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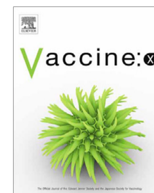

Antigen delivery format variation and formulation stability through use of a hybrid vector

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A B S T R A C T

A hybrid biological-biomaterial antigen delivery vector comprised of a polymeric shell encapsulating an *Escherichia coli* core was previously developed for *in situ* antigen production and subsequent delivery. Due to the engineering capacity of the bacterial core, the hybrid vector provides unique opportunities for immunogenicity optimization through varying cellular localization (cytoplasm, periplasm, cellular surface) and type (protein or DNA) of antigen. In this work, three protein-based hybrid vector formats were compared in which the pneumococcal surface protein A (PspA) was localized to the cytoplasm, surface, and periplasmic space of the bacterial core for vaccination against pneumococcal disease. Furthermore, we tested the hybrid vector's capacity as a DNA vaccine against *Streptococcus pneumoniae* by introducing a plasmid into the bacterial core to facilitate PspA expression in antigen presenting cells (APCs). Through testing these various formulations, we determined that cytoplasmic accumulation of PspA elicited the strongest immune response (antibody production and protection against bacterial challenge) and enabled complete protection at substantially lower doses when compared to vaccination with PspA + adjuvant. We also improved the storage stability of the hybrid vector to retain complete activity after 1 month at 4 °C using an approach in which hybrid vectors suspended in a microbial freeze drying buffer were desiccated. These results demonstrate the flexibility and robustness of the hybrid vector formulation, which has the potential to be a potent vaccine against *S. pneumoniae*.

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1. Introduction

Streptococcus pneumoniae is a major human bacterial pathogen responsible for pneumococcal disease with outcomes spanning pneumonia, otitis media, and bacteremia [1]. Pneumococcal infections are responsible for approximately 445,000 hospitalizations and 22,000 deaths per year in the US alone [2]. Globally, the impact of this pathogen is far greater as it represents one of the primary causes of death in children under five, particularly in low-income regions [3,4]. The current standard of care in the prophylactic treatment against *S. pneumoniae* is glycoconjugate vaccines, such as Prevnar 13, which contain capsular polysaccharides (CPSs) from multiple *S. pneumoniae* serotypes conjugated to a carrier protein (i.e., CRM197) [5]. Although the implementation of pneumococcal conjugate vaccines (PCVs) has significantly reduced the burden of pneumococcal disease in high income countries [6], significant concerns regarding the long term utility of this approach remain. Glycoconjugate vaccines provide potent serotype-specific protection; however, current PCVs cover only 13 of the 90+ disease-causing serotypes of *S. pneumoniae*. Furthermore, the geographic variations in serotype prevalence as well as niche replacement by

Abbreviations: PspA, pneumococcal surface protein A; APCs, antigen presenting cells; CPSs, capsular polysaccharides; PCVs, pneumococcal conjugate vaccines; NVT, non-vaccine type; PhtD, histidine triad protein D; PcpA, pneumococcal choline-binding protein A; CFA, Complete Freund's Adjuvant; LBVs, live bacterial vectors; PAMPs, pathogen-associated molecular patterns; PHV, periplasmic hybrid vector; CHV, cytoplasmic hybrid vector; SHV, surface hybrid vector; ClyA, cytolysin A; pHV, plasmid hybrid vector; LLO, listeriolysin O; CDM, chemically defined bacterial growth medium; SC, subcutaneous; IP, intraperitoneal; IN, intranasal; EHV, empty hybrid vector; AS, aqueous storage; DS, desiccated storage.

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non-vaccine type (NVT) serotypes [7] create a pressing need for pneumococcal vaccines with increasing valency. Manufacturing challenges associated with PCVs limit perpetual expansion of coverage to effectively deal with emergent and less invasive serotypes [8].

The deficiencies of PCVs have encouraged the development of alternative vaccination strategies utilizing conserved protein antigens due to the potential for serotype-independent coverage [9,10]. Furthermore, protein vaccines do not require the chemical synthetic step (reductive amination) required for the scaled manufacture of glycoconjugate vaccines. To date, attempts to develop protein-based pneumococcal vaccines have included histidine triad protein D (PhtD), pneumolysoid, pneumococcal choline-binding protein A (PcpA), and pneumococcal surface protein A (PspA) [11–13]. PspA, in particular, represents a model pneumococcal protein antigen and has been studied extensively for its ability to prompt a humoral response and provide a degree of protection against pneumococcal disease in mice [14–17]. Conventionally, such protein antigens are delivered in conjunction with adjuvants (e.g., Complete Freund's Adjuvant (CFA) or alum) to enhance or modulate the vaccination strategy due to the limited immunogenicity of protein-based subunit vaccines compared to alternative killed or attenuated bacterial options [18]. Such adjuvants often elicit antibody-driven humoral responses; however, recent evidence suggests that cellular immunity also plays an important role in the clearance and prevention of bacterial infections such as *S. pneumoniae* [19,20].

Live bacterial vectors (LBVs) delivering protein antigens represent a promising alternative to traditional adjuvants. Pathogen-associated molecular patterns (PAMPs), which interact with receptors on antigen presenting cells (APCs), are innately associated with bacterial systems and enable the stimulation of a mixed cellular and humoral immune response [21,22]. Furthermore, this approach harnesses cellular machinery to rapidly produce protein antigens within the bacterial cell, thus, serving as a delivery vehicle and providing a consolidated and cost-effective methodology for *in situ* antigen production [21]. To date LBVs, such as attenuated *Salmonella* and lactic acid bacteria (i.e., *Lactococcus* and *Lactobacilli*), have been used to deliver PspA within immunization studies against *S. pneumoniae* [23–26].

Taking advantage of this approach, we developed a hybrid biological-biomaterial vector, hereafter known as the "hybrid vector," comprised of a nonpathogenic *E. coli* core and a cationic polymeric coating [27]. Like LBVs, the hybrid vector naturally expresses PAMPs and is a size optimal for APC uptake (~2 μm) [28], which results in comparable or stronger immune reactivity properties relative to traditional adjuvants [22]. In addition, the delivery and adjuvant properties of this vector were further enhanced by coating the *E. coli* core with a cationic polymer (D4A4), chosen from a library of poly(beta-amino esters) screened for optimal APC delivery, covalently tethered to mannose (D4A4-Man) to accentuate APC recognition and localization through receptor-mediated targeting [27,29–31]. Upon phagocytic uptake by APCs, antigens within the hybrid vector would be selectively delivered via the natural endosomal-lysosomal processing of foreign bodies for downstream immune presentation. Certain versions of the hybrid vector also include heterologous expression of the listeriolysin O (LLO) gene from *Listeria monocytogenes* to prompt endosomal escape upon intracellular uptake and lysosomal processing initiation by APCs [27], with the objective of routing antigen delivery for enhanced cellular presentation (Fig. 1).

Preliminary work with the hybrid vector demonstrated improved protection against pneumococcal pneumonia and sepsis when compared to traditional vaccine formulations (i.e., PspA + alum, PspA + CFA) by delivering PspA accumulated within the bacterial periplasm [22]. We initially tested the improved long-term stability of this particular hybrid vector construct by identifying the storage conditions aligned with cold chain requirements (i.e., 2–8 °C) [32]. In addition, we sought to enhance the immunogenicity and protective efficacy of the hybrid vector to highlight its potential as an effective pneumococcal vaccine. This was accomplished by evaluating the impact subcellular localizations and antigen types had on the hybrid vector's potency. Immunogenicity and protective efficacy of these formulations were analyzed through antibody titers and in pneumococcal challenge models, respectively. The results herein represent the development of a second-generation hybrid vector formulation with the potential to provide strong and lasting protection against pneumococcal disease.

2. Results and discussion

2.1. Storage stability assessment

For many vaccine formulations, vaccine activity can be significantly reduced when stored outside of cold-chain (i.e., temperature-controlled supply chain) temperatures [33], which typically ranges from 2 to 8 °C. For prolonged durations [34], live attenuated vaccines, in particular, have demonstrated pronounced sensitivity to temperature fluctuations [32]. To test the stability of the hybrid vector design, we assessed a version developed previously (the periplasmic hybrid vector [PHV]) [22]. The hybrid vector vaccine demonstrated complete loss in activity after two weeks of storage in phosphate-buffered saline (PBS) at 4 °C (Fig. 2A and B). Therefore, we applied methods known to improve the retention of immunogenicity of the hybrid vector. We tested the impact that several storage buffers (trehalose and a microbial freeze drying buffer [storage buffer]) and desiccation had on the hybrid vector's stability when stored for various durations (1, 7, 14, 28 days) at 4 °C.

Stability of the stored hybrid vector was determined through quantifying the antibody response and protective efficacy of the hybrid vector in a sepsis challenge model for both the aqueous and desiccated storage formats. Long-term aqueous storage in PBS, trehalose, and the storage buffer resulted in a more pronounced loss of immunogenicity (Fig. 2A) and protection (Fig. 2B) when compared to the freshly-prepared formulation. However, desiccating the hybrid vector improved retention of immunogenicity (Fig. 2A) and protective efficacy (Fig. 2C) across all storage solutions. For example, survival in mice immunized with vectors desiccated from PBS and trehalose stored for 1 week increased from 16% to 83% and 67% to 100%, respectively. However, the largest improvement was observed using the storage buffer, which offered complete protection after a month-long storage period in the desiccated format. These results demonstrate that the hybrid vector can retain activity within the cold chain, thus, increasing its utility as a pneumococcal vaccine solution.

2.2. Antigen localization and production within the hybrid vector

The previously developed PHV featured a truncated form of PspA (amino acids 1-302) localized to the periplasm and provided protection against pneumococcal pneumonia in murine models [22]. Since the D4A4-Man polymeric coating portion of the hybrid device had been previously optimized for APC targeting [22], we focused upon the antigen content of the bacterial core in the course of this study. We initially varied the cellular localization of PspA, localizing the truncated gene encoding PspA (from the plasmid used in the PHV construct) to plasmids designed to direct protein products to the cytoplasm and bacterial surface (Fig. 1), thus, generating two new hybrid vector formulations. Cytosolic targeting was achieved using the commercially available plasmid

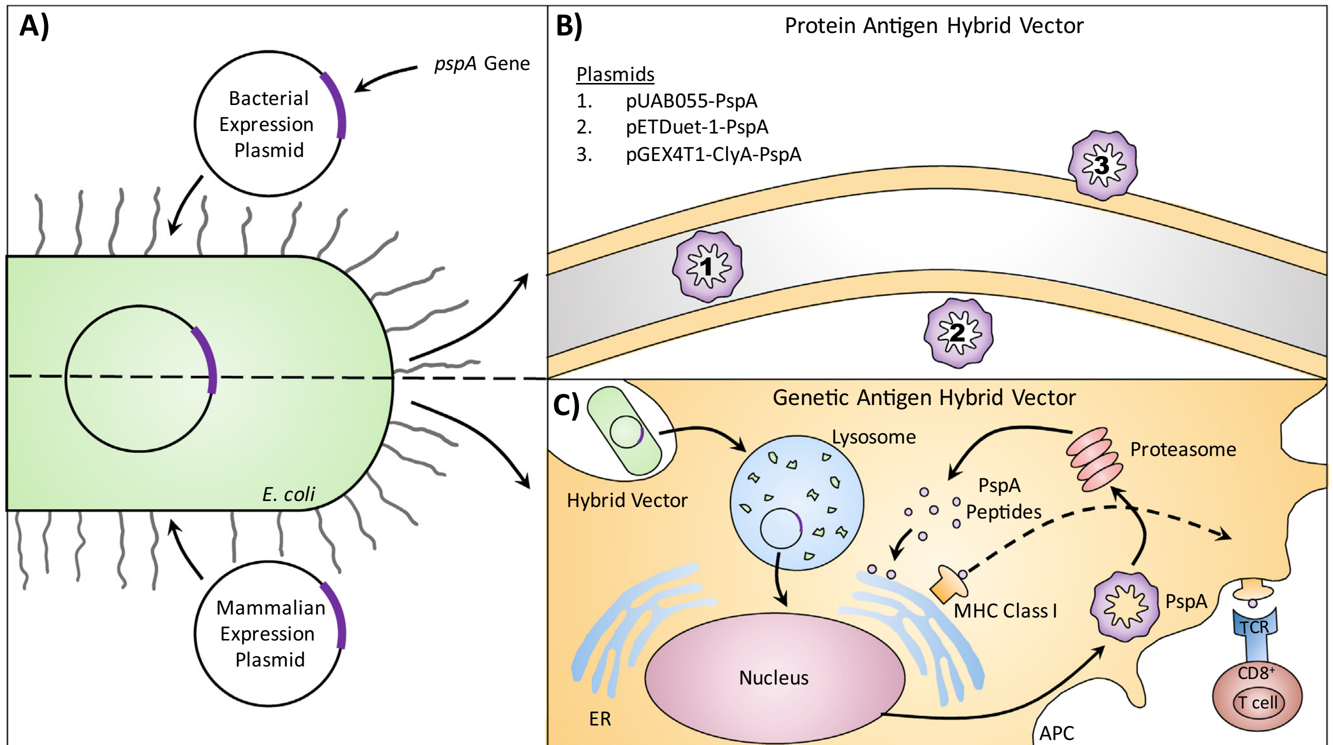

Fig. 1. Schematic of protein and DNA hybrid vector formulations. (A) Transformation of bacterial expression plasmids (protein production and localization) and mammalian expression plasmid (DNA delivery) into the hybrid vector bacterial core. (**B**) Three plasmids were used to localize PspA in various bacterial cellular locations including the periplasm (1), cytoplasm (2), and surface (3). (**C**) A proposed pathway for the uptake and processing of hybrid vectors containing the mammalian expression plasmid encoding PspA.

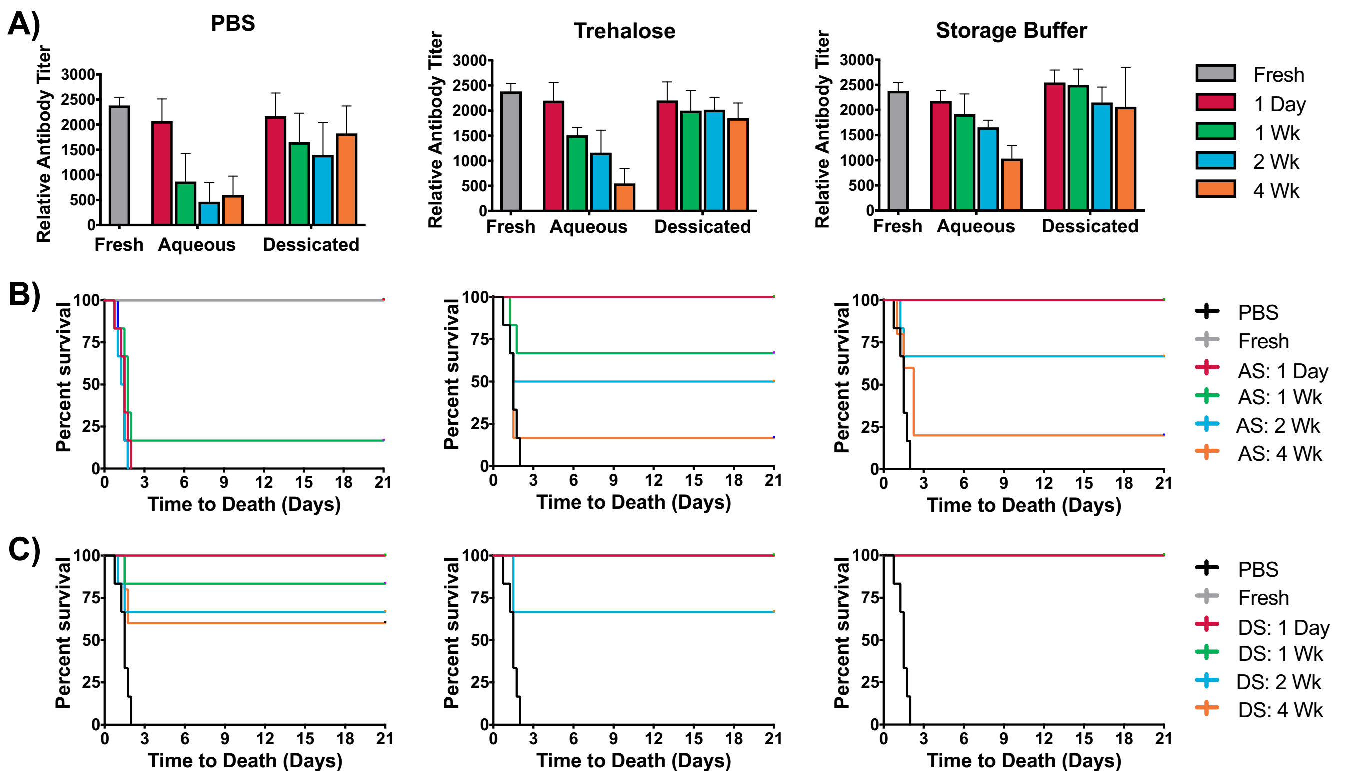

Fig. 2. Evaluation of the periplasmic hybrid vector storage. (A) Relative antibody titers from mice immunized with periplasmic hybrid vector stored in PBS, trehalose, and storage buffer in both aqueous and desiccated formats for varied lengths of time relative to freshly prepared vector. Error bars represent 95% confidence intervals of three replicates. (**B**) Pneumonia challenge model in mice vaccinated with various hybrid vector formulations with PBS (sham) controls. (**C**) Pneumonia challenge model in mice vaccinated with various hybrid vector formulations with sham controls. The 4 Wk samples in trehalose performed identical to the 2 Wk samples. Aqueous storage (AS), desiccated storage (DS).

pETDuet™-1, thus, creating the cytoplasmic hybrid vector (CHV). Alternatively, the surface hybrid vector (SHV) variant achieved surface localization of PspA by fusion to an *E. coli* membrane protein (cytolysin A [ClyA]), which has been shown in recent studies to localize fusion partners such as GFP and β-lactamase to the bacterial surface without altering biological activity [35,36]. In this study, cellular localization of PspA was assumed through use of the aforementioned expression plasmids. However, we acknowledge that future efforts to experimentally confirm PspA bacterial localization (or to confirm alternative antigens) would be needed to further strengthen subsequent results in comparing the final hybrid vector variants.

Once the bacterial strains of the three hybrid vectors had been finalized, culturing conditions (induction time, induction temperature, and isopropyl β-D-1-thiogalactopyranoside (IPTG) inducer concentration) for each of these constructs were varied systematically to optimize the soluble PspA titer by lysing formulated hybrid vectors and quantifying protein in the supernatant using a densitometry analysis of SDS-PAGE gels (Fig. 3A & B). This was compared to the whole-cell protein (all protein contained within the hybrid vector), which includes insoluble protein. Comprehensive results for whole-cell and soluble protein concentrations can be found in Supplementary Table 1. Through this analysis, it was found that the maximum yield of soluble PspA (119.4 μg/OD_{600nm}) was obtained when the CHV was induced with 1 mM IPTG and grown at 37 °C for 8 h (Supplementary Table 1). However, when compared to protein produced at 30 °C for 8 h, the higher temperature conditions generally result in a marked reduction in PspA solubility (Supplementary Table 1). This decrease in overall PspA solubility was most pronounced (76%) for the periplasmic hybrid vector (PHV) after an 8-hour induction using 100 μM IPTG, indicating that PspA thermal stability may be reduced within the bacterial periplasm (Fig. 3C). Interestingly, however, we observed that even with conditions that resulted in insoluble protein antigen content, the associated hybrid vector could still retain vaccine effectiveness (Fig. S1). Induction conditions of 30 °C for 8 h were determined to be optimal for hybrid vectors to retain protein solubility while maximizing protein yield. Finally, in an effort to limit the costs associated with IPTG, used to induce gene expression [37], we decreased the induction concentration to 100 μM which reduced final protein yield by an average of 36%. In this way, we obtained hybrid vectors with optimized PspA production (economically and scientifically) for use in immunogenicity and efficacy experiments.

2.3. Immunogenicity and protective efficacy of protein expressing hybrid variants

The PHV was previously tested as a vaccine option against pneumococcal pneumonia when 10^7 vectors were administered per dose [22]. Consequently, mice were subcutaneously (SC) immunized with 10^7 vectors of PHV, CHV, and SHV formulations, which resulted in PspA ranging from 0.2 to 0.7 μg/dose depending on the formulation (Table 1). It should be noted that despite the presence of lipopolysaccharides (LPS) in the *E. coli* membrane, the hybrid vector did not elicit any adverse effects, a result we have repeatedly observed [22,27] and likely due to the overall reduced toxicity associated with the base bacterial strain of the hybrid vector [38] and the attenuating and complementary adjuvant properties contributed by the surface cationic polymers [27,39]. As shown in Fig. 4A, vaccine immunogenicity following the initial immunization was positively correlated with PspA concentration. However, this trend was not apparent during the secondary immune response (i.e., post booster). When immunized mice were challenged with *S. pneumoniae* D39 (Serotype 2), only the CHV and PHV offered complete (100% survival) protection during the pneumonia challenge model (Fig. 4C). These two formulations also outperformed the SHV formulation in the sepsis challenge model, with the CHV and PHV providing complete or near complete (83% survival) protection, respectively (Fig. 4B). However, the differences in protection provided by the various formulations most likely result from the inconsistent PspA doses administered (Table 1) rather than antigen localization within the hybrid vector.

To eliminate PspA level as a variable, immunizations were standardized to 1 μg soluble PspA that, based on previous results (Fig. 4), is able to provide complete protection using the CHV. The number of cells per dose for each vector is tabulated in Table 1 and ranges from 1.32 to 5.93×10^7. While all hybrid vector formats were capable of eliciting strong relative antibody titers, immunization with the CHV and the SHV represented the most immunogenic formulations, with no statistically significant difference between the two ($p = 0.98$) (Fig. 5A). In the comparison between the CHV and SHV formats, however, it is important to recognize the difference in total vector numbers administered (Table 1). The >4-fold vector number increase for the SHV format relative to the CHV format suggests that vector-specific aspects (e.g., adjuvant capability) may also be contributing to resulting outcomes, while also suggesting that antigen localization influences results on a per antigen and per hybrid vector basis. Variation in immune response due to antigen localization did not have an impact on the efficacy of the hybrid vector at doses of 1 μg soluble protein, as all formulations provided complete protection (time to death ≥ 21 days) in the sepsis challenge model (Fig. 5B). This likely indicates that all hybrid vector formulations delivered a PspA dose greater than that of the minimum effective dose. As such, further experimentation is required to confirm whether localization impacts protection at lower PspA concentrations. Regardless, the hybrid vector demonstrates a strong response when compared to the control 15 μg PspA + CFA/IFA, which only extended the time to death to approximately 5 days, a result that has been observed in previous studies [22]. The only control in this study capable of providing complete protection in pneumococcal pneumonia challenge (100 μg PspA + CFA/IFA; Fig. 4C) was unable to provide complete protection in the sepsis challenge (Fig. 4B). In contrast, the CHV was able to provide complete protection against both sepsis and pneumonia with only 1 μg of PspA. In other words, the hybrid vector can elicit comparable/stronger immune responses with greatly reduced PspA levels relative to standard vaccination strategies, likely due to the inherent adjuvant properties associated with the bacterial and biomaterial components and vector-specific features (hybrid component design, phagocytic size restriction) that heighten antigen delivery.

PspA localization also had an impact on the antibody class distribution, exemplified by the class-switching from immunoglobulin M (IgM) to immunoglobulin G (IgG) within B cells, which was most pronounced for SHV and CHV (Fig. 5C). As IgM to IgG antibody class-switching often signifies the generation of immunological memory [40], and therefore is a strong indication of the long-term success of a vaccination strategy [41], the CHV and SHV demonstrate significant potential for providing potent protection against pneumococcal disease. Despite eliciting varying levels of IgG expression, localization had minimal impact on the IgG subtype profile (i.e., IgG1:IgG2a ratio), as detailed in Table 2. While values ranged from 1.58 to 0.31, the ratios still represent a balanced IgG1:IgG2a response [42]. As IgG1 and IgG2a signify Th2 and Th1 responses, respectively, in mice [42,43], this data also suggest that the hybrid vector generates a balanced Th1/Th2 response. Despite the common belief that clearance of *S. pneumoniae* is primarily dependent on a Th2 response, recent evidence now suggests that this response may limit complement binding to the bacterial surface. In contrast, a balanced Th1/Th2 ratio has been shown to improve complement binding and bacterial clearance [44]. Therefore, as most adjuvants used in anti-bacterial vaccines elicit a primarily Th2 response [45], the balanced response generated by the

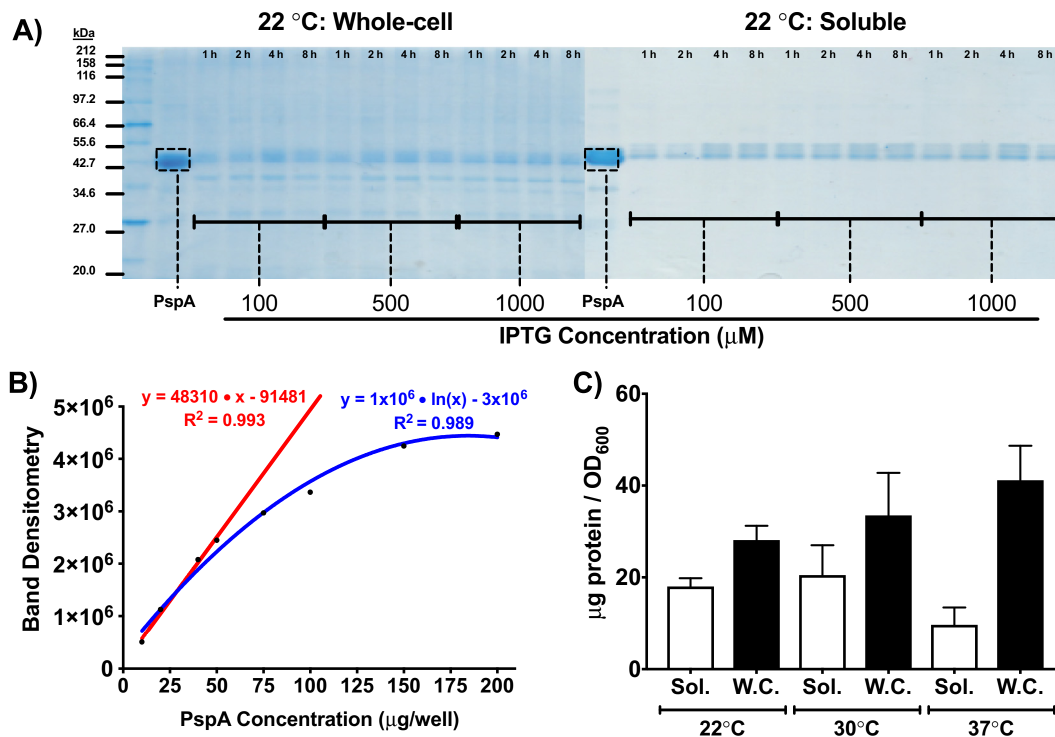

Fig. 3. Panels for periplasmic hybrid vector (pUAB055-*pspA*) showing (A) SDS-PAGE gels for whole-cell and soluble PspA at 22 °C across varied IPTG concentrations and induction times. (**B**) Standard curves for quantity of PspA per well versus SDS-PAGE gel band density using linear (red) and logarithmic (blue) regressions. (**C**) Yield of soluble vs insoluble (difference between whole cell and soluble protein values) PspA across varied induction temperatures (at 100 μM IPTG and 8 h post-induction incubation). Error bars represent the 95% confidence interval of three replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Hybrid vector and PspA administration concentrations.

Hybrid vector variant	Hybrid vector dose for 1 μg PspA	PspA dose (μg/dose) for 10^7 Hybrid Vectors	
		Soluble protein	Insoluble
Cytoplasmic	1.32×10^7	0.70	0.17
Periplasmic	2.39×10^7	0.36	0.10
Surface	5.93×10^7	0.26	0.04

hybrid vector should provide improved protection against pneumococcal disease when compared with standard adjuvants.

Combined, these results suggest that PspA localization to either the bacterial cytoplasm or surface can improve immunogenicity relative to the original PHV formulation. Both the CHV and SHV elicited a more robust immune response with a greater shift towards IgG production. While there was no statistical difference between the immune responses generated by these vectors, the CHV produced 3.5× more soluble PspA than the SHV, indicating that this vector not only has the greatest potential to provide lasting protection against pneumococcal disease, but also provides the best opportunity for dose reduction when dose is defined by hybrid vector count. In other words, the CHV offers the potential for a ~4-fold decrease in the number of hybrid vectors required to achieve complete protection when compared to the SHV. Therefore, we can conclude that the CHV represents the next generation in the development of the hybrid vector for pneumococcal disease application.

2.4. Hybrid vector as DNA vaccine

The hybrid vector was designed to increase uptake and processing by APCs [27,29–31], thus, serving as a potential DNA vaccine delivery vehicle. Although most efforts in DNA vaccine development have been directed towards validating therapeutic vaccines against cancers and intracellular pathogens [46,47], this strategy has recently been applied to extracellular bacterial diseases (e.g., *Pseudomonas aeruginosa* [48]) due to the ability for DNA vaccines to stimulate both humoral and cellular immune responses [49]. Moreover, such approaches have also demonstrated potential for providing protection against *S. pneumoniae* [46,50]. For example, immunization with a DNA vaccine vector expressing the 2/3C-terminal half of PspA resulted in a 75% pneumococcal sepsis survival rate in a murine model [50].

In previous studies, the hybrid vector demonstrated improved results relative to commercially available gene delivery systems such as FuGENE HD, JETPEI, and OmniFect when delivering the gene for green fluorescent protein (GFP) to macrophages [27]. To conduct a comparative assessment of the hybrid vector in a gene delivery format (used to deliver plasmids containing *pspA*) versus the protein antigen hybrid vectors presented earlier in the study, two variants were designed to direct antigen delivery and expression within APCs: (1) the plasmid hybrid vector (pHV) and (2) pHV + LLO. The pHV contains the standard hybrid vector core transformed with the mammalian expression plasmid pcDNA3.3-*pspA*, forming the bacterial strain BL21(DE3)/pcDNA3.3-*pspA*, while the pHV + LLO vector uses the strain YWT7-*hly* (a strain containing an IPTG-inducible T7-*hly* [i.e., LLO] cassette in the BL21(DE3) chromosome) [27,51] transformed with pcDNA3.3-*pspA* (YWT7-*hly*/pcDNA3.3-*pspA*). The pHV + LLO construct has been genetically designed to produce LLO, a pore-forming protein previously shown to facilitate antigen cargo escape from the APC phagosome [51,52]. Therefore, inclusion of LLO within the hybrid vector core was predicted to enhance the escape of plasmids from phagosomes and promote antigen expression by APCs.

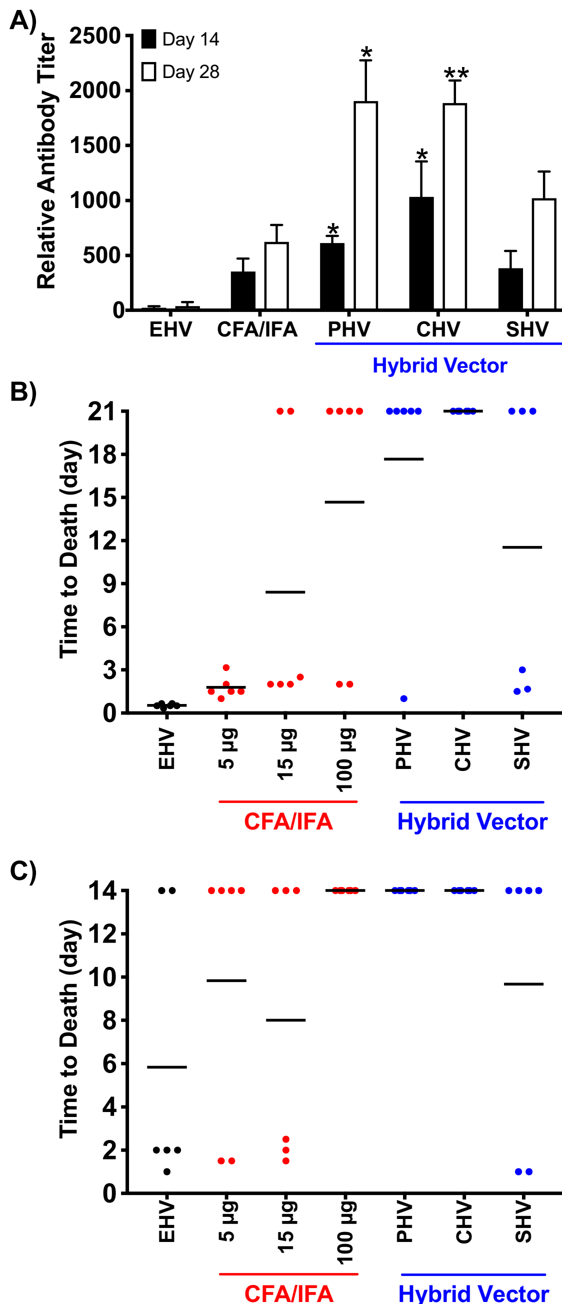

Fig. 4. Immunogenicity and efficacy of the protein hybrid vector variants. (A) Relative antibody titers from mice immunized with 10^7 cells/dose of various hybrid vectors. **P < 0.01, *P < 0.05, relative to control (CFA/IFA – 15 µg PspA) on associated days. Error bars represent 95% confidence intervals of three replicates. Complete protection is represented by a time to death of ≥14 and 21 days for pneumonia and sepsis challenge models, respectively. (**B**) Sepsis challenge model in mice vaccinated with various hybrid vector formulations, PspA + adjuvant, and an empty hybrid vector (EHV) control. (**C**) Pneumonia challenge model in mice vaccinated with various hybrid vector formulations, PspA + adjuvant, and EHV.

Mice were immunized with hybrid vectors containing genetic cargo encoding *pspA*. Both the pHV and pHV + LLO formulations promoted improved immunogenicity relative to the 15 µg PspA + CFA/FA control (Fig. 6A), indicating successful uptake and expression of PspA by APCs. No statistically significant difference (p = 0.31) in resulting antibody levels was observed between the pHV and pHV + LLO hybrid vectors; therefore, we are unable to conclude any impact resulting from the inclusion of LLO upon this metric of delivery assessment. Neither vector was capable of eliciting an antibody class shift (Fig. 6B), possibly due to the intracellular PspA production within the APCs. Antigens produced in this manner are predominantly loaded onto MHC class I molecules, which then activate cytotoxic T cells ($CD8^+$ T cells) instead of the $CD4^+$ T cells needed to trigger the switch from IgM to IgG production within B cells [49,53]. Data in Table 2 did however suggest a balanced Th1/Th2 response when using the DNA versions of the hybrid vector.

When immunized mice were challenged with *S. pneumoniae* D39, the pHV + LLO outperformed the pHV, despite the lack of statistically relevant differences between the antibody responses, in both the sepsis (Fig. 6C) and pneumonia (Fig. 6D) models. Specifically, the addition of LLO improved murine survival from ~6 days to ~12 days in both models. This 2-fold increase in time to death provided by the addition of LLO suggests enhanced release of the mammalian expression plasmid from the APC lysosome, thus, improving PspA gene delivery and eventual production. However, neither DNA hybrid vector provided improved protection when compared to the 100 µg PspA + CFA control and neither was able to confer complete protection (i.e., 21-day and 14-day survival for sepsis and pneumonia, respectively). As such, the DNA hybrid vector variants were unable to match the effectiveness of their protein-producing counterparts.

While the DNA hybrid vectors did not confer complete protection, the immunogenicity of this strategy could be improved by applying established DNA vaccine enhancing techniques [54]. For example, previous studies have demonstrated that priming with a DNA vaccine can improve antibody production following vaccination with recombinant proteins while also providing a strong cellular immune response [54]. This could be applied to the DNA hybrid vectors by either boosting with recombinant PspA or one of PspA-producing hybrid formulations described above. Due to the versatility of the hybrid's *E. coli* core, a protein producing hybrid vector could simultaneously carry the mammalian expression plasmid. This technique, if successful, would represent a unique solution to pneumococcal disease that combines the protective humoral response elicited by the protein hybrid vector with the strong cellular immunity generated by a DNA vaccine.

3. Conclusion

In summary, systematic alteration of growth conditions, antigen localization, and antigen type (i.e., protein and DNA) of the PspA hybrid vectors resulted in complete protection against pneumococcal disease when using the protein delivery variants, regardless of antigen localization. Localization of PspA to the cytoplasm and the bacterial surface improved immunogenicity when compared with the original PHV formulation. Due to its high PspA content (3.5× greater than the SHV), the CHV represented the optimal hybrid formulation developed in this study on a per dose basis. Furthermore, desiccation of the PHV hybrid vector suspended in a microbial freeze drying buffer retained activity even after a 1-month storage period at 4 °C. As such, these results further position the hybrid vector as an effective vaccine delivery solution for pneumococcal disease.

The applications of the hybrid vector, however, are not limited to pneumococcal disease. Due to the innate cellular machinery within the *E. coli* core, this vaccination strategy can easily be altered to immunize against other bacterial targets by replacing PspA with antigens associated with specific pathogens (e.g., M protein for Group A Streptococcus). Furthermore, the potential to induce a cellular immune response by the DNA formulations suggest that the hybrid vector may also be effective at providing protection against intracellular pathogens (e.g., tuberculosis and viral diseases) or cancer. As such, the hybrid vector has the potential to

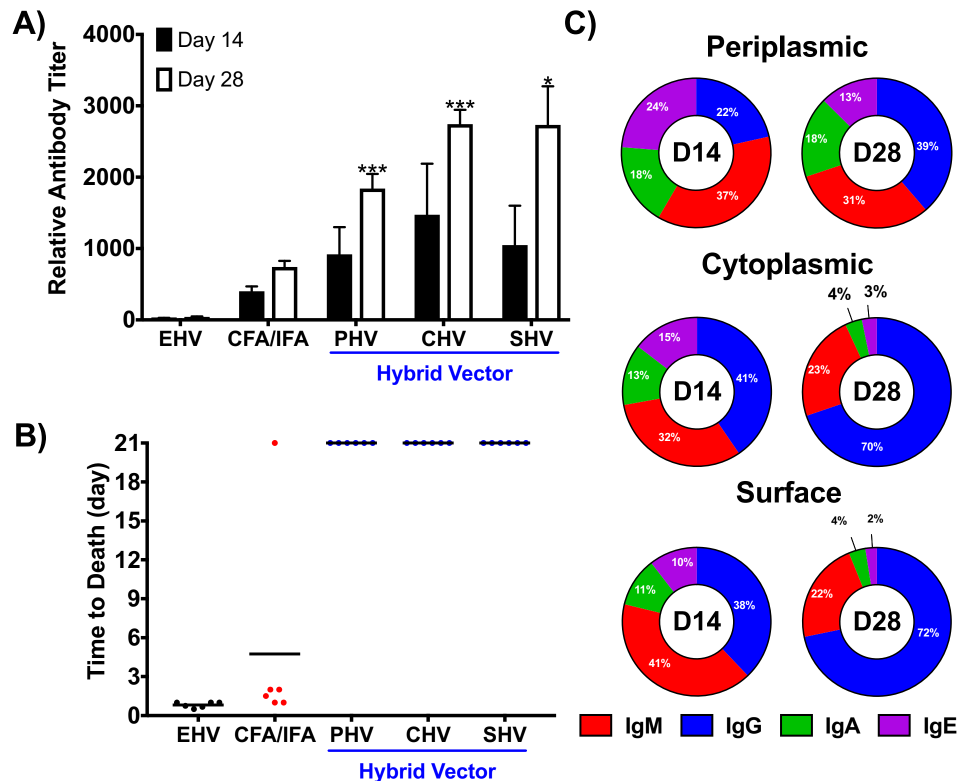

Fig. 5. Effect of PspA localization on hybrid vector immunogenicity. (A) Relative antibody titers from mice immunized with 1 μg of soluble PspA for each hybrid vector formulation. ***P < 0.001, *P < 0.05, relative to control (CFA/IFA – 15 μg PspA) on associated days. Error bars represent 95% confidence intervals of three replicates. Complete protection for sepsis challenge models is represented by a time to death of ≥21 days. (**B**) Sepsis challenge model in mice vaccinated with various hybrid vector formulations compared to EHV control. (**C**) Antibody class distribution in serum from mice immunized with each hybrid vector formulation at days 14 and 28. Antibody class percentages were calculated by comparing individual antibody class titers with total antibody titers.

Table 2
Ratio of IgG1 and IgG2a relative titers.

Hybrid vector variant	IgG1/IgG2a ratio	
	Day 14	Day 28
Cytoplasmic	0.82	1.58
Periplasmic	1.25	0.31
Surface	0.94	0.79
Plasmid	1.54	1.03
Plasmid + LLO	1.19	1.46

act not only as a vaccine option for pneumococcal disease but as a versatile antigen delivery platform for a diverse set of challenging diseases.

4. Materials and methods

4.1. Materials

Reagents utilized to make bacterial and cell culture media were purchased from Thermo Fisher Scientific (Waltham, MA) and Sigma-Aldrich (St. Louis, MO). Chemically defined bacterial growth medium (CDM) was obtained from JRH Biosciences (Lexera, KS). Sheep blood was purchased from Hemostat Laboratories (Dixon, CA). Monomers were purchased from Sigma-Aldrich and TCI (Portland, OR). Acetone (HPLC), chloroform (HPLC), n-hexadecane (99%), DMF (HPLC), and DMSO (≥99.7%) were purchased from Thermo Fisher Scientific. The D4A4-Man polymer used in this study was synthesized as previously described [31,55–57]. PBS was purchased from Life Technologies (Grand Island, NY). Microbial Freeze Drying Buffer was purchased from OPS Diagnostics (Lebanon, NJ). The plasmid pETDuet™-1 was purchased from Novagen (Madison, WI). All remaining chemicals and reagents were purchased from Sigma-Aldrich.

4.2. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee at the University at Buffalo and all conditions for bacterial inoculations and treatments were performed in order to minimize any potential suffering of the animals.

4.3. Strains and plasmids

The gene encoding for a truncated form of PspA (amino acids 1 to 302) from *S. pneumoniae* Rx1 (Accession M74122) was expressed using various plasmids within the bacterial core of the hybrid vector. The *pspA* gene was cloned from pUAB055-*pspA*, a periplasmic localization plasmid derived from pET20b [58], and inserted into either pETDuet-1 or pGEX4T1-ClyA. The commercially available plasmids pETDuet-1 and pGEX4T1-ClyA have been previously designed to produce cytoplasmic- and surface-localized (via fusion to the *E. coli* membrane protein, ClyA) protein products, respectively. Recent studies demonstrate that the fusion of recombinant proteins to the C terminus of ClyA results in the localization of the proteins to the bacterial surface without altering the biological activity of the protein [35,36]. Plasmids were transformed into the *E. coli* strain BL21(DE3) via chemical transformation. For the DNA hybrid vector formulations, *pspA* was cloned into pcDNA3.3,

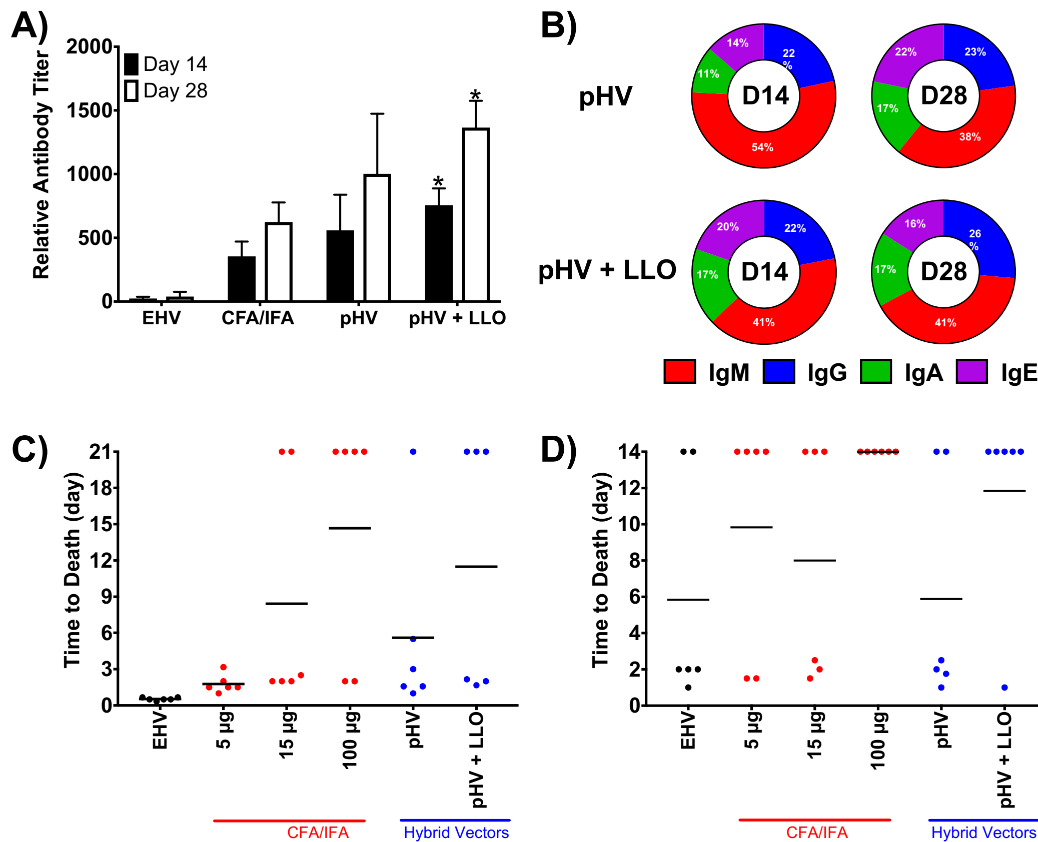

Fig. 6. DNA hybrid vector immunogenicity. (A) Relative antibody titers from mice immunized with DNA hybrid vectors (Plasmid and Plasmid + LLO). *P < 0.05, relative to control (CFA/IFA – 15 μg PspA) on associated days. Error bars represent 95% confidence intervals of three replicates. Complete protection is represented by a time to death of ≥14 and 21 days for pneumonia and sepsis challenge models, respectively. (**B**) Antibody class distributions from mice immunized with DNA hybrid vectors at days 14 and 28. Antibody class percentages were calculated by comparing individual antibody class titers with total antibody titers. (**C**) Sepsis challenge model in mice vaccinated with various hybrid vector formulations, PspA + adjuvant, and EHV. (**D**) Pneumonia challenge model in mice vaccinated with various hybrid vector formulations, PspA + adjuvant, and EHV.

a mammalian expression vector, to produced pcDNA3.3-*pspA* prior to chemical transformation into *E. coli* strain BL21(DE3) to provide the pHV. The background BL21(DE3) strain served as the empty hybrid vector control in these experiments. To enhance hybrid vector escape from endosomes, pcDNA3.3-*pspA* was also transformed into *E. coli* strain YWT7-*hly* (derived from BL21(DE3)), expressing LLO [51], thus facilitating the pHV + LLO formulation.

4.4. Hybrid vector formation and analysis

The *E. coli* strains harboring the various *pspA* expression plasmids were individually cultured at 37 °C and 250 rpm in lysogeny broth (LB) medium. Upon reaching an OD_{600nm} value of 0.4–0.5, cultures were induced with 100, 500, or 1000 μM IPTG (prompting gene expression) and incubated for 0, 2, 4, 6, or 8 h at post-induction temperatures of 22, 30, or 37 °C. The exceptions to this approach were the BL21(DE3) and YWT7-*hly* strains utilized in the DNA vaccine hybrid vector, carrying the *pspA* genetic antigen (pcDNA3.3-*pspA*), which was induced with 100 μM IPTG and grown for 3 hrs at 22 °C post-induction. Cultured bacteria were then washed once and standardized to an OD_{600nm} of 1.0 in 25 mM NaOAc (pH 5.15). D4A4-Man polymer was dissolved in chloroform, desiccated, and resuspended at 1 mg/mL in 25 mM NaOAc (pH 5.15) before being added to bacteria (1:1 v/v) and mixed by vortexing (Analog Vortex Mixer; Fisher Scientific) on setting 5 for 10 s. Following self-assembly of the hybrid vector for 15 min, the solutions were then diluted in PBS to the cell counts specified in Table 1. Protein expression plasmid constructs were analyzed using SDS-PAGE to quantify protein content within the bacterial core of the hybrid devices. Briefly, PspA was purified using a sequence of French press cellular disruption and fast protein liquid chromatography with a Ni-NTA column as described previously [59] and Bradford assay-quantified samples were loaded onto 12% polyacrylamide SDS-PAGE gels run at a constant 120 V to generate a standard curve where gels were visualized using Coomassie staining and densitometry analysis (using ImageJ software [https://imagej.nih.gov/ij/]). The standard curve (Fig. 3B) was then utilized to quantify PspA content from gel band images for various levels of hybrid vector aliquots (subtracting background image data from controls), as we have reported earlier [22].

4.5. Hybrid vector stability testing

After assembly, the various hybrid vector formulations were assessed for retention of immunogenicity over time and at different storage conditions. For these studies, formulations were assembled using the pUAB055-*pspA* hybrid vector, which was induced for 8 h at 1 mM IPTG and 22 °C. Subsequently, these vectors were pelleted and resuspended in various storage media that included PBS, Microbial Freeze Drying Buffer (storage buffer), and trehalose. For dessicated samples, aqueous hybrid vectors, prepared in one of the three buffers, were dried using a desiccation chamber connected to an external vacuum source at room temperature until dry. Vector formulations were then stored at 4 °C for 1,

7, 14, and 30 days prior to characterization. Characterization of vector immunogenicity and protective efficacy was conducted via murine challenge studies described below.

4.6. Immunization of animals

Outbred 6-week-old female CD-1 mice (Harlan Laboratories, Indianapolis, IN) were used in immunization experiments. For experiments with the protein-based hybrid vectors, as well as for the stability studies, mice were immunized by subcutaneous (SC) injection (200 μL) using 10^7 bacterial cells per dose. Associated doses of PspA are listed in Table 1. Further studies used a standardized 1 μg soluble PspA dose, determined using densitometry analysis of SDS-PAGE gels. Concentration of bacterial cells within each hybrid vector dose ranged from 10^7 to 10^8 (determined using OD_{600nm}) and are listed in Table 1. For DNA hybrid vector experiments, mice were immunized with SC injection of 10^7 bacterial cells per dose. Immunization controls included sham (i.e., PBS; all samples were prepared in PBS as the background solution), empty hybrid vector, and PspA + complete Freund's adjuvant (CFA) that was replaced with Incomplete Freund's Adjuvant (IFA) during booster immunizations. CFA and IFA were supplemented with PspA following the adjuvant manufacturer's instructions. Soluble PspA utilized in control samples was purified from recombinant *E. coli* protein production cultures as described above. After 14 days, mice were boosted with the same formulations; however, IFA replaced CFA adjuvant as noted above. At days 14 and 28, samples for antibody analysis were collected by *retro*-orbital bleeding and clarified by centrifugation to collect serum.

4.7. Characterization of antibody production

To quantify antibody titers, an enzyme-linked immunosorbent assay (ELISA) was conducted by coating a 96-well Costar high binding polystyrene plate with 10 μg/mL PspA in tris-buffered saline (TBS) overnight for 4 °C. Available antibody binding sites on the plate were blocked with 3% BSA in TBS-Tween 20 (TBS-T) for one hour at 22 °C. Serum collected from immunized mice was diluted into TBS-T in ratios of 1:1000, 1:5000, 1:7500 and 1:10,000 and added to the plate. The plates were then incubated with mild agitations at 37 °C for three hours. The secondary antibody (Anti-Mouse IgG, IgA, IgM [H + L], IgE, Highly X-Adsorbed-Biotin) was added to the wells in a 1:1000 ratio and agitated for two hours. Streptavidin conjugated with alkaline phosphatase was then added to each well in a 1:1000 ratio and allowed to shake for 30 min. The substrate p-nitrophenyl phosphate was then added to develop the signal and the reaction was quenched using 0.75 M NaOH after 15 min. The signal was quantified using a Synergy 4 Multi-Mode Microplate Reader (BioTek Instruments, Inc.) spectrophotometer at an absorbance of 405 nm.

4.8. Pneumococcal challenge studies

Challenge studies were conducted using the D39 *S. pneumoniae* strain (Serotype 2) due to a notable virulence profile and acceptance as one of the harshest preclinical challenge strains [10]. The strain was initially grown on Todd-Hewitt agar plates supplemented with 0.5% yeast extract and 5% sheep blood and incubated overnight at 37 °C. Single colonies were used to inoculate 5 mL Todd-Hewitt broth containing 0.5% yeast extract and incubated at 37 °C to an OD_{600nm} of 0.6. At this point, *S. pneumoniae* D39 cells used for challenge studies were washed once with and resuspended in PBS. Mice that had been immunized with the hybrid vector formulations as described above were then challenged via intraperitoneal (IP; sepsis) or intranasal (IN) administration with isoflurane (pneumonia) with 1×10^4 and 1×10^6 CFU of *S. pneumoniae* D39, respectively, on day 28 and monitored every four hours for signs of morbidity (huddling, ruffled fur, lethargy, and abdominal surface temperature). Mice found to be moribund were euthanized via CO_2 asphyxiation and subsequent cervical dislocation.

4.9. Statistical evaluation

A two-tailed Student *t* test for unpaired data was used to determine the statistical significance of column comparisons. For multivariance analysis, a one-way analysis of variance (ANOVA) that was corrected using the Bartlett variance test was applied. In addition, the Bonferroni multiple-comparison test was used for multiple comparisons. For all tests, a P value of 0.05 was considered significant. Statistical analysis was performed using the GraphPad Prism software (version 7; GraphPad Software Inc., La Jolla, CA). All data resulted from animal experiments using six subjects per group.

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Competing interests

C.H.J., A.H., and B.A.P. are cofounders of Abcombi Biosciences Inc., a company focused on vaccine design.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jvacx.2019.100012.

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