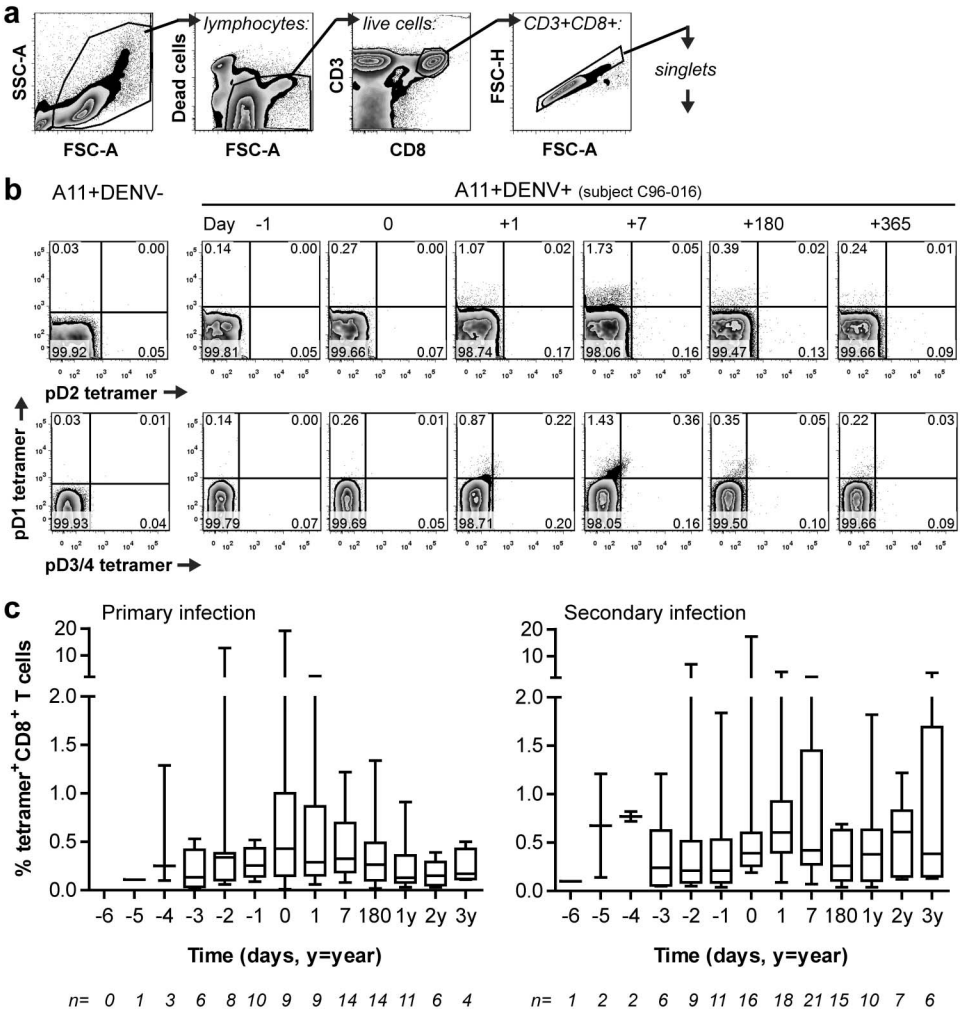

Figure 1 | A11-NS3$_{133}$-specific T cells expand in acute dengue virus infection. a The gating strategy used to identify tetramer$^+$CD8$^+$ T cells selected cells within a generous lymphocyte gate as defined by forward and side scatter profiles. Live cells were next selected by exclusion of the viability marker LIVE/DEAD Aqua. T cells were identified by dual CD3 and CD8 expression followed by gating for singlet cells. b Representative flow plots of triple tetramer staining of PBMC from a healthy HLA-A11$^+$ DENV-naïve control donor (A11+DENV-) as well as a HLA-A11$^+$ DENV-infected (A11+DENV+) individual over the course of acute illness and convalescence. The two rows of plots are different views of live, CD3$^+$CD8$^+$ singlet lymphocytes. c Box and whisker plots show total tetramer-positive T cell frequencies in PBMC from subjects with primary ($n=16$) or secondary ($n=23$) DENV infection over time. Days are relative to the day of defervescence (d0).

keeping the other tetramer concentrations constant. We observed no increase in the frequency of cells that bound to the tetramer variants kept constant (Supplementary Figure S3b). To determine whether tetramer binding reflected functional responses, selected PBMC samples obtained from several of the DENV-3 infected subjects in early convalescence (within 1 week of defervescence) were stimulated ex vivo with each of the A11-NS3$_{133}$ epitope variants in intracellular cytokine staining assays. Cytokine production and/or degranulation by CD8$^+$ T cells in response to heterologous epitope variants was detected (Supplementary Figure S3c), consistent with our prior findings of serotype-cross-reactive epitope-specific CD8$^+$ T cell lines isolated from patients with primary DENV infection[10].

We considered the possibility that the placement of gates could have been inexact in delineating positive and negative tetramer staining and, thereby, our interpretation of serotype-cross-reactivity. Quadrant gates were placed based on staining in positive and negative control specimens. For some subjects, pD1 tetramer-positive T cells seemed to stain weakly with the pD3/4 tetramer, but did not reach the threshold used to count as pD3/4 tetramer-positive (pD1$^+$3/4$^+$; refer to Figure 1b). To account for this, the intensity of pD3/4 tetramer staining of all pD1 tetramer-positive CD8$^+$ T cells was compared across all time points for subjects with primary versus secondary DENV-3 infections (Supplementary Figure S3d). We found no statistically significant differences between these subject groups, further supporting our finding that the extent of serotype-cross-reactivity did not differ according to DENV infection history.

Epitope-specific T cell frequencies peak earlier in primary than in secondary dengue virus infection. We compared the kinetics of A11-NS3$_{133}$-specific T cell expansion in primary versus secondary infection. We found that subjects undergoing primary infection reached their peak tetramer frequency earlier in the course of illness than those with secondary infection ($p=0.02$; Table 3). Given the more rapid proliferation of memory T cells, and the more rapid clearance of viremia in secondary infection[2], this result was not anticipated. However, epitope-specific T cells down-regulated CD45RA expression earlier in secondary infection than in primary infection (Supplementary Figure S4). CD45RA is expressed on some memory T cell populations[27], as shown for A11-NS3$_{133}$-specific T cells in our cohort (Supplementary Figure S4). Therefore, earlier down-regulation of CD45RA supports the reactivation of epitope-specific memory T cells during secondary DENV infection. We did not

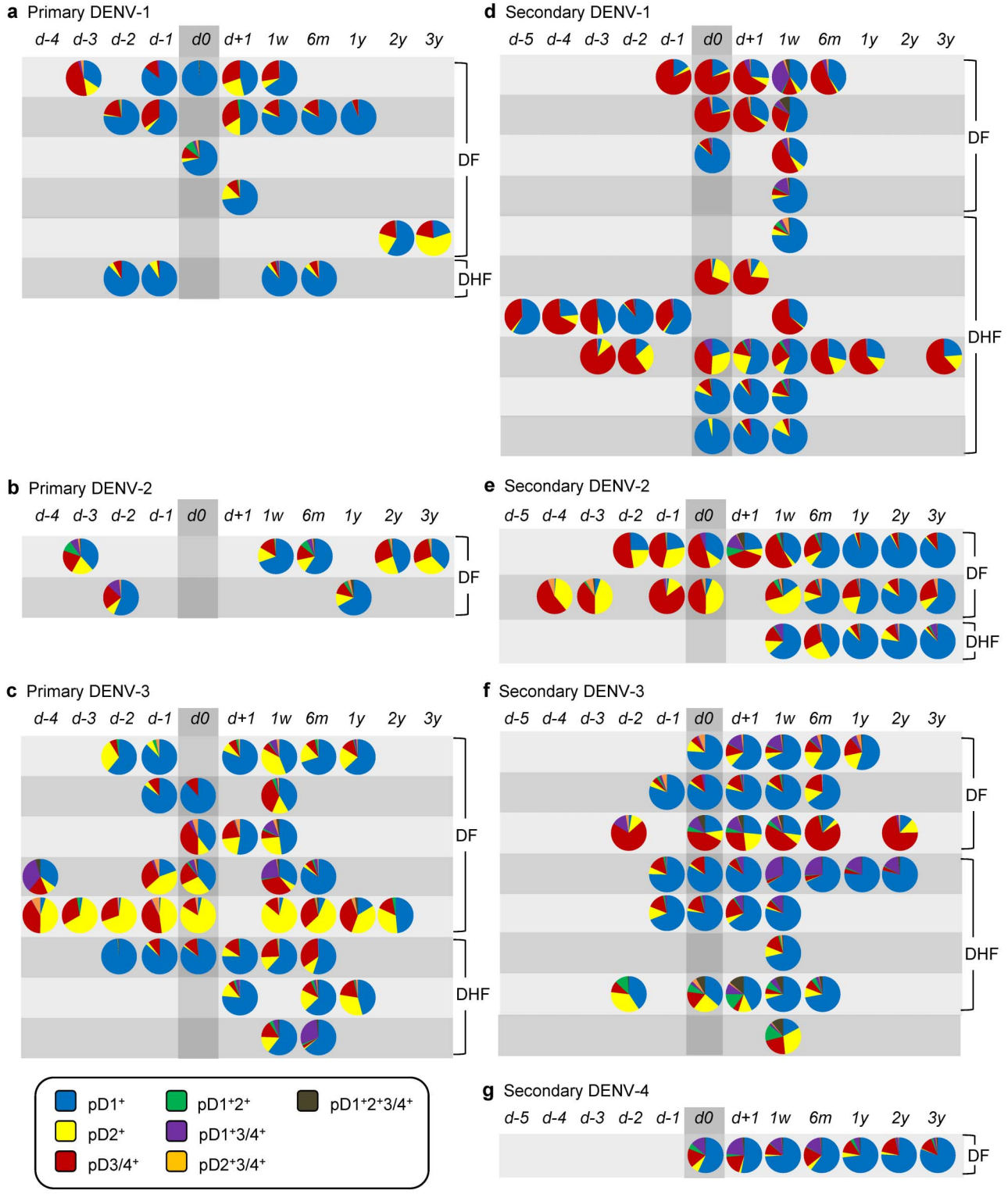

Figure 2 | Tetramer-positive T cells are highly serotype-cross-reactive in subjects with primary or secondary infection. The distribution of sub-populations within the whole population of A11-NS3$_{133}$ tetramer$^+$CD8$^+$ T cells is depicted using pie charts. Tetramer-positive samples from subjects with a (a–c) primary or (d–g) secondary infection with (a, d) DENV-1, (b, e) DENV-2, (c, f) DENV-3 or (g) DENV-4 are shown. No subjects with primary DENV-4 infection were available. Each row represents data from a single donor over time. Time points shown are relative to the day of defervescence (d0), d = day, w = week, m = month, y = year.

detect differences in the timing of peak tetramer frequency between DF and DHF patients (data not shown).

The frequency of A11-NS3$_{133}$-specific T cells does not correlate with clinical findings. We tested whether the frequency of A11-NS3$_{133}$-specific T cells correlated with clinical diagnosis at days 0, +1 and +7 and found no significant differences (data not shown). Therefore, we determined whether the peak frequency of epitope-specific T cells, regardless of timing, correlated with clinical findings. No statistically significant differences were found between subjects

Table 3 | Tetramer frequencies peak earlier in primary DENV infection[a].

	No. subjects	
	Primary	*Secondary*
Fever day ≤0[b]	4	1
Fever day +1	1	8[d]
Fever day +7[c]	1	6

[a]This analysis only considers those subjects for whom we have blood samples from all three of these time points (n=21)
[b]One primary subject had peak tetramer frequency at fever day -2
[c]The actual day post-defervescence differs for each donor (range=4–12, mean=7)
[d]The difference between fever days 0 and +1 is significant by Fisher's exact test (p=0.02)

with DHF and those with DF (Figure 3a). However, among subjects with DF, peak tetramer frequencies were slightly higher in secondary infection than primary infection (p=0.08), and among subjects with secondary DENV infections, peak tetramer frequencies were slightly higher in subjects with DF than those with DHF (p=0.09). We also found no correlations between peak tetramer frequencies and other measures of disease severity, including pleural effusion index (Figure 3b), aspartate aminotransferase values (Figure 3c), change in hematocrit (Figure 3d), and platelet count (Figure 3e).

Antigen-specific CD8+ T cells are highly activated during acute dengue virus infection. We compared CD38 expression on antigen-specific T cells in primary versus secondary infection according to disease severity. High CD38 expression was seen in total $CD8^+$ T cells and even higher expression in epitope-specific T cells was seen during acute infection and shortly after defervescence (Figure 4a). While levels of CD38 expression varied across individuals, the averaged fluorescence intensity of tetramer-positive T cells in PBMC from the four subject groups (primary DF, primary DHF, secondary DF and secondary DHF) showed similar patterns over time (Figure 4b).

T cell expansion in acute dengue virus infection is antigen-specific. We considered that conclusions based on the analysis of a single DENV epitope might not be generalizable or could reflect non-specific rather than antigen-specific T cell expansion. Taking advantage of previously identified epitopes for which we had tetramers available, we identified a subset of $A11^+$ subjects that were also $HLA\text{-}A2^+$ or $HLA\text{-}B7^+$. We stained samples from these

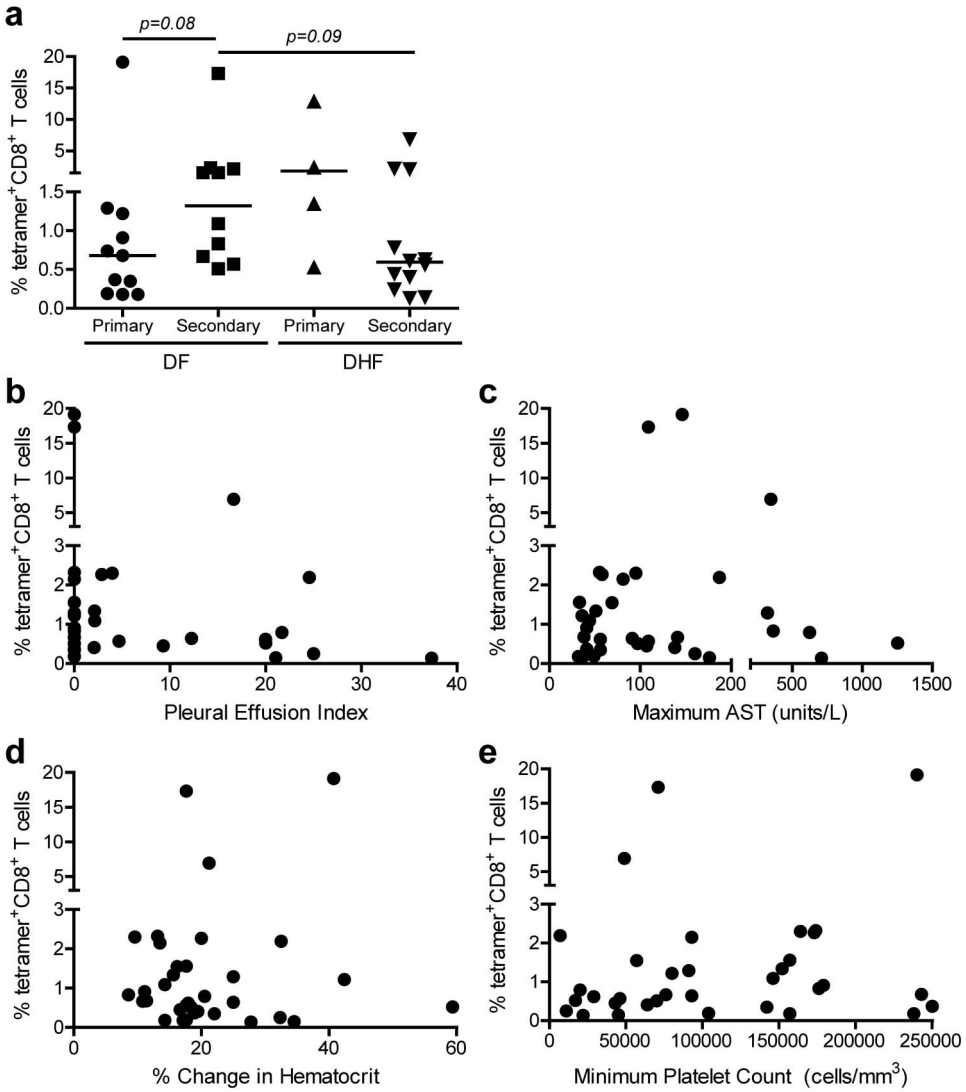

Figure 3 | The magnitude of epitope-specific T cells does not correlate with disease severity. Peak A11-$NS3_{133}$ $tetramer^+CD8^+$ T cell frequencies are plotted versus a clinical diagnosis (DF versus DHF), b pleural effusion index, c maximum aspartate aminotransferase (AST) value, d % change in hematocrit, and e minimum platelet count. No significant correlations were detected by Mann-Whitney (a) or Spearman's test (b–e).

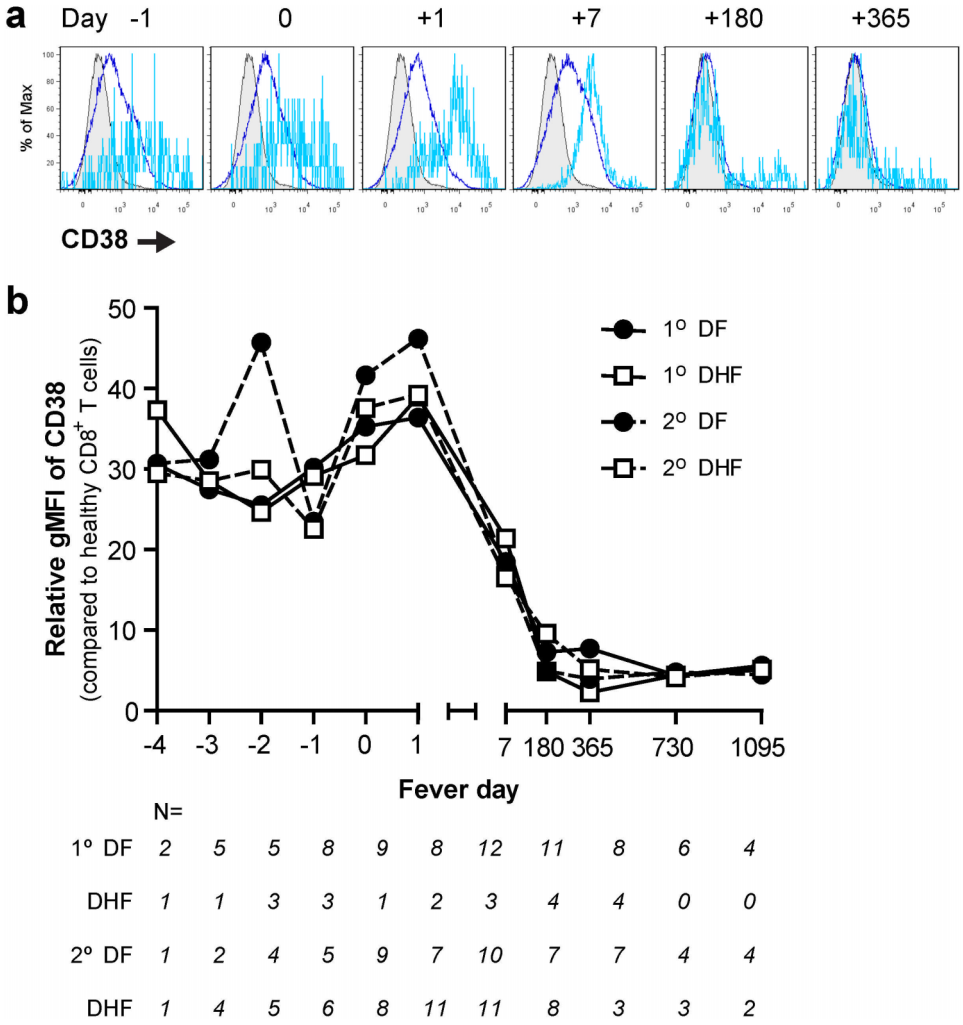

Figure 4 | Antigen-specific T cells are highly activated during acute DENV infection and early convalescence. a Representative histograms show the expression of CD38 over time in total $CD8^+$ T cells from a healthy $A11^+$ donor (shaded) and an $A11^+$ DENV-infected donor (dark blue line) as well as A11-$NS3_{133}$ $tetramer^+CD8^+$ T cells from the same DENV-infected donor (light blue line). b The fold change in geometric mean fluorescence intensity (gMFI) of CD38 staining in A11-$NS3_{133}$ $tetramer^+CD8^+$ T cells from subjects with primary (solid lines) and secondary (dotted lines) DENV infections with either DF (closed circles) or DHF (open squares) over time. Data are presented as means and are relative to the gMFI of CD38 of total $CD8^+$ T cells from a healthy control donor included in each experiment.

subjects with the pD1 tetramer together with a tetramer specific for either the B7-restricted DENV $NS3_{222–231}$ epitope (B7-DENV) or the A201-restricted influenza virus $M1_{58–66}$ epitope (A2-Flu; refer to Table 2).

Similar to pD1-specific T cells, B7-$NS3_{222}$-specific $CD8^+$ T cells expanded during acute infection, followed by a contraction phase in late convalescence (Figure 5a). Frequencies of B7-DENV cells were consistently higher than pD1-specific T cells in 4 of the 5 subjects studied. A2-Flu $M1_{58}$-specific T cells were also present in a subset of our patients, but their frequencies remained stable or decreased during acute DENV infection. Although CD38 expression on A2-Flu $M1_{58}$-specific T cells was slightly increased during acute DENV infection, the average CD38 staining intensity on these cells was lower than on DENV-specific T cells detected by either the A11-$NS3_{133}$ or B7-$NS3_{222}$ tetramers (Figure 5b). These data demonstrate selective activation and expansion of antigen-specific T cells during acute DENV infection and imply that there was minimal contribution of bystander cells to the T cell response to acute infection.

Discussion

Our results demonstrate similar frequencies of serotype-cross-reactive T cells ex vivo in naturally-infected patients with primary and secondary DENV infections. T cell functional responses to heterologous DENV epitopes were observed previously in some subjects following primary DENV infections[8,28], but this is the first demonstration of serotype-cross-reactivity in primary infection based on binding of heterotypic tetramers ex vivo, and illustrates the breadth of the phenomenon. Mongkolsapaya et al. found that A11-$NS3_{133}$-specific T cells in patients with secondary DENV infections preferentially stained ex vivo with pMHC tetramers corresponding to DENV serotypes heterologous to the infecting serotype[13]. We used a similar approach, but extended our analysis to primary DENV infections, an important comparison group absent in the earlier study. Preferential binding to the pD1 tetramer (followed by the pD3/4 tetramer and, finally, the pD2 tetramer) was also seen in our previous study of T cell lines, which we postulated could be explained by their predicted MHC binding ($pD3/4 \geq pD1 \gg pD2$)[10]. Heterotypic tetramer binding for this epitope thus does not reflect the history of prior infection with other DENV serotypes; other possible explanations for this phenomenon are heterologous immunity from prior unrelated infections[29], characteristics of the T cell repertoire[30], or inherent immunogenicity of different epitope variants[8,31]. It is possible that preferential binding to the pD1 tetramer was a reflection of it being the most stable of the three tetramers. However, this preferential

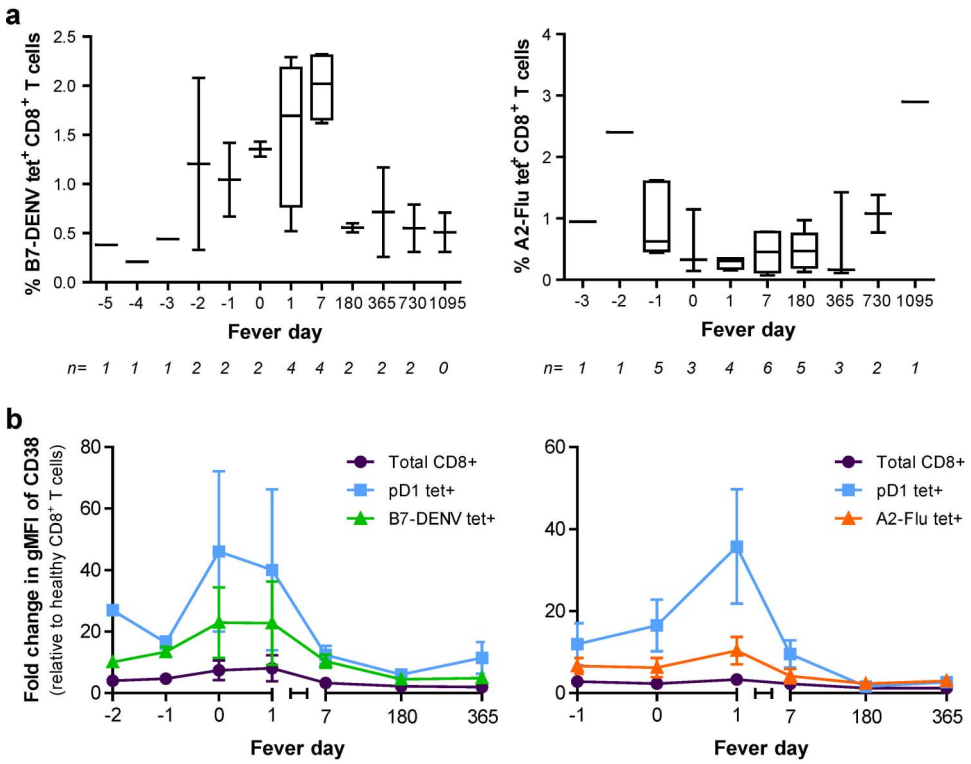

Figure 5 | T cell expansion and activation during dengue virus infection is antigen specific. a Box and whisker plots show the frequency of B7-$NS3_{222}$ DENV epitope-specific or A2-$M1_{58}$ Flu epitope-specific T cells over the course of acute infection and convalescence in HLA-$A11^+B7^+$ (n=5) or HLA-$A2^+A11^+$ subjects (n=6), respectively. b The mean fold change in gMFI of CD38 expression in $pD1^+CD8^+$ T cells (blue), total $CD8^+$ T cells (purple), and B7-$DENV^+CD8^+$ T cells (green) or A2-Flu^+CD8^+ T cells (orange) relative to total $CD8^+$ T cells from healthy control donor PBMC (same donor used in all experiments). Bars indicate standard deviation at each time point.

binding would apply equally to the entire dataset, which would not affect our ability to detect differences in the overall pattern of tetramer staining between various subject groups. Despite donor-to-donor variability, we did note some similarities in patterns of tetramer staining among individuals with the same infection status (DENV serotype, primary versus secondary). This suggests that the sequence of infection does play a role in shaping the DENV-specific memory T cell repertoire, although other factors are also important.

Mongkolsapaya et al. reported low but detectable frequencies of A11-$NS3_{133}$-specific T cells during acute secondary DENV infection in Thai children which peaked in early convalescence[13]. In contrast, Dung et al. reported that A11-$NS3_{133}$-specific T cells were undetectable until after the development of plasma leakage among infected Vietnamese children[25]. Our data (for both the A11-$NS3_{133}$ and B7-$NS3_{222}$ epitopes) are clearly more consistent with the earlier report, and, in fact, several of our subjects had very high frequencies of (activated) epitope-specific T cells during acute infection. These immune responses occurring prior to the onset of plasma leakage therefore have the potential (i.e. are available) to contribute to disease pathogenesis. The finding of antigen-specific T cell expansion and activation during acute illness is also more consistent with observations in other viral diseases[27,32,33] and with other evidence of T cell activation during acute DENV infection[20,23,26]. The divergent findings of Dung *et al.* may reflect differences in experimental technique; a more intriguing possibility is that the expansion of A11-$NS3_{133}$-specific T cells might differ between Thai and Vietnamese patients. In this regard, it is of interest that an association with dengue disease severity has been observed for HLA-A11 in our Thai cohort[34] but was not noted in a study in Vietnam[35] despite the similar genetic backgrounds of these two populations.

We found that the frequency of epitope-specific T cells peaked earlier in subjects with primary infection than in those with secondary infection. Prior studies in which DENV-specific T cells were tracked over time had not differentiated between subjects with primary and secondary infections. Although this result was not anticipated, it is consistent with findings in DENV infection of BALB/c mice[31]. More rapid activation of memory T cells would not necessarily correspond to an earlier peak T cell frequency, since sustained proliferation signals balanced by apoptosis[13,36] would affect the timing of peak responses to a greater extent. Future studies need to incorporate analysis of these mechanisms as well as other immunomodulatory signals such as regulatory T cells[37].

The magnitude of A11-$NS3_{133}$-specific T cells did not correlate with disease severity in this cohort. This held true whether the data were analyzed according to clinical diagnosis (DF versus DHF) or continuous measures of disease severity such as pleural effusion index, hemoconcentration, or platelet nadir. A similar lack of association was reported in a study in Vietnam[25]. Other studies had reported higher frequencies of DENV-specific T cells in patients with DHF, but these associations were found at 2 weeks[13,14] or 6 months[24] post-infection.

The interpretation of our data is subject to several limitations. Although our patient cohort was relatively large, the small number of HLA-$A11^+$ subjects with the same DENV serotype, serologic response (primary/secondary infection), and clinical outcome (DF/DHF) limits the statistical power for important subgroup analyses, and data from HLA association studies suggest that the influence of T cell responses may not be the same for all four serotypes[34]. Additionally, although subjects were followed daily during acute illness, adequate specimens were not available for all of these flow cytometry studies from all time points for all subjects. Given that rapid changes were observed in tetramer-positive T cell frequencies during acute infection, it is likely that peak frequencies were missed

in some subjects. Furthermore, few of the DHF patients in our study experienced shock (DHF grades 3 or 4). As Mongkolsapaya et al. found the highest frequencies of tetramer-positive T cells in patients with shock[13,14], it is possible that the milder disease in our study cohort concealed a relationship to the most severe disease. Alternatively, we cannot exclude the possibility that the A11-$NS3_{133}$ epitope is not representative of the global DENV-specific $CD8^+$ T cell response. Duangchinda et al. recently showed higher cytokine responses in children with DHF compared to DF when overlapping peptides covering the entire NS3 protein were used[38]. However, that study only analyzed PBMC collected several weeks post-infection and the same association was not detected by Simmons et al.[39]. Finally, human studies are limited to analysis of blood samples and we may have missed pathogenic T cells that were bound to the endothelium or located in other tissues.

In summary, our data points to a complex picture of T cell involvement in DENV infection. We found a selective activation and expansion of DENV-specific T cells during acute infection and a diverse pattern of serotype-cross-reactivity in both primary and secondary infections. These findings will need to be taken into account in future studies of T cell responses to natural DENV infection and/or vaccines.

Methods

Study subjects and blood samples. The clinical study design and collection of blood samples have been reported elsewhere[3,40]. Briefly, the study enrolled Thai children 6 months to 14 years of age with acute febrile illnesses. Acute DENV infections were confirmed by serology and virus isolation/detection, and primary and secondary infections were distinguished based on IgM : IgG ratio and hemagglutination-inhibition antibody titer. Blood samples were obtained daily during acute illness, once in early convalescence (~10 days after study entry) and at intervals during late convalescence (6 months – 3 years after study entry), and PBMC were cryopreserved. Time points are reported relative to the day of defervescence (i.e. the day at which fever dissipated and the patient subsequently maintained a temperature below 38°C), which was termed fever day 0 (d0). Fever days -1, -2, etc. occurred before defervescence, and day +1, etc. occurred after defervescence. Written informed consent was obtained from each subject and/or his/her parent or guardian. The study protocol was approved by the Institutional Review Boards of the Thai Ministry of Public Health, the Office of the U.S. Army Surgeon General and the University of Massachusetts Medical School. HLA typing was performed at the University of Massachusetts Medical School or the Department of Transfusion Medicine, Siriraj Hospital, as described[34,41].

Peptide-MHC multimers. Peptides were purchased at >90% purity from AnaSpec, Inc. (San Jose, CA, USA). Sequences are shown in Table 2. pMHC multimers (all referred to as tetramers) were generated at the University of Massachusetts Medical School Tetramer Core as described[42]. The different pMHC monomers were mixed with their respective fluorochrome conjugates at molar ratios of 4 : 1 (A11-$NS3_{133}$ epitope variants), 25 : 1 (Flu $M1_{58}$ epitope) and 50 : 1 (B7-$NS3_{222}$ epitope). Tetramers were conjugated to distinct fluorochromes to allow for staining with multiple epitopes/variants simultaneously (APC-A11-$NS3_{133}$-pD1, PE-Cy7-A11-$NS3_{133}$-pD2, PE-A11-$NS3_{133}$-pD3/4, Qdot-605-A2-Flu $M1_{58}$, Qdot-605-B7-$NS3_{222}$).

Staining and flow cytometry. Cryopreserved PBMC were thawed and rested in RPMI/10% FBS at 37°C for approximately 2 hours. Cells were washed in PBS and stained with LIVE/DEAD Aqua (Molecular Probes, Invitrogen Corp.) according to the manufacturer's instructions. Cells were washed and incubated with 0.5–2μL tetramer for 20 minutes at 4°C. Monoclonal antibodies specific for CD3 (clone UCHT1, Alexa Fluor 700 or clone SK7, PerCP-Cy5.5; BD Biosciences, San Jose, CA, USA), CD8 (clone SK1, PerCP-Cy5.5 from BD Biosciences, clone 3B5, Qdot-655 from Invitrogen Corp., Carlsbad, CA, USA, or clone RAP-T8, Alexa Fluor 700 from BD Biosciences), CD45RA (clone HI100, FITC; BD Biosciences), and CD38 (clone HIT2 conjugated to Qdot-655 from Invitrogen Corp. or to PerCP-Cy5.5 from BD Biosciences) were then added to the cells to incubate at 4°C for 30 minutes. Cells were washed and fixed with BD Stabilizing Fixative (BD Biosciences). Data were collected on a BD FACSAria and analyzed using FlowJo version 7.5.5.

Statistical analysis. For variables with a normal distribution, t-tests were used to compare continuous outcomes at a single time point between two groups and random intercept models were used to make comparisons between multiple groups across time points with the assumption that each individual within a group follows the same slope. For these models, missing values in the CD38 and CD45RA data sets were imputed with the means of all subjects at each time point. For variables that were not normally distributed, the Mann-Whitney rank sum test was used to make comparisons between two groups and Friedman's test was used to make comparisons between three or more groups. Correlations between skewed variables were determined using Spearman's rank correlation. Fischer's exact test was used to compare categorical data. All statistical analysis was performed using Stata Intercooled version 9 (Stata Corporation, College Station, TX, USA) and GraphPad Prism (La Jolla, CA, USA) software packages.

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Author contributions

HF, AM and ALR conceived and designed the experiments and wrote the manuscript text. HF, HB and TTM performed the experiments. HF and JAP analyzed the data. HF prepared the figures. RVG, AN, AS, SG and SK enrolled patients and collected samples. TG contributed reagents and HAFS provided HLA typing data. All authors reviewed the manuscript and agree with the results and conclusions.

Additional information

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