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## T Cell Responses in Dengue Hemorrhagic Fever: Are Cross-Reactive T Cells Suboptimal?

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# T Cell Responses in Dengue Hemorrhagic Fever: Are Cross-Reactive T Cells Suboptimal?<sup>1</sup>

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Gavin Screaton<sup>2\*</sup>

Dengue virus infection poses a growing public health and economic burden in a number of tropical and subtropical countries. Dengue circulates as a number of quasispecies, which can be divided by serology into four groups or serotypes. An interesting feature of Dengue, recognized over five decades ago, is that most severe cases that show hemorrhagic fever are not suffering from a primary infection. Instead, they are reinfected with a virus of different serotype. This observation poses considerable problems in vaccine design, and it is therefore imperative to gain a full understanding of the mechanisms underlying this immunological enhancement of disease. In this study, we examined a T cell epitope restricted by HLA-A\*24, a major MHC class I allele, in Southeast Asia in a cohort of children admitted to a hospital with acute Dengue infection. The cytokine profiles and the degranulation capacity of T cells generated to this epitope are defined and compared across different viral serotypes. Cross-reactive Dengue-specific T cells seem to show suboptimal degranulation but high cytokine production, which may contribute to the development of the vascular leak characteristic of Dengue hemorrhagic fever. *The Journal of Immunology*, 2006, 176: 3821–3829.

**D**engue belongs to the flavivirus family and is transmitted to humans following a bite from an infected mosquito, usually *Aedes aegypti*. Symptoms begin after a typical incubation period of 5–7 days, although ~50% of infections are thought to be asymptomatic (1). Characteristic features of Dengue are a high fever, headache, rash, bone pains, and myalgia. In most patients, the disease is self-limiting and after 5–6 days, the fever remits and symptoms will lessen. These uncomplicated cases are referred to as Dengue fever (DF).<sup>4</sup> In a proportion of cases, the

disease can be more severe with thrombocytopenia, plasma leakage, bleeding, and shock. This is referred to as Dengue hemorrhagic fever (DHF), which occurs in 5–30% of cases, and which can be subdivided on clinical grounds into severity grades I–IV (2).

In the first half of the last century, the geographical spread of Dengue was limited; epidemic activity was low and cases of DHF were relatively unusual. During and after WWII, this changed and the number of cases has steadily increased. Today, some 2.5 billion people are at risk of infection, and there are estimated to be ~50 million Dengue infections per year (2). In the affected regions, this disease has major implications for public health; for instance, in Thailand, there can be >100,000 cases of DHF per annum, all of whom will require hospitalization and will present in an epidemic fashion ([http://w3.whosea.org/en/Section10/Section332\\_1101.htm](http://w3.whosea.org/en/Section10/Section332_1101.htm)).

Following the isolation of Dengue virus in the 1940s and 1950s, it became clear that the virus circulated as a series of distinct subspecies that could be distinguished by serology into four groups: Dengue serotype (Den)-1, Den-2, Den-3, and Den-4. These four serotypes differ in amino acid sequence by 25–30%, and, crucially, infection with one serotype does not confer immunity to infection by other serotypes. Viral and host factors have been proposed to play a role in the development of DHF, but observations of disease activity particularly on island populations, such as Cuba, led to the realization that the majority of DHF cases occur among individuals who are suffering a secondary or sequential infection (3, 4).

Halstead et al. (5, 6) proposed an Ab enhancement hypothesis to explain this phenomenon. This was based on observations that Ab could increase infection of macrophages with the virus presumably by increasing FcR-mediated internalization. Abs generated to a previous infection may not be of high enough avidity or titer to neutralize a second serotype, but may instead enhance infection of macrophages driving higher viral loads.

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<sup>4</sup> Abbreviations used in this paper: DF, Dengue fever; DHF, Dengue hemorrhagic fever; Den, Dengue serotype; RSV, respiratory syncytial virus; LCMV, lymphocytic

choriomeningitis virus; NS3, nonstructural protein 3; FRNT, focus reduction neutralization test; QR, quantum red.

Ab enhancement of Dengue infection has been demonstrated both in vitro and in a maquette model (6, 7), and, furthermore, there is a correlation between peak viral load and disease. However, the timing of the severe illness suggests that viral load per se may not be the sole cause of pathology. Interestingly, virus load falls abruptly as the fever remits, defined as day 0, and it is at this point that the most severe symptoms of plasma leakage, hemorrhage, and shock appear, not at the point of peak virus replication. This suggests that it may be the immune response to the virus rather than the virus itself that causes the pathology.

Such virus-induced immunopathology has been demonstrated in a number of viruses including respiratory syncytial virus (RSV) and lymphocytic choriomeningitis virus (LCMV) in mice (8, 9). In these instances, disease seems to be caused by T cells, and for these reasons, we and others have chosen to study T cell responses in Dengue infection. Such studies have often been conducted on vaccine recipients from nonendemic countries, meaning that studies have been limited by the lack of knowledge of T cell epitopes presented by major MHC alleles expressed by affected populations. In this study, we report the characterization of a Dengue MHC class I-restricted T cell epitope found in the nonstructural protein 3 (NS3) presented by HLA-A\*24 together with an in-depth analysis of the epitope.

## Materials and Methods

### Samples

Blood samples were taken following parental consent and the approval of the ethical committee of Khon Kaen Hospital, Thailand. Acute Dengue infection was identified by RT-PCR-based Dengue gene identification or Dengue-specific IgM capture ELISA (10, 11). Secondary Dengue infection (an acute infection in a patient who had previously encountered Dengue on one or more occasions) was defined as a Dengue-specific IgM:IgG ratio <1.8, by IgM and IgG capture ELISA. Micro-FRNTs (focus reduction neutralization tests) were conducted as described previously (12). The percentage of reduction of foci was plotted against serum dilutions, and the dilution giving 50% reduction in foci was reported as the neutralizing titer.

Disease severity was classified according to the World Health Organization criteria (13): The day of defervescence was defined as day 0, the day before defervescence as day -1, the day after defervescence as day +1, and so forth.

PBMC were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation and cryopreserved until tested. HLA typing was performed by sequence-specific primer PCR (14).

### T cell culture and assays of function

ELISPOT assays were performed as follows: briefly, PBMC were added to polyvinylidene difluoride-backed plates (Millipore) precoated with a mAb to IFN- $\gamma$  (1-DIK; Mabtech). Peptides were then added at a final concentration of 10  $\mu$ M, and the PBMC were cultured overnight. Cells were discarded, and spots were revealed by incubation with biotinylated mAb to IFN- $\gamma$  (Mabtech) followed by streptavidin-conjugated alkaline phosphatase and substrate. The spots were counted using an AID-ELISpot Reader (Autoimmun Diagnostika). The number of specific T cell responders was calculated after subtracting negative control values.

To generate the T cell lines, PBMC were incubated with 100  $\mu$ M peptide for 1 h, after which IL-7 (PeproTech) was added at 25 ng/ml. After day 3, IL-2 (Proleukin) was added at 50 U/ml. Cells were tested for function after 1 wk and restimulated every 2 wk using autologous EBV-transformed B cell lines (BCLs) that had been pulsed with peptide as targets. Dengue-specific T cell clones were generated by the limiting dilution of Dengue-specific T cell lines. T cell clones were expanded by stimulation with PHA in the presence of allogeneic-irradiated (4000 rad) PBMC and IL-2.

Cytotoxicity was measured by a standard 4-h  $^{51}\text{Cr}$  release assay. E:T ratio was 20:1 for CTL lines and 5:1 for CTL clones. The killing (specific  $^{51}\text{Cr}$  release) was calculated as [(experimental release - spontaneous release)  $\div$  (maximum release - spontaneous release)]  $\times$  100. Proliferation was assessed using a [ $^3\text{H}$ ]thymidine incorporation assay. Autologous EBV-transformed BCL was used as APCs. The effectors were cocultured with targets at an E:T ratio of 1:10 for 24 h before being pulsed with 1  $\mu\text{Ci}$ /well [ $^3\text{H}$ ]thymidine for 12 h.

### MHC tetrameric complexes and FACS analysis

Tetramers were prepared as described previously (15). Briefly, the extracellular domain of HLA-A\*24 containing the BirA biotinylation enzyme recognition site at the C terminus and  $\beta_2$ -microglobulin were expressed in *Escherichia coli* as inclusion bodies. Monomeric complexes of peptide, H chain, and  $\beta_2$ -microglobulin (referred to in this study as monomers) were formed by refolding in vitro and then biotinylated using the BirA enzyme (Avidity). Monomers were purified by fast-performance liquid chromatography, and tetramers were formed by mixing biotinylated monomer with fluorochrome-conjugated streptavidin at a 4:1 molar ratio.

PBMC or T cell lines/clones were stained with fluorochrome-labeled tetramer at 37°C for 20 min and then for surface markers at 4°C for 20 min by addition of a fluorochrome-conjugated anti-CD8 Ab and Abs to other surface marker Abs. Cells were washed, fixed, and analyzed by flow cytometry.

### Intracellular cytokine staining and the degranulation assay on double tetramer-stained cells

PBMC were double stained with quantum red (QR)-conjugated NYA Den-1 A\*24 and PE-conjugated NYA Den-2 A\*24 tetramers at 37°C for 20 min. Then, cells were incubated with 1  $\mu\text{g}/\text{ml}$  each of anti-CD28 and anti-CD49d and the appropriate peptide at a concentration of 10  $\mu\text{M}$  in 1 ml. FITC-conjugated CD107a was added to the cells before stimulation. After 1 h of incubation, a mixture of the secretion inhibitors monensin (GolgiStop; BD Pharmingen) and Brefeldin A (Sigma-Aldrich) were added. After another 5-h stimulation, PBMC were washed and fixed/permeabilized using FACS permeabilization buffer II (BD Pharmingen). Cells were washed again and stained with the titrated amount of allophycocyanin-conjugated anti-IFN- $\gamma$ , PE-Cy7-conjugated anti-TNF- $\alpha$ , and allophycocyanin-Cy7-conjugated anti-CD8 mAbs for 30 min, washed, and fixed with 1% formaldehyde in PBS. Six-color FACS analysis was performed using a Cyan flow cytometer (DakoCytometry). All Abs were obtained from BD Pharmingen.

### Cytokine quantitation

Cytokine quantitation was performed on bead-based ELISA using the Bio-Plex system (Bio-Rad) according to the manufacturer's instructions. Briefly, cell culture supernatant was mixed with beads having unique fluorescent intensity and coated with the Abs to various cytokines. Subsequently, the mixture was incubated with biotinylated anti-cytokine Abs. Finally, PE-conjugated streptavidin was added, and the fluorescent signal was detected using a Luminex machine (Bio-Rad). Raw data were initially measured as the relative fluorescence intensity and then converted to cytokine concentration based on a standard curve generated from the reference concentrations supplied in the kit.

Cell culture supernatant was collected after 12-h coculture of T cell clones, and peptide-pulsed autologous EBV transformed BCL at the indicated peptide concentration. The E:T ratio was 1:10.

### Tetramer dissociation assay

These assays were performed as described previously (16). Dengue-specific T cell clones were stained with saturating concentrations of PE-labeled A24-NYA Den-1 or PE-labeled A24-NYA Den-2 tetramers at 4°C for 30 min. The cells were washed three times, and aliquots of cells were removed for FACS analysis. The remaining T cell clones were incubated with a 100-fold excess of unlabeled A24-NYA Den-1 or unlabeled A24-NYA Den-2 tetramer at 4°C. This unlabeled reagent effectively blocks binding/rebinding of PE-labeled tetramer. Further aliquots of cells were removed and analyzed by FACS at appropriate time points. The total fluorescence within the PE-positive gate was plotted against time to give the dissociation curve. This total fluorescence was calculated as the sum of the fluorescence intensities of the tetramer-positive cells normalized per lymphocyte. This was then normalized to percentage of the total fluorescence at the initial time point and plotted on a logarithmic scale.

## Results

### Definition of a Dengue CD8<sup>+</sup> T cell epitope

Most severe Dengue illness in Thailand occurs in children peaking at ~8–10 years of age from whom only relatively small blood samples can be obtained. However, infection is so prevalent that seropositivity in adults is near 100%, presumably representing multiple previous exposures. For this reason, we used a dual approach to define epitopes, studying samples from both convalescent children and healthy immune adults. To study the role of T

cell responses in the pathology of Dengue, we were particularly interested to define epitopes restricted by common HLA alleles found in Southeast Asia, namely for MHC class I, A\*02 (20%), A\*11 (30%), A\*24 (20%), and A\*33 (15%) (17).

Dengue is a negative-strand RNA virus, which encodes a polyprotein of ~3300 aas, which is cleaved into 10 polypeptides by both host and virally encoded proteases. The relatively small size of the Dengue virus allows an overlapping peptide approach to epitope definition. Overlapping peptides, which essentially allow every linear epitope of up to 10 aas in length to be presented, were synthesized as a set of 15-mers overlapping by 10 aas for the envelope and NS3 proteins from the reference Den-2 strain 16681. Pools of peptides were screened against PBMC using an overnight IFN- $\gamma$  ELISpot assay. Many responses were found in the initial screens and could be divided between CD4 and CD8 responses by depletion of either population with magnetic beads coated with either anti-CD4 or anti-CD8 mAbs.

A CD8 response to peptide INYADRRWCFDGVKN from the Dengue NS3 protein restricted by HLA-A\*24 was chosen for further study. T cell lines were created, and a series of truncations of the 15-mer peptide were synthesized to define the minimal epitope for this response (Fig. 1a). The minimal epitope was the 9-mer

556NYADRRWCF564 (A24-NYA). This epitope conforms to the HLA-A\*24 consensus, containing tyrosine at p2 and phenylalanine at the C terminus. We went on to prove that the NYA peptide is restricted by HLA-A\*24 using .221 transfectants expressing HLA-A\*24 (Fig. 1b).

*Variants of the new epitope among the Dens*

Substantial sequence variation, up to 30% difference at the amino acid level, exists between the Dengue virus serotypes, so we searched published Dengue NS3 sequences for variants of A24-NYA. Four additional variants were identified for A24-NYA epitope. These peptides were synthesized, and cytotoxicity of T cell lines for targets pulsed with an increasing titration of peptides were tested by <sup>51</sup>Cr release assays (Fig. 1e). All of these peptides contain the dominant anchor positions and would therefore be likely to bind to MHC class I molecules. We have indirect evidence that this is the case because MHC class I complexes were successfully refolded around the index and variant peptides for HLA-A\*24 (data not shown).

The A24-NYA-responsive lines recognized peptides derived from Den-1 and Den-2 but not to those derived from Den-3 or Den-4. It is notable that the samples used to derive the T cell lines used in these assays have been obtained from patients admitted to our clinical centre in Khon Kaen in North East Thailand, where in recent years there has been substantial activity with Den-1 and Den-2 and less with serotypes 3 and 4.

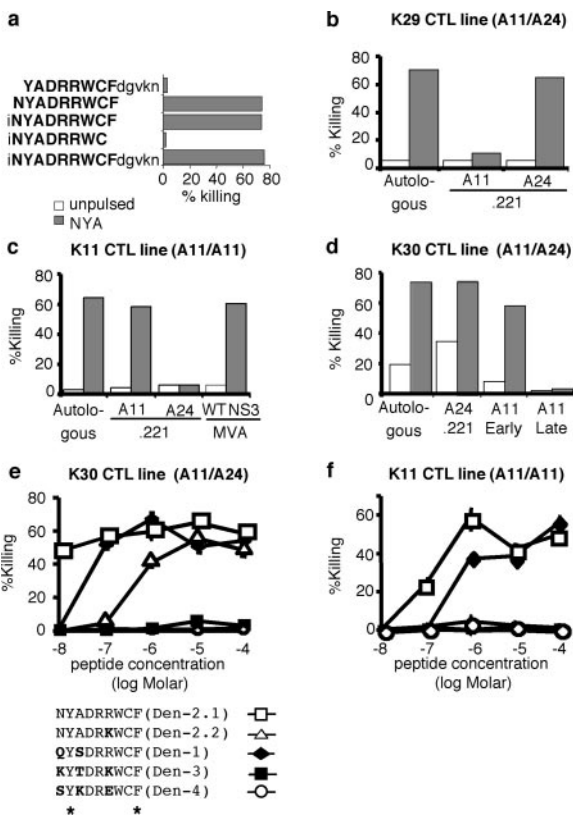
*A24-NYA is also restricted by HLA-A\*11*

While characterizing the A24-NYA-restricted response, we were surprised to find that some of the T cell lines we had derived were from individuals who were actually HLA-A\*24 negative but did have HLA-A\*11 in common. To prove the additional HLA-A\*11 restriction of the NYA epitope, we tested T cell lines against a panel of HLA-A\*11-expressing EBV-transformed cell lines and also against a .221-transfected cell line expressing HLA-A\*11 (Fig. 1c and data not shown). This analysis shows that NYA can indeed be restricted by HLA-A\*11 and that the NYA peptide can be processed from full-length NS3, because cytotoxicity is also seen when HLA-A\*11-expressing BCLs are infected with modified vaccinia virus Ankara-expressing NS3. Interestingly, the peptide specificities of this response were the same as the response to NYA when restricted by HLA-A\*24, i.e., T cells cross-react between peptide variants Den-1 and Den-2 but not to the Den-3- and Den-4-derived peptide sequences (Fig. 1f).

To study this in more detail, T cell lines were derived from three individuals, one HLA-A\*11 homozygote (described above) and two HLA-A\*11/HLA-A\*24 heterozygotes. The T cell line from the HLA-A\*11 homozygote showed restriction to HLA-A\*11 and no cross-reactivity to HLA-A\*24 (Fig. 1c). Of the two T cell lines from the heterozygotes one showed only HLA-A\*24 restriction (Fig. 1b), whereas early passage cultures of the other were initially able to kill both HLA-A\*11 and HLA-A\*24-pulsed target cells, although later in passage this T cell line became exclusively HLA-A\*24-restricted (Fig. 1d). All of the clones that we have derived for the NYA peptide have been either HLA-A\*11 or HLA-A\*24-restricted with no dual restriction, so it seems most likely that these two responses are mutually exclusive at the level of the TCR.

*T cell responses in Dengue-infected individuals*

We went on to study T cell responses to A24-NYA during acute Dengue infection using samples obtained from a clinical center in Khon Kaen, North East Thailand. Consent is obtained from the children's relatives, and following enrollment in the study, blood



**FIGURE 1.** Defining a new HLA-A\*24-restricted T cell epitope. *a*, Results of 4-h <sup>51</sup>Cr release assays using CTL lines on a set of truncated peptides pulsed EBV lines to define the minimal epitope. CTL lines derived from individuals for HLA-A\*11/A\*24 heterozygous (*b* and *d*) and HLA-A\*11 homozygous (*c*) HLA-A\*11 were tested for cytotoxicity on the indicated <sup>51</sup>Cr-pulsed targets. A11Early and A11Late represented early and late passage culture of K30 CTL line. Cross-reactivity of NYA-specific (*f*) HLA-A\*11-restricted or HLA-A\*24-restricted (*e*) CTL line against Dengue variant peptides. CTL lines were cocultured with <sup>51</sup>Cr-labeled autologous EBV-transformed BCLs pulsed with Dengue variant peptides at the indicated concentrations. Amino acids that differ from the index Den-2 sequence are shown in bold, and asterisks indicate the main anchor residue used for MHC-I molecules.

Table I. Comparison of CD8<sup>+</sup> T cells responses against 2 HLA-A\*11-restricted epitopes

| Patient | Severity | Serology  | Den   | SFC <sup>a</sup> million cells |     |
|---------|----------|-----------|-------|--------------------------------|-----|
|         |          |           |       | GTS                            | NYA |
| K11     | DF       | Secondary | Den-1 | 0                              | 238 |
| K12     | DHF I    | Secondary | Den-2 | 125                            | 0   |
| K13     | DHF I    | Secondary | Den-1 | 170                            | 20  |
| K14     | DHF I    | Secondary | Den-1 | 500                            | 0   |
| K15     | DHF I    | Secondary | Den-2 | 34                             | 0   |
| K16     | DHF III  | Secondary | Den-1 | 220                            | 0   |

<sup>a</sup> SFC, Spot-forming cell.

samples (plasma and PBMC) are collected and cryopreserved during each hospital day (average 3 samples) and at 2 wk and 3- to 5-mo follow-up. MHC is typed by PCR on all of the patients and cases who were HLA-A\*24 selected for these studies.

First, we examined the HLA-A\*11-restricted response to NYA and compared them to the response to an epitope we have described previously, A11-GTS (GTS/GSPIIDK), which is also found in the Dengue NS3 protein (16). To prevent confusion with the A24-NYA response, we excluded patients who coexpressed HLA-A\*11 and -A\*24. Six patients were included in this analysis, and ELISPOT responses were tested against all of the peptide variants, which were combined in one tube (Table I). We found that the GTS epitope was strongly dominant in these assays. Interestingly, in one case, the NYA response seemed to be immunodominant to the GTS response.

Next, we examined the A24-NYA response in more detail. Thirteen from our cohort of 90 children were HLA-A\*24 positive, and all but one of these were suffering an acute secondary Dengue infection as evidenced by RT-PCR and serological testing (Table II). The samples were collected during a period of high activity with Dengue virus serotypes one (nine cases) and two (three cases), there was one patient with a Den-3 infection and no cases of Den-4.

ELISPOT analysis was performed using the NYA variant peptides on samples obtained during the hospital admission and during convalescence. At day 0, the day the fever remits, IFN- $\gamma$  ELISPOT responses were low or absent, and they peaked at 2-wk follow-up and declined once more at 3- to 5-mo follow-up (Fig. 2). In some cases, the responses were quite focused, particularly to Den-1 and 2.1 (Fig. 2a), whereas in others, the response was more broadly cross-reactive with the Den-3 (Fig. 2b). Interestingly, although the

Table II. List of patients on A24-NYA study

| Patient | Severity | Serology  | Den   | HLA Class I Type |       |      |
|---------|----------|-----------|-------|------------------|-------|------|
|         |          |           |       | A                | B     | C    |
| K17     | DF       | Primary   | Den-1 | 24/02            | 18/27 | 3/7  |
| K18     | DF       | Secondary | Den-1 | 24/33            | 39/58 | 3/7  |
| K19     | DF       | Secondary | Den-1 | 24/02            | 46/55 | 1/1  |
| K20     | DHF I    | Secondary | Den-2 | 24/02            | 18/46 | 1/7  |
| K21     | DHF I    | Secondary | Den-1 | 24/34            | 15/18 | 4/7  |
| K22     | DHF I    | Secondary | Den-3 | 24/11            | 15/38 | 7/7  |
| K23     | DHF II   | Secondary | Den-2 | 24/02            | 15/46 | 1/12 |
| K24     | DHF II   | Secondary | Den-1 | 24/02            | 13/46 | 1/3  |
| K25     | DHF II   | Secondary | Den-1 | 24/30            | 13/13 | 4/6  |
| K26     | DHF II   | Secondary | Den-1 | 24/33            | 15/58 | 3/8  |
| K27     | DHF II   | Secondary | Den-1 | 24/74            | 15/51 | 7/14 |
| K28     | DHF III  | Secondary | Den-1 | 24/03            | 35/46 | 1/4  |
| K29     | DHF III  | Secondary | Den-2 | 24/11            | 38/46 | 1/7  |

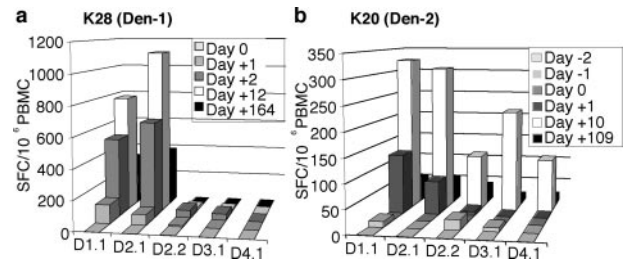


FIGURE 2. T cell responses in two Dengue-infected individuals (a and b). PBMC taken from Den-1- and Den-2-infected patients were tested by IFN- $\gamma$  ELISPOT assay using the variant A24-NYA Dengue epitopes (horizontal axis) at different times across the course of their acute illness and convalescence. Responses are represented as spot-forming cells per million PBMC.

magnitude of responses to the Den-1 and Den-2 variants differed only slightly in these patients, it is noteworthy that the individual infected with Den-1 showed a higher response to Den-2 and vice versa.

#### Measuring NYA-specific T cells using HLA-A\*24/NYA tetrameric complexes

Tetramers loaded with the individual HLA-A\*24-NYA variant peptides Den-1, Den-2, Den-3, and Den-4 described above were constructed. The specificities of these tetramers were determined on the NYA-specific T cell lines and on a number of patient samples. An example of Den-1 staining on a Dengue immune HLA-A\*24-positive individual, Dengue immune HLA-A\*24-negative individual, and a Dengue naive HLA-A\*24-positive individual is shown in Fig. 3a.

Because the pediatric samples are limited in volume, we initially decided to mix all four tetramers and use them together to evaluate NYA-responsive T cells in our cohort of HLA-A\*24-positive children. The frequency of the NYA-specific CD8<sup>+</sup> T cell measured

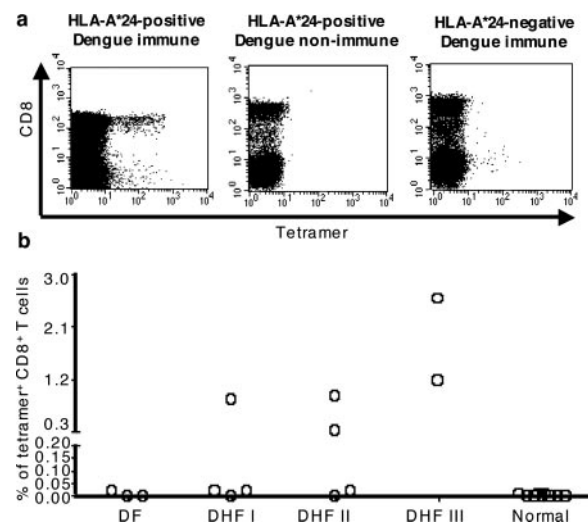


FIGURE 3. Frequency of Dengue NYA-specific CD8<sup>+</sup> T cells. a, Specificity of the A24-NYA tetramer on PBMC taken from a HLA-A\*24-positive Dengue immune individual (left), HLA-A\*24-positive Dengue non-immune individual (middle), and a HLA-A\*24-negative Dengue immune individual (right). b, PBMC were stained with a mixture of tetramers loaded with the NYA variants from all four Dens. The samples were taken at day 14 from Dengue-infected patients having different disease severities and normal healthy Thai control individuals.

by tetramer mirrored those measured by the IFN- $\gamma$  ELISPOT assay, rising from low levels during the acute phase to a peak in the 2-wk follow-up sample (data not shown). The frequency of A24-NYA-specific T cells at 2 wk is shown in Fig. 3*b*. Of the 13 children included in this study, there was no measurable response in 3, a low-level response up to 0.1% circulating CD8<sup>+</sup> T cells in 5, and a higher level response in 5 with a peak frequency of around 2.5%. Because some of the staining was low, we went on to verify the background and specific staining of the tetramer. We first stained 11 control individuals who were Dengue naive or Dengue immune and either HLA-A\*24 positive or negative. In all cases, the staining was between 0 and 0.01% of CD8<sup>+</sup> T cells (data not shown).

Because we were restricted to HLA-A\*24-positive individuals for this analysis, the number of patients included was relatively small. However, there is a clear suggestion that the magnitude of the T cell response follows disease severity; T cell responses were significantly higher in patients who suffered DHF when compared with patients in the DF group (Mann-Whitney *U* test; *p* = 0.049) or normal healthy controls, suggesting a role of the T cell response in disease pathogenesis.

#### Cross-reactivity of the anti-Dengue response in secondary infection

Because most severe Dengue infections occur in individuals who are experiencing a secondary or sequential Dengue infection, it is of interest to determine the viral serotypes to which an individual has been exposed in the past. The virus responsible for the current infection can usually be typed by RT-PCR, and previous infections can sometimes be deduced by the plaque/FRNT. This assay tests the ability of patient serum to neutralize the different Dens using an in vitro infection assay. The results are expressed as the serum dilution, which will achieve a 50% reduction in the plaque/focus count when compared with a nonneutralized control. During a secondary infection any responses previously mounted against the virus will be boosted, a phenomenon referred to as original antigenic sin in the Ab response. The assay can give a highly cross-reactive response where interpretation is difficult. However, we have chosen three cases in which the results are more definitive for further study. Patients K26 and 28 were admitted with Den-1 infection and the FRNT assay implies previous Den-2 exposure, whereas patient K20 was admitted with a Den-2 secondary infection and FRNT implies previous exposure to Den-1 (Table III).

The tetramer analysis described above used a mixture of tetramers loaded with peptides derived from the four viral serotypes to measure the magnitude of the response to all viral serotypes. This analysis does not therefore distinguish between responses to the individual virus serotypes nor does it allow a comparison of the relative avidities, both structural and functional, of the T cells for the different serotype-specific peptides. To further examine the fine specificities of the anti-Dengue T cell response, we went on to perform double tetramer staining assays. In these experiments cells

are stained with two tetramers, loaded with different peptides, which are distinguished from each other because one is conjugated to PE and the other conjugated to QR. Double tetramer staining is thus a competition assay and can be regarded as a measure of relative avidity between the two competing tetramers/peptides.

Representative examples of double tetramer staining using tetramers loaded with Den-1 and Den-2.1 peptides are shown in Fig. 4*a*. These two patients show interesting patterns of reactivity to the two epitopes with populations of cells, which are either fully cross-reactive or which show greater avidity for either of the individual tetramers. Patient K26 was admitted to hospital with a current Den-1 infection, and FRNT suggests that the primary infection was with the Den-2 serotype. It can be seen in this patient that the 68% of T cells are fully cross-reactive between Den-1 and Den-2, there is a substantial population (21%) that reacts preferentially with the previously encountered virus Den-2, and a smaller population (11%) that reacts specifically with the currently infecting virus Den-1. In this case of the total NYA-responsive CD8<sup>+</sup> T cells, 89% are capable of responding to Den-2 the virus encountered on primary infection. This phenomenon whereby a response to a variant epitope is composed predominantly of cross-reactive cells generated to a previously encountered Ag is termed original antigenic sin (18).

#### Functional capacities of specific and cross-reactive T cells

Because original antigenic sin calls upon memory to produce a response to a variant epitope, it is likely that the response can be mounted more quickly, because the precursor frequency of memory cells likely out number naive T cells. However, this advantage can be offset; because memory T cells have a lower threshold for activation, there is the risk that a number of the responding memory clones may have lower avidities for the currently infecting virus, as we have shown above, and may therefore show suboptimal function compared with a response that would have been generated de novo (19, 20).

To examine the functional characteristics of the Dengue-specific T cell response, we designed an assay that involves double tetramer staining and also measures degranulation and cytokine production. PBMC are first stained with two tetramers and then incubated with the Den-1 peptide. To assay for degranulation, an Ab to CD107a is included in the culture. CD107a/Lamp-1 is expressed on the internal surface of cytotoxic granules and upon fusion with the cell membrane/degranulation, this is then exposed at the cell surface and can bind the Ab. CD107a staining is thus an indirect measure of CTL degranulation (21). At the end of the 6-h culture, cells are fixed, permeabilized, and intracellular cytokine staining for IFN- $\gamma$  and TNF- $\alpha$  is performed. CD8, two tetramers, CD107a, IFN- $\gamma$ , and TNF- $\alpha$  can then be read by 6-color FACS analysis. As a control for degranulation and cytokine production and for comparison with the Dengue responses, we performed a similar analysis on a HLA-A\*02-restricted CMV response (NLVPMVATV), although in this case only single tetramer staining is used.

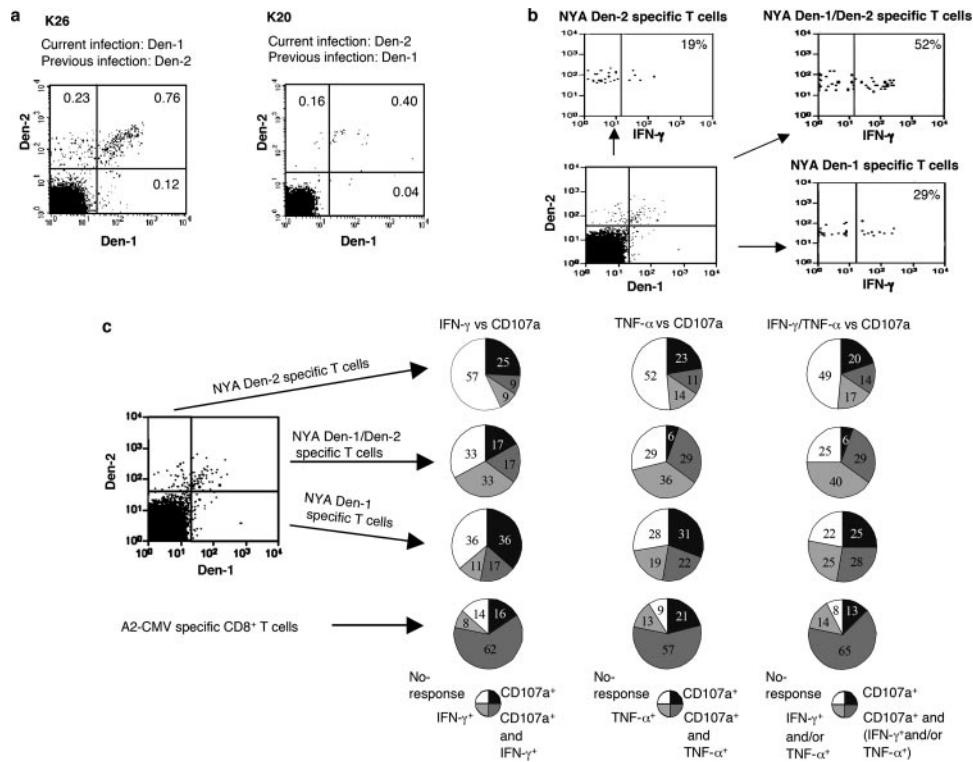
For Dengue, following stimulation with the Den-1 peptide, FACS gates can be set on the cross-reactive (Fig. 4*b*, top right) and monospecific tetramer staining populations (Den-2, top left, and Den-1, bottom right; Fig. 4*b*) to examine the functions of these subsets of T cells. An example of this staining is shown in Fig. 4*b*, where cells have been stimulated with the Den-1 peptide and the IFN- $\gamma$  responses in the different tetramer staining populations are shown.

To reduce the complexity of these multidimensional data, we have broken them down into a series of pair-wise comparisons (Fig. 4*c*). In column 1, we have compared degranulation and IFN- $\gamma$  production in cells that perform either of these functions; in column 2, the same analysis is produced for TNF- $\alpha$  and degranulation; and finally, in column 3, a comparison of CD107a vs cytokine

Table III. FRNT<sup>a</sup>

| Patient | Serotype of Current Infection | Fever Day | FRNT Titre   |               |       |       |
|---------|-------------------------------|-----------|--------------|---------------|-------|-------|
|         |                               |           | Den-1        | Den-2         | Den-3 | Den-4 |
| K20     | Den-2                         | -2        | 2338         | <b>4216</b>   | 984   | 206   |
|         |                               | 99        | <b>40558</b> | 25822         | 4400  | 3876  |
| K26     | Den-1                         | -2        | 980          | <b>13240</b>  | 528   | 112   |
|         |                               | 86        | 13482        | <b>110122</b> | 6084  | 1128  |
| K28     | Den-1                         | 5         | 5252         | <b>56650</b>  | 902   | 2202  |
|         |                               | 164       | 2984         | <b>18976</b>  | 494   | 516   |

<sup>a</sup> The highest FRNT response in each assay is shown in bold.



**FIGURE 4.** Structural and functional avidity of NYA-specific T cells. *a*, K26 and K20 were double stained with a pair of fluorochrome-conjugated NYA A\*24 tetramers (QR-conjugated NYA-Den-1 and PE-conjugated NYA-Den-2). Plots show double tetramer profiles on CD8<sup>+</sup> T cells. The numbers in each quadrant represent the percentage of tetramer<sup>+</sup>CD8<sup>+</sup> T cells within each quadrant. *b*, PBMC from Dengue-infected patient K26 were stained as in (*a*), stimulated with 10  $\mu$ M NYA Den-1 peptide, and then stained with the following: CD107a (a surrogate maker of degranulation), IFN- $\gamma$ , and TNF- $\alpha$ , followed by analysis on a six-color FACS. The *lower left* plot shows a double tetramer profile on CD8<sup>+</sup> T cells. The other three plots show IFN- $\gamma$ <sup>+</sup> staining on the three gated NYA-specific T cell populations. *c*, Analysis of cytokine and CD107a staining in the indicated populations of cells gated from the double tetramer staining. The numbers in the pie charts represent the percentage of cells in each gate that have the functional characteristics represented on the key at the bottom. In the first column, IFN- $\gamma$  is compared with CD107a, in the second TNF- $\alpha$  with CD107a, and in the third, the production of either cytokine IFN- $\gamma$  or TNF- $\alpha$  is compared with CD107a. T cell responses against the A2-CMV epitope, NLVPMVATV, are shown in the *lower row*.

production as a whole is presented (either IFN- $\gamma$  or TNF- $\alpha$  or both).

This analysis is on the patient described above who suffered a primary Den-2 infection and was then secondarily infected with Den-1. Interestingly, only 51% of the cells of low avidity for the Den-1 epitope show any response to stimulation with the currently infecting Den-1 epitope, but these cells showed a greater response to Den-2 with 93% showing either degranulation or cytokine production (data not shown). In contrast, 75% of the Den-1/Den-2 cross-reactive T cells and 78% of the Den-1-monospecific cells react to the Den-1 epitope.

We suggest that the cross-reactive T cells have been generated from memory cells to the original Den-2 infection, whereas the Den-1-monospecific cells have been newly generated by the current Den-1 infection. Interestingly, when the nonresponsive cells are discarded, 53% of the Den-1/Den-2 cross-reactive T cells make a response consisting wholly of cytokine production with no degranulation. There is a reciprocal increase in cells that show only degranulation (8 vs 32%) for the Den-1/Den-2 cross-reactive vs Den-1-specific T cells. In these regards, the Den-1-specific T cells resemble CMV-specific T cells, where function is most often manifest by a combination of cytokine production and degranulation and only 15% of cells show cytokine production without degranulation.

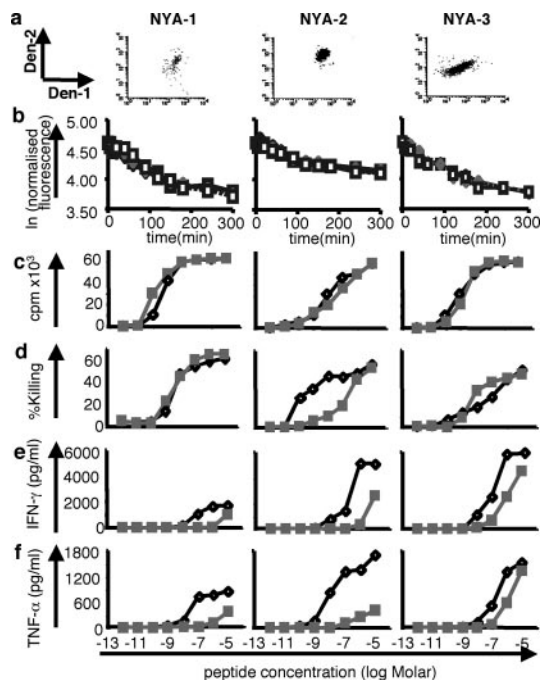
#### Differential T cell programs induced by restimulation

Because the most severe symptoms in Dengue infection are seen at the time of virus control, we and others have suggested that the

severe sequelae, particularly the profound vascular leak leading to shock may be the result of the immune response to the virus rather than virus-mediated cytopathogenicity acting alone, i.e., DHF is the result of immunopathology.

Skewing of a T cell response toward cytokine production and away from direct cytotoxicity/degranulation, as we have mentioned above, may contribute to the damage in secondary Dengue infection. With this in mind, we were interested to determine how Dengue-specific T cells behaved in other aspects of their program to T cell activation, so we generated some A2-NYA-specific T cell clones. Three clones (NYA-1, NYA-2, and NYA-3) were assayed for killing, cytokine production, and proliferation.

All three clones were generated from secondarily infected individuals, and double tetramer analysis showed that they were equally cross-reactive between the Den-1 and Den-2 peptides (Fig. 5*a*). To confirm the finding, we went on to perform tetramer dissociation assays to measure their structural avidities. In this assay, cells were stained with saturating concentrations of PE-labeled tetramer, and cells were then washed and incubated with a high concentration of unlabeled tetramer and tetramer dissociation measured by repeat FACS analysis of aliquots of cells over a time course. This analysis was either performed with PE-labeled Den-1 completed off with unlabeled Den-2 or Den-1 or labeled Den-2 and unlabeled Den-1 or Den-2. For all three clones, the results were similar, i.e., the rates of dissociation of the Den-1 or Den-2 tetramer were similar within a given clone as predicted by the initial double tetramer (Fig. 5*b*). Interestingly, however, although there were apparent similarities in structural avidity as evidenced by



**FIGURE 5.** Phenotype and functional characteristics of three NYA-specific T cell clones. *a*, Three NYA-specific T cell clones were stained with two tetramers and mAb against CD8. *b*, Estimation of the dissociation kinetics of the interaction between the A24-NYA tetramers and the NYA-specific T cell clones. T cell clones were stained with PE-labeled A24-NYA Den-1 tetramer and incubated with 100-fold of unlabeled A24-NYA Den-1 tetramer ( $\diamond$ ) or A24-NYA Den-2 tetramer ( $\blacklozenge$ ). T cell clones were also stained with PE-labeled A24-NYA Den-2 tetramer and incubated with 100-fold of unlabeled A24-NYA Den-1 tetramer ( $\square$ ) or A24-NYA Den-2 tetramer ( $\blacksquare$ ). Cells were analyzed at appropriate time points. The logarithm of the normalized total fluorescence was plotted against time to derive the dissociation curve. The functional T cell clones were assayed following stimulation with the Den-1 ( $\diamond$ ) or Den-2 ( $\blacksquare$ ) peptides for proliferation ( $^3\text{H}$ ]thymidine incorporation) (*c*), cytotoxicity (4-h  $^{51}\text{Cr}$  release) (*d*), or cytokine production IFN- $\gamma$  (*e*) and TNF- $\alpha$  (*f*). Results are representative of at least three independent experiments.

double tetramer staining, there were some clear and reproducible examples in which the clones showed differences in their responses to the two peptides. The proliferation curves for the three clones, although showing different thresholds, did not distinguish between the Den-1 and Den-2 peptides (Fig. 5*c*). Killing was different, with clones NYA-1 and NYA-3 reacting similarly between the two peptides while clone NYA-2 reproducibly showed a 3–4 log difference in its ability to kill targets pulsed with Den-1 vs Den-2 (Fig. 5*d*). Finally, the T cell clones were stimulated with Den-1 or Den-2 peptides and cytokine production; IFN- $\gamma$  and TNF- $\alpha$  in the supernatant were measured (Fig. 5, *e* and *f*). The cytokine production from clone NYA-2 mirrors the results of the killing assay, i.e., it shows a considerably more sensitive response to the Den-1 peptide contrasting with the equal responses measure by proliferation. Another clear difference in T cell responses to stimulation is seen when the NYA-1 and NYA-3 clones are compared; although clone NYA-1 is more sensitive in the killing assay, the production of cytokines is reduced when compared with clone NYA-3.

## Discussion

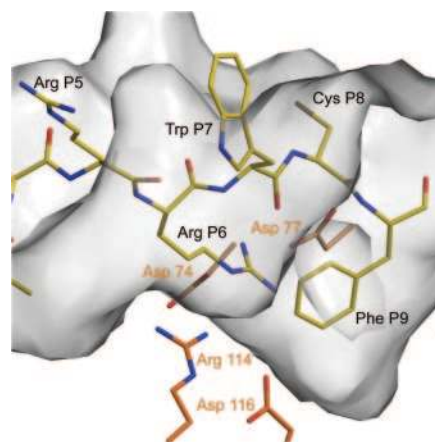
Several T cell epitopes have been identified in Dengue over the last few years. Most of these have been identified in Caucasian vaccines or have been restricted by relatively low frequency oriental HLA alleles, which have limited the power of cross-sectional anal-

yses in endemic countries. In this study, we have reported an epitope restricted by HLA-A\*24 from the NS3 protein of Dengue. Previous studies particularly on CD4 $^+$  T cell responses in Dengue have frequently identified responses to NS3, and although this screen was by its nature biased toward NS3, it is interesting that we have found relatively low-level T cell reactivity to envelope despite the fact that it is 501 aa in length compared with 623 for NS3 (data not shown). The reason for the relative immunodominance of NS3, which is proposed to function as a protease and helicase, is not clear. The genomic RNA of Dengue encodes a polyprotein, and it might therefore be expected that equimolar amounts of the additional 80% of Dengue would be expressed and be equally represented in the T cell response.

While defining the A24-NYA epitope, we were surprised and concerned to find that some individuals responding to this epitope were HLA-A\*24 negative. Inspection of the HLA type of these individuals suggested that the response may be restricted through HLA-A\*11, in addition to HLA-A\*24, which we went on to prove using HLA-A\*11-transfected .221 cells. The response to HLA-A\*11 and HLA-A\*24 were not mutually exclusive because we found both responses in early passage of a T cell line derived from a HLA-A\*11/A\*24 heterozygote, but we found no evidence that a single T cell clone could cross-react between the two MHC's.

The cross-reactivity between HLA-A\*11 and HLA-A\*24 was surprising because these HLA molecules belong to different MHC class I supertypes, and to our knowledge, no peptide cross-reactivity between these two HLAs has been previously reported. The HLA-A\*11 peptide binding consensus differs considerably from the HLA-A\*24 consensus, the former having a lysine at the C terminus, whereas HLA-A\*24 characteristically has aromatic side chains at p2 (F/Y) and an aromatic/hydrophobic (F/W/I/L) residue at the C terminus.

To determine whether and how the NYA peptide would fit into HLA-A\*11, in particular the C terminus of the peptide into the F pocket, a molecular model was created (Fig. 6). This shows that with some repositioning of peptide-MHC charge interactions, the NYA peptide can indeed fit comfortably into HLA-A\*11. The crystal structure of HLA-A\*11 complexed with a nonamer HIV reverse transcriptase peptide (PDB entry 1Q94) was used for this model and shows that the peptide side chains at p2, p6, and p9 can contribute three anchor residues buried within the B, C, and F pockets of the HLA-A\*11 peptide binding groove (22). The B



**FIGURE 6.** Molecular modeling of the HLA-A\*11/NYA complex. A close-up of the interaction between the peptide: Arg p5 to Phe p9 (yellow) and the peptide-binding groove showing pockets C and F (gray). The side chains of the HLA-A\*11 H chain are shown in orange. Red and blue represent negative and positive charges, respectively.



pocket provides a hydrophobic environment to accommodate the A24-consensus p2 tyrosine side chain of the NYA peptides. The F pocket is also large enough for the p9 phenylalanine side chain to be buried comfortably. However, the F pocket has a triad of negative charge contributed by aspartic acid at positions 74, 77, and 116, which provide an electrostatic environment that favors the characteristic C-terminal lysine anchor found in HLA-A\*11 binding peptides. The model suggests that to accommodate the p9 phenylalanine from the NYA peptide, aspartic acid 116 may be stabilized via electrostatic interactions with arginine 114. At the same time, the two other negatively charged side chains of aspartic acid 74 and 77 may be repositioned to be neutralized by interaction with the p6 arginine side chain of the NYA peptide buried within C pocket.

One of the key observations made in Dengue over the last two decades is that the most of the severe disease leading to DHF and Dengue shock syndrome is seen in secondarily infected individuals (3, 4). Some of the best such data comes from observations of island populations, where epidemics can occur through the introduction of a single virus serotype. Such an event occurred in Cuba in 1977 when there was an epidemic caused by Den-1, which was introduced to a population that had previously been protected by an effective mosquito control program and was therefore largely Dengue naive (23). DHF was rare in this epidemic, which petered out over the next few years. It was followed in 1981 by an epidemic of Den-2, and on this occasion there were >10,000 cases of DHF representing ~3% of cases and >150 deaths.

In our cohort of 90 patients, 8 were shown by serology to be suffering a primary infection and the rest were reinfections. A number of years ago, Halstead et al. (6) suggested that Ab enhancement could explain the increase in severity of disease seen upon reinfection with Dengue. It is proposed that, following a primary infection, Ab generated to the primary infecting virus may have suboptimal neutralization capabilities against a second viral serotype, whose envelope protein will show considerable differences in sequence. Rather than neutralize the second virus, this partially cross-reactive serum may instead opsonise the virus and potentiate its uptake via FcR-mediated endocytosis into macrophages, a primary site of virus replication. Ab enhancement can be readily demonstrated *in vitro* (although it often requires several logs of serum dilution) and has also been shown in a primate model (6, 7). Furthermore, Ab-dependent enhancement has also been put forward to explain an excess of DHF in children in the first year of life, who suffer a primary Dengue infection that is proposed to be accentuated by enhancement by passively transferred maternal anti-Dengue Ig (24).

Ab enhancement may well lead to higher viral loads on reinfection, and it has been demonstrated that there is a correlation between the peak viral load and disease severity (25). However, it is now very well established that when the clinical syndrome reaches its peak with shock and hemorrhage, the viral load has fallen precipitously and indeed the virus may be undetectable at this point (26). The coincidence of the severe symptoms with viral control and a storm of proinflammatory cytokine release suggest that the defect in vascular permeability leading to the profound vascular leak may be the result of immune-mediated damage or immunopathology rather than being solely the result of virus-mediated cytopathogenesis. One major source of inflammatory cytokines such as TNF- $\alpha$ , IFN- $\gamma$ , etc. are T cells, meaning that severe disease may be the result of T cell-mediated tissue damage. In addition to the secretion of proinflammatory mediators, cytotoxic CD8<sup>+</sup> T cells may also contribute to the vascular leak by directly lysing infected endothelial cells.

However, one question that is raised is why should the T cell response be more damaging the second time round than it is during the primary infection? First, like Ab responses, boosting or reinfection will lead to a higher amplitude response that is usually generated faster (19). There are a number of animal models of T cell immunopathology in which both CD8<sup>+</sup> and CD4<sup>+</sup> T cells can cause damage when they are produced at high frequencies in the context of a high Ag load, and indeed immunopathology has been recently suggested to play a role in severe acute respiratory syndrome (27). These include models of LCMV immunopathology and also RSV (8, 9). The latter are particularly pertinent in the context of Dengue because they illustrate the potential problems that can be encountered with poorly protective vaccines. In the 1960s, a formalin-inactivated RSV vaccine was tested in children; rather than protecting from infection, this vaccine actually enhanced lung disease when the children became naturally infected (28).

In secondary Dengue infection, there is a further important feature that may contribute to disease; original antigenic sin. Because the viral variants differ by 25–30% in amino acid sequence, most linear 8- to 10-aa<sup>-</sup> long peptide epitopes will contain one or more differences in amino sequence between variants (chance of identical 9-mer assuming 25% variation  $(0.75)^9 = 0.075$ ). Upon secondary encounter with a variant Ag, the T cell response will frequently select from memory a set of T cells that are capable of recognizing the new epitope rather than selecting a novel response from the naive T cell pool. The reason for this is probably that the frequency of memory cells outnumbers naive cells, and, in addition, memory cells have a lower threshold for stimulation than naive cells (19).

This latter feature may prove to be detrimental because there is a danger that a set of T cells with a lower avidity for the currently infecting virus may be expanded in addition to high-avidity cross-reactive cells, and it is also possible that the generation of this population in some way interferes with the kinetics of the response, both delaying the generation of a more avid response from the naive pool and also the generation of truly cross-reactive high-avidity T cells. In this study, we have demonstrated that a cross-reactive response to the A24-NYA epitope is generated and that the response is made up of three populations of cells: those that show preferential avidity for the primarily encountered virus; a population that is cross-reactive between the primary and secondary viruses (both of these populations have probably been generated by original antigenic sin because they show equal or greater avidity for the virus encountered on the primary occasion); and a third group of cells (which show preference for the secondary infecting virus), which we propose have been newly generated during the secondary response from naive T cells.

When we examined the function of these T cell populations, we did indeed find that nearly 50% of the cells with low avidity for the secondarily infecting virus showed no reactivity to stimulation with this epitope. We believe that these nonresponding T cells are not merely bystanders because they have clearly undergone proliferation during the secondary response. One very interesting feature of the cells produced in the secondary response is differences in their ability to produce inflammatory cytokines and to degranulate. A higher proportion of the cross-reactive T cells show cytokine production but do not degranulate when compared with the cells that show single specificity for the secondary infecting virus. These latter cells more resemble the anti-CMV response, where few cells produce cytokines in the absence of degranulation. We propose that the generation of a response skewed toward cytokine production in the absence of degranulation may be detrimental in Dengue infection, meaning that when the virus is finally controlled

it occurs in the context of an expanded T cell response and a cytokine storm.

It is likely that the pathogenesis of DHF is multifactorial, being driven by a variety of host and viral factors. We believe that two of the key drivers are the Ag load and the amplitude and quality of the T cell response. Any factor that leads to the development of a high Ag load concurrent with a high amplitude T cell response may contribute. In this study, we have discussed original antigenic sin in the context of the T cell response but it is perhaps better known for its effects on the Ab response. The effects of original antigenic sin in the Ab response are dramatic and can be seen in the viral neutralization tests that we have shown for the patients analyzed in these studies. Whether original antigenic sin of the Ab response also contributes to disease by steering the secondary response toward low-avidity Ab should also be considered, because this will also be a potential contributor toward a higher Ag load.

Finally, we have discussed cross-reactive T cells in the context of responses to a single pathogen, Dengue. In a series of highly interesting papers, Welsh and colleagues (29, 30) have looked at the potential for cross-reaction in T cell responses between viruses in mouse model. Several examples of cross-reactivity, which in some cases modulate the pattern of disease, have been described between viruses such as LCMV, Pichinde, and vaccinia (29, 30). This leads to the possibility that our response to pathogen challenge may be programmed in part by our previous exposure to diverse infectious agents. To make the situation even more complicated, it seems that in inbred mouse strains there is an idiosyncratic or private specificity to the cross-reactive response (31).

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## Disclosures

The authors have no financial conflict of interest.

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