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Developing a Vaccine Platform for a Balanced Mucosal Immune Response (CB11446), Project Report: Year 1

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Disclaimer

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Developing a Vaccine Platform for a Balanced Mucosal Immune Response (CB11446)

Project Report: Year 1

September 01, 2025

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Introduction

Mucosal vaccines can elicit protective immune responses at the infection site of respiratory or aerosolized pathogens. Achieving balanced mucosal and systemic responses is a key challenge in the design of mucosal vaccines, especially in terms of developing a broadly applicable vaccine delivery platform amenable to a wide range of subunit vaccine antigens. The overarching goal of the project entitled "Developing a Vaccine Platform for a Balanced Mucosal Immune Response" is to develop a pathogen-agnostic vaccine platform that elicits robust and balanced mucosal immune responses upon intranasal vaccination, in the context of a nanolipoprotein particle (NLP) platform. In year one, we investigated NLP-based vaccine formulations incorporating select immune adjuvants (Monophosphoryl Lipid A (MPLA), FSL-1, L18-muramyl dipeptide (MDP), and cholesterol-tagged ODN2006 (cCpG)) along with antigens relevant to plague (LcrV), tularemia (IglC), and Ebola virus (GP). We demonstrated the ability to prepare, characterize, and quantify these formulations. We then carried out studies *in vivo* using a BALB/c mouse model, comparing intranasal and intramuscular administration and systematically evaluated the resulting mucosal and systemic immune responses using ELISpot and ELISAs. All proposed tasks (Table 1) were completed within the twelve-month base period, and the established go/no go metric was successfully achieved. Numerous adjuvant:LcrV:NLP formulations were found to have statistically significant increases in IgA titers in serum and/or lung upon IN administration (compared to IM administration), while also producing robust IgG responses in serum. Some formulations also showed significant IFNγ responses using restimulated splenocytes. The data collected during the base period provide valuable insights for the future design of safe and effective mucosal subunit vaccines while also highlighting areas of research that merit further investigation.

Table 1: All tasks and milestones for CB11446 with projected completion dates for the Year 1 base period. as laid out in the original statement of work.

Tasks and Overall Task Milestones	Base Period											
	Year 1 (month)											
	1	2	3	4	5	6	7	8	9	10	11	12
Task 1: Develop and characterize a panel of adjuvant-bearing NLPs												
Task 1.1: Procure/prepare reagents and assemble adjuvant:NLPs												
Task 1.2 Characterize adjuvant:NLPs												
Task 2: Evaluate antigen conjugation to adjuvant:NLPs and prepare vaccine												
Task 2.1: Evaluate antigen conjugation to adjuvant:NLPs												
Task 2.2: Prepare vaccine formulations												
Task 3: Assessing mucosal and systemic immune responses												
Task 3.1: Approval for animal work												
Task 3.2: Evaluate immunological responses with LcrV antigen												
Task 3.3: Evaluate immunological responses with EBOV GP antigen												
Task 3.4: Evaluate immunological responses with Ft antigens												
M1.1: Develop protocols to incorporate adjuvants into NLPs												
M1.2: Produce at least 200 µg of 7 unique adjuvant:NLP formulations												
M2.1: Develop protocols to conjugate antigens to adjuvant:NLPs												
M2.2: Prepare at least 10 doses of each antigen:adjuvant:NLP formulations												
M3.1: Receive IACUC and ACURO approval for animal work												
M3.2: Immunogenicity measurements of adjuvant:NLPs with LcrV												
M3.3: Immunogenicity measurements of adjuvant:NLPs with GP												
M3.4: Immunogenicity measurements of adjuvant:NLPs with Ft antigens												

Project Results – Year 1

Task 1: Develop and characterize a panel of adjuvant-bearing NLPs

LLNL has an established method for NLP preparation utilizing a strategy in which scaffold proteins and lipid components, initially solubilized in surfactant, undergo dialysis to remove the surfactant and trigger self-assembly. This highly tunable process can be modified slightly to allow for the incorporation of nonpolar, polar, and/or amphiphilic molecules (Figure 1), including the selected adjuvants for this project: cCpG, FSL-1, MDP, and MPLA. We have previously used an add-back approach for incorporation of cCpG into the NLP, taking advantage of its lipophilic cholesterol moiety which self-inserts into the bilayer. The MDP molecule contains a lipophilic stearic acid while the FSL-1 is a diacylated lipoprotein, so it was hypothesized that both molecules should also be amenable to this approach. We successfully conjugated both to the NLP, verifying this using a custom Zeba-column assay, with further characterization and quantification done using reversed phase high performance liquid chromatography equipped with an evaporative light scattering detector (RP-HPLC-ELSD) (Figure 2). Due to its hydrophobic nature, the add-back approach was not suitable for MPLA, and it was instead incorporated during the NLP assembly process. The MPLA:NLPs were characterized by size exclusion chromatography (SEC) and quantified using RP-HPLC-ELSD (Figure 3).

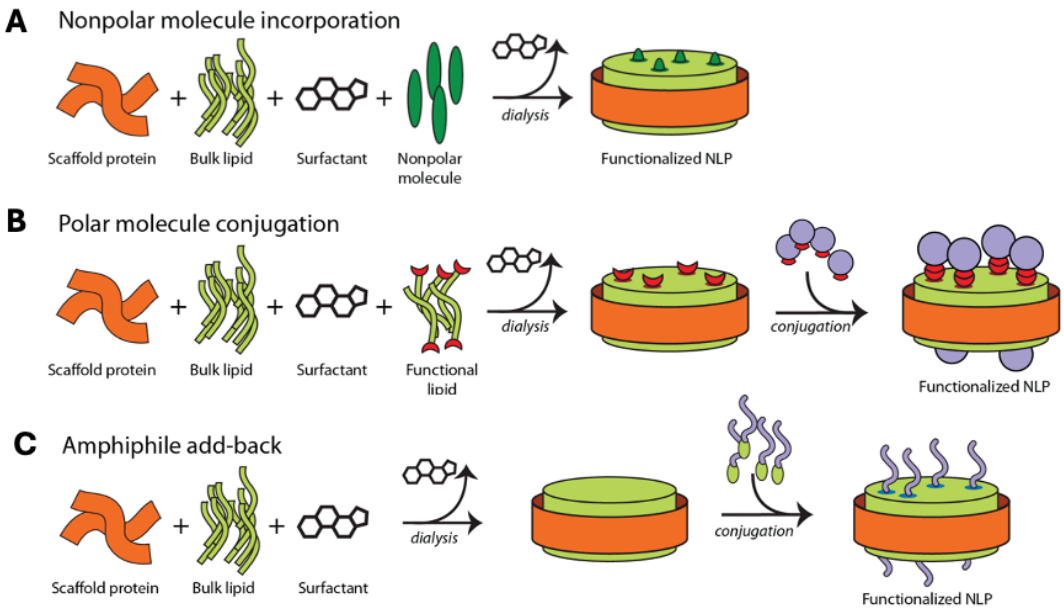


Figure 1. Three distinct strategies allow for the functionalization of NLPs with nonpolar, polar, and amphiphilic molecules. NLPs have defined regions of polarity, allowing for **(A)** incorporation of nonpolar molecules within the lipid bilayer during assembly (e.g. MPLA), **(B)** incorporation of functional lipids during assembly that allow for subsequent conjugation of polar molecules (e.g. Ni-chelating lipid to conjugate His-tagged antigens), and/or **(C)** add-back of amphiphilic molecules that self-insert into the membrane of formed NLPs (e.g. cCpG, MDP, and FSL-1).

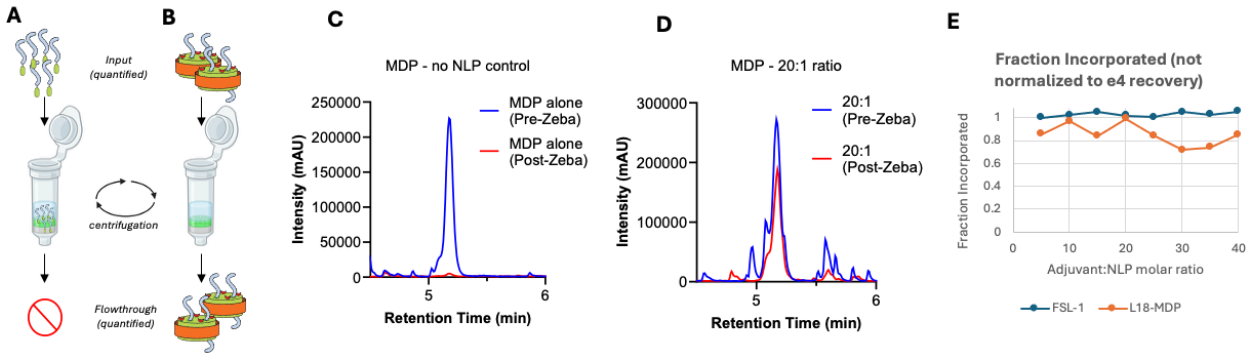


Figure 2. Zeba desalt columns can be used to rapidly assess binding of amphiphilic adjuvants to NLP, and quantification with ELSD demonstrated consistently high conjugation. (A, C) Due to their size**,** free FSL-1 or MDP will be captured in the desalt resin, with none detectable in the "post-Zeba" flow-through after centrifugation. **(B, D)** The larger NLPs will pass through the desalt resin into the flow-through, so any FSL-1 or MDP conjugated to the NLP will also be detectable in the flow-through. Representative graphs are for MDP, but results are identical with FSL-1. **(E)** FSL-1 conjugation (represented by the blue line) is consistently 100% at ratios up to 40 per NLP, with very low inherent variability. MDP conjugation (represented by the orange line) is typically above 80% up to 40 per NLP but has much higher variability.

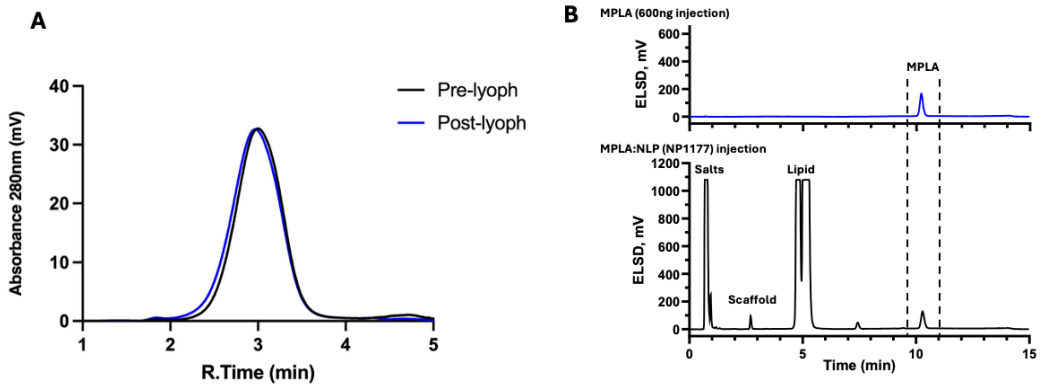


Figure 3. MPLA can be incorporated into NLPs during assembly. (A) MPLA:NLPs can be formed and purified on a 5mg scale, yielding a homogenous population that is stable after lyophilization with trehalose, as assessed by retention time on a Superose 6 column. **(B)** MPLA incorporation into an NLP (noted here as NP1777) can be quantified via RP-HPLC-ELSD, using a standard curve of pure MPLA.

Task 1 - Key Takeaways:

- Protocols were established for forming and fully characterizing NLPs incorporating FSL-1, cCpG, and MDP. These methods should be applicable to other amphiphilic adjuvants/small molecules for future formulations.
- Protocols were established for preparing and fully characterizing MPLA:NLPs. These methods should be applicable to other lipophilic adjuvants for future formulations.
- Task 1 was completed in full.

Task 2: Evaluate antigen conjugation to adjuvanted NLPs and prepare vaccines

Following verification of successful conjugation of the adjuvants to the NLP, we further tested conjugation of the antigens of interest (Lcrv from *Y. pestis*, IglC from *F. tularensis*, and glycoprotein (GP) from Ebola virus) to the adjuvant:NLPs. Conjugation of the three antigens was successful overall, though the size of the resulting NLP complexes varied with antigen and molar ratio of the protein to the NLP (Figure 4). Once antigen conjugation was achieved, vaccine formulations were designed and prepared after careful consideration of dose ranges suitable to the BALB/c mouse model as well as molar ratios of the antigen and adjuvant cargo to the NLP (Figure 5).

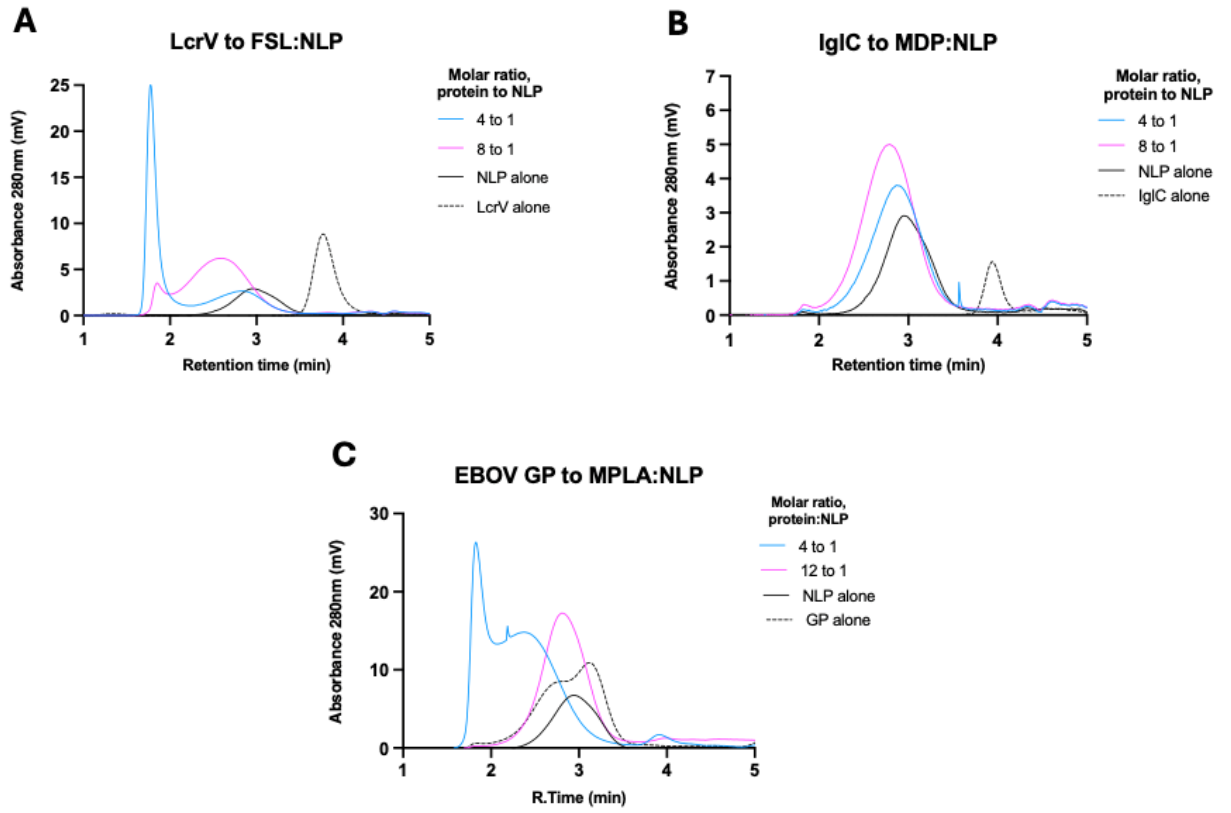


Figure 4. Representative SEC data for conjugation of selected antigens to adjuvanted NLPs. **(A)** Conjugation of LcrV to adjuvanted NLPs was successful but typically resulted in a shift to large NLP structures. **(B)** IglC conjugates as expected to adjuvanted particles, with a shift to slightly larger sizes that is ratio dependent. **(C)** EBOV conjugates successfully to adjuvanted particles, forming large NLP structures or small structures, depending on the molar ratio.

A

	Adjuvant MPLA	Adjuvant chol-CpG 2006	Adjuvant FSL-1	Adjuvant L18-MDP	Antigen LcrV
Dose (µg)	0.5	5	2	1	10
Molar ratio to NLP	3.2	5.8	11.8	12.9	2.6

B

	Adjuvant chol-CpG 2006	Adjuvant FSL-1	Adjuvant Ft LPS	Antigen IglC
Dose (µg)	2	1	5	20
Molar ratio to LPS:NLP	1.1	2.8	15.1	3.8

C

	Adjuvant chol-CpG 2006	Adjuvant FSL-1	Antigen EBOV GP
Dose (µg)	2	1	5
Molar ratio to NLP	10.3	26.1	3.0

D

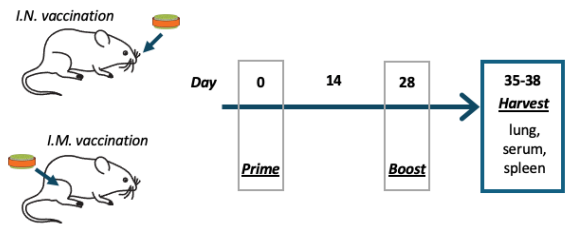


Figure 5. Representative adjuvant and antigen doses, along with their respective molar ratios to the NLP in complete vaccine formulations. The amount of NLP was dictated by the selected 10µg dose of LcrV **(A)**, 20µg dose of IglC **(B)**, and 5µg dose of EBOV GP **(C)**. Molar ratios for the adjuvants were subsequently calculated based on the amount of NLP required to achieve the desired molar ratio for the antigen. The NLP used for IglC studies contained 5µg of lipopolysaccharide from *F. tularensis* (which acts as an additional antigen), and lower doses of cCpG and FSL-1 were used for both the IglC and EBOV formulations, due to adverse effects seen with higher cumulative adjuvant doses in earlier studies. **(D)** Vaccination studies were conducted using female BALB/c mice and a prime-boost regimen, four weeks apart, with samples collected 7-10 days following the boost. Administration was done both intramuscularly and intranasally, for direct comparison of formulations within a study.

Task 2 - Key Takeaways:

- LcrV, IglC, and EBOV GP antigens were successfully conjugated to adjuvant:NLPs.
- Vaccine formulations were prepared for in vivo studies, with antigen and adjuvant amounts selected based on suitable dose ranges and suitable conjugation ratios to the NLP.
- Task 2 was completed in full

Task 3: Assessing mucosal and systemic immune responses

In vivo studies initially focused only on LcrV formulations, while systematically assessing first single adjuvants, then formulations with increasing adjuvant complexity. Lung homogenates and sera collected at the established timepoint (7-10 days post-boost) were then analyzed for IFNγ responses using ELISpot or antibody titers using ELISAs. IFNγ responses were mostly low and non-significant, apart from FSL-1+ cCpG and FSL-1+MDP, which both showed significantly higher responses in the IN groups than the IM groups, with LcrV (Figure 6). ELISAs were used to assess IgG and IgA titers in both lung and serum at the same harvest timepoint. Multi-adjuvant formulations demonstrated robust IgG titers in both serum and lung, noticeably higher than with single adjuvant formulations. No significant differences were seen between the IM and IN groups for IgG (Figure 7) whereas numerous groups showed statistically significant (pairwise T-test between IN vs IM groups) increases in IgA titers in serum and/or lung when the formulations were administered intranasally (Figure 8). The FSL-1+ cCpG formulation was further tested within the context of an Ft vaccine (IglC + LPS) and an EBOV GP vaccine formulation. Significantly higher IFNγ was seen for the adjuvanted Ft vaccine, though there was almost no measurable signal in the same formulation with EBOV. There were no significant titer results for these two formulations (Figure 9). Most of these vaccines were well-tolerated, though select formulations (with FSL-1 doses above 1µg) elicited more severe clinical signs in the animals, so some experiments were modified slightly with adjusted doses or elimination of the boost, due to anticipated morbidity (Figure 10).

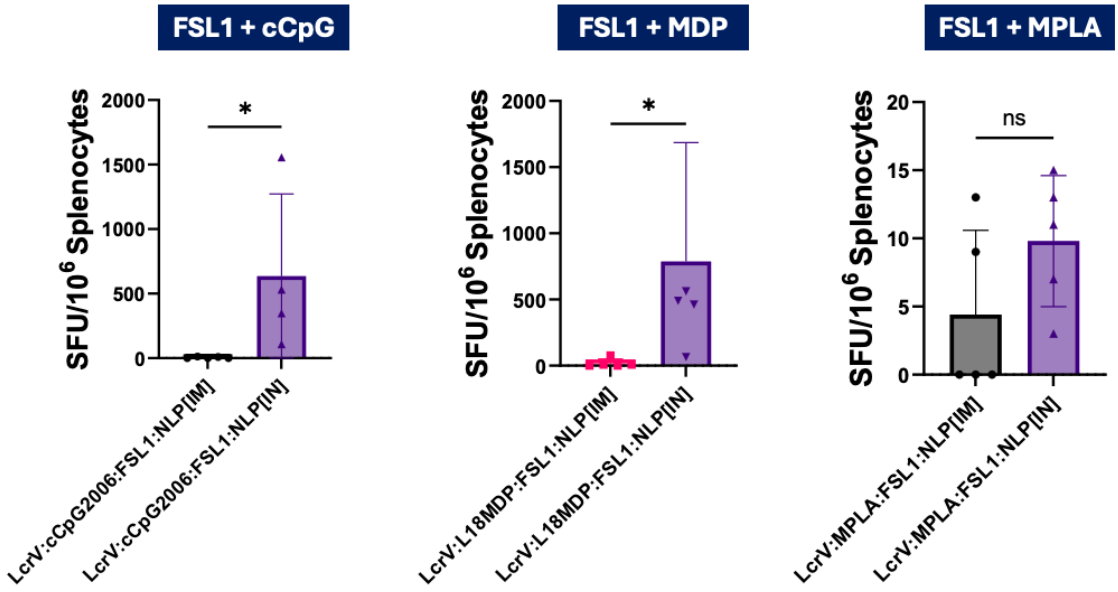


Figure 6: IFNγ ELISPOT of dual formulations in vaccinated animals comparing IM vs IN vaccine administration routes. Splenocytes restimulated with LcrV showed significant IFNγ responses using FSL-1 in combination with cCpG and MDP when vaccine administered intranasally.

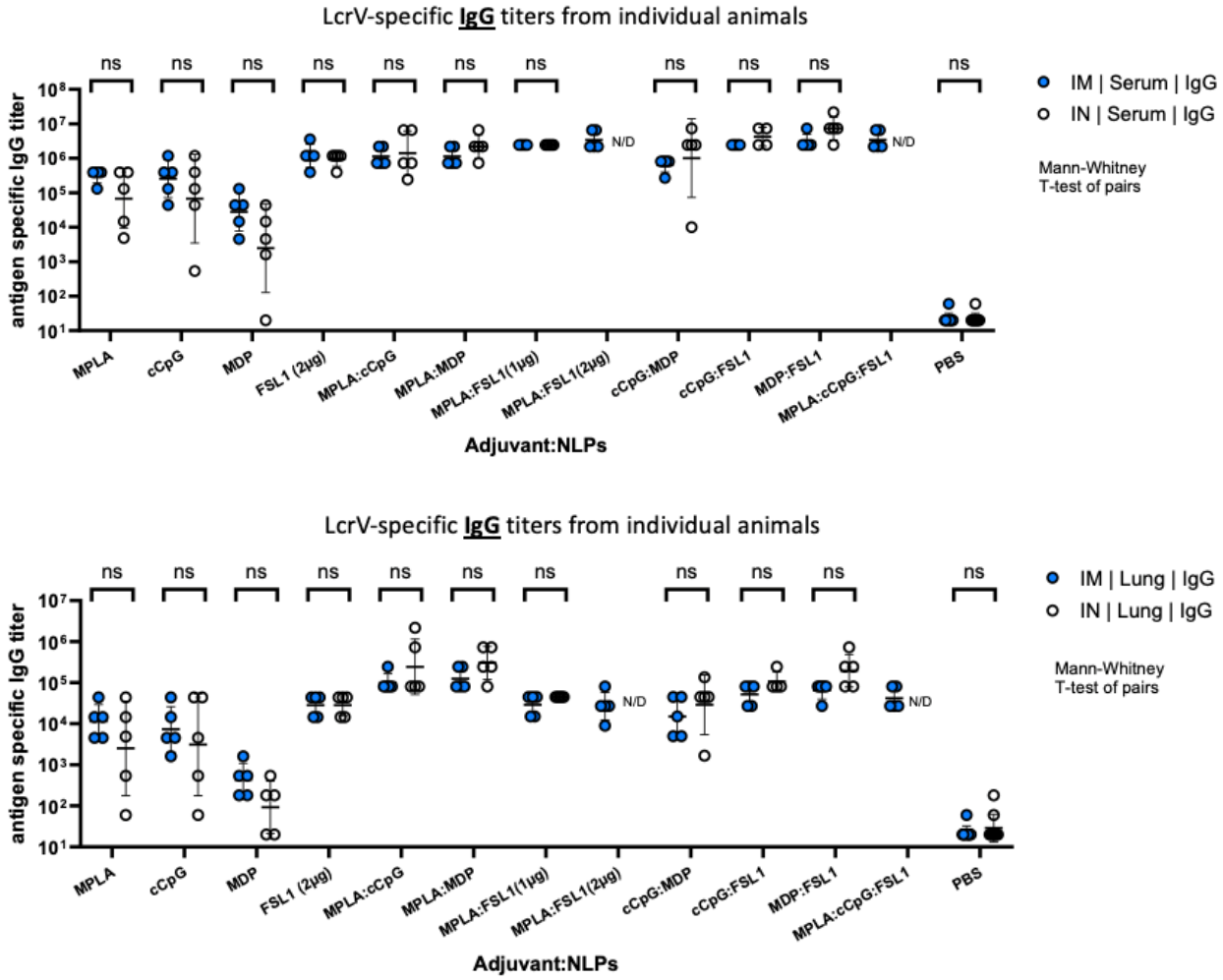


Figure 7. IgG antibody titers against the Yp antigen LcrV in serum and lung. All formulations demonstrated measurable IgG tiers in serum, with no statistical differences between IM and IN administration (top). Dual formulations showed particularly robust titers across the board. IgG titers in the lung were lower than in the serum, as anticipated, but dual formulations still produced robust titers overall (bottom). Mann-Whitney T-test of pairs (IN vs IM); ns = no significance. N/D = not determined.

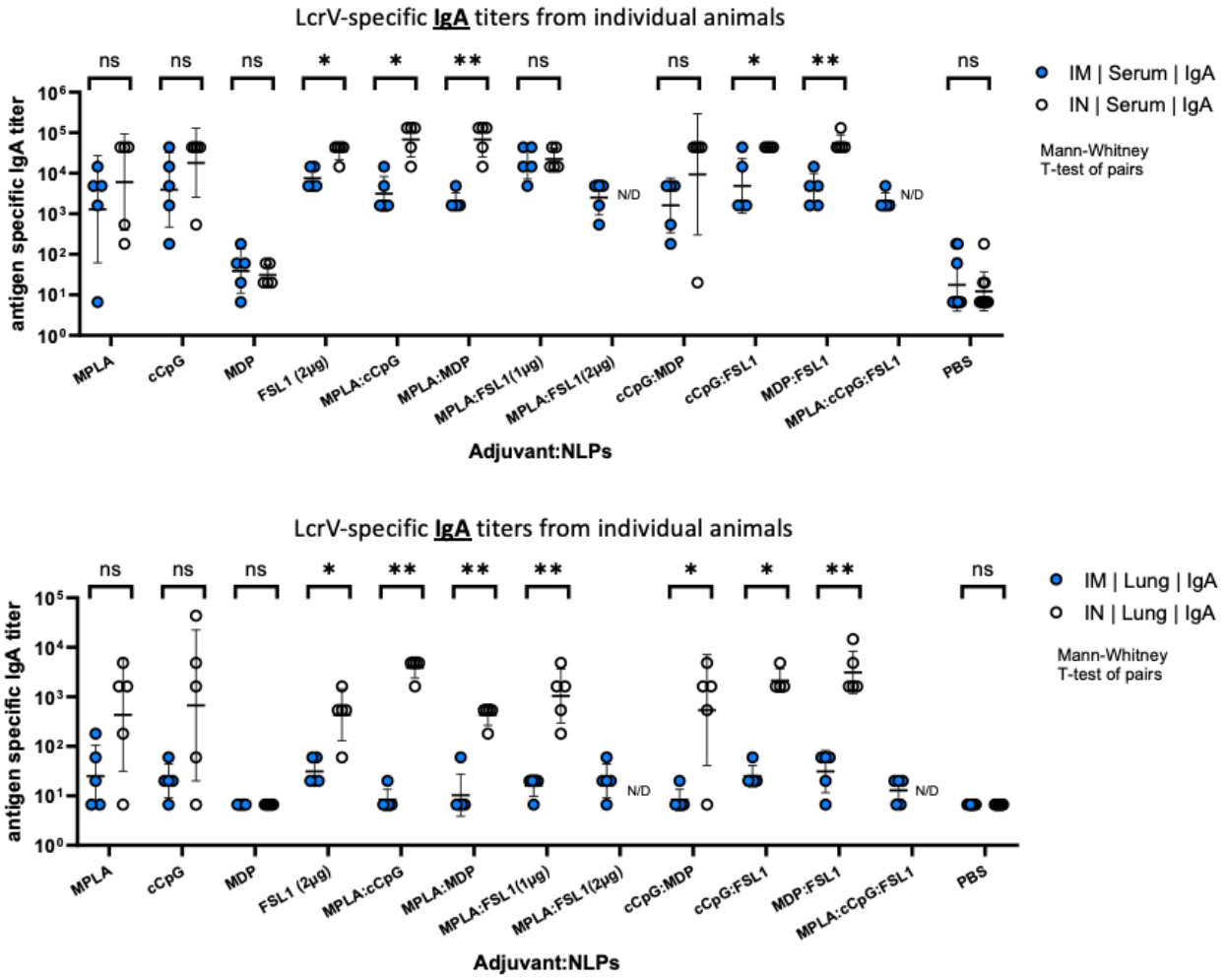
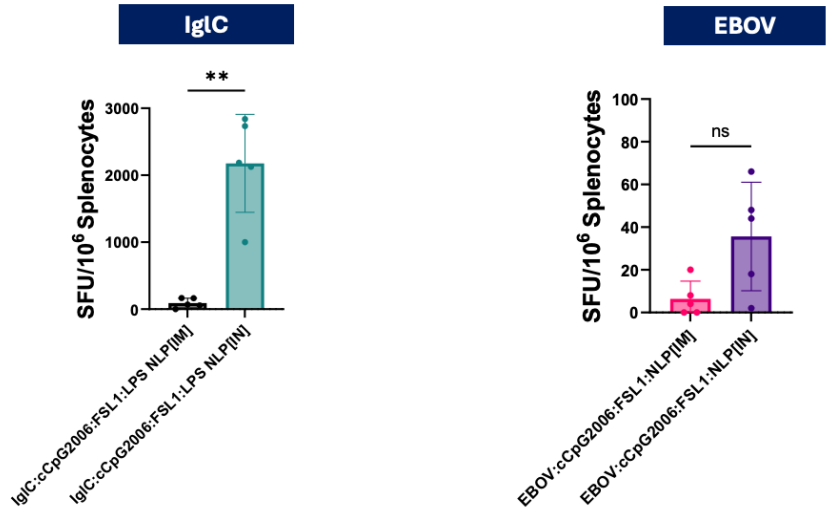


Figure 8. IgA antibody titers against the Yp antigen LcrV in serum and lung. Five formulations have statistically significant increases in serum IgA titers upon IN administration (top). Seven formulations have statistically significant increases in lung IgA titers upon IN administration (bottom). Mann-Whitney T-test of pairs (IN vs IM); ns = no significance * $p < 0.05$, ** $p < 0.01$. N/D = not determined.

A



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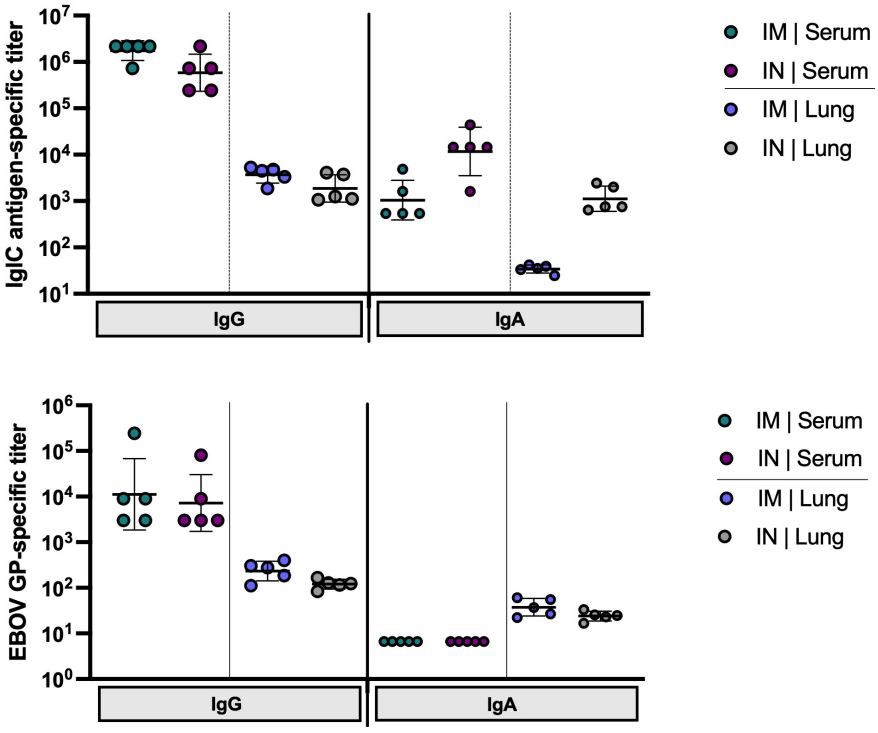

Figure 9: CpG+FSL-1 formulation further investigated with Ft and EBOV antigens. **(A)** Splenocytes restimulated with full protein IgIC showed significant IFNγ responses using adjuvant combination, FSL-1 + cCpG when administered intranasally. Conversely, EBOV antigen-specific IFNγ levels were low regardless of vaccination route. **(B)** Antibody titers were strong for the IgIC formulation, though not significant. The EBOV formulation produced very poor IgG and IgA responses overall.

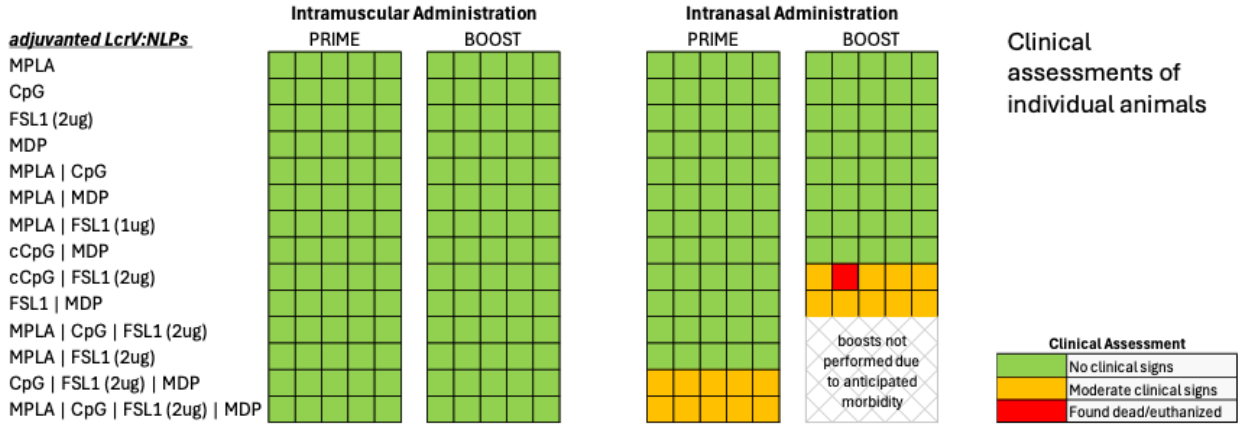

Figure 10. Sensitivity is observed to multi-adjuvant formulations administered intranasally (but not intramuscularly) Combinations that include FSL-1 (at doses above 1µg) elicited more severe clinical signs. Prime vaccinations were tolerated (except for CpG|FSL-1|MDP combinations), but boosts elicited severe effects (or were not performed).

Task 3 - Key Takeaways:

- One formulation (CpG + FSL-1) showed significant IFNγ responses upon IN administration for both LcrV and IglC vaccines, though it produced almost no response for EBOV GP.
- IgG titers were robust overall in both lung and serum, particularly in multi-adjuvant formulations, with no statistical differences between IM and IN groups.
- Five formulations had statistically significant increases in serum IgA titers upon IN administration while seven formulations had statistically significant increases in lung IgA titers upon IN administration.
- Animals demonstrated sensitivity to select formulations using FSL-1 with IN administration, so doses and experiments were modified accordingly.
- The go/no-go metric was successfully achieved.

Conclusions

We have met and exceeded the goal of this project by demonstrating that seven tested formulations elicit superior mucosal immune responses upon intranasal vaccination compared to intramuscular vaccination in the context of an LcrV:NLP vaccine**.** The intranasal vaccinations elicit comparable humoral responses, especially in the dual adjuvant formulations, though some formulations (containing >1µg of FSL-1) induced sensitivity in the mice. A top performer (CpG+FSL-1) was further tested in the context of Ft and EBOV vaccine formulations, yielding significantly higher IFNγ levels for the Ft vaccination for intranasal administration, matching results from the LcrV vaccine, though titer data was not comparable. The platform validation and testing pipeline established during this project can easily be applied to other adjuvants, antigens, and combinations thereof.

In summary, NLPs are an excellent platform for formulating and delivering multiple adjuvant/antigen combinations to elicit strong mucosal immune responses.

In Year 2 of CB11446, the focus will be on evaluating efficacy of IN formulations identified in Y1, using *Y. pestis* and Ebola virus challenge models (depending on DTRA priorities). In addition, in-depth analysis of immunological responses of lead NLP formulations upon IN administration will be conducted by spectral flow cytometry. Additionally, lymphoid tissue and lymphocyte responses in the lung will be investigated.

Acknowledgements

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