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VACCINES AND ANTIVIRAL AGENTS

Patterns of Cellular Immunity Associated with Experimental Infection with rDEN2Δ30 (Tonga/74) Support Its Suitability as a Human Dengue Virus Challenge Strain

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ABSTRACT A deletion variant of the dengue virus (DENV) serotype 2 (DENV2) Tonga/74 strain lacking 30 nucleotides from its 3′ untranslated region (rDEN2Δ30) has previously been established for use in a controlled human DENV challenge model. To evaluate if this model is appropriate for the derivation of correlates of protection for DENV vaccines on the basis of cellular immunity, we wanted to compare the cellular immune response to this challenge strain to the response induced by natural infection. To achieve this, we predicted HLA class I- and class II-restricted peptides from rDEN2Δ30 and used them in a gamma interferon enzyme-linked immunosorbent spot assay to interrogate $CD8^+$ and $CD4^+$ T cell responses in healthy volunteers infected with rDEN2Δ30. At the level of CD8 responses, vigorous *ex vivo* responses were detected in approximately 80% of donors. These responses were similar in terms of the magnitude and the numbers of epitopes recognized to the responses previously observed in peripheral blood mononuclear cells from donors from regions where DENV is hyperendemic. The similarity extended to the immunodominance hierarchy of the DENV nonstructural proteins, with NS3, NS5, and NS1 being dominant in both donor cohorts. At the CD4 level, the responses to rDEN2Δ30 vaccination were less vigorous than those to natural DENV infection and were more focused on nonstructural proteins. The epitopes recognized following rDEN2Δ30 infection and natural infection were largely overlapping for both the CD8 (100%) and CD4 (85%) responses. Finally, rDEN2Δ30 induced stronger CD8 responses than other, more attenuated DENV isolates.

IMPORTANCE The lack of a known correlate of protection and the failure of a neutralizing antibody to correlate with protection against dengue virus have highlighted the need for a human DENV challenge model to better evaluate the candidate live attenuated dengue vaccines. In this study, we sought to characterize the immune profiles of rDEN2Δ30-infected subjects and to compare the profiles with those for subjects from areas where DENV is hyperendemic. Our data demonstrate that T cell responses to rDENV2Δ30 are largely similar to those to natural infection in terms of specificity, highlighting that the response to this virus in humans is appropriate as a model for the T cell response to primary DENV2 infection.

KEYWORDS T cells, dengue virus, human challenge model, vaccine

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The impact of dengue virus (DENV) on human health has dramatically increased in recent years. About 2.5 billion people are at risk of dengue virus transmission, and in more than 100 countries the infection is endemic (WHO and CDC). Primary (first-time) infection with one the four DENV serotypes is often associated with a course of disease relatively less severe than that of secondary infections mediated by different serotypes. Indeed, the development of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) is most frequently associated with such heterologous secondary infections.

Accordingly, the development of a DENV vaccine that simultaneously induces immunity against all four DENV serotypes is of great importance. Several dengue vaccines are currently under clinical evaluation. Among them, Dengvaxia is a live attenuated tetravalent (LATV), chimeric, three-dose vaccine based on the expression of the premembrane (prM) and envelope (E) proteins of DENV serotype 1 (DENV1) to DENV4 within the backbone of the yellow fever virus 17D vaccine strain. Dengvaxia induced neutralizing antibodies against the four dengue virus serotypes, but recent trials have shown variable efficacy against symptomatic dengue and significantly lower efficacy in subjects who were immunologically naive to dengue virus prior to vaccination (1–3). Nonetheless, the Dengvaxia vaccine has been implemented on a restricted basis in some countries and populations. A vaccine that only partially protects from dengue virus infection may be of concern because of potential safety issues, increasing the risk of development of severe dengue disease in areas of endemicity, particularly where rates of transmission are low. In fact, there was a significantly higher risk of hospitalization due to subsequent dengue virus infection in only year 3 of the study for Dengvaxia recipients who were 2 to 5 years of age at the time of vaccination compared to the risk for controls (4).

While no firm correlate of protection against dengue virus has been defined (5), it is possible that optimal vaccination might require, in addition to antibody responses, CD4 and CD8 responses (6, 7). In this case, the use of the yellow fever virus backbone encoding the DENV E and prM antigens might be suboptimal, since DENV-specific CD4 and CD8 responses dominantly target the DENV capsid (C) and nonstructural (NS) proteins, respectively (8), which are not contained in the Dengvaxia vaccine. The DENV C and NS proteins are, however, encoded by tetravalent DENV live attenuated vaccine (TDLAV) constructs.

TDLAV candidates currently in development include the TV003 and the Takeda dengue-dengue chimera (TDV) vaccines (9). The TV003 vaccine is composed of four live attenuated recombinant dengue viruses (a deletion variant of the DENV2 Tonga/74 strain lacking 30 nucleotides from its 3′ untranslated region [rDEN1Δ30], rDEN2/4Δ30, rDEN3Δ30/31, and rDEN4Δ30) and is being developed by the Laboratory of Infectious Diseases at the National Institutes of Health (NIH). The TV003 vaccine has been shown to have acceptable safety, infectivity, and immunogenicity profiles, making it a very promising vaccine candidate (6, 10–12).

A controlled human dengue virus challenge model would more directly allow correlates of protection from DENV infection and disease to be established and would facilitate the evaluation and development of novel vaccine candidates. Previous data have shown that the DENV2 component has the lowest efficacy following Dengvaxia vaccination and also induces the lowest frequency of seroconversion following TV003 vaccination, suggesting that a human challenge model for DENV2 would be of particular practical interest (4, 11).

rDEN2Δ30 derives from a DENV2 isolate identified in the 1974 outbreak in the Kingdom of Tonga and has been characterized as causing mild disease and having a low virus isolation rate (13). rDEN2Δ30 was originally developed as a DENV2 candidate vaccine virus; however, in preclinical studies it was not sufficiently attenuated compared with its parent virus (14). For that reason, rDEN2Δ30 was abandoned as a candidate vaccine, but since individuals vaccinated with rDEN2Δ30 demonstrated consistently detectable low-level viremia, it was suggested that this isolate might be suitable for use as a DENV2 challenge virus (15). Administration of rDEN2Δ30 to

TABLE 1 Homology of rDEN2Δ30 to other DENV sequences

Sequence	Homology of rDEN2Δ30 sequence to PL046 and DENV consensus sequences				Sequence of conserved and mismatched rDEN2Δ30 9- and 10-mer peptides compared to DENV consensus sequences[a]			Sequence of conserved and mismatched rDEN2Δ30 15-mer peptides compared to consensus sequences of four DENV serotypes[b]		
	Total no. of residues	No. of mismatched residues	No. of matched residues	% sequence identity	Total no. of peptides	No. of mismatched peptides	No. of matched peptides	Total no. of peptides	No. of mismatched peptides	No. of matched peptides
DENV1	3,393	936	2,457	72.41	6,765	3,756	3,009	3,377	1,767	1,610
DENV2 consensus	3,391	78	3,313	97.70	6,765	67	6,698	3,377	14	3,363
DENV3 consensus	3,393	946	2,445	72.06	6,765	3,860	2,905	3,377	1,782	1,595
DENV4 consensus	3,393	1,034	2,359	69.53	6,765	4,103	2,662	3,377	1,928	1,449
PL046	3,391	86	3,305	97.46						

[a]Identity, ≥80%.
[b]Identity, ≥85%.

healthy volunteers was associated with detectable transient viremia in 100% of subjects, rash in 80% of subjects, and neutropenia in 40% of the individuals (15). Accordingly, rDEN2Δ30 has since been utilized as a challenge virus in a model of the protection conferred by the TV003 vaccine, which was found to confer complete protection from rDEN2Δ30 challenge (6).

Previous data addressed the suitability of the use of rDEN2Δ30 infection as a model of DENV2 infection predominantly on the basis of clinical parameters and the induction of serological responses. Since T cell responses are also potential correlates of protection in humans (12, 16), we sought to characterize the $CD8^+$ and $CD4^+$ T cell responses elicited in volunteers who were challenged with rDEN2Δ30 and compare the magnitude and specificity of these responses to those observed in the context of natural infection and in subjects from areas where DENV is hyperendemic.

RESULTS

Conservation of potential T cell epitopes in rDEN2Δ30 within DENV2. We compared the rDEN2Δ30 sequence to other DENV2 sequences. The rDEN2Δ30 amino acid sequence is 97.7% homologous to a DENV2 consensus sequence established by aligning the DENV2 sequences available in GenBank as previously described (16) and 97.5% homologous to an attenuated DENV2 sequence PL046 previously used in a murine model of DENV infection (7). In contrast, the homology between the rDEN2Δ30 sequence and the DENV1, DENV3, or DENV4 consensus sequence was, as expected, still appreciable but much lower (72.41%, 72.06%, and 69.53%, respectively; Table 1).

DENV epitopes with more than 2 substitutions (or 80% homology) minimally cross-react at the level of T cell responses (12). Accordingly, we generated all possible 9-, 10-, and 15-mer peptides from the rDEN2Δ30 proteome and compared their sequences to the DENV2 consensus sequence, allowing up to two mismatches for each peptide (which corresponds to >80% and >85% of identity for HLA class I and class II peptides, respectively). Out of the 6,765 total 9- and 10-mers, 6,698 were identical or ≥80% conserved (Table 1). Similarly, only 14 of the 3,377 15-mers were not homologous at the specified identity level (Table 1). As expected, analysis of the DENV1, DENV3, and DENV4 sequences revealed much lower numbers of peptides homologous at the specified identity levels (approximately half). In conclusion, the sequences of the majority of the short peptides encoded by the rDEN2Δ30 sequence are identical or highly homologous to the DENV2 consensus sequence, and thus, this isolate appears to be representative of known DENV2 isolates.

rDEN2Δ30 induces CD8 responses comparable to those seen in natural infection. To assess class I responses, peripheral blood mononuclear cells (PBMCs) from donors infected with rDEN2Δ30 were screened with peptide pools each consisting of 10 predicted class I binders. Pools associated with positive gamma interferon (IFN-γ) responses in an enzyme-linked immunosorbent spot (ELISPOT) assay were deconvoluted to identify the individual epitopes. T cell reactivity was detected in 7 of the 9

TABLE 2 HLA-A, -B, and -DRB1 types of recipients of the rDEN2Δ30 challenge strain analyzed in this study[a]

	HLA type					
LJI identifier	HLA-A	HLA-A	HLA-B	HLA-B	HLA-DRB1	HLA-DRB1
GW0063	A*01:01	A*23:01	B*07:02	B*58:01	DRB1*13:01	DRB1*13:04
GW0064	A*02:01	A*31:01	B*15:01	B*40:01	*DRB1*04:04*	*DRB1*13:01*
GW0065	A*02:60	A*03:01	B*35:01	B*42:01	DRB1*12:01	DRB1*15:03
GW0066	A*23:01	A*23:01	B*15:10	B*82:01	DRB1*11:01	DRB1*11:04
GW0067	A*02:02	A*74:01	B*15:03	B*15:16	DRB1*03:01	DRB1*03:02
GW0068	A*02:01	A*24:02	B*27:02	B*45:01	DRB1*01:02	DRB1*16:02
GW0069	A*02:01	A*29:02	B*44:03	B*45:01	DRB1*07:01	DRB1*13:02
GW0070	A*02:01	A*02:05	B*07:02	B*50:01	DRB1*07:01	DRB1*15:01
GW0071	A*68:01	A*74:01	B*15:03	B*58:01	DRB1*03:01	DRB1*03:02
GW0073	*A*02:02*	*A*74:01*	*B*15:03*	*B*15:16*	DRB1*07:01	DRB1*15:03

[a]HLA alleles tested in this study with a 2-digit or supertype match are underlined. HLA alleles not tested because the sets of predicted epitopes were not available are shown in regular type. HLA alleles not tested because cell numbers were not sufficient for testing are shown in italics.

(78%) individuals immunized with rDEN2Δ30. This is in line with the notion that the two vaccine recipients that did not respond (GW0066 and GW0067) expressed HLA alleles that have previously been associated with low frequencies of responses in natural infection (Table 2) (16).

For each HLA class I allele, Table 3 lists the number of tested donors expressing the indicated HLA molecules, the average number of epitopes detected in those donors, the average magnitude of these responses detected, and the sum total response of all positive responses (averaged over the number of donors tested to normalize for the different number of tested donors expressing the various HLA class I alleles). Considering the 13 HLA alleles studied, the response magnitudes were, on average, 302.1 spot-forming cells (SFC) per allele, with an average repertoire breadth of 1.5 epitopes per HLA per donor and an average magnitude of 106 IFN-γ-producing SFC per epitope.

Next, we compared the responses observed here in rDEN2Δ30-immunized volunteers with the responses observed when the same set of peptides was previously tested in the context of natural infection. Accordingly, in Table 3 we also show for each allele the average number of epitopes identified, the average magnitude of the responses, and the sum total response previously detected and reported in the context of natural infection (16). We observed that the responses to rDEN2Δ30 were associated with similar numbers of epitopes (1.5, on average, versus 1 for natural infection; $P = 0.9886$ by the Mann-Whitney *t* test) and a similar magnitude of response per epitope (106.4

TABLE 3 CD8 immune response per HLA allele in rDEN2Δ30 vaccinees and individuals with natural infection

	Results for rDEN2Δ30 vaccinees				Results for individuals with natural infection			
HLA allele	No. of donors tested	Avg no. of epitopes/donor	Avg response/epitope for positive donors (no. of SFC/10^6 PBMCs)	Total avg response/tested donor (no. of SFC/10^6 PBMCs)	No. of donors tested	Avg no. of epitopes/donor	Avg response/epitope for positive donors (no. of SFC/10^6 PBMCs)	Total avg response/tested donor (no. of SFC/10^6 PBMCs)
A*01:01	1	2.0	210.0	420.0	49	0.1	100.0	8.2
A*02:01	6	2.8	56.8	160.8	55	0.4	102.0	40.9
A*03:01	2	0.0	0.0	0.0	28	0.2	263.0	48.4
A*23:01	2	0.0	0.0	0.0	6	0.0	0.0	0.0
A*24:02	1	0.0	0.0	0.0	75	0.2	124.0	27.3
A*31:01	1	0.0	0.0	0.0	15	0.1	80.0	10.7
A*68:01	1	0.0	0.0	0.0	29	0.3	217.0	102.9
B*07:02	2	5.5	231.7	1,274.2	48	2.1	185.0	392.1
B*15:01	3	0.0	0.0	0.0	68	0.5	54.0	26.5
B*35:01	1	2.0	301.7	603.3	59	6.5	215.0	1,615.5
B*40:01	1	2.0	196.7	393.3	59	0.4	451.0	203.0
B*44:03	1	3.0	221.7	665.0	43	0.3	129.0	47.8
B*58:01	2	2.5	164.3	410.8	37	1.4	208.0	308.5
Avg	1.8	1.5	106.4	302.1	43.9	1.0	163.7	217.8

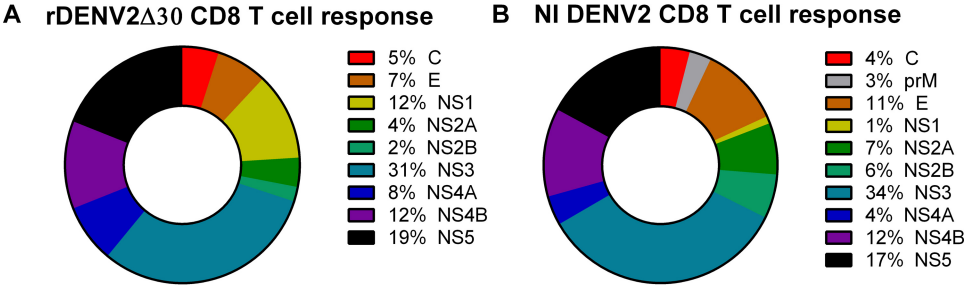

FIG 1 Protein locations of CD8 epitopes from individuals immunized with rDENV2d30 compared to those from individuals with natural infection (NI). The magnitude of the DENV2 response was analyzed by considering the overall protein composition (in percent). (A) Protein composition of the CD8 T cell response in rDEN2Δ30-immunized individuals; (B) protein composition of the CD8 T cell response in DENV2-infected donors in areas of hyperendemicity (16).

versus 163.7 SFC/10^6 PBMC; $P = 0.2817$ by the Mann-Whitney *t* test) and the overall magnitudes of the responses (302.1 versus 217.8 SFC/10^6 PBMC; $P > 0.9999$ by the Mann-Whitney *t* test).

Protein targets of CD8 responses are similar for rDEN2Δ30 and DENV natural infection. Next, we analyzed the specificity of the rDEN2Δ30 immune response at the antigen level. To this end, we calculated the relative fraction of the total magnitude (number of SFCs per 10^6 PBMCs) of the total response directed against each of the 10 DENV proteins, namely, the three structural proteins (the capsid [C], membrane [M], and envelope [E] proteins) and the seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). As shown in Fig. 1A, the CD8 T cell response against rDEN2Δ30 was broad and targeted 9 out of the 10 proteins. The majority of the epitopes were derived from NS proteins and accounted for 88% of the total IFN-γ response observed. Among the seven nonstructural proteins, NS3 and NS5 were the most dominantly targeted, accounting for 31% and 19% of the total IFN-γ response, respectively.

Next, these rDEN2Δ30-specific CD8 responses were compared to the response patterns of recognition following natural infection at the level of DENV2-specific epitopes as previously described (12). In accordance with the previous results, the overall pattern of recognition of CD8 responses to rDENV2Δ30 was remarkably similar to the CD8 response to natural infection, with NS3 and NS5 accounting for 31% and 19% of the total response, respectively (Fig. 1B). NS1 is the only protein for which we observed an increase in the response in individuals inoculated with rDENV2Δ30, in which the response was more than 10% of the total response, whereas the response in naturally infected individuals was 1% of the total response (Fig. 1B).

The rDEN2Δ30 strain-induced CD4 responses are lower than those seen in natural infection. Purified CD4 cells from the rDEN2Δ30-infected donors were also screened for reactivity against HLA class II peptides, as described in Materials and Methods, using an *in vitro* restimulation step (17). Pools associated with positive responses in the IFN-γ ELISPOT assay were deconvoluted to identify the individual epitopes. T cell reactivity was detected in 8 of the 9 (89%) rDEN2Δ30 recipients.

For each HLA allele, Table 4 lists the number of donors expressing the HLA-DRB1 allele that were tested, the average number of epitopes detected, the average response magnitude, and the sum total response of all positive responses restricted by that allele normalized for the number of donors expressing the specific HLA-DRB1 allele. Considering the seven HLA alleles studied, response magnitudes were, on average, 539.2 SFC/allele, with an average repertoire breadth of 2 epitopes per HLA and an average magnitude of 1,541.1 SFC/epitope.

Since the same sets of peptides tested in the present study were also previously tested in the context of natural infection, this allowed a comparison of the magnitude of the responses observed for the various peptide sets in HLA-matched donors in the context of natural infection. Accordingly, we observed that the responses to rDEN2Δ30 were associated with lower numbers of epitopes (2, on average, versus 8 for natural

TABLE 4 CD4 immune response per HLA allele in rDEN2Δ30 vaccinees and individuals with natural infection

	Results for rDEN2Δ30 vaccinees				Results for individuals with natural infection			
HLA allele	No. of donors tested	Average no. of epitopes/donor	Avg response/epitope for positive donors (no. of SFC/10^6 PBMCs)	Total avg response/tested donor (no. of SFC/10^6 PBMCs)	No. of donors tested	Avg no. of epitopes/donor	Avg response/epitope for positive donors (no. of SFC/10^6 PBMCs)	Total avg response/tested donor (no. of SFC/10^6 PBMCs)
DRB1*01:01	1	2.0	556.7	1,113.3	10	7.0	607.5	5,447.0
DRB1*03:01	2	2.0	714.4	1,470.0	10	3.0	656.7	1,858.0
DRB1*07:01	4	5.0	756.5	4,317.8	9	10.0	1,042.8	11,159.0
DRB1*11:01	1	0.0	0.0	0.0	8	2.0	743.9	1,421.0
DRB1*12:01	1	2.0	626.7	1,253.3	11	19.0	1,222.4	24,759.0
DRB1*13:01	1	2.0	530.0	1,060.0	18	10.0	989.6	11,732.0
DRB1*15:01	3	2.7	590.0	1,573.3	12	8.0	1,064.4	11,476.0
Avg	2	2.0	539.2	1,541.1	11	8.0	903.9	9,693.1

infection; $P < 0.0087$ by the Mann-Whitney test) and a lower magnitude of response per epitope (539.2 versus 903.9 SFC/10^6 PBMC; $P < 0.0175$ by the Mann-Whitney test) and lower overall magnitudes of responses (1,541.1 versus 9,693.1 $P < 0.007$ by the Mann-Whitney test) (Table 4).

Overlap in protein targets of CD4 responses recognized in rDEN2Δ30 and natural infection. Next, we investigated to what extent CD4 responses associated with rDEN2Δ30 infection would mimic immunity resulting from naturally occurring DENV infections. As shown in Fig. 2A, the immune response against rDEN2Δ30 was also broad, with 9 out of 10 proteins being recognized. The majority of the epitopes were derived from NS3, NS2A, and C, and these accounted for 24%, 20%, and 16% of the total IFN-γ response observed, respectively.

Next, these rDEN2Δ30-specific responses were compared to the patterns of recognition observed following natural infection at the level of DENV2-specific epitopes, as previously described (12) and shown here in Fig. 2B for reference purposes. While all proteins were recognized in both cases, certain differences were noted. The NS3 antigen was recognized at similar levels in rDEN2Δ30-immunized individuals and individuals with natural infection, while NS2A was less dominantly recognized in individuals with natural infection. Further, the responses generated during primary natural infection showed that the C protein was recognized 2-fold more prominently after natural infection than after rDEN2Δ30 immunization (16% for rDEN2Δ30 immunization versus 39% for natural infection).

rDEN2Δ30 induces stronger CD8 responses than other, more attenuated DENV isolates. The data presented above demonstrate that the rDEN2Δ30 isolate induces CD8 responses similar in magnitude to those induced by natural infection. We previously reported an analysis of the CD8 responses induced by immunization with monovalent components of the tetravalent DENV live attenuated vaccine (DLAV)

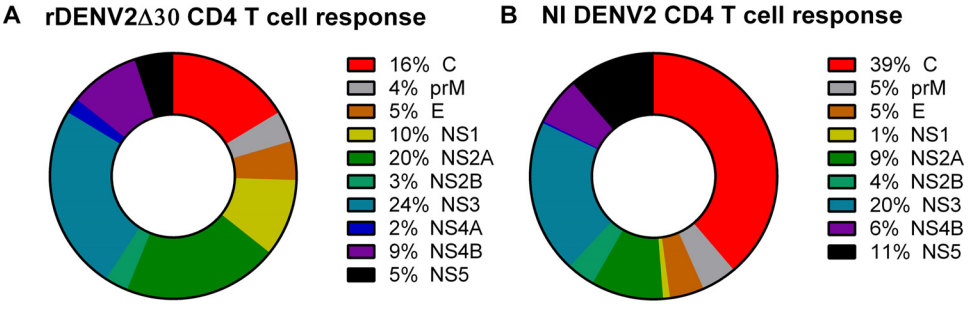

FIG 2 Protein locations of CD4 epitopes from individuals immunized with rDENV2d30 compared to those from individuals with natural infection. The magnitude of the DENV2 response was analyzed by considering the overall protein composition (in percent). (A) Protein composition of the CD4 T cell response in rDEN2Δ30-immunized individuals; (B) protein composition of the CD4 T cell response in DENV2-infected donors in areas of hyperendemicity (17).

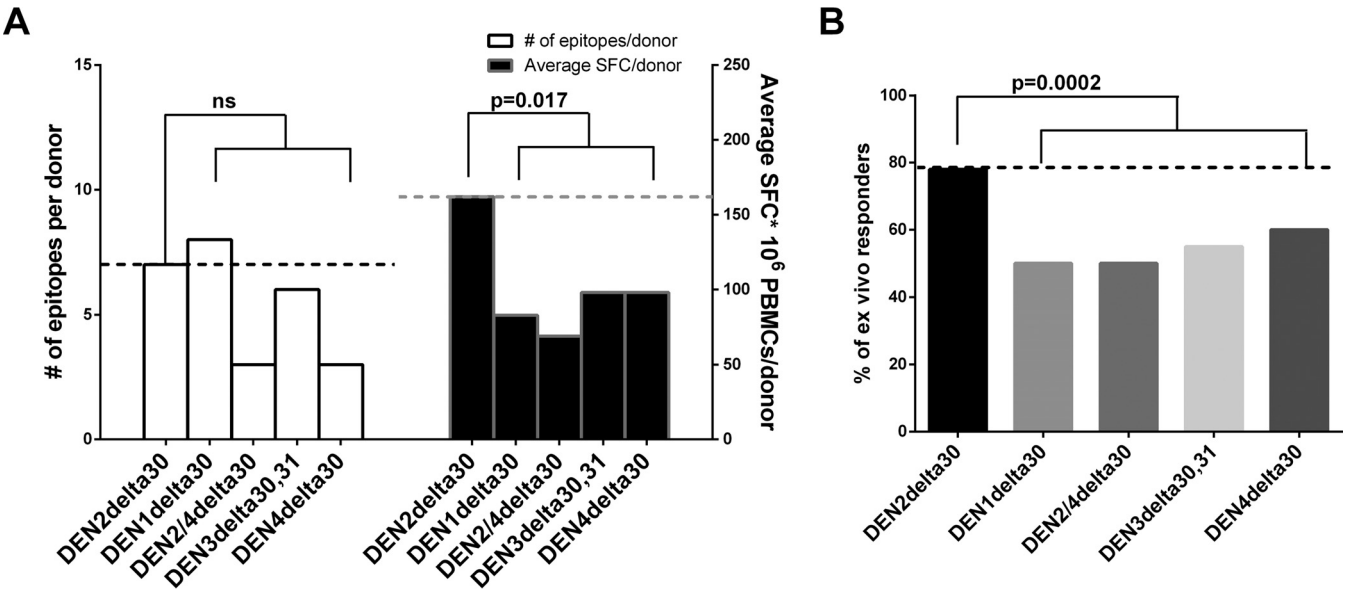

FIG 3 CD8 immune response to rDEN2Δ30 compared to that to attenuated DENV vaccine strains. (A) Number of CD8-specific epitopes per donor in response to rDENV2Δ30 and the magnitude of the response in terms of the average number of SFC per donor. (B) Percentage of donors responding to rDENV2Δ30 with respect to that to other monovalent attenuated vaccine strains (12).

developed by the NIH (11). Here we compared the magnitude and breadth of the response and the response rates of CD8 T cells observed in the monovalent DLAV studies (12) with those of CD8 T cells observed in the current study. A similar analysis at the level of CD4 responses was not feasible since the CD4 immunogenicity of the attenuated viruses was evaluated only with the tetravalent formulation (8).

If the number of epitopes restricted by all HLA-A and HLA-B loci were taken into consideration, the breadth of the response induced by rDEN2Δ30 (mean, 7 epitopes per donor; range, 1 to 9 epitopes per donor) was comparable to that induced by other monovalent DLAVs (mean, 6 epitopes per vaccine; range, 3 to 8 epitopes per vaccine) (Fig. 3A). However, the magnitude of the response was higher for the rDEN2Δ30 isolate (average, 162 SFC/10^6 PBMCs) than the other DLAVs (mean for the various DLAVs, 95 SFC/10^6 PBMCs; range, 76 to 12,395 SFC/10^6 PBMCs; $P = 0.017$ by the one-sample *t* test) (Fig. 3A). *Ex vivo* reactivity was detected in seven out of the nine infected volunteers (Fig. 3B) of the nine infected volunteers studied. This value was also higher than the values observed for the other DLAVs ($P = 0.0002$ by a one-sample *t* test).

Substantial overlap between epitope specificity of the CD4 and CD8 responses observed in rDEN2Δ30- and other DLAV-immunized individuals and those with natural infection. Based on the results presented above, we hypothesized that a substantial overlap might exist between the epitopes recognized in the context of rDEN2Δ30 experimental challenge/infection and those recognized in the context of DLAV vaccination and natural infection. A complete list of the $CD8^+$ and 28 $CD4^+$ T cell epitopes detected in the present study is provided in Table 5, which lists, for each epitope, the antigen of origin, the sequence, and the putative HLA restriction.

We next analyzed, for each of these epitopes, whether the same epitope reactivity was previously detected in the context of natural infection (16) or DLAV vaccination (12). We found that 100% of the CD8 epitopes (37 out of 37) and approximately 85% of the CD4 epitopes (26 out of 28) were previously observed in one or both of these systems.

Correlation of rDEN2Δ30 cellular responses with antibody titer and viremia. As shown in Table 6, antibody titers and viral loads were also determined for all vaccinees. The humoral response peak occurred on day 28 after vaccination, and no significant correlation between the antibody titer and the magnitude of the cellular immune response was observed (for CD4, $r = 0.3167$ and $P = 0.4101$; for CD8, $r = 0.4286$ and

TABLE 5 HLA class I and II epitopes detected in this study

HLA class and protein[a]	Sequence	HLA	Previously detected[b]
HLA class I			
$C_{43–52}$	GPMKLVMAFI	HLA-B*0702	NI
$E_{553–562}$	MSSGNLLFTG	HLA-B*5801	NI
$E_{631–639}$	RLITVNPIV	HLA-A*0201/0203	NI
$E_{723–732}$	AIYGAAFSGV	HLA-A*0201/0203/0206	NI
$E_{738–746}$	ILIGVIITW	HLA-B*5801	NI
$E_{738–746}$	ILIGVVITW	HLA-B*5801	NI
$NS1_{968–976}$	RAVHADMGY	HLA-B*5801	NI
$NS1_{1042–1051}$	GPWHLGKLEL	HLA-B*0702	NI
$NS2A_{1146–1155}$	FLEEMLRTRV	HLA-A*0201	NI
$NS2A_{1161–1169}$	ILLVAVSFV	HLA-A*0201	NI
$NS2A_{1191–1200}$	TMTDDIGMGV	HLA-A*0201	NI
$NS2A_{1230–1239}$	MMATIGIALL	HLA-A*0201	NI
$NS2A_{1242–1251}$	LSIPHDLMEF	HLA-A*0101	NI
$NS2A_{1251–1259}$	LIDGISLGL	HLA-A*0101	NI
$NS2A_{1271–1279}$	YQLAVTIMA	HLA-A*0201/0206	NI
$NS2B_{1464–1473}$	TAAAWYLWEV	HLA-A*0201/0206/6802	NI
$NS3_{1889–1897}$	SEMGANFKA	HLA-B*4403	NI
$NS3_{1902–1910}$	DPRRCLKPV	HLA-B*0702	NI
$NS3_{1978–1987}$	TPEGIIPALF	HLA-B*0702/3501/5301	NI
$NS3_{1978–1987}$	TPEGIIPALF	HLA-B*3501	NI
$NS3_{2070–2078}$	RPRWLDART	HLA-B*0702	NI
$NS4A_{2207–2216}$	LEFFLMVLLI	HLA-B*4403	NI
$NS4B_{2232–2241}$	YVVIAILTVV	HLA-A*0201/6802	NI
$NS4B_{2280–2288}$	RPASAWTLY	HLA-B*0702	NI
$NS4B_{2286–2295}$	TLYAVATTFV	HLA-A*0201	NI
$NS4B_{2322–2331}$	LMGLGKGWPL	HLA-A*0201	NI
$NS4B_{2359–2367}$	LLLVAHYAI	HLA-A*0201	NI
$NS4B_{2419–2428}$	MLLILCVTQV	HLA-A*0201	NI
$NS4B_{2420–2428}$	LLILCVTQV	HLA-A*0201	NI
$NS5_{2677–2685}$	VLNPYMPSV	HLA-A*0201	NI
$NS5_{2609–2618}$	IPMSTYGWNL	HLA-B*0702	NI
$NS5_{2609–2618}$	IPMATYGWNL	HLA-B*0702	NI
$NS5_{2919–2928}$	EAVEDGRFWE	HLA-B*5801	NI
$NS5_{2921–2929}$	VEDEDFWKL	HLA-B*4001	NI
$NS5_{3058–3066}$	KLAEAIFKL	HLA-A*0201	NI
$NS5_{3110–3118}$	MEVQLVRQM	HLA-B*4001	NI
$NS5_{3200–3209}$	QQVPFCSHHF	HLA-B*4403	NI
HLA class II			
$C_{45–59}$	LRLFMALVAFLRFLT	HLA-DRB1*1501	NI
$C_{70–84}$	WGTIKKSKAINVLRG	HLA-DRB1*0701	NI/DLAV
$E_{512–526}$	WIQKETLVTFKNPHA	HLA-DRB1*1501	NI/DLAV
$E_{513–527}$	NRKELLVTFKNAHAK	HLA-DRB1*1501	NI/DLAV
$E_{547–561}$	GATEIQMSSGNLLFT	HLA-DRB1*0301	
$M_{242–256}$	ILRHPGFTIMAAILA	HLA-DRB1*0701/HLA-DRB1*1501	NI/DLAV
$M_{242–256}$	ILRHPGFTLMAAILA	HLA-DRB1*1501	NI
$M_{264–278}$	FQRVLIFILLTAVAP	HLA-DRB1*0701	NI/DLAV
$NS1_{860–874}$	VKLTIMTGDIKGIMQ	HLA-DRB1*0301	NI
$NS1_{861–875}$	KLTIMTGDIKGIMQA	HLA-DRB1*0301	NI/DLAV
$NS2A_{1197–1211}$	GMGVTYLALLAAYKV	HLA-DRB1*0101	NI
$NS2A_{1199–1213}$	GVTYLALLAAFKVRP	HLA-DRB1*0101	NI
$NS2A_{1231–1245}$	LLTIGLSLVASVELP	HLA-DRB1*0701	NI/DLAV
$NS2A_{1257–1271}$	LGMMVLKIVRNMEKY	HLA-DRB1*1202	NI
$NS2A_{1284–1298}$	NTIFTLTVAWRTATL	HLA-DRB1*0701	NI/DLAV
$NS2B_{1355–1369}$	IMAVGIVSILLSSLL	HLA-DRB1*0701	NI/DLAV
$NS2B_{1356–1370}$	MAVGMVSILASSLLK	HLA-DRB1*0701	NI/DLAV
$NS3_{1521–1535}$	GTFHTMWHVTRGAVL	HLA-DRB1*0701	NI/DLAV
$NS3_{1523–1537}$	FHTMWHVTRGAVLTY	HLA-DRB1*0701	NI/DLAV
$NS3_{1523–1537}$	FHTMWHVTRGAVLMH	HLA-DRB1*0701	NI/DLAV
$NS3_{1534–1548}$	VLMHKGKRIEPSWAD	HLA-DRB1*1301	
$NS3_{1620–1634}$	KGKVVGLYGNGVVTR	HLA-DRB1*1501	NI
$NS3_{1621–1635}$	GKIVGLYGNGVVTTS	HLA-DRB1*1501	NI
$NS3_{1850–1864}$	TNCLRKNGKRVIQLS	HLA-DRB1*1301	NI
$NS4A_{2179–2193}$	LGMCCIITASILLWY	HLA-DRB1*0701	DLAV
$NS4B_{2417–2431}$	QIMLLILCTSQILLM	HLA-DRB1*0701	NI/DLAV
$NS4B_{2423–2437}$	LCAVQLLLMRTSWAL	HLA-DRB1*1202	NI/DLAV
$NS5_{2874–2888}$	RWLWGFLSRNKKPRI	HLA-DRB1*1501	NI/DLAV

[a]Subscript numbers indicate the amino acid positions of the indicated protein.
[b]NI, natural infection; DLAV, DENV live-attenuated vaccine.

TABLE 6 Antibody titers and levels of viremia in the rDEN2Δ30 vaccinees analyzed in this study

	Antibody (DENV2) titer on day:				Level of viremia (log_{10} no. of PFU/ml) on day:							
LJI identifier	0	28	56	180	0	2	4	6	8	10	12	14
GW0063	0	158	133	46	0	0	1.2	2.5	1.6	1.5	0.5	0
GW0064	0	1,185	109	29	0	0	1.7	2.7	2.3	0.5	0	0
GW0065	0	1,071	519	38	0	0	NA[a]	2.8	2.3	0	0	0
GW0066	0	459	147	38	0	0	0	2	2.4	1.5	0.5	0
GW0067	0	764	244	72	0	0	0.5	1.8	2.4	2.6	1.3	0
GW0068	0	678	469	53	0	0	1.5	1.5	1	0	0	0
GW0069	0	1,966	820	NA	0	0	1	2.6	2	0.5	0	0
GW0070	0	1,435	272	NA	0	0	1.5	2.8	2.1	0	NA	0
GW0071	0	107.3	113.9	NA	0	0	0	0	1	1.5	0.5	0.5
GW0072	0	120	169.8	NA	0	0	0.5	1.7	1.3	0.5	0.5	0
GW0073	0	120.4	53.6	NA	0	0	0	2.2	2.1	1.5	0.5	0

[a]NA, not available.

$P = 0.3536$). The viral load peak was observed at day 6 in 8 out of 10 donors, while only 2 donors showed viral load peak after 8 days of inoculation. A significant negative correlation between the viral load and the magnitude of the CD8 response was observed ($r = -0.8310$, $P = 0.0071$), while no significant correlation was observed when CD4 responses were considered ($r = -0.3504$, $P = 0.3536$).

DISCUSSION

The rDEN2Δ30 isolate cross-reacts with other DENV2 isolates at the serological level (12, 14, 16). While serological cross-reactivity can be dependent on the conservation of conformational determinants on viral surface proteins, cross-reactivity at the level of CD8 and CD4 immunity is dependent on the homology of 9- or 10- and 15-residue peptides, respectively. Here we show that the majority of the peptides that were of 9 to 15 amino acid residues and that thus were potentially recognized by CD8 and CD4 T cells are conserved between rDEN2Δ30 and other DENV2 isolates, thus providing support for the notion that rDEN2Δ30 might induce T cell responses with specificities similar to those induced by DENV2 in the context of natural infection.

In terms of CD8/class I responses, we observed that the overall magnitude and breadth of the responses were very comparable to those observed and previously reported in the context of natural infection with PBMC donations derived from the area of Sri Lanka where dengue is hyperendemic (16). A similar pattern of immunodominance was observed for most of the NS proteins in rDEN2Δ30 vaccinees and those with natural infection, suggesting similar patterns of immune recognition of rDEN2Δ30 and DENV2. In particular, NS3 and NS5 are dominantly recognized in both contexts. The main differences in terms of response were observed in the response to the premembrane (prM) and NS1 proteins. It is possible that reduced viral replication due to the attenuation of rDEN2Δ30 leads to small differences in the protein recognition pattern. Overall, these data suggest that rDEN2Δ30 infection mimics wild-type DENV2 infection in terms of the immunodominance of MHC class I responses.

In contrast to the similar immunogenicity observed in the case of class I, the class II-restricted responses to rDEN2Δ30 infection were relatively less vigorous than those to natural DENV infection. Furthermore, the relative immunodominance observed in the case of rDEN2Δ30 was, in the case of class I-restricted responses, essentially superimposable with the pattern observed in the case of natural infection. Interestingly, a much less stringent correlation was observed in the case of the class II response. Here the main difference was that the C antigen, which is dominant in natural DENV infection, was still recognized in the context of rDEN2Δ30-induced responses but in a much less dominant manner, while, on the contrary, a stronger response against the NS1 protein was observed in rDEN2Δ30 infection. These observations may suggest that rDEN2Δ30 and the Tonga/74 wild-type strain in general have reduced rates of propagation (~100 PFU/ml) (3, 9, 18) compared to those of other wild-type DENV strains (106 to 109

PFU/ml) (19). If this is true, fewer virions containing the viral structural proteins would be released from infected cells and available for the exogenous pathway of antigen processing and presentation, which is most prominently involved in the generation of class II-restricted peptides. This would also reduce structural protein availability and recognition by the immune system, increasing the responses against the nonstructural proteins. In addition, this may also explain why the Tonga/74 strain was described to cause more mild illness (13).

We have previously studied the CD8 T cell response elicited by each of the monovalent DLAVs currently included in the TV003 vaccine (6). A comparison of the T cell responses elicited by each of the monovalent vaccines with vaccine-specific peptides from their corresponding serotypes demonstrated that the magnitude and the breadth of responses elicited by rDEN2Δ30 were higher. The higher immunogenicity of rDEN2Δ30 is consistent with the lower degree of attenuation (2.2 $\log_{10}$ focus-forming units/ml for monovalent DLAVs versus ~0.5 $\log_{10}$ focus-forming units/ml for rDEN2Δ30) (5, 14, 20) and with the significant negative correlation with the viral load observed in the case of CD8 responses, possibly suggesting that more viral replication results in at least moderately higher levels of immunogenicity. This effect has been reported in the context of other viral systems. A similar comparison of the various DLAVs and rDEN2Δ30 at the level of CD4 responses was not feasible since the CD4 immunogenicity of the attenuated viruses was evaluated only with the tetravalent formulation (8). Since no clinical evaluation of monovalent DLAVs is currently ongoing, samples for study were no longer available for our analysis.

To use the rDEN2Δ30 virus isolate as a DENV2 challenge agent, in the context of vaccination with other DLAVs, the epitopes recognized following experimental challenge/infection with rDEN2Δ30 must largely overlap those recognized in the context of DLAV vaccination and natural infection. The results obtained in this study demonstrate that this is indeed the case, providing an additional piece of evidence supporting the feasibility of using rDEN2Δ30 in a human DENV2 challenge model.

MATERIALS AND METHODS

Sequence conservation analysis. Full-length polyprotein sequences of each DENV serotype were retrieved from the NCBI Protein database as described previously (16). To eliminate geographical bias, the number of isolates from any one country was limited to 10. Sequences were considered unique if they varied by at least 1 amino acid from all other sequences. A total of 162 DENV1, 171 DENV2, 169 DENV3, and 53 DENV4 sequences were retrieved from the NCBI Protein database and utilized to evaluate the variability of the sequence of the rDEN2Δ30 strain compared with the sequences of the respective serotypes. All the sequences were aligned using MAFFT software online (v.7) (21), and consensus sequences were generated using BioEdit software (v.7.0) (22). Each consensus sequence was separately aligned to the rDEN2Δ30 sequence and analyzed for the number of matches and mismatches.

Human subjects. Volunteers previously infected with rDEN2Δ30 (ClinicalTrials.gov registration no. NCT01931176) were recalled to donate blood for this study (6). Full-blood units from 10 volunteers were collected 4 to 7 months after inoculation at the Johns Hopkins University Bloomberg School of Public Health (JHSPH) in Baltimore, MD, after informed consent was granted by the volunteers under a JHSPH Institutional Review Board (IRB)-approved protocol (IRB no. 00006691). PBMCs were purified, frozen, and shipped to the La Jolla Institute (LJI) for further analysis. Antibody titers and levels of viremia after vaccination were determined as previously described (14).

HLA typing. HLA typing for class II DRB1 was performed by an ASHI-accredited laboratory at Murdoch University (Western Australia, Australia) using locus-specific PCR amplification of genomic DNA. Patient-specific barcoded primers were used for amplification. The amplified products were quantitated and pooled by subject, and products from up to 48 subjects were pooled. An unindexed library (454 8-lane runs) or an indexed library (8 indexed MiSeq runs) was then quantitated using kappa universal quantitative PCR library quantification kits. Sequencing was performed using either a Roche 454 FLX+ sequencer with titanium chemistry or an Illumina MiSeq sequencer using 2 × 300 paired-end chemistry. Reads were quality filtered and passed through a proprietary allele-calling algorithm and analysis pipeline using the latest international ImMunoGeneTics project (IMGT) HLA allele database as a reference. Table 2 lists the HLA types of all donors used in this study.

HLA binding predictions. Major histocompatibility complex class I binding predictions and peptide selections were conducted using the Immune Epitope Database (IEDB) analysis resource (www.iedb.org) (23) as previously described (16, 24). The 9-mer and 10-mer peptides selected for synthesis were within the top 1% of the predicted binders for HLA class I A and B alleles frequently expressed in the study population. On average, 300 peptides (range, 275 to 332 peptides) were tested for each HLA class I allele. These peptides sets were subdivided into pools of 10 individual peptides and were previously utilized to characterize CD8 responses in the areas of Colombo, Sri Lanka, and Managua, Nicaragua, where dengue

is hyperendemic and in the context of vaccination with the tetravalent DENV live attenuated vaccine (TDLAV) (12, 16, 25). These peptide sets were previously utilized to characterize the CD4 responses in the areas of Colombo, Sri Lanka, and Managua, Nicaragua, where dengue is hyperendemic and in the context of vaccination with TDLAV (8, 12, 16).

To analyze DENV-specific HLA-restricted CD4 responses, we utilized sets of DENV peptides predicted to bind HLA-DRB1* allelic variants expressed in the donor population as previously described (8, 17). These sets of peptides included 15-mer HLA class II allele-restricted peptides (average, 130 peptides; range, 108 to 172 peptides) subdivided into pools of 20 individual peptides previously utilized to characterize CD4 responses in the area of Colombo, Sri Lanka, where dengue is hyperendemic and in the context of vaccination with TDLAV (8, 17).

Epitope predictions were performed using the consensus prediction methods publicly available through the IEDB analysis resource (www.iedb.org) (23, 26, 27). For each allele, we synthesized peptides predicted to bind at the 2% consensus threshold, after removing redundant peptides overlapping by 8 residues or more. This roughly corresponds to the top 10% of 15-mers overlapping by 10 residues (Mimotopes, Victoria, Australia).

***In vitro* expansion of DENV-specific T cells to study CD4 responses.** PBMCs were thawed, and $CD4^+$ T cells were isolated by magnetic bead negative selection and cultured separately in RPMI 1640 (Omega Scientific) supplemented with 5% human serum (Cellgro) at a density of 2×10^6 cells per ml in 24-well plates (BD Biosciences). Cells were cocultured with autologous antigen-presenting cells at a density of 1×10^6 cells/ml and stimulated with DENV-specific pools (average, 20 peptides per pool). Cells were kept at 37°C in 5% CO_2, and additional interleukin-2 (10 U/ml; eBioscience) was added 4, 7, and 11 days after the initial antigenic stimulation. On day 14, cells were harvested and screened for reactivity against individual DENV-specific peptides.

ELISPOT assays. PBMCs from immunized donors (2×10^5 cells/well) were incubated with 2 μg/ml of HLA-matched peptides in plates coated with the anti-human IFN-γ antibody 1-D1K (5 μg/ml; Mabtech) at 37°C for 20 to 24 h. The plates were then developed as previously described (16).

Strategy for assessment of CD8 and CD4 T cell responses. To assess the responses elicited by rDEN2Δ30, we used an approach previously utilized to characterize the responses to DLAV candidates (12) and to characterize the responses induced by natural infection in the area of Sri Lanka where dengue is endemic (16). This experimental design was chosen to enable comparison of the responses elicited by rDEN2Δ30 to those induced by DLAV or natural infection. This strategy is based on the testing of sets of peptides predicted to bind specific HLA alleles for recognition by PBMCs from HLA-matched donors.

Here, we utilized the same sets of peptides previously utilized in the DLAV and natural infection studies (8, 16, 17) and supplemented these sets of peptides with any additional predicted peptides uniquely found in the nucleic acid sequence of rDEN2Δ30. Full-blood units were collected from 10 rDEN2Δ30-infected volunteers 4 to 7 months following inoculation at JHSPH in Baltimore, MD. PBMCs were purified, frozen, and shipped to LJI for further analysis. PBMCs were tested with sets of peptides predicted to bind the HLA alleles expressed in the particular donor.

In the case of two donors, limited cell numbers were available, and accordingly, only predicted class I (donor GW0064) or class II (donor GW0073) epitopes were tested. Sets of predicted binders were available for 30 out of 36 instances in which a given HLA-A/B allele was expressed in the nine donors analyzed. Likewise, sets of predicted binders were available for 12 out of 18 instances in which a given HLA-DRB1 allele was expressed in the nine donors analyzed (Table 2).

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