Cell-Mediated Immunity Generated in Response to a Purified Inactivated Vaccine for Dengue Virus Type 1

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Cell-Mediated Immunity Generated in Response to a Purified Inactivated Vaccine for Dengue Virus Type 1

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ABSTRACT Dengue is the most prevalent arboviral disease affecting humans, and a vaccine appears to be the most rational means of control. Dengue vaccine development is in a critical phase, with the first vaccine licensed in some countries where dengue is endemic but demonstrating insufficient efficacy in immunologically naive populations. Since virus-neutralizing antibodies do not invariably correlate with vaccine efficacy, other markers that may predict protection, including cell-mediated immunity, are urgently needed. Previously, the Walter Reed Army Institute of Research developed a monovalent purified inactivated virus (PIV) vaccine candidate against dengue virus serotype 1 (DENV-1) adjuvanted with alum. The PIV vaccine was safe and immunogenic in a phase I dose escalation trial in healthy, flavivirus-naive adults in the United States. From that trial, peripheral blood mononuclear cells obtained at various time points pre- and postvaccination were used to measure DENV-1-specific T cell responses. After vaccination, a predominant CD4+ T cell-mediated response to peptide pools covering the DENV-1 structural proteins was observed. Over half (13/20) of the subjects produced interleukin-2 (IL-2) in response to DENV peptides, and the majority (17/20) demonstrated peptide-specific CD4+ T cell proliferation. In addition, analysis of postvaccination cell culture supernatants demonstrated an increased rate of production of cytokines, including gamma interferon (IFN-γ), IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Overall, the vaccine was found to have elicited DENV-specific CD4+ T cell responses as measured by enzyme-linked immunosorbent spot (ELISpot), intracellular cytokine staining (ICS), lymphocyte proliferation, and cytokine production assays. Thus, together with antibody readouts, the use of a multifaceted measurement of cell-mediated immune responses after vaccination is a useful strategy for more comprehensively characterizing immunity generated by dengue vaccines.

IMPORTANCE Dengue is a tropical disease transmitted by mosquitoes, and nearly half of the world’s population lives in areas where individuals are at risk of infection. Several vaccines for dengue are in development, including one which was recently licensed in several countries, although its utility is limited to people who have already been infected with one of the four dengue viruses. One major hurdle to understanding whether a dengue vaccine will work for everyone—before exposure—is the necessity of knowing which marker can be measured in the blood to signal that the individual has protective immunity. This report describes an approach measuring multiple different parts of immunity in order to characterize which signals one candidate vaccine imparted to a small number of human volunteers. This approach was designed to be able to be applied to any dengue vaccine study so that the data can be compared and used to inform future vaccine design and/or optimization strategies.

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Dengue is a mosquito-borne disease caused by any of four genetically distinct types of dengue virus (dengue virus serotype 1 [DENV-1] to DENV-4). At least one-third of the world’s population lives in regions where dengue is endemic, making it the most important arboviral disease globally (1). The virus continues to spread geographically, and as the footprints of the four types increasingly overlap, the threat of severe disease rises. It is estimated that at least 50 million cases of dengue fever occur annually, including over 25,000 deaths due to dengue hemorrhagic fever and dengue shock syndrome. A major risk factor for severe disease is the presence of serotype cross-reactive but not cross-protective immunity after the initial or primary DENV infection, which in some individuals leads to enhanced viral replication upon subsequent infection with a different (heterologous) serotype (2). In order to mitigate this risk, a safe and effective DENV vaccine may need to confer protective immunity against all four virus types after a single immunization. Although risk for severe disease is highest after secondary DENV infection, it decreases dramatically for tertiary and quaternary dengue infections (3, 4), a pattern that reinforces the need for a tetravalent dengue vaccine. Simultaneous circulation of serologically distinct but antigenically cross-reactive DENV-1 through DENV-4, coupled with immune enhancement of disease, presents a unique problem for development of an efficacious vaccine. The prerequisite for a dengue vaccine to generate multivalent protection is further complicated by the lack of a precisely defined immunological correlate of protection. Hence, comprehensive evaluation of immune responses generated by candidate dengue vaccines is essential.

Traditionally, flavivirus vaccine development relied on antibody-based assays to demonstrate immunogenicity. For Japanese encephalitis virus, yellow fever virus, and tick-borne encephalitis virus, the generation of neutralizing antibodies (NAbs) closely correlates with vaccine efficacy (5–8). Many successful vaccines also generate strong cell-mediated immunity (CMI) in addition to a broad array of antibody specificities and functions (6, 9–11). Evidence suggests that the high efficacy and long-lived immunity generated by vaccines such as vaccinia virus or YFV-17D are critically dependent upon the strong CMI that is generated alongside potent humoral immunity (9–13). CMI encompasses the responses of T and B cells, among other cell types, which are influenced by the type of antigen introduced by a vaccine. For example, a live attenuated virus vaccine typically engages both CD4+ and CD8+ T cells, whereas a purified inactivated virus (PIV) vaccine predominantly generates a CD4+ T cell response. A variety of assays, including enzyme-linked immunosorbent spot (ELISpot), flow cytometry-based intracellular cytokine staining (ICS), and lymphocyte proliferation assays, have been employed in different vaccine clinical trials in order to evaluate CMI responses (14–20). Each of these technologies has advantages and disadvantages based on sample availability, information gained, scalability, cost, time requirements, and other logistical restraints. As numerous candidate dengue vaccines enter clinical development, it is important to develop a monitoring strategy that interrogates all aspects of CMI in order to capture a comprehensive picture of vaccine-induced immunity that can be used to compare data across various vaccine platforms.

Sanofi Pasteur used YFV-17D as the backbone for a chimeric dengue vaccine (CYD), exchanging the YFV premembrane (prM) and envelope (E) genes for those from DENV-1 to DENV-4, and the resulting product, Dengvaxia, has been licensed for use in several countries where dengue is endemic (21, 22). However, this vaccine appears poorly effective at protecting against disease in dengue-naive individuals, and furthermore, the risk for severe disease resulting from natural DENV infections several years after vaccination appears to be increased (23, 24). Surprisingly, the presence of high NAb titers, the presumed strongest correlate with immunity, did not in this case correlate with protection from infection or disease, suggesting that other immunological markers may be equally if not even more important (23, 24).

With the goal of making a tetravalent PIV vaccine formulation, the Walter Reed Army
In the early 2000s, the Institute of Research developed a monovalent PIV vaccine against DENV-1 as a proof of concept. This vaccine was tested in a phase I dose escalation trial in which 20 healthy, flavivirus-naive adults were immunized in a two-dose (month 0 and month 1) regimen. Vaccine-induced antibody responses were examined with regard to binding, neutralization capacity, and avidity in that study. This study sought to characterize the CMI response to the PIV-1 vaccine. Peripheral blood mononuclear cells (PBMC) prepared from whole-blood samples collected at various time points pre- and postvaccination were used to measure vaccine-induced DENV-specific T cell responses. Here, we report results from multiple assay platforms, including gamma interferon (IFN-\(\gamma\)) and interleukin-2 (IL-2) ELISpot assays, multiplexed enzyme-linked immunosorbent assays (ELISA; Luminex), ICS assays, and flow cytometry-based proliferation assays. The results demonstrate that DENV PIV-1-induced CD4\(^+\) T responses were detectable in most subjects and that the profile of these cells was consistent with a helper T cell phenotype.

**RESULTS**

Twenty healthy, flavivirus-naive adults were vaccinated on day 0 and then boosted on day 28 with a PIV vaccine against DENV serotype 1 (DENV PIV-1). Subjects were stratified into two groups of 10 subjects, each receiving either 2.5 \(\mu\)g/0.5 ml (low-dose group) or 5.0 \(\mu\)g/0.5 ml (high-dose group) of alum-absorbed DENV PIV-1. Comparable NAb responses were generated in the high-dose and low-dose groups, and the responses peaked at day 56. We hypothesized that for an inactivated vaccine, the T cell response would be mediated primarily by CD4\(^+\) T cells, as opposed to CD8\(^+\) T cells. To investigate this, we first screened the vaccinees using ELISpot assays that detected secretion of IFN-\(\gamma\) or IL-2 in response to overnight stimulation with peptide pools specific for the DENV-1 envelope (E-1) and capsid/premembrane (CM-1) proteins. At day 56 (28 days after dose 2), the IL-2 responses detected were of both greater frequency and greater magnitude than the IFN-\(\gamma\) responses (Table 1; see also Fig. 1). The results showed median IL-2 postvaccination responses of 54 and 38 spot-forming cells (SFC)/10\(^6\) PBMC for the CM-1 and E-1 peptide pools, respectively. The frequencies of IL-2 responders after vaccination were 55% for the CM-1 peptide pool and 45% for the E-1 peptide pool (65% to either peptide pool), whereas the corresponding response rates for IFN-\(\gamma\) were 25% for the CM-1 peptide pool and 30% for the E-1 peptide pool (30% to either peptide pool). Responses were similar between the two vaccine dosage groups, with no significant differences in response rate or magnitude (see Fig. S1 in the supplemental material).

We next performed a flow cytometry-based intracellular cytokine staining (ICS) assay, which permitted the assessment of a broader number of functions as well as of the multifunctionality of responses at the individual T cell level. We measured degranulation (CD107a expression), T helper function (CD40L, also known as CD154), and cytokine production (IFN-\(\gamma\), IL-2, tumor necrosis factor alpha [TNF-\(\alpha\)], and MIP-1\(\beta\)) in response to DENV-1 peptide stimulation in PBMC collected at day 0 (prevaccination) and day 56 (postvaccination).
and day 56 (28 days after dose 2). Compared to day 0, the PIV-1 vaccine induced multifunctional CD4^+ T cell responses at day 56 to peptides representing DENV structural proteins E-1 and CM-1 (Fig. 2; see also Table 2). The functional subsets that expanded in response to peptide stimulation were, at a minimum, IL-2^+ TNF-α^+ doubly positive but also included cells that coexpressed IFN-γ and CD154 (Fig. 2; see also Fig. S2). Vaccination did not appear to induce NS1-specific responses (Fig. S3), nor did it induce any measurable DENV-specific CD8^+ T cell response (Fig. S3). No differences between the low-dose and high-dose groups were observed (Fig. S3). These data corroborated the ELISpot data wherein IL-2 was more readily detectable than IFN-γ.

Lymphocyte proliferation is a sensitive and informative surrogate measure of memory T cell differentiation. A flow cytometry-based dye dilution proliferation assay was used to determine the precursor frequency of antigen-specific T cells generated by vaccination with PIV-1. Panel A of Fig. 3 shows an example of antigen-specific CD4^+ T cell proliferation of day 0 versus day 56 PBMC for one subject in response to CM-1 and E-1 peptide pools. For the group of 20 subjects as a whole, high response rates and statistically significant increases in precursor frequencies of antigen-specific CD4^+ T cells were observed in PBMC from day 56 in comparison with day 0 PBMC (Fig. 3B; see also Table 3). Robust responses to both antigens (CM-1 and E-1) were detected, and no significant difference was observed between the high-dose and low-dose groups (Fig. S4). A time course analysis of the T cell proliferative response was performed using
PBMC from 10 subjects (Fig. 3C). Strong CD4+ T cell proliferative responses to CM-1 and E-1 antigens were detected as early as 14 days after the first dose of vaccine in 1/10 and 2/10 subjects, respectively, and increased after the second (boost) dose in 9/10 subjects. The median CD4+ T cell precursor frequency peaked at day 42 for both antigens (CM-1 = 0.30% and E-1 = 0.22%) and showed only a slight diminution by day 90 (CM-1 = 0.18% and E-1 = 0.14%). There was no significant difference between the median responses at day 42 and day 56, indicating that day 56 was an appropriate time point for this and other assays of T cell responses. These data are consistent with the ELISpot and ICS assays, which demonstrated low but readily detectable levels of IL-2-producing cells that would therefore be capable of supporting T cell proliferation. Of particular note, the peptide pool representing the CM proteins was recognized at least as frequently as the E protein-derived peptide pool. This observation is consistent with both the ELISpot and ICS data as well.

Culture supernatants from the proliferation assay were harvested 7 days after stimulation and tested for cytokine secretion by multiplexed ELISA. In unstimulated cul-

![Image](https://msphere.asm.org/)
tures, numerous cytokines, chemokines, and growth factors were produced constitutively in response to vaccination, some with peaks that were more distinct and higher at day 35 (7 days postboost) than at day 7, although this was not supported statistically (Fig. S5). At day 56, less IL-2 was detected in the culture supernatants stimulated by DENV peptide pools than in unstimulated culture supernatants (Fig. 4A), likely because IL-2 is consumed by proliferating cells. The levels of IFN-\(\gamma\) produced in response to CM-1 and E-1 peptide pools at day 56 were higher than those seen in unstimulated cultures (Fig. 4B). DENV-specific IL-5 (Fig. 4C) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig. 4D) were also expressed at significantly high levels at day 56, indicative of antigen-specific T helper function supporting NAb production.

To determine whether CMI responses correlated with NAb responses, we compared both total DENV-specific IgG titers and 50% microneutralization (MN50) titers at day 56 (25) to corresponding DENV-specific CD4\(^+\) T cell frequencies as determined by the proliferation assay (Fig. 5). As would be predicted for T cell-dependent antigens, we saw positive correlations between CD4\(^+\) T cell precursor frequency and both total IgG and NAb response magnitudes at day 56.

**DISCUSSION**

Dengue is a complicated disease in that pathogenesis appears to involve a complex interaction of factors specific to the virus, the individual host, and the host immune response to infection. Identifying the factors that directly influence clinical outcome has proven challenging, and the factors appear to differ from person to person. While numerous studies have assessed the role of CMI in natural infection, few have applied comprehensive testing strategies for CMI in the context of vaccination. Vaccine trials provide a controlled environment in which to study immunity generated to a specific antigen at defined time points postexposure. Our laboratory has developed a suite of standardized assays (including ELISpot, ICS, flow-based proliferation, and Luminex) for assessment of CMI generated by candidate vaccines, which we applied here to expand immunogenicity testing of the DENV PIV-1 vaccine.

We demonstrated that a two-dose regimen of PIV-1 elicited a detectable CD4\(^+\) T cell response in the majority of vaccine recipients by using different assay formats measuring T cell function. We found IFN-\(\gamma\)-producing CD4\(^+\) T cells after vaccination, albeit at low levels. IFN-\(\gamma\) production has been linked to a positive clinical outcome for dengue (26, 27), which is encouraging for this vaccine. Additionally, the induction of IL-2-producing proliferative CD4\(^+\) T cells by PIV-1 vaccination suggests the generation of a T cell response that supports antibody production. The most sensitive of the assays for detection of T cell responses to this vaccine was the proliferation assay. As the magnitude of the T cell response is dependent on its ability to expand in response to antigen, this assay reflects an important feature of vaccine-induced CMI (28–30).

### TABLE 2 Number of subjects with CD4\(^+\) T cells expressing at least IL-2 and TNF-\(\alpha\) as determined by ICS assay in response to PIV-1 vaccination

<table>
<thead>
<tr>
<th>Study day</th>
<th>Antigen</th>
<th>No.(%) of responders(^a)</th>
<th>Median frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (prevaccination)</td>
<td>CM-1</td>
<td>0/16 (0)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>E-1</td>
<td>0/17 (0)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Any</td>
<td>0/16 (0)(^a)</td>
<td></td>
</tr>
<tr>
<td>56 (postvaccination)</td>
<td>CM-1</td>
<td>6/16 (38); (P = 0.0177)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>E-1</td>
<td>4/17 (24); NS</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Any</td>
<td>6/16 (38); (P = 0.0177)(^b)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)A responder was defined as a subject with an antigen-stimulated response that was \(\geq 2\times\) higher than that seen with the respective negative (no stimulation) control and was \(\geq 0.02\%\) after subtraction of the respective negative (no stimulation) control data. Where indicated, \(P\) values represent results from Fisher’s exact tests comparing the number of responders to the number seen under the same stimulation conditions between day 0 and day 56; NS, not significant.

\(^b\)Only subjects whose samples had sufficient numbers of cells to enable testing against both the CM-1 and E-1 peptide pools were included in this analysis.
use of carboxyfluorescein succinimidyl ester (CFSE) dye to track cells proliferating in response to DENV peptides allowed us to take advantage of the multiparametric capability of flow cytometry so that we could phenotype the responding cells and calculate precursor frequencies, neither of which is possible with the more traditional proliferation assays (28). Supernatants collected from these cultures prior to flow cytometric analysis revealed IL-5, GM-CSF, and IFN-γ upregulation as the result of antigen-specific stimulation. The absence of IL-2 in culture supernatants likely reflects its consumption by activated and proliferating CD4+ T cells (31). The detection of both TH1 and TH2 cytokines indicates that the alum-adjuvanted PIV-1 vaccine does not appreciably bias the cytokine profile of the CD4+ T cell response toward either phenotype.

The ability of this vaccine to elicit CD4+ T cells that secrete IL-2 and exhibit a high proliferative capacity, coupled with the production of IL-5 and GM-CSF by proliferating cells, is suggestive of antibody helper capacity (32). Importantly, there was a positive correlation of both total binding antibody (IgG) and NAb titers with the CD4+ T cell precursor frequency 28 days post dose 2. These observations are in agreement with prior studies of CMI generated by other inactivated vaccine products such as the seasonal influenza

FIG 3 PIV-1 vaccination induced DENV-specific CD4+ T cell proliferation. (A) Representative flow cytometric plots show the CD4+ T cell proliferative response to CM-1 and E-1 peptide pools prevaccination (Day 0) and at 56 days postvaccination. Frequencies in red are back-calculated precursor frequencies (refer to Materials and Methods). (B) Precursor frequency analysis of all 20 subjects showed significant CD4+ T cell proliferation responses to CM-1 and E-1 peptide pools 56 days postvaccination with PIV-1. Wilcoxon signed-rank tests were performed comparing day 0 and day 56 responses to the CM-1 and E-1 peptide pools, respectively. P-values of less than 0.05 were considered significant. NS, not significant. (C and D) A kinetic analysis of PBMC from a subset of subjects (n = 10) demonstrated CD4+ T cell proliferation in response to CM-1 (C) and E-1 (D) peptide pools throughout the postvaccination period. Gray lines represent individual subjects; the red line indicates the median response. Arrows indicate the days of vaccination (days 0 and 28).
vaccine. Inactivated influenza vaccine titers have been shown to be positively correlated with measurable CD4\(^+\) T cell parameters, including in vivo expansion of CD4\(^+\) T cells proximal to vaccination (33), increases in abundances of both total and antigen-specific CD4\(^+\) T cells with a follicular helper-like phenotype (CXCR5\(^+\)/ICOS\(^+\)/IL-21\(^+\)) (34, 35), and the maintenance of long-term influenza virus strain cross-reactive T cell responses following repeated vaccinations (36, 37). Studies performed with other nonreplicating vaccine products, such as recombinant proteins and virus-like particles (VLPs), have demonstrated the presence of de novo-generated CD4\(^+\) T helper responses that correlate directly with enhanced antibody titers and vaccine performance (38–44).

As expected, no appreciable CD8\(^+\) T cell response was detected after DENV PIV-1 vaccination. Vaccine-elicited CD8\(^+\) T cell responses have particular requirements for priming, such as proinflammatory conditions that are provided only in the context of viral vector replication (45–47), the presence of viral nucleic acids (48, 49), or specific adjuvants (50). These data therefore suggest that the T cell response induced by PIV-1 vaccination is limited to antigen-specific CD4\(^+\) T helper cells which support the production of anti-DENV antibodies.

### TABLE 3 CD4\(^+\) T cell proliferation responses and rates of response to PIV-1 vaccination

<table>
<thead>
<tr>
<th>Study day</th>
<th>Antigen</th>
<th>No.(%) of responders(^a)</th>
<th>Median precursor frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (prevaccination)</td>
<td>CM-1</td>
<td>1/20 (5)</td>
<td>0.02%</td>
</tr>
<tr>
<td></td>
<td>E-1</td>
<td>3/20 (15)</td>
<td>0.01%</td>
</tr>
<tr>
<td></td>
<td>Any</td>
<td>3/20 (15)</td>
<td></td>
</tr>
<tr>
<td>56 (postvaccination)</td>
<td>CM-1</td>
<td>16/20 (80); (P &lt; 0.0001)</td>
<td>0.25%</td>
</tr>
<tr>
<td></td>
<td>E-1</td>
<td>13/20 (65); (P = 0.0031)</td>
<td>0.20%</td>
</tr>
<tr>
<td></td>
<td>Any</td>
<td>17/20 (85); (P &lt; 0.0001)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)A responder was defined as a subject with an antigen-stimulated precursor frequency that was \(\geq 2\times\) higher than the respective negative (no stimulation) control AND \(\geq 0.1\%\). Where indicated, \(P\) values represent results from Fisher’s exact tests comparing the number of responders to the number seen under the same stimulation conditions between day 0 and day 56.

**FIG 4** Cytokine analysis of cell culture supernatants indicates induction of CD4\(^+\) T helper cells after PIV-1 vaccination. Cell culture supernatants from the peptide pool-stimulated proliferation cultures (Fig. 3) were harvested after 7 days and tested using a 30-plex Luminex-based kit for the presence of various cytokines, chemokines, and growth factors. IL-2 (A), IFN-\(\gamma\) (B), IL-5 (C), and GM-CSF (D) were all produced by PBMC (\(n = 10\)) in response to CM-1 and E-1 peptide pools at day 56. Wilcoxon signed-rank tests were performed comparing NC and CM-1 or E-1 responses; \(P\) values of less than 0.05 were considered significant. NC, negative (no stimulation) control; NS, not significant.
While the data presented here suggest that PIV-1 vaccination induces a T cell response that is similar to that induced by other vaccines known to confer protection, whether the PIV-1 product, or its tetravalent counterpart (51), is efficacious for preventing dengue is unknown. Previous studies in nonhuman primates demonstrated that tetravalent PIV vaccination followed by live DENV challenge resulted in most animals developing breakthrough RNAemia (52). In that same study, individual animals showed higher viremia than unvaccinated controls as well as differences from the unvaccinated controls in cytokine responses; the low number of animals with enhanced viremia prevented statistically significant conclusions, but in light of the Dengvaxia experience, this finding is of potential concern. It is worth noting that no CMI studies were performed in that study and that the role of CMI in the dengue nonhuman primate model in general is unclear. CD8⁺ T cell-mediated protection has been demonstrated in mouse models of DENV infection (53–56), and correlative evidence in humans also supports a likely protective role for CD8⁺ T cells, or for functional subsets thereof (27, 57). The lack of CD8⁺ T cell responses induced by the PIV-1 vaccine, while such responses are anticipated given its antigenic nature, may therefore be cause for concern. Given the similar absence of DENV-specific CD8⁺ T cell responses generated after immunization with Dengvaxia (58), which incorporates a yellow fever virus backbone, researchers in the dengue field may need to consider the possibility that vaccines which do not incorporate antigens capable of inducing a robust T cell response are missing a critical component for generating protective immunity. Thus, future studies of this—and other—vaccines should ideally include a comprehensive analysis of multiple components of immunity, including T cell responses. Subsequent efficacy and/or virus challenge studies will then be needed to link the characterized responses to clinical outcome data.

In summary, using a comprehensive suite of assays, we detected low-level T cell responses induced by a DENV monovalent PIV vaccine. These were dominated by

![Graph A](image1)

**FIG 5** Frequencies of DENV-specific CD4⁺ T cells correlate with circulating antibody responses post-vaccination. CD4⁺ T cell precursor frequencies for CM-1 and E-1 peptide pools were added and compared to (A) total DENV-1-specific IgG titers or (B) DENV-1 neutralizing antibody titers for all 20 subjects at day 56 postvaccination. Spearman correlation analyses were performed, with P values of less than 0.05 considered significant (indicating a correlation).
IL-2-producing, proliferating, multifunctional CD4+ T cells, which was expected due to the inactivated nature of the vaccine antigen. Such a response is consistent with induction of an antigen-specific T helper population that provided support for the development of neutralizing antibodies in these subjects. Future immunogenicity studies in the context of clinical trials of tetravalent PIV formulations will need to determine the serotype-specific valency of the response as well as exploit in-depth phenotyping techniques to explore the relationship between the CD4+ T cell response and the magnitude and durability of the antibody response.

MATERIALS AND METHODS

Clinical trial design. The samples used in this study were collected during clinical trial WRAIR protocol 1856 ["A Phase 1 Trial of the Walter Reed Army Institute of Research (WRAIR) Dengue Serotype 1 Purified Inactivated Virus Vaccine (DENV-1 PIV) in Flavivirus Antibody Naive Adults"], the details of which were previously reported (25). Briefly, 2.5 μg/0.5 ml (low dose) or 5 μg/0.5 ml (high dose) purified inactivated DENV-1 Nauru/West Pac/1974 adsorbed to alum was used to immunize 20 subjects (10 per group) during a two-dose vaccination regimen (days 0 and 28). Blood draws were performed prevaccination (day 0) and on days 7, 14, 28, 35, 42, 56, and 90 postvaccination. PBMC were isolated from whole blood collected in cell preparation tubes containing sodium citrate (BD Biosciences). The cells were placed in fetal bovine serum containing 10% dimethyl sulfoxide (DMSO) and cryopreserved in vapor-phase liquid nitrogen until use. The protocol was approved by the institutional review board, U.S. Army Human Subjects Research Review Board, Office of the Surgeon General. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Peptides. Pools of 12mer to 20mer peptides with 10 to 12 amino acids of overlap and corresponding to the full-length envelope (E) and nonstructural 1 (NS1) proteins of DENV-1 Nauru/West Pac/1974 were obtained from BEI Resources. Peptide pools (16mers with 11 amino acids of overlap) covering both the capsid (C) and precursor membrane (M) proteins of DENV-1 were purchased from JPT Peptide Technologies. Peptide pools corresponding to the hexon protein of adenovirus serotype 5 (AdHex) and the pp65 protein of human cytomegalovirus (HCMV) were also purchased from JPT Peptide Technologies and used as controls. Peptide pool stocks were reconstituted in 100% DMSO at a concentration of 200 μg/ml/peptide and stored at –80°C.

T cell ELISpot assay. Cryopreserved PBMC were thawed and placed in RPMI 1640 medium supplemented with 10% heat-inactivated normal human serum (NHS; catalog no. 100-318; Gemini Bio-Products), 1-glutamine, penicillin, and streptomycin. After an overnight rest at 37°C, the PBMC were washed, resuspended in serum-free medium (X-Vivo 15; Lonza), and 1×10^6 to 2×10^6 cells were plated per well of a 96-well plate (Millipore; catalog no. MAIPSWU) coated with anti-IFN-γ or anti-IL-2 antibodies as instructed in the respective ELISpot kit manuals (3420-2HW-Plus and 3440-2HW-Plus; Mabtech Inc.). Peptide pools were added to the cells to reach a final concentration of 1 μg/ml/peptide prior to incubation at 37°C overnight. The negative control was serum-free medium containing 0.5% DMSO. Positive controls included staphylococcal enterotoxin B (SEB) and 1 μg/ml/peptide of the AdHex or HCMV peptide pools. The ELISpot plates were developed the next day using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and read using a CTL ImmunoSpot plate reader (Cellular Technology Limited).

Intracellular cytokine staining (ICS) assay. Cryopreserved PBMC were thawed and placed in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1-glutamine, penicillin, and streptomycin ("R10"). Approximately 1×10^6 PBMC were plated per well of a 96-well plate in a total volume of 200 μl R10 along with anti-CD28, anti-CD49d, and fluorescein isothiocyanate (FITC)-conjugated anti-CD107a antibodies (BD Biosciences) and 1 μg/ml/peptide of the relevant peptide pool. R10 containing 0.5% DMSO was used as a negative control. Positive controls included 50 ng/ml phorbol myristate 13-acetate (PMA) plus 1 μg/ml ionomycin and 1 μg/ml/peptide AdHex. Cells were incubated at 37°C for 1 h prior to addition of brefeldin A and monensin (BD Biosciences) and then left to continue incubating overnight. The next day, cells were washed and stained with LIVE/DEAD Aqua (Invitrogen, Life Technologies) followed by the surface antibodies Brilliant Violet 785-conjugated anti-CD3 (BV785-CD3), BV605-CD4, BV650-CD8 (BioLegend), Alexa 700-CD14, and Alexa 700-CD19 (BD Biosciences). After fixation in 4% formaldehyde, cells were permeabilized and stained with the antibodies eFluor450–IFN-γ, phycoerythrin (PE)–Cy7–TNF-α, PE–MIP-1β, allop hurcyanin (APC)–IL-2, and PE-Cy5–CD154 (BD Biosciences). Data were collected using a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed using FlowJo Version 7 software (FlowJo, LLC).

CFSE-based proliferation assay. Cryopreserved PBMC were thawed and rested overnight in RPMI 1640–10% NHS. Cells were then washed in Hanks’ balanced salt solution (HBSS) and labeled with 5 μm carboxyfluorescein succinimidyl ester (CFSE)–HBSS at room temperature for 10 min. After addition of an equal volume of 100% NHS for 5 min, the labeled cells were washed and plated (1×10^6 per well) in RPMI 1640–10% NHS with 1 μg/ml/peptide of the DENV-1 CM or E peptide pool. The negative control was RPMI 1640–10% NHS plus 0.5% DMSO, and the positive control was 1 μg/ml each of anti-CD3 and anti-CD28 antibodies. Cells were cultured at 37°C for 7 days, and the supernatants were saved for cytokine analysis (with storage at –80°C). Then, cells were washed and stained with LIVE/DEAD Aqua as well as BV785-CD3, BV605-CD4, BV650-CD8, and Alexa 700-CD19. After fixation in 4% formaldehyde, samples were run on a BD LSRFortessa flow cytometer and data analyzed with FlowJo software. Responses to anti-CD3/CD28 stimulation were used to model the different generations of proliferating cells, and the resultant generational gates were used to define the different generations of cells present in the
DENV-specific proliferation data from the same sample to calculate CM- or E-specific T cell precursor frequencies. Precursor frequencies were calculated as described previously (28–30). This assay was performed on a subset of 10 subjects, including 6 from the low-dose group and 4 from the high-dose group, based on sample availability.

Luminex assay. Supernatants collected from the CFSE assay cultures were thawed and tested using a human cytokine 30-plex panel kit (catalog no. LHC6003M; Invitrogen Inc.) according to the manufacturer’s instructions, resulting in quantification of the presence of the following molecules: epidermal growth factor (EGF), hepatocyte growth factor (HGF), IFN-α, IFN-γ, IL-1 receptor antagonist (IL-1RA), IL-1α, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, IP-10, monocyte chemottractant protein 1 (MCP-1), MIG, MIP-1α, MIP-1β, RANTES, TNF-α, and vascular endothelial growth factor (VEGF). The culture supernatants were plated undiluted in 96-well Multiscreen HTS filter plates (catalog no. MSBVS1210; EMD Millipore Corp.) containing the 30-plex antibody-coated beads and incubated at room temperature on a shaker platform (MmixMate; Eppendorf) at 550 rpm for 2 h. The plates were washed using a MultiScreen HTS vacuum manifold (Millipore). The analyte-bound beads were tagged with the biotin-conjugated antibodies included in the kit, washed, and visualized with streptavidin-conjugated PE. The data were acquired on a Luminex 200 instrument (Luminex Corp.) with Luminex 100 Integrated System 2.3 software (Luminex Corp.). Protein standards were provided in the kit, and standard curves were generated with eight standard dilutions (undiluted, 1:3, 1:9, 1:27, 1:81, 1:243, 1:729, 1:2,187) using a five-parameter logistic curve fit and 1/y weighted function. The data were then exported and further analyzed using Microsoft Excel and GraphPad Prism software packages. This assay was performed on a subset of the samples collected from the CFSE cultures; n = 10 at study days 0 and 56 and n = 4 (2 each from the low-dose and high-dose groups) at all other time points.

Statistics. All statistical analysis was performed using GraphPad Prism 6, FlowJo 7.0, and Microsoft Excel 2007 software packages. Nonparametric tests were used as the default to compare effects (frequencies of responsive cells or concentrations of cytokine/chemokine produced) between different test and control groups. Where appropriate, corrections for multiple comparisons were made. Fisher’s exact test was used for determination of statistical significance in contingency-table-based data (number of responders to a given stimulation condition).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 1.1 MB.

FIG S2, TIF file, 0.8 MB.

FIG S3, TIF file, 0.8 MB.

FIG S4, TIF file, 0.1 MB.

FIG S5, TIF file, 0.6 MB.

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