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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 2).

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: 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.	
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Milestone(s) Achieved: Having set the optimal dose and route of administration for PpSP15 and LdNH36 vaccine.	12
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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.
  - 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 have been developed.
- For Year 2:
  - We have demonstrated reproducibility of the upstream and downstream production processes for PpSp15 in three reproducibility runs.
  - 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.

Subtask 4: Activity 1.3.4. Three reproducibility runs of PpSP15 10 L fermentation

As reported in 2018, in Year 1, we completed the expression and purification of the recombinant LdNH36 parasite antigen. We also completed cloning and small-scale expression for the recombinant PpSp15 vector antigen. Moreover, a process for the production and purification was developed. In Year 2, we were tasked with demonstrating that the developed process was reproducible with low variance. To this avail, three reproducibility production and purification runs were conducted and characterized.

One vial of Research Seed Stock was used to inoculate 1 L of BMG medium in a 2 L shake flask and incubated overnight at 30°C with agitation at 250 +/- 5 rpm. After the OD_{600} reached a value of between 5 and 10, this seed culture was used to inoculate 4.5 L of sterile basal salt medium (BSM) in the fermenter, yielding a starting OD_{600} of 0.4. Prior to inoculation, PTM1 trace salts and 0.02% Biotin were added to the fermentation at 4.35 ml/L BSM, and 10% antifoam 204 was added at 1 ml/L. The pH of the BSM was adjusted to 5.0 ± 0.1 prior to inoculation by adding 14% NH_4OH.

Fermentation was conducted in a Celligen 310 Bioreactor with a 14 L vessel (Eppendorf), controlled by the Eppendorf Bio Command software. Cell expansion was continued at 30°C with a dissolved oxygen set point of 30%. Following the DO spike, a fed-batch was initiated with a 50% glycerol feed at 15 ml/L/hr for 5 hrs. During the last hour of the fed-batch phase, the pH was increased from 5.0 to 5.2, and the temperature was decreased from 30 to 26°C. After the fed-batch phase, the methanol induction was initiated. The 100% methanol included 12 ml/L PTM4 trace salts and 12 ml/L 0.02% biotin. A methanol feed was increased from 1 to 12 ml/L/hr over eight hours. After the methanol ramp, the feed rate was maintained at 12 ml/L/hr for approximately 21 hours, and then the methanol pump speed was increased from 12 to 14 ml/L/hr over a four-hour period to compensate for the added vessel volume. The total induction time was approximately 68-72 hours. Biomass was removed by centrifugation at 12,227 x g for 30 minutes at 4°C, and the supernatant was filtered through 0.45 µm Polyethylene sulfone (PES) filters. Approximately 5.5 L fermentation supernatant (FS) were stored at -80°C for about 2-4 weeks until purification could be performed.

Subtask 5: Activity 1.3.5. Three reproducibility runs of PpSP15 downstream purification

Three liters FS stored at -80 °C were thawed at 4°C over 2-3 days. The thawed FS was centrifuged at 12,227 x g for 20 minutes at 4°C to remove precipitation and then filtered with 0.22 µm PES filters prior to tangential flow filtration. The clarified fermentation supernatant was concentrated to approximately 1 L before diafiltration using two Pellicon® 3 Cassettes w/Ultracel (Millipore, 3K MWCO, 0.22 m^2 membrane area). PpSP15 was then buffer exchange into 0.1 M Potassium Phosphate at pH 6 for 3-4 diavolumes, until a target pH of 6.0 ± 0.1 and a target conductivity of 9.8 ± 0.6 ms/cm were achieved.

SP Sepharose XL chromatography

Cation exchange chromatography was used to capture the target protein and remove most of the host cell protein contaminants. SP Sepharose XL resin (GE healthcare) was packed into a Vantage L Column VL 44 x 250 (Millipore, column volume (CV) 226 ml, bed-height: 14.9 cm). The column was primed with 0.5 M NaCl and equilibrated with 0.1 M Potassium Phosphate at pH 6. The buffer exchanged fermentation supernatant was loaded onto an SPXL column at a flow rate of 38.5 mL/min (153 cm/h using AKTA Avant 150 (GE Healthcare)). After sample loading, the column was washed for 5 CVs with 0.1 M Potassium phosphate buffer at pH 6, and then eluted with 5 CVs of 30% elution buffer (0.1 M potassium phosphate pH 6.0, 1 M NaCl). The entire elution was collected as one pool and kept at 2-4°C before the next purification step.

Hydrophobic Interaction Chromatography

Phenyl Sepharose 6 Fast Flow high sub (PS6FF) chromatography was performed as a final polishing step to remove additional contaminants. PS6FF resin was packed into a HiScale 50/40 column (GE healthcare) to a column volume of 324 ml and a bed height of 16.5 cm. The column was primed with 0.5 M NaCl prior to use and equilibrated with 0.1 M potassium phosphate pH 6.0 containing 2 M ammonium sulfate.

The SPXL elution pool was mixed with ammonium sulfate to reach a target concentration of 2 M, filtered through a 0.22 µm PES filter, and then loaded onto the PS6FF column at a flow rate of 55.63 mL/min. A wash step was performed for 2 CVs with 0.1 M potassium phosphate pH 6.0 containing 2 M ammonium sulfate before the target was eluted with 50% of 0.1 M potassium phosphate pH 6.0. The elution was collected as one pool and stored at 2-4^{0}C before the next purification step.

The PS6FF elution pool was concentrated to approximately 0.8 L using two Pellicon® 3 Cassettes w/Ultracel (Millipore, 3K MWCO, 0.22 m^2 membrane area). PpSP15 was then dialyzed into 150 mM sodium citrate at pH 6.0, until a target pH of 6.0 ± 0.1 and a target conductivity of 23 ± 0.5 ms/cm were achieved.

Three purification runs (PDD031819/PDD050619/PDD052019) were successfully executed under the same conditions and with similar results. Three lots of purified PpSP15 were generated from three reproducibility runs: PpSP15-220319ZXL-1 (Run 1), PpSP15-100519ZXL-1 (Run 2) and PpSP15-240519ZXL-1 (Run 3). The results (yields, recovery and purity) are presented in the **Table 1**. The average yield was 0.65 g of PpSP15 per L of fermentation supernatant (determined by A280) and 0.75 g of PpSP15 per L of fermentation supernatant (determined by SDS-PAGE densitometry). Each 10 L run yielded 3 -4 grams of PpSP15. The final purity was 97% by SDS-PAGE and the final average recovery was 81%. All CV (coefficient of variation) values were below 10%.

Overall, the results indicated the process was reproducible as the purity and recovery for each in-process step showed comparable results with the previous process development runs.

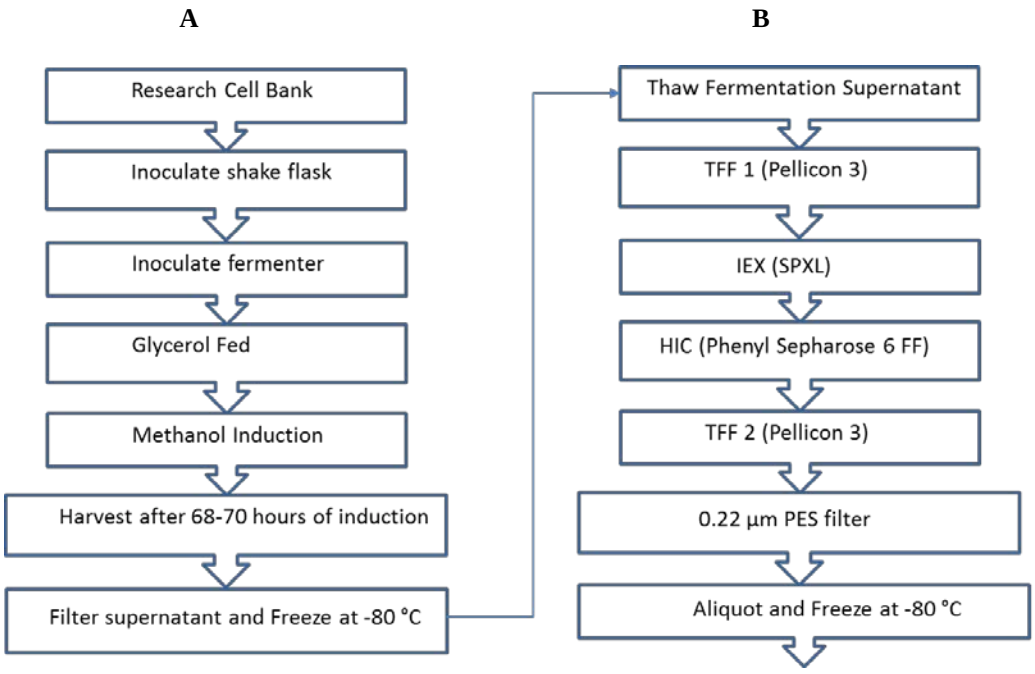


Figure 1: Process Schematic Overview. (A) Schematic of the fermentation process of PpSP15. (B) Schematic of the purification process of PpSP15.

Table 1: Mass Balance Report for the purification of PpSP15 from three 10 L fermentations in *P. pastoris.* CV: Coefficient of Variance, $\bar{X}$: Average, SD: Standard Deviation

	Yield (g/L of Fermentation Supernatant)						Recovery [%]						Purity assessed on reduced Bis-Tris Gels [%]						Purity assessed on non-reduced Bis-Tris Gels [%]					
Step	**Run 1**	**Run 2**	**Run 3**	**$\bar{X}$**	**SD**	**CV [%]**	**Run 1**	**Run 2**	**Run 3**	**SD**	**$\bar{X}$**	**CV [%]**	**Run 1**	**Run 2**	**Run 3**	**SD**	**$\bar{X}$**	**CV [%]**	**Run 1**	**Run 2**	**Run 3**	**SD**	**$\bar{X}$**	**CV [%]**
FS	0.91	0.92	0.94	0.92	0.01	1							75	64	71	70	5	7	90	91	85	89	3	3
TFF 1	0.89	0.92	0.87	0.89	0.02	2	99	99	93	97	3	3	81	77	76	78	2	3	91	93	91	92	1	1
SPXL	0.82	0.93	0.82	0.86	0.05	6	92	102	94	96	4	4	97	98	96	97	1	1	98	98	97	98	0	0
PS6FF	0.74	0.83	0.82	0.80	0.04	5	91	89	100	93	5	5	98	97	97	97	0	0	98	98	96	97	1	1
TFF 2	0.73	0.84	0.68	0.75	0.07	9	99	102	84	95	8	8	97	97	97	97	0	0	97	98	96	97	1	1
Total Process Recovery							81	91	72	81	8	10												

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.

We have developed a panel of biophysical and biochemical assays to characterize PpSP15 protein and monitor its long-term stability as well as its stability under duress (accelerated or short-term). The three lots of purified PpSP15 were visually clear, non-viscous, and colorless, with a pH of 5.97 ± 0.03 and a concentration of between 2.0 and 2.5 mg/mL (**Table 2**). After SDS-PAGE and staining with Coomassie blue, PpSP15 migrated as a single band with a molecular weight of approximately 16 kDa and a purity of approximately 96%. Other more sensitive and specific assays were also applied to study purity, including silver-stained SDS-PAGE (**Figure 2**) as well as anti-PpSP15 Western blotting (**Figure 3a**) and anti-*P. pastoris* X33 HCP Western blotting (**Figure 3b**). The results suggested that only trace amounts of HCP, product-derived impurities, or degraded PpSP15 were present in all three lots. RP-HPLC results indicated that the purity of the three lots of protein was consistent (96%). Finally, host cell protein slot blot analysis suggested that there was only a trace amount of yeast host-cell protein (<0.66%) in each lot. Collectively, therefore the data indicate that three independent runs yielded comparable lots with a coefficient of variation of less than 6% for all parameters tested.

Table 2. Characterization of PpSP15 from three reproducibility runs.

Test	**Run 1** PDD031819	**Run 2** PDD050619	**Run 3** PDD052019	**Mean**	**% CV**
Color and appearance	clear	clear	clear	-	-
pH	6.00 ± 0.01	5.93 ± 0.01	5.99 ± 0.03	5.97 ± 0.03	0.52%
Concentration, mg/mL	2.21	2.49	1.99	2.23 ± 0.20	9.18%
SDS-PAGE/Coomassie (Non-reduced)					
MW, kDa	15.0 ± 0.2	15.2 ± 0.4	16.3 ± 0.2	15.5 ± 0.6	3.69%
Purity, %	97.2 ± 0.4	93.5 ± 1.1	96.9 ± 0.2	95.9 ± 1.7	1.75%
SDS-PAGE/Coomassie (Reduced)					
MW, kDa	15.4 ± 0.8	15.6 ± 0.2	18.4 ± 0.8	16.5 ± 1.4	8.32%
Purity, %	97.1 ± 0.1	95.4 ± 0.8	96.8 ± 0.4	96.4 ± 0.7	0.77%
Purity by Reverse-phase HPLC, %	96.2 ± 0.02	97.3 ± 0.1	96.6 ± 0.1	96.7 ± 0.5	0.47%
Host cell protein slot blot, %	< 0.47	0.78	0.73	<0.66	-

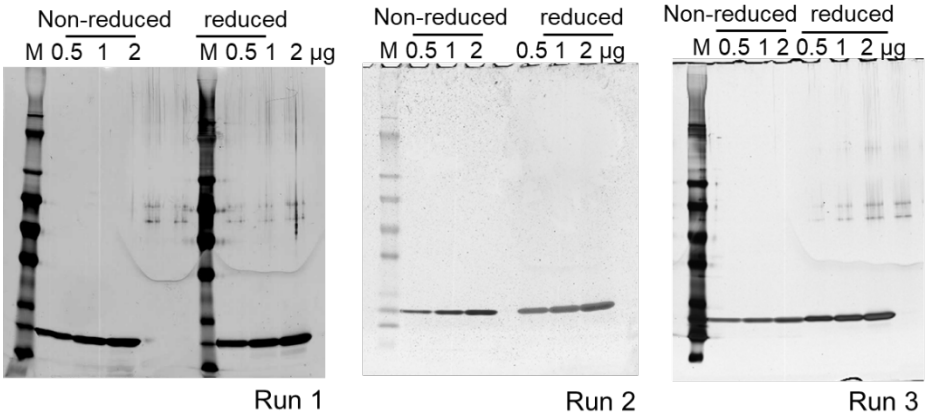


Figure 2. Silvered stained SDS-PAGE gel. Different amounts of purified PpSP15 from three reproducibility runs were loaded.

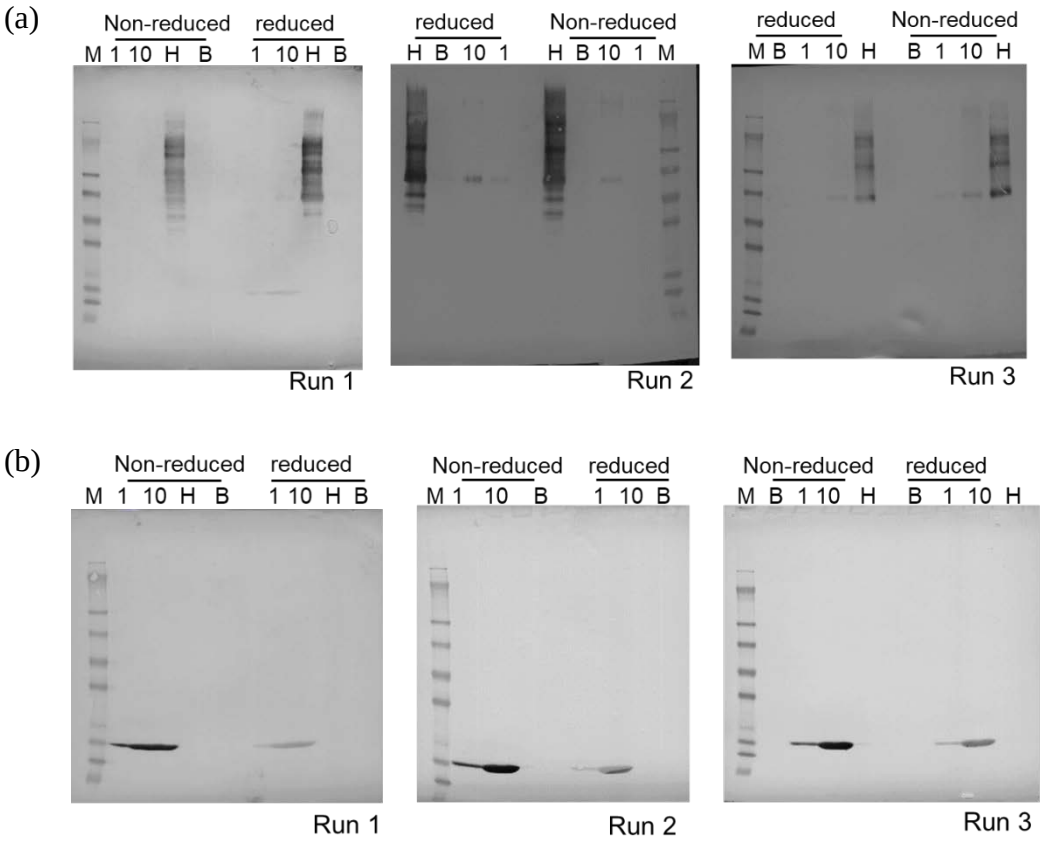
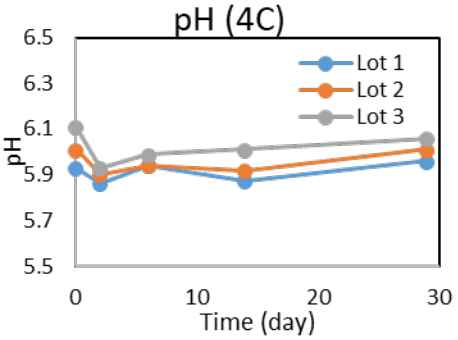


Figure 3. Western blot. Different amounts of PpSP15 from three different reproducibility runs were loaded on SDS-PAGE gels and transferred to PVDF membranes. The membranes were then blotted with (a) anti-PpSP15 antisera, or (b) anti-*P. pastoris* X33 antibodies. HCP (H) and BSA (B) were also included as control.

Accelerated stability of all three reproducibility lots, as measured by color and appearance, pH, UV spectrophotometry, SDS-PAGE, and RP-HPLC, was evaluated over approximately 30 days at 4°C, room temperature, and 37°C. All three lots remained visually clear, non-viscous, and colorless over approximately 30 days at 4°C, room temperature, and 37°C. The pH was stable at 5.85- 6.11 at all temperatures. No significant changes were observed over approximately 30 days on SDS-PAGE gels stained with Coomassie blue, and purity remained above 95%, except for lot 2, the purity dropped to approximately 94% when stored at 37°C for more than 15 days. The molecular weight of the intact antigen remained constant at approximately 16 kDa. With respect to RP-HPLC analysis, it was noted that there was a systemic RP-HPLC issue which caused an apparent purity drop to approximately 91-92% on day 2; moreover, very slight degradation at 37°C was detected in all lots by reverse-phase HPLC, with purity decreasing from 97-98 % to 93-94 %. (**Figure 4**).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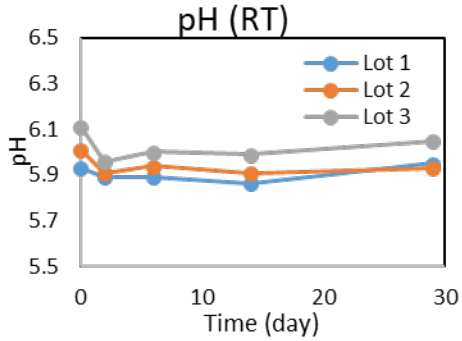
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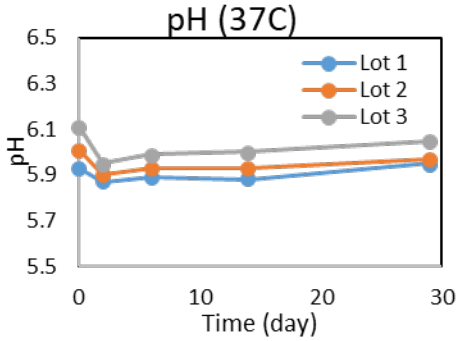
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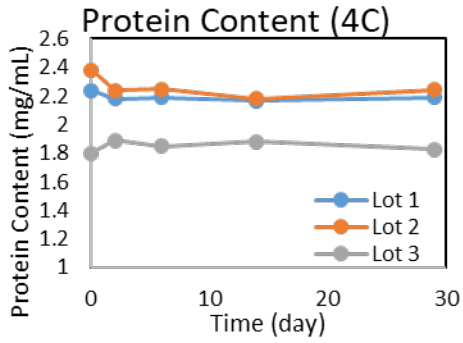
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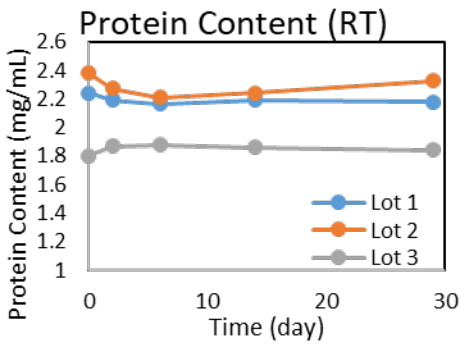
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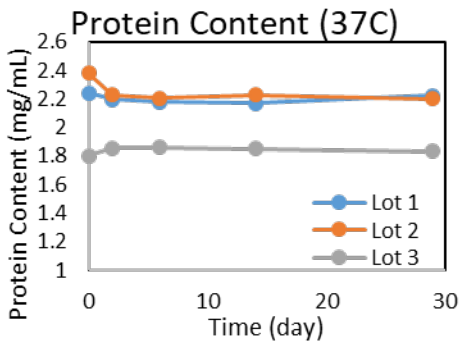
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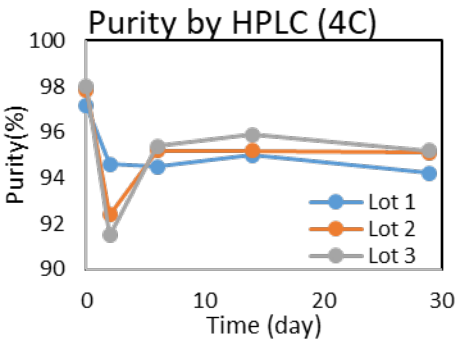
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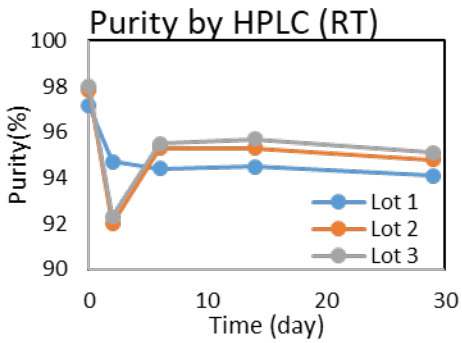
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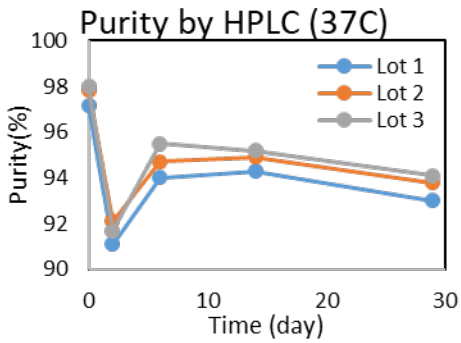
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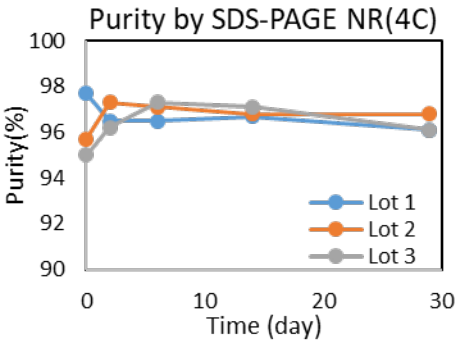
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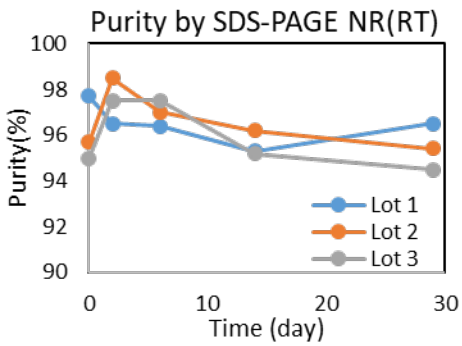
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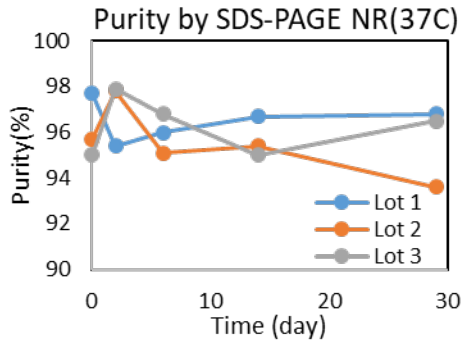
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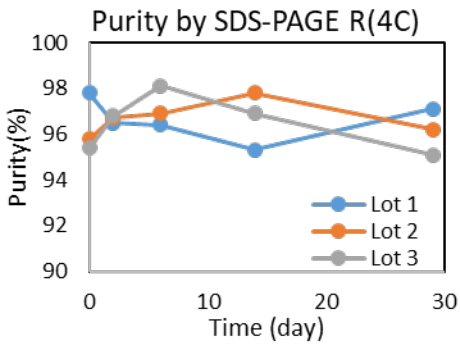
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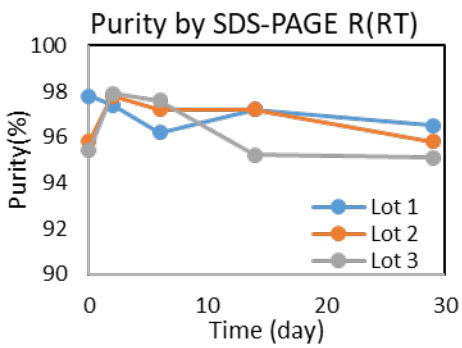
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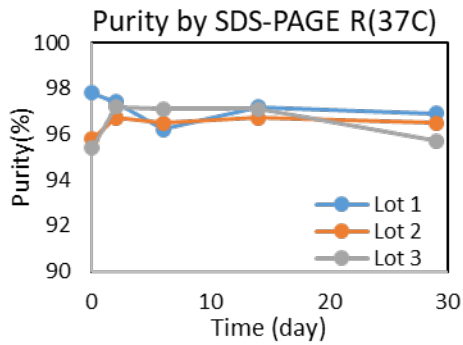
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Figure 4. Accelerated stability of PpSP15 from three reproducibility runs was performed at 4**°**C, room temperature (RT) and 37**°**C, and was monitored by different stability indicators, including pH, protein content by absorbance at 280 nm, purity on RP-HPLC, and purity on non-reduced (NR) SDS-PAGE and reduced (R) SDS-PAGE gels.

Collectively, the data indicate that the process for the yeast-expressed PpSP15 reproducibly yielded products with comparable analytical characteristics good stability throughout the accelerated stability study. The antigen is stable at 4°C, room temperature and 37°C for approximately 30 days with a very slight decrease in purity at 37°C.

Since all three lots shared similar characteristics and stability, the lot from run ID# PDD031819, lot# PpSP15220319ZXL-1 was selected as the reference standard due to its slightly higher purity and slightly better stability. This reference standard stored at -80°C is being tested for its long-term stability. So far, the protein has remained stable for at least 3 months.

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

The overall goal is to develop an effective vaccine that incorporates a vector salivary protein (PpSP15) in an anti-*Leishmania* (LdNH36) vaccine formulation.

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: During year 2 we addressed Major Task 8, testing the role of GLA-SE adjuvant in C57/B6 mice vaccinated with recombinant PpSP15 and LdNH36 (**Table 3**). We achieved the milestone of determining the need to add the TLR4 agonist GLA-SE for more potent immunogenicity of the combined antigen PpSP15 and LdNH36 immunization. From the experiments in year one we selected three doses and the route of administration that gave optimal cellular immune responses.

Table 3. *Study group design for determining the effect of GLA-SE adjuvant on the immune response in mice with LdNH36 and PpSP15*

	+ GLA-SE	-GLA-SE
5ug LdNH36/5ug PpSp15 SQ	5 mice	5 mice
5ug LdNH36/5ugPpSp15 ID	5 mice	5 mice
Prime 5ug LdNH36/0.5 ug PpSp15 SQ	5 mice	5 mice
Controls		
PBS ID	5 mice	
PBS SQ	5 mice	
GLA-SE ID	5 mice	
GLA-SE SQ	5 mice	

*2 μg GLA-SE were used

Immunizations were performed 3 times, at one-month intervals at the NIH Twinbrook vivarium. The injected antigen preparations were well tolerated by the mice. No adverse effects were seen. Mice were bled by tail nick pre-vaccine and then 3 weeks after each immunization to assess the humoral response to both antigens. Two weeks after the last immunization, mice were euthanized, bled by cardiac puncture, and the spleen was harvested.

We first checked if mice developed any immune response against the administered antigens. We showed very high levels of anti-LdNH36 or anti-PpSp15 total IgG with all the vaccination preparations given with GLA-SE (**Figure 5**). The IgG2 subset was > IgG1, suggesting a Th1 like response.

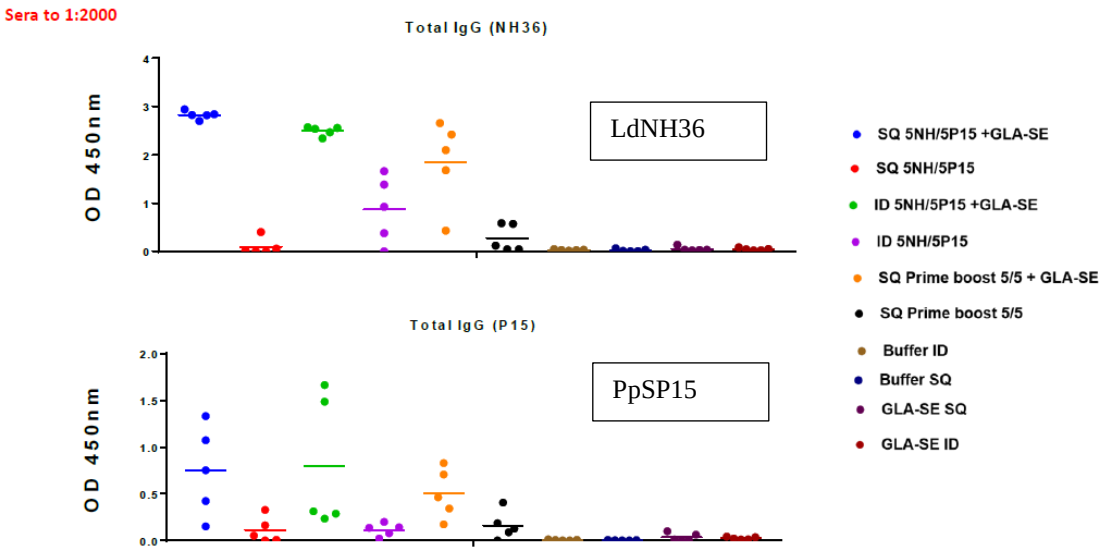

Figure 5. High levels of IgG were seen with all antigen combinations with GLA-SE while much lower responses were seen without GLA-SE. Note sera diluted 1:2,000.

The cellular immune response was assessed using antigen specific stimulated splenocytes, with supernatant studied by ELISA for interferon gamma production, Luminex for 17 cytokine profile, and flow cytometry (**Figure 6**). The below gating of CD4 shows increased interferon gamma production when GLA-SE is added to the vaccine (*vs.* vaccine without adjuvant in the lower panels). CD4 T cells were primarily responsible for interferon gamma release (**Figure 7**).

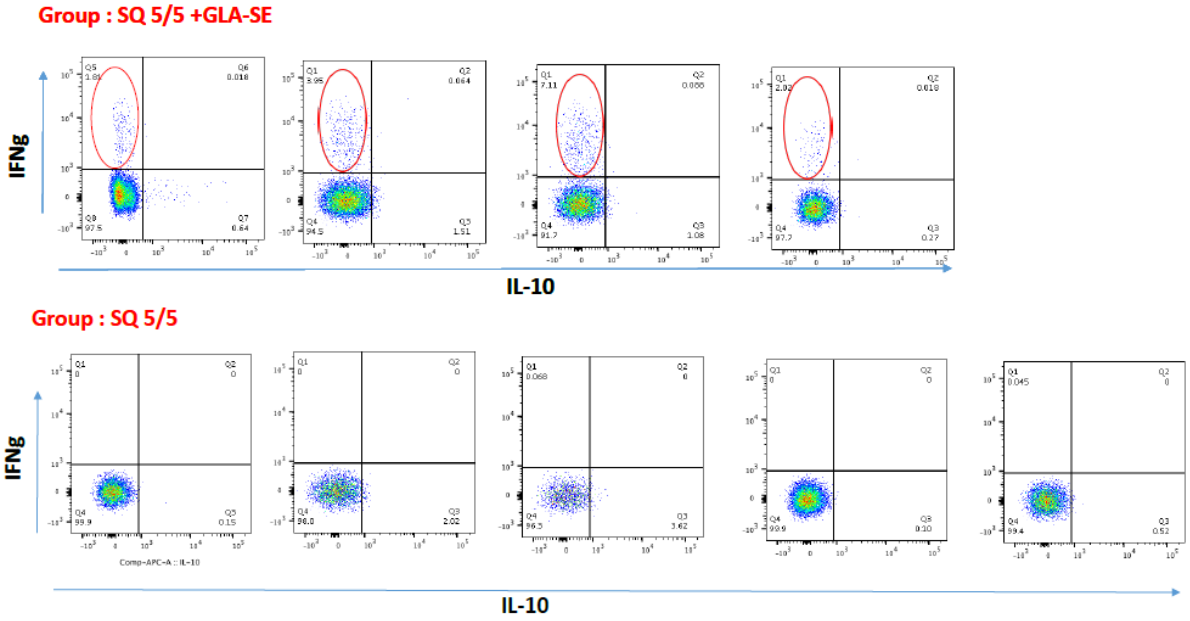

Figure 6. Interferon gamma and IL-10 production in CD4 T cells from the group vaccinated SQ with 5 µg LdNH36 and 5 µg PpSP15 +/- GLA-SE.

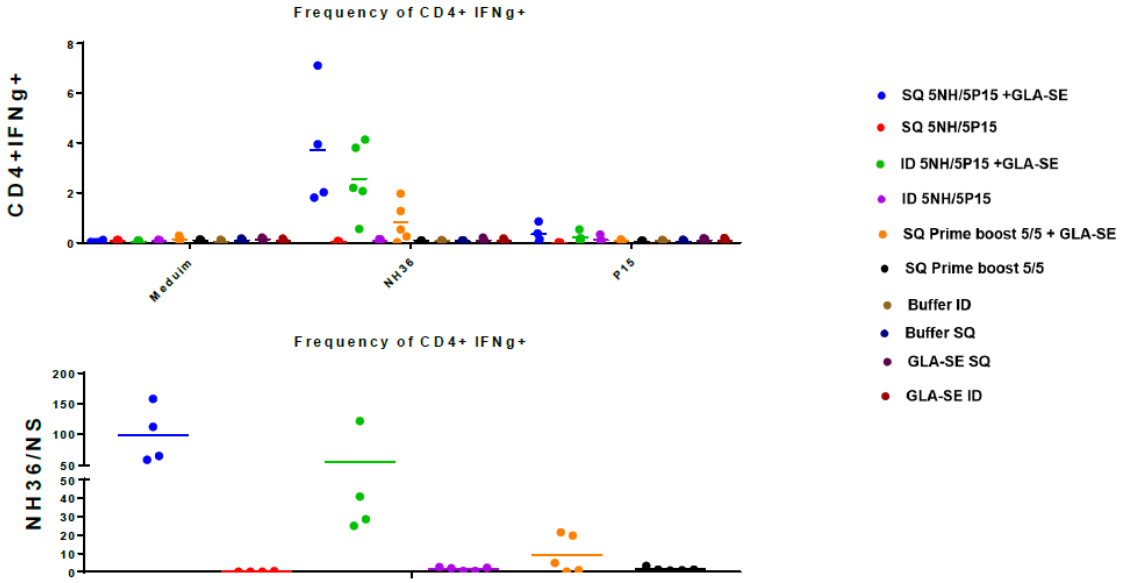

Figure 7. In the presence of GLA-SE, antigen combination vaccination induces high proportion of CD4+ IFNγ cells

We further assessed cellular immune function using Luminex to assess for 17 chemo- and cytokines (**Figure 8**). LdNH36 was associated with higher levels of cytokines, especially IFNγ, compared to PpSP15 antigen stimulation. Only the GLA-SE group data is shown here because it was consistently higher than antigen without adjuvant.

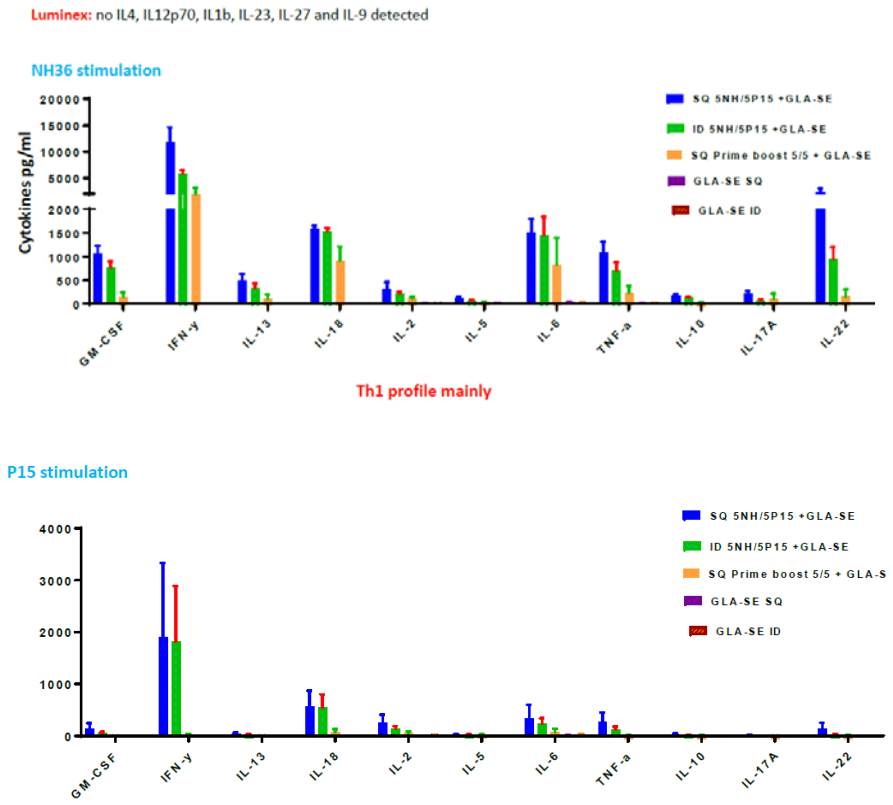

Figure 8. Luminex data: profile of the cellular immune response induced by antigens LdNH36 combined with PpSP15 vaccination.

In the next experiment we selected the SQ (blue in above graphs) 5 µg LdNH36 + 5 µg PpSP15 vaccination and compared it to the individual antigens with/without GLA-SE. The mice were immunized as above (**Table 4**), but this experiment was done in the USUHS vivarium. No adverse events were noted.

Table 4: Comparison of combined versus single antigens +/- GAL-SE

	+GLA (2ug)	-GLA
NH	5ug	5ug
P15	5ug	5ug
NH+P15	5+5	5+5
GLA	2ug	
Buffer	citrate	

As in prior experiments IgG2> IgG1 isotype in this experiment (**Figure 9**).

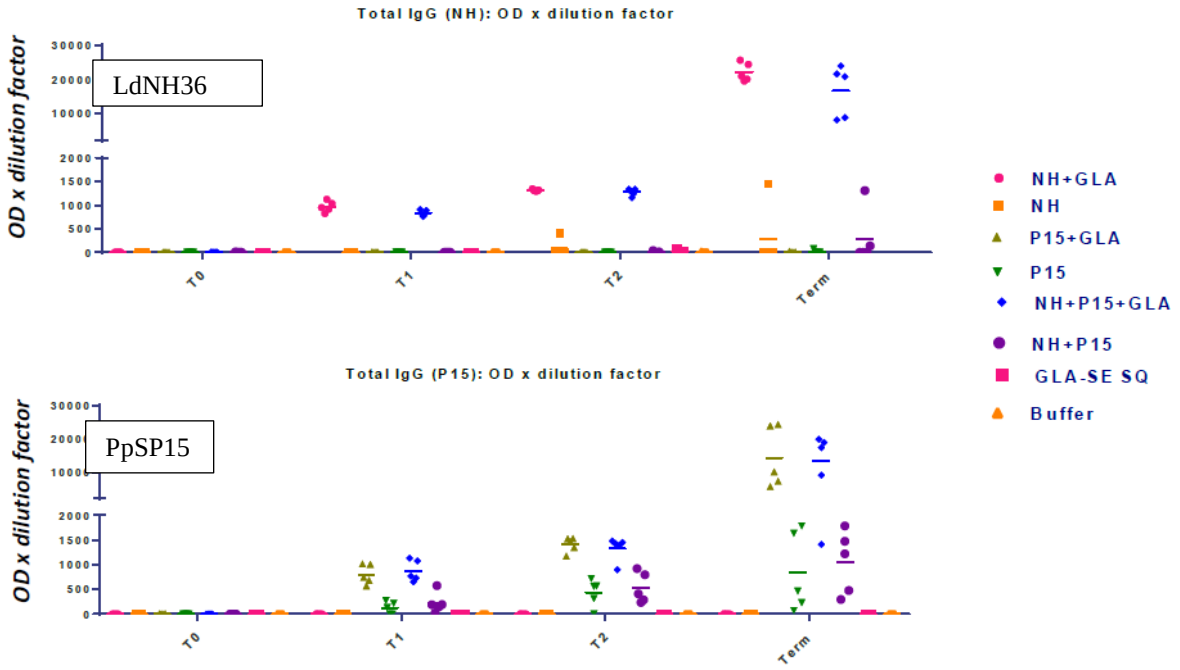

Figure 9. Antigen combinations + GLA associated with higher levels of IgG (ELISA)

Cellular immune responses were measured by IFNγ ELISA and Luminex (**Figure 10**). Of note, particularly high levels of IFNγ were seen with GLA-SE adjuvant and LdNH36.

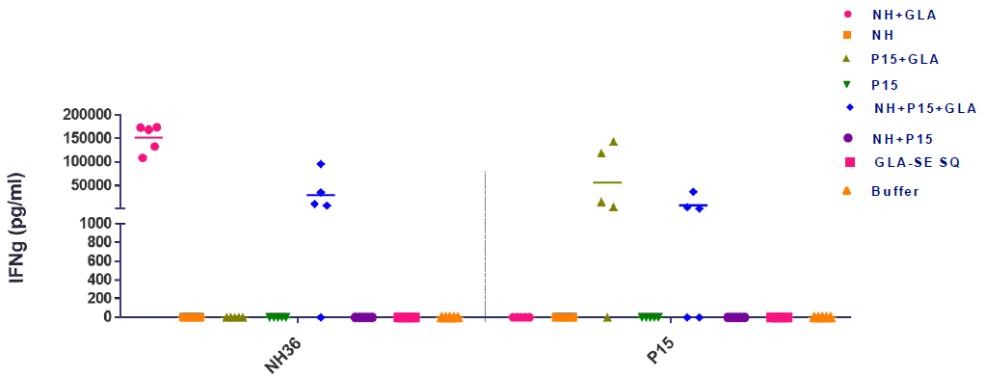

Figure 10. IFN gamma levels measured from antigen stimulated splenocyte culture supernatants. LdNH36 on left, PpSP15 antigen on right of figure.

Profiling the cellular immune response using Luminex (**Figure 11**), a predominantly Th1 type response is noted, with some suggestion that the combination antigen is actually inhibitory and that the LdNH36 antigen has the larger effect on this immune response. This will need to be assessed further in the context of the *Leishmania* challenge experiments.

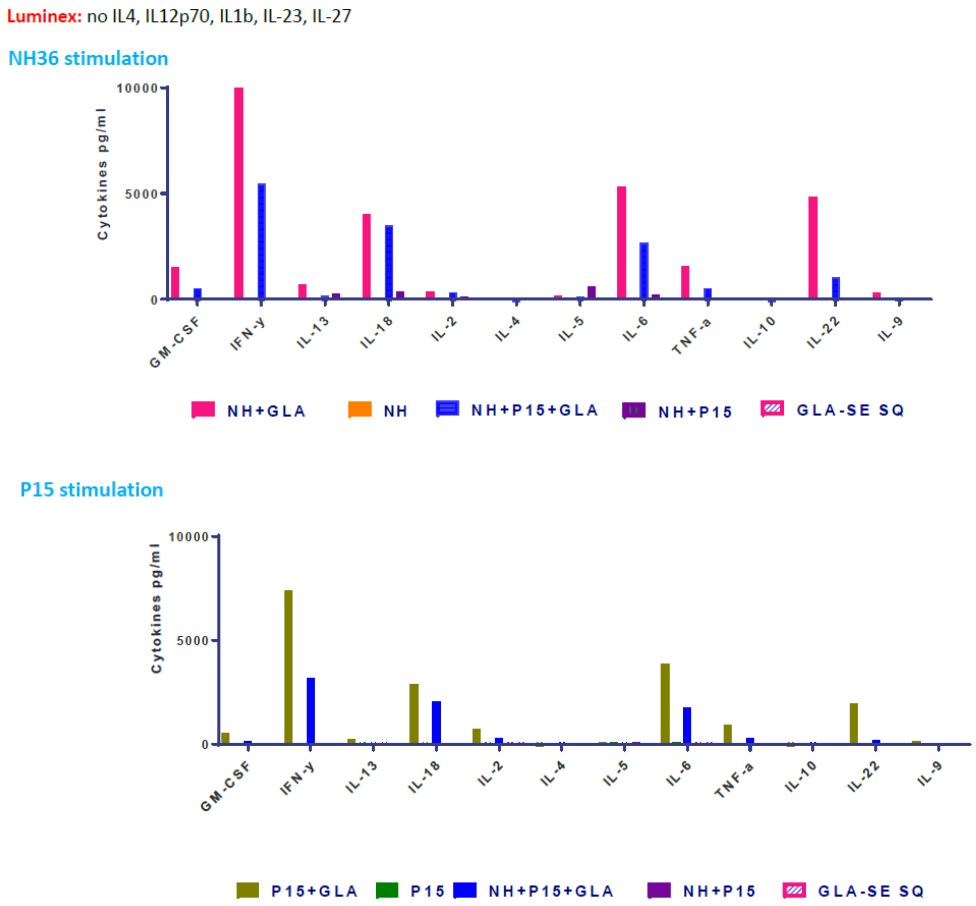

Figure 11. Cytokine profiles from antigen stimulated splenocytes show that GLA-SE is consistently associated with higher levels as is NH36 antigen. Surprisingly the combination antigens plus GLA-SE (blue in both graphs) performed less well than antigen alone plus GLA-SE.

In summary, we found this 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. Given that the salivary antigen (PpSP15) is best tested with sand fly saliva, we are awaiting the results of Specific Aim 3 where we plan to also look at the antigens separately as well as in combination. In addition, we are working to develop an ADCC assay to further elucidate that immune response to these antigens.

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.

The Vector Molecular Biology Section (VMBS) at The National Institute of Allergy and Infectious Diseases (NIAID), U.S. National Institutes of Health (NIH) will collaborate with USUHS to ensure technical consistency is maintained throughout the project. Animals at NIAID will be immunized with both antigens (produced in Aim 1) using the best dose and route of immunization determined in Aim 2. The VMBS will 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 will also update the infrastructure necessary for the infection of *P. papatasi* and maintain a virulent parasite stock for the natural challenge of the vaccinated mice. Three to four weeks after the last immunization, control and vaccinated mice will be challenged with *L. major*-infected sand flies. Animals will be followed-up for cutaneous lesion development

at the site of bites and analyzed at specific time points to determine the parasite burden and the immunological parameters related to development of protective immunity or disease.

Major Task 9, Activity 3.1: Update the insectary facility to have a high capacity Phlebotomus papatasi *sand fly colony for transmission experiments.*

We secured a second sand fly insectary chamber to grow the sand fly *P. papatasi*. We further secured four insectary incubators to grow the sand fly larvae in the new insectary. Moreover, we hired personnel to be dedicated to grow sand flies in the LMVR/NIAID insectary.

With those factors in place, we have now started to expand the sand fly, *P. papatasi*, colony. We started with less than 100 sand flies per week and are now producing about 1,500 sand flies per week.

The immune response generated at 3-4 weeks after the final immunization and 3 weeks after challenge with infected sand flies was determined using measurement of antigen specific IgG by ELISA.

<u>Round 1:</u> For round 1 of the project, antigen specific IgG was measured via ELISA 4 weeks after the final immunization and confirmed that immunized SP15/NH36/GLA-SE mice had higher specific antibody levels when compared to SP15/NH36 and naïve mice (**Figure 12**). Unfortunately, the challenge of vaccinated mice using *L. major* infected sand flies could not be completed due to poor *P. papatasi* infections (**Figure 13**). For a successful sand fly transmission, *P. papatasi* infections need to reach a minimum threshold of 75% metacyclic promastigotes prior to transmission.

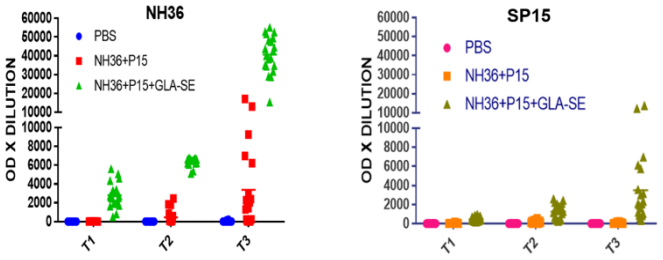

Figure 12. Results of antigen specific ELISA measuring IgG levels for weeks 3, 7, and 12 post- immunization. Comparison of groups immunized with NH36/SP15/GLA-SE, NH36/SP15, or naïve mice (PBS) against NH36 or PpSP15. T1 = Week 3, T2 =Week 7, T3 = Week 12.

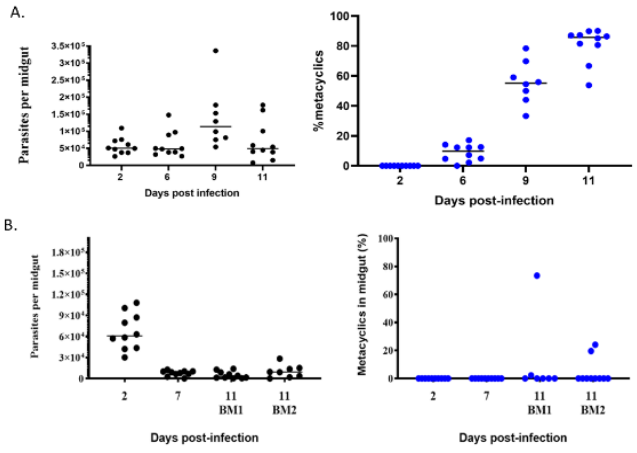

Figure 13. A. Representative transmissible infection of *L. longipalpis/L. major* with parasites reaching 80% metacyclic promastigote threshold 11 days post-infection. B. Representative poor Infection *L. longipalpis/L. major* with parasites not reaching the 80% metacyclic threshold by day 11 even with a second blood meal to boost the infection.

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 14A**). Using transgenic parasites is a

valuable technique to better visualize the course of disease and protection (**Figure 14B**) and may be employed in future studies for this project.

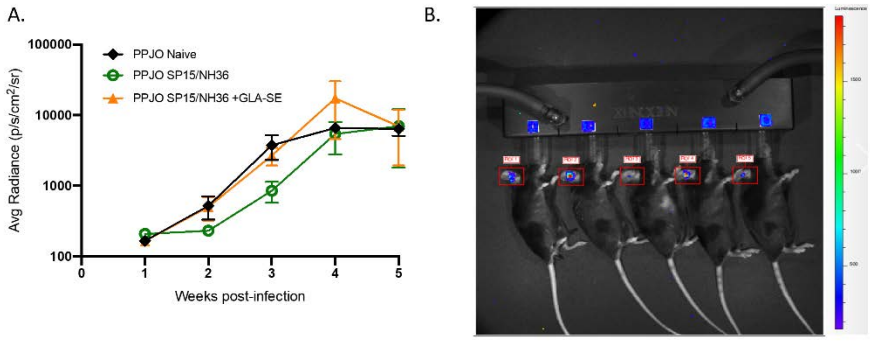

Figure 14 A. Parasite burden post-needle challenge measured via bioluminescence for 5 weeks. SP15/NH36/GLA-SE, SP15/NH36, or naïve mice were challenged by needle with *P. papatasi* (PPJO) salivary gland homogenate and 1000 (*L. major (Iraq) R-Fluc+tdt*$^{+}$) metacyclic promastigotes. **B.** Respective 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 15**). Immunological assessment of Th1/Th2 response at 3-week post-challenge from the draining lymph nodes and spleen and cytokine transcripts will be performed in round 2.

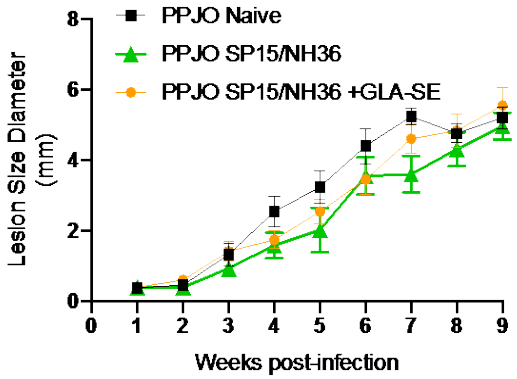

Figure 15. Lesion size diameter for immunized SP15/NH36/GLA-SE, SP15/NH36, or naïve mice challenged by needle with *P. papatasi* (PPJO) salivary gland homogenate and 1000 *L. major* metacyclic promastigotes. Lesions were followed for a period of 9 weeks.

Round 2: For round 2, we have completed the three immunizations and have collected serum for week 3 and week 7. ELISAs to detect specific levels of IgG for weeks 3 and 7 are being performed at USUHS, and we are currently waiting to collect serum for week 12. As originally intended, four weeks after the last immunnization, immunized and naïve mice will be challenged using the natural model *P. papatasi* sand flies infected with *L. major*. 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 (**Figure 16**). We have now reached the minimum threshold of 75% metacyclic promastigotes and can move forward with the proposed plan for challenge. The new conditions

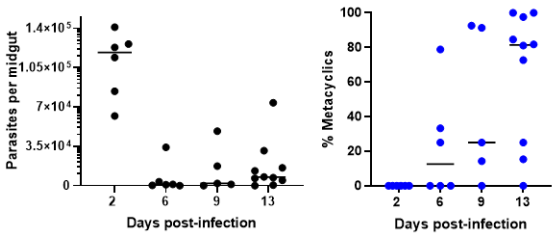

Figure 16. Representative transmissible infection of *P. papatasi / L. major* after troubleshooting various conditions. Now 80% metacyclic promastigote threshold is reached by 13 days post-infection.

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.

Activity 3.4. Evaluation of the efficacy of PpSP15 and LdNH36 in protecting C57Bl/6 mice against sand fly transmitted leishmaniasis. Immunized animals will be challenged by NIAID in one ear with *L. major*-infected *P. papatasi* sand flies to determine the protective efficacy of this novel vaccine. Development of cutaneous lesions at the site of bites representing disease progression will be monitored bi-weekly for up to 4 months. Three to four weeks following challenge with infected sand flies and at the study endpoint, the parasite burden will be assessed in the ears and draining lymph node by RT-PCR and limiting dilution assay, respectively.

Round 1: After challenge, we completed weekly ear thickness and ear lesion measurements until week 9 using a digital caliper (Figure 3). Analysis of the parasite burden for each group is currently in progress and will be measured via limiting dilution assays.

Round 2: Mice have been immunized three times and mice will be challenged in one ear with *L. major*-infected *P. papatasi* sand flies to determine the protective efficacy of the vaccine. Ear thickness and ear lesion diameter will be measured on a weekly basis for up to 4 months. Three to four weeks following challenge with infected sand flies and at the study endpoint, parasite burden will be assessed in the ears and draining lymph node by RT-PCR and limiting dilution assay, respectively.

Activity 3.4.1. Exposure of vaccinated animals to *L. major* infected sand flies and follow up of lesion development. Up to ten *L. major*-infected sand flies will be allowed to bite mice immunized with PpSP15 and LdNH36 or control vaccine. Mice will be anesthetized, and individual ears will be 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 will be placed in the vial. They will be allowed to feed for up to two hours, in the dark. Animals will be checked at least once every 30 minutes, and the feeding rate of applied flies will be determined. Measurements of potential cutaneous lesions due to Leishmania major will be obtained using a Vernier caliper (Mitutoyo, Baltimore, MD). Lesion size will be measured weekly as the diameter of the skin lesion. Potential lesions will be photographed. For Parasite load, total genomic DNA will be extracted from mice ears using the DNeasy tissue kit. A total of 100 ng will be amplified by real time PCR using primers JW11 and JW12 and 18S primers as a housekeeping gene with the FastStart Sybr green I kit (Roche). Expression levels will be normalized to 18S DNA and corrected for the weight of the whole ear. **Deliverable**: Having determined the protective efficacy of PpSP15 as a vaccine against cutaneous leishmaniasis following vector-challenge.

Round 1: Challenge with *L. major* infected sand flies could not be completed due to poor infections. Instead, we executed ear intradermal injections (n=10 per group) with 1,000 purified *L. major* metacyclic promastigotes and one pair of *P. papatasi* SGH using a 27-gauge needle. Weekly lesion measurements by size diameter and representative images of immunized and control group at week six are shown in **Figure 17**. Parasite load limited dilution assay (LDA) is currently in process.

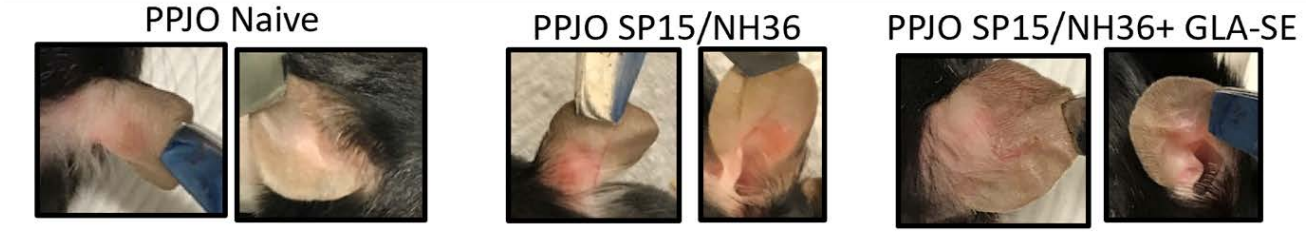

Figure 17. Representative images of ear lesions from immunized SP15/NH36/GLA-SE, SP15/NH36, or naïve mice that were needle challenged with *P. papatasi* (PPJO) salivary gland homogenate and 1000

Round 2: Moving forward for round 2 of the project, we have troubleshooted various conditions for *P. papatasi* infections and have found favorable conditions that should allow us to transmit using the sand flies. We have now reached the minimum threshold of 75% metacyclic promastigotes and can move forward with the proposed plan for challenge. After challenge we will complete weekly lesion size diameter measurements and will photograph lesions. We will continue the rest of the experiments as previously planned.

Activity 3.5. Immunological assessments post-infection. This activity will be focused in the characterization of the correlates of protection of vaccinated or control animals post sand fly challenge. Humoral and cellular immune responses will be measured in protected and non-protected mice.

Round 1: For round 1 of the project, we measured antigen specific IgG via ELISA four weeks after the final immunization and confirmed that immunized SP15/NH36/GLA-SE mice had higher specific antigen levels when compared to SP15/NH36 immunized mice and naïve mice (**Figure 12**).

Round 2: For round 2, we have completed the three immunizations and have collected serum for week three and week seven from all groups. We are running ELISAs to detect levels of IgG for these weeks and we are currently waiting to collect serum for week 12 to test IgG levels as well. After the last serum collection (week 12), we will measure cellular immune responses from ear and spleen by flow cytometry in immunized/challenged mice.

Activity 3.5.1. Antibody measurements of vaccinated animals after infected sand fly challenge. Antibody measurements will be performed by ELISA. Microtiter plates will be coated with recombinant PpSP15 (2 μg/ml) or LdNH36 (2 μg/ml) overnight at 4 °C. Plates will be blocked with Tris-buffered saline, 4% bovine serum albumin, 0.05% Tween 20 for 2 hours. Sera will be diluted (1:100) and incubated for 2 hours at 37°C. After washing, plates will be incubated with alkaline phosphatase–conjugated anti-mouse IgG (Promega, Madison, WI), IgG1 (BD Biosciences, Sparks, MD), or IgG2a (BD Biosciences) antibody (1/1,000). Binding will be detected using alkaline phosphate substrate (Promega). Absorbance will be recorded at 405 nm in a microtiter plate reader (Molecular Devices).

Round 1: Total IgG levels were measured from all groups post-immunizations.

Round 2: ELISA against IgG, IgG1, and IgG2a for weeks three and seven post-immunizations are currently being processed. Furthermore, sera from immunized mice will be collected post-challenge to assess the humoral immune response.

Activity 3.5.2. Ear tissue preparation. Mouse ear tissue will be processed to test for immunological parameters. For *ex vivo* experiments, 2 million cells will be cultured for 4 hours with Brefeldin A (BD Golgi Plug; BD Pharmingen, Sparks, MD). For experiments involving overnight stimulation, 2 million cells will be cultured with bone marrow–derived dendritic cells with or without PpSP15 (4 μg/ml), LdNH36 (4 μg/ml) or soluble *Leishmania* antigen (100 μg/ml) at 37°C and 5% CO2 for 18 hours. Brefeldin A will be added during the last 4 hours of culture. Cells will be harvested to test in various immunological assays. **Deliverable**: Having determined the correlates of protection for sand fly-transmitted *L. major* infection in PpSP15-immunized animals.

Round 1: Mouse ears (n=10) were collected from immunized mice and naïve group at week 9 post-challenged. Then, single cell suspensions were processed and analyzed by flow cytometry analysis. Briefly, cells were stimulated overnight with 100 μg/ml of soluble *Leishmania* antigen, followed by PMA/ION and Brefeldin A for the last 4 h of culture. Parasite load by LDA is still underway.

Round 2: Ear tissue will be collected four weeks after the last immunization, and at weeks 2 and 6 post-challenge. Cytokine levels will be measured via Luminex, flow cytometry, and parasite load via LDA will be measured.

Activity 3.5.3. Flow cytometry. The following antibodies will be used for cell staining: PerCP or FITC-labeled anti-CD4 (RM4-5 and GK1.5), PerCP-labeled anti-CD8 (53-6.7), antigen-presenting cell-labeled anti-TCR-β (H57-597), FITC-labeled anti-IFN-γ (XMG 1.2), and phycoerythrin-labeled anti-tumor necrosis factor-α (MP6-XT22). A minimum of 100,000 cells will be acquired using a FACSCalibur cytometer (BD Biosciences). Data will be analyzed with the Flow Jo software version 9.4.10. **Deliverable**: Having determined the correlates of protection for sand fly-transmitted *L. major* infection in PpSP15-immunized animals.

Round 1: Gating strategy for flow cytometry analysis of immunized mice or naïve group at 9-weeks post-challenge (**Figure 18**). Mouse ears were collected, and ear lysates were macerated using Medimachines (BD biosciences). Briefly, cells were stimulated overnight with 100 µg/ml of soluble *Leishmania* antigen, followed by PMA/ION and Brefeldin A for the last 4 h of culture. Single cell suspensions were then incubated with anti-mouse CD16/32 (TruStain FcX, clone 93; Biolegend), CD4 (GK1.5), anti-CD8 (53-6.7), anti-TCR-β (H57-597), IFN-γ (XMG 1.2), IL-10 (JE55-16E3), and IL-4 (11B11) for 30 min. Dead cells were excluded by staining LIVE/DEAD fixable yellow dead cell stain kit (Thermo Fisher Scientific). A minimum of 100,000 events were acquired by MACSQuant flow cytometer (Miltenyi Biotec) and analyzed by FlowJo software. Flow analysis indicates high secretion IFNγ and low secretion of IL-10 and IL-4 by $CD4^+$ T-Cells. These results are indicative of a Th1 dominant response.

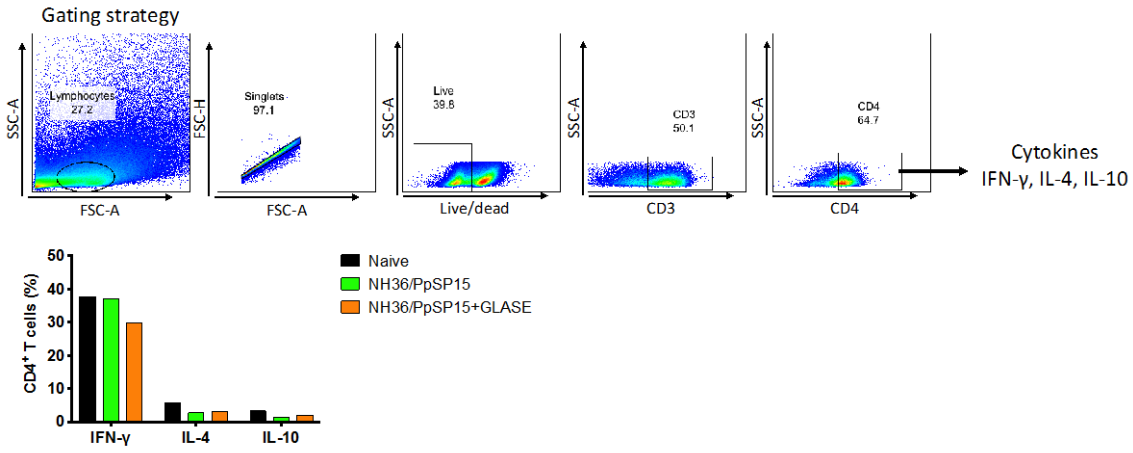

Figure 18. Gating strategy and frequency (%) of $CD4^+$ T cells secreting IFNγ, IL-4 and IL-10 from immunized SP15/NH36/GLA-SE, SP15/NH36, or naïve mice at 9-weeks post-challenge.

Round 2: Flow cytometry will be completed on whole ear tissue and splenocytes four weeks after the last immunization, and at weeks 2 and 6 post-challenge.

Activity 3.5.4. *In vitro* stimulation of spleen cells and cytokine ELISA. Spleen cells (5 × 106ml–1) of vaccinated or control mice will be cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 5×10^{-5} M 2-mercaptoethanol in the presence of 20 µg/ml of PpSP15 or LdNH36. Cells will be incubated at 37°C with 5% CO2 for 72 hours. Supernatants will be collected and analyzed by ELISA. After 72 hours of stimulation with recombinant antigens, IFN-γ, IL-10, and IL-4 levels were measured in supernatants using specific sandwich ELISA (BD Biosciences). **Deliverable**: Having determined the correlates of protection for sand fly-transmitted *L. major* infection in PpSP15-immunized animals.

Round 2: *In vitro* stimulation of splenocytes from of vaccinated or control mice (n=15 per group) will be processed 4 weeks after the last immunization and at weeks 2 and 6 post-sand fly challenge. Cells will be cultured in the presence of 20 µg/ml of PpSP15 or LdNH36, or 100 µg/ml of soluble *Leishmania* antigen,

respectively. Supernatant will be collected after 72 hrs and a panel of 11 Th1/Th2/Th17 cytokines will be analyzed via Luminex.

Potential problem areas and alternative methods and approaches:

One potential problem is that *P. papatasi* sand flies may not achieve a threshold of infectivity to be used to challenge vaccinated animals. This happens rarely in our laboratory and in other laboratories, however, this is something we have previously experienced. For a successful sand fly transmission, *P. papatasi* infections need to reach a minimum threshold of 75% metacyclic promastigotes and more than 10, 000 parasites in the gut of the insect prior to transmission, if this threshold is not achieved an efficient transmission will not occur. An alternative method is to perform a needle challenge using *L. major* parasites in combination with *P. papatasi* salivary gland homogenate. We have successfully used this approach as it mimics sand fly transmitted *Leishmania* infection.

To help de-risk investment in the proposed project, cGMP manufacture of the recombinant protein antigens and the animal toxicology testing will be delayed until adequate preclinical project results are obtained and completed. A subsequent application may include these activities, as well as a Phase 1 first-in-humans clinical trial at either the USUHS Clinical Research Unit or the Walter Reed Army Institute of Research (WRAIR) Clinical Trial Center to determine a safe, non-reactogenic dose of the vaccine in healthy human volunteers.

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. **How were the results disseminated to communities of interest?**

Beyond the scientific papers, 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):

1: Bailey F, Mondragon-Shem K, Haines LR, Olabi A, Alorfi A, Ruiz-Postigo JA, Alvar J, Hotez P, Adams ER, Vélez ID, Al-Salem W, Eaton J, Acosta-Serrano Á, Molyneux DH. Cutaneous leishmaniasis and co-morbid major depressive disorder: A systematic review with burden estimates. PLoS Negl Trop Dis. 2019 Feb 25;13(2):e0007092. doi: 10.1371/journal.pntd.0007092. eCollection 2019 Feb. PubMed PMID: 30802261; PubMed Central PMCID: PMC6405174.

2. Hotez PJ. Immunizations and vaccines: a decade of successes and reversals, and a call for 'vaccine diplomacy'. Int Health. 2019 Sep 2;11(5):331-333. doi: 10.1093/inthealth/ihz024.

3: Rees CA, Hotez PJ, Monuteaux MC, Niescierenko M, Bourgeois FT. Neglected tropical diseases in children: An assessment of gaps in research prioritization. PLoS Negl Trop Dis. 2019 Jan 29;13(1):e0007111. doi: 10.1371/journal.pntd.0007111. eCollection 2019 Jan. PubMed PMID: 30695020; PubMed Central PMCID: PMC6368333.

4: Hotez PJ. The rise of leishmaniasis in the twenty-first century. Trans R Soc Trop Med Hyg 2018 Sep 1;112(9):421-422. doi: 10.1093/trstmh/try075.PMID: 30239944

Press outreach. We conducted several interviews for leading websites and newspapers on neglected tropical diseases and vaccine diplomacy, including an interview with NATURE Middle East on the emergence of leishmaniasis among refugees fleeing conflict zones. https://www.natureasia.com/en/nmiddleeast/article/10.1038/nmiddleeast.2019.74. Dr. Hotez, in his role as a member of the Board of Governors of the US Israel Binational Science Foundation with the US State Department, has also highlighted the importance of the leishmaniasis vaccine in the Middle East and Central Asia.

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 and plenaries at the following outlets:

1. Pediatric Infectious Disease Society (PIDS, Wash, DC)
2. Clinical Immunology Society (Atlanta, GA)
3. APIC (Association of Professionals in Infection Control), Minneapolis MN
4. ASTMH Pre-meeting course on leishmaniasis
5. World Health Assembly (Geneva, Switzerland)
6. World Vaccine and Immunotherapy Congress (SF)
7. Mexican Pediatric Society (Monterrey)
8. London School of Hygiene and Tropical Medicine
9. Univ Alabama Birmingham (UAB) AOA Lecture
10. Wistar Institute Philadelphia PA
11. Hospital for Sick Children, University of Toronto
12. University of Oklahoma (Norman OK)
13. University of Massachusetts Amherst
14. Columbia University (New York, NY)
15. University of California Berkeley (California)
16. Univ Vermont, Larner College of Medicine (Burlington VT)
17. Northeastern University (Boston, MA)

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

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 are developing an ADCC assay to further assess the immune responses given the high levels of IgG.

Under Major Task 9, 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 8000 sand flies per week. We have also trained personnel to dissect sand fly salivary glands to be used for this study. 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 75% metacyclic promastigotes are needed in the insect to efficiently transmit the *Leishmania* parasite to animals. The new conditions for transmissible infection include: 5x107 promastigotes/mL, rabbit blood, not heparin treated, and heat inactivated, with 1 mg/mL soybean trypsin inhibitor and no antibiotics in the bloodmeal. We will use this threshold and the new conditions in the next set of experiments.

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 discussed in last annual report USUHS moved its second experiment to the NIH Twinbrook vivarium in Fall 2018 (due to a parvovirus outbreak), then in spring 2019 we conducted a third experiment here in the USUHS vivarium. All studies went smoothly.

Actual or anticipated problems or delays and actions or plans to resolve them.

Unfortunately, the challenge of vaccinated mice using *L. major* infected sand flies could not be completed due to poor *P. papatasi* infections. For a successful sand fly transmission, *P. papatasi* infections need to

reach a minimum threshold of 75% 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 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 Leishmania infection.

Briefly, immunized mice were challenged intradermally in the ear with 1000 purified L. major metacyclic promastigotes expressing luciferase and TdTomato (*L. major* (Iraq) R-Fluc+tdt^{+}) and 1 pair of *P. papatasi* salivary gland homogenate (SGH). Of note, 1,000 purified metacyclic promastigotes have been routinely used in lab to mimic natural infection and observe protection in other vaccine candidates. Additionally, we hoped using transgenic parasites would provide a visual reference of parasite burden and corroborate bioluminescence data to lesion size diameter.

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

7. Participants & Other Collaborating Organizations

Uniformed Services University of the Health Sciences	
Name:	*Ines Elakhal Naouar*
Project Role:	*Associate Investigator, Junior Scientist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*5*
Contribution to Project:	*Dr. Elakhal Naouar has performed all the mouse vaccination and immunogenicity experiments in Task 7.*
Funding Support:	
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:	*6*
Contribution to Project:	*Ms. Nurmukhambetova assisted Dr. Elakhal Naouar with mouse work and 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):	*https://orcid.org/0000-0002-5589-9450*
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):	*https://orcid.org/0000-0001-8770-1042*
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):	*https://orcid.org/0000-0002-8429-0476*
Nearest person month worked:	*1*
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:	*Coreen Beaumier*
Project Role:	*Assistant Professor*
Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0002-7095-6214*
Nearest person month worked:	*0.2*
Contribution to Project:	*Dr. Beaumier served as the project manager for part of this project.*
Funding Support:	
Name:	*Jeroen Pollet*
Project Role:	*Director of Formulation*
Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0003-1420-4015*
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):	*https://orcid.org/0000-0002-6884-9452*
Nearest person month worked:	*0.6*
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):	*https://orcid.org/0000-0001-9455-7683*
Nearest person month worked:	*0.3*
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:	*C. Patrick McAtee*
Project Role:	*Director, Upstream Development*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.2*

Contribution to Project:	*Dr. McAtee conducted the initial antigen purification.*
Funding Support:	
Name:	*Zhuyun Liu*
Project Role:	*Director, Downstream processing*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.8*
Contribution to Project:	*Ms. Liu took over the execution of the reproducibility runs.*
Funding Support:	
Name:	*Nyon Mun Peak*
Project Role:	*Postdoctoral Researcher*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.7*
Contribution to Project:	*Initial downstream purification of PpSP15.*
Funding Support:	
Name:	*Wen-Hsiang Chen*
Project Role:	*Director, Quality Control*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Chen oversaw all quality control for PpSP15 and LdNH36. He supervised Dr. Biter*
Funding Support:	
Name:	*Amadeo Biter*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.4
Contribution to Project:	*Dr. Biter worked on assay development for the vaccine antigens, as well as on stability and compatibility studies.*
Funding Support:	
Name:	*Portia Gillespie*
Project Role:	*Laboratory manager*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*0.3*
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 in support of Dr. Beaumier and later Dr. Strych*
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:	*An Nguyen*
Project Role:	*Senior Project Coordinator, Quality Assurance*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4.8*
Contribution to Project:	*Ms. Nguyen provided quality assurance services during the performance of the process development and quality control testing activities*
Funding Support:	
Name:	*Shannon McKim*
Project Role:	*Project Coordinator, Quality Assurance*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.8*
Contribution to Project:	*Ms. McKim provided quality assurance services during the performance of the process development and quality control testing.*
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: Chagas Disease Vaccine Development Program – End date extended to 12/31/2019
- Title of the project: Developing and Testing a novel, low-cost, effective HOOKworm VACcine to Control Human Hookworm Infection in endemic countries – Ended 3/31/2019
- Title of the project: U.S.-Malaysian Neglected Tropical Disease Initiative – Ended 12/31/2018
- Title of the project: West Nile Virus vaccine development – End date extended to 12/31/2019
- Title of the project: Chagas Vaccine Initiative: Accelerated Development of the First Therapeutic Vaccine for Chagas Disease – Ended 12/31/2018
- 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: Combatting Next Generation Lethal Epidemics in Saudi Arabia - New addition to active support; Ended 4/30/2019
- Title of the project: A novel mucosal trichuriasis vaccine for Th2 immune protection - New addition to active support; Effort at 10%
- Title of the project: Phase I/IIb Testing of the Sm-TSP-2 Schistosomiasis Vaccine in Uganda - New addition to active support; Effort at 2%
- Title of the project: Chagas Vaccine Initiative - New addition to active support; Effort at 8.33%

Changes for Coreen Beaumier:

- Title of the project: West Nile Virus vaccine development – Effort reduced to 0%Title of the project: U.S.-Malaysian Neglected Tropical Disease Initiative – Ended 12/31/2018Title of the project: The Slim Initiative for the Development of the firs Human Chagas Disease vaccine – Effort reduced to 0%
- Title of the project: Chagas Vaccine Initiative: Accelerated Development of the First Therapeutic Vaccine for Chagas Disease – Ended 12/31/2018
- Title of the project: Chikungunya Recombinant Subunit Vaccine – Effort reduced to 0%Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease – Effort reduced to 0%
- Title of the project: A vaccine to prevent leishmaniasis – Effort reduced to 0%
- Title of the project: Combatting Next Generation Lethal Epidemics in Saudi Arabia – Effort reduced to 0%

Changes for Maria Elena Bottazzi:

- Title of the project: Chagas Disease Vaccine Development Program – End date extended to 12/31/2019
- Title of the project: Development of a novel adjuvant for vaccine sparring – Ended 12/31/2018
- Title of the project: Developing and Testing a novel, low-cost, effective HOOKworm VACcine to Control Human Hookworm Infection in endemic countries – Ended 3/31/2019
- Title of the project: U.S.-Malaysian Neglected Tropical Disease Initiative – Ended 12/31/2018
- Title of the project: West Nile Virus vaccine development – End date extended to 12/31/2019
- Title of the project: Chagas Vaccine Initiative WO3 - Effort changed to 10%
- Title of the project: Chagas Vaccine Initiative: Accelerated Development of the First Therapeutic Vaccine for Chagas Disease – Ended 12/31/2018
- 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: Combatting Next Generation Lethal Epidemics in Saudi Arabia - Ended 4/30/2019
- Title of the project: A novel mucosal trichuriasis vaccine for Th2 immune protection - New addition to active support; Effort at 5%
- Title of the project: Phase I/IIb Testing of the Sm-TSP-2 Schistosomiasis Vaccine in Uganda - New addition to active support; Effort at 2%
- Title of the project: Chagas Vaccine Initiative - New addition to active support; Effort at 5%

Changes for Jeroen Pollet:

- Title of the project: Developing and Testing a novel, low-cost, effective HOOKworm VACcine to Control Human Hookworm Infection in endemic countries – Ended 3/31/2019
- Title of the project: U.S.-Malaysian Neglected Tropical Disease Initiative – Ended 12/31/2018
- Title of the project: Development of a novel adjuvant for vaccine sparring – Ended 12/31/2018
- Title of the project: Chagas Vaccine Initiative: Accelerated Development of the First Therapeutic Vaccine for Chagas Disease – Ended 12/31/2018
- 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 WO3 - Effort changed to 0%
- Title of the project: Combatting Next Generation Lethal Epidemics in Saudi Arabia - Ended 4/30/2019
- Title of the project: Chagas Vaccine Initiative - New addition to active support; Effort at 8.33%

Changes for Ulrich Strych:

- Title of the project: U.S.-Malaysian Neglected Tropical Disease Initiative – Ended 12/31/2018
- Title of the project: The Slim Initiative for the Development of the first Human Chagas Disease vaccine – Effort Changed to 10%
- Title of the project: Chagas Vaccine Initiative: Accelerated Development of the First Therapeutic Vaccine for Chagas Disease – Ended 12/31/2018
- 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: Combatting Next Generation Lethal Epidemics in Saudi Arabia - Ended 4/30/2019
- Title of the project: Chagas Vaccine Initiative - New addition to active support; Effort at 8.33%

Changes for Bin Zhan:

- Title of the project: Development of a novel adjuvant for vaccine sparing – Ended 12/31/2018
- Title of the project: Chagas Vaccine Initiative: Accelerated Development of the First Therapeutic Vaccine for Chagas Disease – Ended 12/31/2018
- Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease –Effort reduced to 4%
- Title of the project: Combatting Next Generation Lethal Epidemics in Saudi Arabia - Ended 4/30/2019
- Title of the project: A novel mucosal trichuriasis vaccine for Th2 immune protection - New addition to active support; Effort at 10%

Changes for Naomi Aronson:

- Title of the project: Surveillance for visceral leishmaniasis in Iraq/Afghanistan previously deployed service members currently on immunosuppressive therapies - New addition to active support; Effort at 15%

- Title of the project: Latent visceral leishmaniasis among Afghanistan deployed US military personnel - New addition to active support; Effort at 15% effort

Changes for Ines Elakhal Naouar:

- Title of the project: A vaccine to prevent leishmaniasis - Effort decreased to 0%

8. Special Reporting Requirements

Not applicable

9. Appendices

Nothing to report

References:

1. McAtee CP, Seid CA, Hammond M, Hudspeth E, Keegan BP, Liu Z, Wei J, Zhan B, Arjona-Sabido R, Cruz-Chan V, Dumonteil E, Hotez PJ, Bottazzi ME. Expression, purification, immunogenicity and protective efficacy of a recombinant nucleoside hydrolase from Leishmania donovani, a vaccine candidate for preventing cutaneous leishmaniasis. Protein expression and purification. 2017;130:129-36. doi: 10.1016/j.pep.2016.10.008. PubMed PMID: 27773761.

2. Oliveira F, Rowton E, Aslan H, Gomes R, Castrovinci PA, Alvarenga PH, Abdeladhim M, Teixeira C, Meneses C, Kleeman LT, Guimaraes-Costa AB, Rowland TE, Gilmore D, Doumbia S, Reed SG, Lawyer PG, Andersen JF, Kamhawi S, Valenzuela JG. A sand fly salivary protein vaccine shows efficacy against vector-transmitted cutaneous leishmaniasis in nonhuman primates. Sci Transl Med. 2015;7(290):290ra90. doi: 10.1126/scitranslmed.aaa3043. PubMed PMID: 26041707.