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Nanolipoprotein Particles (NLPs) as Versatile Vaccine Platforms for Co-delivery of Multiple Adjuvants with Subunit Antigens from Burkholderia spp. and F. tularensis - Annual Technical Report

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April 16, 2015

Disclaimer

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CBM Annual Progress Report

Proposal/Contract #: CBCALL12-PLAT2-2-0010

Principal Investigator: Nicholas O. Fischer, Ph.D.

Applicant Organization: Lawrence Livermore National Laboratory

Period Covered by this Report: April 01, 2014 to March 31, 2015

Title of Project: Nanolipoprotein particles (NLPs) as versatile vaccine platforms for co-delivery of multiple adjuvants with subunit antigens from *Burkholderia spp*. and *F. tularensis*

Current TRL: 3

DUAL USE RESEARCH OF CONCERN

Select Agent/Toxin: Please indicate the select agents or toxins studied in this project.

☐ Does not involve use of virulent select agent, toxin or genetic components

☐	*Bacillus anthracis*	☐	Ebola virus
☒	*Francisella tularensis*	☐	Marburg virus
☐	*Yersinia pestis*	☐	Variola major virus
☒	*Burkholderia mallei*	☐	Variola minor virus
☒	*Burkholderia pseudomallei*	☐	Rinderpest virus
☐	Botulinum neurotoxin	☐	Foot-and-mouth disease virus
☐	Toxin producing strains of *Clostridum botulinum*	☐	Avian Influenza virus (highly pathogenic)
		☐	Reconstructed 1918 Influenza virus

Experiments of Concern: The following categories of experiments will be used to determine dual use research of concern. Please indicate the type of research conducted from the list below.

☒ Research conducted does NOT involve any of the listed experiments

☐	Enhances the harmful consequences of the select agent or toxin
☐	Disrupts immunity or the effectiveness of an immunization against the select agent or toxin without clinical and/or agricultural justification
☐	Confers to the select agent or toxin resistance to clinically and/or agriculturally useful prophylactic or therapeutic interventions against that select agent or toxin or facilitate their ability to evade detection methodologies
☐	Increases the stability, transmissibility, or the ability to disseminate the select agent or toxin
☐	Alters the host range or tropism of the select agent or toxin
☐	Enhances the susceptibility of a host population to the select agent or toxin
☐	Generates or reconstitutes an eradicated or extinct biological agent or toxin from select agent or toxins listed above

Abstract

The goal of this proposal is to demonstrate that co-localization of protein subunit antigens and adjuvants on nanolipoprotein particles (NLPs) can increase the protective efficacy of recombinant subunit antigens from *Burkholderia spp*. and *Francisella tularensis* against an aerosol challenge. NLPs are are biocompatible, high-density lipoprotein mimetics that are amenable to the incorporation of multiple, chemically-disparate adjuvant and antigen molecules. We hypothesize that the ability to co-localize optimized adjuvant formulations with subunit antigens within a single particle will enhance the stimulation and activation of key immune effector cells, increasing the protective efficacy of subunit antigen-based vaccines. While *Burkholderia spp.* and *F. tularensis* subunit antigens are the focus of this proposal, we anticipate that this approach is applicable to a wide range of DOD-relevant biothreat agents. The F344 rat aerosol challenge model for *F. tularensis* has been successfully established at Battelle under this contract, and Year 3 efficacy studies performed at Battelle demonstrated that an NLP vaccine formulation was able to enhance survival of female F344 rats relative to naïve animals. In addition, Year 3 focused on the incorporation of multiple *Burkholderia* antigens (both polysaccharides and proteins) onto adjuvanted NLPs, with immunological analysis poised to begin in the next quarter.

Task 1

The initial phase of Task 1, namely the preparation of *F. tularensis* and *Burkholderia spp*. subunit antigens, was completed in Year 1 of this effort. As indicated in the Year 1 annual report, initial difficulties in the expression and purification of two *Burkholderia* antigens (BimA and BopA) were encountered. Subsequent work has identified an alternative approach to the expression and purification of these antigens. However, the decision to focus solely on *F. tularensis* has put this work on hold for the foreseeable future. As such, all work related to Task 1 in year two was in preparation for testing *F tularensis* antigens and NLP formulations in the F344 rat model. No additional work on Task 1 was conducted in Year 3, aside from occasional expression and purification of the *F. tularensis* antigens using protocols established in Years 1 and 2 for vaccination studies.

Task 2

Task 2 involved the production of master cell banks, characterization of the aerosol exposure system, and pathogen dosing optimization in BALB/c mice under the original proposed SOW. As this task was primarily for establishing the *B. mallei* aerosol model in BALB/c mice at Battelle, and the current focus *is F. tularensis*, no additional work on this task was conducted in Year 3. However, Battelle is poised to finalize both characterization of the aerosol exposure system with *B. mallei* China 7 and assessing the aerosol LD_{50} dose in BALB/c mice if deemed necessary.

Task 3

Task 3 involved the testing of NLP-based vaccine formulations in enhancing protective efficacy of subunit antigens against aerosol pathogen challenges in the BALB/c model. As the focus of the revised SOW has shifted the work to assessing efficacy in the F344 model, Task 3 has been completed and no work on this task has been conducted in Year 3. Final study report by Battelle was submitted to DTRA.

Task 4

Task 4.1

This Task was aimed at providing a systematic immunological assessment of various NLP formulations in order to advance top candidates for protective efficacy testing in the F344 rat model at Battelle. Both humoral and cellular immune responses were to be assessed and used to determine those formulations with the greatest likelihood of providing protection in an aerosol challenge model. Three different adjuvant:NLP formulations would be tested in this Subtask. Cholesterol-modified CpG ODN1826 is the CpG molecule utilized during Year 1 of this effort. This CpG molecule has been widely cited in the literature, and is classified as a Class B CpG ODN. This class of CpG strongly activates B cells, but only weakly stimulates INF-α secretion. To expand the breadth of immune stimulation, we have also chosen to study cholesterol-modified CpG ODN2395, a Class C CpG ODN. This class of CpG is characterized by the ability to promote both strong B cell responses and IFN-α production from plasmacytoid dendritic cells (pDCs). In addition to CpG molecules, the third adjuvant to be tested was MPLA, which has been successfully incorporated into NLPs and has provided stimulation of both humoral and cellular immune responses in mice. Importantly, MPLA is the only TLR adjuvant that has been approved for use in a human vaccine (Cervarix). By assessing various formulations of NLPs incorporating these adjuvants, the goal was to identify those formulations that might provide the best possible opportunity to protect the animals against a lethal aerosol SCHU S4 challenge. Preliminary immunological assessments on effect of administration route on overall antibody titer production were completed in Year 2, and based on these results (see Year 2 annual report), the i.n. and i.m. routes were chosen for use in the full immunological assessment of our NLP formulations.

The large immunological assessment study was initiated at LLNL in 03.2014, and consisted of 8 distinct NLP formulations: 20 μg CpG ODN2395, 5 μg CpG ODN2395, 1 μg CpG ODN2395, 20 μg CpG ODN1826, 5 μg CpG ODN1826, 1 μg CpG ODN1826, 1 μg MPLA, and 0.5 μg MPLA (see Figure 1). Each formulation included 10 μg each of IglC, DnaK, and KatG. Control groups received only PBS vehicle. To accommodate appropriate numbers of adjuvant (1 to 8 adjuvant molecules per NLP) and antigen (1 to 15 protein molecules per NLP), the final concentration of NLP varied between formulations. However, all final amounts of NLP (and rat E422k) were below 35 μg per administration. Animals were vaccinated three times at 4-week intervals. Each group consisted of 8 F344 rats (4 male / 4 female). Rats vaccinated i.m. received 100 μL into the hind thigh muscle. Rats vaccinated i.n. were anesthetized with isofluorane, and 50 μL of the vaccine formulation was administered (25 μL per nare).

Serum samples were collected serially approximately 4 weeks after each vaccination (via submandibular bleed), and serum was assessed for antigen-specific antibody titers. At the 12-week time point, spleen and lymph node tissues were collected from a subset of animals (4 animals per group) for subsequent in vitro re-stimulation assays.

Systemic humoral immune responses elicited by our vaccine formulations were quantitated over time in the context of our prime-boost-boost regimen. Robust antigen-specific IgG antibody production was observed with the NLP vaccine formulations for each antigen (IglC, DnaK, and KatG) vaccinated either i.m. or i.n. (Figure 1). However, animals vaccinated i.m. exhibited more robust humoral immune responses (greater than 10^6) with less intra-animal variability compared with those rats vaccinated i.n. Vaccine formulations containing CpG (ODN1826 and ODn2395) consistently elicited markedly higher systemic antigen specific IgG titers in rats vaccinated i.n. or i.m. compared with those formulations containing the TLR4 adjuvant MPLA (Figure 1).

Both local (BAL) and systemic (Serum) mucosal immune responses where quantitated. IgA titers were quantified in both serum (for IM and IN groups) and bronchoalveolar lavage (BAL; IN groups only) 4 weeks after the third (and final) inoculation. As illustrated in Figure 2, serum IgA levels were highest for IglC, primarily when formulated on mCpG:NLPs, regardless of administration route. While antigen-specific titers were lower for DnaK and KatG, those

formulated with mCpG:NLPs exhibited the highest serum IgA titers. Importantly, vaccine formulations containing ODN1826 elicited a more robust, localized IgG and IgA response in the BAL for all three antigens (Figure 3), indicating the ability of our NLP-based vaccine formulations to induce immune responses at the lung mucosa. Assessment of IgG isotypes and IgA titers is currently underway.

Quantification of cytokines known to mediate T-cell proliferation and downstream inflammation was performed using a Luminex multiplexed cytokine array in a classic antigen re-stimulation assay. A representative panel of three cytokines is shown in Figure 4. The NLP vaccine formulations significantly modulate both the magnitude and quality of T-cell responses in vivo, as assessed by secretion of IL-2, IFN-γ and IL-17A, with ODN1826 eliciting significantly more cytokines upon restimulation with IglC, KatG and DnaK than either ODN2395 or MPLA. We observed similar trends for IP-10, IL-10 and RANTES but did not observe detectable levels of IL-12p70 or IL-5 (data not shown). Taken together, the NLP vaccine formulations and the route of vaccination appear to contribute to both the magnitude and quality of the T cell responses in vivo. It is interesting to note that antigen-specific T cell responses appear similar between the i.n. and i.m. vaccinated rats (with the exception of IL-17A), which is in contrast to what we observed with respect to systemic antigen-specific antibody generation where i.m. vaccination resulted in markedly more systemic IgG compared with i.n. vaccination. Therefore, downselection of vaccine formulations that move to the challenge phase should likely include quantitative analyses of both humoral and cell mediated immune responses.

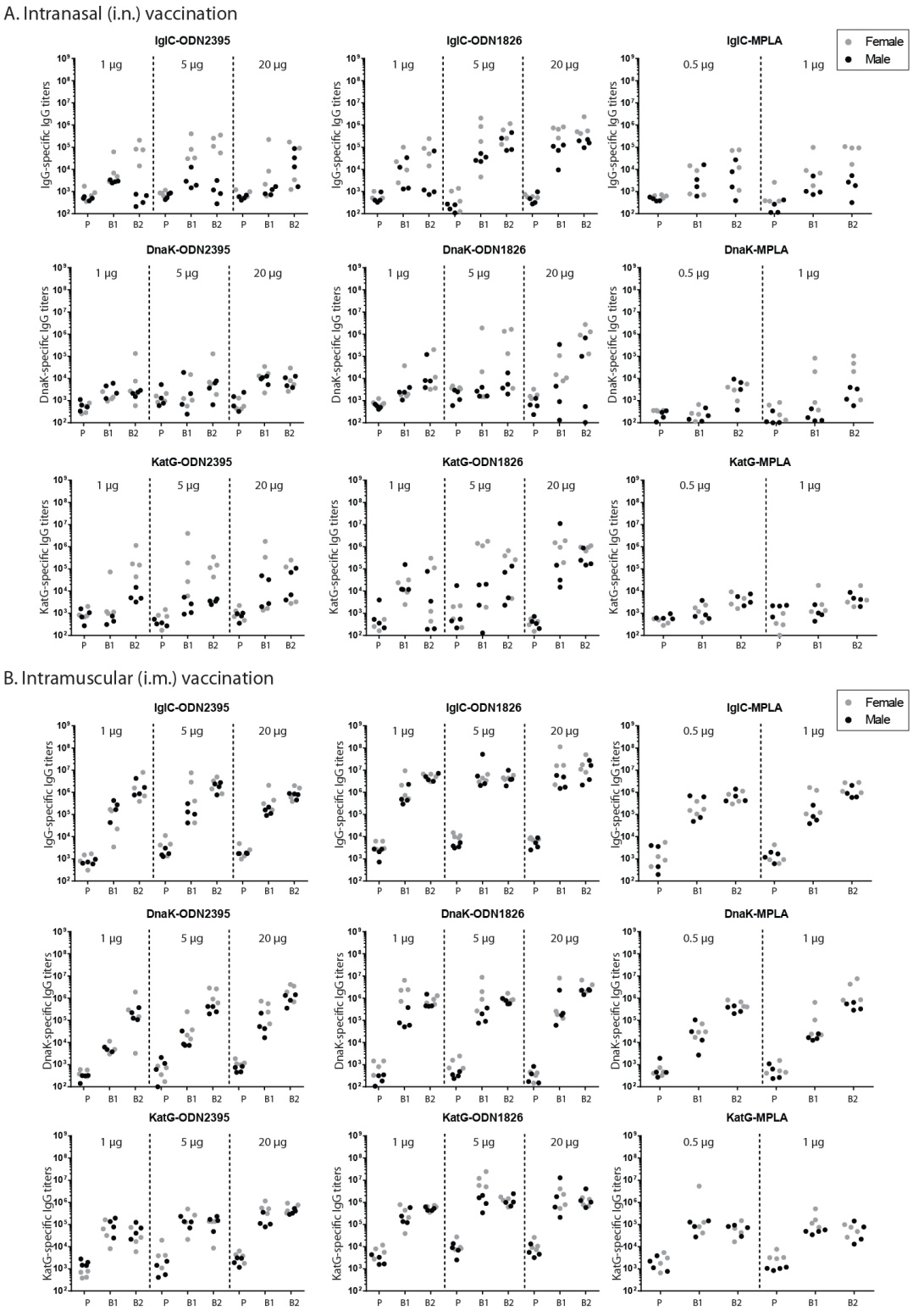

Figure 1. Compilation of antigen specific titers via (A) intranasal or (B) intramuscular route. Each symbol represents titers from individual animals (males = black circles; females = gray circles). Animals were primed (P) and boosted on weeks 4 (B1) and 8 (B2). Adjuvant amount administered per dose is indicated at the top of each graph.

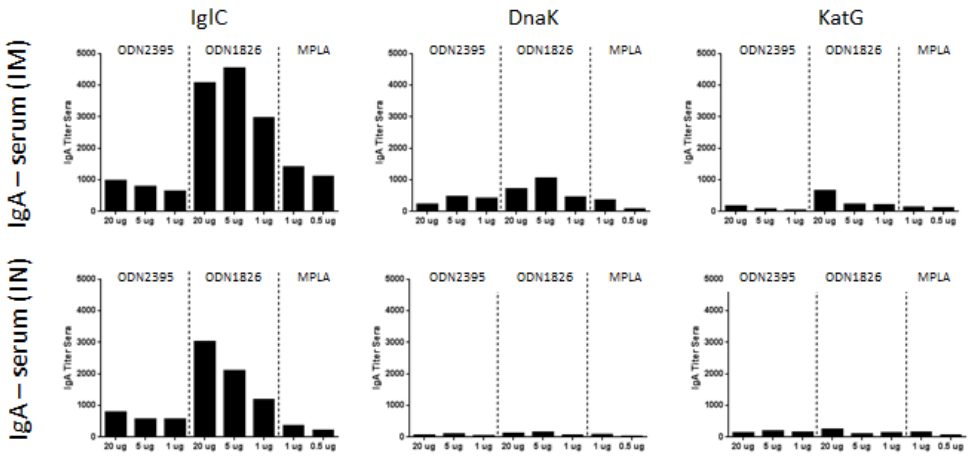

Figure 2. Quantification of antigen-specific serum IgA titers. Sera from animals were collected 12-weeks post-prime (4-weeks after third vaccination), pooled, and analyzed for IgA titers. Data are groups according to antigen (IglC, DnaK, KatG), adjuvant formulation, and dose (5, 20 µg CpG or 1 µg MPLA).

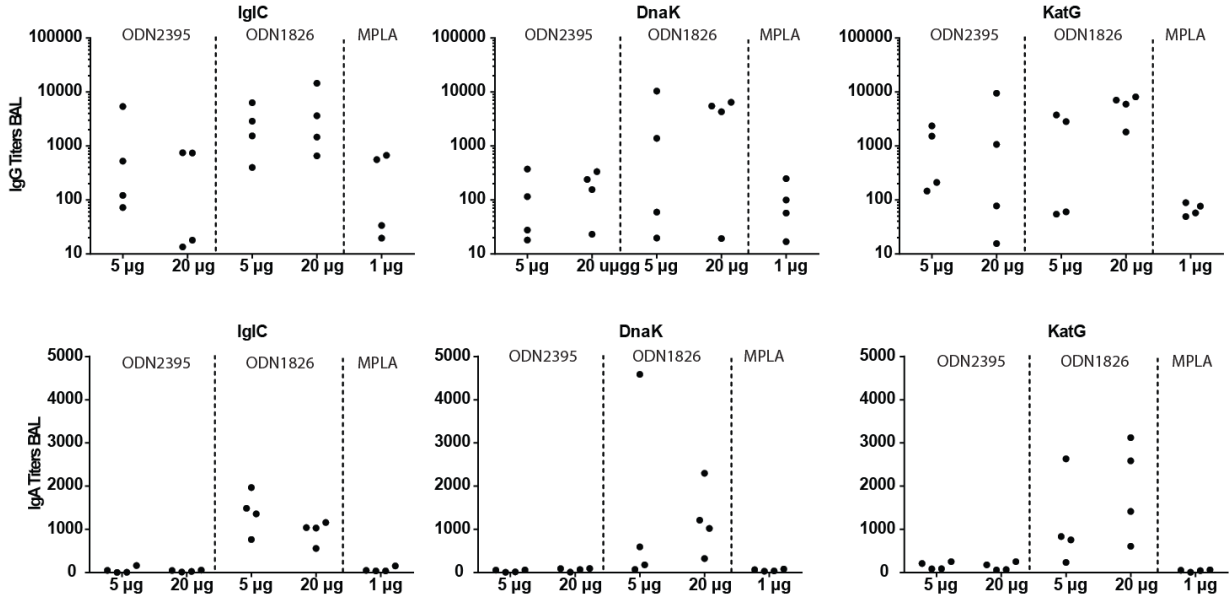

Figure 3. Quantification of IgG and IgA in BAL of animals (n=4) immunized via i.n. Antigen-specific titers were determined for individual animals. Data are groups according to antigen, adjuvant formulation, and dose (5, 20 µg CpG or 1 µg MPLA).

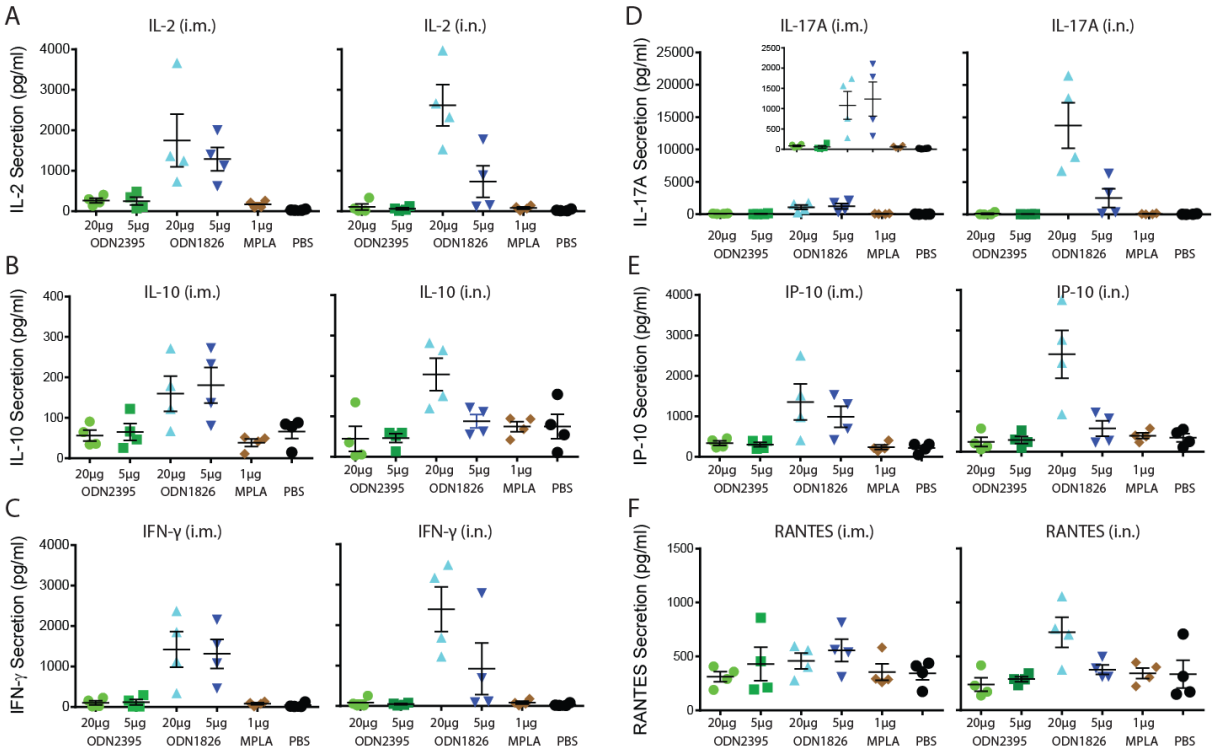

Figure 4. Quantification of IL-2, IFN-γ, and IL-17A upon splenocyte restimulation with antigen cocktail. Antigen specific T cell activation was assessed in 6 groups (4 rats/group) four weeks after the second boost (B2). Briefly, splenocytes were harvested and stimulated with 10 ug of each antigen and supernatants were collected 72 hrs post antigen restimulation. Cytokine levels for individual animals are plotted with the SEM.

Task 4.2:

This Task established the *F. tularensis* Schu S4 aerosol LD_{50} in F344 rats at Battelle to ensure that the appropriate challenge dose will be delivered during the vaccine efficacy experiments. Based on the statistical analyses, an LD50 value of **32.5 cfu** was calculated (with 95% confidence intervals of 14.8 and 72.6). This task was completed in Year 2, and no work on this Task was conducted in Year 3.

Task 4.3:

This Task encompassed the in vivo phase of aerosol F. tularensis SCHU S4 challenge experiments in the F344 rat model. Based on the data obtained in Task 4.1, the CpG adjuvant ODN 1826 was deemed to elicit the greatest humoral and T-cell responses in the animals. As both i.m. and i.n. vaccination elicited both humoral and cell mediated responses (albeit with disparate magnitudes), it was decided to utilize both routes (same formulations, but separate groups) for vaccinating the challenge animals. Again, only CpG ODN1826 formulations were chosen for this study, as these formulations consistently elicited robust antibody and T cell responses. Table 1 outlines the experimental design for the challenge experiment carried out at Battelle. Animals were vaccinated three times every 4 weeks with adjuvanted (ODN1826) NLPs co-formulated with all three protein antigens (IglC, DnaK, KatG). Animals were vaccinated i.n. or i.m. Three unique NLP formulations were tested, varying in amount of adjuvant

Table 1. Experimental groups and design for Task 4.3

Group #	Vaccine Formulation	Vaccination Route	Number of F344 Rats		Challenge Dose (cfu)	Vaccination Points	Blood Collection Schedule (Study Day)	Post-Challenge Observations	Endpoint
			Male	Female					
Challenge with *F. tularensis* Strain Schu S4									
1	CpG:NLP (Adj5/Ag10)	IM	10	10	10xLD$_{50}$ (320 cfu)	I_1 = Day 0 I_2 = Day 28 I_3 = Day 56	21, 77	Twice Daily Clinical Observations Through Study Day 112	Survival, time-to-death
2	CpG:NLP (Adj5/Ag20)	IM	10	10					
3	CpG:NLP (Adj20/Ag10)	IM	10	10					
4	CpG:NLP (Adj5/Ag10)	IN	10	10					
5	CpG:NLP (Adj5/Ag20)	IN	10	10					
6	CpG:NLP (Adj20/Ag10)	IN	10	10					
7	PBS	IM	10	10					
8	LVS	IM	10	10		I_1 = Day 56	77		

I_1: First Immunization
I_2: Second Immunization
I_3: Third Immunization
Immunization Injection Volume: IM. = 100 µL; IN. = 50 µL (25 µL/nare)
Vaccination Site: Groups 1, 2, 3, 7, 8 = IM. ; Groups 4, 5, 6 = IN.

and antigen material: 5 µg CpG + 10 µg each antigen (5/10), 5 µg CpG + 20 µg each antigen (5/20), and 20 µg CpG + 10 µg each antigen (20/10). In addition, a group was vaccinated 4 weeks prior to challenge with 3 X 10^7 LVS (I.M.) to serve as a positive control for protective vaccination. A group of unvaccinated naïve animals served as a negative control for protection. Vaccine material was prepared by LLNL and shipped at 2-8°C to Battelle at least two days prior to vaccination (fresh material was prepared for each vaccination). It was decided that Battelle would anesthetize animals using an injectable ketamine/xylazine mixture, due to limitations at Battelle to anesthetize using isofluorane.

All experimental groups consisted of 20 animals (10 males / 10 females), 8-10 weeks of age. Rats were initially randomized by weight into vaccine groups. Prior to aerosol challenge, rats were further randomized by weight, vaccine group, and sex into a challenge order such that each 10 minute aerosol run of 30 rats would consist of as even a match as possible of sex, vaccine group, and weight. This second round of randomizations was conducted due to the weight differences between the male and female rats on study. The final randomization would help to ensure that male and female rats get dosed appropriately based on their weight differences. Animals were bled on Days 21 and 77, and sera were shipped to LLNL for evaluation of antigen-specific antibody titers.

On study Day 84, rats were challenged with a target dose of 320 colony forming units (cfu) (10 X LD_{50}) of *F. tularensis* Schu S4 via a nose only exposure chamber. Suspensions of *F. tularensis* SchuS4 were aerosolized by a 6-jet Collison nebulizer and delivered to the exposure system. The *F. tularensis* concentration (based on enumerations) of the nebulizer and impinger samples was used to determine the actual exposure dose for each challenge group. Using Guyton's formula and the concentration of *F. tularensis* collected from air sampled from the exposure system, the inhaled dose was determined from the enumeration results. Based on these

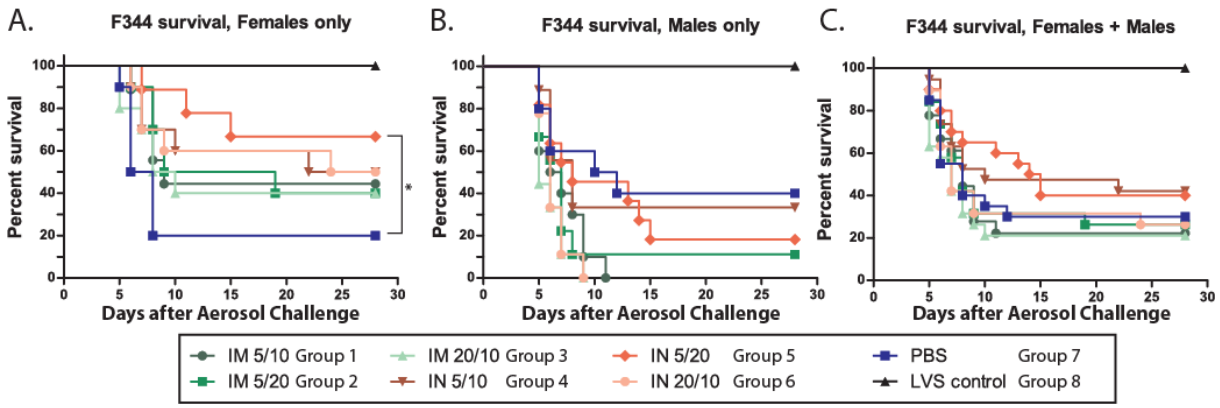

Figure 5. In vivo aerosol challenge experiments comparing NLP-vaccinated F344 rats with naïve and LVS vaccinated animals. Kaplan-Meier graphs depicting survival of (A) females, (B) males, or (C) combined females + males over the 28-day observation period. Survival in females was enhanced upon vaccination with NLP formulations, and significant survival relative to naïve animals was observed in females receiving intranasal inoculation of 5ug CpG and 20ug of each antigen (IN 5/20 group) (*is p = 0.013, log-rank test).

enumerations, the mean challenge dose was 1,364 (± 199) cfu *F. tularensis* (Schu S4, BBRC Lot FTS773) (42 ± 6 aerosol LD_{50}).

Within two weeks post-challenge, significant differences in the survival rates between females and males were observed (see Figure 5). As such, statistical analyses were conducted on individual genders. For males, survival proportions ranged from 0 to 33 percent for groups vaccinated with NLP:CpG formulations. Forty percent of males vaccinated with naïve PBS survived while all males vaccinated with LVS survived. For females, survival proportions ranged from 40 to 67 percent for groups vaccinated with NLP:CpG formulations. Twenty percent of females vaccinated with naïve PBS survived, while all females vaccinated with LVS survived.

Table 2 presents both the unadjusted and Bonferroni-Holm adjusted p-values from the pairwise group log-rank tests between the non-vaccinated control group and each of the vaccinated groups for all animals, males, and females, respectively, testing whether the survival probabilities throughout the study (i.e., survival rates combined with times to death) significantly differ between the groups at p<0.05. When considering all animals, there were no significant differences in survival probabilities between the non-vaccinated PBS control group (Group 7) and any of the NLP:CpG vaccinated groups. However, the survival probabilities for the LVS group (Group 8) were significantly greater than that of the non-vaccinated control group (Group 7). For males, the survival probabilities for Groups 1, 3, and 6 were all significantly less than those of the group vaccinated with PBS (Group 7) with all males in Groups 1, 3, and 6 succumbing by Day 11. In addition, the survival probabilities for Group 8 (LVS) were significantly greater than those of the negative controls (Group 7). When the p-values were adjusted via the Bonferroni-Holm method, the only difference in survival probabilities occurred between the groups vaccinated with PBS and LVS. The differences in male survival probabilities can be seen visually in Figure 2, which displays the Kaplan Meier curves for each group. For females, the survival probabilities for Group 5 and Group 8 (LVS) were significantly greater that of the group vaccinated with PBS (Group 7). When the p-values were adjusted via the Bonferroni-Holm method, the only difference in survival probabilities occurred between the groups vaccinated with PBS and LVS. The differences in female survival probabilities can be seen visually in Figure X, which displays the Kaplan Meier curves for each group. Of particular note are the female animals receiving I.N. administration of 5µg CpG / 20µg antigens (5/20) (Group 5). These animals exhibited >65% survival (p = 0.013 relative to naïve group).

Table 2: Results of the unadjusted and adjusted Log-Rank tests comparing survival probabilities (survival rates combined with time to death) between each vaccinated group and the non-vaccinated control group.

Group Comparison		All Animals			Males		Females	
		Unadjusted P-Value	Adjusted P-Value		Unadjusted P-Value	Adjusted P-Value	Unadjusted P-Value	Adjusted P-Value
CpG:NLP (Adj5/Ag10)	PBS	0.7296	1.0000		0.0213*	0.1065	0.0894	0.3934
CpG:NLP (Adj5/Ag20)	PBS	0.8574	1.0000		0.0841	0.2524	0.0804	0.3934
CpG:NLP (Adj20/Ag10)	PBS	0.3830	1.0000		0.0085*	0.0510	0.3178	0.4655
CpG:NLP (Adj5/Ag10)	PBS	0.5804	1.0000		0.7899	1.0000	0.2327	0.4655
CpG:NLP (Adj5/Ag20)	PBS	0.2521	1.0000		0.5108	1.0000	**0.0129***	0.0774
CpG:NLP (Adj20/Ag10)		PBS	0.9161	1.0000	0.0250*	0.1065	0.0787	0.3934
LVS		PBS	<0.0001*	0.0001**	0.0063*	0.0438**	0.0010*	0.0071**

As seen in Figure 6, median survival time was greatly enhanced, particularly in animals receiving I.N. administration of NLP-based vaccine formulations. Based on our survey of the current literature, these findings represent the first demonstration of significant protection against aerosolized SCHU S4 using purified, recombinant subunit protein antigens. While we cannot currently explain the lack of protection in males we appreciate that addressing this fact is important. However, all published protective efficacy assessments have been conducted using only female rats, making it impossible to compare our current survival results of the male groups with previously published data. These findings underscore the need to assess protective efficacy in both male and female test animals.

Following the final vaccination at Battelle, individual serum samples were assessed for antigen-specific IgG titers at LLNL (Figure 7). All serum IgG titers in I.M. groups were consistently higher than in corresponding I.N. groups. In addition, IgG titers in I.N. groups ranged widely between individual animals in contrast to I.M. groups. We believe that this variability (and lower overall titers) is not an inherent property of an immune response elicited by I.N. vaccination, but rather an inconsistency in the systematic delivery of the vaccine formulation to the lung via I.N. administration. Recent tests at LLNL have shown that, in contrast to mice, I.N. inoculation in the rat results in greater amounts of inoculum in the stomach, rather than the lungs. Based on these observations, we believe that animals in this study inoculated by I.N. received a significantly lower effective dose of the vaccine formulations. Importantly, even with this lower effective dose, protection via I.N. was observed, leading to the hypothesis that ensuring direct deposition of the NLP inoculum to the lung (*e.g.* intratracheal administration) will afford the protection levels sought by the DoD.

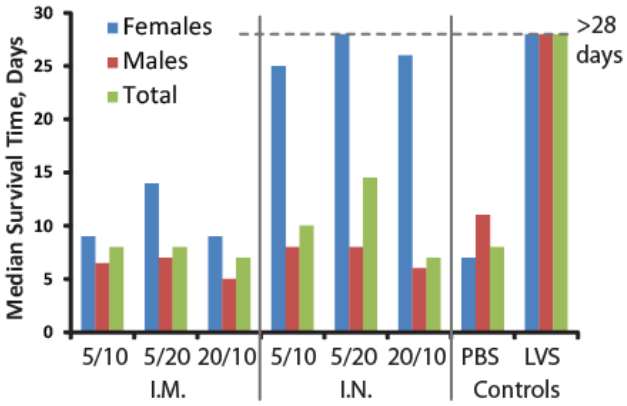

Figure 6. Median survival time for all groups, represented as females, males, and combined. Experimental observation period was 28 days (dotted line).

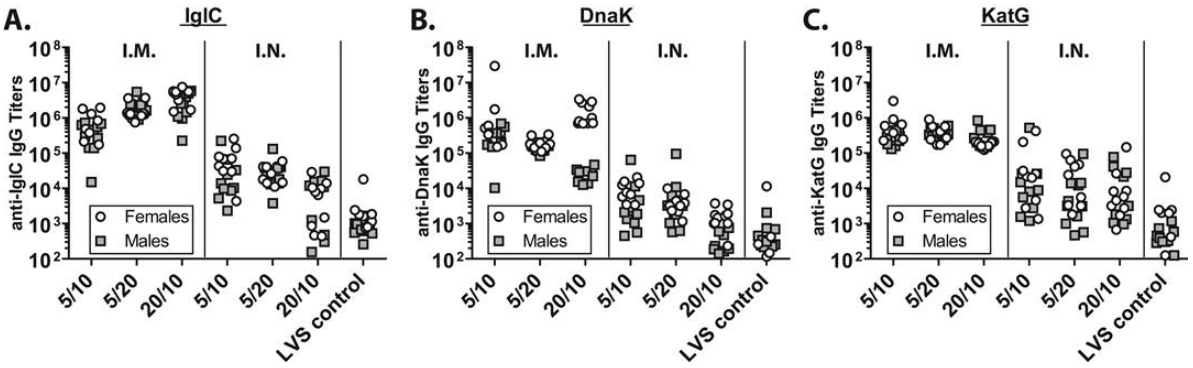

Figure 7. Antigen-specific IgG titers in serum were assess in the vaccinated animals one week prior to aerosol challenge. Titers for individual animals were assessed, against each antigen in the vaccine formulation. A) IglC, B) DnaK, C) KatG.

Task 4.4:

This task was aimed at evaluating the downselected NLP formulation from Task 4.3 in a full efficacy assessment. However, in consultation with the DTRA PM and in light of unexpected costs increases for Task 4.3, this task was removed from the SOW and replaced with Task 5 (see Programmatic and Technical Challenges section).

Task 5:

The goals of this Task are to prepare and assess immunogenicity of adjuvanted NLPs formulated with *Burkholderia* capsular polysaccharides (CPS) and protein antigens. In collaboration with Dr. Paul Brett (University of South Alabama), the objective is to assess the immunological effect of adjuvants and Burkholderia antigens co-localized on the NLP platform. The antigens that will be tested have been shown to be important for the virulence of *Burkholderia* in vivo and hold great promise in protecting against infection. These antigens include 1) the type VI secretion system (T6SS) protein Hcp1, 2) the ubiquitin-specific protease protein TssM, and 3) purified *B. pseudomallei* capsular polysaccharides.

Task 5.1:

This Task is aimed at conjugating *Burkholderia* CPS and protein antigens to adjuvanted NLPs. Recombinant, His-tagged protein antigens (Hcp1 and TssM) were provided by Dr. Brett for use in Tasks 5.1 and 5.2

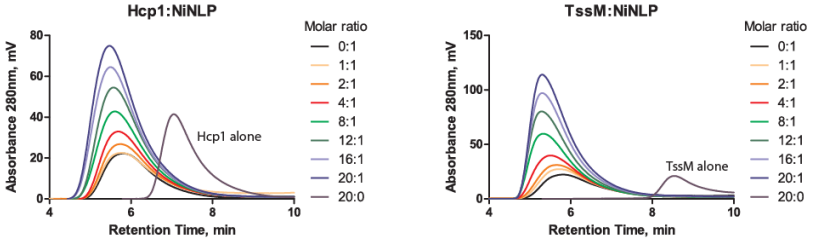

Figure 8. SEC was used to assess conjugation of His-tagged Hcp1 and TssM to Ni-chelating NLPs. NLPs were incubated with increasing molar ratios of protein (up to 20:1, antigen:NLP). Absorbance was monitored at 280nm. No unconjugated protein was observed, indicating that all protein was conjugated to the NLP.

The proteins were initially assessed by SEC to verify molecular weight and purity, and both proteins were determined to be pure. The SEC analysis indicated that TssM eluted as a monomer, whereas Hcp1 eluted as an oligomer with a molecular weight of 145kDa, mirroring published reports that Hcp1 forms a hexamer in solution (data not shown). While His-tagged, oligomeric proteins can negatively impact NLP stability (by forming extended aggregates due to multiple His-tag interactions with multiple NiNLPs), the Hcp1 hexamer subunits are all identically oriented, such that the His-tags are on the same face of the hexamer. Preliminary conjugation screens verified the ability to conjugate multiple copies of each protein to the NiNLPs (Figure 8). At least 20 molecules of either protein can be accommodated on a single NLP. Successful conjugation is evidenced by both an increase in absorbance signal intensity at 280nm and a shift to shorter SEC retention times upon increasing loading ratios. The successful conjugation of the Burkholderia protein antigens was an important first step. Next, the ability to conjugate the *B. pseudomallei* CPS molecule developed by Dr. Brett was investigated.

To investigate the feasibility of CPS conjugation to NLP, and the effect the conjugation has on the antigenicity of the CPS, we decided to utilize the same conjugation strategy as with our protein antigens, namely relying on a His-tagged:nickel interaction. This approach, rather than a covalent modification, was chosen for three reasons: 1) this conjugation approach has been successfully used for conjugation of biomolecules of disparate sizes (15-100 kDa), 2) the conjugation efficiency we routinely observe between His-tagged biomolecules and NiNLP is extremely high (relative to covalent conjugation schemes), and 3) the same NiNLP composition can be used for all vaccine formulations, ensuring consistency between groups. As such, a variant of *Burkholderia* capsular polysaccharide was prepared at USA, featuring a 16 amino acid peptide containing six N-terminal histidines covalently linked to the CPS molecule. This construct was extensively purified by dialysis, and provided as a lyophilized powder.

It was important to compare this novel CPS construct to unmodified CPS, so two independent preparations of unmodified CPS were similarly provided by Dr. Brett (labeled CPS-A and CPS-B for this report). The initial goal of the CPS analysis was four-fold: 1) utilize LLNL capabilities to determine an apparent molecular weight, 2) determine the size polydispersity, 3) demonstrate consistency between CPS formulations, and 4) evaluate His-CPS relative to unmodified CPS. One of the key difficulties in analyzing and characterizing polysaccharides is that they have extremely low intrinsic absorbance, meaning that spectrophotometric detection cannot be used for routine monitoring during chromatographic separations. We have an evaporative light scattering detector as part of our liquid chromatography instrumentation, which measures mass of total solute in the eluate. In this manner,

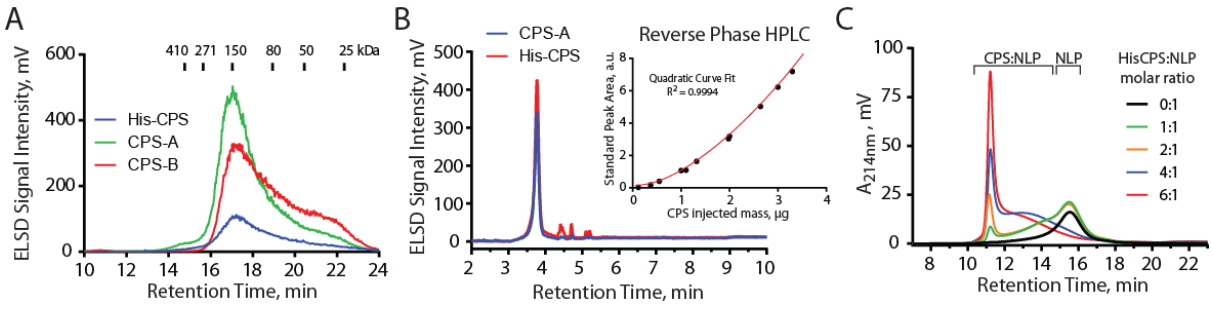

Figure 9. *Burkholderia* CPS characterization and preliminary conjugation to NLPs. A) Two unmodified CPS preparations (CPS A, CPS B) and His-CPS were analyzed by SEC (Superose 6 gel filtration column) using ELSD to monitor CPS elution. Demarkations on top of chromatogram indicate elution times of dextran molecular weight standards. B) Unmodified CPS (CPS-A) and His-CPS have identical elution profiles by RP-HPLC. CPS-A was used to prepare a standard curve (insert) to enable direct quantification of His-CPS stock solution by RP-HPLC. C) Preliminary validation of successful conjugation between His-tagged CPS and NiNLPs (on Superose6 gel filtration column). The shift to lower retention time indicates the formation of a larger species (i.e. conjugation of His-CPS to NiNLP).

polysaccharides can be readily visualized during SEC and RP-HPLC. First, a standard curve was prepared using commercial linear dextran standards, which serve as adequate molecular weight markers for polysaccharides. As seen in Figure 9A, CPS-A, CPS-B, and His-CPS were each characterized by a main species *ca* 150kDa size. The relative broad shoulder to the right of the main elution peak in each sample indicates that a significant number of smaller-sized populations are present, ranging from 150 kDa to 25 kDa. In spite of this polydispersity, however, these formulations are very consistent in overall size and distribution.

Quantification of the CPS material is an important parameter to ensure that vaccine formulations are well characterized. The unmodified CPS material can be readily weighed after preparation using a microbalance, as typically >50mg are purified at a time. However, the His-CPS pilot conjugation reaction yielded less than 4mg of material, making weight measurements more difficult and less reliable. The broad peaks typically observed with polysaccharides using size exclusion preclude any type of quantitative assessment of the material. However, RP-HPLC provides very clean and sharp elution peaks with a linear water:acetonitrile gradient that can be readily used for quantification. As such, RP-HPLC was used to generate a standard curve of the unmodified CPS material to facilitate quantification of the His-CPS material. As seen in figure 9B, both CPS-A and His-CPS have identical elution profiles, and the standard curve generated in using CPS-A (insert) was readily used to quantitate the His-CPS. This approach to CPS quantitation may also be useful in quantifying CPS material in NLP:CPS conjugates.

Conjugation of His-CPS to NiNLPs was assessed by SEC (Figure 9C). Increasing amounts of His-CPS were incubated with a fixed amount of NiNLP. After 30 minutes, the conjugated material was assessed by SEC (Superose 6 column). At all ratios of His-CPS:NiNLP, a peak at 11min is observed, as well as a broadening of the NLP peak to larger size (i.e. lower retention times). We hypothesize that the sharp peak at 11 min correlates to NLPs to which more than 1 His-CPS molecule has bound. As the amount of His-CPS is increased, this intensity of this peak is significantly increased. Importantly, the peak corresponding to NLP (15.5 min) is no longer present, indicating that all NLPs have successfully conjugated the His-CPS material.

In preparation for the immunological assessments of the Burkholderia antigens conjugated to the NLPs, a verification of simultaneous conjugation of both protein and His-CPS antigens was conducted (Figure 10). These tests were conducted using ratios that reflect the final antigen ratios in the vaccine material (10 µg of protein antigen and 5 µg of CPS antigen). These analyses were conducted using an analytical Superdex 200 SEC column, which provides greater resolution between smaller species (e.g. proteins), rather than NLPs and NLP conjugates. This separation media was used to verify that all protein material incubated with the NLPs is conjugated, and that

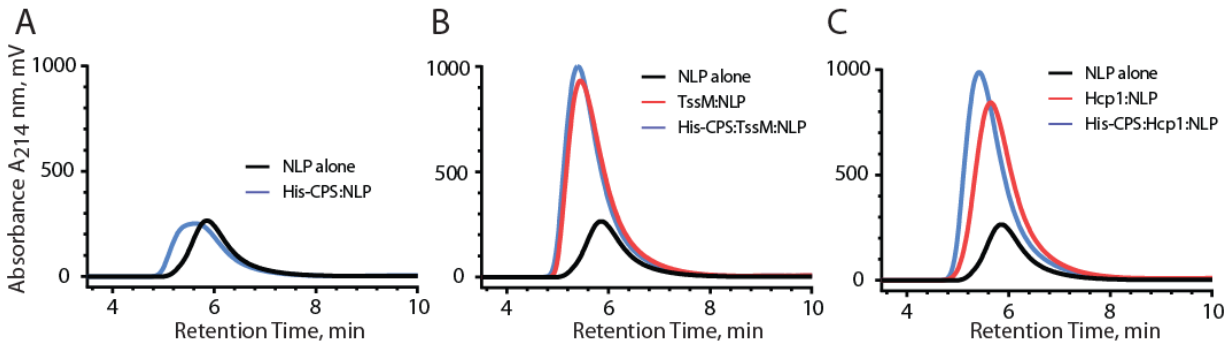

Figure 10. Preparation of *Burkholderia* vaccine formulations. Formulations of NiNLPs with (A) His-CPS alone, (B) TssM and TssM:HisCPS, and (C) and Hcp1 and Hcp1:HisCPS were prepared at the ratios intended for vaccination. Characteristic shift to lower retention time is observed in all cases, indicating successful conjugation of antigenic material.

no material remains free (i.e. unconjugated) (see Figure 8 for direct comparisons). As expected, incubation of the NiNLPs resulted in a shift to large species upon incubation with His-CPS (Figure 10A). This shift was mirrored in both with both TssM and Hcp1 (with and without His-CPS) (10B and 10C, respectively). Importantly, no free protein was observed when both TssM or Hcp1 were incubated with His-CPS, indicating that the NLP surface can readily accommodate conjugation and retention of these molecules. The final component of the vaccine material, CpG ODN 1826 modified with a cholesterol tag, was also readily incorporated into the NLP (data not shown).

Task 5.2:

This Task is aimed at assessing immunogenicity of NLPs co-formulated with *Burkholderia* antigens and adjuvant. LLNL IACUC and DoD ACURO approval has been obtained to begin in vivo immunological assessment of NLP-based vaccine formulations. The initial experiment (outlined in Table 3 below) will be initiated on April 09, 2015. The goal of this experiment will be to determine if conjugation of the CPS to the NLP elicits sufficient to elicit robust titers against both CPS and protein antigens.

Table 3: Experimental groups for evaluation of NLP formulations co-localizing CPS, protein antigens, and adjuvant

Group	Sample Description	Formulation				Route	# Animals	Volume, uL
		Platform	Adjuvant	CPS formulation[1]	Protien antigen[2]			
A	I.N.: - CPS:Ag:CpG1826:NLP	NLP	Chol-modified ODN1826	His-CPS	TssM + Hcp1	I.N.	8	30 (2x15)
B	I.N. - CPS:Ag:CpG1826	n/a	Chol-modified ODN1826	His-CPS	TssM + Hcp1	I.N.	8	30 (2x15)
C	I.N. - CRM-CPS:Ag:CpG1826	n/a	Chol-modified ODN1826	CRM197-CPS	TssM + Hcp1	I.N.	8	30 (2x15)
D	I.N. - PBS	n/a	n/a	n/a	n/a	I.N.	8	30 (2x15)
E	I.M.: - CPS:Ag:CpG1826:NLP	NLP	Chol-modified ODN1826	His-CPS	TssM + Hcp1	I.M.	8	100
F	I.M. - CPS:Ag:CpG1826	n/a	Chol-modified ODN1826	His-CPS	TssM + Hcp1	I.M.	8	100
G	I.M. - CRM-CPS:Ag:CpG1826	n/a	Chol-modified ODN1826	CRM197-CPS	TssM + Hcp1	I.M.	8	100
H	I.M. - CRM-CPS:Ag:CpG2006:ALUM	n/a	ODN2006 + Alhydrogel	CRM197-CPS	TssM + Hcp1	I.M.	8	100
I	I.M. - PBS	n/a	n/a	n/a	n/a	I.M.	8	100

[1]: 5µg CPS/dose
[2]: 10µg of each protein antigen/dose

Programmatic and Technical Challenges

During the course of this study, it had been suggested by DTRA PMs and SMEs that blood be collected from the vaccinated animals at Battelle to be analyzed at LLNL for antigen-specific serum titers. This is a very important metric for understanding the anticipated results from the upcoming challenge experiments. However, the costs for blood draws and training animals were not included in the original SOW or Battelle budget. To enable these activities, as well preparing LVS as a positive experimental control, the Battelle subcontract budget was increased for this study, reciprocally reducing the budget for the final Battelle efficacy study. The activities required for this additional work included training of Battelle personnel, cost of training animals, blood collection from

experimental animals, sample handling, and shipping to LLNL. Modifications to the SOW were prepared and approved by the DTRA PM. No increase in budget was requested. Based on the decrease in funding available for the final Battelle efficacy study, a further modification to the SOW was approved by DTRA to use the remaining funds to generate preliminary data on the immunological response to adjuvanted NLPs co-localizing *Burkholderia* CPS and protein antigens (Task 5).

Discussion and Significance

NLPs represent a versatile, biocompatible antigen and adjuvant delivery platform for the formulation of recombinant subunit antigen vaccines against biothreat agents. The flexibility in accommodating a wide range of chemically disparate biomolecules, ranging from hydrophobic to very polar, provides a means of co-localizing multiple adjuvants and antigens onto a single delivery platform. In the course of this funded research, we have demonstrated the expression and purification of a number of relevant recombinant antigens for *Burkholderia* spp. and *F. tularensis* vaccine formulations. These recombinant antigens were pure (>90%) and could be readily conjugated to the NLP platform. In Year 1 of this effort, NLP vaccine formulations were prepared for efficacy challenge studies against *B. pseudomallei* and *F. tularensis* (BALB/c models, single antigen formulations) conducted at Battelle. While these studies did not demonstrate increased protection against the aerosolized agents, no conclusion regarding the efficacy of the NLP platform can be discerned. Currently, no purified subunit antigens have been demonstrated as effective against the aerosolized pathogen strains used in these studies. The lack of protection can be attributed to a number of potential issues, including the animal model, identity of the antigens and their formulation (e.g. multivalent formulations). In Year 2 of this effort, we revised the SOW with the goal of testing the protective efficacy of NLP vaccine formulations containing all three *F. tularensis* antigens in the Fischer 344 rat model. F344 rats have recently emerged as a more relevant platform for evaluation of putative tularemia vaccine candidates, in large part because these rats are more resistant than mice to *F. tularensis* infection and the pathogenesis mimics human disease progression more closely than what has been observed in mice. In Year 2, optimization of the vaccine route of administration was completed at LLNL, and Battelle established the *F. tularensis* Schu S4 aerosol LD50 in F344 rats to ensure that the appropriate challenge dose will be delivered during the vaccine efficacy experiments.

Year 3 of this effort focused on optimizing the vaccine formulations at LLNL by comparing humoral and cell mediated immune responses. Three different adjuvants (at varying concentrations) were assessed, and ultimately the CpG adjuvant ODN1826 outperformed ODN2395 and MPLA in all metrics. Interestingly, i.n vaccination elicited only marginal humoral immune responses relative to i.m. vaccination. In addition, a larger variability in the levels of antigen-specific antibody titers was observed between animals vaccinated i.n compared with i.m. This is in contrast with our observations in the mouse model, where i.n. typically outperformed all other routes of administration (data not shown) based on systemic antibody titers. However, cell-mediated immune responses elicited by i.n. vaccination were consistently equal or superior to i.m. vaccination in our current study. As such, both routes were included in the protective efficacy challenge experiment at Battelle. An additional formulation not originally screened at LLNL (5 µg of CpG with 20 µg of each antigen colocalized on the NLP; CpG:NLP 5/20) was included (see Table 1 for complete description of all NLP vaccine formulations), in part due to recent published studies using antigen amounts exceeding 10 µg.

As demonstrated in Task 4.3, greater than 65% protection was afforded to female F344 rats vaccinated i.n. with the CpG:NLP 5/20 formulation. To our knowledge, this level of protection against an aerosol challenge with fully virulent *F. tularensis* using a vaccine formulation based on subunit antigens has not previously been reported.

Interestingly, three key observations came out of our most recent challenge studies, and serve as the basis for the proposed work: 1) the demonstrated protection in the F344 rats was only observed in females, as all vaccinated males rats succumbed to infection at rates similar to PBS controls, 2) significant protection was only afforded in female rats vaccinated i.n. (i.m. groups succumbed to infection at rates similar to not significantly different than PBS controls) and 3) all i.m. vaccinated rats exhibited significant levels of antigen specific antibodies in sera against all three subunit antigens, yet these robust humoral immune responses did not correlate with protection in our study. Correlating these findings back to the initial immunological assessment conducted at LLNL (Task 4.1), a few additional observations could be made: 1) rats vaccinated i.n. had significantly enhanced mucosal IgA in the lung and 2) i.n. vaccination resulted in a significant enhancement in both the magnitude and quality of T cell responses as assessed by restimulation assays. These findings all point to the importance of delivering the vaccine formulations directly to the lung mucosa to elicit robust IgA and cell-mediated immune responses in vivo while simultaneously promoting long-lived immunity. However, we postulate that i.n. administration, particularly in the rat, will require extensive optimization for eliciting optimal mucosal immunity. As observed in the titer data from both Battelle and LLNL samples, the i.n. titers are highly variable (especially relative to i.m.), suggesting that variable amounts of the vaccine material may actually be appropriately deposited in the deep lung tissue. Subsequent distribution experiments at LLNL in the F344 rat have verified this observation, and have demonstrated that the majority of vaccination material is not deposited in the lung upon i.n. administration. In light of these observations, ***we hypothesize that direct delivery of the vaccine material to the lung via intratracheal administration will overcome the outlined obstacles and lead to greatly enhance humoral and cell-mediated immune responses and provide enhanced protection in both female and male F344 rats.***

With regards to the newly initiated collaboration with current DTRA performers Dr. Paul Brett and Dr. Mary Burtnick (U. South Alabama), we hypothesize that the colocalization of both *Burkholderia* protein and CPS antigens on a single adjuvanted particle will provide the enhanced mucosal, humoral and cellular immune responses that will be needed to protect against aerosolized *Burkholderia* challenges. NLPs offer a number of advantages as a delivery platform in the context of *Burkholderia* vaccines. 1) Adjuvants can be incorporated into the NLPs to enable co-localization of both antigen and adjuvant into a single particle. Co-localization strategies have been demonstrated to provide a more robust immune response compared to co-administration formulations. 2) The NLP and associated protein antigens can assume the role of “carrier protein” for the CPS by providing conjugated T-cell epitopes and enhancing APC uptake. 3) Intranasal delivery of the NLP promotes lung retention, elicits IgG and IgA responses in both serum and mucosa, and elicits IFN-γ. Experiments are currently ongoing, but preliminary findings with regards to vaccine formulation are encouraging, as protein antigens, CPS antigen, and adjuvant are readily conjugated to the NLP platform.