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TITLE: Systems Biology of the Immune Response to Live and Inactivated Dengue Virus Vaccines

PRINCIPAL INVESTIGATOR: Dr. Alan L Rothman

RECIPIENT: University of Rhode Island

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Fort Detrick, Maryland 21702-5012

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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

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

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:** Provide a brief list of keywords (limit to 20 words).

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:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Please see **Attachment #1** for the status of major tasks, subtasks, and milestones.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Please see **Attachment #2** for a description of accomplishments.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

A manuscript describing cellular immune responses to PIV/LAV prime-boost vaccination is in preparation.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Data collection for this project is now complete and we are continuing the analysis of the extensive genomic and immunologic datasets to identify the strongest correlates of vaccine immunogenicity and to compare responses to different regimens of live and inactivated vaccines.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The genomic and immunologic data generated from the ADVP-003 study are being used to guide parallel studies of alternative vaccine regiments (e.g., ADVP-004 study of live and inactivated vaccines in a heterologous prime-boost regimen), dengue human challenge experiments, and natural dengue virus infections.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

The project team reviewed the results of the initial set of RNA-seq data in June 2019. The team assessed that extending the RNA-seq studies by testing additional subjects and additional timepoints would be a more cost-effective strategy to complete the project objectives than the Nanostring and luminex analyses originally proposed.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Preparation of libraries for RNA-seq analysis was more time-consuming than our original plan (for Nanostring analysis); this has led to a delay in completing Tasks 3, 4, and 9 and in reaching project milestones #2, 3, 5, and 6. We are requesting an additional no-cost extension to the project until 30 Mar 2020 to complete these tasks.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

None; all of the project funds have been expended.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

None during the most recent reporting period.

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: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

See **Attachment #3** for a full list of individuals who have worked on this project.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

New Active Support- new PRMRP grant W81XWH1920023 was awarded to SUNY Upstate Medical University (PI Stephen Thomas); Dr. Rothman is Co-Investigator at URI on this project.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

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

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Attachment #1

STATEMENT OF WORK – Month/Day/Year
START DATE Sept 1, 2016
INTERIM PROGRESS DATE Sept 30, 2019

Site 1:	Site 2:	Site 3:
University of Rhode Island (URI)	Walter Reed Army Institute of Research (WRAIR)	University of Massachusetts Medical School (UMMS)
80 Washington St.	503 Robert Grant Ave.	55 Lake Ave. North
Providence, RI 02903	Silver Spring, MD	Worcester, MA 01655
Initiating PI: Dr. Rothman	Partnering PI: Dr. Currier	Co-Investigator: Dr. Fitzgerald

Specific Aim 1: Compare the innate immune responses activated by primary and booster immunizations with inactivated and live attenuated dengue vaccines	**Timeline** (months)	**Task and Milestone Status** (Completion date or delay issue)
Major Task 1: Obtain institutional approvals and select specimens for analysis		
Subtask 1: File amendment with WRAIR IRB	1-2	Completed January 2017
Subtask 2: Review sample inventory and select subjects and specimens for testing	1-3	Completed January 2017
Milestone #1: Institutional approvals obtained, specimens for analysis identified	2-3	Completed August 2017; IRB required that subjects be re-consented for gene expression analyses
Major Task 2: RNA-seq analysis on early PBMC samples from subset of study population		
Subtask 1: Isolate RNA from PBMC and assess quality	3-6	Completed September 2018; delayed due to requirement to re-consent subjects
Subtask 2: Prepare RNA for RNA sequencing and submit to service core facility	4-7	Library preparation completed October 2018, sequencing completed December 2018

Subtask 3: Bioinformatics analysis • UMMS: Quality control of RNA-seq reads, Alignment to reference genome, differential Expression- statistical testing, Systems Biology analysis • URI: Systems Biology analysis	7-12	Analysis of the pilot data set completed April 2019
Milestone #2: Prepare manuscript on RNA sequencing data	8-14	Postponed- manuscript will incorporate more extensive RNA-seq dataset (Major Task 3)
Major Task 3: Nanostring analysis of candidate gene expression in full trial cohort		
Subtask 1: Selection of codeset for Nanostring analysis	13-14	Experimental strategy revised July 2019- samples submitted for RNA-seq rather than Nanostring
Subtask 2: Isolate RNA from PBMC and assess quality	6-14	Completed October 2019
Subtask 3: Perform Nanostring analyses	15-18	RNA-seq performed- completed November 2019
Major Task 4: Measure serum cytokine levels		
Subtask 1: Perform Luminex assays	3-6	Experimental strategy revised July 2019- additional analyses performed using RNA-seq rather than Luminex (Major Task 3)
Subtask 2: Analyze data	7-12	In progress- anticipated completion March 2020
Milestone #3: Prepare manuscript on innate immune response (PBMC gene expression and serum cytokines)	18-24	Not yet initiated- will follow completion of RNA-seq data analysis, anticipated completion May 2020
<u>Specific Aim 2:</u> 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	**Timeline** (months)	**Task and Milestone Status** (Completion date or delay issue)

Major Task 5: Ex vivo flow cytometry analysis of T and B lymphocyte specificity and phenotype		
Subtask 1: Prepare fluorescently labeled DENV	1-6	Completed July 2018
Subtask 2: Perform HLA typing	3-6	Completed January 2019
Subtask 3: Obtain HLA-peptide tetramers	3-12	Peptides ordered, tetramers will be requested from NIAID Core Facility when peptides available, anticipated completion January 2020
Subtask 4: Perform ex vivo flow cytometry	9-15	Completed August 2019
Subtask 5: Analyze data	10-18	Completed August 2019
Major Task 6: Flow cytometry analysis of peptide-specific T lymphocyte responses		
Subtask 1: Perform ICS assays to identify immunodominant epitopes	4-12	Completed April 2017 (using ELISPOT assays)
Subtask 2: Analyze data	4-14	Completed September 2018
Milestone #5: Prepare manuscript- ex vivo flow cytometry and ICS assays	13-15	In progress- anticipated completion May 2020
Major Task 7: Analyze gene expression in sorted T and B lymphocyte populations		
Subtask 1: Perform fluorescence-activated cell sorting	10-15	Completed August 2019
Subtask 2: Isolate RNA	11-16	Completed August 2019
Subtask 3: Perform Nanostring analysis of candidate gene expression	13-18	Completed August 2019 (using 10X Genomics Chromium platform)
Subtask 4: Data analysis	15-20	Completed August 2019
Major Task 8: Perform TCR-effector linkage sequencing analysis of peptide-stimulated PBMC		
Subtask 1: Select samples for analysis	10-16	Completed June 2019

Subtask 2: Stimulate PBMC and generate single cell emulsions	11-18	Completed August 2019 (using 10X Genomics Chromium platform)
Subtask 3: Perform linkage PCR	11-18	Completed August 2019 (using 10X Genomics Chromium platform)
Subtask 4: Deep sequencing of PCR products	13-20	Completed August 2019 (using 10X Genomics Chromium platform)
Subtask 5: Data analysis	15-22	Completed August 2019 (using 10X Genomics Chromium platform)
Milestone #6: Prepare manuscript- Nanostring and TELS analyses	21-24	In progress- anticipated completion May 2020
<u>Specific Aim 3:</u> 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	**Timeline** (months)	**Task and Milestone Status** (Completion date or delay issue)
Major Task 9: Perform integrated data analysis		
Subtask 1: Develop coordinated database (neutralizing antibody, T and B cell ELISPOT, gene expression, and flow cytometry) • URI: Create and house database, import data from external sources • WRAIR/UMMS: Input on database organization, provide data sources for inclusion	3-16	Completed March 2019
Subtask 2: Statistical analyses	16-24	In progress- anticipated completion May 2020

Attachment #2

Scientific Accomplishments – September 2019

Accomplishments summary: The following tasks in the Statement of Work were addressed during the most recent reporting period:

1. We completed a first round of RNA-seq analysis of PBMC samples from 6 time points from 21 subjects. Systems biology analysis of these data identified gene signatures of the different vaccines (live versus inactivated) and preliminary correlates of immune responses (neutralizing antibody and IFN-γ ELISPOT responses).
2. We performed a second round of RNA-seq analysis of a total of 288 PBMC samples from all study subjects who consented to genetic testing. These data are currently being processed through our computational pipeline. Preliminary analyses indicate that we exceeded our targeted sequencing depth and quality for all of the libraries tested. Systems biology analysis of these data will be initiated once the initial processing is complete.
3. We completed molecular HLA typing from all study subjects who consented to genetic testing. These data have been analyzed in combination with peptide mapping ELISPOT data, and have identified several strong candidate epitopes for more detailed characterization including HLA-peptide tetramers.
4. We performed detailed flow cytometry and single-cell genomic analysis of PBMC from multiple time points after live virus vaccination in a representative study subject. Systems biology analysis of these data demonstrated peak gene expression responses within the monocyte population during the second week after vaccine administration; these data were used to inform our selection of PBMC samples for the second round of bulk PBMC RNA-seq analysis.

Major Accomplishments: Additional details on major accomplishments during year 2 are listed below by specific aim and major task.

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

Major Task 2: RNA-seq analysis on early PBMC samples from subset of study population

We isolated RNA from 126 PBMC samples collected on days 0, 3, and 7 around both vaccine doses from a total of 21 subjects (5 in vaccination groups 1-3 and 6 in vaccination group 4). RNA-seq libraries were prepared using TruSeq Stranded Total RNA LP Gold kits (Illumina), and the libraries were then pooled and sequenced on an Illumina NovaSeq instrument at WRAIR.

Raw sequence reads were processed and analyzed through our computational pipeline to align the sequences with the human genome and assemble read counts for each sample. Differential gene expression analysis was then performed in R using DEseq2.

Analyses conducted to date have shown greater changes in gene expression on day 3 after PIV (inactivated vaccine) administration and on day 7 after LAV (live vaccine) administration (Figures 1, 2). Using a cutoff p value of <0.05 to identify candidate genes for further study, we found >2000 genes with altered expression on day 3 after PIV and >1000 genes with altered expression on day 7 after LAV. Candidate genes whose expression correlated with DENV-specific neutralizing antibody responses and/or DENV peptide-specific IFN-γ T cell responses were also identified.

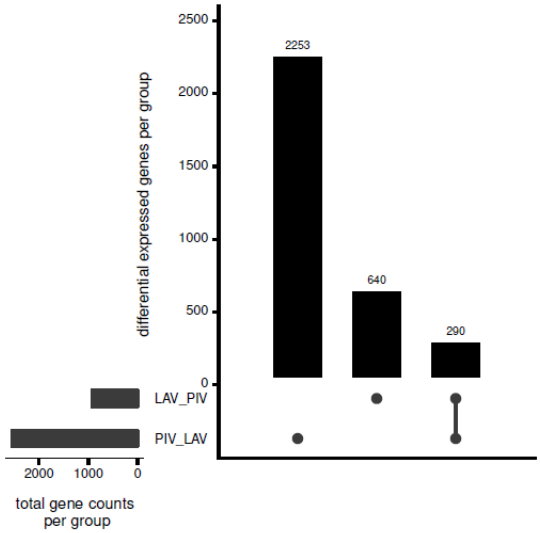


Figure 1. Number of genes differentially expressed ($p<0.05$) between days 0 and 3. LAV_PIV group received LAV on day 0; PIV_LAV group received PIV on day 0.

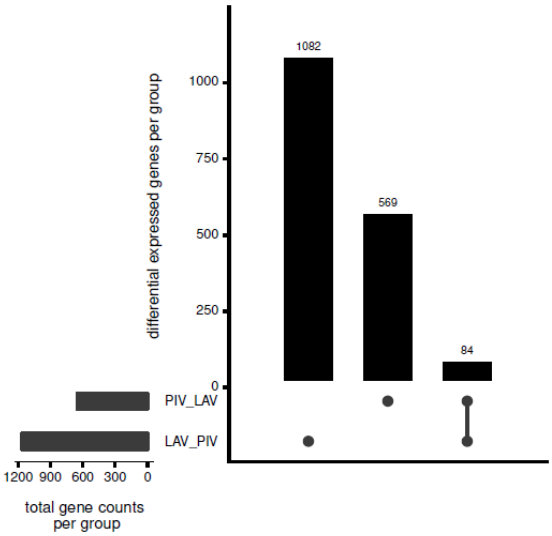


Figure 2. Number of genes differentially expressed ($p<0.05$) between days 0 and 7. LAV_PIV group received LAV on day 0; PIV_LAV group received PIV on day 0.

Major Task 3: Nanostring analysis of candidate gene expression in full trial cohort

After reviewing the results of the initial RNA-seq analysis described above, we revised our plan for testing the larger trial cohort, favoring additional RNA-seq over Nanostring. This decision was based on the large number of differentially expressed genes and the relative cost of RNA-seq on the WRAIR Novaseq versus Nanostring.

We therefore selected 288 additional PBMC samples collected on days 0, 3, 7, 10, 14, and 28 around both vaccine doses from all of the 50 subjects who had consented to such testing (Figure 3). (Subjects tested in the first RNA-seq run had the additional time points tested in run #2.) Preparation of

libraries, sequencing, and processing of raw sequence data were conducted as described above, except that pooling of libraries was performed at URI.

Processing and analysis of this larger RNA-seq dataset is still in progress. Analyses conducted to date have shown read quality and quantity were higher than our target for all libraries.

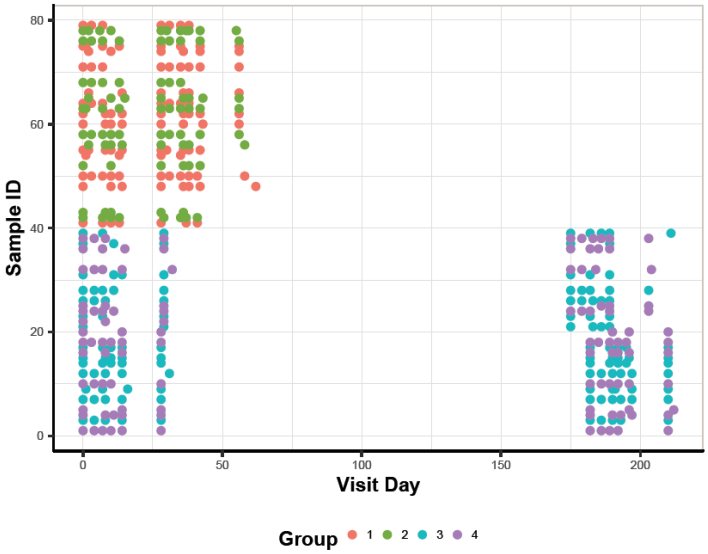


Figure 3. PBMC samples analyzed by RNA-seq, by subject number (Sample ID) and visit day. Vaccine groups 1 and 3 received LAV followed by PIV; vaccine groups 2 and 4 received PIV followed by LAV. The interval between vaccinations was ~28 days for groups 1 and 2 and ~180 days for groups 3 and 4.

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.

Major Task 5: Ex vivo flow cytometry analysis of T and B lymphocyte specificity and phenotype

We performed molecular HLA typing of the HLA-A, -B, -C, -DRB1, -DPB1, and -DQB1 gene loci from the 50 subjects who consented to genetic testing. Genomic DNA was isolated from PBMC samples and subjected to targeted gene amplification by PCR. Sequencing libraries were prepared using Nextera Flex DNA kits (Illumina) and sequenced on an Illumina MiSeq instrument at URI. Sequence data were analyzed and mapped to HLA alleles using HISAT-genotype. HLA alleles found commonly (>25%) in the study cohort included 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 next aligned the HLA typing data with the results of peptide mapping IFN-γ ELISPOT assays. This analysis identified several candidate immunodominant T cell epitopes (Table 1), which are being evaluated further.

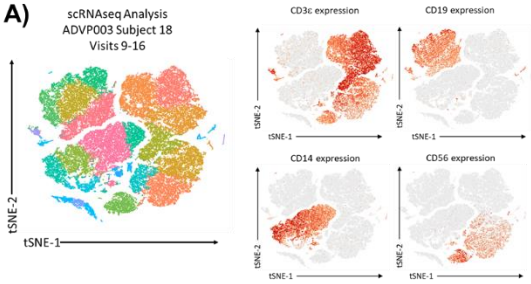
Table 1. Immunodominant T cell epitopes identified in study subjects and candidate restricting HLA-A or -B alleles.

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

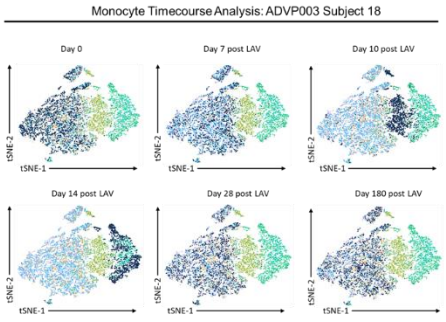
Major Task 7: Analyze gene expression in sorted T and B lymphocyte populations:

We performed single cell RNA-seq (scRNA-seq) to assess at a more granular level the specific cell types associated with the gene expression changes detected in the first round of bulk PBMC RNA-seq analyses (see above). For this purpose, we utilized the 10x Genomics Chromium Single Cell Analyzer and the Drop-Seq approach, which generates cDNA libraries tagged with unique cell-specific barcode tag, allowing for downstream deconvolution of pooled samples. We first 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 from one subject; upfront flow cytometry and cell sorting permitted both phenotypic analysis of PBMC and balancing of the cell populations to provide equivalent breadth and depth of scRNA-seq for these different cell populations. The barcoded single-cell cDNA libraries were then processed to generate single-cell sequencing libraries for both traditional gene expression analysis and for immunoreceptor (full-length TCR and BCR) analysis using the 10x Chromium reagent kits. Results of gene expression analysis are shown in Figure 4. T cells, B cells, monocytes, and NK cells could be readily defined within the scRNA-seq dataset (Figure 4A). Time course analysis showed the largest changes in gene expression within the monocyte population, and these changes were most marked during the second week (days 10 and 14) post-vaccination (Figure 4B). Differentially expressed genes (Figure 4C) reflect an innate immune response, with a predominance of IFN-stimulated genes.

Figure 4: scRNAseq gene expression analysis of 8 longitudinal samples from a single vaccine recipient post LAV administration. **A)** tSNE projection of 53,706 cells derived from 8 time points post LAV administration in a WRAIR 2136 study participant. Total T cells (CD3+ CD19-) B cells (CD3- CD19+) and non T/B cells (CD3- CD19-) were sorted from cryopreserved PBMC and re-pooled at a 1:1:1 ratio prior to analysis by scRNAseq. All samples were sequenced to an average depth of 150,000 reads/cell, resulting in a median recovery of 1,427 unique genes per cell. CD3ε, CD19, CD14, and CD56 gene expression is highlighted to show the distribution of T cells, B cells, monocytes and NK cells within the aggregate dataset. **B)** Time-course analysis of the differential gene expression elicited by LAV administration in monocytes captured by scRNAseq analysis. All captured monocytes are shown in each panel, with the cells corresponding to the indicated time point highlighted in dark blue. Spatial localization within the tSNE graph corresponds to differential gene expression elicited by vaccination. **C)** Top 10 most differential expressed genes between monocytes captured on day 0 post LAV administration and Day 14 post LAV administration



B)



C)

		Day 0 vs Day 14	
Ensembl ID	**Gene Name**	**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

Attachment #3

Individual Contributors – September 2018

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Nearest person month worked:	8
Contribution to Project:	Dr. Gabriel supervised RNA/DNA isolation and preparation of sequencing libraries, and contributed to the analysis of NGS data.