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14. ABSTRACT
The objective of this project is to elucidate the immunological mechanisms induced by live-attenuated and purified inactivated dengue virus vaccines administered in a heterologous prime-boost regimen. Innate and adaptive (T and B cell) responses will be measured using molecular and cellular approaches and the data analyzed using a systems biology approach. During the third project year, we performed genomic characterization of study subjects using molecular HLA typing, RNA-seq of unfractionated PBMC, and single-cell RNA-seq of PBMC. Genomic data were analyzed for associations with vaccine regimen and measures of vaccine immunogenicity. Preliminary findings from these analyses are being explored further.

15. SUBJECT TERMS
Dengue, vaccine, immune response

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1. INTRODUCTION:

This Investigator-Initiated Research Award project addresses the FY15 PRMRP Topic Area of Dengue. Dengue, a mosquito-borne viral disease, represents a global health concern that affects the US military because of the risk of illness in personnel deployed to endemic areas in Asia, Central and South America, and the Middle East. The development of an effective vaccine against dengue has been given a high priority by the WHO, NIH, and DoD. Results of phase III clinical trials of the most advanced dengue vaccine candidate, a chimeric dengue-yellow fever live virus vaccine, indicate that this vaccine may not be suitable for DoD use due to a prolonged (12-month) dosing regimen and poor efficacy in dengue-naïve subjects. To mitigate this concern, the DoD's Alternate Dengue Vaccine Program (ADVP) has conducted clinical trial ADVP-003, a four-arm study using a heterologous prime-boost dosing regimen involving live attenuated virus (LAV) and purified inactivated virus (PIV) vaccine formulations in both sequences with two different intervals between doses. The ADVP-003 trial is a critical first step towards testing this vaccine strategy, to be followed by downselection of one or more regimens for more extensive testing. The short-term impact of this project will be to elucidate the immunological mechanisms induced by live-attenuated virus (LAV) and purified inactivated virus (PIV) based Dengue vaccines and thereby guide the design of subsequent clinical trials. The long-term impact of this project will be to advance understanding of dengue vaccines in general and provide a framework for assessment of next generation dengue vaccines.

2. KEYWORDS:

Dengue virus; cell-mediated immunity; systems biology; transcriptomics; innate immunity; adaptive immunity; correlates of immunity; live-attenuated; purified inactivated; biomarkers; T-cell; B-cell; epitope

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The objective of the original project was to apply a comprehensive suite of immunological assays to blood samples collected in a clinical trial of different regimens of live and inactivated dengue virus vaccines, in order to a) identify the immunological pathways activated by each vaccine component, and b) identify the immunological pathways that most strongly predict the magnitude of post-vaccination dengue virus (DENV)-specific antibody and T cell responses. The Specific Aims were:

1. Compare the innate immune responses activated by primary and booster immunizations with inactivated and live attenuated dengue vaccines
2. Compare the frequency, phenotypes, antigen specificity, and gene expression of activated T and B lymphocytes during the acute response to primary and booster immunizations with inactivated and live attenuated dengue vaccines

3. Determine the associations between early innate and adaptive immune activation and the levels, antigen specificity, and durability of DENV-specific antibody and memory T and B cell responses after primary and booster immunizations

The Major Tasks and Milestones under these Aims that were listed in the approved Statement of Work and their statuses are shown in the table below.

Task/Milestone	Time (mos)	Status
Specific Aim 1		
Major Task 1: Obtain institutional approvals and select specimens for analysis	3	Completed
Milestone #1: Institutional approvals obtained, specimens for analysis identified	3	Completed
Major Task 2: RNA-seq analysis on early PBMC samples from subset of study population	14	Laboratory work completed
Milestone #2: Prepare manuscript on RNA sequencing data	14	50% completed
Major Task 3: Nanostring analysis of candidate gene expression in full trial cohort	18	Completed
Major Task 4: Measure serum cytokine levels	24	Experimental work completed Data analysis 75% completed
Milestone #3: Prepare manuscript on innate immune response (gene expression, cytokines)	24	50% completed
Specific Aim 2		
Major Task 5: Ex vivo flow cytometry analysis of T and B lymphocyte specificity and phenotype	18	Completed
Major Task 6: Flow cytometry analysis of peptide-specific T lymphocyte responses	15	Laboratory work completed
Milestone #5: Prepare manuscript- ex vivo flow cytometry and ICS assays	15	50% completed
Major Task 7: Analyze gene expression in sorted T and B lymphocyte populations	20	Completed
Major Task 8: Perform TCR-effector linkage sequencing analysis of peptide-stimulated PBMC	24	Laboratory work completed (using single-cell RNA-seq)
Milestone #6: Prepare manuscript- Nanostring and TELS analyses	24	50% completed
Specific Aim 3		
Major Task 9: Perform integrated data analysis	24	Database setup completed

Task/Milestone	Time (mos)	Status
		Data analysis 75% completed

What was accomplished under these goals?

The ADVP-003 clinical trial compared immune responses to four different heterologous prime/boost vaccine regimens; subjects received one of two sequences of vaccines- live attenuated vaccine (LAV) prime followed by purified inactivated vaccine (PIV) boost or PIV prime followed by LAV boost- at intervals of 28 d or 6 mo. Blood samples collected during the trial, as well as the primary immunogenicity read-outs (neutralizing antibody titers and IFNγ ELISPOT responses at 6 mo post-vaccination) were completed and available to this project.

Specific Aim 1: Compare the innate immune responses activated by primary and booster immunizations with inactivated and live attenuated dengue vaccines.

An amendment to the ADVP-003 study protocol was submitted to address the transcriptional profiling studies. While genetic studies were proposed in the original study protocol, the IRB determined that it was necessary to re-consent subjects before the proposed studies could begin. Fifty-two subjects consented to genetic testing.

We analyzed serum levels of 59 cytokines and immune activation markers by multiplex immunoassays from a subset of study subjects. We found elevated serum levels of several cytokines and activation markers in the acute phase after receipt of LAV. In contrast, there was very little change in levels of these markers after receipt of PIV; Figure 1 shows levels of soluble TNF receptor 2 (sTNFR2) as one example. These results suggest that LAV induced greater immune activation than PIV.

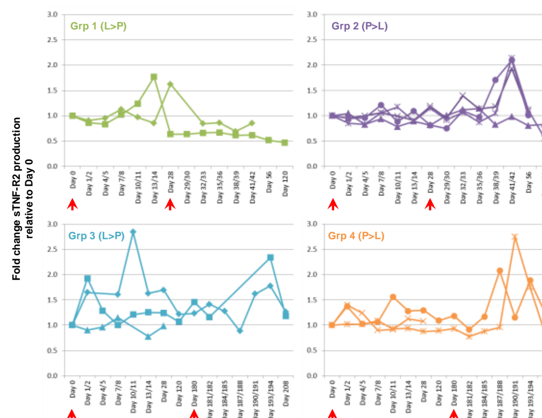


Figure 1. Changes in serum levels of sTNF-R2 after vaccination, according to dosing group. Lines represent individual subjects and values are fold change from baseline levels. Dates of vaccination are marked with red arrows. L = LAV, P = PIV.

We performed transcriptomic analysis using RNA-seq. Total cellular RNA was isolated from PBMC samples of 50 study participants (13, 10, 14, and 13 subjects from each vaccine group) and used to prepare Illumina-compatible libraries. We studied time points immediately before and in the early period after initial (prime) and boost immunization. Sequencing of the

libraries was performed on an Illumina NovaSeq 6000 sequencer, and differential gene expression analyzed using the statistical software environment R. We used mixed effects modeling to identify genes that showed significant differences in gene expression around vaccination with LAV or PIV. We used these associations to define biological processes showing the most significant associations with vaccination group (Table 1). As anticipated, a number of immune response pathways were differentially activated by these vaccines. The kinetics of expression of *fcgr1a* are shown in Figure 2 as an example of these associations. The inclusion of metabolic pathways in the list of biological processes associated with vaccine group is consistent with experimental evidence that cellular metabolism is critical in regulating the development of immunological memory. These results identify promising gene sets for studies of long-term vaccine immunogenicity.

Table 1. Biological processes showing enrichment in gene expression associated with vaccination group in the ADVP-003 trial at false discover rate (FDR) <0.05. Gene expression changes were evaluated in a multivariable model and significant changes were analyzed using the PANTHER analysis tool.

Gene ontogeny	Fold enrichment	FDR
pentose-phosphate shunt	9.5	0.00149
NADPH regeneration	8.23	0.0028
response to peptidoglycan	7.72	0.00977
negative regulation of IL-12 production	6.36	0.0211
positive regulation of TCR signal pathway	6.17	0.0499
TLR4 signaling pathway	5.4	0.0392
positive regulation of antigen receptor-mediated signaling	5.37	0.0207
T cell selection	4.78	0.00156
positive regulation of p38MAPK cascade	4.75	0.0351
negative regulation of viral transcription	4.41	0.0493
dendritic cell differentiation	4.21	0.035
positive regulation of T cell differentiation	3.02	0.00687
positive regulation of lymphocyte differentiation	2.94	0.00433
positive regulation of leukocyte cell-cell adhesion	2.76	1.21 E-05
leukocyte activation involved in immune response	2.72	2.31 E-15

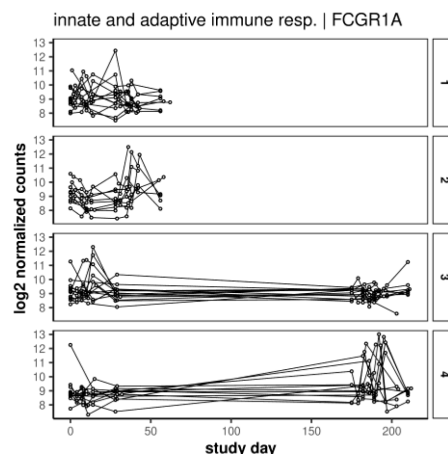


Figure 2. Expression of fcgr1a by RNA-seq, by vaccination group.

Specific Aim 2: Compare the frequency, phenotypes, antigen specificity, and gene expression of activated T and B lymphocytes during the acute response to primary and booster immunizations with inactivated and live attenuated dengue vaccines.

We used flow cytometry, intracellular cytokine staining, and IFN-γ ELISPOT assays to measure the expression of activation markers and functional responses to DENV peptides. We detected increases in the frequency of antigen-specific T cells after vaccination, and these cells expressed multiple effector functions (Figure 3).

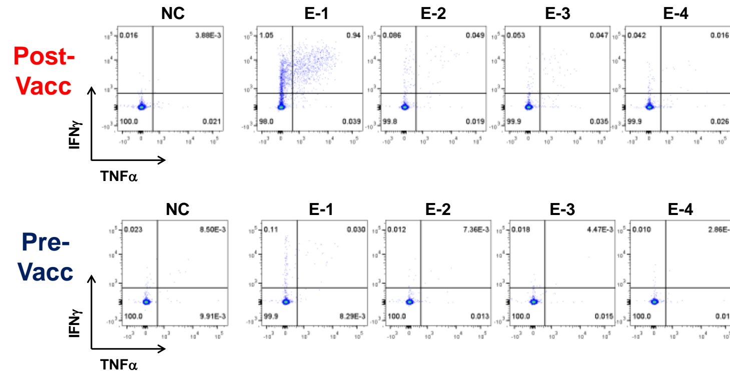


Figure 3. Representative cytokine flow cytometry comparing responses to E peptide pools of each DENV serotype in PBMC collected pre-vaccination versus post-vaccination. Top right quadrants indicate the frequency of T cells producing both IFNγ and TNFα. Peptide pools used for stimulation are noted at the top of each plot. NC = negative (unstimulated) control.

We optimized laboratory protocols for fluorescent staining of DENV for multicolor assays. We successfully labeled viruses with four distinct fluorochromes: DL405, DL488, DL594, and DL650, and we demonstrated simultaneous detection of the four colors in both flow cytometry and fluorospot assay formats. These methods represent an advance over our previous work by facilitating the 4-way analysis of serotype crossreactivity of memory B cells.

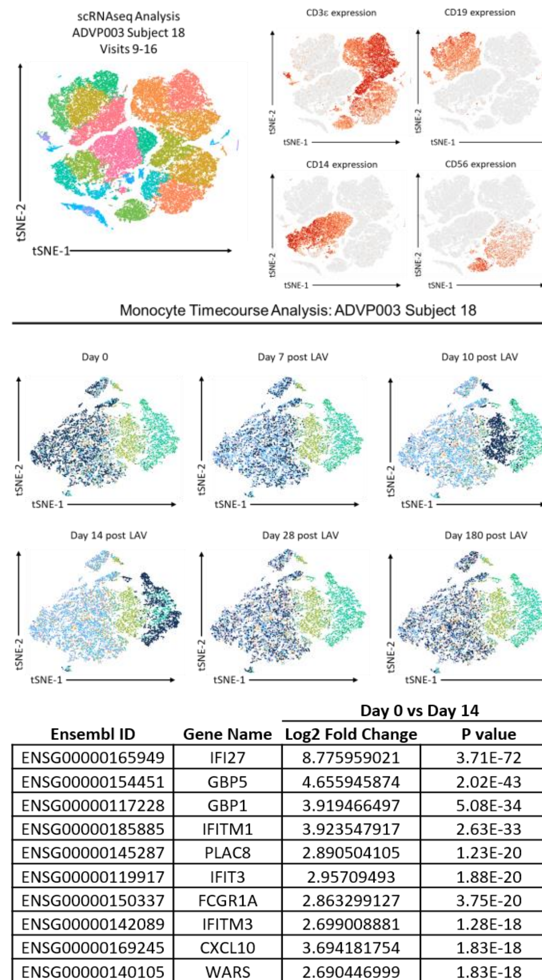
We performed molecular HLA typing of the HLA-A, -B, -C, -DRB1, -DPB1, and -DQB1 gene loci by targeted gene amplification of genomic DNA isolated from PBMC. Sequencing libraries were prepared using Nextera Flex DNA kits (Illumina) and sequenced on an Illumina MiSeq instrument. Sequence data were analyzed and mapped to HLA alleles using HISAT-genotype. We identified HLA alleles that were found commonly (>25%) in the study cohort: HLA-A*02 (17 subjects), A*30 (13), B*07 (15), C*03 (14), C*06 (13), C*07 (30), DRB1*11 (14), DRB1*13 (13), DRB1*15(19), DPB1*02 (15), DPB1*04 (27), DQB1*02 (21), DQB1*03 (24), and DQB1*06 (26).

We aligned the HLA typing data with the results of peptide mapping IFN-γ ELISPOT assays. This analysis identified several novel candidate immunodominant T cell epitopes restricted by common HLA alleles (Table 2). These peptides will be useful for detailed characterization of DENV-specific T cells in this and other study cohorts.

Table 2. Immunodominant T cell epitopes identified in study subjects and candidate restricting HLA-A or -B alleles.

Peptide(s)	HLA class I alleles
NS2b-21, NS2b-22	A*23:01, A*30:01, B*53:01
E-28	B*57:01
NS5-58, NS5-59	A*02:01, A*03:01, B*07:02; B*15:01
NS1-20	B*07:02

We performed single cell RNA-seq (scRNA-seq) analysis to assess the specific cell types associated with gene expression changes (Figure 4). We utilized the 10x Genomics Chromium Single Cell Analyzer and the Drop-Seq approach. We sorted T cells, B cells, and non-T/non-B cells (mainly NK cells and monocytes) from cryopreserved PBMC collected at eight time points before and after LAV vaccination; flow cytometry and cell sorting provided parallel phenotypic data. Barcoded single-cell cDNA libraries were processed to generate single-cell sequencing libraries for both gene expression and immunoreceptor (full-length TCR and BCR) analysis. T cells, B cells, monocytes, and NK cells were readily defined within the scRNA-seq dataset (Figure 4 top). Time course analysis showed the most significant changes in gene expression within the monocyte population during the second week post-vaccination (Figure 4 middle). Differentially expressed genes in monocytes (Figure 4 bottom) reflected a predominance of IFN-stimulated genes. These results established suitable experimental protocols for further studies and identified candidate gene sets for additional analyses.



Ensembl ID	Gene Name	Day 0 vs Day 14	
		Log2 Fold Change	P value
ENSG00000165949	IFI27	8.775959021	3.71E-72
ENSG00000154451	GBP5	4.655945874	2.02E-43
ENSG00000117228	GBP1	3.919466497	5.08E-34
ENSG00000185885	IFITM1	3.923547917	2.63E-33
ENSG00000145287	PLAC8	2.890504105	1.23E-20
ENSG00000119917	IFIT3	2.95709493	1.88E-20
ENSG00000150337	FCGR1A	2.863299127	3.75E-20
ENSG00000142089	IFITM3	2.699008881	1.28E-18
ENSG00000169245	CXCL10	3.694181754	1.83E-18
ENSG00000140105	WARS	2.690446999	1.83E-18

Figure 4. scRNAseq analysis of blood samples collected before and after LAV administration from one ADVP-003 subject. (**top**) tSNE projection of 53,706 cells derived from 8 time points. T cells (CD3+ CD19-), B cells (CD3- CD19+), and non T/B cells (CD3- CD19-) were sorted and pooled (1:1:1 ratio) for scRNAseq analysis. CD3e, CD19, CD14, and CD56 gene expression is highlighted to show the distributions of T cells, B cells, monocytes and NK cells in the dataset. (**middle**) Differential gene expression in monocytes in sequential blood samples. All captured monocytes are shown in each tSNE plot, with the cells corresponding to the indicated time point highlighted in dark blue. (**bottom**) Ten most differentially expressed genes between monocytes captured on day 0 (pre-vaccination) and day 14 (post-vaccination).

Specific Aim 3: Determine the associations between early innate and adaptive immune activation and the levels, antigen specificity, and durability of DENV-specific antibody and memory T and B cell responses after primary and booster immunizations

We established a centralized SQL database for the project. Datatables were configured for each of the assays performed and linked by sample. We configured queries to merge datasets, as illustrated above (Table 2). The database has the capability of incorporating additional datasets as they become available and will serve as a resource for future exploratory analyses of ADVP-003 trial data.

Summary: We generated a detailed characterization of immune activation and antigen-specific immune responses in subjects from the ADVP-003 trial. These data have identified candidate early markers of vaccine immunogenicity/efficacy which are available to be used in future studies.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Two manuscripts describing a) cellular immune responses to PIV/LAV prime-boost vaccination and b) RNA-seq analysis of responses to PIV/LAV prime-boost vaccination are in preparation.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

We generated a detailed characterization of immune activation and antigen-specific immune responses in subjects from the ADVP-003 trial, and these data have identified candidate early markers of vaccine immunogenicity/efficacy. The findings are being applied to guide the design of studies of alternative vaccine regimens, dengue human challenge experiments, and natural dengue virus infections, for example, in setting the time points for collection of blood samples for analysis. The findings are also being used in the planning of new research initiatives to prospectively test candidate markers of vaccine immunogenicity and efficacy for vaccines against dengue and other viral infections.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

As reported previously, we revised our original experimental strategy to rely more heavily on global gene expression analysis using RNA-seq rather than targeted gene expression using Nanostring. This change in strategy was based on preliminary analyses of our initial experimental results as well as revised cost estimates for the more extensive global gene expression analyses.

Actual or anticipated problems or delays and actions or plans to resolve them

Requests to the NIAID Tetramer Core for HLA-peptide tetramers and work on project manuscripts were delayed during the final year due to work restrictions related to COVID-19.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS

- **Publications, conference papers, and presentations**

Journal publications.

Gabriel B, Medin C, Fitzgerald K, Waickman A, Rothman A, Currier J, Friberg H. Activation of innate and adaptive immune response pathways by live attenuated and purified inactivated dengue vaccines administered in a heterologous prime-boost regimen. Manuscript in preparation.

Friberg H, Waickman A, Mathew A, Gabriel B, Rothman A, Currier J. Cellular activation and dengue virus-specific immune responses induced by heterologous prime-boost dengue vaccination. Manuscript in preparation.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

WRAIR (Silver Spring, MD):

Name: Jeffrey R. Currier – No change
Project Role: Co-Principal Investigator

Name: Heather Friberg – No change
Project Role: Co-Investigator

Name: Adam Waickman – No change
Project Role: National Research Council Fellow

University of Rhode Island (Providence, RI):

Name: Alan L Rothman – No Change
Project Role: Co-Principal Investigator

Name: Anuja Mathew – No Change
Project Role: Co- Investigator

Name: Barbara Payne – No Change
Project Role: Co- Investigator

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Organization name: Walter Reed Army Institute of Research (WRAIR)
Location of Organization: Silver Spring, MD
Partner's contribution to the project: Collaboration (WRAIR is a partner institution on this collaborative award)

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES

None