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"Distinct Activation Phenotype of a Highly Conserved Novel HLA-B57-Restricted Epitope during Dengue Virus Infection."
Distinct Activation Phenotype of a Highly Conserved Novel HLA-B57-Restricted Epitope during Dengue Virus Infection

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Short Title: CD71 expression and dengue-specific CD8 T cells

Key words: CD8 T cells, Dengue, HLA-B57, CD71, Transferrin

Abbreviations:
DENV-Dengue Virus
B-LCLs-B-lymphoblastoid cell lines
DF/DHF-Dengue Fever/Dengue Hemorrhagic Fever
MHC-Major histocompatibility complex
ADE- antibody dependent enhancement

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ABSTRACT

Variation in the sequence of T cell epitopes between dengue virus (DENV) serotypes is believed to alter memory T cell responses during second heterologous infections. We identified a highly conserved, novel, HLA-B57-restricted epitope on the DENV NS1 protein. We predicted higher frequencies of B57-NS1_{26-34}-specific CD8\(^+\) T cells in PBMC from individuals undergoing secondary rather than primary DENV infection. However, high tetramer-positive T cell frequencies during acute infection were seen in only 1 of 9 subjects with secondary infection. B57-NS1_{26-34}-specific and other DENV epitope-specific CD8\(^+\) T cells, as well as total CD8\(^+\) T cells, expressed an activated phenotype (CD69\(^+\) and/or CD38\(^+\)) during acute infection. In contrast, expression of CD71 was largely limited to DENV epitope-specific CD8\(^+\) T cells. In vitro stimulation of cell lines indicated that CD71 expression was differentially sensitive to stimulation by homologous and heterologous variant peptides. CD71 may represent a useful marker of antigen-specific T cell activation.

Key words: CD8 T cells, Dengue, HLA-B57, CD71, Transferrin
INTRODUCTION

Dengue virus (DENV), a member of the flavivirus family, consists of four distinct serotypes. Many DENV infections are asymptomatic and the majority of cases present as an acute febrile illness, dengue fever (DF). A small percentage of individuals develop dengue hemorrhagic fever (DHF), which is characterized by plasma leakage and bleeding tendency coincident with resolution of fever and clearance of viremia. While host-dependent factors and virus-dependent factors may influence the risk of developing DHF, prospective cohort studies have identified secondary infection with a heterologous DENV serotype as the major risk factor. Additionally, it has been suggested that the order of infections modulates the risk of developing DHF.

Antibodies and T cells are proposed to contribute to the development of severe dengue disease. Non-neutralizing antibodies, through antibody dependent enhancement (ADE), may enhance viral load and immune activation. Other studies have reported higher frequencies of CD8+ T cells expressing CD69, and higher levels of immune activation markers in individuals with DHF as compared to those with DF. Several studies have reported associations between specific HLA class I alleles and disease severity; these epidemiological links provide additional support for a role of CD8+ T cells in contributing to clinical outcome.

HLA-B57 has been associated with slow progression following HIV infection, the clearance of acute HCV infection and is strongly associated with a number of type 2 idiosyncratic adverse drug reactions. The relative ability of HLA-B57 to control HIV infection correlated with unique peptide-binding characteristics that affect thymic development of CD8+ T cells. A larger proportion of the naïve repertoire of T cells
restricted by HLA-B57 recognized HIV viral epitopes compared to other HLA alleles. Extended human major histocompatibility complex (MHC) haplotypes containing TNF-4 and LTA-3, together with HLA-B*48, HLA-B*57, and HLA-DPB1*0501, were detected only in patients with secondary DHF.\textsuperscript{15}

We identified a highly conserved 9aa epitope on the NS1 protein recognized by HLA-B57-restricted T cells. We hypothesized that B57-NS1\textsubscript{26-34}-specific CD8\textsuperscript{+} T cells would be preferentially expanded during secondary infection since the epitope sequence would be identical to that seen in primary infection. Using PBMC samples from Thai children with primary or secondary DENV infection\textsuperscript{24}, we found that frequencies of B57-NS1\textsubscript{26-34} tetramer-positive T cells were elevated during acute infection. Only one subject with secondary infection had particularly high frequencies of B57-NS1\textsubscript{26-34} T cells (~20\% of CD8\textsuperscript{+} T cells). Consistent with previous studies, expression of the activation markers CD69 and CD38 was upregulated on the total CD8\textsuperscript{+} T cell population as well as on DENV-specific T cells. In contrast, the expression of the transferrin receptor CD71 was significantly upregulated on B57-NS1\textsubscript{26-34}, A2-E\textsubscript{213-221} and A11-NS3\textsubscript{133-142} T cells, but not on total CD8\textsuperscript{+} T cells. In vitro studies demonstrated that, while stimulation with homologous and heterologous peptides induced similar levels of CD69 expression, the intensity of CD71 expression was differentially sensitive to variant peptide stimulation.
MATERIALS AND METHODS

Study subjects and blood samples. The study design for patient recruitment and collection of blood samples has been reported in detail elsewhere ², ²⁴-²⁶. Briefly, the subjects enrolled were Thai children 6 months to 14 years of age with acute febrile illnesses (<72 hrs) diagnosed as DF or DHF according to WHO guidelines ²⁷. Serology and virus isolation were used to confirm acute DENV infections, and primary and secondary infections were distinguished based on serologic responses ². For donors undergoing a secondary infection it is not possible to accurately determine what the previous serotype(s) were due to the activation of broadly cross-reactive DENV neutralizing antibodies ²⁸. Blood samples were obtained daily during acute illness, once in early convalescence, and at intervals during late convalescence. Informed consent was obtained from each subject and/or his/her parent or guardian and the study 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 (UMMS). PBMC were isolated by density gradient centrifugation, cryopreserved, and stored at −70°C. The samples are numbered relative to the day of defervescence (designated Fever Day 0). Serologic HLA class I typing was performed on blood from immune Thai donors or healthy subjects for use as B57⁺ dengue naive controls. HLA typing was performed at UMMS or the Department of Transfusion Medicine, Siriraj Hospital, as previously described ¹⁴, ²⁵.

Cytotoxicity assay. Cytotoxicity was assessed as previously described ²⁵. Briefly, HLA-B*57⁺ B-lymphoblastoid cell lines (BLCLs) targets were labeled with ⁵¹Cr and pulsed with 10μg/mL of the indicated peptides or infected with recombinant vaccinia viruses at an
moi=5. Primary dendritic cells from HLA-B*57+ healthy individuals were generated and infected with DENV-1-4 at a moi of 5. Peptide-pulsed or virus-infected target cells were cultured with T cells at an effector-to-target ratio of 10:1. After 4 hours, supernatants were harvested and ⁵¹Cr content was measured in a gamma counter. Percent specific lysis was calculated:

\[
\text{\% lysis} = \frac{(\text{experimental } ⁵¹\text{Cr release} - \text{minimum } ⁵¹\text{Cr release})}{(\text{maximum } ⁵¹\text{Cr release} - \text{minimum } ⁵¹\text{Cr release})} \times 100.
\]

**Flow cytometry.** Cryopreserved PBMC were thawed and washed in RPMI before resting in RPMI/10% FBS at 37°C for 2 hours. Cells were washed in PBS and stained with 1µL of 1:80 dilution of the dead cell marker LIVE/DEAD® Green (Molecular Probes, Invitrogen Corp.). Cells were then washed with FACS Buffer (PBS/2% FBS/0.1% sodium azide) and incubated with 0.5-2µL pMHC tetramer for 20 minutes at 4°C. Monoclonal antibodies specific for CD3, CD8, CD45RA, CCR7, CD69, CD38, CD57, CD71, CD28 or CD56, CD19, and CD14 were then added to the cells to incubate at 4°C for an additional 30 minutes (Supplementary Table 2). Cells were washed and fixed with BD Stabilizing Fixative™ (BD Biosciences). Data were collected on a BD FACSaria™ and analyzed using FlowJo version 10 and Gemstone (Verity House, Topsham, ME).

**Peptide stimulation of T cell lines.** At day 16 of culture approximately 2x10⁵ T cells were cultured with 2x10⁴ HLA matched B-LCLs, which had been pre-incubated for 30 minutes with peptide at the concentrations indicated, at 37°C for 0–24 hours. Cells were washed in PBS and stained with antibodies to CD8, CD19, CD69, CD38, and CD71 for 30 minutes at 4°C (Supplementary Table 2). Finally, cells were washed and placed in fixative until data
collection. All peptides were synthesized at >90% purity from AnaSpec, Inc. (Fremont, CA) or 21st Century Biochemicals (Marlboro, MA).

**Peptide-MHC tetramers.** Peptide-MHC tetramers were generated at the UMMS and the NIAID Tetramer Core. The different peptide-MHC multimers were conjugated to distinct fluorochromes (APC-A11-NS3133 or Qdot605-A11-NS3133, PE-B57-NS126-34, APC A2-E213-221).

**Intracellular Cytokine Staining.** 2x10^5 T cells were mixed with 2x10^4 HLA matched BLCLs with peptide or PHA in the presence of anti-CD107a antibodies and BD Golgi Stop/Golgi Plug™ for 6hrs. Cells were washed in PBS and stained with 1µL of 1:80 dilution of the dead cell marker LIVE/DEAD® Green (Molecular Probes, Invitrogen Corp.). Cells were washed with FACS Buffer (PBS/2% FBS/0.1% sodium azide) and incubated with surface antibodies specific for CD3, CD8, and CD19 and incubated at 4°C for 30 minutes (Supplementary Table 2). The cells were washed with 2mLs of FACS buffer then fixed and permeabilized using BD Cytofix/CytoPerm™ for 20 minutes at 4°C. Cells were washed with 1mL of BD Perm Wash buffer™. The cells were stained with intracellular antibodies against IFN-γ, TNF-α and MIP-1β and incubated at 4°C for 30 minutes. Cells were then washed with 1mL BD Perm Wash Buffer™ and fixed with 100µL of BD Stabilizing Fixative (1:3) and keep at 4°C until flow analysis.
RESULTS

Identification of a highly conserved HLA-B57-restricted DENV epitope

We previously identified HLA-B57-restricted CD8\(^+\) T cell lines, which recognized the DENV NS1 or NS2a protein, using convalescent PBMC from a Thai patient with DF\(^25\). As shown in Figure 1A, two representative T cell lines, 3C11 and 3F2, lysed autologous B-LCLs infected with a recombinant vaccinia virus expressing the DENV-2 NS1/2a proteins. We used pools of overlapping peptides from the NS1 protein and identified a minimal 9mer epitope recognized by these T cell lines corresponding to aa 26-34 (HTWTEQYKF) (Figure 1 B, C). Restriction of this epitope by HLA-B57 was confirmed by cytotoxicity assays using partially HLA-matched B-LCLs (data not shown). We determined the degree of conservation of NS1\(_{26-34}\) using the FLAVIdB database (http://cvc.dfci.harvard.edu/flavi/); this epitope was >99% conserved across >2600 sequences from all four serotypes of DENV. Comparison to previously identified CD8+ DENV epitopes indicated that this was the only epitope with such a high degree of homology (Supplemental Table 1).

T cell lines lysed virus-infected primary dendritic cells from an HLA*B57\(^+\) individual (one of four T cell lines shown) (Figure 1D) indicating that this epitope can be recognized by T cells in the context of DENV infection. Differences in percent specific target cell lysis likely reflect differences in the percentage of DCs that were infected with each DENV serotype.

For ex vivo analysis of epitope-specific T cells, we obtained an HLA-B5701/NS1\(_{26-34}\) tetramer. We confirmed the specificity of this tetramer by showing binding to the DENV-specific T cell line 3C11, but not to an HLA-B57-restricted HIV-specific T cell line. The
DENV-specific T cell line did not bind a previously described HIV-B57 tetramer (TW10-Gag; TSTLQEIQGW) (Figure 1E).

**Detection of B57-NS126-34 tetramer-positive T cells in PBMC collected during acute infection**

We used this B57-NS126-34 tetramer together with activation and phenotypic markers and performed a longitudinal analysis of B57-NS126-34-specific T cells in PBMC from HLA-B*57+ subjects. We tested samples obtained at multiple time points during and after acute DENV infection from eleven HLA-B*57 children, two with primary and nine with secondary DENV infection (Table 1).

Figure 2A shows our gating strategy. Each experiment included PBMC from a healthy subject, PBMC from an HLA-B*57+ DENV-naïve subject as a negative control (Supplementary Fig 1A) and healthy donor PBMC spiked with an epitope-specific T cell line as a tetramer-positive control (Supplementary Fig 1B). Figures 2B and 2C show tetramer frequencies for two subjects over time. Subject KPP94-037 had a very high frequency of B57-NS126-34-specific T cells reaching ~20% at fever day +7. Frequencies of B57-NS126-34-specific T cells in subject CHD06-029 were more representative of the staining observed in the remaining donors. **Expansion** of B57-NS126-34+ T cells during infection was detected with contraction during convalescence in PBMC from every dengue subject tested. Peak frequencies ranged from 0.5- 20% (Figure 2D). Only subject KPP94-037 with secondary DENV infection had high B57-NS126-34-specific T cell frequencies (Figure 2D). Excluding this subject, frequencies of B57-NS126-34+ T cells were not higher in those with secondary infection compared to primary infection (Figure 2D).
We used tetramers for two other DENV CD8 T cell epitopes (A11-NS3<sub>133-142</sub> or A2-E<sub>213-221</sub>) to compare the frequencies of tetramer-positive cells in subjects who were HLA-B*57<sup>+</sup> and HLA*A11<sup>+</sup> or HLA*A2<sup>+</sup> (Figure 2E). T cell frequencies were similar for all of epitopes in PBMC from the 7 subjects tested.

**Antigen-specific CD8<sup>+</sup> T cells are highly activated during acute DENV infection**

Using antibodies to CD69 and CD38, we followed CD8<sup>+</sup> T cell activation over the course of acute dengue illness. Frequencies of CD69<sup>+</sup>CD8<sup>+</sup> T cells were elevated early in acute illness compared to early (1 wk after defervescence) or late (6-12 months after illness) convalescence (p<0.001), with the peak frequencies (10.7%-46.3%) occurring at or before fever day −4 (Figure 3A, B). Peak frequencies of B57-NS1<sub>26-34</sub><sup>+</sup>CD69<sup>+</sup> cells (Figure 3C) and A2-E<sub>213-221</sub><sup>+</sup>CD69<sup>+</sup> or A11-NS3<sub>133-142</sub><sup>+</sup>CD69<sup>+</sup> cells (Figure 3D) were 10.5%-48.5% and 15.4-50.3%, respectively. CD38 expression peaked later than CD69 expression, on fever days −1 and 0 (Figure 3E). Frequencies of CD38<sup>+</sup> cells in the total CD8<sup>+</sup> population were between 2.45%-57.3%. Peak frequencies of B57-NS1<sub>26-34</sub><sup>+</sup>CD38<sup>+</sup> cells (Figure 3F) and A2-E<sub>213-221</sub><sup>+</sup>CD38<sup>+</sup> or A11-NS3<sub>133-142</sub><sup>+</sup>CD38<sup>+</sup> cells (Figure 3G) were 15.8%-92.4% and 10%-77.8%, respectively. The pattern of CD38 and CD69 expression on all tetramer-positive T cells followed the same pattern as the expression on the total CD8 positive population.

**Increased frequencies of CD71-expressing cells on the DENV-specific B57-NS1<sub>26-34</sub><sup>+</sup>, A11-NS3<sub>133-147</sub><sup>+</sup> and A2-E<sub>213-221</sub><sup>+</sup> T cell populations.**

We assessed CD71 expression, a marker associated with cell cycle activity<sup>30</sup>, on total CD8 T cells and DENV-specific T cells. Figure 4G shows representative staining of
CD71 on PBMC from a subject during acute infection. CD71 expression was low on total CD8+ T cells with a mean frequency of 2.1% during acute illness (fever day -4 through fever day +3) (Figure 4A). In contrast, the mean frequency of B57-NS126-34+ T cells expressing CD71 was 18.39% and of A11-NS3133-147+ or A2-E213-221+ T cells was 12.21% during acute illness (Figure 4B, C). The mean frequencies of CD71-expressing cells during acute illness were significantly higher in the CD8+DENV-specific T cells compared to the total CD8+ population with p-values <0.001 (Table 2). There were no statistically significant differences between the B57-NS126-34+ and the A11-NS3133-147/A2-E213-221-specific T cell populations.

The peak frequency, as determined for each donor during acute illness, of CD71+DENV-specific CD8 T cells was also significantly higher than that of the total CD8+ T cells (p <0.005). Frequencies of CD71+ DENV-specific T cells remained higher compared to the total CD8 T cell population 1 year following infection (Figure 4A, B, C) (p <0.0001), but were lower than the peak CD71 frequencies during acute infection in most donors. Interestingly, mean and peak frequencies of CD38 expression during acute illness were significantly higher in B57-NS126-34+, but not A11-NS3133-147+/ A2-E213-221+, specific T cells. CD69 expression was minimally increased only in A11-NS3133-147+ T cells (Figure 3 and Table 2). We also compared the geometric mean fluorescence intensity (gMFI) of CD71 expression between populations (Figure 4D, E, F) and again found significant differences in the intensity of CD71 staining on the CD71+ cells during acute illness between the DENV-specific populations and total CD8+ T cells (p<0.05).

To further evaluate the expression of CD71 and its relationship to T cell activation by antigen, we stimulated a B57-NS126-34-specific T cell line 3C11 with different
concentrations of the NS1<sub>26-34</sub> peptide and measured the frequencies and intensity of CD71 expression. Figure 4H shows representative staining of CD71 expression on cell line 3C11 at 24 hours after stimulation with peptide. We detected CD71 upregulation from base line expression as early as 1 hr post stimulation with the peptide and the MFI of CD71 expression depended both on the concentration of peptide and the duration of incubation (Figure 4I).

**CD71, CD69, CD107a and cytokine expression in epitope-specific T cell lines.**

Since the NS1<sub>26-34</sub> epitope is highly conserved with only rare variants, we next assessed CD71 expression on other DENV-specific cell lines where epitope variants are more common. We used a well characterized A11-NS3<sub>133-147</sub> epitope-specific cell line 10C11, which was cross reactive for the pD1 and pD3/4 variant peptides but did not recognize the pD2 variant in tetramer staining and ICS assays<sup>31</sup>. We stimulated 10C11 with three variant peptides for 6 hrs and evaluated the expression of CD107a, CD69, and CD71 (Figure 5A). We detected similar CD69 upregulation following stimulation with the pD1 and pD3/4 variant peptides. CD107a staining was more uniform following stimulation with the pD3/4 variant compared to the pD1 variant. A higher frequency of the 10C11 cell line upregulated CD71 following stimulation with the pD3/4 variant compared to the pD1 variant peptide (Figure 5A). We did not detect CD69, CD107a and CD71 upregulation after stimulation with the pD2 variant of the A11-NS3<sub>133-147</sub> epitope.

We also stimulated an A2-E<sub>213-221</sub> epitope-specific cell line P1A07 with four peptide variants. Cell line P1A07 had similar upregulation of CD69 following stimulation with all four peptide variants (Figure 5B). In contrast, there was stronger upregulation of CD71 and CD107a with the pD1 and pD2 variants compared to the pD3 and pD4 variant peptides.
(Figure 5B). We found the largest production of TNF-α and IFN-γ following stimulation with pD1 and pD2 variants and significant production following stimulation with the pD4 variant (Figure 5C) which matched CD71 and CD107a expression patterns. MIP-1β production was upregulated with pD4 ≈ pD1 > pD2 variant peptide stimulation. The pD3 variant peptide did not induce cytokine production (Figure 5C). Together, our data using cell lines suggest that CD71 expression was differentially sensitive to stimulation by homologous and heterologous variant peptides.
DISCUSSION

We analyzed the frequency, kinetics, and phenotype of T cells specific for a novel HLA-B57-restricted epitope, B57-NS1_{26-34} over the course of acute DENV infection. Alignment of over 2610 strains of DENV from all four serotypes revealed >99% homology in the epitope. This conservation led us to hypothesize that it might be an important target for DENV control in HLA-B*57-positive individuals. Variation in the sequence of T cell epitopes between DENV serotypes has been shown to influence the effector functions of DENV-specific memory T cells\textsuperscript{31, 32}. Since the sequence of this epitope in a secondary DENV infection would be identical to the sequence from an earlier primary DENV infection, we predicted that PBMC from donors with secondary infection would have particularly strong secondary responses to the B57-NS1_{26-34} epitope. While we detected tetramer-positive T cells in all subjects tested, their frequencies in subjects with secondary infections were not higher than in subjects with primary infections, with one exception. Frequencies of B57-NS1_{26-34}^{+} T cells were similar to those of A11-NS3_{133-142} and A2-E_{213-221}^{+} T cells in the same subjects and to the frequencies of A11-NS3_{133-142}^{+} T cells reported elsewhere\textsuperscript{12, 33}. One donor had a peak frequency of B57-NS1_{26-34}^{+} T cells at day 180. While we may have missed the peak frequency during acute illness a second subclinical infection at the 6 month time point cannot be ruled out.

Interestingly this linear NS1_{26-34} epitope has been demonstrated to be an antibody epitope in mice\textsuperscript{34}. NS1 is unique among the DENV non-structural proteins because it is secreted and expressed on cell surfaces\textsuperscript{35}. We are unaware of other linear dengue B cell epitopes which map exactly to a CD8 T cell epitope. Since peptides presented by class I MHC come from cytosolic proteins in virally-infected cells and not from phagocytized
soluble NS1, antibodies to NS1_{26-34} are unlikely to affect CD8 T cell responses. It is also unlikely that T cells are able to recognize this epitope on the surface or soluble NS1 since presentation of peptides on MHC molecules are critical for T cell recognition \(^{36}\).

One possible explanation for the lower-than-expected frequency of tetramer-positive cells could be differential processing and presentation of this epitope between the four serotypes. Differential processing of HIV epitopes has been shown to result in striking differences in CTL recognition \(^{37}\). We demonstrated that B57-NS1_{26-34}-specific cell lines were able to lyse cells infected with any of the four DENV serotypes in vitro. Whether there is differential processing of the four serotypes for this epitope in vivo is unknown. Alternatively, a yet unidentified factor may dampen the activation of B57-NS1_{26-34}^+ T cells during a second infection.

Previous studies have used a number of cell surface markers to phenotype CD8^+ T cells in DENV infection \(^{12, 13, 26, 33, 38}\). We included a diverse panel of surface markers including some that have not previously been studied in DENV infection, such as CD71. The timing of expression of CD69 in this cohort was consistent with previous reports \(^{13}\). While Akondy et al reported that CD38, HLA-DR, and Ki-67 are specific markers of activation when present in combination, there were a significant proportion of cells that expressed only CD38 \(^{39}\). Friberg et al found lower intensity of CD38 expression on influenza tetramer-positive cells compared to A11-NS3_{133-142} tetramer-positive cells during DENV infection \(^{33}\). The findings of Akondy et al and Friberg et al, suggest that the intensity of CD38 staining correlates with the specificity of activation and that bystander cells which are activated become CD38^+, but not CD38 high. The high frequency of CD38 expression in our T cell population is consistent with these findings.
Our study is the first to assess CD71 (transferrin receptor) expression on CD8\(^+\) T cells in the context of an acute viral illness. Over the course of DENV infection we observed upregulation of CD71 predominantly on DENV-specific CD8\(^+\) T cells and not on total CD8\(^+\) T cells. This was in contrast to CD69 and CD38 expression, which was similar between B57-NS1\(_{26-34}\)^+ T cells, A2-E\(_{213-221}\)^+ or A11-NS3\(_{133-142}\)^+ T cells and total CD8\(^+\) T cells during acute DENV infection. CD71 is required for DNA synthesis and cell division and is upregulated on dividing cells \(^{30,40,41}\). Upon cell activation, CD71 is recruited to the immunological synapse coincident with upregulation of surface CD71 \(^{42}\). Salmeron et al demonstrated that CD71 plays a role in the phosphorylation of TCR\(\zeta\) chain following CD3 and CD28 stimulation \(^{43}\), and anti-CD71 mAb abrogates CTL responses to alloantigens \(^{44}\). Upregulation of CD71 on DENV-specific T cells may therefore indicate that these cells had a more productive activation and are more cytolytic. Our data suggest that CD71\(^{hi}\) expression more accurately identifies DENV-specific T cells compared to expression of CD69 and/or CD38 with significant differences in both frequency and MFI of CD71 expression between the total CD8\(^+\) T cell population and the DENV-specific populations. Previous in vitro work showed upregulation of CD71 following \(\alpha\)CD3 or mitogen stimulation \(^{45,46}\). We are the first to show robust expression of CD71 on T cell lines after peptide stimulation in vitro. Unlike CD69, the extent of CD71 upregulation was dependent on the peptide variant used and for the most part matched CD107a expression These in vitro experiments support our ex vivo observation and suggest that CD71 expression may reflect qualitatively different signaling in the T cell response to DENV infection. We noted high levels of CD71 in B57-NS1\(_{26-34}\)^+ and A11-NS3\(_{133-147}\)^+/ A2-E\(_{213-221}\)^+-specific T cell populations in many donors at days 180 and 365. We do not have an explanation for
persistent elevation in convalescence but CD71 expression was generally lower than the peak frequency during acute infection. We have similarly found that antigen-specific cell lines have marked levels of CD71 2-3 weeks after in vitro culture (data not shown). It is possible that certain subsets of memory cells have slightly higher baseline levels of CD71 but further studies are needed to confirm these findings.

Our study population, although small, included subjects with primary and secondary infections, DF and DHF, and each of the four DENV serotypes. This small sample size precluded comparing the magnitude of B57-NS1_{26-34}-specific T cells during primary and secondary infections. Previous work has provided conflicting data on the role of CD8^{+} T cells in the development of severe dengue disease and has focused heavily on responses to the HLA-A11-restricted NS3_{133-142} epitope \(^{12,38}\). The number of consecutive blood draws at early time points during illness and consistency of patient care during acute illness are important strengths of this cohort. Additionally, our data suggest that even within 72hrs of fever onset immune responses are well underway, and therefore potentially important early events may not be captured.

In summary, we found modestly increased frequencies of HLA-B57-restricted NS1-specific T cells in PBMC from the majority of Thai donors with secondary DENV infection. The absence of a stronger B57-NS1_{26-34}-specific response leads us to believe that other factors may be involved in influencing the magnitude of the response to this highly conserved epitope. The finding of a novel and distinct phenotype (CD71^{+}) in these epitope-specific T cells suggests differential activation that merits further investigation.
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FIGURE LEGENDS

Figure 1. **Identification of the HLA-B57-restricted DENV epitope.** (A) Cell lines 3C11 and 3F2, generated from PBMC of donor KPP94-037, were used in a $^{51}$Cr release assay using B-LCLs infected with vaccinia virus recombinants expressing DENV-2 NS1/2a as target cells. (B) $^{51}$Cr release assay using B-LCLs pulsed with peptide pool 1A and individual 15 mer peptides covering pool 1A of NS1. (C) Identification of the minimal 9mer epitope B57-NS1$_{26-34}$ recognized by cell line 3C11. (D) Lysis of DENV-infected dendritic cells (DCs) by B57-NS1$_{26-34}$-specific cell line 3F11. (E) Validation of B57-NS1$_{26-34}$ tetramer staining using a B57-NS1$_{26-34}$-specific T cell line and an HIV gag-specific HLA-B57-restricted T cell line.

Figure 2. **Expansion of DENV specific T cells during acute infection.** (A) Gating strategy used to identify tetramer-positive CD8$^+$ T cells started by selecting cells within the lymphocyte gate as defined by forward and side scatter profiles followed by gating for singlet cells. Live CD14$^-$ CD19$^-$ cells were next selected by exclusion of the viability marker LIVE/DEAD® Green along with αCD14-FITC and αCD19-FITC. CD8$^+$ T cells were identified by CD8 expression. (B) Kinetics of B57-NS1$_{26-34}^+$ frequencies in PBMC from donor KPP94-037 and (C) donor CHD06-029 over the course of acute illness and convalescence. (D) B57-NS1$_{26-34}^+$ CD8$^+$ T cell frequencies versus fever day in PBMC from study subjects. Symbols distinguish subjects with primary ($n$=2, grey symbols) versus secondary ($n$=9, black symbols) DENV infections and lines distinguish those with DF ($n$=6, black line) versus DHF ($n$=5, dashed line). (E) PBMC from subjects who were also HLA*A2- or HLA*A11-positive ($n$=6) were stained with A2-E$_{213-221}$ or A11-NS3$_{133-142}$.
tetramers. Two of these subjects had primary infections (grey symbols) and one subject had DHF (dashed line). Fever Day is defined as the day of defervesence (Fever Day 0).

Figure 3. **Antigen-specific T cells are highly activated during acute DENV infection and early convalescence.** (A) Representative staining of CD69 and CD38 on total CD8+ T cells during acute infection and in convalescence from 1 subject. (B and E) Staining of CD69 and CD38 on total CD8 cells, (C and F) B57-NS126-34+ T cells and A11-NS3133-142+ or (D and G) A2-E213-221+ T cells over the course of acute DENV infection and convalescence, respectively. PBMC from 11 subjects with primary (grey symbols) or secondary (black symbols) infection and DF (black lines) or DHF (dashed lines) were tested.

Figure 4. **CD71 expression on total CD8 and DENV-specific CD8 T cells.** Frequency of CD71+ cells in (A) total CD8+ cells, (B) B57-NS126-34+ T cells and (C) A11-NS3133-142+ or A2-E213-221+ T cells over the course of acute DENV infection and convalescence. MFI of CD71 expressed on CD71+ (D) CD8+ cells, (E) B57-NS126-34+ T cells and (F) A11-NS3133-142+ or A2-E213-221+ T cells over the course of acute DENV infection and convalescence. (G) Representative staining of CD71 on CD8+ T cells at fever day -2 from a subject with primary infection. (H) Representative staining of CD71 on a CD8+ T cell line 24 hours after stimulation with (black) or without (NS, grey) peptide stimulation. (I) CD71 expression of a B57-NS126-34+-specific cell line following stimulation with 10, 1, 0.1 and 0.01µg/mL NS126-34 peptide HTWTEQYKF.
Figure 5. **CD71 expression and effector functions on epitope-specific T cell lines.**

CD107a, CD69 and CD71 expression after in vitro stimulation of cell line (A) 10C11 for 6 hrs with 10µg/mL A11-NS3\textsubscript{133-142} variant peptides pD1, pD2, and pD3/4 and cell line (B) P1A07 for 6 hrs with 10µg/mL A2-E\textsubscript{213-221} variant peptides pD1, pD2, pD3, and pD4. NS= no peptide stimulation. C) Intracellular cytokine staining (ICS) of cell line P1A07 with variant peptides pD1, pD2, pD3, and pD4 at 10µg/mL. NS= no peptide as the negative control. Data are displayed as histograms with the gMFI of each parameter listed.

Supplemental Figure 1. **Tetramer Staining Controls.** (A) PBMC from DENV naïve HLA B57\textsuperscript{+}, A2\textsuperscript{+} and A11\textsuperscript{+} individuals were stained with B57-NS1\textsubscript{26-34}, A2-E\textsubscript{213-221} or A11-NS3\textsubscript{133-147} tetramers. (B) PBMC spiked with the appropriate epitope-specific cell line were stained with B57-NS1\textsubscript{26-34} or A2-E\textsubscript{213-221} or A11-NS3\textsubscript{133-147} tetramer.
**TABLE 1:** Clinical, viral and immunogenetic profiles of the study subjects

<table>
<thead>
<tr>
<th>Donor</th>
<th>Serology&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Serotype&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Diagnosis&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>P</td>
<td>DENV-1</td>
<td>DF</td>
<td>HLA-A1,11 HLA-B56,57</td>
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<td>DENV-3</td>
<td>DF</td>
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<td>DENV-2</td>
<td>DHF-1</td>
<td>HLA-A33,34 HLA-B57,75</td>
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<td>DENV-2</td>
<td>DHF-3</td>
<td>HLA-A1,11 HLA-B57,60</td>
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<td>DF</td>
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<td>CHD02-073</td>
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<td>HLA-A1,33 HLA-B57,35</td>
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<sup>a</sup> Primary (P) versus secondary (S) infection as determined by IgM/IgG ratios

<sup>b</sup> Of current infection

<sup>c</sup> According to WHO guidelines 1997; DF = dengue fever, DHF = dengue hemorrhagic fever
Table 2: Statistical analysis of activation markers on CD8+ T cells

<table>
<thead>
<tr>
<th>Populations compared</th>
<th>CD69</th>
<th>CD38</th>
<th>CD71</th>
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<tbody>
<tr>
<td>Mean frequency</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total CD8+ vs. B57-NS1_{26-34}^+</td>
<td>N.S.</td>
<td>0.0017</td>
<td>&lt;0.0001</td>
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<tr>
<td>Total CD8+ vs. A11-NS3_{133-142}^+/A2-E_{213-221}^+</td>
<td>N.S.</td>
<td>N.S.</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>B57-NS1_{26-34}^+ vs. A11-NS3_{133-142}^+/A2-E_{213-221}^+</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
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<tr>
<td>Peak frequency</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total CD8+ vs. B57-NS1_{26-34}^+</td>
<td>N.S.</td>
<td>0.0115</td>
<td>0.0021</td>
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<tr>
<td>Total CD8+ vs. A11-NS3_{133-142}^+/A2-E_{213-221}^+</td>
<td>0.04</td>
<td>N.S.</td>
<td>0.0005</td>
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<td>B57-NS1_{26-34}^+ vs. A11-NS3_{133-142}^+/A2-E_{213-221}^+</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
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</tbody>
</table>

*Mean frequency* = average frequency of CD69, CD38 and CD71+ cells for all times points between fever day -4 to fever day +3.

*Peak frequency* = average of the peak frequency of CD69, CD38 and CD71 between fever day -4 to fever day +3.

*N.S.* = not significant
Figure 1

A

% Specific Lysis

rVV DENV-2 NS1/2a
rVV Control
Media

Cell Lines

B

% Specific Lysis

Pool 1A

Peptides

C

% Specific Lysis

DENV2 #3
DENV2 #4
DENV2 NS1 9 mer
Media

Infected DCs

D

% Specific Lysis

Uninfected DCs
BLCLs w/peptide
DENV-1
DENV-2
DENV-3
DENV-4

Infected DCs

E

DENV Cell Line

HIV Cell Line

CD8
Figure 2

A. Lymphocytes, Single Cells, Live CD14-CD19-, CD8+, B57+

B. Fever Day 0, +1, +7, +365

B57 TETRAMER

C. Fever Day 1, +6, +180, +365

B57 TETRAMER

D. HLA-B57 Tetramer+ T cells

E. HLA-A2, A11 Tetramer+ T cells

- Primary
- Secondary
- DF
- DHF

Tetramer Frequency

Fever Day
Figure 3

A

Fever Day -1
34.5

Fever Day 365
1.74

Fever Day 0
10.7

Fever Day 365
2.08

B

CD8+ T cells

CD69

CD38

C

HLA-B57 Tetramer+ T cells

D

HLA-A2,-A11 Tetramer+ T cells

E

CD8+ T cells

F

HLA-B57 Tetramer+ T cells

G

HLA-A2,-A11 Tetramer+ T cells

Primary
Secondary
DF
DHF
Figure 4

A) CD8+ T cells
   - Primary
   - Secondary
   - DF
   - DHF

B) HLA-B57 Tetramer+ T cells

C) HLA-A2/A11 Tetramer+ T cells

D) CD8+CD71+ T cells

E) HLA-B57 Tetramer+CD71+ T cells

F) HLA-A2/A11 Tetramer+CD71+ T cells

G) CD71 staining on Thai PBMC

H) CD71 staining on 3C11

I) 3C11 (B57 NS1 specific)

MFI (FC)

Fever Day

CD71

B57 Tetramer

CD71

CD8

CD8

CD71

Peptide Dose
Figure 5

A. 10C11 (A11 NS3 specific) 10µg/mL peptide

B. P1A07 (A2 E specific) 10µg/mL peptide

C. P1A07 (A2 E specific) 10µg/mL peptide
Supplemental Figure 1

A

B57^+ DENV naïve Donor PBMC

A2^+ DENV naïve Donor PBMC

A11^+ DENV naïve Donor PBMC

B

3C11 spiked into PBMC

P1A07 spiked into PBMC

10C11 spiked into PBMC

4.50x10^-3

7.7x10^-3

0.1

17.6

38.9

29.8
**Supplemental Table 1:** Conservation of amino acid sequences among known CD8+ DENV-specific T cell epitopes

<table>
<thead>
<tr>
<th>EPITOPE</th>
<th>SEQUENCE</th>
<th>HLA RESTRICTION</th>
<th>No. of SEQ</th>
<th>% CONSERVATION</th>
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<tbody>
<tr>
<td>E 211-219</td>
<td>FFDLPLPWT</td>
<td>A02</td>
<td>1148</td>
<td>100 78 99 100 100 100 100 95 n/a</td>
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<td>100 100 100 100 100 51 100 89 100 n/a</td>
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Sequence conservation of a sample of known CD8+ T cell epitopes among 4 serotypes of human DENV strains calculated using sequence and variability analysis tool on flavidB database [http://cvc.dfci.harvard.edu/flavi/index.php](http://cvc.dfci.harvard.edu/flavi/index.php). A NCBI search revealed two strains with a variant B57 epitope. n/a indicated epitopes containing only 9aa.
**Supplemental Table 2:** Antibodies used for flow cytometry studies.

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