Human T cell responses to Japanese encephalitis virus in health and disease

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Abbreviations:

C	Core protein
CSF	Cerebrospinal fluid
CFSE	Carboxyfluorescein succinimidyl ester
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DMSO	Dimethylsulphoxide
E	Envelope protein
IISc	Indian Institute of Science
IQR	Interquartile range
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
MIP	Macrophage inflammatory protein
NAb	Neutralising antibody
NIMHANS	National Institute of Mental Health and Neurosciences
NS	Non-structural protein
prM	Pre-membrane protein
PRNT	Plaque reduction neutralisation titre
SFC	Spot forming cells
TCL	T cell line
WNV	West Nile virus
VIMS	Vijayanagar Institute of Medical Science
YFV	Yellow fever virus
ZIKV	Zika virus

Abstract

Japanese encephalitis (JE) virus (JEV) is an important cause of encephalitis in children of South and Southeast Asia. However, the majority of individuals exposed to JEV only develop mild symptoms associated with long-lasting adaptive immunity. The related flavivirus dengue (DENV) co-circulates in many JEV endemic areas and clinical data suggest cross-protection between DENV and JEV. To address the role of T cell responses in protection against JEV, we conducted the first full-breadth analysis of the human memory T cell response using a synthetic peptide library. *Ex-vivo* interferon-γ responses to JEV in healthy JEV-exposed donors were mostly $CD8^+$ and targeted non-structural (NS) proteins, whereas interferon-γ responses in recovered JE patients were mostly $CD4^+$ and targeted structural proteins and the secreted protein NS1. Amongst patients, a high quality, polyfunctional $CD4^+$ T cell response was associated with complete recovery from JE. T cell responses from healthy donors showed a high degree of cross-reactivity to DENV which was less apparent in recovered JE patients, despite equal exposure. These data reveal divergent functional $CD4^+$ and $CD8^+$ T cell responses linked to different clinical outcomes of JEV infection, associated with distinct targeting and broad flavivirus cross-reactivity including epitopes from DENV, West Nile and Zika virus.

Introduction

Japanese encephalitis (JE) virus (JEV) is a member of the family *Flavivirus*, genus *Flaviviridae*. JEV is an arthopod-borne virus (arbovirus) endemic to rural parts of South and Southeast Asia. JE is the most commonly diagnosed encephalitis in Asia, and is an important cause of disability and death in children in this region. Only around 0.1-1% of JEV infections of humans result in encephalitis (Halstead and Grosz, 1962). The remainder are clinically silent or result in a mild febrile illness (Watt and Jongsakul, 2003). The overwhelming majority of JEV-exposed individuals thus develop long-lasting naturally induced adaptive immunity, although this wanes in later life (Solomon, 2004).

JEV is a single stranded, positive sense RNA virus; the single 10 kb open reading frame (ORF) encodes three structural proteins, envelope (E), (pre-)Membrane (prM/M) and core (C); and seven non-structural (NS) proteins designated NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Sumiyoshi et al., 1987). There are an estimated 68 000 cases of JE annually, and probably around 17 000 deaths (Campbell et al., 2011). Clinical outcomes vary from complete recovery, through recovery with neuropsychiatric sequelae to death (Solomon et al., 2002). The role of the immune response to JEV in determining whether infection is asymptomatic or results in disease is incompletely understood.

Protection by neutralising antibody (NAb) to JEV in animal models is well established, whether passively administered, pre-formed by immunisation, or rapidly developed after challenge by a memory response (Gao et al., 2010; Konishi et al., 1999; Larena et al., 2011; Van Gessel et al., 2011). Higher titres of JEV NAb correlate with better protection (Lubiniecki et al., 1973). Development of JEV NAb after vaccination correlates with protection in humans (Hoke et al., 1988) and an anamnestic antibody response is associated with good outcome from disease (Libraty et al., 2002; Winter et al., 2004). Conversely, low JEV antibody levels in serum and cerebrospinal fluid (CSF) of patients with JE are associated with death (Libraty et al., 2002; Solomon et al., 2002).

Cell mediated immunity to JEV is less well studied. Observations from animal models suggest that T cells play a subsidiary role in protection from JE but could also contribute to immunopathology (Larena et al., 2011). Adoptive transfer of primed T cells can protect

147 animals from intracerebral JEV challenge (Murali-Krishna et al., 1996), even in the
148 absence of antibody in some cases (Ashok and Rangarajan, 1999).
149
150 Lymphocyte responses to JEV have been detected in humans for many years (Chaturvedi
151 et al., 1979; Konishi et al., 1995). Studies of healthy JEV-exposed residents of endemic
152 areas demonstrated $CD4^+$ and $CD8^+$ T cell responses to several JEV proteins dominated
153 by NS3, especially amino acids 193 - 324 (Kumar et al., 2004c). In recovered JE patients
154 IFNγ responses to NS3 amino acids 193 - 324 were infrequent and smaller than in healthy
155 JEV-exposed controls, despite proliferative responses being preserved (Kumar et al.,
156 2004b). Low IFNγ production was associated with poor outcome, indicating a role for IFNγ
157 in recovery from JE. However, other cytokine responses and responses to other JEV
158 proteins have not been examined in JE patients.
159
160 JEV co-circulates with other flaviviruses in much of South and Southeast Asia, notably the
161 four serotypes of dengue virus (DENV). Prior DENV exposure reduces the severity of JE
162 (Edelman et al., 1975; Libraty et al., 2002); however the effect of JEV immunity on
163 subsequent DENV infection is less clear. A large study of an inactivated JE vaccine in
164 Thailand showed those patients who developed dengue haemorrhagic fever (DHF) had
165 lower illness severity scores if they had received JE vaccine, suggesting a beneficial effect
166 (Hoke et al., 1988). On the other hand, a later careful prospective study in the same
167 location showed that the presence of JEV NAb at the study entry (in the absence of DENV
168 NAb) gave a 2.75 fold increase in the risk of dengue fever (DF) (Anderson et al., 2011).
169
170 DENV infection primes broadly cross-reactive T cell responses; such responses between
171 DENV serotypes have been studied extensively, particularly with reference to DHF
172 (Rothman, 2010). Limited data suggest that cross-reactive T cell responses between JEV
173 and DENV exist in inactivated JE vaccine recipients (Aihara et al., 1998). However, the
174 potential for cross-reactive T cell responses between JEV and other flaviviruses after
175 natural infection and their potential for protection is not known.
176
177 Taken together, the available data suggest that T cell responses could be protective in
178 human JE; however, no study to date has compared responses across the entire length of
179 the JEV polyprotein between encephalitis and asymptomatic infection. Moreover, many of
180 the published DENV epitopes show marked similarity to the corresponding sequences in

JEV, suggesting that immunological cross recognition by T cells is theoretically possible. Given that 1) prior DENV infection is protective in JE and 2) DENV infection primes broadly cross-reactive T cells, we hypothesised that, in regions endemic to both viruses, patients who had suffered from JE would have memory T cell responses that had less cross-reactivity with DENV than healthy JEV-exposed subjects. Therefore, we undertook a study to systematically map JEV epitopes using a full length synthetic peptide library for JEV. We assessed the specificity, function and magnitude of the T cell response and measured the degree of T cell receptor cross-reactivity in healthy JEV-exposed donors and recovered JE patients.

Results

Subjects

The study took place in Karnataka State, South India, a JE endemic area. One hundred and three participants were recruited into the study (table I). Fifty-one were healthy Indian residents of JEV endemic areas, 39 were recovered JE patients. Healthy donors were recruited in Bangalore and at Vijayanagar Institute of Medical Sciences (VIMS), Bellary; recovered JE patients were recruited at VIMS. Thirty-five healthy donors (69%) were defined as JEV-exposed on the basis of residence in a JEV endemic area during childhood and a positive NAb assay. A training cohort of 13 participants was recruited in the UK, comprising a mixture of flavivirus exposed and unexposed subjects. Two subjects recruited in the UK reported classical dengue illness (1 DF, 1 DHF) with positive DENV IgM, and one more without diagnostic testing; all three had acquired infection in dengue endemic areas and were anti-DENV NAb positive.

T cell responses in healthy JEV-exposed subjects are dominated by diverse, highly cross-reactive, $CD8^+$ cells

Thirty-five JEV NAb+ healthy South Indian donors were screened by enzyme linked immunospot (ELISpot) assay or whole blood intracellular cytokine staining (WB ICS) for JEV T cell responses. Seventeen (49%) showed positive *ex-vivo* IFNγ assays (Fig 1 A & B). In a subset of 24 subjects tested, 22 (92%) showed proliferative responses (Fig 1 C). IFNγ responses were predominantly directed against NS3, NS4 and NS5 (Fig 1 A) and were modest in size (Fig 1 B), whereas proliferative responses recognised all viral proteins (Fig 1 C). Flow cytometric analysis showed 80% of *ex-vivo* IFNγ responses were $CD8^+$ (Fig 1 D).

Clinical data suggest cross protection between DENV and JEV. Two subjects with documented dengue illness (but who were unlikely to have been JEV-exposed) and one JEV NAb negative volunteer showed IFNγ ELISpot responses to the JEV peptide library (shown in red in Fig 1 B) - no responses were detected in healthy DENV and JEV unexposed controls (unpublished data). The two subjects reporting dengue were also

positive for JEV NAb, though anti-DENV titres were higher, consistent with prior DENV infection (JEV 50% plaque reduction neutralization titer [$PRNT_{50}$] 1 in 266 and 1 in 85 and DENV $PRNT_{50}$ 1 in 4,515 [DENV1] and 1 in 12,413 [DENV3], respectively). Therefore, we set out to determine whether JEV and DENV responses cross-react. First, responses were mapped by ELISpot or by expanding short term T cell lines from donors showing *ex-vivo* responses followed by deconvolution of pools in ICS assays. Next, cross-reactivity was tested using variant peptides from DENV (and other flaviviruses) corresponding to the mapped peptides of JEV.

Using this approach, we first studied two naturally JEV-exposed subjects (H001/1 and H008/4) and one reporting DF (H001/4) in detail. $CD8^+$ T cell responses were identical in size and functional characteristics to peptide sequence variants from other flaviviruses (Fig 2 A top panels & B). T cell lines showed similar responses in functional assays whichever peptide was tested (Fig 2 A bottom panels), irrespective of which peptide was used to expand the line (Fig 2 C). Titrations of variant peptides showed responses detectable in the nanomolar range and that cross-reactivity was not limited to high peptide concentration (figures 2B & C), although there was some variation in efficiency of individual peptides.

We then extended this analysis across the cohort; using peptides of West Nile virus ((WNV), a flavivirus closely related to JEV); DENV2, and E, NS1, NS3 and NS5 proteins from DENV1, 3 and 4 (see materials and methods). Once library peptides were mapped, the same T cell lines were then tested against the equivalent peptides from DENV1-4 and WNV, based on a ClustalW alignment of the library polyprotein sequence (an example is shown in Fig 2 D). Responses to the variant peptides were normalised across different assays by dividing the result by the value for JEV peptide in the same assay, a cross-reactivity index of 1 indicating an equal response to JEV and variant peptides. In five subjects, cross-reactive responses tested both *ex-vivo* and on T cell lines showed good agreement (Fig 2 E). Next, we studied 10 healthy JEV-exposed donors in whom 15 epitopes were mapped. In all but three, responses were highly cross-reactive (Fig 2 F) and were not significantly different from the hypothetical value of 1 (indicating equivalent responses) by Wilcoxon signed rank test. Eight out of the 10 donors showing responses that recognised peptides from at least two other flaviviruses (often more) as efficiently as JEV.

Cross-reactivity was confirmed by dual tetramer staining between the JEV epitope and three variant epitopes from WNV, DENV-1 and DENV-2/3 (Fig 2 G), at least in one individual. Cross-reactivity occurred at the single cell level with apparently equal affinity (Fig 2 H). Finally, to determine past exposure to DENV, $PRNT_{50}$ to DENV1-4 were measured in those subjects who had cross-reactivity assays performed; the cohort was extensively DENV exposed (Fig 2 I).

Overall, these experiments confirmed clear JEV-specific T cell memory responses in JEV NAb positive subjects from a JE endemic area. These responses were predominantly $CD8^+$, highly cross-reactive among flaviviruses and mostly targeted NS proteins, including in donors with clear DENV exposure.

$CD8^+$ T cell responses in healthy JEV-exposed subjects are polyfunctional and show a cytotoxic phenotype

We next addressed the functionality of these responses. We asked whether cytokines were co-secreted by JEV-specific T cells using WB ICS assays; example flow cytometry data are shown in Fig 3 A. The $CD8^+$ T cell response was dominated by $IFN\gamma^+/TNF\alpha^+/MIP\text{-}1\beta^+$ and $IFN\gamma^+/TNF\alpha^+$ cells (Fig 3 B). Overall 75% of the responding cells made >1 cytokine. The small number of $CD4^+$ T cell responses precluded this analysis for this population; $CD4^+$ T cell responses were smaller as well as less frequently detected (Fig 3 C).

To address the production of other cytokines, bead array assays were performed in a subset of 13 donors on day 2 and day 5 supernatants from proliferation assays showing the largest responses, which were often different pools from the *ex-vivo* IFNγ assays (Fig 3 D, data for day 5). This showed some evidence of more diverse cellular responses with release of IL10 (the cytokine most frequently detected in these assays) in 11 (85%) of the donors (Fig 3 D & E); IL4 and IL13 were also detected but in much smaller amounts and less frequently. Other T cell derived cytokines and chemokines were detected infrequently; five subjects (39%) made either CCL3 or CCL5 responses and only two subjects (15%) made IL17A (Fig 3 E).

In selected individuals, where re-sampling was possible after $CD8^+$ T cell responses were mapped, the cytotoxic capacity of these cells was studied in more detail. JEV-specific $CD8^+$ T cell responses degranulated in response to peptide stimulation (Fig 4 A) and degranulation responses were cross-reactive (Fig 4 A - C). Responses were detectable to at least 10nM peptide *ex-vivo* indicating that they are likely to be functional *in-vivo* (Fig 4 C). Responding cells expressed perforin and granzyme B (figures 4D - F) consistent with killing capacity. To confirm these results short term T cell lines were expanded to responding peptides: these too degranulated (Fig 4 G) and were cross-reactive between JEV and DENV (Fig 4 H). Cross-reactive degranulation responses were equivalent in overall functionality between JEV and DENV, with 55-60% of responding cells degranulating in both cases (Fig 4 I). Finally, cross-reactive $CD8^+$ T cell lines were able to kill peptide loaded cells, whether expanded to JEV or DENV peptides (Fig 4 J).

Screening for T cell responses in convalescent JE patients

We next addressed T cell responses in recovered JE patients. Thirty-nine recovered JE patients were screened by ELISpot or WB ICS; 28 (72%) showed *ex-vivo* IFNγ responses. Sample flow cytometry data are shown in Fig 5 A. *Ex-vivo* IFNγ responses targeted all JEV proteins (Fig 5 B), with slightly more responses against the C/prM and NS1/NS2 pools. Around 80% of the IFNγ response came from $CD4^+$ T cells (Fig 5 C), several donors showed exclusively $CD4^+$ responses. Proliferative responses were similarly distributed among JEV proteins (Fig 5 D & E). Responding peptides were mapped using short term T cell lines using the same strategy as the healthy JEV-exposed donors, and 14 peptides from 7 recovered patients were tested in cross-reactivity assays (Fig 5 F). Mapped responses in recovered JE patients were mostly JEV-specific, and showed a cross-reactivity index significantly different from the hypothetical value of 1 (indicating equivalent responses) by Wilcoxon signed rank test in all cases except DENV1, which approached significance despite fewer variant peptides tested. Importantly, NAbs to DENV were present in all the recovered JE patients with peptides mapped and cross-reactivity assays performed (Fig 5 G and table I), suggesting the lack of cross-reactivity in recovered JE patients is not a result of lack of DENV exposure. In the same subset, JEV NAb titres were also tested and showed no difference between the good outcome and poor groups (Fig 5 H).

A polyfunctional, IFNγ-dominated CD4$^+$ T cell response correlates with better clinical outcome in convalescent JE patients

JE patients were categorised into good outcome, (complete recovery with no neurological deficit; Liverpool outcome score 5) or poor outcome (incomplete recovery - Liverpool outcome score 2-4 or abnormal neurological examination, see methods). There was no difference in targeting of viral proteins or proportion of the response expressed by CD4$^+$ T cells when analysed by outcome (unpublished data). IFNγ ICS responses, however, were larger in patients with good outcome (Fig 6 A). TNFα production was equivalent between the two groups (Fig 6 B), but was derived mostly from TNFα single positive cells in those with poor outcome (Fig 6 C). Complete recovery was strongly associated with a polyfunctional IFNγ$^+$/IL2$^+$/TNFα$^+$ CD4$^+$ T cell response, indicating that a poor quality memory response, which is pro-inflammatory, but lacking in anti-viral/helper capacity, is associated with recovery from JE with long term neurological damage.

To explore cytokine production further, in a subgroup of 11 patients, secreted cytokine responses were assayed from the day 2 and day 5 supernatants of proliferation assays as for the healthy JEV-exposed group (Fig 6 D, data for day 5). In these assays across both time-points IL10 and IL13 were detected in several patients from both groups (Fig 6 D & E) and proinflammatory TNFα responses were frequent. Similar to the healthy group, other T cell derived cytokine and chemokine responses were rare. Three subjects made CCL3 or CCL5 responses and three made IL17A. Looking specifically at CD8$^+$ T cell responses in recovered JE patients by WB ICS, these were mostly polyfunctional and characterised by IFNγ$^+$/TNFα$^+$/MIP-1β$^+$ and IFNγ$^+$/TNFα$^+$ cells (Fig 6 F).

Importantly, all the patients were sampled at least 6 months from acute illness, by which time most recovery is expected to have occurred. Time from illness to sampling was the same in both groups (Fig 6 G), indicating that this variable did not confound the results. Interestingly, the observed defect in CD4$^+$ T cell cytokine production was not apparent in proliferative responses which were equal in magnitude between the two groups; CD8$^+$ proliferative responses were slightly larger in those with poor outcome (Fig 5 E). Overall, these results indicated that T cell responses in patients who have recovered from JE were predominantly CD4$^+$ and JEV-specific. Smaller IFNγ responses in conjunction with poor quality, pro-inflammatory (TNFα$^+$) CD4$^+$ responses were associated with poor outcome.

T cell responses are differentially targeted in JE patients and healthy donors

During screening, *ex-vivo* IFNγ responses in healthy JEV-exposed donors were directed mostly at NS3, NS4 and NS5 (Fig 1 A), whereas recovered JE patients responded to all JEV proteins (Fig 5 B). Mapped peptides were plotted according to their location in the JEV polyprotein for healthy, JEV-exposed donors (Fig 7 A), recovered JE patients (Fig 7 B) and two UK participants reporting dengue illness (Fig 7 C). Sequences of all the JEV peptides identified are in table II and the variant peptides showing responses in cross-reactivity assays are shown in table III. Almost all the mapped responses in structural proteins were identified in JE patients, and one healthy JEV-exposed donor showed a $CD4^+$ response to E protein of JEV (Fig 7 A & B, table II). In contrast, the responses in healthy JEV-exposed donors were focused on NS3 and NS5, and only one peptide was successfully mapped in NS3 in a JE patient. This was despite multiple attempts at expanding T cell lines to NS3 in *vitro*, which failed to produce responses in all except one individual. The two subjects reporting dengue illness showed similar findings to the healthy JEV-exposed donors (Fig 7 C). The average Shannon entropy (H Index) of Indian isolates of JEV and DENV corresponding to the position of JEV peptides eliciting T cell responses in recovered JE patients was significantly greater than in healthy JEV-exposed donors, $p = 0.0003$ (Fig 7 F), indicating that heathy donors target conserved regions of the flavivirus polyprotein.

The absence of T cell responses in NS1 in the healthy JEV-exposed donors could not be explained by a lack of potential HLA-binding motifs. The HLArestrictor server (Erup Larsen et al., 2011) identified 122 HLA class I binding motifs for the alleles identified ranked "strong binder" with a predicted accuracy of > 80%. A more limited analysis of the HLA class II DRB1 alleles 03:01, 04:01, 07:01, 11:01 and 15:01 using SYFPETHI (Rammensee et al., 1999) showed 197 15-mer peptides with score ≥ 20.

Few peptides were identified in more than one individual despite overlap of HLA alleles across the cohort (table II). One exception was peptide TMAMTDTTPFGQQRVFK at position 2869 to 2885 of the viral polyprotein (NS5 amino acids 342 - 358) which showed $CD8^+$ T cell responses in three healthy JEV-exposed donors and one DENV-exposed donor. This corresponds to a highly conserved 20 amino acid segment which is nearly

identical across all the medically important mosquito borne flaviviruses, visible as a trough in Shannon entropy (H index) in NS5 (Fig 7 D). In our study, three healthy HLA A*68:01 positive JEV-exposed donors who recognised JEV NS5 342 - 358 also recognised DENV peptides that were truncated for the C terminal three amino acids (Fig 2 D), suggesting they were recognising DTTPFGQQR, previously described as one of the most immunogenic DENV $CD8^+$ T cell epitopes out of 408 identified (Weiskopf et al., 2013). In a fourth, DENV exposed, HLA B*35:01 positive subject (H010/4), the JEV NS5 342 - 358 response was mapped to TPFGQQRVF (table II), also previously shown to be presented by this allele (Rivino et al., 2013).

These data suggest that T cell responses from recovered JE patients target JEV-specific regions that are less conserved among flaviviruses (high H index). Healthy JEV-exposed donors made responses against conserved regions, that were highly cross-reactive among different flaviviruses (Fig 2 F).

Discussion

In this study we have presented data showing that across the entire JEV polyprotein memory T cell responses in healthy JEV-exposed donors are (i) predominantly directed against NS3, NS4 and NS5, (ii) are $CD8^+$ and (iii) cross-react extensively between flaviviruses. Recovered JE patients, on the other hand, mount mostly JEV-specific $CD4^+$ T cell responses and target epitopes predominantly within the JEV structural proteins and the secreted protein NS1. Among the recovered JE patients, the quality of the *ex-vivo* $CD4^+$ T cell response was associated with outcome; in individuals with poor outcome, the presence of a significant $TNF\alpha^+$-only $CD4^+$ T cell population suggests a potential mechanism whereby T cells might contribute to damaging inflammation in JE. These data are compatible with the notion, supported by animal studies (Larena et al., 2011; Larena et al., 2013), that T cells may play a role both in protection and immunopathology in JE. In addition this study describes the first polyfunctional T cell responses to JEV, the first minimal epitopes of JEV in humans and the first demonstration of cytotoxic $CD8^+$ T cell responses to JEV in naturally exposed individuals.

Anti-viral T cells exhibiting multiple functions are well described after many viral infections, and have been observed after infection or vaccination with several flaviviruses (Akondy et al., 2009; Friberg et al., 2011b; Piazza et al., 2010). In this study we have described polyfunctional T cell responses to JEV in both $CD4^+$ and $CD8^+$ subsets and found that the quality of the $CD4^+$ T cell response was the factor most strongly associated with complete recovery from JE. Evidence for the protective capacity of polyfunctional T cell responses in humans is largely correlative (Betts et al., 2006), as is the case here. On average, >50% of the responding cells expressed two or more cytokines and the subjects in this study were sampled a median of 6 years or more after the viral encounter. Therefore, these responses likely reflect a long lived stable memory population rather than newly responding or terminally differentiated single cytokine positive cells (Seder et al., 2008).

In a subgroup of subjects, $CD8^+$ T cell degranulation responses were studied. Sampling limitations meant we could not test these responses in the whole cohort, but in all the subjects tested, $CD8^+$ T cells degranulated in response to JEV peptides in the 10nM range. Degranulation, perforin and granzyme B expression correlates closely with the

ability to kill (Betts et al., 2003; Pardo et al., 2004); which we have confirmed using peptide loaded cells. These cytotoxic responses also cross-react between JEV and DENV; in other words a DENV primed cell could in principle kill a JEV infected target or vice versa. Killing of cells expressing Pox virus vectored JEV prM/E/NS1 proteins has previously been demonstrated for $CD4^+$ T cell clones and $CD8^+$ short term T cell lines expanded from PBMC from JE vaccinated subjects, rather than natural JEV exposure (Aihara et al., 1998; Konishi et al., 1998). Although we have not shown killing of virally infected target cells (itself a surrogate marker of protection), the data presented here make it highly likely that these responses would be capable of killing JEV infected cells. T cell killing of JEV infected cells, to our knowledge, has only been shown for murine and never for human cytotoxic T lymphocytes (Murali-Krishna et al., 1994).

Previous work in the same location found a higher frequency of responses to NS3 (Kumar et al., 2004a; Kumar et al., 2004b), likely accounted for by the time interval between exposure and testing. The patients in our study were sampled on average 6 years after JE, whereas in the studies of Kumar *et al*. (2004b) they were sampled around 6 months after disease. In the case of DENV, when patients are sampled two weeks after illness, the frequency of responses to NS3 reported is as high as 90% (Duangchinda et al., 2010). In recovered JE patients, IFNγ ELISpot responses to NS3 were small (unpublished data), and repeated attempts at *in vitro* expansion of responding T cells to NS3 consistently failed to produce responses in $IFN\gamma^+$/TNFα ICS assays. Taken together, our results are consistent with earlier findings, that JE patients had defective IFNγ but preserved proliferation responses to JEV NS3 amino acids 193-324 (Kumar et al., 2004b). Now we have also shown reduction in IFNγ is a general phenomenon associated with poor outcome from JE, and occurs in response to all JEV proteins not only NS3, increasing the likelihood that this is a biologically relevant result. In addition, we have extended these findings to show that an unbalanced $CD4^+$ T cell response characterised by pro-inflammatory TNFα production, in the absence of sufficient additional helper/anti-viral IL2 and IFNγ, is strongly associated with poor outcome from JE.

We observed a striking degree of cross-reactivity in the NS protein specific short term $CD8^+$ T cell lines from healthy JEV-exposed donors. The average Shannon entropy of sequences recognised by this group was low, indicating they were highly conserved. A recent study on dengue also found the majority of conserved $CD8^+$ T cell epitopes are

found in the NS proteins (Weiskopf et al., 2015). Short term T cell lines from some healthy donors in our study recognised peptides from flaviviruses for which the chance of exposure was extremely remote, such as tick borne encephalitis virus and St Louis encephalitis virus (unpublished data), and some sequences were in common with geographically diverse viruses such as Murray Valley encephalitis virus and Zika virus, suggesting that this phenomenon goes beyond simply exposure and is largely accounted for by selective targeting of conserved regions. The dominant responses in recovered JE patients, on the other hand, cross-reacted much less with variant peptides from DENV and WNV. DENV NAb assays revealed this was not simply due to a lack of exposure. $CD4^+$ T cells dominated the responses in recovered JE patients, this cannot account for the lack of cross-reactivity because $CD4^+$ T cells are known to mount cross-reactive responses to flaviviruses (Aihara et al., 1998; Kurane et al., 1991). Although the cross-reactivity of T cell responses appeared to differ between healthy JEV-exposed donors and recovered patients, the retrospective nature of this study and the likely difference in time between exposure and sampling for these different groups limits our ability to assign a protective role to these responses.

Both serotype specific and serotype cross-reactive T cell responses are readily detectable after a single DENV infection (Friberg et al., 2011a), and, over subsequent DENV exposures, the response may be skewed towards more conserved epitopes (Weiskopf et al., 2013). The exposure to DENV and the highly cross-reactive T cell responses in the healthy JEV-exposed donors in this study is consistent with these findings, and suggest this phenomenon is not restricted to DENV. Given the protective effect of DENV on JEV (Edelman et al., 1975), one possibility is that exposure to DENV primes JEV cross-reactive T cell responses. Although there are well described examples of pathology mediated by DENV cross-reactive T cells (Mongkolsapaya et al., 2003), not all studies on dengue have confirmed the relationship between cross-reactive T cells and disease (Friberg et al., 2011a), and some observations imply a protective role for cross-reactive memory T cells (Hatch et al., 2011; Weiskopf et al., 2013). Similarly, animal models of sequential T cell priming and challenge can show either impaired or enhanced protection (Welsh et al., 2010). JE pathogenesis is distinct from some other viral infections in that viraemia is typically very low and often clinically inapparent; therefore an early, modestly sized, cross-reactive $CD8^+$ T cell response in the periphery may prevent dissemination to the central nervous system and limit the virus to a compartment where it is not particularly pathogenic.

The retrospective nature of this study means we are unable to address this question directly, but this could be the subject of future prospective studies.

There are several possible reasons for the limited flavivirus cross-reactive T cell responses seen in recovered JE patients even years after disease and despite DENV exposure. There may be intrinsically pathogenic responses in the context of disease that are different from those in health, and age at exposure or variation in innate immunity may affect the character of responses. Also, the order of priming may affect the cross-reactivity of T cell responses. Support for this is found in epidemiological observations that DENV infection is at least partially protective against JE (Edelman et al., 1975; Libraty et al., 2002) and that JEV NAb (but not vaccination) has a detrimental effect on DENV (Anderson et al., 2011). However, a weakness of our study (common to many) is that we cannot determine the order in which different flaviviruses were encountered and therefore cannot provide direct evidence that JEV priming modifies DENV responses. Future prospective studies could address this question, and investigate differences in the T cell response as a potential mechanism.

Ex-vivo responses in recovered JE patients against the structural proteins and the pool containing NS1 were frequently observed, but were rare in the healthy JEV-exposed donors. It is hypothesized based on observations in DF that structural proteins and the secreted protein NS1 interact with B cells and elicit $CD4^+$ responses, whereas the remaining non-structural proteins elicit mostly $CD8^+$ T cell responses (Rivino et al., 2013), consistent with what we have seen in this study. The reasons underlying the absence of responses to NS1 in the healthy JEV-exposed donors are not clear, but previous work in this region identified T cell responses against NS1 in healthy children (Kumar et al., 2004a) so anti-NS1 responses are likely not intrinsically pathogenic.

Whilst the dominant responses mapped in recovered JE patients were directed against less conserved viral proteins, we nevertheless identified a number of IFNγ responses against NS3, NS4 and NS5. One $CD8^+$ T cell response from a recovered JE patient (JE098/2) targeted a conserved epitope (ELGEAAAIFMTATPP, NS3 306-320), which showed a cross-reactive response in subject H013/3 (these two subjects shared HLA 02:01 and B40:01, as well as being closely matched at B35:01 and B35:03; table II). This response was not further tested in JE098/2 (due to insufficient cells), but it is likely that this

response would have been cross-reactive. Although the predominance of JEV-specific responses in recovered JE patients is likely explained by variation in amino acid sequences between JEV and DENV, in some cases single amino acid changes (aside from anchor residues) had very profound effects on responses. For example, changing Ala at position 5 of GEAAAIFMT to Gly completely abolished $CD8^+$ T cell recognition in donor H013/3. In donor JE054/2, changing Leu to Ile (a very modest alteration) at position 12 of peptide AISGDDCVVKPLDDRF made no difference whilst changing Asp to Glu (also similar amino acids) at position 14 eliminated the $CD8^+$ T cell response. On the other hand, two amino acid changes from the HLA B*08:01 restricted epitope DLMCHATF (DENV) to D**V**MCHAT**L** (JEV) gave rise to identical $CD8^+$ T cell responses in donor H001/4 (who reported dengue illness) and a wide range of single amino acid variants from Gln at position 8 of the HLA B*58:01 restricted epitope MTTEDMLQVW (to Ser, Thr, Asp or Glu) were also recognised by $CD8^+$ T cells in donor H008/4. Therefore, the number of amino acid variants alone cannot explain the variation in cross-reactive responses seen here, and the reasons for this remain to be determined.

A limitation of our study is that the healthy JEV-exposed donors were older than the recovered JE patients. Practically, it is difficult to draw blood from children who have not been ill in this setting. Nevertheless, both groups were well matched for viral exposure, likely were exposed to JEV years earlier with ongoing exposure year on year, and we are not drawing direct comparisons between the two groups. Our within-group comparison of outcome from JE does not suffer from this limitation. Ideally the two groups should be matched for the time elapsed since first exposure to JEV, although within groups we could not observe a signal indicating a change in responsiveness of cross-reactivity over time/age (unpublished data). The optimum way to address this question would be using a prospective study design, but given the high rate of asymptomatic JEV infection, relatively low incidence of JE, and existence of many other conditions which are hard to differentiate from JE, such a study would be impractical.

A second shortcoming is the small number of subjects with peptide mapping and cross-reactive T cell response data, despite the screening of larger numbers. Although the clinical setting introduces some limitations, our data demonstrate a clear lack of cross-reactive responses in recovered JE patients. A technical concern of our cross-reactivity experiments was that these were not conducted on optimally defined peptides, as optimal

T cell epitopes located within a larger peptide can affect detection of the response (Draenert et al., 2004). The need to deconvolute the peptide pools in quick succession using single T cell lines, making maximal use of scarce samples, precluded minimal epitope mapping and HLA restriction in each case. Despite this, cross-reactive responses were readily detectable using library peptides. Because of the closed-ended nature of the HLA class I peptide binding groove, this would reduce detection of cross-reactive $CD8^+$ T cell responses more than $CD4^+$, the converse of what we have observed. Lastly, we justified the use of short term T cell lines by showing in a subset of donors that cross-reactive responses *ex-vivo* correlated well with cross-reactive responses in T cell lines.

In summary, using the first full breadth analysis of T cell responses to JEV in humans, we have demonstrated that a high quality IFNγ dominated $CD4^+$ T cell response is associated with good recovery from JE in humans. JEV T cell responses are dominated by broadly cross-reactive $CD8^+$ T cells in most healthy JEV-exposed donors. Cross-reactive responses in recovered JE patients, on the other hand, are much less frequent, though uncertainty remains regarding the true significance of this finding. In much of South and Southeast Asia, where JEV and DENV co-circulate, the effect of JEV immune responses could be significant in subsequent natural infection and in response to vaccines. Therefore, the sequence and timing of flavivirus infections and their effect on immunopathogenesis and potentially vaccine responses are worthy of further study. In the context of the current outbreak of Zika virus, this cross-reactivity at the T cell level, as we have identified here, could be of relevance to disease pathogenesis.

Materials and Methods

Setting

The study was conducted at the Indian Institute of Science (IISc) and National Institute for Mental Health and Neuroscience (NIMHANS) in Bangalore and Vijayanagar Institute of Medical Sciences (VIMS), Bellary; all in Karanataka State, India. Karnataka is endemic for JE; the disease occurs in rural areas but is uncommon in districts around Bangalore. Recovered JE patients were therefore recruited at VIMS medical centre. For most experiments, blood samples were transported overnight by train for processing in a laboratory in Bangalore.

Study subjects

Healthy donors with no history of neurological illness were recruited at IISc, NIMHANS and VIMS. Recovered JE patients were recruited at dedicated outpatient clinics held at the VIMS Medical Centre. Healthy donors were taken from family members of patients and other members of the local community. JE patients were drawn from previous cohorts studied in this location (Lewthwaite et al., 2010b; Lewthwaite et al., 2010c), recruited based on hospital records of positive CSF JEV IgM samples and follow up of acute JE cases recruited during the study period (October 2011 to March 2013). All recovered JE patients had been admitted to VIMS medical centre with a clinical illness compatible with encephalitis (fever plus one of the following: clouding of consciousness, seizures or focal neurological signs) and a positive enzyme linked immunosorbent assay for JEV IgM in CSF. Patients had been admitted a median of 6 years earlier (mean 5.98, range 6 months to 14 years). A small cohort was also recruited in the UK. Age, sex, travel history/previous area of residence, flavivirus vaccine history and medical history (focused on flavivirus illness) was collected from all participants. Recovered JE patients underwent detailed neurological examination and disability assessment using the Liverpool outcome score (Lewthwaite et al., 2010a).

Ethics statement

This study was carried out in accordance with the principles of the declaration of Helsinki. The experimental studies in India were approved by the IISc Institutional Human Ethics Committee (IHEC, ref 5/2011). All Indian studies were also approved by the Liverpool school of Tropical Medicine research ethics committee (ref 10.59). The component of the study conducted at VIMS medical centre was further reviewed by the VIMS ethics committee. The UK set up phase of the study was approved by the National Research Ethics Committees Northwest 7 (NREC NW7, ref 10/H1008/23). All participants gave informed consent, or their parent/guardian gave consent if aged under 18. Minors aged 12 to 18 were able to give assent in addition to the guardian's consent if they wished.

Peptide libraries

All available JEV complete genome sequences in the NCBI nucleotide database were downloaded in April 2010. Open reading frames were translated, aligned, and a consensus amino acid sequence was generated using Se-AL carbon v2.0a11 (http://tree.bio.ed.ac.uk/software/seal/). Because only JEV genotype III was identified as circulating in South India until April 2010 the sequence used for the JEV peptide library was the consensus sequence of JEV genotype III. However, genotype III sequences predominated in the database and a consensus sequence generated using all the available sequences was identical. The JEV consensus sequence was used to generate a library of peptides 18 amino acids long overlapping by 10. In addition, if the C terminal amino acid residue of the peptide was not an HLA class I anchor residue the peptide was truncated until an "allowed" residue was reached or until a length of 15 amino acids (the overlap was kept constant at 10) to improve the sensitivity for $CD8^+$ responses (Draenert et al., 2003; Draenert et al., 2004). Peptides were synthesised by Mimotopes Ltd as a PepSet™ (Supplementary information). Peptides were dissolved in dimethyl sulphoxide (DMSO) and pooled as indicated. The following peptide sets were obtained through Biodefense and Emerging Infection (BEI) Resources, NIAID, NIH: DENV1 Singapore/S275/1990 E (NR4551), NS1 (NR2751), NS3 (NR2752) NS5 (NR4203); DENV2 New Guinea C (NGC) C (NR505), prM (NR506), E (NR507), NS1 (NR508), NS2a (NR2747), NS2b (NR2748), NS3 (NR509), NS4a (NR2749), NS4b (NR2750), NS5 (NR2746); DENV3 Sleman 1978 E (NR511), DENV3 Philippines/H87/1956 NS1 (NR2753), NS3 (NR2754), NS5 (NR4204); DENV4 Dominica/814669/1981 E (NR512), DENV4 Singapore/8976/1995 NS1 (NR2755), NS3 (NR2756) NS5 (NR4205); West Nile vrus

NY99-flamingo382-99 C (NR432), prM (NR433), M (NR434), E (NR435), NS1 (NR436), NS2a (NR437), NS2b (NR438), NS3 (NR439), NS4a (NR440), NS4b (NR441) and NS5 (NR442). BEI resources peptides were dissolved using the same strategy as for JEV.

IFNγ ELISpot assay

ELISpot assays were performed using anti-human IFNγ capture and biotinylated detection antibodies from Mabtech, according to the manufacturers instructions. Development was by detection with goat anti-biotin-HRP and NBT/BCIP one step substrate (Fisher). PBMC were plated at 2 x 10^5 per well and incubated overnight in 100µl RPMI supplemented with 2mM L-glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin (sRPMI) with 10% fetal calf serum (FCS, R10), and peptides at a final concentration of 3-6µg/ml. The positive control was concanavalin A at a final concentration of 5µg/ml, negative control was DMSO at equivalent concentration to the peptide pools.

ELISpot assays were considered positive if there were at least 50 spot forming cells (SFC)/10^6 PBMC and double the background value. In cases close to the cut-off value, confirmation by another assay or mapping to a peptide was used. In 7 JEV antibody negative UK residents, no subject gave a response using these criteria, and studies on other members of the family *Flaviviridae* have used similar/idenitcal thresholds (Appanna et al., 2007; Barnes et al., 2012; Parsons et al., 2008). Healthy donors were screened using 11 pools of peptides (1 pool for C/prM, 2 for E, 1 each for NS1 & NS2, 2 for NS3, 1 for NS4 and 3 for NS5). Recovered JE patients, where available samples were smaller, were screened using 6 pools as indicated in Fig 1 A-C. For the healthy donors, responses to adjacent pools that were above the threshold were summed to create data comparative to the 6 pools used in recovered JE patients. Pools which did not reach the cut-off value were considered to be zero to prevent the creation of apparent responses by addition of two sub-threshold values.

Carboxyfluorescein succinimidyl ester (CFSE) labelling and proliferation assays

PBMC were used fresh or thawed and rested overnight. Eight to 10 million PBMC were labelled with CFSE (Molecular probes, Life Technologies) at a concentration of 1µM in 1ml pre-warmed PBS in the dark at 37°C for 10 minutes. Labelling was terminated by addition

of 5 volumes of ice cold R10 followed by incubation on ice for 3-4 minutes. Labelled cells were washed twice then plated at 0.5 million cells per well in 250µl sRPMI with 10% human serum (Valley Biomedical, California, USA), and stimulated with peptide pools at a final concentration of 3µg/ml. The positive control was concanavalin A at a final concentration of 2.5µg/ml, negative control was DMSO at equivalent concentration to the peptide pools. Cells were incubated for 8 days with removal and replacement of 75µl medium for cytokine assay after 48 h and 5 days. After incubation the cells were stained with near infra-red (IR) cell viability marker (Molecular probes, Life Technologies), fixed in 2% formaldehyde in PBS then frozen at -80°C in 1% BSA, 10% DMSO in PBS until analysis by flow cytometry. Prior to analysis cells were stained with CD3 Alexa Fluor 700 (clone UCHT-1), CD4 PE (clone RPA-T4), CD8 APC (clone RPA-T8) and CD38 PE-Cy7 (clone HIT2); all from BD Pharmingen. Responses were considered positive if there were at least 1% of cells in the $CD4^+$ or $CD8^+$ gate were $CFSE^{lo}/CD38^{hi}$ and this value was at least double the background.

Ex-vivo peptide stimulation and intracellular cytokine staining (ICS)

Whole blood was stimulated with JEV peptide pools in the presence of 10µg/ml brefeldin A (Sigma Aldrich) and incubated for 6h in a water bath at 37°C using the field-suitable method of Hanekom *et al.* that does not require a CO_2 incubator (Hanekom et al., 2004). Power interruptions were frequent at VIMS but the ambient temperature was 34-35°C, so the water bath maintained ≥36°C, within the normal range of human body temperature (Mackowiak et al., 1992). After 6 hours the stimulation was stopped by placing the tubes on ice followed by transport back to Bangalore the same night. Subjects studied in Bangalore followed an identical protocol with a 6 hour stimulation terminated by placing the samples at 4°C overnight. Red blood cells were lysed the following morning. Freshly isolated PBMC were stimulated with peptides overnight, 10µg/ml brefeldin A was added after 1 hour. PMA (25ng/ml) and ionomycin (250ng/ml) (both Sigma Aldrich) served as the positive control; the negative control was the equivalent concentration of DMSO to the peptide pools. Cells stimulated by either method were stained with near IR cell viability marker then fixed and frozen prior to analysis. Cells were permeabilised (BD Perm/Wash) and stained with CD3 (clone UCHT-1), CD4 (clone RPA-T4), CD8 (clone RPA-T8), IFNγ (clone 4S.B3), TNFα (clone MAb11), IL2 (clone 5344.111), macrophage inflammatory protein (MIP)-1β (clone D21-1351) and CD14 APC-Cy7 (clone MΦP9, dump channel). In

some experiments CD107a (clone H4A3) was added at the start of the stimulation to assess for degranulation (Betts et al., 2003). Anti-MIP-1β was from R&D systems, all other antibodies were from BD Pharmingen. Cells were acquired on a BD Canto II cytometer with photomultiplier tube voltages set using a single batch of setup & tracking beads (BD Bioscience) throughout. Data were analysed using FlowJo v8.8.6 (TreeStar), polyfunctional T cell distributions were analysed and presented using SPICE v5.35, with pre-processing in Pestle v1.7 (Roederer et al., 2011). The SPICE statistical function "compare pies" was used for comparison of T cell function patterns (Fig 6 C).

Whole blood ICS (WB ICS) responses were considered positive if the responding population was at least 0.02% of the parent gate and double the background (example data are given in Fig 2 A). Background was subtracted prior to analysis.

Expansion of short term T cell lines

Short term T cell lines were expanded to JEV peptide pools or individual peptides showing *ex vivo* responses. Fresh or frozen rested PBMC were stimulated with peptide pools at 3µg/ml (up to a maximum of 1% DMSO). Where responding peptides were known, 10µg/ml peptide was used to expand cells for cross-reactivity assays. Cells were cultured at 2 million cells per well in 24 well plates in 1ml of RPMI with 10% human serum, 10% Natural T cell growth factor/IL2 ("T-stim") (Helvetica healthcare) and 20ng/ml IL7 (R&D systems) for 8 to 10 days. Before assay the cells were rested overnight in R10 then stimulated with peptides or pools of peptides for 6 hours in the presence of brefeldin A. Cells were fixed, permeablised, stained and analysed by flow cytometry using the same method as for *ex vivo* ICS.

Cytotoxicity assay

Cytotoxicity assays were conducted as previously described (Kurioka et al., 2015). Briefly, appropriately HLA matched peptide loaded B cell lines were used as target cells and labelled with CFSE according to the manufacturer's protocol. Cells were pulsed with peptide at the indicated concentrations for one hour followed by three washes with R10. Non-peptide pulsed cells were labelled with Cell Trace Violet (CTV, Molecular probes). Pulsed and unpulsed cells were mixed in a 1:1 ratio and incubated with peptide specific

short term T cell lines for 4 hours at an effector:target ratio of 10:1 in duplicates. CD107a (clone H4A3) was added at the start of the stimulation. Following incubation, cells were stained with near-IR viability marker, CD3 (clone UCHT1; eBioscience), CD8 (clone RPA-T8; eBioscience), and CD19 (clone LT19; Miltenyi Biotec). The average % survival of CFSE labelled cells in wells containing no effectors was used to calculate the expected frequency of target cells in each well: expected ratio (ER) was calculated as % $CFSE^+$/% CTV^+. The specific killing was then calculated as:

% Specific killing = 100 x [(ER x % CTV^+ cells) - % $CFSE^+$ cells]/(ER x % CTV^+ cells).

HLA typing

HLA typing was done using commercially available kits at the TTK Rotary blood bank, Bangalore (India) or the Churchill Hospital, Oxford (UK). Not all subjects in the cohort were HLA typed, typing focused on those subjects where peptides were mapped. HLA class I A, B and class II D related (DR) typing was done on subjects who showed predominantly CD4 responses, and HLA class I A, B and C typing was done on those showing predominantly CD8 responses.

HLA class I peptide tetramers

HLA class I peptide tetramers were supplied by the NIH tetramer core facility. JEV peptide-tetramers were labelled with PE and variant peptide-tetramers were labelled with APC. The reagents used were: HLA B*58:01-MTTEDMLQVW-PE (JEV NS5 809 - 818), HLA B*58:01-MTTEDMLSVW-APC (DENV1 NS5 803 - 812), HLA B*58:01-MTTEDMLTVW-APC (DENV2/3 NS5 804 - 813) and HLA B*58:01-MTTEDMLEVW-APC (WNV NS5 808 - 817). Fresh viable PBMC or T cell lies were stained for 30 minutes at 37°C before being stained with cell viability maker and other surface antibodies as described in the ICS method. Cells were fixed prior to analysis on a BD LSR II cytometer.

Bead array

Supernatants collected from proliferation assays on day 2 and day 5 were assayed using the Bio-Plex 14-plex human cytokine/chemokine kit (Bio-Rad) on the Bio-Plex 200 platform

according to the manufacturers instructions. Supernatants were selected on the basis of the largest proliferation responses per subject. Responses were considered positive if the value secreted was at least double the amount detected from unstimulated cells and the background was subtracted before analysis.

Viruses and cell lines

JEV 0423 (derived from SA_{14}-14-2), DENV1 16007, DENV2 16681, DENV3 16562, DENV4 C0036/06, C6/36 cells and LLC-monkey kidney $(MK)_2$ cells for use in 50% plaque reduction neutralisation titre ($PRNT_{50}$) determination were supplied by AFRIMS virology dept, Bangkok. JEV P20778, used for screening assays, and PS cells were grown in house at NIMHANS, Bangalore. LLC-MK_2 cells were maintained in M199 medium, C6/36 and PS cells were maintained in minimum essential medium (MEM). All media were supplemented with 2mM L-glutamine, 10% FCS and 100U/0.1mg/ml penicillin/streptomycin. LLC-MK_2 cells and PS cells were maintained at 37°C with 5% CO_2, C6/36 cells were maintained at 28°C with 5% CO_2. All viruses were passaged once prior to use in C6/36 cells, titred and stored in aliquots at -80°C. Epstein Barr virus transformed HLA types B cell lines for HLA restriction experiments were kindly provided by Prof. Philip Goulder, University of Oxford, Oxford, UK.

Plaque reduction neutralisation titres

Sera of healthy donors were screened for JEV NAb by incubating 2-fold dilutions down to 1 in 16 of heat inactivated sera with 100 plaque forming units (PFU) of JEV P20778 for one hour at 37°C. Mixtures were inoculated onto monolayers of PS cells with rocking at 37°C for one hour to allow virus adsorption followed by removal of inoculum and addition of minimum essential medium (MEM) with 2% FCS and 1% low melting point (LMP) agarose. After three days, destruction of the monolayer was assessed by crystal violet staining after fixing with 10% formalin in saline for 30 minutes. $PRNT_{50}$ were subsequently assayed in a subset by a standard method (Russell et al., 1967). Briefly, heat inactivated sera were diluted serially four-fold from 1 in 10 to 1 in 2650, incubated with 30-50 PFU of JEV or DENV1 to 4 at 37°C for one hour then inoculated onto duplicate monolayers of LLC-MK_2 cells with rocking at room temperature (25-28°C) for one hour followed by removal of inoculum and addition of 1ml phenol red free overlay medium with 0.9% LMP

agarose. Plates were incubated for 4 days (JEV, DENV1 and DENV3), 6 days (DENV4) or 7 days (DENV2) prior to addition of a further 1ml overlay medium containing 4% neutral red. Plaques were visualised and counted after a further 24 hours incubation. $PRNT_{50}$ was calculated by probit regression.

Statistical and bioinformatic methods

All statistical analyses were done using R version 3.1.2 (www.r-project.org). Differences between non-parametric unpaired variables were assessed with a two-sided Mann Whitney U (Wilcoxon rank sum) test. Categorical variables across groups were assessed by Fisher's exact test. Correlations between *ex-vivo* and *in-vitro* expanded T cell responses were assessed by Spearman's test. P values < 0.05 were considered significant. Shannon entropy of aligned JEV and DENV polyprotein sequences was calculated using the protein variability server of the Immunomedicine group, Universidad Complutense, Madrid (http://imed.med.ucm.es/PVS/) (Garcia-Boronat et al., 2008). Sequence data were obtained from the NIAID Virus Pathogen Database and Analysis Resource (ViPR) online through the web site at http://www.viprbrc.org by searching for all JEV and DENV complete genomes restricted to India as country of origin (Pickett et al., 2012). The polyprotein sequences of the JEV and BEI resources DENV1 to 4 peptide libraries used were also included. Nucleic acid sequences were downloaded, edited to remove 5' and 3' untranslated regions and translated using SeaView v4.4.2 (Gouy et al., 2010). Polyprotein alignments were performed using ClustalW (Larkin et al., 2007). HLA-peptide binding predictions were conducted using the software of the Bioinformatics and Molecular Analysis Section (BIMAS), NIH (http://www-bimas.cit.nih.gov/molbio/hla_bind/); SYFPEITHI (http://www.syfpeithi.de/) and HLArestrictor 1.2 Server (http://www.cbs.dtu.dk/services/HLArestrictor/) (Erup Larsen et al., 2011; Parker et al., 1994; Rammensee et al., 1999).

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Figures

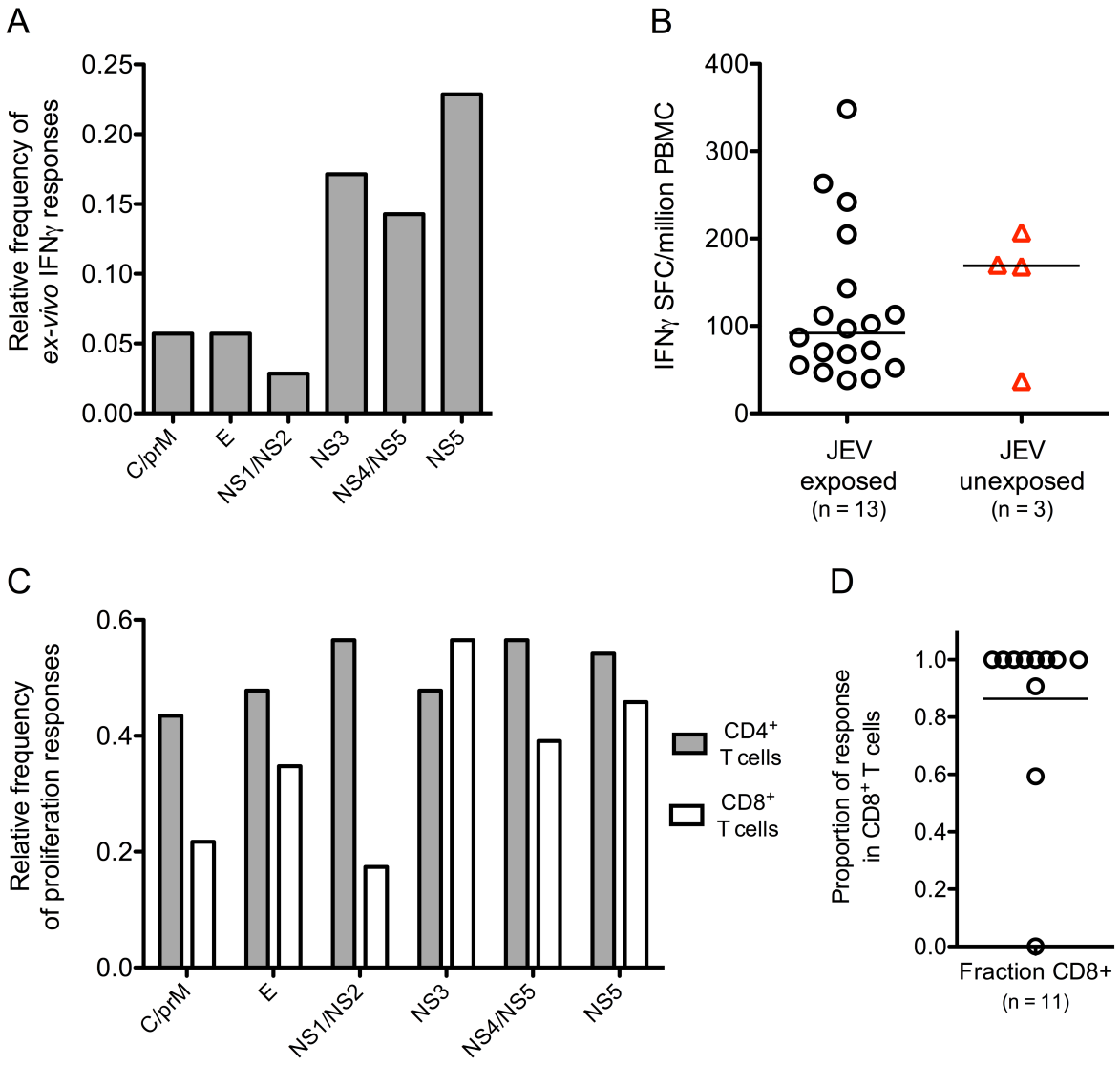

Figure 1. Breadth of the T cell response to peptide pools of JE virus in JEV-exposed healthy donors.

(A) Relative frequency of *ex-vivo* IFNγ responses were measured by ELISpot or intracellular cytokine staining (ICS) in JEV-exposed healthy donors (n = 35, 29 ELISpot and 6 ICS). Peptide pools are shown grouped by viral proteins (C = core, prM = pre membrane, E = envelope, NS = non-structural protein). For a subset of 5 subjects ICS and ELISpot was performed at least three times with consistent results. (B) Spot forming cells (SFC) per million PBMC were measured by ELISpot in 13 healthy JEV-exposed donors (18 responses, black circles) and three DENV exposed subjects (four responses, red triangles). (C) Proliferative responses were measured by CFSE dilution and flow cytometry

in healthy JEV-exposed donors once per subject. Data are relative frequency (n = 24) for $CD4^+$ (grey bars) and $CD8^+$ T cells (open bars). (D) Based on data from ICS assays the proportion of the total IFNγ response produced by $CD8^+$ T cells in each healthy JEV-exposed donor was calculated; bar depicts the median.

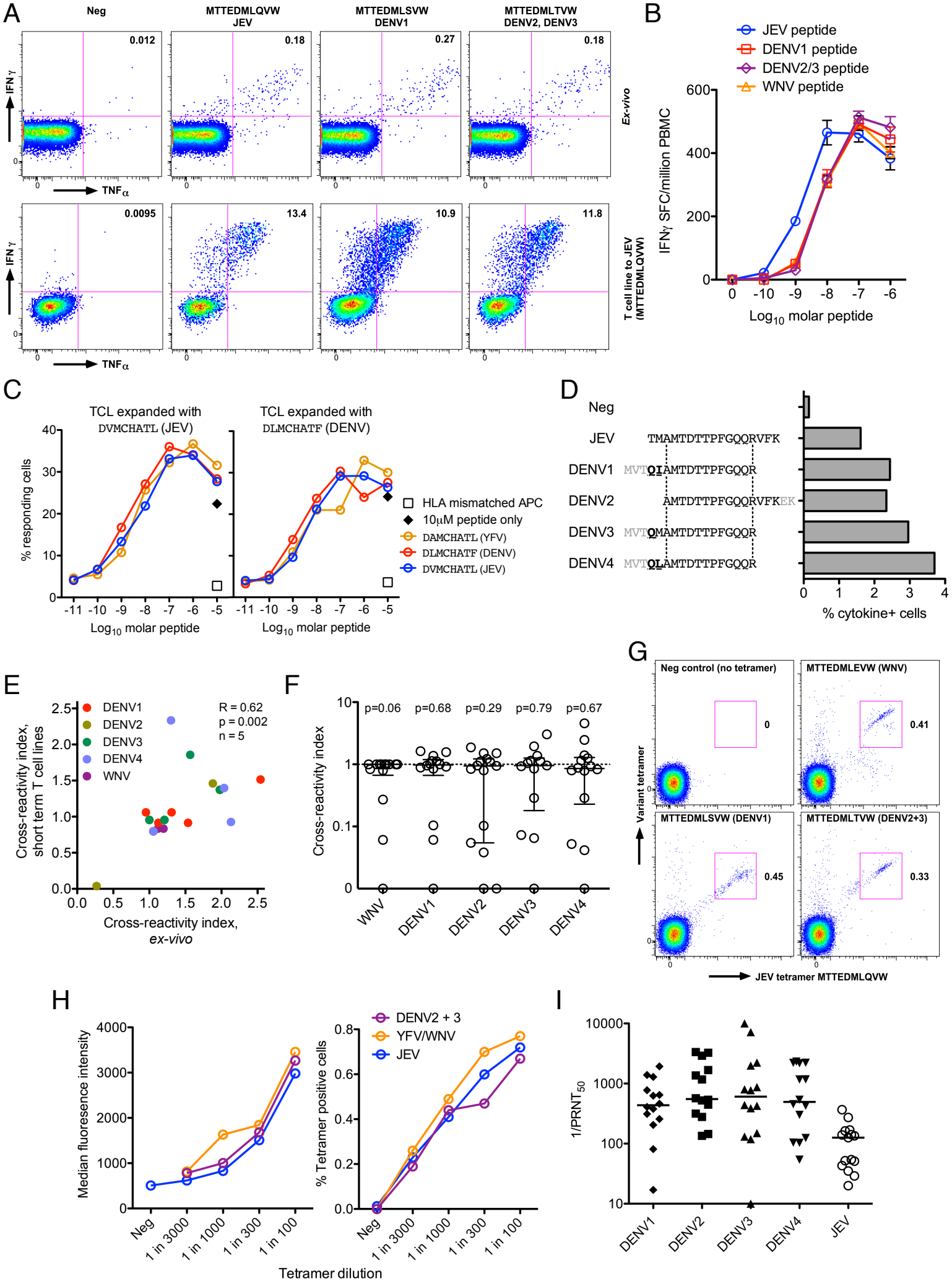
A
Neg
MTTEDMLQVW
JEV
MTTEDMLSVW
DENV1
MTTEDMLTVW
DENV2, DENV3
0.012
0.18
0.27
0.18
0.0095
13.4
10.9
11.8
IFN γ
TNFα
Ex-vivo
T cell line to JEV
(MTTEDMLQVW)
B
JEV peptide
DENV1 peptide
DENV2/3 peptide
WNV peptide
IFNγ SFC/million PBMC
Log10 molar peptide
C
TCL expanded with
DVMCHATL (JEV)
TCL expanded with
DLMCHATF (DENV)
% responding cells
Log10 molar peptide
HLA mismatched APC
10μM peptide only
DAMCHATL (YFV)
DLMCHATF (DENV)
DVMCHATL (JEV)
D
Neg
JEV
DENV1
DENV2
DENV3
DENV4
TMAMTDTTPFGQQRVFK
MVTQLAMTDTTPFGQQR
AMTDTTPFGQQRVFKEK
MVTQMAMTDTTPFGQQR
MVTQLAMTDTTPFGQQR
% cytokine+ cells
E
DENV1
DENV2
DENV3
DENV4
WNV
R = 0.62
p = 0.002
n = 5
Cross-reactivity index,
short term T cell lines
Cross-reactivity index,
ex-vivo
F
p=0.06
p=0.68
p=0.29
p=0.79
p=0.67
Cross-reactivity index
WNV
DENV1
DENV2
DENV3
DENV4
G
Neg control (no tetramer)
MTTEDMLEVW (WNV)
MTTEDMLSVW (DENV1)
MTTEDMLTVW (DENV2+3)
0
0.41
0.45
0.33
Variant tetramer
JEV tetramer MTTEDMLQVW
H
Median fluorescence intensity
% Tetramer positive cells
DENV2 + 3
YFV/WNV
JEV
Neg
1 in 3000
1 in 1000
1 in 300
1 in 100
Tetramer dilution
I
1/PRNT50
DENV1
DENV2
DENV3
DENV4
JEV

Figure 2. CD8$^+$ T cell responses are highly flavivirus cross-reactive in healthy JEV-exposed donors.

(A) ICS assays were used to detect IFNγ$^+$/TNFα$^+$ cells from healthy JEV-exposed donor H008/4. Example flow cytometry data from an *ex-vivo* assay (top panels) and a short term T cell line (bottom panels) show responses to variant peptides of JEV NS5 MTTEDMLQVW, gated on live, CD3$^+$, CD8$^+$ cells, representative of three experiments. Similar results were obtained with DENV4 and West Nile virus (WNV) peptides (not depicted). Axes are $\log_{10}$ fluorescence units. (B) IFNγ responses to peptide titrations of the same NS5 peptides in (A) and WNV peptide MTTEDMLEVW were measured by *ex-vivo* ELISpot, representative of two independent experiments. (C) Cytokine (IFNγ$^+$, TNFα$^+$ or MIP-1β$^+$ in any combination) responses to NS3 peptide titrations of JEV, DENV1-4 and YFV presented on a B cell line (BCL) matched for HLA B*08:01 were measured by ICS. Responding cells were CD8$^+$ T cell lines (TCL) from a subject reporting dengue illness, yellow fever vaccination but not JEV exposure (H001/4), expanded with JEV (left panel) or DENV (right panel) peptides, each assayed against all three peptides. Black diamonds indicate peptide with no BCL. Open squares indicate a peptide pulsed HLA-mismatched BCL. Peptide titrations by *ex-vivo* ELISpot or ICS gave similar results and expansion of T cell lines from two further independent samples showed equal cross-reactivity. (D) IFNγ$^+$/TNFα$^+$ cells of a CD8$^+$ T cell line from healthy JEV-exposed donor H007/3 were measured by ICS to a JEV NS5 peptide and the DENV variant peptides indicated, showing epitope conservation in variant peptides. The same experiment was performed twice *ex-vivo* with similar results. (E) Cross-reactivity of *ex-vivo* responses and short term T cell lines were measured by ICS. Cross-reactivity index (variant response ÷ JEV response from the same assay) between JEV and DENV1-4/WNV in 5 subjects of *ex-vivo* measurements correlated with T cell lines (Spearman's $R = 0.62$, $p = 0.002$). *Ex-vivo* assays were performed at least twice in all donors except one with similar results. (F) Cross-reactivity of short term T cell lines expanded with JEV peptides with WNV and DENV1-4 was measured by ICS. Data are cross-reactivity indices as in (E) of all T cell responses identified in healthy JEV-exposed donors. Wilcoxon signed rank tests showed no significant differences from a hypothetical value of 1 (indicating an equal response to JEV and variant peptides); p = 0.06 (WNV), 0.68 (DENV1), 0.29 (DENV2), 0.79 (DENV3), 0.67 (DENV4). In two subjects these assays were repeated three times with good agreement. (G) CD8$^+$ T cells from donor H008/4 were stained with with JEV peptide-HLA class I tetramers (x axis) and variant peptide tetramers (y axis) and analysed by flow

cytometry. Data are representative of two independent experiments. (H) $CD8^+$ T cells were tetramer stained and analysed as in (G). Titrations of three different tetramers are shown: JEV (MTTEDMLQVW, blue), WNV (MTTEDMLEVW, yellow) and DENV2+3 (MTTEDMLTVW, purple, common epitope). Data are median fluorescence intensity (left) and $\%CD8^+$ T cells stained (right). Repeating this experiment using a short term T cell line expanded with JEV peptide gave similar results. (I) Neutralising antibodies against JEV and all four DENV serotypes were measured by 50% plaque reduction ($PRNT_{50}$). Bars depict the median; four subjects had some assays repeated with similar results.

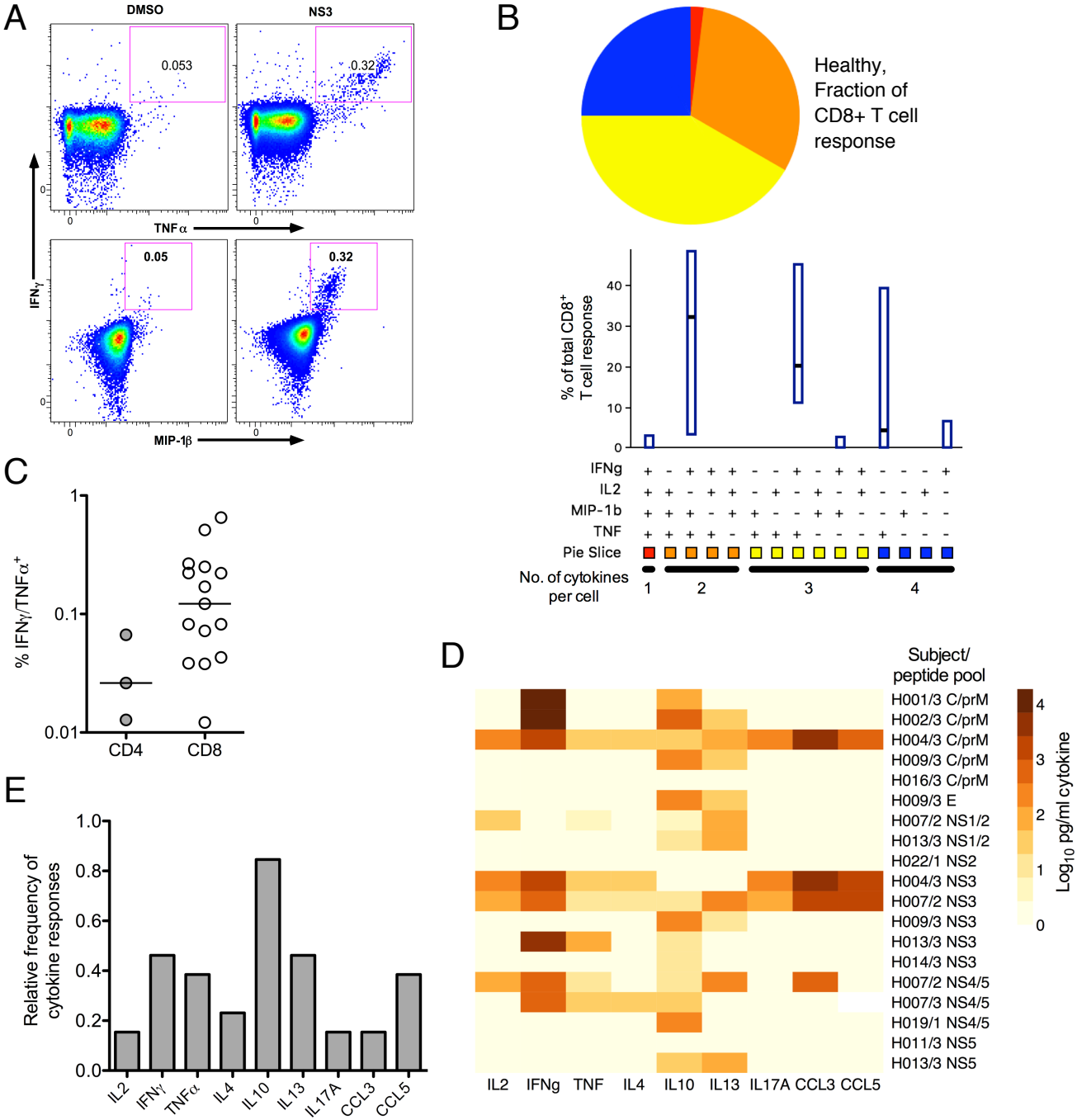
A
DMSO
NS3
0.053
0.32
TNFα
IFNγ
0.05
0.32
MIP-1β
B
Healthy,
Fraction of
CD8+ T cell
response
% of total CD8+
T cell response
IFNg
IL2
MIP-1b
TNF
Pie Slice
No. of cytokines
per cell
1
2
3
4
C
% IFNγ/TNFα+
CD4
CD8
D
Subject/
peptide pool
H001/3 C/prM
H002/3 C/prM
H004/3 C/prM
H009/3 C/prM
H016/3 C/prM
H009/3 E
H007/2 NS1/2
H013/3 NS1/2
H022/1 NS2
H004/3 NS3
H007/2 NS3
H009/3 NS3
H013/3 NS3
H014/3 NS3
H007/2 NS4/5
H007/3 NS4/5
H019/1 NS4/5
H011/3 NS5
H013/3 NS5
IL2
IFNg
TNF
IL4
IL10
IL13
IL17A
CCL3
CCL5
Log10 pg/ml cytokine
E
Relative frequency of
cytokine responses
IL2
IFNγ
TNFα
IL4
IL10
IL13
IL17A
CCL3
CCL5

Figure 3. Function of T cell responses to peptide pools of JE virus in healthy JEV-exposed donors.

(A) Whole blood ICS (WB ICS) assays were used to measure cytokine$^+$ cells from 11 healthy JEV-exposed donors. Example data from a WB ICS experiment (donor H014/3) are shown. Axes are $\log_{10}$ fluorescence units. Top panels: IFNγ vs TNFα, bottom panels: IFNγ vs MIP-1β. (B) WB ICS data were analysed using Simplified Presentation of Incredibly Complex Evaluations (SPICE) software for ten donors; one donor did not have the full flow cytometry panel performed. Bars indicate median and interquartile range (IQR); pie slices indicate the fraction of response of the group that expresses four cytokines (red), three (orange), two (yellow) and one (blue) in any combination. (C) Magnitude of $CD4^+$ and $CD8^+$ WB ICS $IFN\gamma^+/TNF\alpha^+$ responses in healthy JEV-exposed donors measured by ICS (n = 11, 15 $CD8^+$ and 3 $CD4^+$ T cell responses). IFNg, IFN-gamma; MIP-1b, MIP-1beta. (D) Secreted cytokine concentrations were measured by bead array from 12 healthy JEV-exposed donors after 5 days of stimulation with JEV peptide pools. The heatmap depicts $\log_{10}$ transformed pg/ml. Some subjects had >1 peptide pool assayed. Data are depicted after subtraction of values obtained from unstimulated cells. This experiment was performed once. (E) Relative frequency of secreted cytokine responses from the same data in (D) after 2 and 5 days of stimulation with JEV peptide pools. Subjects were considered positive if any pool was positive at either time-point.

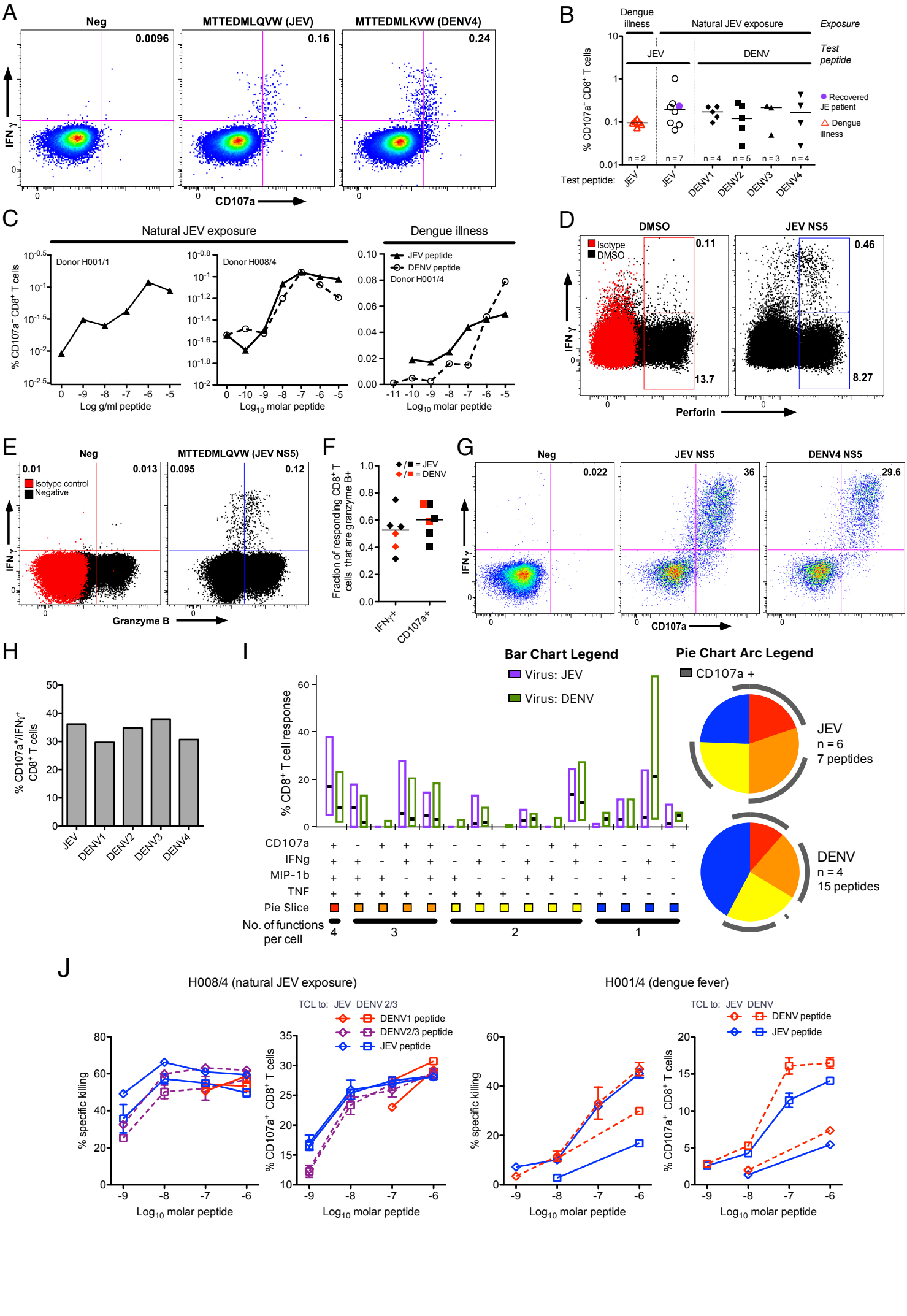
A
Neg
MTTEDMLQVW (JEV)
MTTEDMLKVW (DENV4)
0.0096
0.16
0.24
IFN γ
CD107a
B
Dengue illness
Natural JEV exposure
Exposure
Test peptide
JEV
DENV
Recovered JE patient
Dengue illness
% CD107a+ CD8+ T cells
n = 2
n = 7
n = 4
n = 5
n = 3
n = 4
Test peptide: JEV JEV DENV1 DENV2 DENV3 DENV4
C
Natural JEV exposure
Dengue illness
Donor H001/1
Donor H008/4
Donor H001/4
JEV peptide
DENV peptide
% CD107a+ CD8+ T cells
Log g/ml peptide
Log10 molar peptide
Log10 molar peptide
D
DMSO
JEV NS5
Isotype
DMSO
0.11
13.7
0.46
8.27
IFN γ
Perforin
E
Neg
MTTEDMLQVW (JEV NS5)
0.01
0.013
0.095
0.12
Isotype control
Negative
IFN γ
Granzyme B
F
JEV
DENV
Fraction of responding CD8+ T cells that are granzyme B+
IFNγ+
CD107a+
G
Neg
JEV NS5
DENV4 NS5
0.022
36
29.6
IFN γ
CD107a
H
% CD107a+/IFNγ+ CD8+ T cells
JEV
DENV1
DENV2
DENV3
DENV4
I
Bar Chart Legend
Virus: JEV
Virus: DENV
Pie Chart Arc Legend
CD107a +
% CD8+ T cell response
CD107a
IFNg
MIP-1b
TNF
Pie Slice
No. of functions per cell
JEV
n = 6
7 peptides
DENV
n = 4
15 peptides
J
H008/4 (natural JEV exposure)
H001/4 (dengue fever)
TCL to: JEV DENV 2/3
DENV1 peptide
DENV2/3 peptide
JEV peptide
TCL to: JEV DENV
DENV peptide
JEV peptide
% specific killing
% CD107a+ CD8+ T cells
Log10 molar peptide

Figure 4. CD8$^+$ T cell responses from JEV-exposed donors show a cytotoxic phenotype.

(A) Degranulation of CD8$^+$ T cells in response to JEV and DENV variant peptides was measured by CD107a staining/flow cytometry. Representative data from one of two experiments in healthy JEV-exposed donor (H008/4). (B) Degranulation responses to variant flavivirus peptides were measured as in (A) in naturally JEV-exposed subjects (n = 7; 6 healthy, 1 recovered JE patient) and subjects reporting dengue illness (recovered JE and dengue illness are indicated). (C) Degranulation responses were measured as in (A) to titrations of JEV and DENV variant peptides in two healthy JEV-exposed donors (H001/1, left panel, H008/4, centre panel) and a subject reporting dengue illness (H001/4, right panel). (D, E) JEV specific CD8$^+$ T cells were identified by IFNγ ICS and co-stained for (D) perforin and (E) granzyme B. Representative data from one (perforin, JE054/2; granzyme B H008/4) of four subjects are shown. (F) IFNγ$^+$/granzyme B$^+$ and CD107a/granzyme B$^+$ double positive CD8$^+$ T cells were detected in the same experiments as (D & E) in one healthy JEV-exposed and two dengue-exposed donors in response to all peptides tested; four JEV (black) and two DENV peptides (red). (G) Degranulation of a short term T cell line from a healthy JEV-exposed donor (H007/2) in response to JEV and DENV variant peptides measured as in (A), representative of five donors tested. (H) Data from the same experiment as (G) showing cross-reactivity for DENV peptides. Cross-reactivity was observed in two independent experiments, one included CD107a staining. (I) CD8$^+$ T cell IFNγ, TNFα, MIP-1β, and CD107a responses were measured by ICS and analysed using SPICE. Six healthy JEV-exposed donors (7 JEV responses), four of whom had variant DENV peptides tested (15 DENV peptides), were included. In two subjects repeat experiments showed similar results. Bars indicate median and IQR, pie slices the fraction of response showing four (red), three (orange), two (yellow) and one (blue) function(s) in any combination. (J) Peptide pulsed, CFSE labelled, HLA matched targets were incubated with CD8$^+$ T cell line effector cells and % specific killing was measured by flow cytometry in response to JEV (blue) and DENV (red/purple) peptides. Peptides were: donor H001/4, DVMCHATL (JEV) and DLMCHATF (DENV); donor H008/4 MTTEDMLQVW (JEV), MTTEDMLSVW (DENV1) and MTTEDMLTVW (DENV2/3). Diamonds indicate lines expanded with JEV peptide, squares DENV peptide, assays were performed once for each T cell line/peptide pair.

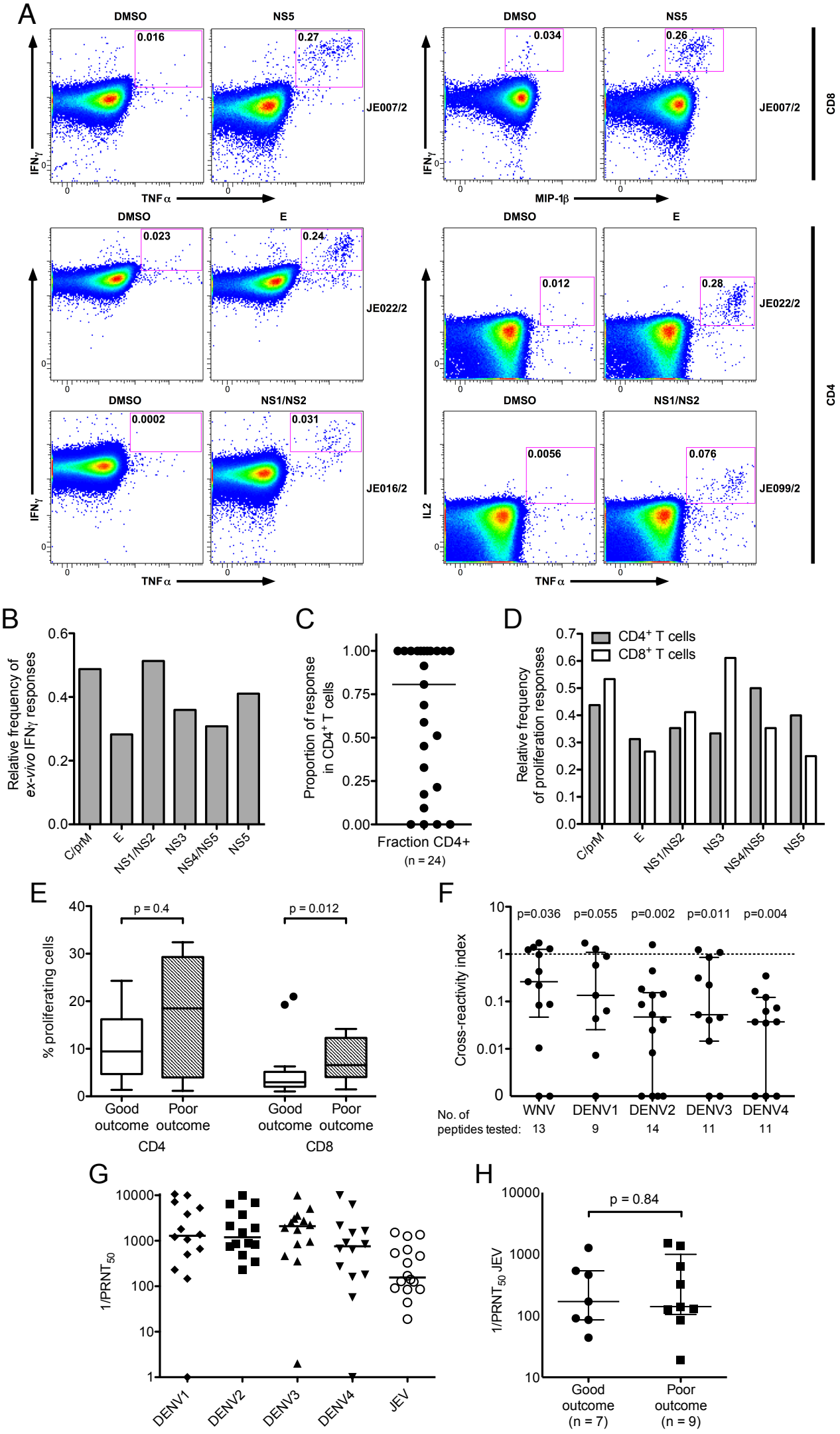
A
DMSO
NS5
0.016
0.27
JE007/2
IFNγ
TNFα
DMSO
NS5
0.034
0.26
JE007/2
CD8
IFNγ
MIP-1β
DMSO
E
0.023
0.24
JE022/2
DMSO
E
0.012
0.28
JE022/2
CD4
DMSO
NS1/NS2
0.0002
0.031
JE016/2
IFNγ
TNFα
DMSO
NS1/NS2
0.0056
0.076
JE099/2
IL2
TNFα
B
Relative frequency of ex-vivo IFNγ responses
C/prM
E
NS1/NS2
NS3
NS4/NS5
NS5
C
Proportion of response in CD4+ T cells
Fraction CD4+
(n = 24)
D
CD4+ T cells
CD8+ T cells
Relative frequency of proliferation responses
C/prM
E
NS1/NS2
NS3
NS4/NS5
NS5
E
% proliferating cells
p = 0.4
p = 0.012
Good outcome
Poor outcome
Good outcome
Poor outcome
CD4
CD8
F
Cross-reactivity index
p=0.036
p=0.055
p=0.002
p=0.011
p=0.004
No. of peptides tested:
WNV 13
DENV1 9
DENV2 14
DENV3 11
DENV4 11
G
1/PRNT50
DENV1
DENV2
DENV3
DENV4
JEV
H
p = 0.84
1/PRNT50 JEV
Good outcome (n = 7)
Poor outcome (n = 9)

Figure 5. Breadth of the T cell response to JEV peptide pools in recovered JE patients.

(A) WB ICS assays were used to measure cytokine$^+$ cells in recovered JE patients, example flow cytometry data from four patients are shown. Axes are $\log_{10}$ fluorescence units. Top panels: CD8$^+$ responses, IFNγ vs TNFα (left panels) IFNγ vs MIP-1β (right panels); bottom panels: CD4$^+$ responses, IFNγ vs TNFα (left panels) and IL2 vs TNFα (right panels). (B) *Ex-vivo* IFNγ responses from 39 recovered JE patients were measured by ELISpot (n = 17) or ICS (n = 22) and relative frequency of responses to each peptide pool was calculated. (C) Relative proportion of the cytokine response produced by CD4$^+$ T cells in individual patients was measured by ICS in the same experiments as (A) and (B); bar depicts the median. (D) Proliferation responses were measured by CFSE dilution and flow cytometry in 18 recovered JE patients of whom 15 showed responses; relative frequency for CD4$^+$ T cells (grey bars) and CD8$^+$ T cells (open bars) is shown. (E) Proliferative responses from the same experiments as (D) were equal in size between good outcome (open bars) and poor outcome (hatched bars) for CD4$^+$ cells (25 responses in 8 patients with good outcome and 13 responses in 4 patients with poor outcome, p = 0.4, Mann Whitney U test); CD8$^+$ responses were larger in poor outcome (23 responses in 8 patients with good outcome and 17 responses in 6 patients with poor outcome, p = 0.012). Bars depict median and IQR, whiskers 1.5 x IQR and outliers are shown as points. (F) Cross-reactivity of short term T cell lines expanded with JEV peptides with WNV and DENV1-4 was measured by ICS. Data are cross-reactivity indices (as per Fig 2 E) of 14 T cell responses identified in 7 recovered JE patients. Wilcoxon signed rank tests showed significant differences from a hypothetical value of 1 (i.e. the response to JEV and variant peptides was not equivalent) for all viruses except DENV1; p = 0.036 (WNV), 0.055 (DENV1), 0.002 (DENV2), 0.011 (DENV3), 0.004 (DENV4). (G) Neutralising antibodies against JEV and all four DENV serotypes were measured by 50% plaque reduction ($PRNT_{50}$). Bars depict the median; assays were generally performed once per person, nine subjects had some assays repeated with similar results. (H) JEV $PRNT_{50}$ were the same in JE patients with good (n = 9) and poor (n = 7) outcome (p = 0.84, Mann Whitney U test). Bars depict the median and IQR.

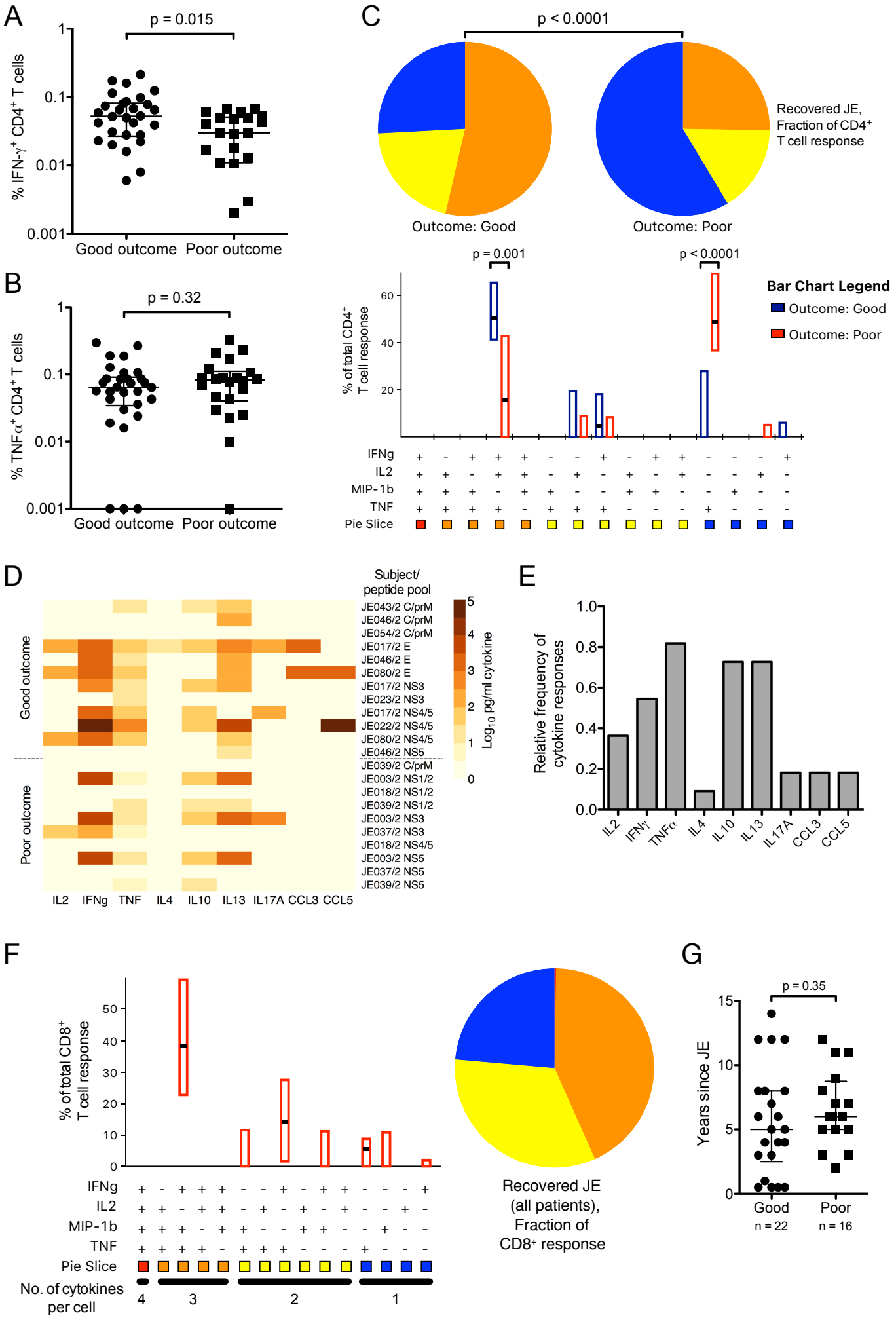
A
p = 0.015
% IFN-γ+ CD4+ T cells
1
0.1
0.01
0.001
Good outcome
Poor outcome
B
p = 0.32
% TNFα+ CD4+ T cells
1
0.1
0.01
0.001
Good outcome
Poor outcome
C
p < 0.0001
Outcome: Good
Outcome: Poor
Recovered JE, Fraction of CD4+ T cell response
p = 0.001
p < 0.0001
% of total CD4+ T cell response
60
40
20
Bar Chart Legend
Outcome: Good
Outcome: Poor
IFNg
IL2
MIP-1b
TNF
Pie Slice
D
Subject/ peptide pool
Good outcome
Poor outcome
JE043/2 C/prM
JE046/2 C/prM
JE054/2 C/prM
JE017/2 E
JE046/2 E
JE080/2 E
JE017/2 NS3
JE023/2 NS3
JE017/2 NS4/5
JE022/2 NS4/5
JE080/2 NS4/5
JE046/2 NS5
JE039/2 C/prM
JE003/2 NS1/2
JE018/2 NS1/2
JE039/2 NS1/2
JE003/2 NS3
JE037/2 NS3
JE018/2 NS4/5
JE003/2 NS5
JE037/2 NS5
JE039/2 NS5
IL2 IFNg TNF IL4 IL10 IL13 IL17A CCL3 CCL5
Log10 pg/ml cytokine
5
4
3
2
1
0
E
Relative frequency of cytokine responses
1.0
0.8
0.6
0.4
0.2
0.0
IL2
IFNγ
TNFα
IL4
IL10
IL13
IL17A
CCL3
CCL5
F
% of total CD8+ T cell response
50
40
30
20
10
0
IFNg
IL2
MIP-1b
TNF
Pie Slice
No. of cytokines per cell
4
3
2
1
Recovered JE (all patients), Fraction of CD8+ response
G
p = 0.35
Years since JE
15
10
5
0
Good
n = 22
Poor
n = 16

Figure 6. T cell cytokine responses to JEV peptide pools in recovered JE patients. Recovered JE patients showing $CD4^+$ T cell responses were categorised into good outcome (complete recovery, n = 16) and poor outcome (recovery with residual neurological deficit/disability > 6 months after disease, n = 11). Cytokine responses were measured by WB ICS once per subject; 30 responses were detected in the good outcome group and 22 responses in the poor outcome group. (A) IFNγ responses were smaller in those with poor outcome ($p = 0.015$, Mann Whitey U test). (B) TNFα responses showed no difference with outcome ($p = 0.32$, Mann Whitey U test). (C) $CD4^+$ T cell responses were analysed using SPICE according to outcome from JE. Patients with poor outcome had fewer polyfunctional cells ($p < 0.0001$, SPICE compare pies function, 10 000 replicates), with the difference largely accounted for by $IFN\gamma^+/TNF\alpha^+/IL2^+$ cells ($p = 0.001$, Mann Whitney U test); and more $TNF\alpha^+$ cells ($p < 0.0001$). Data in (C) were analysed for individual responses; summing by subjects retained the strong significance of the result. (D) Secreted cytokine concentrations were measured by bead array from 11 recovered JE patients after 5 days of stimulation with JEV peptide pools. The heatmap depicts $\log_{10}$ transformed pg/ml. Some subjects had >1 peptide pool assayed. Data are depicted after subtraction of values from unstimulated cells and are displayed according to outcome from JE (top, good outcome n = 7; bottom, poor outcome, n = 4). This experiment was performed once. (E) Relative frequency of secreted cytokine responses from the same experiments as (D) after 2 and 5 days of stimulation with JEV peptide pools. Subjects were considered positive if any pool was positive at either time point. (F) $CD8^+$ T cell responses from recovered JE patients were assayed once per subject by WB ICS (n = 14, 18 responses) and analysed using SPICE. (G) There was no difference in years since admission with clinical JE and sampling for this study between the good and poor outcome groups ($p = 0.35$, Mann Whitney U test).

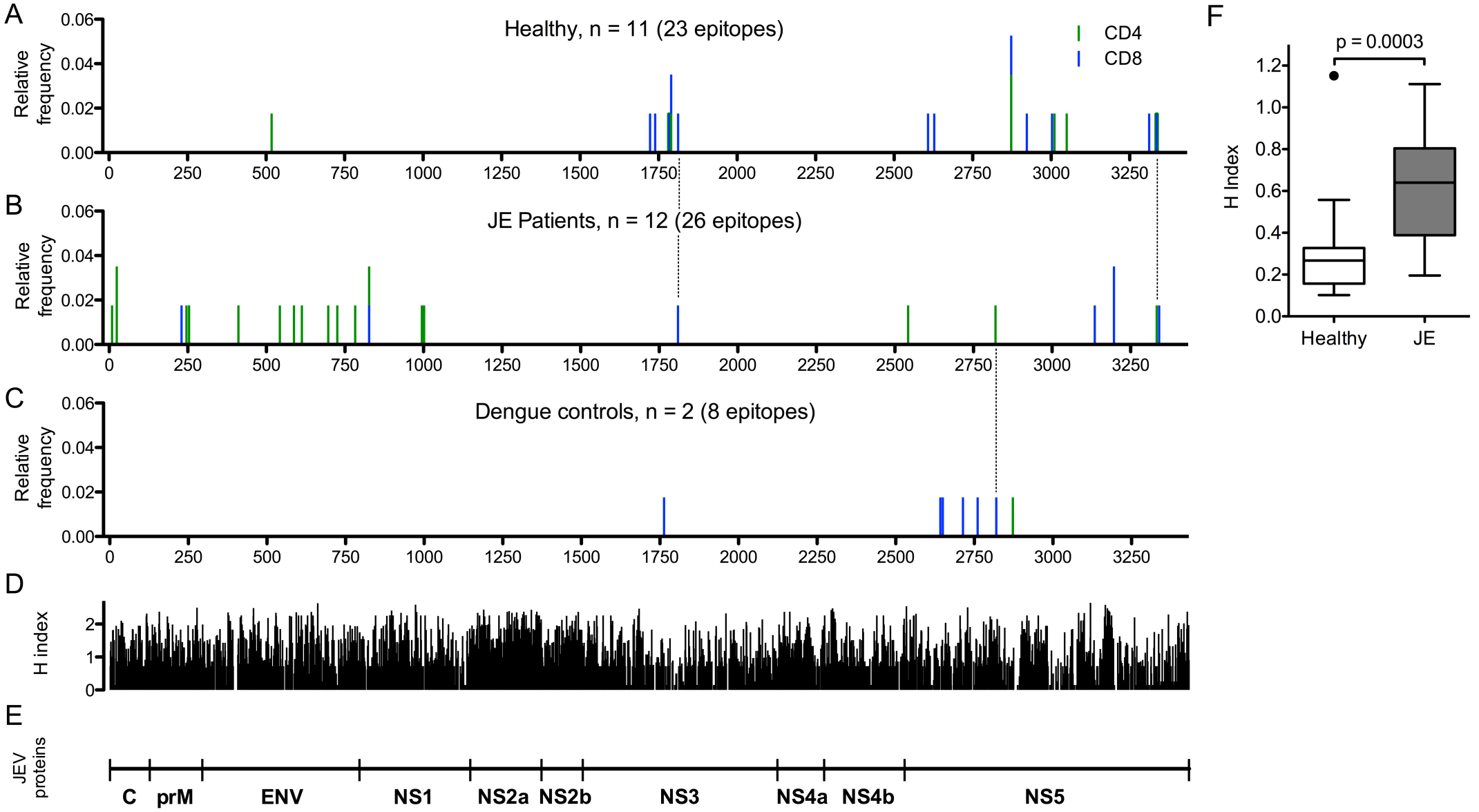
A
Healthy, n = 11 (23 epitopes)
CD4
CD8
Relative frequency
0.06
0.04
0.02
0.00
0 250 500 750 1000 1250 1500 1750 2000 2250 2500 2750 3000 3250
B
JE Patients, n = 12 (26 epitopes)
Relative frequency
0.06
0.04
0.02
0.00
0 250 500 750 1000 1250 1500 1750 2000 2250 2500 2750 3000 3250
C
Dengue controls, n = 2 (8 epitopes)
Relative frequency
0.06
0.04
0.02
0.00
0 250 500 750 1000 1250 1500 1750 2000 2250 2500 2750 3000 3250
D
H index
2
1
0
E
JEV proteins
C
prM
ENV
NS1
NS2a
NS2b
NS3
NS4a
NS4b
NS5
F
p = 0.0003
H Index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Healthy
JE

Figure 7. Peptide epitopes of JEV identified in healthy JEV-exposed donors, recovered JE patients and DENV exposed donors.

Peptide were identified in ICS assays of short term T cell lines expanded with peptide pools showing *ex-vivo* responses, or by *ex-vivo* ELISpot. Both peptides that have been mapped down to the minimal epitope (8 peptides) and library peptides of 15-18 amino acids in are shown. $CD4^+$ responses are green, $CD8^+$ blue. Location of peptide responses in (A) healthy JEV-exposed donors (n = 11), (B) recovered JE patients (n = 12) and (C) two subjects reporting dengue illness. Three peptides in common between JE patients and either healthy group are marked by dotted lines. (D) Shannon entropy (H index) was calculated using all the available DENV and JEV complete polyprotein sequences from India (13 DENV1 sequences, 10 DENV2 sequences, 8 DENV3 sequences, 5 DENV4 sequences and 8 JEV sequences), and the sequences of the DENV1-4 and JEV peptide libraries used in this study. The H index varies from 0, corresponding to a single conserved amino acid residue at that position, to 4.322, where all 20 amino acids are represented equally. (E) Schematic representation of JEV proteins corresponding to their size. (F) Average Shannon entropy (H Index) of flavivirus regions corresponding to 15 unique peptides identified in the healthy JEV-exposed group and 25 unique peptides identified in the recovered JE group (using the same virus polyprotein sequences in (D)) was significantly lower in the epitopes identified from recovered JE patients ($p = 0.0003$, Mann Whitney U test). Bars depict median and IQR, whiskers 1.5 x IQR and outliers are shown as points.

TABLE I. Characteristics of the study participants

		Recovered JE patients	Healthy, JEV NAb positive Indian controls	Healthy, JEV NAb negative Indian controls	UK cohort
N		39	35	16	13
Median age (range)		13 (1 -25)	26 (7 - 55)	25 (22 - 55)	26 (23 - 60)
Sex		22M, 17F	24M, 11F	10M, 6F	7M, 6F
Years since JE		6 (0.5 - 12)	NA	NA	NA
Number tested for DENV NAb		14	14	3	8
DENV NAb positive (any serotype)		14	14	3	3
Dengue illness		0	3	0	4
Residence in a JE endemic area		All	All	All	1
Travel to a JE endemic area		NA	NA	NA	8
JE vaccine		0	0	0	6[a]
Liverpool outcome score	5	24	NA	NA	NA
	4	5			
	3	7			
	2	3			

The Liverpool outcome score is a five-point score grading recovery from encephalitis in children and adolescents (Lewthwaite et al., 2010a). 5 = full recovery, 4 = minor disability, 3 = moderate disability, 2 = severe disability, 1 = death (not applicable here). Six subjects in the UK cohort received JE vaccine ([a]), four before and two during the study period.

TABLE II. JEV peptides eliciting T cell responses identified in this study

Subject	Peptide	Subset	JEV protein	Amino acid location	Type	HLA Restriction		HLA Class I		HLA Class II
							A	B	C	DR
JE082/2	GKNRAINMLKRGLPRVF	CD4	C	9 - 25	JE		11:01, 33:01	13:01, 44:02		DR12, DR14, DR52
	VFPLVGVKRVVMSLLDGR	CD4	C	24 - 41						
JE093/2	MLKRGLPRVFPLVGVKRVVMSLLDGR	CD4	C	16 - 41	JE		24:02, 24:02	13:01, 27:02		DR15, DR51
JE043/2	SLVNKKEAWLDSTKATRY	CD8	M	230 - 247	JE		01:01, 11:01	52:01, 57:01		DR3, DR15, DR52, DR51
	WIIRNPGYAFLAAVLGWM	CD4	M	254 - 271						
JE101/2	RYLMKTENWIIRNPGYAF	CD4	M	246 - 263	JE		30:01, 33:01	13:01, 44:02		DR7, DR53
JE017/2	AKFSCTSKAIGRTIQ	CD4	E	411 - 425	JE		03:01, 11:02	07:02, 35:01		DR4, DR11, DR52, DR53
	KQSVVALGSQEGGLHQAL	CD4	E	543 - 560						
	CRLKMDKLALKGTTYGMCTEKFSF	CD4	E	581 - 604						
	HGTVVIELSYSGSDGPCK	CD4	E	613 - 630						
	LGKAFSTTLKGAQRLAAL	CD4	E	697 - 714						
	WDFGSIGGVFNSIGKAVHQVFGGAF	CD4	E	719 - 735						
	GVLVFLATNVHADTGCAI	CD4	E	783 - 801						
H021/1	PWTSPSSTAWRNRELLM	CD4	E	518 - 534	HC		02:01, 11:01	07:02, 52:01		DR15, DR51
JE003/2	KYLPETPRSLAKIVHK	CD4 + CD8	NS1	827 - 842	JE		03:01, 33:01	15:01, 37:01		DR10, DR15, DR51
	AVHSDLSYWIESRYNDTWKLERAVF	CD4	NS1	987 - 1011						
	WIESRYNDTWKLERAVFGEVKSCTW	CD4	NS1	995 - 1019						
JE001/2	EAWVDRYKYLPETPRSLAKIVHK	CD4	NS1	820 - 842	JE		11:01, 31:01	35:01, 52:21		DR4, DR15, DR53, DR51
	TYALNTFTNIAVQLVRLM	CD8	NS5	3136 - 3153						
H014/3	TAVLAPTRVVAAEMAEAL	CD8	NS3	1723 - 1740	HC		03:01, 30:01	07:02, 13:01	06:02, 07:01	
H001/1	ALRGLPVRY	CD8	NS3	1739 - 1747	HC		11:01, 32:01	15:01, 55:01	01:02, 04:01	
H001/4	DVMCHATL	CD8	NS3	1763 - 1770	D	B*08:01	01:01, 03:01	08:01, 44:02	05:01, 07:01	DR8, DR17, DR52
H019/1	NLFVMDEAHFTDPASI	CD8	NS3	1784 - 1799	HC		02:01	38:01, 40:06	07:01, 15:02	
	EAHFTDPASIAARGYI	CD8	NS3	1790 - 1805						
H013/3	NLFVMDEAHFTDPASI	CD4	NS3	1784 - 1799	HC		02:01, 24:02	35:03, 40:06	02:21, 15:02	
	EAHFTDPASIAARGYI	CD4 + CD8	NS3	1790 - 1805						
	GEAAAIFMT	CD8	NS3	1812 - 1820						
JE098/2	ELGEAAAIFMTATPP	CD8	NS3	1810 - 1824	JE		02:01	35:01, 40:06	04:01, 07:01	
H005/1	GCGRGGWSYYAATLKKV	CD8	NS5	2608 - 2624	HC		02:01, 30:01	13:01, 38:01	06:02, 12:02	
	RGYTKGGAGHEEPMLM	CD8	NS5	2628 - 2643						
H010/4	LMQSYGWNL	CD8	NS5	2642 - 2650	D		01:01, 02:01	08:01, 35:01	04:01, 07:01	
	LVSLKSGVDVFYKPS	CD8	NS5	2650 - 2664						
	YMPKVIEKMEV	CD8	NS5	2714 - 2724						
	NMTSQVLLGRMDRTVWR	CD8	NS5	2761 - 2777						
	HPYRTWTY	CD8	NS5	2827 - 2834		B*35:01				
	TPFGQQRVF	CD4 + CD8	NS5	2873 - 2884						
JE080/2	KLNAMSREEFFKYRREAIIEVDR	CD4	NS5	2542 - 2564	JE		02:01, 33:01	15:05, 44:02		DR7, DR53
	TWHKDPEHPYRTWTYHGSYEVK	CD4	NS5	2820 - 2841						

H007/2	TMAMTDTTPFGQQRVFK	CD4 + CD8	NS5	2869 - 2885	HC		01:01, 68:01	57:01		DR53, DR7
H011/3	TMAMTDTTPFGQQRVFK	CD8	NS5	2869 - 2885	HC		02:01, 68:01	37:01, 51:01	06:02, 15:02	
H007/3	TMAMTDTTPFGQQRVFK	CD4 + CD8	NS5	2869 - 2885	HC		11:01, 68:01	07:02, 52:02	07:01, 12:02	
	CTKEEFIKKVNSNAAL	CD8	NS5	2923 - 2938						
	DIAGKQGGKMYADDTAGW	CD4	NS5	3050 - 3067						
H002/3	IWFMWLGARY	CD8	NS5	3003 - 3012	HC		01:01, 29:01	07:05, 57:01	06:02, 15:04	
	RYLEFEALGFL	CD4	NS5	3011 - 3021						
JE054/2	AISGDDCVVKPLDDRFATALHFL	CD8	NS5	3191 - 3213	JE		11:01, 24:02	15:25, 51:01	07:01, 16:01	
JE074/2	CVVKPLDDRFATALHFL	CD8	NS5	3197 - 3213	JE					
H008/4	MTTEDMLQVW	CD4 + CD8	NS5	3336 - 3345	HC	B*58:01	01:01, 33:03	58:01, 15:17	07:01, 03:04	DR13, DR15, DR51, DR52
	CSAVPVDW	CD8	NS5	3312 - 3319		B*58:01				
JE446/2	GEWMTTEDMLQVWNRVWI	CD4	NS5	3333 - 3350	JE		01:01, 33:01	52:01, 58:01		DR14, DR15, DR52, DR51
	MLQVWNRVWIEENEWMM	CD8	NS5	3341 - 3357						

In total 57 responses to 40 peptides (or adjacent pairs of peptides) were identified. JE = recovered JE patient; H = healthy JEV exposed donor; D = dengue exposed.

TABLE III. Variant peptides eliciting T cell responses.

JEV Peptide sequence	Subject type	Viruses with identical sequence	Subset	Variant	Virus/BEI peptide location
CSAVPVDW	H	DENV1	CD8	CS**S**VPVDW	MVEV
MTTEDMLQVW	H		CD8	MTTEDML**R**VW	JEV
				MTTEDML**S**VW	DENV1
				MTTEDML**T**VW	DENV2+3
				MTTEDML**K**VW	DENV4
				MTTEDML**E**VW	WNV, YFV
				MTTEDML**D**VW	TBEV
ALRGLPVRY	H	ZIKV	CD8	ALRGLP**I**RY	WNV, DENV2+4
NLFVMDEAHFTDPASI EAHFTDPASIAARGYI	H	DENV2+3	CD8	NYN**MII**MDEAHFTDPA	DENV1 NS3 p49
				IMDEAHFTDPASIA**R**RG	DENV1 NS3 p50
				NYNL**II**MDEAHFTDPASI	DENV2 NS3 p38
				NYNL**II**MDEAHFTDPA	DENV3 NS3 p48
				PNYNL**I**VMDEAHFTD	DENV4 NS3 p49
				L**I**VMDEAHFTDP**S**S**V**AA	DENV4 NS3 p50
				AHFTDP**S**S**V**AARGYIST	DENV4 NS3 p51
TMAMTDTTPFGQQRVFK	H	WNV	CD4+CD8	MVT**QI**AMTDTTPFGQQR[a]	DENV1 NS5 p60
				AMTDTTPFGQQRVFKEK[a]	DENV2 NS5 p61
				MVT**Q**MAMTDTTPFGQQR[a]	DENV3 NS5 p60
				MVT**QL**AMTDTTPFGQQR[a]	DENV4 NS5 p60
GEAAAIFMT	H	WNV, DENV1,3+4, ZIKV	CD8	MGEAAAIFMTATPPGSV[a]	DENV1 NS3 p54, DENV3 NS3 p52
				VEMGEAAAIFMTATPPG[a]	DENV4 NS3 p54
TAVLAPTRVVAAEMAEAL	H	WNV	CD8	NVRT**LI**LAPTRVVA**S**EM	DENV1 NS3 p38
				LRT**LI**LAPTRVVAAEM	DENV2 NS3 p30
				T**LI**LAPTRVVAAEM**E**EA	DENV3 NS3 p38, DENV4 NS3 p39
DVMCHATL	D	WNV, MVEV, SLEV	CD8	DLMCHATF	DENV1-4, ZIKV
				DAMCHATL	YFV
AVHSDLSYWIESRYNDTW	JE		CD4	NLA**I**HSDLSYWIESR**L**	WNV NS1 p26
WIESRYNDTWKLERAVF	JE		CD4	R**L**NDTWKLERAVLGEVK	WNV NS1 p28
				IES**EK**N**E**TWKL**A**RA**S**FI	DENV NS1 p36
KQSVVALGSQEGGLHQAL	JE		CD4	HATKQSV**I**ALGSQEG**A**LH	WNV E p34
				ALGSQEG**A**LHQALAGAI	WNV E p35
				VV**V**LGSQEG**AM**H**T**ALTG	DENV1 E p44
				HAKKQ**D**VV**V**LGSQEG**AM**H	DENV2 E p34
				VLGSQEG**AM**H**T**ALTGA	DENV2 E p35
				HAKKQ**E**VV**V**LGSQEG**AM**H	DENV3 E p35, DENV1[b]
				VLGSQEG**AM**H**T**ALTGA	DENV3 E p36,
				HAK**R**Q**D**VT**V**LGSQEG**AM**H	DENV4 E p36
				VLGSQEG**AM**H**S**ALAGA	DENV4 E p37
HGTVVIELSYSGSDGPCK	JE		CD4	HGTVV**L**EL**Q**Y**T**G**T**DGPCK	WNV E p44
LGKAFSTTLKGAQRLAAL	JE		CD4	HKSGSS**I**GKAF**T**TTLKGA	WNV E p55
				KAF**T**TTLKGAQRLAAL	WNV E p56
WDFGSIGGVFNSIGKAV	JE		CD4	WDFGS**V**GGVF**T**S**V**GKAVH	WNV E p59
GVFNSIGKAVHQVFGGAF				VF**T**S**V**GKAVHQVFGGAFR	WNV E p60
AISGDDCVVKPLDDRF CVVKPLDDRFATALHFL	JE		CD4+CD8	A**V**SGDDCVVKPLDDRFA	WNV NS5 p91+92[c]
				VVKPLDDRFAT**S**LHFLNA	
				ISGDDCVVKP**I**DDRFAT	DENV1 NS5 p115+116[c]
				VVKP**I**DDRFATAL**TA**LN	
				ISGDDCVVKPLDDRFA	DENV2 NS5 p116+117[c]
				CVVKPLDDRFA**S**AL**TA**L	
				MAISGDDCVVKPIDDRF	DENV3 NS5 p115+116[c]
				DCVVKP**I**DDRFA**N**AL**LA**	
				MAISGDDCVVKPLDERF	DENV4 NS5 p116+117[c]
				DCVVKPLD**E**RF**S**T**S**L**L**F	

Variant peptides were synthesised or obtained from BEI resources; their location within each peptide set (see materials and methods) is given in the last column. Bold/underlined amino acids represent differences from the JEV sequence. [a] = Likely to represent conserved epitope

DTTPFGQQR (see Fig 2 D). [b] = Identical viral sequences, but corresponding peptides vary slightly. [c] = Pairs of peptides tested together in the same assay. JE = recovered JE patient, H = healthy JEV exposed, D = dengue exposed. MVEV = Murray Valley encephalitis virus, WNV= West Nile virus, YFV = yellow fever virus, TBEV = tick borne encephalitis virus, SLEV = St. Louis encephalitis virus, ZIKV = Zika virus.