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14. ABSTRACT

Building on extensive preliminary data and an established consortium of academic, government, military, and product development partners, we propose to develop a production process for a novel, bivalent vaccine against leishmaniasis, a serious neglected tropical disease (NTD) of military and civilian personnel now spreading rapidly in conflict zones of the Middle East and Central Asia. The proposed Cutaneous Leishmania Vaccine (CL-Vax) is a bivalent, recombinant protein-based vaccine that will be comprised of a specific Leishmania parasite antigen together with a novel sand fly salivary antigen, co-administered at bedside with an adequate adjuvant. The components of the vaccine will be extensively characterized, and their immunogenicity and efficacy will be confirmed in animal models.

15. SUBJECT TERMS

None Listed

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1. Introduction

This proposal seeks to develop an effective, safe, and innovative vaccine to combat cutaneous leishmaniasis (CL), a neglected tropical disease caused by protozoan parasites of the genus *Leishmania* and transmitted to humans by the bite of a phlebotomine sand fly. Building on extensive preliminary data, we are developing a production process for a novel, bivalent vaccine against leishmaniasis and test its immunogenicity and efficacy in a mouse model of disease. The Cutaneous Leishmania Vaccine, CL-Vax, is based on recombinant proteins and is comprised of a specific *Leishmania* parasite antigen (LdNH36) together with a novel sand fly salivary antigen (PpSP15), co-administered at bedside with an adequate adjuvant (GLA-SE). Our hypothesis is that vaccination with CL-Vax is much more efficacious in reducing the lesion size caused by the infection and in reducing the parasite count at the infection site, than vaccination with just the *Leishmania vaccine* antigen alone. CL-Vax would induce a robust immune response to two independent antigens that are co-localized at the site of infected bites. Our approach builds on more than a decade of preliminary studies and publications.

2. Keywords

Sand fly; cutaneous leishmaniasis; LdNH36; PpSP15; Process development; neglected tropical diseases; bivalent vaccine; salivary antigen

3. Accomplishments (For activities scheduled for Year 3).

What were the major goals of the project?

Specific Aim 1 To develop a scaled-up process for the production of the sand fly antigen, PpSP15, and to produce PpSP15 and LdNH36 for immunogenicity and efficacy studies.	**Timeline (Months)**
Major Task 5, Activity 1.5. Execution of three successive process development runs at the 10 L scale.	
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Milestone Achieved: Process is shown to be reproducible with low variance	18
Milestone Achieved: Protein available for Aims 2 and 3.	18
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Specific Aim 2. To test the immunogenicity of PpSP15 and LdNH36 given at various dosages.	
Major Task 7, Activity 2.1: Immunological assessment of C57Bl/6 mice vaccinated with recombinant PpSP15 and LdNH36: route and dose ranging evaluation.	
Subtask 1: Write animal protocol and submit for review	1
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Milestone(s) Achieved: Local IACUC Approval	3
Milestone(s) Achieved: ACURO Approval	6
Milestone(s) Achieved: Having set the optimal dose and route of administration for PpSP15 and LdNH36 vaccine.	12
Major Task 8, Activity 2.2: Immunological assessment of C57Bl/6 mice vaccinated with recombinant PpSP15 and LdNH36: testing the role of GLA-SE adjuvant.	
Subtask 1: Select optimized doses and route of administration and test the effect upon immunogenicity by adding GLA-SE adjuvant	12-18
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Specific Aim 3. To evaluate the efficacy of PpSP15 and LdNH36 as a vaccine in protecting mice challenged by the natural mode of transmission, L. major infected sand flies.	
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What was accomplished under these goals?

Specific Aim 1: To develop a scaled-up process for the production of the sand fly antigen, PpSP15, and to produce PpSP15 and LdNH36 for immunogenicity and efficacy studies.

We had already developed and recently published a low-cost process for the expression and purification for the recombinant LdNH36 antigen from *Escherichia coli* (McAtee *et al.*, 2017)[1]. For this proposal, we sought funding for a 10 L research grade production run to produce the necessary LdNH36 protein in quantities sufficient to evaluate its co-administration with PpSP15 in the animal model. In addition, for PpSP15, activities under this objective include the development and optimization of a production process at the 10 L scale, including up to three reproducibility runs. Overall quality management and documentation oversight will be provided to ensure seamless future technology transfer to a contract manufacturer. Throughout this objective, we expect to generate the PpSP15 protein production process and formulation to be used in the subsequent aims. Furthermore, the material generated will be used to establish product characteristics and short- and long-term stability profiles.

We have successfully accomplished the following deliverables stated in the original proposal:

- For Year 1:
  - Protein LdNH36 (~100 mg) was produced and provided to our collaborator at USUHS.
  - Both *E. coli* and yeast expression systems have been evaluated for PpSP15 protein expression and *E. coli* was downslected due to insolubility of PpSP15. Proof-of-feasibility for expressing PpSP15 in the yeast *Pichia pastoris* platform was provided.
  - A protocol for the production of PpSP15 has been developed. Prior to optimization during months 15-18 of the grant, the current yield is >700 mg per liter of fermentation supernatant. The stated deliverable in the grant of 60 mg PpSP15 per liter of fermentation has thus been exceeded by a factor >10.
  - More than 600 mg purified PpSP15 protein were produced per liter of fermentation supernatant, which exceeded the deliverable of 20-25 mg by a factor of >24.
  - A set of assays suitable for the characterization of PpSP15 has been developed (Chen *et al.*, 2020), biophysical and biochemical analyses demonstrated that the protein was not only highly pure (>97%) and monomeric, but also maintained a well-defined secondary structure.
  - Buffer pH screening was conducted and demonstrated that PpSP15 was stable between pH 4.4 and pH 9.0, a range that is wider than the typical pH range suitable for parental vaccination (pH 5.5- pH 9.0).
  - Technical reports have been completed for all activities.
- For Year 2:
  - We have demonstrated reproducibility of the upstream and downstream production processes for PpSp15 in three reproducibility runs (Liu *et al.*, 2020).
  - The average yield was further increased to 750 mg PpSP15 per liter of fermentation supernatant with a coefficient of variation (CV) of only 7.7%
  - The overall average recovery of the process was 81% (CV=8.6%)
  - The average final purity of PpSP15 was > 97% (CV = 0.5%)
  - In accelerated stability testing, PpSp15 was found to be stable at 4°C, room temperature and 37°C for over approximately 30 days with only a slight decrease in purity at 37°C.

- A reference standard has been selected for its long-term stability, and the current results suggested that PpSP15 stored at -80°C remained stable for at least 3 months.
- Technical reports have been completed for all activities.

- For Year 3:
  - At BCM, we have monitored the stability of the in-house reference protein lots that we selected using the toolbox of analytical and biophysical assays developed specifically for this project (Chen *et al.*, 2020). Current data indicates that PpSP15 recombinant protein stored at -80°C remains stable for at least 12 months. From the work performed at BCM during years 1 and 2, two manuscripts were published (see below).

Major Task 6, Activity 1.6. Formulation and stabilization of PpSP15 and LdNH36 with GLA.

Subtask 1, Activity 1.6.1. Stability studies for PpSP15. (BCM)

In Year 2, we have characterized and performed the accelerated stability for the three PpSP15 lots from the reproducibility runs. Based on the data, we selected an in-house reference lot# PpSP15220319ZXL-1.

In Year 3, the long-term stability for protein reference lot PpSP15220319ZXL-1 stored at -80°C has been monitored using a panel of established assay, including Color and Appearance, pH, UV spectrophotometry, SDS-PAGE, and RP-HPLC. The Color and Appearance indicated that the protein solution remained clear, colorless and homogenous over 12 months. Trend analysis was performed for all the other quantifiable assays, and the results are reported in **Figure 1**. During the 12-month testing period, the measured protein content remained ~2.2 mg/mL and pH remained 5.9-6.0. A slight downward trend was observed for purity assessment analyzed by RP-HPLC (96% at month 0 to 94% at month 12); however, the purity analyzed by SDS-PAGE remained ~97±1%. Collectively, all the testing results are still within the proposed specifications, indicating that this protein lot remained stable for at least 12 months when stored at -80°C. We will continue to monitor the stability for this protein lot and build up the information of its shelf life.

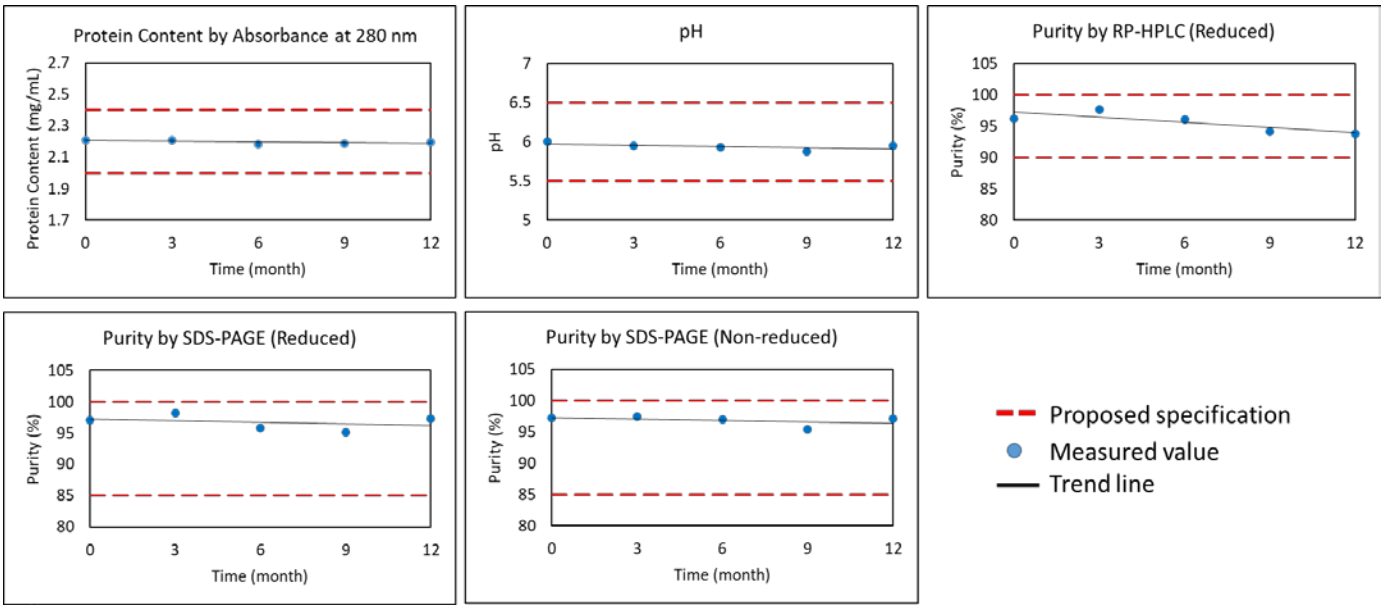


Figure 1 Long-term stability data for PpSP15 lot# PpSP15220319ZXL-1.

<u>Specific Aim 2: To test the immunogenicity of PpSP15 and LdNH36 given at various dosages.</u>

Year 1: For the first-year reporting period we completed Major Task 7, an immunological assessment of C57Bl/6 mice vaccinated with the recombinant PpSP15 and LdNH36 vaccine (CL-Vax), evaluating route and dose. Our objective was to set the optimal dose and route of administration for CL-Vax, the combined PpSP15 and LdNH36 vaccine, by testing the humoral response against antigen, adjuvant, and the cell mediated immune responses in the spleen. We showed that a high level of anti-LdNH36 or anti-PpSP15 total IgG were found with the following combinations: 5 μg LdNH36 + 5μg PpSP15 SQ or ID, 5 μg LdNH36 + 0.5 μg PpSP15 SQ or ID and Prime boost 5 μg LdNH36 + 0.5 μg PpSP15 SQ.

Protection from *Leishmania* related diseases is associated with the cellular immune response. We assessed splenocyte IFNγ secretion after 72 hr stimulation with both LdNH36 and PpSP15 antigens using ELISA. The combination giving the highest IFNγ results were in the SQ arm: 5 μg LdNH36 + 5 μg PpSP15, Prime

boost 5 μg LdNH36 + 0.5 μg PpSP15 and Prime boost 20 μg LdNH36 + 0.5 μg PpSP15. For the ID route, immunizations with 5 μg LdNH36 + 5 μg PpSP15 or Prime boost 20 μg LdNH36 + 5 μg PpSP15 were associated with the highest levels of IFNγ secretion. Culture supernatants from the vide listed vaccine conditions were analyzed by Luminex™ using the Th1/Th2/Th9/Th17/Th22/Treg Cytokine 17-Plex Mouse ProcartaPlex™ Panel. We determined the highest ratios of IFNγ/IL-10 as a predictor of vaccine success. Three antigen doses/routes met our selection criteria and were chosen to be tested in association with GLA-SE: 5 μg LdNH36 + 5 μg PpSP15 SQ, 5 μg LdNH36 + 5 μg PpSP15 ID, Prime 5 μg LdNH36 + 0.5 μg PpSP15 SQ.

Year 2: We found the combination of parasite and vector sand fly antigens to be immunogenic. A Th1 type immune response was consistently boosted using GLA-SE adjuvant. Interestingly the combination vaccine was associated with less cytokine production compared to either LdNH36 or PpSP15 alone, raising the issue that the combination could have an inhibitory effect on the cellular immune response.

Year 3: Immunogenic responses to vaccination were further studied during the sand fly challenge experiments and are reported later in this report. Vaccine antigen structure: We have resolved the structure of L. donovani NH36 in apo form (without substrate analog) in two crystal forms. These structures are very similar and will be compared to the published structure of NH36 from Leishmania major (1999, J Biol Chem). For SP15 we obtained crystals that refracted to low resolution so the structure could not be solved and have tried to obtain further crystals without success. We plan in next year to use additional strategies to coax SP15 to crystallize.

ADCC: Given the extremely high levels of antigen-specific IgG obtained with our combined vaccine we sought to assess antibody dependent cell mediated cytotoxicity. We developed a modified ELISA based protocol detecting CD107a as a surrogate marker of natural killer cell mediated degranulation (as marker for ADCC) adapting to mice the method of Galit Alter in HIV with interferon gamma readout. We also studied the vaccinated mouse sera with flow cytometry using markers of CD3, interferon gamma, CD107a, NK 1.1, CD49b. Some increase in NK activation at the terminal timepoint was seen in the mice receiving NH36 solely but less in the combined antigen stimulation or SP15 alone. (**Figure 2**). Targets were mouse splenocytes as well as mouse natural killer cells. Both assays compared NH36 (group A), SP15 (Group C), and combined vaccine (group E) at pre, time 1(2 weeks) and terminal bleed timepoints. Overall, some indication that NH36 alone had increasing ADCC by terminal bleed, especially when combined with SP15.

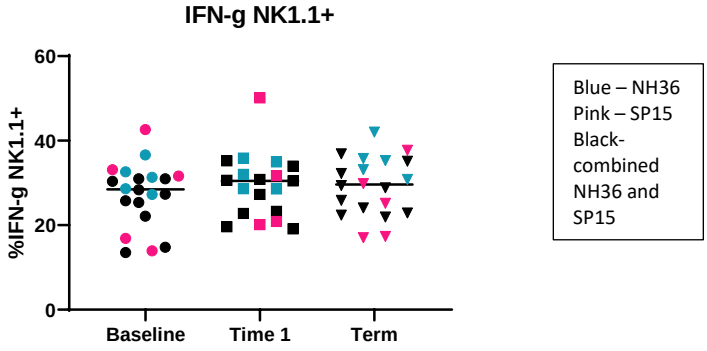


Figure 2. Assessment of NK activation over time in mouse splenocytes by vaccinated mouse sera by flow cytometry

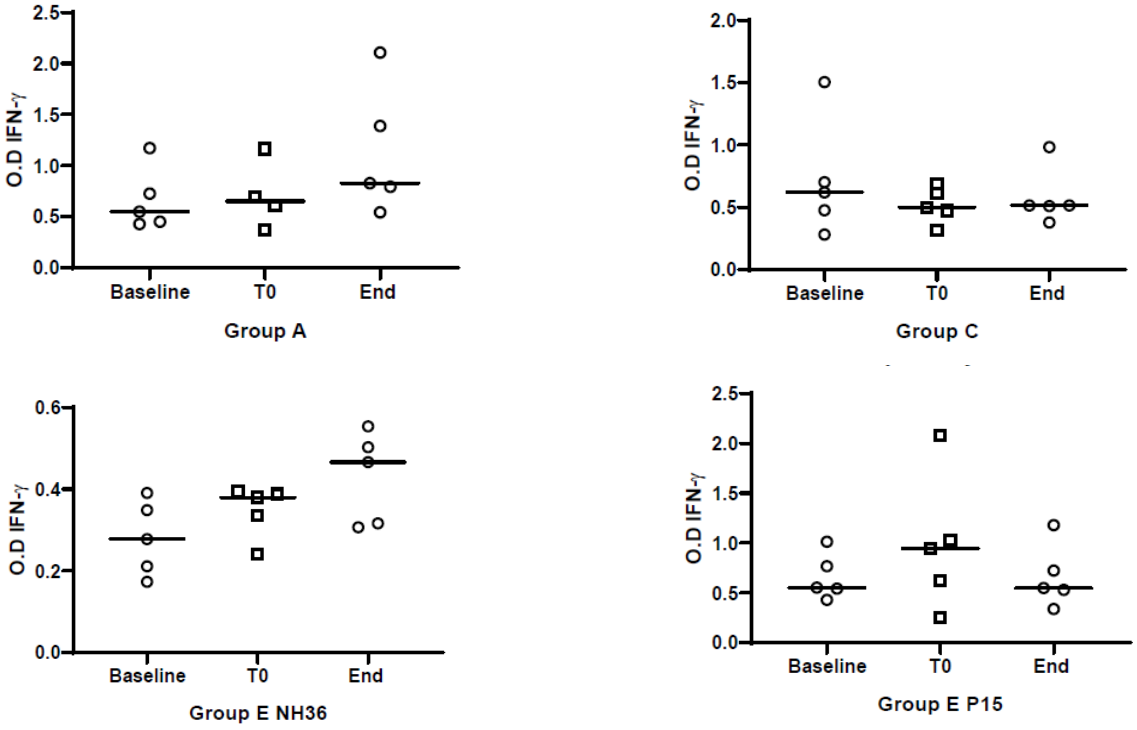


Figure 3. IFNγ response after 24 hour stimulation of mouse splenocytes in modified ADCC assay. Baseline, 2 week and post 3 vaccination samples are assessed. Group A is NH36, Group C is SP15, Group E is combined antigen vaccination

<u>Specific Aim 3. To evaluate the efficacy of PpSP15 and LdNH36 as a vaccine in protecting mice challenged by the natural mode of transmission, *L. major* infected sand flies.</u>

The VMBS setup an insectary dedicated to the growth and maintenance of the sand fly *P. papatasi*, a natural vector of *L. major*, a model previously used at a low scale at the VMBS. The VMBS updated the infrastructure necessary for the infection of *P. papatasi* and maintained a virulent parasite stock for the natural challenge of the vaccinated mice.

Major Task 11, Activity 3.3. Challenge of vaccinated mice using Leishmania major infected sand flies and characterization of post-challenge immunity.

During year 3 we evaluated the efficacy of PpSP15 and LdNH36 in protecting C57Bl/6 mice against sand fly transmitted leishmaniasis. Overall, no protective efficacy was observed when compared to non-immunized challenged mice after sand fly challenge with PpSP15/LdNH36 in the presence or not of the GLA-SE adjuvant. We have now completed three rounds as follows:

<u>Round 1:</u> For round 1 of the project, mice were immunized for a total of three times. Antigen specific IgG was measured via ELISA 3, 7 and 12 weeks post-immunization and confirmed that immunized SP15/NH36/GLA-SE mice had higher specific antibody levels when compared to SP15/NH36 and naïve mice (**Figure 4**). Unfortunately, the challenge of vaccinated mice using *L. major* infected sand flies could not be completed due to poor *P. papatasi*

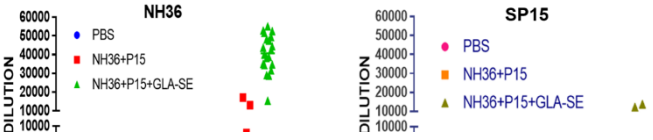


Figure 4. Results of antigen specific ELISA measuring IgG levels for weeks 3, 7, and 12 post- immunization. Comparison of groups immunized with NH36/SP15/GLA-SE, NH36/SP15, or naïve mice (PBS) against NH36 or PpSP15. T1 = Week 3, T2 =Week 7, T3 = Week 12.

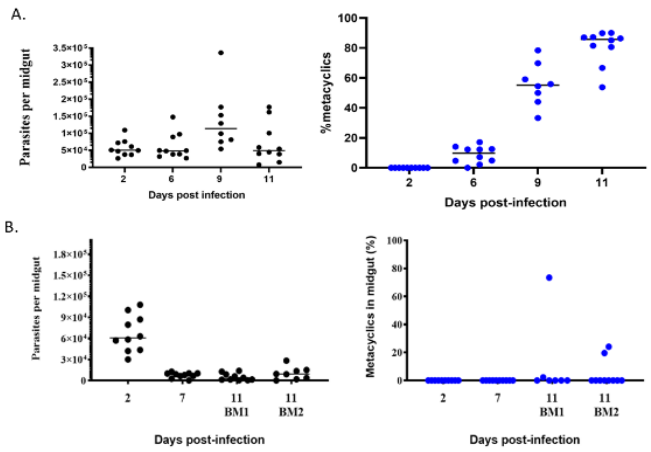


Figure 5. A. Representative transmissible infection of *L. longipalpis/L. major* with parasites reaching 80% metacyclic promastigote threshold 11 days post-infection. B. Representative poor Infection *L. longipalpis/L. major* with parasites not reaching the 80% metacyclic threshold by day 11 even with a second blood meal to boost the infection.

infections (**Figure 5**). For a successful sand fly transmission, *P. papatasi* infections need to reach a minimum threshold of >65% metacyclic promastigotes prior to transmission.

Out of 13 sand fly infections only 3 were successful; this is very unusual, however, refractoriness to *Leishmania* infection happened a few years ago in our sand fly colony, thus, we decided to move forward with needle challenge that includes *L. major* parasites and salivary gland homogenate of *P. papatasi* sand flies to mimic as closely as possible sand fly transmitted infection. Briefly, immunized mice (n=30) were challenged intradermally in the ear with 1,000 purified *L. major* metacyclic promastigotes expressing luciferase and TdTomato (*L.major* (Iraq) R-Fluc+tdt^{+}) and one pair of *P. papatasi* salivary gland homogenate (SGH). Of note, 1,000 purified metacyclic promastigotes have been routinely used in the laboratory to mimic natural infection and observe protection in other vaccine candidates. Additionally, these parasites provide a visual reference of parasite burden and corroborate bioluminescence data to lesion size diameter. Bioluminescence data via IVIS imaging software was measured for a period of 5 weeks post-needle challenge (**Figure 6A**). Using transgenic parasites is a valuable technique to better visualize the course of disease and protection (**Figure 6B**) and may be employed in future studies for this project.

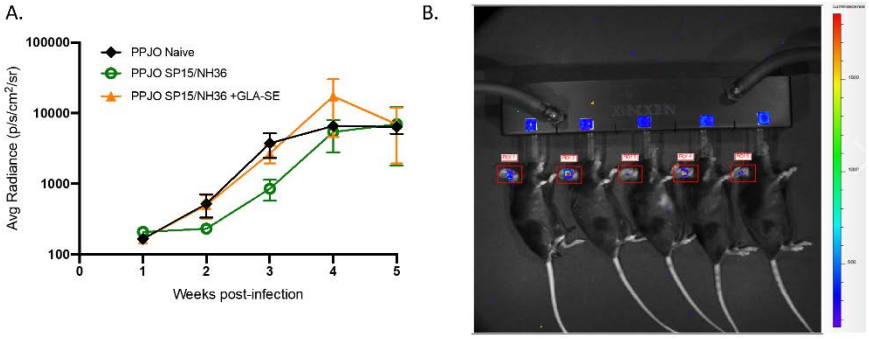


Figure 6 A. Parasite burden post-needle challenge measured via bioluminescence for 5 weeks. SP15/NH36/GLA-SE, SP15/NH36, or naïve mice were challenged by needle with *P. papatasi* (PPJO) salivary gland homogenate and 1000 (*L. major (Iraq) R-Fluc+tdt*$^{+}$) metacyclic promastigotes. **B.** Respective image of parasite burden as observed via the IVIS imaging software.

Finally, weekly lesion measurements of vaccinated or naïve mice were followed up to week 9 post-challenge, and no significant difference was observed among groups (**Figure 7**).

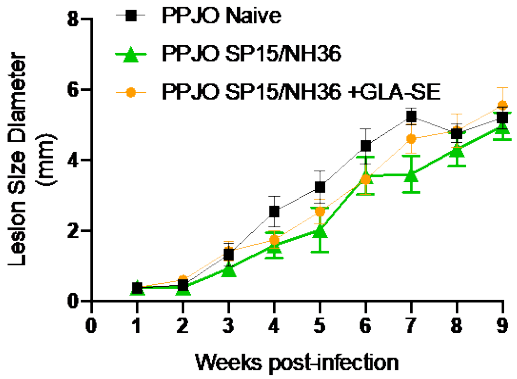


Figure 7. Lesion size diameter for immunized SP15/NH36/GLA-SE, SP15/NH36, or naïve mice challenged by needle with *P. papatasi* (PPJO) salivary gland homogenate and 1000 *L. major* metacyclic promastigotes. Lesions were followed for a period of 9 weeks.

<u>Round 2</u>: For round 2, mice were immunized three times and serum was collected at week 3, week 7 and week 12 post-immunizations. ELISAs to detect specific levels of IgG confirmed that immunized SP15/NH36/GLA-SE mice had higher specific antibody levels when compared to SP15/NH36 and naïve mice (**Figure 8)**.

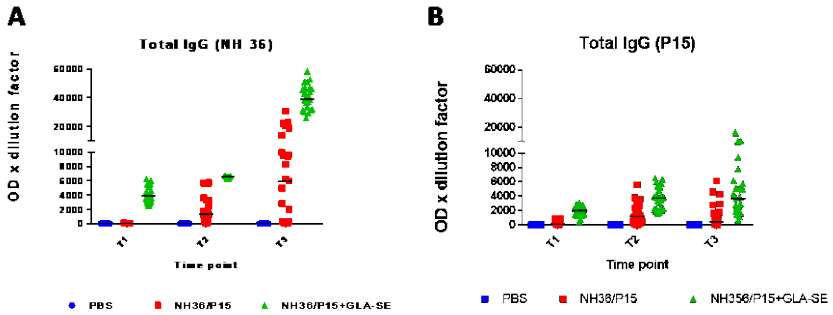


Figure 8. Results of antigen specific ELISA measuring IgG levels for weeks 3, 7, and 12 post- immunization. Comparison of groups immunized with NH36/SP15/GLA-SE, NH36/SP15, or naïve mice (PBS) against NH36 or PpSP15. T1 = Week 3, T2 = Week 7, T3 = Week 12.

To assessed the protective efficacy of the vaccine five weeks after the last immunization, immunized and naïve mice (n=15/group) were challenged with ten *L. major-P. papatasi* infected sand flies to assess the protective efficacy of the vaccine. We had troubleshooted various conditions for *P. papatasi* infections and have found favorable conditions that should allow us to transmit using the sand fly model. We have now reached the minimum threshold of 65% metacyclic promastigotes and can move forward with the proposed plan for challenge. The new conditions for transmissible infection include: $5x10^7$ promastigotes/mL, rabbit blood, not heparin treated, and heat inactivated, with 1 mg/mL soybean trypsin inhibitor and no antibiotics in the bloodmeal. The infection status of *P. papatasi* infected sand flies use to challenge the vaccinated or naïve groups for Round 2 is shown in **Figure 9**.

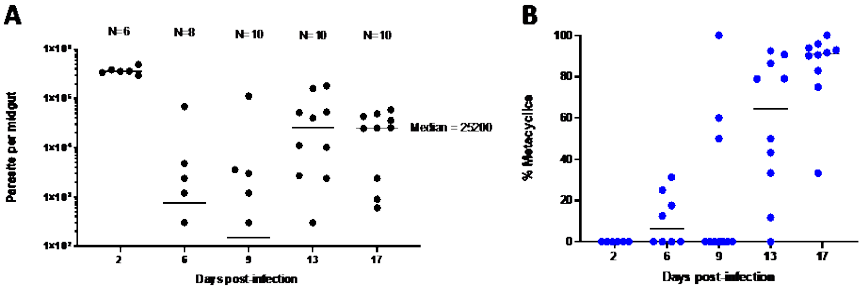


Figure 9. A. Infection status of *P. Papatasi* sand flies infected with rabbit defibrinated blood spiked with 5 x 10^7 *L. major* (Iraq) promastigotes per mL. **A).** Number of parasites per midgut and **B)** Percent of metacyclic promastigotes per midgut infection.

L. major-infected sand flies were allowed to bite mice immunized with PpSP15 and LdNH36 or control vaccine. Briefly, mice were anesthetized, and individual ears were pressed flat against the nylon mesh covering of small plastic vials containing the flies using specially designed clamps that hold the vial and ear in place between support arms adapted at the ends with a flat rubber surface. Up to ten sand flies were

placed in the vial. Sand flies were allowed to feed for up to two hours, in the dark. Animals were checked at least once every 30 minutes, and the feeding score among groups was determined (**Figure 10**).

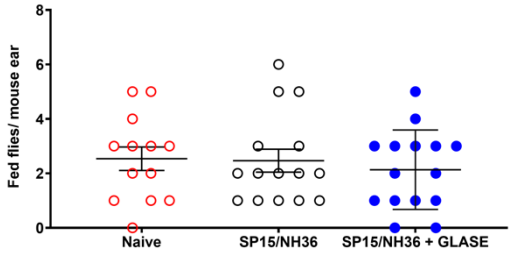


Figure 10. Naïve or immunized groups were exposed to infected *P. papatasi* sand flies (n = 10) for 2hr and the number of fed flies per mouse ear was recorded among groups. No significance was observed among groups.

Ear thickness and ear lesion diameter was measured on a weekly basis for up to 7 weeks using a Vernier caliper (Mitutoyo, Baltimore, MD). At 7 weeks post-challenge with infected sand flies mice (n=6-7/group) were sacrificed and parasite burden was assessed in the ears by limiting dilution assay. No significance was observed in the lesion diameter or parasite burden among vaccinated groups when compared to control group (**Figure 11**).

Figure 11. Evaluation of protective efficacy of SP15/NH36 +/- GLA-SE after sand fly challenge A) Lesion size diameter for immunized SP15/NH36/GLA-SE, SP15/NH36, or naïve mice challenged with 10 *P. papatasi* sand flies. Lesions were followed for a period of 7 weeks. **B)** Representative images of vaccinated SP15/NH36 mice with or withouth GLA-SE adjuvant at week 7 post-challenge. **C)** Limited dilution assay at week 7 post-challenge.

<u>Round 3:</u> For round 3, mice were immunized three times as previously described in Round 2, however due to the shutdown of the NIH campus because of COVID-19 an additional boost was administrated 3 months after the 3rd immunization. Serum was collected at week 3, 7,12 and 24 post-immunizations and ELISAs to detect specific levels of IgG confirmed that immunized SP15/NH36/GLA-SE mice had higher specific antibody levels when compared to SP15/NH36 and naïve mice (**Figure 12**).

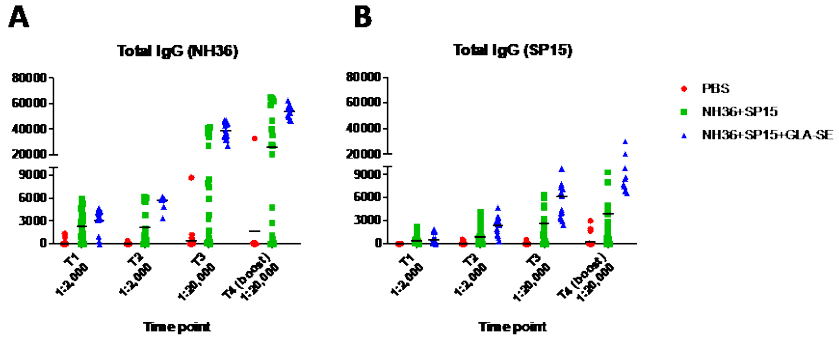


Figure 12. Results of antigen specific ELISA measuring IgG levels for weeks 3, 7, 12 and 24 post- immunization. Comparison of groups immunized with NH36/SP15/GLA-SE, NH36/SP15, or naïve mice (PBS) against NH36 or PpSP15. T1 = Week 3, T2 = Week 7, T3 = Week 12, T4 = Week 24.

Five weeks post-last immunization mice (n=8/group) were challenged in one ear with *L. major*-infected *P. papatasi* sand flies (n=15) and the protective efficacy of the vaccine were determined. The infection status of the infected *P. papatasi* sand flies is shown in **Figure 13**.

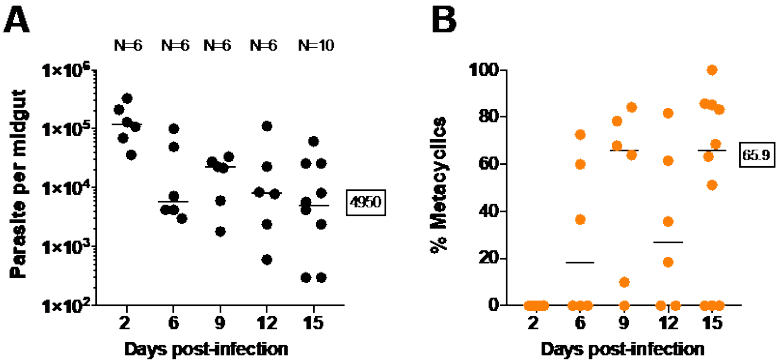


Figure 13. Infection status of *P. Papatasi* sand flies infected with rabbit defibrinated blood spiked with 5 x 10^7 *L. major* (Iraq) promastigotes per mL. **A).** Number of parasites per midgut and **B)** Percent of metacyclic promastigotes per midgut infection.

The sand fly feeding score after transmission was recorded (**Figure 14**) and no difference was observed among groups.

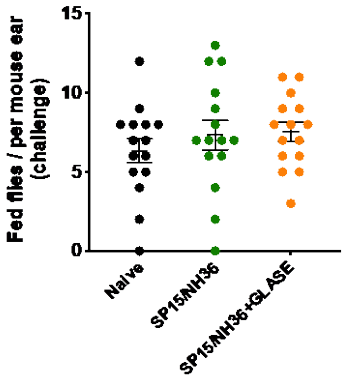


Figure 14. Naïve or immunized groups were exposed to infected *P. papatasi* sand flies (n = 15) for 2hr and the number of fed flies per mouse ear was recorded among groups. No significance was observed among groups.

Next, we evaluated the protective efficacy by measuring the ear lesion diameter weekly for up to 7 weeks and parasite burden was assessed in the ears by limiting dilution assay and no and no significant difference was observed among groups (**Figure 15**).

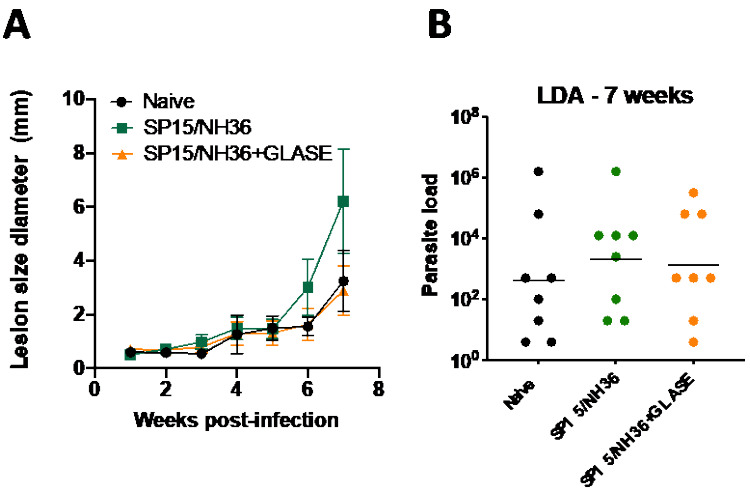


Figure 15. Evaluation of protective efficacy of SP15/NH36+/-GLA-SE after sand fly challenge A) Lesion size diameter for immunized SP15/NH36/GLA-SE, SP15/NH36, or naïve mice challenged with 15 *P. papatasi* sand flies. Lesions were followed for a period of 7 weeks. **B**) or limited dilution assay at week 7 post-challenged showing no significance between groups.

In summary, as shown in the cumulative data from Round 2 and Round 3 when compared to control group, no difference was observed between PpSP15/NH36 immunized groups in the presence or not of GLA-SE (**Figure 16**).

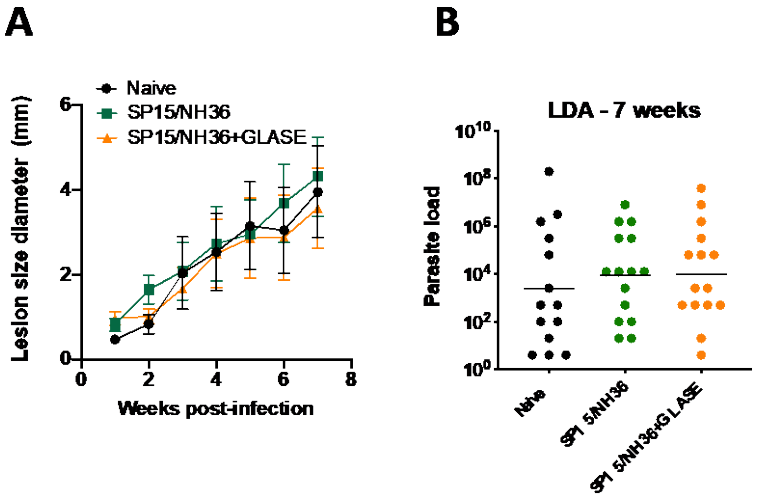


Figure 16. Cumulative results of Round 2 and 3 after sand fly challenge A) Lesion size diameter for immunized SP15/NH36/GLA-SE, SP15/NH36, or naïve mice challenged with 15 *P. papatasi* sand flies. Lesions were followed for a period of 7 weeks. **B**) or limited dilution assay at week 7 post-challenged showing no significance between groups.

Potential problem areas and alternative methods and approaches:

As an alternative approach we currently are looking at ways to improve recombinant protein vaccine efficacy based on the new data summarized in this report. For instance, because of previous success with DNA vaccines, we are exploring multiple pathways to include DNA-protein prime boosts along with single antigen immunizations in order to establish synergistic responses and/or rule out immunological interference.

These data reported here are also allowing us to identify specific protective mechanisms that go beyond two dimensional Th1 vs Th2 responses. We remain optimistic about the prospects of a recombinant protein-based vaccine as a suitable preventive strategy and how optimizations reported in this grant will be instrumental to mechanisms that maximize parasite reductions.

What opportunities for training and professional development has the project provided?

At the NIH/NIAID, we have trained postdoctoral fellows to perform vaccine studies and trained them in many aspects of vector biology, specifically the use of infective insects to transmit disease to animals for vaccine studies.

At USU, a postdoctoral fellow was mentored to develop a new type of cytotoxicity assay and a graduate student was trained to develop the NH36 crystals and analyze their structure.

How were the results disseminated to communities of interest?

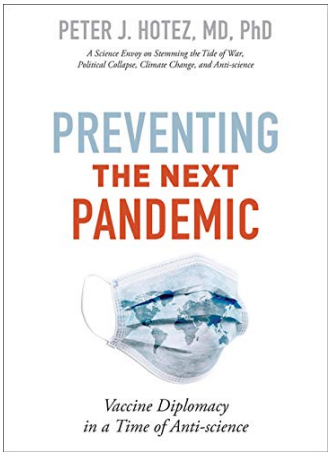
Two major scientific papers were published:

Liu Z, Kundu R, Damena S, Biter AB, Nyon MP, Chen WH, Zhan B, Strych U, Hotez PJ, Bottazzi ME. A scalable and reproducible manufacturing process for Phlebotomus papatasi salivary protein PpSP15, a vaccine candidate for leishmaniasis. Protein Expr Purif. 2020 Sep 10;177:105750. doi: 10.1016/j.pep.2020.105750.

Chen WH, Nyon MP, Poongavanam MV, Liu Z, Biter AB, Kundu RT, Strych U, Hotez PJ, Bottazzi ME. Process Characterization and Biophysical Analysis for a Yeast-Expressed Phlebotomus papatasi Salivary Protein (PpSP15) as a Leishmania Vaccine Candidate. J Pharm Sci. 2020 May;109(5):1673-1680. doi: 10.1016/j.xphs.2020.02.004.

In addition, Dr. Hotez completed a new single-author book featuring work on leishmaniasis:

Hotez PJ. Preventing the Next Pandemic: Vaccine Diplomacy in a Time of Anti-Science (Johns Hopkins University Press)



Beyond the scientific papers and the book, during this past year our group published several key advocacy documents in order to raise the profile of cutaneous leishmaniasis, and highlight both its hidden disease burden due to scarring, social stigma, and mental health and how and why this disease is emerging in global areas of conflict and political instability and why this disease is of military importance. Dr. Hotez also refers regularly to the leishmaniasis vaccine project as an important example of "vaccine diplomacy":

Press outreach. Much of the public outreach this past year was focused on COVID-19 with Dr. Hotez conducting interviews and appearances almost daily on CNN, MSNBC, or Fox News. In addition, we conducted several interviews on leishmaniasis and leishmaniasis vaccines, including the following:

- http://outbreaknewstoday.com/leishmaniasis-in-kenya-dozens-of-new-cases-reported-96308/
- https://www.infectiousdiseaseadvisor.com/home/topics/vector-borne-illnesses/cases-of-infection-with-leishmania-parasites-on-the-rise-in-us/
- https://www.natureasia.com/en/nmiddleeast/article/10.1038/nmiddleeast.2019.74

Dr. Hotez also received several key awards:

- Ronald McDonald House Charities, Medical Award of Excellence 2019
- Award for Leadership in Advocacy for Vaccines, American Society of Tropical Medicine and Hygiene (ASTMH)
- Fred L Soper Lecturer, American Society of Tropical Medicine and Hygiene (ASTMH)
- Hagler Institute for Advanced Study at Texas A&M University

Public lectures. Dr. Hotez, the Principal Investigator, spoke extensively about leishmaniasis vaccine efforts: His public lectures and speeches about the leishmaniasis vaccine project (and its potential role in US vaccine diplomacy), included keynotes – such as medical grand rounds at Walter Reed National Military Medical Center (January 2020) and plenaries at the following outlets:

September 21, 2020	WorldLeish-7 Congress (Cartegena, Colombia, Via Zoom)
September 17, 2020	UCSF, Institute for Global Health Sciences (San Francisco, CA),
September 16, 2020	Yale University School of Medicine and Yale-New Haven Children's Hospital, Pediatric Grand Rounds (New Haven, CT, Via Zoom
September 15, 2020	Baylor University BIO 2201, Biology of Global Health (Waco, TX), "Global Vaccines"
September 14, 2020	Baylor College of Medicine, Introduction to Health Policy, MEETH-413 – "Global Vaccines"
September 14, 2020	Institute for Science & Policy, Denver Museum of Nature & Science (Denver, CO),

September 10, 2020	2020 Rotary Zone Institute (Houston, TX), “Preventing the Next Pandemic”
September 9, 2020	Brockman Foundation Scholars Program, Texas A&M University Evening Lecture (College Station, TX), “Preventing the Next Pandemic”
August 20, 2020	National Academies of Science, Engineering, and Medicine, Forum on Microbial Threats, The Critical Public Health Value of Vaccines – Tackling Issues of Access and Hesitancy: A Workshop (Washington DC, Via Zoom)
August 18, 2020	US State Department and US Embassy in Pakistan, US-Pakistan COVID-19 Seminar Series (Karachi, Pakistan, Via Zoom), “Preventing the Next Pandemic””
August 17, 2020	Uniformed Services University of the Health Sciences, Out of the Horse’s Mouth Lecture Series (Bethesda, MD Via Zoom), “My life in science and science policy”
August 5, 2020	Martin Kleiman Lecture, Pediatric Grand Rounds, Indiana University School of Medicine and Riley Children’s Hospital (Indianapolis, IN), “Preventing the Next Pandemic”
July 28, 2020	ASM Chat, American Society of Microbiology (Wash DC, via Zoom) with Dr. Jennifer Gardy, Gates Foundation,
July 21, 2020	Cook Children’s Hospital, Pediatric Grand Rounds (Fort Worth TX, Via Zoom), “Preventing the Next Pandemic”
July 17, 2020	2nd International Research Team COV-IRT Symposium, Rice University, “COVID19 Global Health Vaccines” (Via Zoom, Houston TX)
June 29, 2020	James Cook University, International Day of the Tropics 2020 (Townsville, Australia, Via Zoom), “Preventing the next pandemic”
June 29, 2020	Oregon Health Sciences University Brain Institute (Portland OR Via Zoom)
June 22, 2020	American Society of Microbiology, ASM Microbe (Wash DC Via Zoom), “Preventing the Next Pandemic”
June 12, 2020	Annual Southeastern Pediatric Research Conference, Emory University (Atlanta GA, via zoom), “Preventing the Next Pandemic”
June 10, 2020	Oklahoma Children’s Hospital and University of Oklahoma Health Sciences Center Department of Pediatrics (Oklahoma City OK, via zoom), “Preventing the Next Pandemic”
June 10, 2020	US State Department and Model Organization of the Islamic Conference (Istanbul, Turkey, via Zoom), “Preventing the Next Pandemic”
May 26, 2020	Milton Markowitz Lecture, Pediatric Grand Rounds, Connecticut Children’s Medical Center, University of Connecticut (Hartford, CT via zoom), “Preventing the next pandemic”
May 21, 2020	Cambridge Healthtech Institute, Webinar (Houston, Texas via zoom), “COVID19 Vaccines”
May 6, 2020	Research!America, Alliance Member Webinar (Wash DC via zoom), “Preventing the Next Pandemic”
May 5, 2020	Houston Methodist Hospital and Weil Cornell Medical College, Medical Grand Rounds (Houston, TX via zoom), “Preventing the Next Pandemic”
April 22, 2020	London School of Hygiene and Tropical Medicine, NTD Course (London, UK) via Zoom, “The NTDs”
March 4, 2020	ISNTD, International Society for Neglected Tropical Diseases (London, UK via skype)
February 27, 2020	Hagler Institute for Advanced Study and College of Veterinary Medicine & Biosciences (College Station, TX), Class on 21st Century Global One Health, “Vaccines and public health in the 21st Century”
February 25, 2020	Cleveland Clinic, Pediatric Grand Rounds (Cleveland OH), “Vaccine diplomacy in an age of war, political unrest, antiscience, and climate change”
February 12, 2020	Bar-Ilan University, Azrieli Faculty of Medicine (Sefad, Israel), “Vaccine and neglected disease diplomacy in an age of war, political collapse, climate change, and antiscience”

January 31, 2020	Walter Reed National Military Medical Center, Grand Rounds (Bethesda MD), "Vaccine and Neglected Tropical Disease Diplomacy in an Age of War, Political Collapse, Climate Change and Antiscience"
January 30, 2020	Johns Hopkins School of Medicine, Hopkins Medicine Distinguished Speakers Series (Baltimore, MD), "Vaccines in an age of conflict, global instability, climate change, and antiscience"
January 24, 2020	University of California Davis Children's Hospital, Pediatric Grand Rounds (Sacramento, CA), "Vaccine diplomacy in an age of war, political collapse, climate change, and antiscience"
January 10, 2020	UT Health McGovern Medical School, Department of Microbiology & Molecular Genetics, MBID (Molecular Biology of Infectious Diseases) Annual Retreat, "Vaccine and Neglected Disease Diplomacy in an Age of War, Political Collapse, Climate Change, and Antiscience"
December 12, 2019	Commencement Address, The College of Health Professions (Houston, TX)
December 11, 2019	University of California Irvine (Irvine, CA), Infectious diseases research group, "Vaccine diplomacy in an age of war, political collapse, climate change and antiscience"
December 10, 2019	National Academy of Sciences, Engineering and Medicine, Beckman Conference Center (Irvine, CA), Life 2.0 the promise and challenge of a crispr path to a sustainable planet, "Public Health Challenges of Infectious Diseases: New Infectious Disease Technologies vs The "Anthropocene"
December 4, 2019	World Vaccine and Immunotherapy Congress (San Francisco, CA), Roundtable leader for discussion on the antivaccine movement
November 21, 2019	Fred L Soper Lecture, American Society of Tropical Medicine & Hygiene, Annual Meeting (Washington DC), "Vaccine and neglected tropical disease diplomacy in our Anthropocene epoch"
November 21, 2019	American Society of Tropical Medicine and Hygiene, Annual Meeting (Washington DC), "Meet the professor"
November 18, 2019	Pediatric Scientist Training and Development Program, Texas Children's Hospital and Baylor College of Medicine (Houston, TX), "Dinner remarks"
November 15, 2019	Sigma Xi Annuarl Meeting: Our Changing Global Environment (Madison, WI), keynote: "Vaccine diplomacy in an age of war, political collapse, climate change, and antiscience"
November 5, 2019	Baylor University, Department of Biology (Global Health Biology Major) (Waco, TX via webex), "Vaccine diplomacy in an age of war, political collapse, climate change, and antiscience"
October 31, 2019	Rice University, School of Engineering, Bioengineering Innovation Symposium (Houston, TX), Keynote Presentation
October 15, 2019	Feinstein Institute of Medical Research, Elmezzi School of Graduate Molecular Medicine (Great Neck, NY), "Vaccines and public health in the Anthropocene Epoch"
October 7, 2019	Georgetown University Medical Center, Department of Microbiology and Immunology (Wash DC), "Vaccine diplomacy in an age of war, political collapse, climate change and antiscience" "Vaccine diplomacy in an age of war, political collapse, climate change and antiscience"
October 7, 2019	Uniformed Services University of the Health Sciences, Department of Pathology (Bethesda MD),
October 6, 2019	IDWeek2019 Keynote (Wash DC), "Vaccine & Neglected Disease Diplomacy in an Age of War, Political Collapse, Climate Change, and Antiscience"
October 4, 2019	Pediatric Infectious Diseases Society, Annual ID Week Dinner (Wash DC), "The Pediatric Infectologist: A New Generation Vaccine Ambassador"
October 2, 2019	Baylor College of Medicine, MD PhD Medical Scientist Training Program (MSTP,

	Houston, Texas), "Vacines and vaccine diplomacy"
October 2, 2019	Texas Vaccines for Children (TVFC) Annual meeting and awards ceremony (Houston, TX), "Combating a 21st Century Antiscience Movement"
September 17, 2019	European Congress on Tropical Medicine & International Health ECTMIH 2019 (Liverpool, UK), Lord Soulsby Lecture: "One Health in an Age of War, Political Unrest, Climate Change, and Antiscience"

What do you plan to do during the next reporting period to accomplish the goals?

We have received a one year no-cost extension to complete the work delayed due to the COVID-19 pandemic. At BCM, we will continue the stability evaluation of the PpSP15 antigen. We will also continue to provide our partners with additional protein, PpSP15 as well as LdNH36.

At USU we will work to complete the crystal structure analysis during a one year no-cost extension.

For activities scheduled for Major Tasks 1-4, as described above, we remain on schedule to achieve the milestones set in the original statement of work. Major Task 6 (formulation and stability studies) is ongoing as scheduled and will be completed before the end of Year 3. With these activities we will have a) provided proteins to our partners for immunogenicity and efficacy studies, and b) developed a production process for both vaccine antigens

We completed Major Task 8, Activity 2.2 as scheduled, the immunological assessment of C57Bl/6 mice vaccinated with recombinant PpSP15 and LdNH36: testing the role of the GLA-SE adjuvant. The USUHS deliverable was to select optimized CL-Vax doses and route of administration and test the effect upon immunogenicity by adding GLA-SE adjuvant.

We completed Major Task 9-11, we have established a large colony of the sand fly *Phlebotomus papatasi* for vaccine studies in our insectaries. We have increased significantly the number of sand flies from 500 sand flies per week to 8,000 sand flies per week. We have troubleshooted various conditions for *P. papatasi* infections and have found favorable conditions that should allow us to transmit using the sand fly model. We have learned that the minimum threshold of 65% metacyclic promastigotes are needed in the insect to efficiently transmit the *Leishmania* parasite to animals. The new conditions for transmissible infection include: $5x10^7$ promastigotes/mL, rabbit blood, not heparin treated, and heat inactivated, with 1 mg/mL soybean trypsin inhibitor and no antibiotics in the bloodmeal. We completed two rounds of experiments using the natural model of *L. major* transmission, *P. papatasi* bites. In summary, the combination of the two recombinant proteins with or without the addition of GLA-SE adjuvant failed to protect after sand fly challenge.

4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**
  - We are further advancing the concept that it is feasible to develop a recombinant vaccine to prevent cutaneous leishmaniasis, one of the leading neglected tropical disease of military importance
- **What was the impact on other disciplines?**
  - We are advancing the concept that simultaneously targeting both the infectious organism + its vector is a promising vaccine strategy to combat vector-borne infectious diseases.
- **What was the impact on technology transfer?**

- We are optimizing processes for the express purpose of technology transfer of two recombinant protein antigens. These processes appear to be robust and express the proteins at high yield. Our purification processes produce proteins at high levels of purity.

- **What was the impact on society beyond science and technology?**

  - The major impact is that we are developing an innovative vaccine for a serious infection emerging in areas of conflict and political instability. We hope that our leishmaniasis vaccine will not only have an important military use, but also will find use in preventing this disease among highly vulnerable populations, including refugee populations.

5. Changes/Problems

Changes in approach and reasons for change

As highlighted above we are working to modify our vaccine and vaccine strategies to explore DNA-protein prime-boost approaches as well as single antigen experiments to rule out antigen-antigen immunological interference. **Actual or anticipated problems or delays and actions or plans to resolve them.**

The COVID pandemic restricted access to the laboratories at all three sites for part of the year. This has delayed the proposed work. We therefore requested a no-cost extension which was granted on July 15, 2020.

Changes that had a significant impact on expenditures.

Significant changes in use or care of human subjects, vertebrate animals, biohazard

Significant changes in use or care of human subjects

Not applicable

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. Products

LdNH36 protein

PpSP15 protein

Publications:

[1] W.H. Chen, M.P. Nyon, M.V. Poongavanam, Z. Liu, A.B. Biter, R.T. Kundu, U. Strych, P.J. Hotez, M.E. Bottazzi, Process Characterization and Biophysical Analysis for a Yeast-Expressed *Phlebotomus papatasi* Salivary Protein (PpSP15) as a Leishmania Vaccine Candidate, Journal of pharmaceutical sciences, 109 (2020) 1673-1680.

[2] Z. Liu, R. Kundu, S. Damena, A.B. Biter, M.P. Nyon, W.H. Chen, B. Zhan, U. Strych, P.J. Hotez, M.E. Bottazzi, A scalable and reproducible manufacturing process for *Phlebotomus papatasi* salivary protein PpSP15, a vaccine candidate for leishmaniasis, Protein expression and purification, 177 (2020) 105750.

7. Participants & Other Collaborating Organizations

Uniformed Services University of the Health Sciences	
Name:	*Naomi Aronson*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Aronson supervised the mouse work, participated in all consortium calls, completed most of the regulatory documents and subaward, agreement, paperwork allowing this project to proceed*
Funding Support:	
Name:	*Saule Nurmukhambetova*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Ms. Nurmukhambetova completed the processing of tissue samples, including ELISAs for NIAID.*
Funding Support:	
NIH	
Name:	*Jesus G. Valenzuela*
Project Role:	*Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Valenzuela has performed work in conditioning an insectary room to grow sand flies for vaccine challenge study.*
Funding Support:	
Baylor College of Medicine	
Name:	*Peter J. Hotez*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Hotez has directed the team at BCM and has guided the experimental design and reviewed the data analysis.*
Funding Support:	
Name:	*Maria Elena Bottazzi*
Project Role:	*Co-Director Vaccine Center*

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.6*
Contribution to Project:	*Dr. Bottazzi supervised the upstream and downstream process development teams, as well as the quality control unit at the vaccine center. She also reviewed all technical reports.*
Funding Support:	
Name:	*Jeroen Pollet*
Project Role:	*Director of Formulation*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.3*
Contribution to Project:	*Dr. Pollet was involved in assay development and stability assessment of the vaccine antigen candidates*
Funding Support:	
Name:	*Bin Zhan*
Project Role:	*Director, Molecular Biology and Antigen Discovery*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.3*
Contribution to Project:	*Dr. Zhan was responsible for ensuring the scale-up of the PpSP15 production process.*
Funding Support:	
Name:	*Ulrich Strych*
Project Role:	*Director, Reporting*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.6*
Contribution to Project:	*Project coordination, Scientific review of all experimental designs at BCM, Composition of annual report. After Dr. Beaumier's departure, Dr. Strych took over her responsibilities on program management.*
Funding Support:	
Name:	*Wen-Hsiang Chen*
Project Role:	*Director, Quality Control*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.82*

Contribution to Project:	*Dr. Chen oversaw all quality control for PpSP15 and LdNH36.*
Funding Support:	
Name:	*Portia Gillespie*
Project Role:	*Laboratory manager*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.37*
Contribution to Project:	*Laboratory management*
Funding Support:	
Name:	*Diane Nino*
Project Role:	*Project Manager*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.6*
Contribution to Project:	*Project coordination*
Funding Support:	
Name:	*Junfei Wei*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Wei worked under supervision of Dr. Zhan on the engineering of the PpSP15 constructs.*
Funding Support:	
Name:	*Ghada Launey*
Project Role:	*Project Manager*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Project coordination*
Funding Support:	
Name:	*Amadeo Biter*
Project Role:	*Quality Control*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*1*
Contribution to Project:	*Dr. Biter conducted the quality control assay for PpSP15.*
Funding Support:	

Has there been a change in the active other support of PD/PI(s) or senior/key personnel since the last reporting period?

Changes for Peter Hotez:

- Title of the project: West Nile Virus vaccine development – End date extended to 6/30/2020
- Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease – Effort changed to 5%
- Title of the project: A novel mucosal trichuriasis vaccine for Th2 immune protection - Effort changed to 2.5%
- Title of the project: Chagas Vaccine Initiative - Effort changed to 5%
- Title of the project: Generation of the COVID-19 RBD protein: characterization, immunogenicity, and cross-reactivity with anti-SARS-CoV serum. New active support. Effort at 5%
- Title of Project: SARS-CoV Vaccine Development. New active support, effort at 3%
- Title of Project: Advancing an RBD-based vaccine against COVID-19. New active support, effort at 5%
- Title of Project: A Vaccine to prevent Leishmaniasis. End date extended to 9/14/2021
- Title of Project: MERS-CoV RBD Vaccine - Technology transfer and cGMP manufacture. New active support, effort at 5% (later)

Changes for Maria Elena Bottazzi:

- Title of the project: West Nile Virus vaccine development – End date extended to 6/30/2020
- Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease – Effort changed to 5%
- Title of the project: A novel mucosal trichuriasis vaccine for Th2 immune protection - Effort changed to 2.5%
- Title of the project: Chagas Vaccine Initiative - Effort changed to 5%
- Title of Project: Identification of the mechanism of protecting against a B. burgdorferi CspZ for the prevention of Lyme disease. New active support, effort at 3%
- Title of Project: Generation of the COVID-19 RBD protein: characterization, immunogenicity, and cross-reactivity with anti-SARS-CoV serum. New active support, effort at 5%
- Title of Project: SARS-CoV Vaccine Development. New active support, effort at 3%
- Title of Project: Advancing an RBD-based vaccine against COVID-19. New active support, effort at 10.5%
- Controlled Infection for Testing Efficacy of Hookworm Vaccines in Brazil. New active support, effort at 1%
- Title of Project: MERS-CoV RBD Vaccine - Technology transfer and cGMP manufacture. New active support, effort at 5% (later)

Changes for Jeroen Pollet:

- Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease – Effort changed to 1%
- Title of Project: Passive Immunization of Foals with mRNA-Abs against R. equi. New active support, effort at 1%
- Title of Project: SARS-CoV Vaccine Development. New active support, effort at 5%

- Title of Project: Advancing an RBD-based vaccine against COVID-19. New active support, effort at 25%
- Title of Project: MERS-CoV RBD Vaccine - Technology transfer and cGMP manufacture. New active support, effort at 10%

Changes for Ulrich Strych:

- Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease –Effort changed to 5%
- Title of the project: Chagas Vaccine Initiative - Effort changed to 15%
- Title of Project: SARS-CoV Vaccine Development. New active support, effort at 5%
- Title of Project: Advancing an RBD-based vaccine against COVID-19. New active support, effort at 20%
- Title of Project: MERS-CoV RBD Vaccine - Technology transfer and cGMP manufacture. New active support, effort at 5% (later)
- Title of Project: Generation of the COVID-19 RBD protein: characterization, immunogenicity, and cross-reactivity with anti-SARS-CoV serum. New active support, effort at 5%

Changes for Bin Zhan:

- Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease –Effort changed to 4%
- Title of the project: A novel mucosal trichuriasis vaccine for Th2 immune protection - Effort changed to 2.5%
- Advancing an RBD-based vaccine against COVID-19. New active support, effort at 20%
- Title of Project: Generation of the COVID-19 RBD protein: characterization, immunogenicity, and cross-reactivity with anti-SARS-CoV serum. New active support, effort at 10%

Changes for Naomi Aronson:

- Title of the project: BCG Vaccine to Prevent COVID-19 in Military Health System Healthcare Workers. – New Addition to active support. Effort 20%

8. Special Reporting Requirements

Not applicable

9. Appendices

Nothing to report