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Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever

Juthathip Mongkolsapaya[1,2,7], Wanwisa Dejnirattisai[2,7], Xiao-ning Xu[1], Sirijitt Vasanawathana[3], Nattaya Tangthawornchaikul[4], Aroonrung Chairunsri[2], Siraporn Sawasdivorn[6], Thaneeya Duangchinda[1], Tao Dong[1], Sarah Rowland-Jones[1,5], Pa-thai Yenchitsomanus[2], Andrew McMichael[1], Prida Malasit[2,4] & Gavin Screaton[1,5]

Dengue virus presents a growing threat to public health in the developing world. Four major serotypes of dengue virus have been characterized, and epidemiological evidence shows that dengue hemorrhagic fever (DHF), the more serious manifestation of the disease, occurs more frequently upon reinfection with a second serotype. We have studied dengue virus–specific T-cell responses in Thai children. During acute infection, few dengue-responsive CD8$^+$ T cells were recovered; most of those present showed an activated phenotype and were undergoing programmed cell death. Many dengue-specific T cells were of low affinity for the infecting virus and showed higher affinity for other, probably previously encountered strains. Profound T-cell activation and death may contribute to the systemic disturbances leading to DHF, and original antigenic sin in the T-cell responses may suppress or delay viral elimination, leading to higher viral loads and increased immunopathology.

Dengue is an arthropod-borne flavivirus that can be subdivided into four major serotypes (Den-1, Den-2, Den-3 and Den-4). Symptoms begin 5–7 d after a bite from an infected mosquito. Patients develop a high fever for 2–7 d, which corresponds to a period of high viremia[1]. When the fever remits on the defervescent day, defined as day 0, patients can get worse and in severe cases can develop shock. After defervescence the viral load falls to undetectable levels by 48 h, at which time the patient will generally begin to improve. The majority of infections are asymptomatic or cause a self-limiting febrile illness known as dengue fever. The more severe form of dengue infection, DHF, is characterized by high fever, hemorrhagic phenomena and plasma leakage, and may be life threatening. DHF is classified into four grades, the more severe of which (grades III and IV) are associated with dengue shock syndrome[2]. There are hundreds of thousands of cases of DHF each year, and without appropriate treatment mortality can be over 20%. With hospitalization and supportive care, mainly careful fluid management, this can be reduced below 1%.

The pathogenesis of DHF is not well characterized, but key epidemiological studies indicate that it often occurs when a dengue-immune person becomes secondarily infected with a virus of a different serotype[3,4], although severe disease on primary infection has been reported, particularly in neonates[5]. Halstead proposed a model of antibody-dependent enhancement whereby, upon secondary infection, pre-existing non-neutralizing antibodies may opsonize the virus and enhance its uptake and replication in macrophages. This has been shown to lead to higher viral loads both *in vitro* and in an *in vivo* primate model[6,7]. As was pointed out by the author of the *in vivo* study, however, an important challenge is to establish a connection between an infection of mononuclear cells and pathophysiologic changes[6].

To this end, others have suggested that the disease may be caused by T-cell activation; the levels of several cytokines such as tumor necrosis factor-α, as well as the magnitude of T-cell responses, have been correlated with disease severity[8,9]. So far, only a few peptide epitopes have been isolated for CD8$^+$ cytotoxic T lymphocytes (CTLs). The latter have often been from Western volunteers who do not possess the common Asian human leukocyte antigen (HLA) types. To date, no well-defined epitopes have been defined for HLA-A*11, which is found in around 30% of the population in southeast Asia, or for the other common Asian HLA alleles A*24 (20%) or A*33 (15%)[10]. The lack of understanding of such epitopes has meant that dengue virus–specific CD8$^+$ T-cell responses have not been studied over the time course of acute dengue.

RESULTS

Identification of a new HLA-A*11–restricted T-cell epitope

Because of the relatively small volume of blood samples available from acutely infected children, we decided to search for dengue CTL epitopes in healthy Thai adults, the majority of whom have been infected with dengue on one or more occasions. Previous studies have

[1]MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, OX3 9DS, UK. [2]Medical Molecular Biology Unit, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok noi, Bangkok 10700, Thailand. [3]Pediatric Department, Khon Kaen Hospital, Ministry of Public Health, Khonkaen 40000, Thailand. [4]Medical Biotechnology Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA) Patumthani 12120, Thailand. [5]Nuffield Department of Medicine, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, UK. [6]Queen Sirikit National Institute of Child Health, Bangkok 10400, Thailand. [7]These authors contributed equally to this work. Correspondence should be addressed to G.S. (screaton@molbiol.ox.ac.uk) or P.M. (sipml@mahidol.ac.th).

suggested that the envelope and NS3 proteins are frequent targets of immune responses in flavivirus infections, including dengue[11,12]. Peripheral blood mononuclear cells (PBMCs) from 20 healthy donors were screened with a panel of 210 overlapping peptides (15-mers overlapping by ten amino acids) spanning the envelope and NS3 proteins of dengue serotype 2 strain 16681. Pools of peptides were screened against PBMCs using an interferon (IFN)-γ Elispot assay. We found a T-cell response to peptide FSPGTSGSPIIDKKG (FSP), which covers NS3 residues 130–144, in two of the donors. Using a set of truncated peptides, the highest responses were found to the 11-mer peptide GTSGSPIIDKK (11GTS), although there were also responses to the 10-mer peptide GTSGSPIIDK (10GTS), truncated by one amino acid at the C terminus (**Table 1a**). Both of the donors expressed HLA-A*11 and the epitope conformed to the A11 consensus, which predicts peptides with a positively charged C-terminal anchor, frequently lysine, that are 10 or 11 residues in length. An FSP-specific CTL line was generated from one of the donors by stimulation with the FSP 15-mer peptide. This line was used to confirm the HLA restriction (**Fig. 1a**).

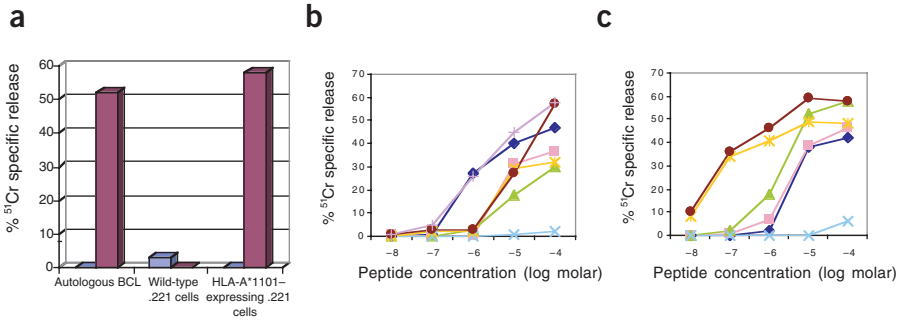

Figure 1 Defining a new HLA-A*11–restricted T-cell epitope. (**a**) HLA-A*1101 restriction of the GTSGSPIIDKK-specific response shown by a 4-h chromium-release assay using a GTS-specific CTL line from donor 1 (effector/target ratio = 10:1). Targets were an EBV-transformed autologous B-cell line (BCL), wild-type .221 cells and HLA-A*1101–expressing .221 cells pulsed with GTSGSPIIDKK (D2.1; ■) or no peptide (■). (**b**,**c**) The FSP-specific CTL line was cocultured with a ^{51}Cr-labeled B-cell line (E/T ratio = 20:1) pulsed with 11-mers (**b**; D1.1 (✳), D2.1 (◆), D2.2 (■), D2.3 (▲), D2.4 ( ) D3.1 (●) and D4.1(+)) and 10-mers (**c**; D1.1 (✳), D2.1 (◆), D2.2 (■), D2.3 (▲), D2.4 ( ) or D3.1/D4.1(●)).

Variants of the HLA-A*11–restricted GTS epitope

In general there is a 30–40% difference in amino acid sequences between dengue serotypes, so we searched for variants of the GTS epitope among the published dengue NS3 sequences. Six variants were identified: three from Den-2 (D2.2, D2.3 and D2.4) and one each from the other serotypes (D1.1, D3.1 and D4.1; **Table 1b**). All of these variants were synthesized as 10- and 11-mers and tested on the FSP-specific T-cell line that had been generated by stimulation with the D2.1 epitope (**Fig. 1b,c**). All variants, apart from D2.4, stimulated T-cell responses at high concentrations, but there were marked differences in the titration curves, where the lower concentrations of peptide were probably more representative of the concentrations achieved *in vivo*. D2.4, which did not stimulate the cell line, has a relatively conservative alanine substitution at position 8. D1.1, D2.3 and D3.1 worked better as 10-mers than 11-mers (extra C-terminal amino acid), suggesting they were using the arginine at position 10 as the C-terminal anchor.

Different patterns of response during acute infection

Next, we studied responses in hospitalized children suffering an acute or current dengue virus infection. Samples were collected during the acute hospitalization phase and at the 2-week and 2-month follow-ups. Acute dengue infection was diagnosed by detection of the dengue viral genome from acute sera by RT-PCR or serological testing[13–16]. All but 2 of 73 patients were shown by serology to be suffering a secondary or sequential infection. The 19 patients expressing HLA-A*11 were included in this study; all had secondary infections, and 12 were infected with Den-1, 6 had Den-2 and 1 had Den-3 (**Table 1c**). The disease severity was classified according to World Health Organization guidelines[17].

PBMCs were screened with all dengue variant epitopes by IFN-γ Elispot assays. Unexpectedly, we found low or absent Elispot responses in samples taken during acute hospital admission. In contrast, all the follow-up samples taken at 2 weeks and 2 months gave responses (**Fig. 2a,b** and **Supplementary Fig. 1** online). A similar pattern is seen in hepatitis C virus infection: antigen-specific T cells, although relatively prevalent, do not produce IFN-γ during an acute infection and the response returns 10 weeks later, after viral clearance[18]. Similarly, antigen-specific CD8$^+$ T cells in mice exposed to lymphocytic choriomeningitis virus (LCMV) proliferate rapidly at first and produce IFN-γ, but the cells later become 'stunned' and cease producing IFN-γ. Production of this cytokine is eventually regained, however[19,20].

The magnitude, timing, pattern of response and peptide titration curves to the variant peptides varied between patients. These results suggest that there is some cross-reactivity between the different epitopes but that the cross-reactivity is only partial. To gain further insight into this cross-reactivity, we did peptide titrations of fresh *ex vivo* PBMCs with the variant peptides. As expected, there were differences in the peptide titration curves, suggesting differences in functional avidity in dengue-specific T cells (**Fig. 2c,d** and **Supplementary Fig. 2** online).

Using MHC class I tetramers to study the acute response

To study dengue-specific responses in more detail, we made class I major histocompatibility complex (MHC) peptide tetrameric complexes containing the following dengue variant peptide sequences: D2.2 10-mer, D1.1 11-mer and D3.1 10-mer (10-mers derived from D3.1 and D4.1 sequences are the same). FACS analysis showed that all tetramers stained the FSP CTL line (data not shown). Based on this and the Elispot results, the D3.1 10-mer was selected for further studies. Its specificity was confirmed by staining PBMCs taken from individuals that were HLA-A*11–positive and dengue-immune, HLA-A*11–positive and dengue-nonimmune, or HLA-A*11–negative and dengue-immune (**Fig. 3a**)

Acute and follow-up PBMCs from dengue-infected patients were then stained with the tetramers. The average of the relative frequencies of GTS-specific CD8$^+$ T cells is shown in **Figure 3b**. The responses peaked at 2 weeks and mirrored the results of the IFN-γ Elispot assay. Unlike the Elispot results, however, almost all of the patients had low but detectable levels (0.02–2.5%) of GTS-specific CD8$^+$ T cells during the acute phase. When we compared disease severity to the magnitude of the GTS-specific CD8$^+$ T-cell response at 14 d, we found that dengue-infected patients had higher circulating GTS-specific CD8$^+$ T cells than normal dengue-immune individuals (**Fig. 3c**). The three patients with dengue shock syndrome (DHF grade III) all had peak responses greater than 2% (mean ± s.d. was 4.26 ± 2.03; $n = 3$) com-

pared with patients with DHF grades I or II (mean ± s.d. was 0.84 ± 0.82; $n = 11$).

During the acute phase, the GTS-specific T cells showed an activated effector phenotype, with almost all of them expressing CD45RO, CD27, CD38 and HLA-DR, but not CCR7 (**Supplementary Fig. 3** online). They also stained with Ki67, indicating that the cells were proliferating. As expected, the activation markers were reduced in the follow-up samples, which also showed a reduction in CD28 and some re-expression of CD45RA (**Supplementary Fig. 3** online).

Massive apoptosis in dengue T cells

We were interested to determine why, in the early stages of the illness, the frequencies of antigen-specific T cells were so low in contrast to other virus infections such as HIV or Epstein-Barr virus[21]. Three possibilities exist: the cells may not have had time to proliferate fully, they may have been sequestered in peripheral tissues or lymphoid organs, or they may have been dying at an increased rate. PBMCs taken from eight patients were stained with tetramer followed by TUNEL (a marker for DNA fragmentation). During acute infection, the majority of GTS-specific T cells were TUNEL-positive (**Fig. 4b**). In contrast, fewer than 20% of tetramer-negative $CD8^+$ T cells were apoptotic. In the follow-up samples, a minority of $CD8^+$ T cells, both tetramer-positive and tetramer-negative, were positive for TUNEL staining. Costaining experiments showed that the GTS-specific T cells expressed little or no tumor necrosis factor receptor-1, death receptor-4 or death receptor-5 (data not shown). However, most of the GTS-specific T cells expressed the death receptor Fas and had low levels of the antiapoptotic protein Bcl-2 (**Fig. 4c**). It is usually difficult to detect circulating apoptotic cells, as they are very rapidly removed from the circulation. It is possible that there is a deficiency in the clearance of apoptotic cells in acute dengue virus infection.

The very high percentage of apoptotic cells detected here, combined with the expression of the proliferation marker Ki67 by 75–100% of cells (**Fig. 4c** and **Supplementary Fig. 3** online), suggests that there is huge proliferation balanced by massive apoptosis in the acute setting. Apoptosis is reduced upon viral clearance, allowing a rebound in CTL numbers at 2 weeks, which reduces by 2 months as memory is established.

Table 1 Dengue NS3 peptides and patient details

a

Peptide sequence	SFCs per million PBMCs
FSPGTSGSPIIDKKG	92
GTSGSPIID	12
TSGSPIIDK	0
GTSGSPIIDKK	148
TSGSPIIDKK	4
PGTSGSPIIDK	0
GTSGSPIIDK	84

b

Variant	Dengue serotype	Sequence	Number of sequences found[a] per total[b]
D2.1	2	GTSGSPIIDKK	29/39
D2.2	2	GTSGSPI**V**D**R**K	6/39
D2.3	2	GTSGSPI**V**DKK	3/39
D2.4	2	GTSGSPI**A**DKK	1/39
D1.1	1	GTSGSPI**VNRE**	5/5
D3.1	3	GTSGSPII**NRE**	2/2
D4.1	4	GTSGSPII**NR**K	2/2

c

Patient	Diagnosis	Serology	Dengue serotype	HLA class I type		
				A	B	C
K05	Dengue fever (DF)	Secondary	Den-1	11/33	44/58	3/7
K14	DF	Secondary	Den-1	11/24	13/40	3/7
K17	DF	Secondary	Den-1	11/02	46/–	1/1
K21	DF	Secondary	Den-1	11/30	15/35	4/4
K237	DF	Secondary	Den-1	11/02	15/55	7/8
K01	DHF grade I	Secondary	Den-1	11/29	7/18	7/15
K19	DHF grade I	Secondary	Den-2	11/02	13/15	4/8
K203	DHF grade I	Secondary	Den-1	11/24	27/40	7/15
K221	DHF grade I	Secondary	Den-1	11/02	27/40	3/7
K239	DHF grade I	Secondary	Den-2	11/02	40/40	3/7
K24	DHF grade II	Secondary	Den-2	11/24	27/51	3/14
K201	DHF grade II	Secondary	Den-1	11/24	15/46	1/8
K206	DHF grade II	Secondary	Den-1	11/68	27/40	8/8
K213	DHF grade II	Secondary	Den-2	11/24	27/40	3/15
K214	DHF grade II	Secondary	Den-3	11/24	46/51	1/14
K234	DHF grade II	Secondary	Den-1	11/24	35/46	1/4
K10	DHF grade III	Secondary	Den-2	11/2	15/15	8/8
K22	DHF grade III	Secondary	Den-1	11/2	27/46	1/12
K27	DHF grade III	Secondary	Den-2	11/2	18/46	1/7

(**a**) Truncated Den-2 peptides were used to define the optimum peptide. SFCs, spot-forming cells. (**b**) The sequence of D2.1 was used to search for variant dengue sequences published in GenBank. [a]Number of sequences found in GenBank. [b]Total number of GenBank-published NS3 sequences for each dengue serotype. (**c**) Summary of dengue-infected patients.

T-cell original antigenic sin during secondary infection

To search for serotype-specific and serotype–cross-reactive responses, we stained samples with pairs of variant tetramers (**Fig. 5a** and **Supplementary Fig. 4** online) Although this double staining assay is more a measure of relative avidity than of absolute specificity, it clearly shows the existence of multiple dengue-specific T-cell populations in a single individual. The 10GTS-D3.1 tetramer was the most cross-reactive, confirming the Elispot data. Staining with either the D1.1 or D2.2 tetramers showed the existence of dual populations with high and low staining.

Particularly relevant is the finding that in secondary infection with Den-1, many of the T cells showed a preference for Den-3 tetramers, and in Den-2 infection, many of the cells reacted better with the Den-1 and Den-3 tetramers. The measurable tetramer response was low in the early acute samples and increased in the later samples, so these cells had clearly been stimulated to proliferate by the currently infecting

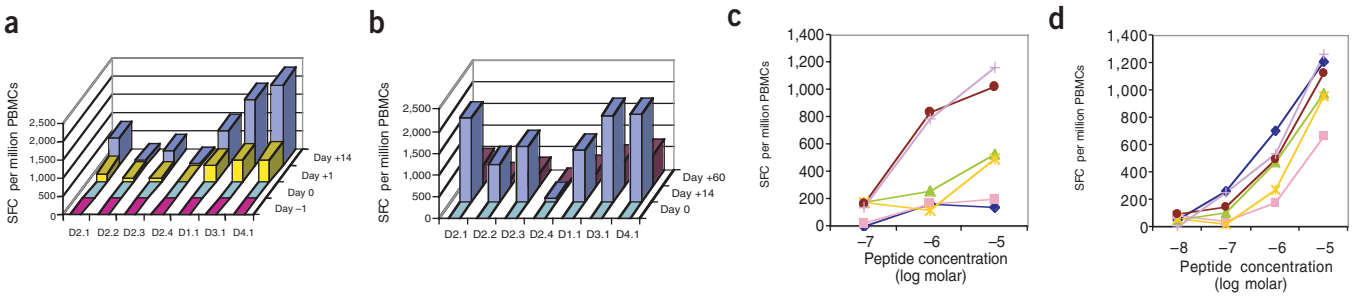

Figure 2 T-cell responses in dengue-infected patients. (**a**,**b**) PBMCs taken from dengue-infected patients K22 (**a**, Den-1 infection) and K10 (**b**, Den-2 infection) were stimulated with seven variant 11GTS epitope peptides (D2.1, D2.2, D2.3, D2.4, D1.1, D3.1 and D4.1). Samples were taken at the indicated time points. (**c**,**d**) PBMCs taken from dengue-infected patients K237 (**c**, Den-1 infection) and K27 (**d**, Den-2 infection) were stimulated with the indicated concentrations of variant 11GTS epitope peptides D1.1 (*), D2.1 (◆), D2.2 (■), D2.3 (▲), D3.1 (●) and D4.1 (+). Responses were measured as spot-forming cells (SFCs) per million PBMCs by IFN-γ Elispot assay.

virus. In other words, a single virus can stimulate the proliferation from memory of a spectrum of clones with different affinities and cross-reactivities. The finding that secondary infection with a virus carrying a similar but distinct epitope can stimulate the proliferation of cross-reacting, low-affinity clones is consistent with the phenomenon of original antigenic sin. We believe this is the first direct demonstration of this phenomenon in T cells for a human pathogen.

It would be interesting to know which viruses were responsible for the infections preceding the acute infection examined in this study. Unfortunately, the current methods to define this are not optimal and in many cases it is impossible to extract more information, above using PCR to identify the currently infecting virus and using serology to establish that the infection is secondary. Original antigenic sin has been reported for dengue antibody responses, and cross-reactivity in virus neutralization tests can sometimes allow deconvolution of a person's previous dengue exposure[22]. In an attempt to gain more information, we did virus serotype neutralization assays in which patient serum is used to inhibit infection by the four dengue serotypes of a monolayer on an LLC-MK2 cell line. The results of this analysis must be interpreted with caution, but they indicate that the patient with Den-1 infection (**Fig. 5a** and **Supplementary Table 1** online) may have been exposed to at least Den-3, and the patient with Den-2 infection may have been exposed to Den-1, Den-3 or both.

Finally, to further show differences in structural avidity between dengue-specific T cells, we performed tetramer dissociation assays. In these assays, cells were incubated with saturating concentrations of phycoerythrin-labeled variant tetramers at 4 °C. The cells were then washed and incubated with an excess of unlabeled tetramer, and the rate of tetramer dissociation was assayed by fluorescence-activated cell sorting (FACS). Three representative examples of CTL lines are shown in **Figure 5b**. The assays show a spectrum of responses ranging from almost complete cross-reactivity to much lower cross-reactivity. The Den-2 tetramer dissociated faster than the Den-1 and Den-3 tetramers from cells derived (and cloned in the absence of peptide stimulation) from a patient with a current Den-2 infection. Scatchard analysis confirmed this finding, as the Den-2 tetramer showed a K_d of 10.6 M, whereas Den-1 and Den-3 had K_d values of 1.6 and 1.2 M, respectively (data not shown).

DISCUSSION

Dengue virus infections are now one of the biggest threats to public health in a number of developing countries. Over 2.5 billion people are at risk, and there are up to 50 million people infected per year. Epidemics are common in southeast Asia, South America and the western Pacific[2]. In addition to the mortality and morbidity associated with infection, dengue also imposes a considerable burden on the

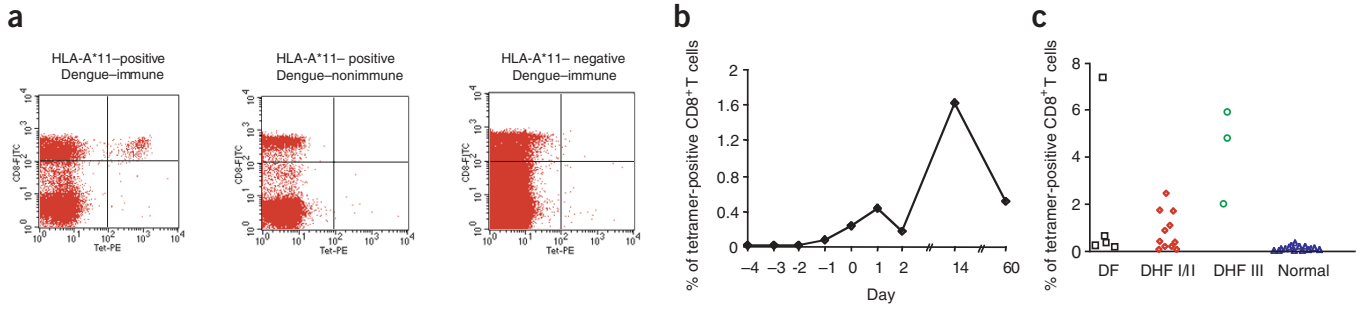

Figure 3 Analysis of responses using an HLA-A*11 tetramer. (**a**) Specificity of the GTS A11 tetramer. PBMCs were double-stained with phycoerythrin-conjugated GTS A11 tetramer (Tet-PE) and FITC-conjugated monoclonal antibody to CD8 (CD8-FITC). PBMCs were obtained from an HLA-A*11–positive, dengue-immune individual (left), an HLA-A*11–positive, dengue-nonimmune individual (center) or an HLA-A*11–negative, dengue-immune individual (right). (**b**) Mean frequency of GTS-specific CD8+ T cells during the course of acute dengue infection and follow-up. (**c**) Frequency of dengue GTS-specific CD8+ T cells at 14 d, from patients with different severities of disease (dengue fever (DF), DHF grades I and II, and DHF grade III) compared with the frequencies in 15 normal healthy individuals (mean ± s.d. of 1.18 ± 0.19 in patients versus 0.12 ± 0.1 in healthy individuals; Fisher's exact test P value = 0.0014).

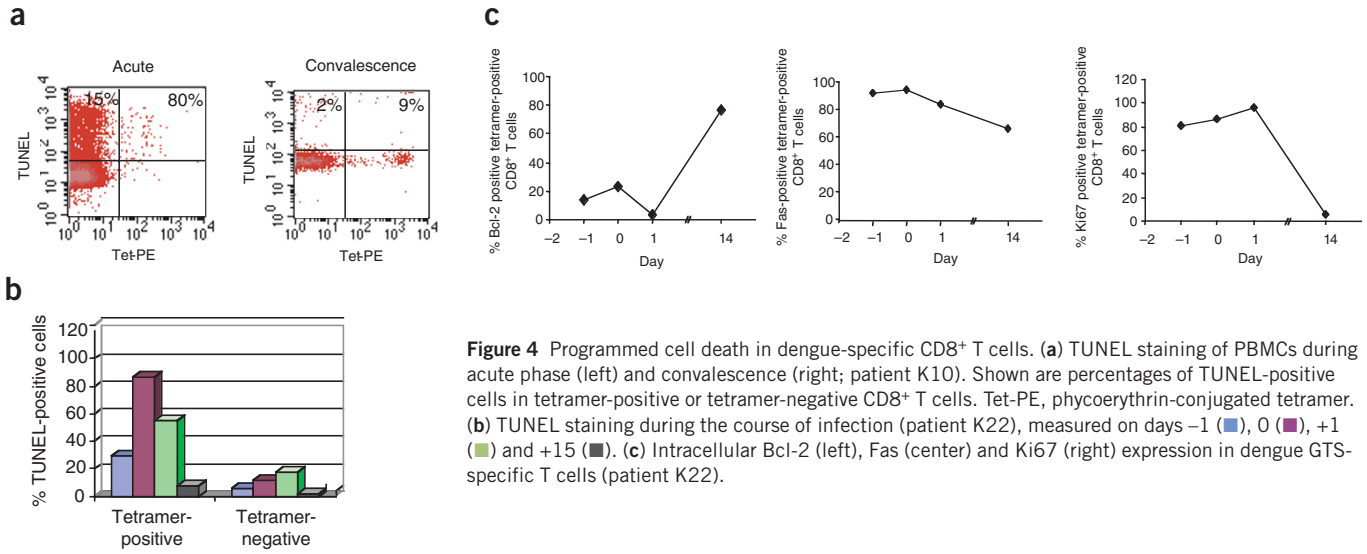

Figure 4 Programmed cell death in dengue-specific CD8+ T cells. (**a**) TUNEL staining of PBMCs during acute phase (left) and convalescence (right; patient K10). Shown are percentages of TUNEL-positive cells in tetramer-positive or tetramer-negative CD8+ T cells. Tet-PE, phycoerythrin-conjugated tetramer. (**b**) TUNEL staining during the course of infection (patient K22), measured on days –1 (■), 0 (■), +1 (■) and +15 (■). (**c**) Intracellular Bcl-2 (left), Fas (center) and Ki67 (right) expression in dengue GTS-specific T cells (patient K22).

finances and infrastructure of the health-care systems in these countries. Because of the scale of the problem, there is currently much interest in the generation of a dengue vaccine. These efforts have been hampered by two major factors: first, the lack of an animal model for severe dengue infection, and second, the epidemiological evidence for secondary infection in severe dengue. A better understanding of the pathogenesis of DHF would enhance our ability to develop safe vaccines, and would also expedite clinical trials by reducing their perceived risks.

In this study, we carried out a detailed analysis of a CTL response during acute dengue, and our results suggest an immunopathogenic role for T cells in DHF. Up to 7% of circulating CD8+ T cells react with the A11-restricted GTS epitope, and there is an association between the magnitude of the T-cell response and disease. The three patients with dengue shock syndrome (DHF grade III) all had peak responses of greater than 2% compared with 1 of 11 patients with DHF grades I and II, and 1 of 5 patients with dengue fever (Fisher's exact *P* value of 0.01 for dengue shock syndrome versus dengue fever and DHF grades I and II). These results are in agreement with a recent report showing a correlation between disease severity and T-cell responses to an HLA-B*07–restricted NS3 epitope[9].

A number of investigators have studied the potential for T cells to cause damage in animal models. Much of this work has been done in the mouse LCMV model. LCMV is a noncytopathic virus, but when an infection leads to a high antigen load combined with a large CTL response, immune damage to infected tissues can ensue[23]. There are a number of factors that can modify disease in this model, including viral dose and strain, previous infection, site of immunization or inoculation, age of the mice and presence of circulating antibodies[24].

Original antigenic sin was first shown for antibody responses, and implies that the response to a secondary challenge is dominated by the proliferation of cross-reacting memory cells induced by the primary infection, which may be of lower affinity for the secondary challenging antigen[25]. In this report we chose to use the term 'original antigenic sin' to denote the resurrection from memory of a response, without prejudice as to whether this will be to the benefit or the detriment of the host. Antigenic sin has the advantage that it can lead to the rapid

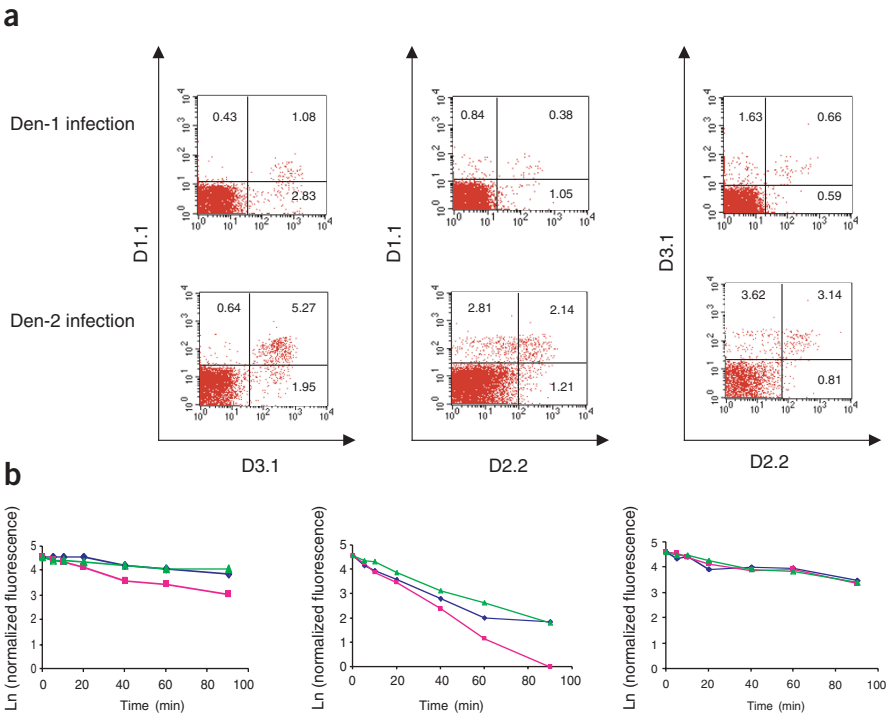

Figure 5 Differential avidities of T cells for dengue serotypes. (**a**) Double tetramer staining of three pairs of tetramers (D1.1/D2.2, D1.1/D3.1 and D2.2/D3.1) used to stain PBMCs from patients with acute Den-1 (K22) and Den-2 infection (K10). Plots represent double tetramer staining gated on CD8+ T cells. Percentage of cells is indicated in the three tetramer-positive quadrants. (**b**) Dissociation kinetics of the interaction between A1101-GTS D1.1 (◆), D2.2 (■) or D3.1 (▲) tetramers with a GTS-specific CTL line derived from healthy donors 20 (left) and 12 (right) and a dengue-specific CTL line from patient K213 (center; Den-2 infection) cultured in the absence of peptide.

mobilization of a memory response, which in many instances may be advantageous. Memory cells have a much lower threshold for activation compared with naive cells[26], however, so there is a risk that clones activated by original antigenic sin may have lower affinity and be less effective at clearing the secondary challenge[27], and indeed may promote immunopathology.

Recently, original antigenic sin has been shown to occur in T cells upon infection of mice with related strains of LCMV[28]. In these experiments, reinfection with certain viral strains recalled the less-effective primary response to the detriment of a *de novo* response to secondary antigen, leading to impaired viral clearance[28]. In addition, infection with heterologous virus may recall a memory response to a different primary virus challenge. This may also alter immunopathology such as the fat necrosis, necrotizing bronchiolitis and bronchiolitis obliterans seen in LCMV-immune mice upon infection with vaccinia virus, although in this instance heterologous immunity enhanced viral control[29,30]. In dengue there is also the possibility of cross-reaction with other flaviviruses, as dengue cocirculates with Japanese encephalitis virus in southeast Asia and yellow fever virus in South America.

In dengue virus infection, there is a correlation between viral load and disease severity[1], and the high viral load has been attributed to antibody-dependent enhancement[6,7]. In this study, we saw expansion of T cells with relatively lower affinity for the currently infecting virus and higher affinity for serotypes presumed to be previously encountered. This skewing of the response could result from the detrimental effects of original antigenic sin. An alternative explanation would be that the high antigenic load associated with a second dengue infection, when immune enhancement occurs, may preferentially drive high-affinity T cells into apoptosis, which would increase the frequency of lower-affinity cells.

Dengue can also infect macrophages and dendritic cells, where it can proliferate and activate the dendritic cells[31,32]. The effects of antibody-dependent enhancement leading to high viral loads, together with enhanced antigen presentation, result in extensive T-cell activation. Acutely, the CTLs are stunned with low IFN-γ expression and, for the reasons described above, a relatively high proportion of the activated clones may be of lower affinity and may not be very effective at clearing virus, especially in the absence of IFN-γ [27,33], yet they may contribute to damage. A dynamic equilibrium is established between T-cell activation and proliferation and cell death. The activation and death of these cells leads to cytokine release and immune-mediated tissue damage, which has been shown to occur by provoking massive activation-induced cell death of $CD4^+$ and $CD8^+$ T cells in several mouse models[23,34].

In this study we have identified and studied a CTL response restricted by one of the major southeast Asian MHC class I subtypes. Larger studies will identify more CD8 epitopes, and it is reasonable to expect that some of these will be serotype-specific. If we can identify enough of these, it may be possible to incorporate them into a dengue vaccine that would improve safety by avoiding potential antibody enhancement or cross-reactivity in T-cell responses.

METHODS

Samples. Blood samples were taken after patient consent and approval from the ethical committee of Khonkaen hospital, Thailand, and normal healthy subjects from Bangkok. Acute dengue infection was identified by RT-PCR–based dengue gene identification or dengue-specific IgM capture ELISA and hemagglutination inhibition test against all four dengue serotypes[13–17]. 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, or a ≥4-fold rise in hemagglutination inhibition antibody titer against any dengue serotype in paired acute and convalescent sera[14–17]. Plaque-reduction neutralization tests were done as previously described[35]. Percent reduction of plaques was plotted against serum dilutions. The dilution giving 50% plaque reduction was reported as the neutralizing titer.

Disease severity was classified according to World Health Organization criteria[17]. 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.

PBMCs were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation and cryopreserved until tested. HLA typing was performed by sequence-specific primer PCR[36].

FACS analysis. PBMCs were stained with phycoerythrin-labeled tetramer at 37 °C for 20 min and then for surface markers at 4 °C for 20 min by addition of a panel of conjugated antibodies. CCR7 staining was indirect. For intracellular staining, tetramer-stained cells were fixed and permeabilized in FACS permeabilization buffer (BD Pharmingen). Apoptosis was assessed using the TUNEL assay according to the manufacturer's protocol (Roche).

Elispot assay and T-cell culture. Briefly, PBMCs were added to polyvinylidene difluoride–backed plates (Millipore) precoated with a monoclonal antibody to IFN-γ (1-DIK; MABTECH). Peptides were then added at a final concentration of 10 M and the PBMCs were cultured overnight. Cells were discarded and spots were revealed by incubation with biotinylated monoclonal antibody to IFN-γ (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 line, PBMCs were incubated with 100 M of peptide for 1 h, after which IL-7 (Peprotech) was added at 25 ng/ml. After 3 d, IL-2 was added at 50 U/ml. Cells were tested for function after 1 week and restimulated every 2 weeks using autologous Epstein-Barr virus–transformed B-cell lines pulsed with peptide as targets. Chromium-release assays were done and specific ^{51}Cr release was calculated as [(experimental release – spontaneous release) (maximum release – spontaneous release)] 100.

MHC tetrameric complexes. Tetramers were prepared as previously described[37]. Briefly, the extracellular domain of HLA-A*1101 (heavy chain) containing the BirA biotinylation enzyme recognition site at the C terminus and β2-microglobulin was expressed in *E. coli* as inclusion bodies. Monomeric complexes of peptide, heavy chain and β2-microglobulin (referred to here as 'monomers') were formed by refolding *in vitro* and then biotinylated using BirA (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. The tetramer dissociation assay was done as previously described[38,39]. Dengue-specific CTL lines were stained for 30 min at 4 °C with saturating concentrations of phycoerythrin-labeled tetramer. CTL lines were then washed three times and an aliquot of cells was removed for FACS analysis. The remaining PBMCs were incubated with a 100-fold excess of unlabeled tetramer at 4 °C to block the binding and rebinding of phycoerythrin-labeled tetramer. Further aliquots of cells were removed and analyzed by FACS at appropriate time points. The total fluorescence within the phycoerythrin-positive gate was plotted against time to give the dissociation curve. The total fluorescence is the sum of the fluorescence intensity of tetramer-positive cells normalized per lymphocyte. This was then normalized to the percentage of the total fluorescence at the initial time point and plotted on a logarithmic scale.

Scatchard analysis. The assay to estimate the apparent K_d values for the interaction between tetramer and peptide-specific CTL lines was done as previously described[38,39]. Dengue-specific CTL lines were stained for 3 h at ambient temperature (22 °C) with a range of subsaturating concentrations of phycoerythrin-labeled tetramer. CTL lines were then washed three times and analyzed by FACS. Tetramer concentration (M) was plotted against bound tetramer (total fluorescence). In addition, Scatchard plots of bound tetramer (total fluorescence) versus bound tetramer/free tetramer (fluorescence units per M) were plotted. Apparent K_d values were derived from the negative reciprocal of the slope of the line fit to Scatchard plots of bound tetramer versus bound tetramer/free tetramer.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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