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AWARD NUMBER: W81XWH-16-2-0032

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

PRINCIPAL INVESTIGATOR: Dr. Jeffrey Currier

RECIPIENT: Geneva Foundation
Tacoma, WA 98402

REPORT DATE: September 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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6. AUTHOR(S)
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The Geneva Foundation
917 Pacific Ave Suite 600
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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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	25	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

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.

We will perform RNA-sequencing of bulk PBMC pellets and single-cell preparations of sorted T and B lymphocytes and monocytes following the broad objectives of the original proposal. We have converted to using the 10X Chromium platform for single-cell analyses. Nanostring studies will be initiated once the RNA-seq data on bulk PBMC have been analyzed.
We will continue to upload laboratory data into the project database and perform statistical analyses as additional data become available.

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 were used to inform the design of the subsequent ADVP-004 study. The ADVP-004 study was initiated and is in progress.

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.

There were no significant changes in objectives or scope of the project during year 2. New technical capabilities were established for single-cell sequencing at WRAIR using the 10X Chromium platform and Illumina NovaSeq instrument. After successful preliminary experiments on this equipment, we have decided to use this method for single-cell analysis rather than the TELS platform.

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.

We reported a delay in IRB approval during year 1 of the project. There were no additional delays encountered during year 2.

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.

Due to delays previously reported, we requested an extension of the project to September 2019.

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

Amendment of WRAIR protocol #2136 and the IRB requirement to re-consent subjects for genetic testing was reported previously. During the past year, we obtained consent for this additional testing from 52 subjects.

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- grant P01 AI034533 was funded by the NIH; Dr. Rothman is Program Director, and Drs. Medin, Mathew, Currier, and Friberg are Co-Investigators.

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, 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		
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	In progress- library preparation underway with anticipated completion late September or early October 2018, sequencing will be

		performed at WRAIR in October 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	In progress- delayed due to requirement that subjects be re-consented for this analysis, pipelines have been established, analysis of project data to begin once sequencing complete with anticipated completion December 2018
Milestone #2: Prepare manuscript on RNA sequencing data	8-14	Not yet initiated- will follow Subtask 3
Major Task 3: Nanostring analysis of candidate gene expression in full trial cohort		
Subtask 1: Selection of codeset for Nanostring analysis	13-14	Not yet initiated- Major Task 2
Subtask 2: Isolate RNA from PBMC and assess quality	6-14	In progress- RNA isolation completed, quality assessment in progress with anticipated completion September 2018
Subtask 3: Perform Nanostring analyses	15-18	Not yet initiated- will follow Subtask 1
Major Task 4: Measure serum cytokine levels		
Subtask 1: Perform Luminex assays	3-6	In progress- first-round testing of 20 subjects completed March 2017, second-round testing anticipated completion January 2019
Subtask 2: Analyze data	7-12	In progress- preliminary analyses of first-round data performed, additional analyses will follow Subtask 1
Milestone #3: Prepare manuscript on innate immune response (PBMC gene expression and serum cytokines)	18-24	Not yet initiated- will follow completion of Major Tasks 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	**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	In progress- delayed due to requirement to re-consent subjects, anticipated completion January 2019
Subtask 3: Obtain HLA-peptide tetramers	3-12	Not yet initiated- will follow Subtask 2
Subtask 4: Perform ex vivo flow cytometry	9-15	In progress- initial testing completed March 2017, further testing (incorporating Subtask 3) was delayed due to the requirement to re-consent subjects, anticipated completion August 2019
Subtask 5: Analyze data	10-18	In progress- will follow Subtask 4
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 January 2019
Major Task 7: Analyze gene expression in sorted T and B lymphocyte populations		
Subtask 1: Perform fluorescence-activated cell sorting	10-15	In progress- delayed due to requirement to re-consent subjects for genetic analyses, anticipated completion August 2019

Subtask 2: Isolate RNA	11-16	In progress- being done in combination with single-cell analyses using 10X Chromium method, anticipated completion August 2019
Subtask 3: Perform Nanostring analysis of candidate gene expression	13-18	In progress- using 10X Chromium method (combined with Subtask 2); Nanostring studies will follow completion of Major Task 2, anticipated completion August 2019
Subtask 4: Data analysis	15-20	In progress- anticipated completion August 2019
Major Task 8: Perform TCR-effector linkage sequencing analysis of peptide-stimulated PBMC		
Subtask 1: Select samples for analysis	10-16	In progress- delayed due to requirement to re-consent subjects for genetic analyses, anticipated completion June 2019
Subtask 2: Stimulate PBMC and generate single cell emulsions	11-18	In progress- now using 10X Chromium method, anticipated completion August 2019
Subtask 3: Perform linkage PCR	11-18	In progress- now using 10X Chromium method (combined with Subtask 2), anticipated completion August 2019
Subtask 4: Deep sequencing of PCR products	13-20	In progress- now using 10X Chromium method (combined with Subtask 2), anticipated completion August 2019
Subtask 5: Data analysis	15-22	In progress- anticipated completion August 2019
Milestone #6: Prepare manuscript- Nanostring and TELS analyses	21-24	Not yet initiated- will follow completion of Major Tasks 7 and 8
Specific Aim 3: Determine the associations between early innate and adaptive immune activation and the levels, antigen specificity, and durability of DENV-	**Timeline** (months)	**Task and Milestone Status** (Completion date or delay issue)

specific antibody and memory T and B cell responses after primary and booster immunizations		
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	In progress- delayed due to status of other Major Tasks, anticipated completion March 2019
Subtask 2: Statistical analyses	16-24	Not yet initiated- will follow completion of Specific Aims 1 and 2

Attachment #2

Scientific Accomplishments – September 2018

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

1. We attempted to contact all ADVP-003 study subjects who had previously consented to future studies. Fifty-two subjects consented to genetic testing; this cohort is adequate to meet the study objectives for RNA-sequencing and gene expression studies.
2. We completed the isolation of RNA and DNA from all samples planned for gene expression studies. RNA quality has been checked and we have identified the samples to have RNA-seq libraries prepared. Library preparation was initiated.
3. We performed further optimization of methods to label DENV with fluorochromes for 4-color flow cytometry assays.
4. We conducted preliminary studies to establish the laboratory and data analysis pipelines for HLA typing and bulk PBMC and single-cell RNA-seq data. An alternative strategy using the 10X Genomics platform and Illumina NovaSeq sequencer was tested; based on our preliminary results (see below) we have determined that this strategy should replace our previous plans to use the TELS assay.
5. We uploaded additional project datasets to the SQL database at URI.

Major Accomplishments: Additional details on major accomplishments during year 2 are listed below 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.

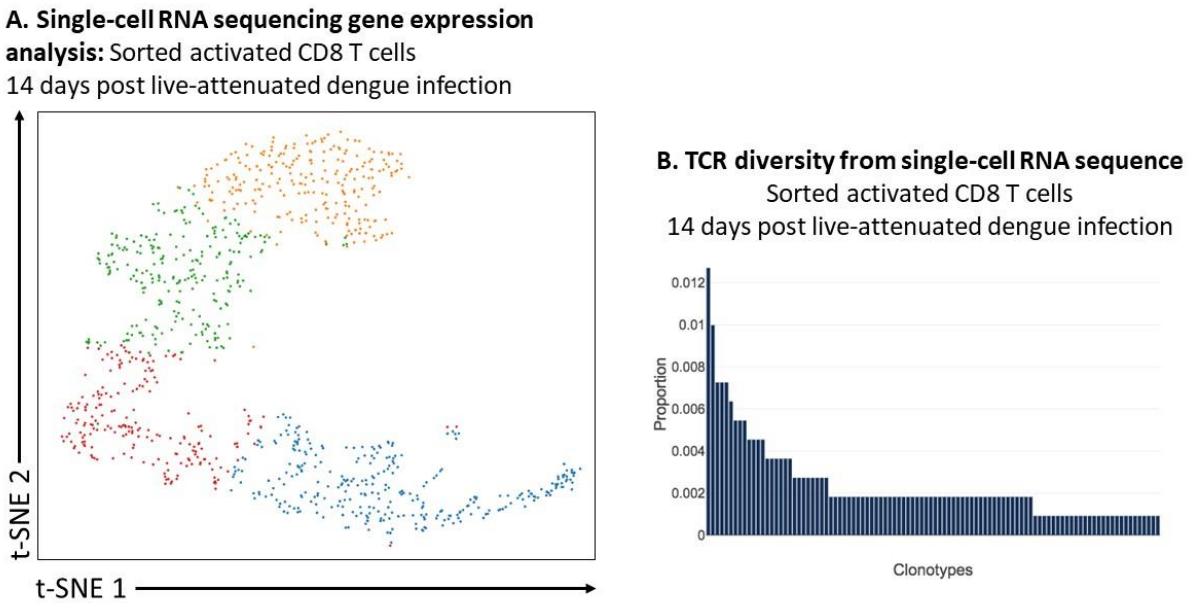
Major Task 7: Analyze gene expression in sorted T and B lymphocyte populations:

We utilized the 10x Genomics Single Cell Analyzer partnered with the Illumina NovaSeq 6000 NGS platform to analyze the gene expression profile and immunoreceptor (TCR/BCR) diversity of PBMCs following vaccination with single-cell resolution. 6,000 to 8,000 individual cells - including T cells, B cells, monocytes and NK cells - were analyzed from 8 samples collected at various time points following vaccination in one subject.

Preliminary results demonstrate that we are able to ascertain at the single cell level gene transcription profiles and co-incident T cell receptor, B cell receptor and HLA data from a single experimental run. In addition, by sorting and processing additional PBMC subpopulations, we have been able to include simultaneous analysis of monocytes and NK cells to create a complete picture of immune activation in all PBMC during the acute phase of vaccination. Preliminary data from the WRAIR VDB laboratory demonstrates the feasibility and data output of the technology. The scRNA-seq used here provides a molecular barcoding and analysis that delivers cell-by-cell 5’ counting of mRNA transcripts for many tens of thousands of cells per run. Data is presented as a 2-dimensional t-SNE plot of activated, FACS sorted and transcriptionally profiled single CD8 T cells obtained 14 days after vaccination with a live-attenuated dengue vaccine product (Figure 1A). Parallel analysis of the TCR clonotype diversity of the cells from this time-point (Figure 1B) demonstrated that the clonal diversity of the acutely activated CD8 T cells is greater than expected. Further analysis of the sequencing data is ongoing. These data provide unparalleled insight into the immune response generated by DENV infection, and also afford the opportunity to identify and track DENV-specific memory T cells from first appearance in the periphery immediately post-vaccination.

As a further proof-of-concept for the study of samples from the ADVP-003 study, we have successfully performed scRNA-seq on PBMC collected during the febrile and post-febrile stages of a subject with natural secondary DENV-1 infection. This assessment focused upon the total T and B cell populations and has provided us with an unprecedented resolution of cellular activation and responsiveness to DENV secondary infection. As summarized in Figure 2 we can generate a cellular transcriptome landscape that is based upon lineage and prevailing activation conditions. We can differentiate, for example, B cell plasmablasts from all other lymphocyte populations, including non-activated memory B cells. Furthermore, we can identify in parallel full-length transcripts matching the immunoglobulin genes that are clonally expressed in the activated B cells. Selected immunoglobulin heavy and light chain genes were cloned and expressed. Several recombinant monoclonal antibodies were tested and shown to possess potent DENV neutralization capacity. This experiment showcases the power and utility of this technology. The WRAIR VDB has the complete in-lab capacity to proceed from a single vial of PBMC through FACS sorting and isolation of specific cells of interest, and then perform the entire scRNA-seq workflow through library generation, high throughput NGS and data analysis. This work has been spearheaded by Dr. Adam Waickman, a senior NRC fellow in the Immunology Section of the Viral Diseases Branch. This project has provided Dr. Waickman with a thorough training process that has permitted him to grow as scientist into a team leader for running the in-lab work for this project. This workflow and sample analysis pipeline will be directly applicable to study PBMC samples from the ADVP-003 study.

Figure 1



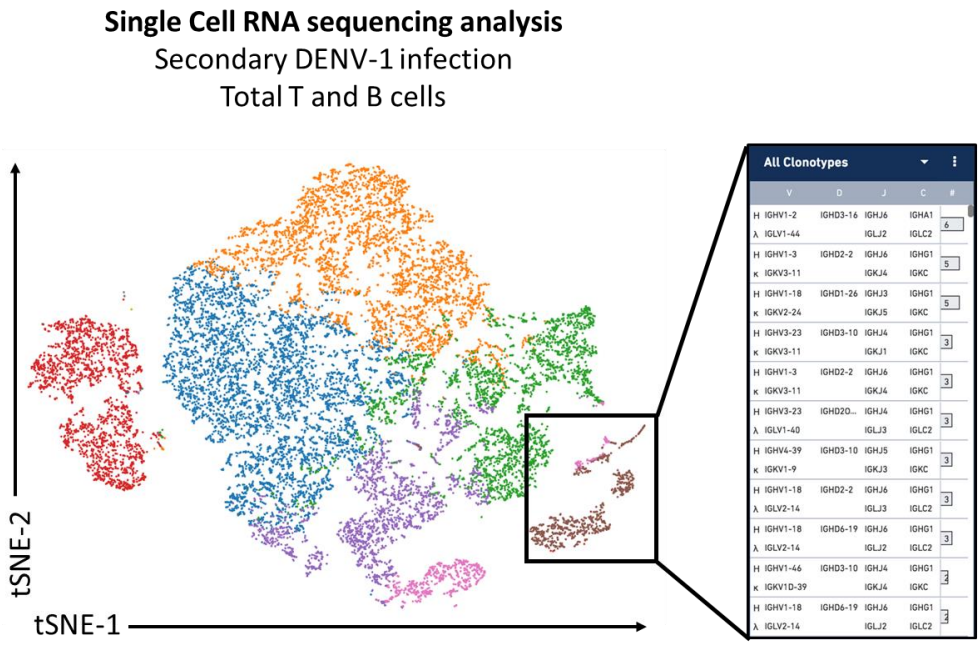


Figure 2. Single cell RNA sequencing analysis of sorted total T and B cells from a pediatric secondary dengue 1 infection. A total of 16,000 cells from 5 timepoints (Days -2, -1, 0, 1, 5, 180 relative to defervescence) were analyzed using the Chromium Single Cell Analyzer and Illumina NovaSeq platforms. Clonotype information highlighted from transcriptionally defined plasmablasts, demonstrating the ability to recover full-length, paired heavy/light chains from BCRs and paired alpha/beta chains from TCRs.

Attachment #3

Individual Contributors – September 2018

WRAIR (Silver Spring, MD):

Name:	Jeffrey R. Currier – No change
Project Role:	Co-Principal Investigator
Name:	Heather Friberg – No change
Project Role:	Co-Investigator
Researcher Identifier:	ORCID ID: 0000-0001-5173-2701
Name:	Adam Waickman
Project Role:	National Research Council Fellow
Researcher Identifier:	ORCID ID: N/A
Nearest person month worked:	3
Contribution to Project:	Dr. Waickman has led the development and implementation of single cell RNA sequencing experiments, within the Viral Diseases Branch.
Funding Support:	N/A
Name:	Kristin Hatch – No change
Project Role:	Research Associate
Name:	Kaitlin Victor
Project Role:	Research Associate
Researcher Identifier:	ORCID ID: N/A
Nearest person month worked:	3
Contribution to Project:	Ms. Victor has performed library preparations for the single cell RNA sequencing experiments, T cell ELISpot assays and flow cytometric assays in support of the project.
Funding Support:	N/A

University of Rhode Island (Providence, RI):

Name:	Alan L Rothman – No Change
Project Role:	Co-Principal Investigator
Name:	Carey Medin – No Change
Project Role:	Co- Investigator
Name:	Anuja Mathew – No Change
Project Role:	Co- Investigator
Name:	Barbara Payne – No Change
Project Role:	Co- Investigator
Name:	Diane Lang
Project Role:	Research Associate
Researcher Identifier:	ORCID ID: 0000-0001-7546-0464

Nearest person month worked: 6
Contribution to Project: Ms. Lang has isolated RNA from cell samples for molecular analyses.
Funding Support: N/A

Name: Amy Princiotto
Project Role: Research Associate
Researcher Identifier: ORCID ID:
Nearest person month worked: 2
Contribution to Project: Ms. Princiotto has isolated RNA and DNA from cell samples for molecular analyses.
Funding Support: N/A

Name: Luis Sanchez Vargas
Project Role: Post-Doctoral Research Associate
Researcher Identifier: ORCID ID:
Nearest person month worked: 11
Contribution to Project: Dr. Sanchez Vargas has performed assays of DENV-specific T cells and contributed to the analysis of data on DENV-specific T and B cell responses.
Funding Support: N/A

Name: Benjamin Gabriel
Project Role: Post-Doctoral Research Associate
Researcher Identifier: ORCID ID:
Nearest person month worked: 1
Contribution to Project: Dr. Gabriel has supervised RNA/DNA isolation and preparation of sequencing libraries, and has contributed to the analysis of NGS data.
Funding Support: N/A

Name: Nathaniel Awkerman
Project Role: Research Assistant (part-time)
Researcher Identifier: ORCID ID:
Nearest person month worked: 2
Contribution to Project: Mr. Awkerman has assisted in establishing pipelines for analysis of NGS data.
Funding Support: N/A