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

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13. SUPPLEMENTARY NOTES

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 first project year, IRB approvals, inter-institutional agreements, and measurements of dengue virus-specific neutralizing antibody titers and frequencies of cytokine-producing CD4 and CD8 T cells were completed. Flow cytometry of cellular activation and measurement of serum cytokine levels were completed on a subset of subjects. Pilot studies were done for RNA extraction and analysis of B cells and T cell repertoires.

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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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 proposed SoW for the study. Milestones that have been completed are highlighted in GREEN; those that are ongoing are highlighted in YELLOW and those that have been delayed are highlighted in RED. Milestones for experimental procedures that were not scheduled to start until year 2 (after October 1, 2017) are not highlighted.

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 the immunogenicity of the prime-boost vaccination approach using PIV/LAV is in preparation. Two abstract/poster presentations summarizing the B-cell data and the plasma cytokine profiling were presented at the American Association of Immunologists conference, May 2017.

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

Subjects will be re-consented to allow gene expression profiling and HLA typing.
Laboratory testing of innate and adaptive (T and B cell) responses will be performed as in our original plan.
Laboratory data will be uploaded into the project database and statistical analyses will be performed.

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 humoral and cell-mediated immunity immunogenicity data generated from the AVDP-003 study has been used to inform the design of the subsequent ADVP-004 study (ADVP = Alternative Dengue Vaccine Program). The PIV-prime/LAV-boost combination separated by 6 months was the strategy which generated the most robust humoral and cellular adaptive immunity. This strategy will be used to vaccinate more subjects with the long-term goal of challenging with a well-characterized dengue virus strain to study vaccine-induced protection.

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.

While there have been no changes to the overall experimental strategy and scope of work, we have encountered one problem that has impacted our timeline significantly. Institutional approvals from the WRAIR HSPB/IRB and from HRPO took 9 months to acquire. Stringent interpretations of the "Common Rule" by the WRAIR IRB/HSPB to conclude that transcriptional profiling is genetic testing necessitated re-consent of all subjects who participated in ADVP-003 to agree to testing that included "genetic testing". The initiation of the re-consent process by the WRAIR Clinical Trials Center has taken another 3 months. These regulatory requirements have resulted in delays of over 9 months in the initiation of work that is the core of our proposal. All work involving transcriptional profiling such as RNA-seq, Nanostring analysis and HLA-typing has been postponed until all appropriate approvals have been obtained. Any work not involving "genetic testing" was given the go-ahead by the the WRAIR/IRB and HRPO.

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.

As stated previously, appropriate regulatory approvals and informed consent were a necessary source of delay for our study timeline. We amended the clinical protocol for ADVP-003 (WRAIR #2136) as required to reflect the genetic testing component. Regulatory approval was obtained for all study objectives in August of 2017**.** Re-consent of all subjects from the clinical protocol has been initiated and we anticipate that by October 2017 we will know exactly how many subjects have agreed to the genetic testing component of the study.

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.

Hiring of a post-doctoral fellow at URI and assignment of existing staff to perform project assays were both delayed as a results of the regulatory delays and re-consent process discussed above. Although we anticipate a potential delay of 6 months in the execution of experiments, these developments are not expected to alter the overall costs of executing the scientific studies that were planned.

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

As outlined above, WRAIR protocol #2136 was amended to provide further information concerning the transcrional profiling studies and to provide an amended Informed Consent Form to include provisions for genetic testing. Beyond the requirement to re-contact and re-consent subjects, there are no other significant changes from the use of human subjects originally proposed.

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.*

We presented data from the prime-boost trial (ADVP-003) at the American Association of Immunologists conference May, 2017 in Washington DC. The following two abstracts were presented:
1) Analysis of Cytokines and Chemokines Circulating in Plasma Obtained from a Phase I Prime-Boost Dengue Vaccine Trial Fessehazion A, Currier JR, Koren, M, Keiser, P, Lin L, Jarman RG, Friberg H.
2) A Prime-Boost Dengue Vaccination Strategy Induces Durable Multivalent Memory B Cell Responses Hatch K, Friberg H, Koren, M, Keiser, P, Lin L, Jarman RG, Currier JR.

- **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.

Nothing to report.

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:
<u>Organization Name:</u>
<u>Location of Organization: (if foreign location list country)</u>
<u>Partner's contribution to the project</u> (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, 2017

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		Completed August 2017
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	Awaiting final re-consent of subjects prior to initiating any transcriptional profiling experiments
Major Task 2: RNA-seq analysis on early PBMC samples from subset of study population		Pilot experiments ongoing to determine sample and method suitability
Subtask 1: Isolate RNA from PBMC and assess quality	3-6	RNA isolation method and PBMC quality assessments ongoing
Subtask 2: Prepare RNA for RNA sequencing	4-7	Delayed due to requirement for subject re-consent for sample use
Subtask 3: Bioinformatics analysis • UMMS: Quality control of RNA-seq reads, Alignment to reference genome, differential Expression- statistical testing, Systems Biology analysis	7-12	Delayed due to requirement for subject re-consent for sample use

• URI: Systems Biology analysis				
Milestone #2: Prepare manuscript on RNA sequencing data	8-14	Delayed due to requirement for subject re-consent for sample use		
Major Task 3: Nanostring analysis of candidate gene expression in full trial cohort				
Subtask 1: Selection of codeset for Nanostring analysis	13-14	Medin		Fitzgerald
Subtask 2: Isolate RNA from PBMC and assess quality	6-14	Medin		
Subtask 3: Perform Nanostring analyses	15-18			Fitzgerald
Major Task 4: Measure serum cytokine levels				
Subtask 1: Perform Luminex assays	3-8	First-round screening of 20 subjects completed March 2017		
Subtask 2: Analyze Luminex data	9-14	Analysis delayed until completion of second-round testing		
Milestone #3: Prepare manuscript on innate immune response (PBMC gene expression and serum cytokines) in full study cohort	18-24	Medin	Currier	Fitzgerald
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	**Timeline** (months)	All standard immune monitoring studies for vaccine immunogenicity have been completed and a manuscript is in preparation. Completed March 2017		
Major Task 5: Flow cytometry analysis of T and B lymphocytes				
Subtask 1: Prepare fluorescently labeled DENV		Labeling methods established for DENV viruses May 2017		
Subtask 2: Obtain HLA-peptide tetramers		Delayed due to requirement for subject re-consent for sample use		
Subtask 3: Perform ex vivo flow cytometry		Initiated May 2017 (Analysis ongoing)		
Subtask 4: Perform cytokine flow cytometry assays		Completed March 2017 (Analysis ongoing)		

Subtask 5: Analyze data		Analysis of flow cytometry ongoing		
Milestone #5: Prepare manuscript		Manuscript in preparation for description immunity generated by the prime-boost (PIV/LAV) approach		
Major Task 6: Analyze gene expression in sorted T and B lymphocyte populations				
Subtask 1: Perform fluorescence-activated cell sorting			Currier	
Subtask 2: Isolate RNA		Medin		
Subtask 3: Perform Nanostring analysis of candidate gene expression				Fitzgerald
Subtask 4: Data analysis				
Major Task 7: Perform TCR-effector linkage sequencing analysis of peptide-stimulated PBMC				
Subtask 1: Select samples for analysis		Rothman Payne	Currier	
Subtask 2: Stimulate PBMC and generate single cell emulsions		Payne		
Subtask 3: Perform linkage PCR		Payne		
Subtask 4:				
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	**Timeline** (months)	**Site 1** (URI)	**Site 2** (WRAIR)	**Site 1** (UMMS)
Major Task 8: Prepare combined database of immune response data				
Subtask 1:				

Major Task 9: Perform integrated data analysis				
Subtask 1:				

Attachment #2

Scientific Accomplishments – September 2017

Accomplishments summary: The following accomplishments according to the proposed Statement of Work have been met:

1. Institutional approvals from the WRAIR HSPB/IRB and from HRPO have been granted. The re-consent of subjects to obtain appropriate informed consent for performance of the transcriptional profiling and RNA-sequence analysis is ongoing. Institutional approvals were a critical major milestone for the study. The re-consent of subjects will be the final hurdle to initiation of all scientific studies.
2. A three-way CRADA between WRAIR/URI/UMASS has been finalized and signed. This CRADA provides the framework for sample and data sharing among the institutions.
3. Comprehensive immune monitoring studies that measure and characterize the humoral and cell-mediated immunity generated by the PIV/LAV prime-boost strategy have been completed. Immunogenicity of the vaccine(s) delivered in a prime-boost approach was exceptional. Humoral immunity demonstrated vaccine seroconversion for 100% of subjects in 2 arms of the trial. Magnitudes of neutralizing antibodies suggest that the ordering of the vaccines (either PIV first or LAV first) may impact immunogenicity and hence reflect differential engagement of innate system. Potent cell-mediated immunity (CMI), particularly CD4 and CD8 T cell responses were also demonstrated in all arms of the trial, with several subjects demonstrating no detectable response. Generation of differential adaptive immunity in both the humoral and CMI compartments instills confidence that the transcriptional profiling studies will reveal important signatures associated with vaccination. These data justify the strategic approach to sample PBMC and plasma "early and often" immediately after vaccination. Furthermore, the immunogenicity data has been used to guide the next phase of clinical testing of the prime-boost approach by enabling selection of the best order and temporal spacing of vaccine delivery.
4. Shipping of all PBMC pellets to URI (Providence, RI) for RNA extraction has been completed.
5. Pilot studies of RNA extraction procedures for optimizing RNA yields from PBMC pellets have been initiated.
6. Successful demonstration of fluorescent DENVs as reagents for identifying antigen-specific B cells in both flow cytometry and ELISpot format assays.
7. The multiplexed soluble factor screening assays for circulating cytokine/chemokine profiles of plasma at time-points proximal to vaccination (first 4 weeks) have been completed. Surprisingly, the signals for only three or four (of a total of 59) soluble factors displayed any kind of increase. Kinetics of the response was studied for 20 subjects at 16 time-points post-vaccination. Further validation of this data will be required prior to selection of a specific time-point for further testing.
8. A memory B cell ELISpot assay was developed and used to demonstrate the frequencies of memory B-cells and the isotype usage of the antibody response.
9. A project-specific SQL database has been set up on a server based at URI.

Major Accomplishments: Major accomplishments are detailed as follows and listed by specific aim and major task.

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.

Subtask 1 of Major Task 5: Prepare fluorescently labeled DENV. Time line 1-6 months.

Strains of sucrose gradient DENV for all four serotypes were received from WRAIR. These strains were aliquoted based on concentration. Labeling kits were purchased from Novus Biologicals. We selected 4 dyes DL405, DL488, DL594, DL650 with distinct fluorescence to label each of the four serotypes of DENV.

We initially labeled **DENV-4 with DL 488** and we tested the labeled DL488 DENV-4 preparation for binding to beads that were coated with negative control antibodies (CD28), dengue-serotype cross-reactive antibodies (4G2 and 2H2). We also performed additional validation on beads coated with a DENV-4 specific monoclonal Ab to further confirm we have a labeled preparation. We found a significant shift in fluorescence with dengue-specific antibodies and not control antibodies. We infected susceptible cells (U937 DC SIGN) with DL DENV-4 preparation and detected fluorescence intracellularly 24 and 48 hours later. We have also successfully labeled **DENV-3 with DL594** and **DENV-1 with DL650** using similar protocols.

We labeled DENV-2 with DL405 and we tested the labeled DL405 DENV-2 preparation for binding to beads that were coated with negative control antibodies, and dengue-specific antibodies (4G2 and 2H2). We found a modest shift in fluorescence using beads coupled to DENV reactive Abs. We have therefore been exploring different dyes to enable labeling of a fourth DENV to a distinct fluor that can be cleanly separated from the other three labeled preparations. We used a biotinylation kit to label DENV-2 and were successful with detecting a shift in signal following indirect staining on functionalized beads with Streptavidin BV421. We were also successfully able to label DENV-2 directly with DL-680 with good separation in fluorescence using beads labeled with DENV-specific Abs compared to beads labeled with CD28 Abs. However, when tested with DL650 DENV-1, the spectral overlap was significant when assessed by flow cytometry. We labeled the DENV-2 preparation with CF-750, a near infra-red dye supplied by Biotium. While we observed a significant shift in fluorescence with some DENV cross-reactive Ab coated beads we have noticed that other DENV cross-reactive Ab coated beads appear to be masked by the CF750 labeling process.

We have now further optimized the labeling process of **DENV-2 with DL405**. We find a shift with DENV functionalized beads that allows for the best separation of all four labeled preparations. These preparations are currently being tested on dengue immune and naïve PBMC for further validation and have been sent to WRAIR for further testing.

We have also performed memory B cell ELISpot measurements on the 6 month post-vaccination samples derived from ADVP-003. Memory B cells specific for all four serotypes of DENV and capable of secreting IgG were detected in all arms at 6 moths post-vaccination. Of note is that little correlation with contemporaneous MN_{50} data was found. Utilizing a three color Fluorospot assay to measure DENV-specific IgG, IgA, and IgM secreting cells, we tested 6-months post second dose memory B cell cultures to determine if the response was tetravalent and if a change in vaccination strategy affected the valency. We also investigated how memory B cell responses correlated to neutralizing antibody data. Results show that a majority of subjects have a robust multivalent DENV-specific IgG memory B cell response at 6 months post-vaccination. A better understanding of memory B cell responses and how they relate to neutralizing antibody will help find a suitable DENV vaccine that provides durable tetravalent immunity.

Subtask 3 of Major Task 5: Perform *ex vivo* flow cytometry

Direct *ex vivo* flow cytometric analysis of all major subsets of circulating lymphocytes and monocytes was performed on PBMC samples from the ADVP-003 study. A total of 6 flow cytometry panels (10-12 parameters each), and each focused on a specific subset of lymphocytes or monocytes, was used to interrogate the phenotypic immune activation profiles of the circulating immune cell repertoire in over the

course of 6 months post-vaccination. We have identified distinct populations of T cells (both $CD4^+$ and $CD8^+$, as well $CD4^-/CD8^-$ DN cells), NK cells and B cells that are transiently activated and return baseline after receipt of the LAV component of the vaccine. These results validate our original hypothesis that we would be able to identify and directly sort immune cells that are activated/expanded post vaccination and perform in-depth systems biology analysis of the cells. Once appropriate consent from the subjects is obtained we will use these data to guide our cell sorting and transcriptional profiling studies.

Subtask 4 of Major Task 5: Perform cytokine flow cytometry assays

As part of the immune monitoring studies and to identify high/low responders to the different vaccination strategies we performed cytokine flow cytometry studies on most subjects in each arm of the trial. We used our standard 11 parameter, 6 function ICS panel to determine the frequency, phenotype and functional profiles of antigen-specific T cells generated in the ADVP-003 study. These measurements, as well as the IFN-γ ELISpot data, will be used for correlative studies with the transcriptomics data in order to define expression profiles and pathways that indicate a good vaccine response. One month after completion of the two-dose vaccination schedule ICS was performed using peptide pools corresponding to the complete DENV-2 proteome. High frequencies of multifunctional $CD4^+$ and $CD8^+$ T cells were detected in all four vaccination arms. Of note is that low/non-responders by CMI were detected in all four arms. These data will be critical for interpreting the transcriptional profiling and innate immunity studies.

Subtask 1 of Major Task 4: Measure serum cytokine levels by Luminex-based multiplex assay.

Plasma samples were collected pre-vaccination and at multiple time points immediately post-vaccination to assess the kinetics of the acute phase soluble factor response to vaccination. We tested the plasma samples using Luminex-based magnetic bead kits measuring 59 different chemokines and cytokines. Our results reveal that different cytokine profiles were induced in subjects depending on the vaccine modality and whether it was given as the prime or boost dose. LAV appeared to be most immunogenic, particularly when received as a booster dose after PIV priming. Production of cytokines, including sCD30, APRIL, sTNF-R2, and IL-1RA, occurred in the majority of subjects tested approximately 10-14 days post-vaccination. This study supports other data from this trial demonstrating that the type of vaccine and the order in which it is received impacts immunogenicity. Correlations of soluble factor responses with traditional adaptive immune responses, such as neutralizing antibody and IFN-γ ELISpot assays, will be performed subsequently.

Subtask 2 of Major Task 7: Clonal expansion of a single T-cell receptor clonotype following DENV3 in vitro stimulation of PBMC from a DENV-immune donor.

We developed a technique to identify and track unique T cell responses following immunization or exposure to DENV using next generation sequencing of the T-cell receptor and subsequent analysis of clonal frequency. This technique will allow unambiguous identification of T-cell clones responding to DENV serotypes and is suitable for cryopreserved or freshly collected samples. Briefly peripheral blood mononuclear cells (PBMC) are isolated prior to RNA extraction. RNA is mixed with forward primers specific for 23 distinct T-cell beta chain variable regions and reverse primers specific for the T-cell receptor constant region. A series of PCR reactions modify the amplicons to include a unique molecular identifier and Illumina-specific tags for use in next generation sequencing. We have developed a pipeline for T-cell receptor repertoire analysis of the data generated. PBMC from an individual immunized with DENV3 >10 years previously were thawed, counted and plated in the presence or absence of DENV3. Aliquots were removed at days 0, 3 and 7 for T-cell repertoire analysis. The 100 most prevalent clones were identified in the stimulated cultures. The clones were also present, albeit at a very low frequency in

the unstimulated cultures. The identical clone was expanded in a second independent experiment. This method holds promise for understanding the maturation of the DENV-specific cellular responses in the setting of vaccine trials, observational cohorts, or human infection models.

Attachment #3

Individual Contributors – September 2017

WRAIR (Silver Spring, MD):

Name: Jeffrey R. Currier
Project Role: Co-Principal Investigator
Researcher Identifier ORCID ID: N/A
Nearest person month worked: 1
Contribution to Project: Dr. Currier has supervised the research activities at WRAIR and has coordinated project activities and data exchange with the URI research team.
Funding Support: N/A

Name: Heather Friberg
Project Role: Co-Investigator
Researcher Identifier ORCID ID: N/A
Nearest person month worked: 1
Contribution to Project: Dr. Friberg has overseen the immune monitoring studies for the ADVP-003 study at WRAIR and has coordinated sample exchange with the URI team.
Funding Support: N/A

Name: Kristen Hatch
Project Role: Research Associate
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Contribution to Project: Ms. Hatch has performed B cell ELISpot assays and flow cytometric assays in support of the project.
Funding Support: N/A

Name: Kaitlin Victor
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Funding Support: N/A

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Funding Support: N/A

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Contribution to Project: Dr. Medin has overseen organization and RNA isolation of samples
Funding Support: N/A

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Contribution to Project:
Funding Support: N/A

Name: Barbara Payne
Project Role: Co- Investigator
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Nearest person month worked: 1
Contribution to Project: Dr. Payne has conducted preliminary in vitro stimulation experiments to validate the use of T-cell repertoire analysis to detect clonal T cell populations expanded in response to DENV exposure.
Funding Support: N/A

Name: Diane Lang
Project Role: Research Associate
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Nearest person month worked: 2 months
Contribution to Project: Ms. Lang has isolated RNA from cell samples for molecular analyses.
Funding Support: N/A

Name: Jeremiah Alves
Project Role: Research Assistant (part-time)
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Nearest person month worked: 2
Contribution to Project: Mr. Alves has assisted in the setup of the SQL database and analysis pipeline for gene expression data.
Funding Support: N/A

Name: Sierra Valois
Project Role: Research Assistant (part-time)
Researcher Identifier ORCID ID:
Nearest person month worked: 2
Contribution to Project: Ms. Valois has performed RNA isolations from cell samples for molecular analyses.
Funding Support: N/A