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14. ABSTRACT
Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis, remains a major public health threat worldwide, yet effective *Mtb* vaccines remain elusive. *Mtb* antigens ESAT-6 and Ag85B have shown promise as the basis of vaccines. However, developing effective immune responses to such antigens requires co-administration of immune boosters known as adjuvants, which complicates vaccine development. Another limitation of many current *Mtb* 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 *Mtb* vaccine candidates. First, the introduction of the 2CARD signaling domains from the pattern recognition receptor RIG-I confers self-adjuvanting activity to the eVLPs, such that they elicit vigorous immune responses as compared to standard eVLPs. Second is the inclusion of proven *Mtb* antigens that can elicit beneficial immunity into the 2CARD-eVLP platform. To date, we have successfully produced highly purified eVLPs that incorporate the ESAT-6 *Mtb* antigen, and that incorporate 2CARD with ESAT-6 when fused to eVP40. We have shown that 2CARD-ESAT-6-eVP40 causes robust activation of innate immune signaling when expressed in cells, and that "infection" of cells with VLPs containing this construct also activates innate immune responses. This puts us in good position to progress to the proposed in vivo (mouse) immunogenicity studies in year 2.

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

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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 cytokine, and obtaining sufficient quantities of highly purified eVLPs to commence 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

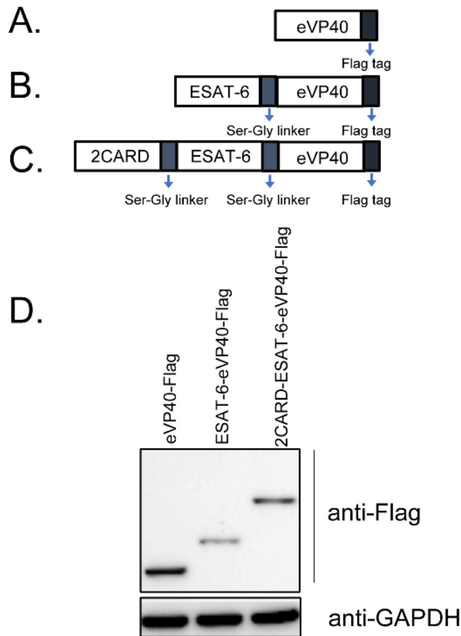

Figure 1. Successful expression of ESAT6 fused to eVP40 and 2CARD-ESAT6 fused to eVP40. The following proteins were transiently expressed: **A.** Ebola VP40 with a C-terminal FLAG tag (eVP40-Flag), **B.** ESAT-6 fused to Ebola VP40 with a C-terminal FLAG tag (ESAT-6-eVP40 –Flag), or **C.** 2CARD and ESAT-6 fused to Ebola VP40 with a C-terminal FLAG tag (2CARD-ESAT-6-eVP40–Flag). Serine Glycine linkers are indicated by gray boxes and are labeled Ser-Gly linker. The Flag tag at the C-terminus of each construct is depicted by a black box, as indicated. **D.** Western blot for each of the constructs. A GAPDH western blot served as a loading control.

ACCOMPLISHMENTS

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.

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

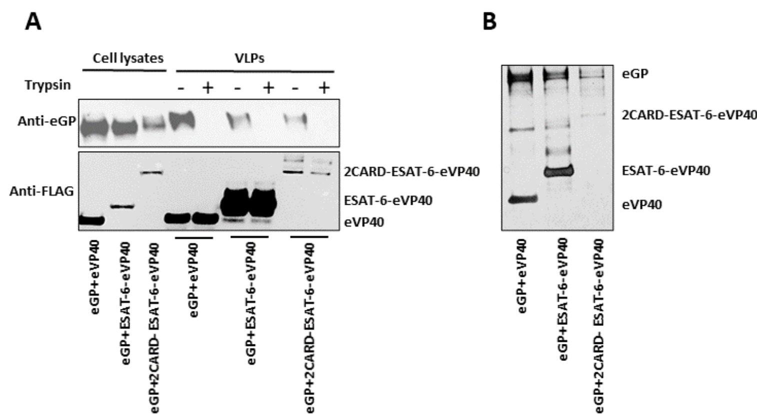

Figure 2. Successful production of VLPs possessing ESAT-6, with and without the 2CARD signaling domain. A. Western blots performed with anti-Ebola glycoprotein (eGP) and anti-Flag antibodies on transfected cell lysates and virus-like particles (VLPs) collected from cell culture supernatants. eGP was co-expressed with Flag-tagged Ebola VP40 (eVP0), with Flag-tagged ESAT-6-fused to eVP40 and with Flag-tagged 2CARD-ESAT-6-eVP40. Blots of cell lysates and of VLPs are indicated. VLPs were either not treated or treated with tryspin to degrade non-membrane bound protein. B. Silver stained gel of purified VLP preparations produced with the indicated proteins.

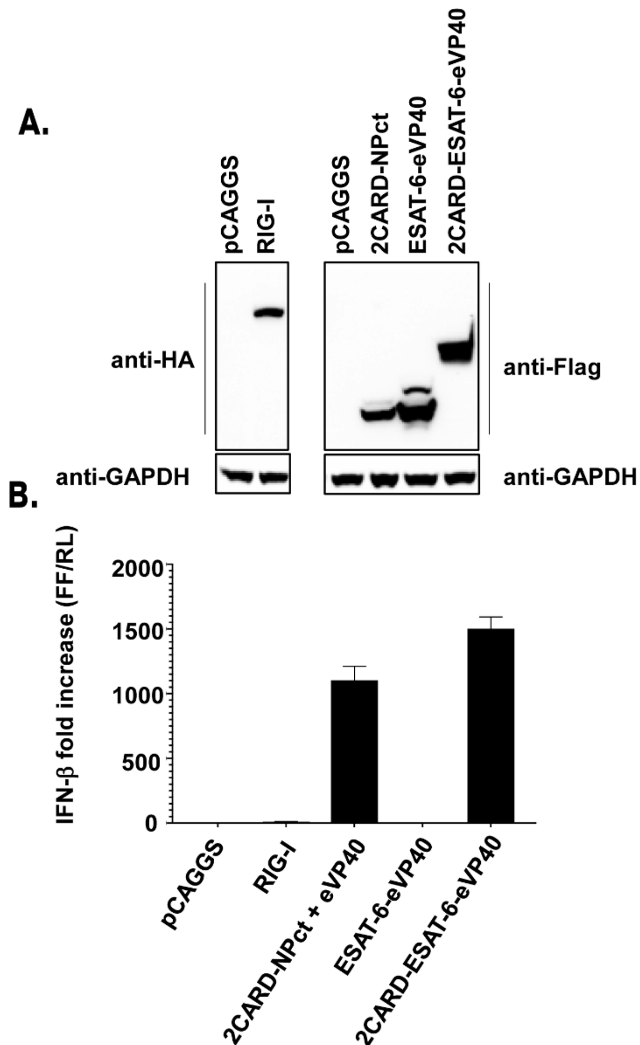

Figure 3. The 2CARD-ESAT6-eVP40 fusion activates the IFN beta promoter, as measured by a reporter gene assay. Cells were transfected in triplicate with an interferon beta (IFN-β)-firefly luciferase reporter plasmid; a constitutively-expressing *Renilla* luciferase reporter plasmid; and either empty expression plasmid (pCAGGS) or plasmids expressing full-length RIG-I (RIG-I), the RIG-I 2CARD domain fused to the Ebola virus nucleoprotein C-terminus (2CARD-NPct), ESAT6 fused to Ebola virus VP40 (ESAT6-eVP40) or 2CARD-ESAT6-fused to Ebola VP40 (2CARD-ESAT6-eVP40). Each of the proteins expressed was Flag-tagged. **A.** Western blot with anti-HA antibody to detect expression of RIG-I and anti-Flag antibody to detect expression of the indicated Ebola fusion proteins. An anti-GAPDH western blot served as a loading control. **B.** Reporter assay to detect activation of the IFN-β promoter. 24 hours post-transfection, dual luciferase assays (Promega) were performed. Firefly luciferase activity was normalized to *Renilla* luciferase. Shown are the means and standard deviations normalized to the pCAGGS control.

could be incorporated into the VLPs. For the Mtb vaccine, we first needed to identify how best 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.

Our initial attempts to express Mtb antigens involved fusing these to NP_{ct}, with and without the 2CARD domain. We also explored fusing Mtb antigen to VP40. Our best success has come by fusing ESAT-6 to EBOV VP40 (eVP40). We have also successfully expressed a construct with 2CARD and ESAT-6 fused to eVP40 (**Fig. 1**). For each fusion, we placed a Ser-Gly linker between the partner proteins to facilitate proper folding of the different domains. When co-expressed with EBOV GP (eGP), ESAT-6-eVP40 and 2CARD-ESAT-6-eVP40 were expressed, albeit to somewhat lower levels than eVP40 (**Fig. 2**). When VLPs were collected from the cells expressing these constructs and concentrated and partially purified by centrifugation through a 20 percent sucrose cushion, VLPs containing GP and eVP40, ESAT-6-VP40 and 2CARD-ESAT-6-eVP40 were found to be produced. Interestingly, ESAT-6-eVP40 was very highly represented in the VLPs. Relative to eGP amounts, ESAT-6-eVP40 is present at much higher levels than eVP40. 2CARD-ESAT-6-eVP40 was also present in VLPs but at lower levels (**Fig. 2A**). To further confirm that the proteins detected are present in virus-like particles, these were treated with the protease trypsin. We expect most of the eGP protein should be on the outside of the VLP membrane and the eVP40 to be within the membrane. Therefore, in intact VLPs, eGP should be susceptible to

trypsin digestion and eVP40 and its fusion partners should be resistant. For each VLP preparation, eGP signal was lost and the eVP40, ESAT-6-eVP40 or 2CARD-ESAT-6-eVP40 signal was largely retained following tryspin treatment. We conclude that we can successfully produce VLPs with ESAT-6 incorporated, with or without 2CARD, and that ESTA6-eVP40 incorporates Mtb antigen at very high levels into the VLPs.

The basis for the self-adjuvanting activity of our Ebola VLPs is the presence of the 2CARD domain. the 2CARD should enhance immune responses by activating signaling pathways that trigger type I IFN and cytokine production, mimicking a natural viral infection. To test whether 2CARD is functional in the context of the 2CARD-ESAT-6-eVP40 fusion, we tested its capacity to activate the IFN-β promoter, as measured by a reporter gene assay, upon transfection into mammalian cells. As negative controls, where promoter activation would not be expected, we transfected empty expression plasmid (pCAGGS), full-length RIG-I (which should only become active when exposed to dsRNA), and ESAT-6-eVP40. As a positive control, we transfected 2CARD fused to NP_{ct}, because we previously demonstrated the capacity of this construct to activate the IFN-β promoter. As shown in **Fig. 3**, each of the negative controls failed to activate the IFN-β promoter, but 2CARD-NPCT and 2CARD-ESTAT6-eVP40 similarly stimulated the IFN-β promoter.

Having demonstrated that 2CARD-ESAT-6-eVP40 can activate innate immune signaling when expressed in cells, it was next of interest to determine whether "infection" of cells with VLPs containing this construct would activate innate immune responses that should enhance VLP immunogenicity. Therefore, we produced VLPs with eGP+eVP40, GP+eVP40+2CARD NP_{ct} (which we previously have demonstrated will activate IFN responses, data not shown), GP+ESAT-6-eVP40 and GP+2CARD-ESAT-6-eVP40. We then measured levels of host cell RIG-I mRNA, expression of which is induced by IFN, and the IFN beta mRNA by quantitative RT-PCR. The data are reported as relative copy numbers, based on normalization to cellular beta-actin mRNA levels. At 12 hours post addition of VLPs, activation of the response was seen only with the GP+eVP40+2CARD NP_{ct} and GP+2CARD-ESAT-6-eVP40 conditions. Signaling by 2CARD is expected to require the presence of the cellular signaling molecule MAVS. Consistent with our

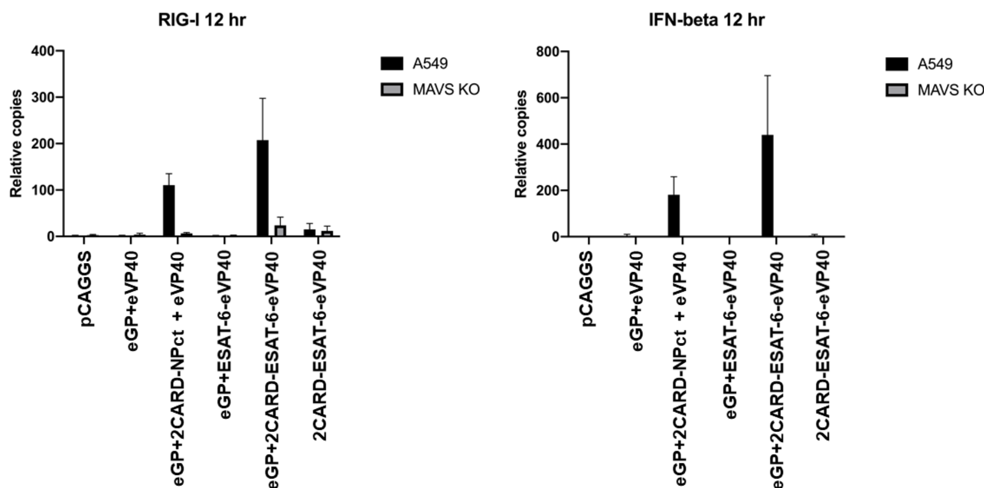

Figure 4. Infection of cells with VLPs containing 2CARD-ESAT-6-eVP40 activates an IFN response in a MAVS-dependent manner. VLPs were produced by co-expressing eGP with eVP40, 2CARD-NPct +VP40, ESAT-6-eVP40 or 2CARD-ESAT-6-eVP40. Controls included empty vector and VLPs produced by expressing 2CARD-ESAT-6-eVP40 in the absence of eGP. The lack of eGP is expected to prevent the VLPs from entering cells. These VLP preparations were added in triplicate to wildtype A549 cells with an intact type I IFN response and MAVS knockout A549 cells which are defective for response to RIG-I. 12 hrs post VLP addition, RNA was harvested from the cells and quantitative RT-PCR was performed to determine levels of RIG-I and IFN-β mRNAs. The data for RIG-I and IFN-β mRNA levels were normalized to β-actin mRNA levels and are reported as relative copies. Left. RIG-I mRNA levels. Right. IFN-β mRNA levels.

VLP-delivered 2CARD activating IFN responses by the canonical signaling pathways, IFN-β and RIG-I mRNA upregulation is abrogated in the absence of MAVS.

Based on these data, we conclude that we can produce Ebola VLPs that incorporate Mtb antigen and that we can introduce 2CARD with the ESAT-6 antigen when fused to eVP40. Therefore, we are in position to progress to proposed in vivo (mouse) immunogenicity studies. Because of the very high ESAT-6-eVP40 incorporation into VLPs, we are currently exploring two strategies to produce VLPs with this construct and that also have 2CARD adjuvanting activity. First, we are testing whether 2CARD-NP_{ct} can be incorporated into ESAT-6-eVP40-containing VLPs. We will also try mixing eGP+ESAT-6-eVP40 VLPs with eGP+eVP40+2CARD-NP_{ct} VLPs. Because GP is present on both VLPs, we would expect the same cell types to be targets by both components of the mixed VLP preparation.

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 due to a pause in non-COVID-19-related research this past Spring. The project resumed in early summer and is now progressing.

We originally planned to test not only ESAT-6 as a representative Mtb antigen, but also Ag85B. In our initial studies with Ag85B, we found that Ag85B fusion proteins expressed well but, when co-expressed with 2CARD constructs, IFN responses were not induced. Because initial troubleshooting did not resolve this issue, we have decided to proceed by focusing primarily on the ESAT-6-containing VLPs. If we can show that these elicit robust immunity, we will return to examine Ag85B and/or other possible Mtb antigens in our system.

We initially planned to use Huh7 cells for all of our studies in cell lines. However, compared to our past experience studying the Ebola VLP platform, yields from Huh7 cells are low relative to HEK293T cells. Because HEK293T cells are a commercially-available, non-stem cell cell line, we have provided to HRPO documentation from our IRB that the use of HEK293T cells does not constitute human subjects research, with the hope and expectation that we can use HEK293T cells going forward. This will facilitate production of VLPs in sufficient quantities to allow the proposed mouse studies to proceed.

PRODUCTS

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

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	JoAnn Tufariello
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Tufariello designed the overall strategy, planned the experiments, and directed and supervised the work performed by the postdoctoral fellow and technician. 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:	3
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.
Funding Support:	N/A

Name:	Freddy Sanchez
Project Role:	Lab manager/Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person	4

month worked:	
Contribution to Project:	Mr. Sanchez has assisted the post-doctoral fellow with cloning, and facilitated the work by preparing growth media and maintaining cultures.
Funding Support:	N/A

OTHER SUPPORT
TUFARIELLO, JOANN
ACTIVE
R21 AI146682 06/01/2020-05/31/2022
NIH/NIAID 2.4 calendar months
Intrinsically-Enhanced Ebola Virus Like Particles for Increased Potency and Immune Memory
Seeks to develop a novel Ebola and Marburg virus vaccine using enhanced virus-like particle approaches.
Role: PI

Since the submission of the Department of Defense-funded project described herein, the PI has received funding for the proposal above, which was listed as "pending" support on the initial application. The above R21 presents no overlap with the current proposal, as it seeks to develop vaccines for Ebola and Marburg viruses, and the project described here has the objective of developing a vaccine for M. tuberculosis.

We also note that, since submission of the Department of Defense-funded project described herein, funding for the following grant has ended.
1R03AI133172-01A1 (Magee) 02/05/2018 - 01/31/2020 NIAID
Latent tuberculosis infection and risk of type 2 diabetes mellitus in US veterans.
The goal of this project is to understand the epidemiological link between Mycobacterium tuberculosis infection and type 2 diabetes. It has no overlap with the current application.
Role: co-investigator

SPECIAL REPORTING REQUIREMENTS
Not applicable

APPENDICES
None