AWARD NUMBER: W81XWH-20-1-0099

TITLE: A Novel Self-Adjuvanting cc Particle-Based Tuberculosis Vaccine

PRINCIPAL INVESTIGATOR: Tshidi Tsibane

CONTRACTING ORGANIZATION: Georgia State University Research Foundation Inc., Atlanta, GA

REPORT DATE: February 2023

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE *(DD-MM-YYYY)* | 2. REPORT TYPE | 3. DATES COVERED *(From - To)* |
|---|---|---|
| February 2023 | Annual | 01Feb2022- 31Jan2023 |

**4. TITLE AND SUBTITLE**

A Novel Self-Adjuvanting cc Particle-Based Tuberculosis Vaccine

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-20-1-0099

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

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Atlanta, GA 30303-2921

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

*Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis, remains a major public health threat yet effective vaccines remain elusive. *Mtb* antigens ESAT-6 and Ag85B have shown promise as the basis of vaccines. However, developing effective immune responses to these antigens requires co-administration of immune boosters known as adjuvants, which complicates vaccine development. Another limitation of many current vaccines is the need for multiple immunizations to induce strong immunity. This proposal seeks to develop a unique Ebola virus-like particle-(eVLP)-based approach comprised of Ebola virus proteins and built-in adjuvanting activity that has several advantages over existing vaccine candidates. First, introduction of the 2CARD signaling domains from the pattern recognition receptor RIG-I confers self-adjuvanting activity to the eVLPs, resulting in vigorous immune responses as compared to standard eVLPs. Second is inclusion into the platform of proven *Mtb* antigens that can elicit beneficial immunity. We have successfully produced highly purified eVLPs that incorporate ESAT6 and Ag85B, as well as the 2CARD domain, in various configurations. We have previously shown that "infection" of cells with VLPs containing the constructs causes robust activation of innate immune responses. We have built on these findings by demonstrating rapid induction of antibody responses to the GP surface antigen of the eVLPs following immunization of C57BL/6 mice; this puts us in good position to progress to assessment of T cell responses to the *Mtb* antigens in the coming year.

**15. SUBJECT TERMS**
*Mycobacterium tuberculosis*, vaccine, Ebola virus-like particles (eVLPs), ESAT-6, antigen, innate immunity, dendritic cells, RIG-I, CARD domains, self-adjuvanting

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
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| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRDC |
| U | U | U | UU | 13 | 19b. TELEPHONE NUMBER *(include area code)* |

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## INTRODUCTION

*Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB), is associated with an estimated 10 million infections and 1.6 million deaths per year, according to the WHO (1). Drug resistance is an increasing problem and imposes major obstacles to TB care, rendering treatment lengthier and more difficult. Despite intense efforts over decades, we do not have vaccines that effectively prevent pulmonary TB. While numerous approaches have been tried, a commonly employed strategy to immunize against Mtb has been to express individual or fusions of two or more protein antigens and to administer these together with adjuvant. Typically, multiple immunizations are required to provoke a robust response. A vaccine that elicits potent and sustained immunity that is protective after a single dose would be highly preferable. Further, in prior studies, choice of adjuvant appears to be important for efficacy, suggesting that activation of robust and appropriate innate immune responses is required to elicit optimal adaptive immunity (2). Therefore, developing vaccines that appropriately and adequately activate innate immune signaling is also critical. Our project focuses on a novel approach to an Mtb vaccine, based on our hypothesis that a vaccine platform capable of eliciting rapid and robust immunity will prove superior to previously described strategies in terms of eliciting protection from Mtb infection. We have developed a novel, non-replicating virus-like particle (VLP)-based vaccine platform, that uses Ebola virus proteins and is specifically engineered to incorporate an innate immune signaling module, the signaling domain from the pattern recognition receptor RIG-I, which consists of two caspase recruitment domains (2CARD). The 2CARD domain provokes potent activation of cytoplasmic pattern recognition receptor (PRR) signaling. This results in rapid development of robust adaptive immune responses. Because the Ebola virus glycoprotein (GP) directs the VLPs to dendritic cells (DCs) and macrophages- antigen presenting cells are preferred infection targets of Ebola virus- the vaccine delivers antigen to the most relevant cells for inducing T cell immunity. By delivering proven Mtb antigens to the appropriate cell types and robustly activating in these cells the appropriate innate immune program, we hypothesize that our novel a platform will prove superior to previously studied Mtb vaccines. Success will pave the way for further development of our Mtb vaccine. In this report we describe our progress in terms of optimizing the incorporation of Mtb antigens into eVLPs, assessing induction of IFN and cytokines, obtaining sufficient quantities of highly purified eVLPs and initiation of animal studies.

1. WHO. Global tuberculosis report 2018 World Health Organization. Geneva: 2018.
2. Van Der Meeren O, Hatherill M, Nduba V, Wilkinson RJ, Muyoyeta M, Van Brakel E, Ayles HM, Henostroza G, Thienemann F, Scriba TJ, Diacon A, Blatner GL, Demoitie MA, Tameris M, Malahleha M, Innes JC, Hellstrom E, Martinson N, Singh T, Akite EJ, Khatoon Azam A, Bollaerts A, Ginsberg AM, Evans TG, Gillard P, Tait DR. Phase 2b Controlled Trial of M72/AS01E Vaccine to Prevent Tuberculosis. N Engl J Med. 2018;379(17):1621-34. doi: 10.1056/NEJMoa1803484. PubMed PMID: 30280651; PMCID: PMC6151253.

**KEYWORDS**
Mycobacterium tuberculosis, vaccine, VLP (virus-like particle), Ebola virus, dendritic cells, innate immunity, pattern recognition receptor (PRR), retinoic acid inducible gene I (RIG-I), caspase recruitment domains (CARD), ESAT-6, antigen

**ACCOMPLISHMENTS**

As outlined in our prior Technical Reports, the goal of this project is to develop and evaluate the immunogenicity of a novel *Mycobacterium tuberculosis* (Mtb) vaccine based on a self-adjuvanting Ebola virus-like particle (VLP) system. The VLPs are not able to replicate and therefore are not infectious. The novelty of our VLP system is two-fold. First, we make the VLPs "self-adjuvanting" by including in the particles a signaling domain (2CARD) that is derived from the cellular RIG-I pattern recognition receptor. The isolated 2CARD signaling domain will, upon introduction into cells, trigger type I interferon (IFN) and cytokine responses that we expect to promote TH1 immunity. Second, this would be the first example of Ebola VLPs that deliver an Mtb antigen. An advantage of the Ebola VLP platform is that the particles possess the Ebola virus glycoprotein (GP). GP naturally targets antigen presenting cells, such as dendritic cells and macrophages, and therefore will deliver antigen to the desired cell types to elicit robust immunity.

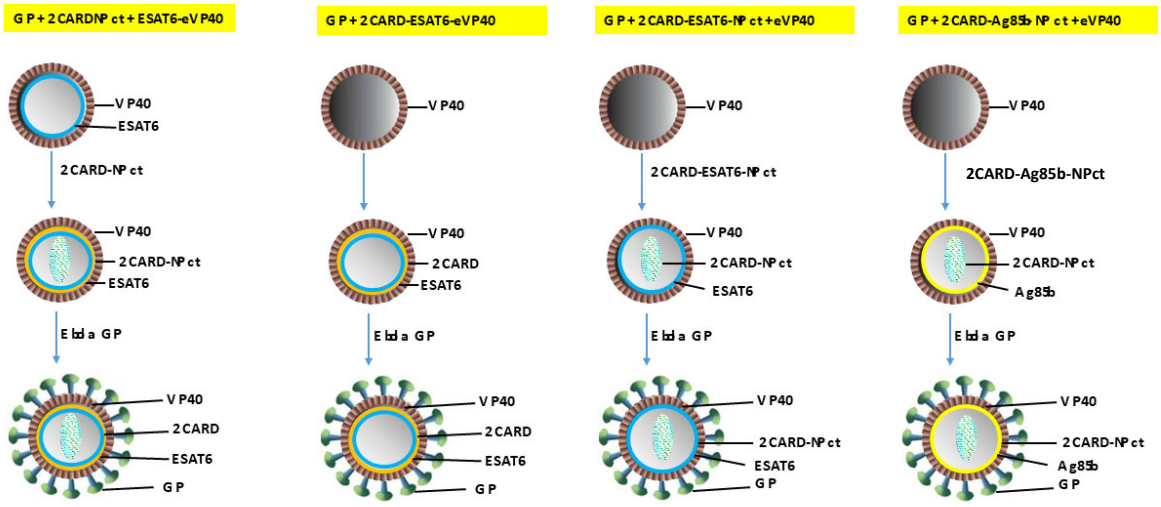


**Figure 1. Diagram of structure of VLPs expressing MTB antigens.**

The first step towards development of the VLP-based Mtb vaccine was to build the expression plasmids that are required to produce the proteins needed for VLP production. Expression of the Ebola virus matrix protein eVP40 is sufficient for VLPs to form and bud from cells. Co-expression of GP results in VLPs that have GP on the VLP membrane and eVP40 within the particle. Additional expression of the Ebola virus nucleoprotein (NP) results in NP incorporation within the VLPs. We had additionally demonstrated that we could fuse the 2CARD domain to the C-terminal domain of NP (2CARD-$NP_{ct}$) and that this fusion could be incorporated into the VLPs. For the Mtb vaccine, our approach was to fuse Mtb antigen to the VLP proteins such that expression would be sufficient and incorporation of Mtb antigen into the VLPs

would occur. We also needed to define the best way to introduce 2CARD into the system that expresses Mtb antigens. For these initial studies we tested the Mtb antigens ESAT-6 and Ag85B.

As described in our prior Technical Reports, we have successfully produced VLPs that incorporate ESAT6 into the VLPs. This was achieved by fusing ESAT-6 to EBOV VP40 (eVP40). We have also successfully expressed a construct with 2CARD fused to ESAT-6-NPct, and another construct with 2CARD-ESAT-6 fused to eVP40 (**Fig. 1**). Previously reported difficulties in incorporating Ag85B into the VLPs were overcome by generating VLPs in which Ag85b is fused at its N-terminus with the 2CARD domain and at its C-terminus with the $NP_{CT}$ domain (**Fig. 1**). For each fusion, we placed a Ser-Gly linker between the partner proteins to facilitate proper folding of the different domains. Expression of antigens was confirmed by Western blotting of lysates from transfected cells.

As we previously reported, the basis for the self-adjuvanting activity of our Ebola VLPs is the presence of the 2CARD domain, which should enhance immune responses by activating signaling pathways that trigger type I IFN and cytokine production. We have demonstrated that addition to cell culture of 2CARD-containing VLPs results in the desired type I IFN response and further verified that this response requires the presence of the 2CARD domain and is absent when cells lacking the MAVS signaling molecule, which is downstream of RIG-I in the IFN induction signaling pathways and required for RIG-I signaling (data not shown). Therefore, the Mtb-VLPs elicit the desired immune enhancing response.

As described in the prior Technical Report, we generated large batches of the VLPs and assessed their immunogenicity in mice. Prior to immunization, contents of the VLPs were evaluated by Western blot to confirm the presence of the expected proteins (data not shown). The VLPs were also examined by SDS-PAGE followed by silver staining to determine relative levels of proteins in the VLPs and the purity of the VLPs (**Figure 2).**

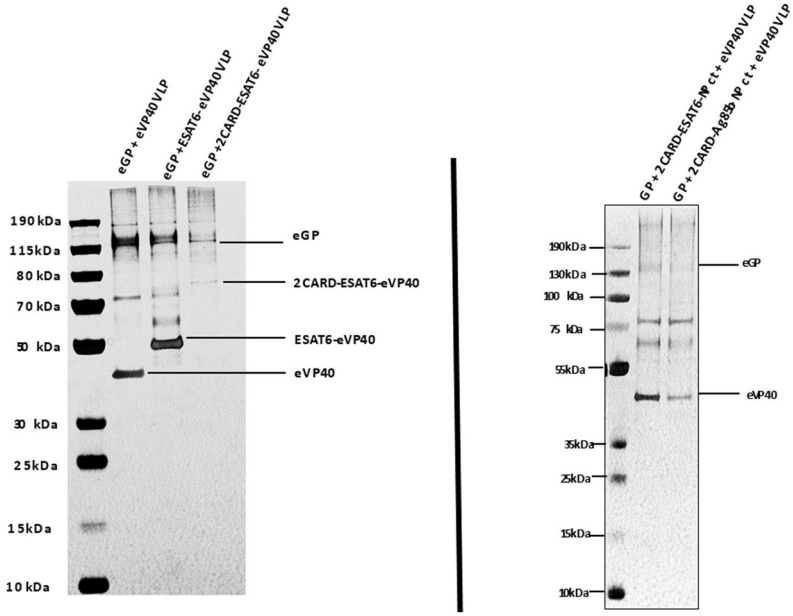


**Figure 2. Representative silver stained gels of the protein contents of Mtb VLPs are provided.**

As described and illustrated in our prior Technical Report, we measured antibody responses in C57BL/6 mice to GP in bleeds after one (3 weeks sera) or two (6 weeks sera) immunizations by ELISA. As expected, mice receiving PBS did not possess anti-GP antibodies. Each of the VLP preparations, which all contain GP, elicited anti-GP responses at 3 weeks, after one immunization. After a second immunization, when animals were bled at 6 weeks, all of the anti-GP responses were boosted from the 3-week values. These findings indicate robust immunogenicity for the Mtb VLPs. Because we are interested in immunity to ESAT6 and Ag85B, we also performed ELISAs using plates coated with recombinant ESAT6 or Ag85B protein. These studies did not detect anti-ESAT6 or anti-Ag85B antibodies. This is not surprising, given that the ESAT6 and Ag85B are present internally in the VLPs and are not directly exposed to immune cells in the same way as GP, which is present on the surface of the VLPs.

The most desirable immune responses to ESAT6 and Ag85B are T cell responses. We therefore immunized mice a third time and two weeks after this, harvested spleen cells. These have been frozen to enable T cell assays. Ongoing work involves validation of ELISpot-based T cell assays. We plan to measure IFNγ cytokine production by T cells and also measure pro-inflammatory cytokine production following restimulation of splenocytes with control peptide or peptides derived from GP, ESAT6 and Ag85B. Each of the peptides to be used has previously been demonstrated to re-stimulate T cell responses in C57BL6 mice. Since our last Technical Report, we have purchased the relevant Mtb peptides required for these assays, and we also have the necessary VLP preparations in hand. We have reviewed the experimental plan with an experienced immunologist at Mount Sinai whose lab routinely performs ELISpot assays, and he has offered to provide guidance and technical support as needed for this phase of the work, including use of his laboratory's equipment for reading the ELISpot plates and running the cytokine analyses. We have ordered and are waiting for one additional control peptide to arrive before starting this experiment.

**IMPACT**

We have completed the first steps towards testing our novel Mtb vaccination approach. Successful development of an Mtb vaccine would address a global health concern that also poses threats to American military personnel. Successful development of this vaccine would also suggest that the Ebola VLP platform could be developed for other infectious diseases of concern.

**CHANGES/PROBLEMS**

Some delays were encountered because Dr. Tufariello moved from Georgia State University (GSU) to Icahn School of Medicine in New York (Mount Sinai) in December of 2021. The move necessitated the appointment of Dr. Tshidi Tsibane as PI of the grant at GSU and the extension of a subcontract to Mount Sinai to enable the work to continue. This has slowed progress, and has prevented access to grant funds since December of 2021. However, the PI change has been approved by the sponsor, as has the subcontract. Mount Sinai grants specialists have been in contact with colleagues at GSU regarding establishing the subaward. We hope that this will soon be finalized, to enable continuation of this work.

**PRODUCTS**

We developed expression plasmids that enable introduction of Mtb antigen into Ebola VLPs. These are pCAGGS-ESAT-6-eVP40, pCAGGS-2CARD-ESAT-6-eVP40, pCAGGS-2CARD-ESAT6-NPct and pCAGGS-2CARD-Ag85B-NPct. We also developed techniques that enable production of VLPs incorporating the proteins produced by these plasmids.

**PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

| | |
|---|---|
| Name: | Tshidi Tsibane |
| Project Role: | PI |
| Researcher Identifier (e.g. ORCID ID): | N/A |
| Nearest person month worked: | N/A (no access to funds during establishment of subaward) |
| Contribution to Project: | Dr. Tsibane will serve primarily as the administrative PI, overseeing budget, compliance and reporting to the funding agency at GSU. She will also provide scientific input by interpreting data and suggesting research directions, as needed. |
| Funding Support: | Please see "Other Support" information provided below. |

| | |
|---|---|
| Name: | JoAnn Tufariello |
| Project Role: | PI of subaward |
| Researcher Identifier (e.g. ORCID ID): | N/A |
| Nearest person month worked: | N/A (no access to funds during establishment of subaward) |
| Contribution to Project: | Dr. Tufariello designed the overall strategy, planned the experiments, and directed and supervised the work performed by the postdoctoral fellow.<br><br>Progress was reviewed during scheduled weekly lab meetings as well as frequent informal discussions in the lab. |
| Funding Support: | Please see "Other Support" information provided below. |

| | |
|---|---|
| Name: | Naveen Thakur |

| | |
|---|---|
| Project Role: | Post-doctoral Fellow |
| Researcher Identifier (e.g. ORCID ID): | N/A |
| Nearest person month worked: | N/A (no access to funds during establishment of subaward) |
| Contribution to Project: | Dr. Thakur built the vaccine constructs expressing the Mtb antigens as fusions with VP40 and/or 2CARD-NPct, verified expression and induction of cytokine responses, and generated and purified the VLPs and conducted the mouse immunization studies. |
| Funding Support: | N/A |

**OTHER SUPPORT**

Dr. Tsibane has no other support to report.

**OTHER SUPPORT**

for Dr. Tufariello

**Icahn School of Medicine at Mount Sinai**

Full-Time 1.000 FTE

Appointment Title: Associate Professor

**Other Support - Projects/Proposals**

ACTIVE

*Title: VPS34 inhibitors at SARS-CoV-2 antivirals

Aims: This project seeks to evaluate inhibitors of the cellular kinase as anti-SARS-CoV-2 antivirals and to define their mechanism of antiviral action.

*Status of Support: Active

Project Number: R21AI161104

Name of PD/PI: Basler

Role: Co-I

*Supporting Agency: NIH/NIAID

*Primary Place of Performance: ISMMS

Project/Proposal Start and End Date: (MM/YYYY) (if available): 1/1/2022 - 7/31/2023

*Total Award Amount (including Indirect Costs):

Contracts/Grants Officer: Amanda Amescua

Contact Address: 1 Gustave L. Levy Place, Box 1075, New York, NY 10029

*Person Months (Calendar/Academic/Summer) per budget period:

| **Year (YYYY)** | **Calendar Months** |
|---|---|
| 2. 2023 | 1.200 |

*Title: Understanding how the MERS Coronavirus protein ORF4b interactions with importin alpha modulate innate immunity

Aims: The major goals of this project are to characterize MERS-CoV ORF4b protein and NF-kB p50 interactions with importin alpha proteins and to test the hypothesis that ORF4b competes with NF-kB for binding importin alpha proteins to block activation of NF-kB-responsive genes.

*Status of Support: Active

Project Number: R21AI164080

Name of PD/PI: Basler

Role: Co-I

*Supporting Agency: NIH/NIAID

*Primary Place of Performance: ISMMS

Project/Proposal Start and End Date: (MM/YYYY) (if available): 12/15/2021 - 6/30/2023

*Total Award Amount (including Indirect Costs):

Contracts/Grants Officer: Amanda Amescua

Contact Address: 1 Gustave L. Levy Place, Box 1075, New York, NY 10029

*Person Months (Calendar/Academic/Summer) per budget period:

| **Year (YYYY)** | **Calendar Months** |
|---|---|
| 2. 2023 | 1.320 |

*Title: Intrinsically-enhanced Ebola and Marburg virus like particles for increased potency and

immune memory

Aims: This grant seeks to develop novel, self-aduvanting virus-like particle based vaccines for Ebola and Marburg virus.

*Status of Support: Active

Project Number: R21AI146682

Name of PD/PI: Tufariello

Role: PI

*Supporting Agency: NIH/NIAID

*Primary Place of Performance: ISMMS

Project/Proposal Start and End Date: (MM/YYYY) (if available): 3/25/2022 - 5/31/2023

*Total Award Amount (including Indirect Costs):

Contracts/Grants Officer: Amanda Amescua

Contact Address: 1 Gustave L. Levy Place, Box 1075, New York, NY 10029

*Person Months (Calendar/Academic/Summer) per budget period:

| **Year (YYYY)** | **Calendar Months** |
|---|---|
| 3. 2023 | 1.800 |

*Title: Novel VP30-host Interactions that Negatively Regulate Ebola Virus Infection

Aims: Major Goals: To biophysically characterize newly identified host factors and their interactions with Ebola virus VP30 protein as well as their functional consequence that will inform on the molecular basis for host-VP30 interactions during viral infection.

*Status of Support: Active

Project Number: R01AI143292

Name of PD/PI: Amarasinghe, Basler, Krogan

Role: Co-I

*Supporting Agency: NIH/Washington University - St. Louis

*Primary Place of Performance: ISMMS

Project/Proposal Start and End Date: (MM/YYYY) (if available): 3/1/2022 - 2/29/2024

*Total Award Amount (including Indirect Costs):

Contracts/Grants Officer: Amanda Amescua

Contact Address: 1 Gustave L. Levy Place, Box 1075, New York, NY 10029

*Person Months (Calendar/Academic/Summer) per budget period:

| Year (YYYY) | Calendar Months |
|---|---|
| 4. 2023 | 1.200 |
| 5. 2024 | 1.200 |

PENDING

*Title: Ebola virus VP35 targets E3 ubiquitin ligase MIB2 to block innate immune signaling Aims: Our studies will clarify how a key filoviral protein, VP35, interacts with host protein MIB2 to counteract immune responses and promote virus replication and virulence, thereby providing insight into what makes these viruses such deadly pathogens and suggesting therapeutic approaches.

*Status of Support: Pending

Project Number: R01AI178897

Name of PD/PI: Basler

Role: Co-I

*Supporting Agency: NIH/NIAID

*Primary Place of Performance: ISMMS

Project/Proposal Start and End Date: (MM/YYYY) (if available): 7/1/2023 - 6/30/2028

*Total Award Amount (including Indirect Costs):

Contracts/Grants Officer: Amanda Amescua

Contact Address: 1 Gustave L. Levy Place, Box 1075, New York, NY 10029

*Person Months (Calendar/Academic/Summer) per budget period:

| Year (YYYY) | Calendar Months |
|---|---|
| 1. 2024 | 1.200 |
| 2. 2025 | 1.200 |
| 3. 2026 | 1.200 |
| 4. 2027 | 1.200 |
| 5. 2028 | 1.200 |

**Other Support - In Kind Contributions**

ACTIVE

None

PENDING

None

OVERLAP
*No scientific, budgetary, or commitment overlap.*

**SPECIAL REPORTING REQUIREMENTS**
Not applicable

**APPENDICES**
None