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Immunogenicity and protective efficacy of a prototype pneumococcal bioconjugate vaccine

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Abstract

Capsular polysaccharides (CPSs), with which most pathogenic bacterial surfaces are decorated, have been used as the main components of glycoconjugate vaccines against bacterial diseases in clinical practice worldwide. Pneumococcal conjugate vaccines (PCVs) are administered globally to prevent invasive pneumococcal disease (IPD). While PCVs have played important roles in controlling IPD in all age groups, their empirical, and labor-intensive chemical conjugation yield poorly characterized, heterogeneous, and variably immunogenic vaccines, with poor immune responses in high-risk populations such as the elderly and patients with weak immune systems. We previously developed a method that bypasses the dependency of chemical conjugation and instead exploits prokaryotic glycosylation systems to produce pneumococcal conjugate vaccines. The bioconjugation platform relies on a conjugating enzyme to transfer a bacterial polysaccharide to an engineered carrier protein all within the lab safe bacterium *E. coli*. In these studies, we demonstrate that a serotype 8 pneumococcal bioconjugate vaccine is highly immunogenic and

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Author Contributions.

JA, CMH, and FYA designed the study. JA, AVP, CJK, LSR optimized the assays and performed the experiments. NES performed MS analysis. JA, CMH and FYA wrote the manuscript. All authors participated in the editorial process of the manuscript. All authors have approved the manuscript.

Competing Interest Statement

CJK, LSR, MFF, and CMH have a financial stake in Omniose, a for-profit entity developing bioconjugate vaccines using patented technology derived from the data presented in this and other published manuscripts.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

elicits functionally protective anti-serotype 8 antibody responses. Specifically, using multiple models we show that mice immunized with multiple doses of a serotype 8 bioconjugate vaccine elicit antibody responses that mediate opsonophagocytic killing, protect mice from systemic infection, and decrease the ability of serotype 8 pneumococci to colonize the nasopharynx and disseminate. Collectively, these studies demonstrate the utility of bioconjugation to produce efficacious pneumococcal conjugate vaccines.

Keywords

Bioconjugation; Bioconjugate; Pneumococcus; Capsular polysaccharide; Conjugate vaccine

1. Introduction

Streptococcus pneumoniae (Spn) is a Gram-positive commensal bacterium capable of invading sensitive host tissues and propagating invasive pneumococcal disease (IPD). IPD can be in the forms of pneumonia, bacteremia, meningitis, or otitis media and result in millions of hospitalizations every year, or even death. In fact, the latest WHO report from 2019 recognizes IPDs as responsible for approximately 14% of all deaths of children under 5 despite the existence of multiple vaccines [1]. The multivalent, serotype-based glycoconjugate vaccines overall work well to reduce IPD caused by serotypes included [2]; however, the empirical and taxing nature of the vaccine in regards to polysaccharide purification, chemical conjugation, or total manufacturing cost [3-5] makes it difficult for the current vaccine model to be comprehensive or expansive to the now over 100 identified serotypes [6]. Furthermore, there are observations of serotype replacement or switching, as well as certain serotypes not exhibiting the downward trend in incidence rates since the deployment of the conjugate vaccines in clinic [7,8]. These caveats have invited new approaches to combat the serotypes unphased by the existing conjugate vaccines [9] and a need for a knowledge-based vaccine design [10].

One unique form of conjugate vaccine design that has grown in recent years is an alternative approach to chemical conjugation known as bioconjugation where bacterial protein glycosylation systems are harnessed for *in vivo* conjugation. Specifically, the oligosaccharyltransferase (OTase)-dependent pathways in Gram-negative bacteria have been well described to transfer long-chain polysaccharides onto engineered acceptor proteins [11]. PglB in *C. jejuni* [12] and PglL in *N. meningitidis* [13] have been functionally characterized in *E. coli* for over a decade now and more recently, a new class of OTase coined PglS was identified as the first system to transfer glycans containing a glucose residue at the reducing end [14]. The ability to express acceptor protein, OTase, and polysaccharide in the same organism and produce a homogeneous bioconjugate vaccine has removed multiple fiscal and procedural challenges associated with the conjugate vaccine manufacturing [15].

Here, we report production improvements and vaccine dosage efficacy of a first-in-class bioconjugate vaccine produced through PglS bioconjugation in *E. coli* that targets the pneumococcal serotype 8. Immunogenicity, opsonophagocytic killing assay (OPKA),

and multiple challenge studies demonstrate the protective capability of the serotype 8 pneumococcal bioconjugate vaccine across all doses tested.

2. Methods

Bacterial strains, plasmids, and growth conditions.

Streptococcus pneumoniae type 8 (ATCC 6308) was obtained from American Type Culture Collection (Rockville, MD) and was cultured aerobically at 37 °C on tryptic soy agar with 5% sheep blood (TSAB) or in Todd-Hewitt broth plus 0.5% yeast extract (THY). *E. coli* strain SDB1 [16] was used for all bioconjugate vaccine production and was cultured at 37 °C on L-agar or as described below.

Serotype 8 pneumococcal bioconjugate vaccine production.

Glycoengineered *E. coli* strain SDB1 hosting the pB8 [17], pVNM124 [18], and pVNM13 [18] plasmids were cultured in a 10 L bioreactor system (Solida Biotech GmbH, Munich, Germany) for large-scale bioconjugate production using a fed-batch method modified from previous examples for producing bioconjugate vaccines [19]. Colonies from LB plates were inoculated into 500 mL Terrific Broth (TB) starter media supplemented with Ampicillin 100 µg/mL, Tetracycline 10 µg/mL, and Spectinomycin 50 µg/mL and grown overnight while shaking at 200 RPM at 30 °C. The next day, the starter was added to 6.5 L of bioreactor media containing 20 g/L Soytone (Difco), 10 g/L yeast extract (Difco), 25 g/L glycerol, 5 g/L NH₄SO₄, 90 mM potassium phosphate pH 7.2, 5 mg/L thiamine-HCl, trace metals, and antibiotics. The trace metal solution was prepared 100-fold concentrated and contained 10 mM CaCO₃, 25 mM FeCl₃, 5 mM MnCl₂, 1 mM H₃BO₃, 1 mM CoCl₂, 1 mM CuSO₄, 5 mM ZnCl₂, 1 mM Na₂(MoO₄), 1 mM Na₂O₄Se, 1 mM NiCl₂, 51 mM EDTA, and 0.1% HCl. Phosphate salts, thiamine, and concentrated trace metals were added to the bioreactor media after autoclave sterilization and cooling to room temperature. Throughout the run, the dissolved O₂ concentration was maintained at 10% by regulating the speed of the mixer between 400 RPM and 1000 RPM and by bubbling O₂ gas directly into the vessel. The media pH was maintained at 7.2 throughout by the addition of 4 M KOH and 25% (v/v) phosphoric acid. The culture was maintained at 30 °C throughout the run. The cells were grown until the optical density at 600 nm (OD₆₀₀) reached 30 and then induced with 1 mM IPTG. After induction, 2 L of feed media was added at 80 mL/hr for the next 24 h. The feed media contained 12 g/L Soytone, 24 g/L yeast extract, 250 g/L glycerol and other nutrient and antibiotic components at the same concentrations as the starting media. After 48 h the cells were harvested via centrifugation and frozen at -80 °C.

Serotype 8 pneumococcal bioconjugate vaccine purification.

E. coli SDB1 cells expressing the serotype 8 pneumococcal bioconjugate vaccine were lysed via sonication and clarified by centrifugation. Clarified lysates were filtered and purified via Nickel affinity chromatography as previously described [18]. Nickel affinity purified bioconjugates were concentrated and buffer exchanged into 20 mM Tris pH 8.0 and loaded onto a Mono Q 5/50 GL column. Bioconjugates were eluted using a linear gradient of increasing sodium chloride concentration in 20 mM Tris pH 8.0. Fractions containing high molecular weight CPS8-EPA bioconjugates were pooled, concentrated, and loaded onto

a Superdex 200 Increase 10/300 GL column equilibrated with phosphate buffered saline. Serotype 8 bioconjugates were identified by Coomassie staining of SDS-PAGE resolved Superdex 200 fractions, pooled, concentrated and stored at -80°C .

Western blotting.

The purified serotype 8 pneumococcal bioconjugate was separated on a 7.5% precast TGX sodium dodecylsulfate polyacrylamide gel (Bio-Rad) and then transferred to nitrocellulose membranes with a $0.2\ \mu\text{m}$ pore size (Bio-Rad). Membranes were blocked with Licor TBS blocking buffer for one hour, incubated with primary antibody for an additional hour, washed three times with TBS containing 0.05% Tween-20, incubated with secondary antibodies for one hour, washed three times, and subsequently imaged using an Odyssey Infrared Imaging System (LiCor Biosciences). Primary antibodies, used at 1:1000 concentration, included reference serotype 8 antisera from Statens Serum Institut (catalog number 16751) and mouse anti 6X-His antibodies from Invitrogen. Secondary antibodies, used at 1:10,000 concentration, included IRDye 680RD goat anti-mouse and IRDye 800CW goat anti-rabbit secondary antibodies (Li-Cor).

3. Intact protein analysis

Intact mass analysis was performed as previously described [14,20]. Briefly, samples were re-suspended in 2% acetonitrile, 0.1% trifluoroacetic acid and loaded onto a Jupiter 300 C5 column ($2\ \text{mm} * 50\ \text{mm}$, Phenomenex) using an Agilent 1200 HPLC at a flow rate of 0.25 mL/min. Five μg of the CPS8-EPA^{iGTcc} bioconjugate was desalted on the column for 2 min with Buffer A (2% acetonitrile 0.1% formic acid) before being separated by altering the percentage of Buffer B (80% acetonitrile, 0.1% formic acid) 0% to 100% over 15 min. The column was then held at 100% Buffer B for 1 min before being equilibrated for 2 min with Buffer A for a total run time of 20 min. Samples were infused into a 6520 Accurate mass Q-TOF mass spectrometer (Agilent) by ESI with MS1 mass spectra acquired with a mass range of 300–3000 m/z at 1 Hz. Intact mass deconvolution of the observed protein elution peaks was then performed using MassHunter B.06.00 (Agilent).

Mice.

Eight-week-old female and male BALB/c mice were acquired from The Jackson Laboratory (Bar Harbor, ME) and housed in the Coverdell Rodent Vivarium at the University of Georgia. Mice were kept in microisolator cages and handled under biosafety level 2 (BSL2) hoods. All mice experiments were conducted in compliance with the University of Georgia Institutional Animal Care and Use Committee under an approved animal use protocol.

Immunizations.

Immunizations consisted of the CPS8-EPA^{iGTcc} bioconjugate in sterile phosphate-buffered saline (PBS) and mixed with 2% alhydrogel (Invitrogen) to form an emulsion equating to either 0.2 μg polysaccharide, 1 μg polysaccharide, or 2 μg polysaccharide per 200 μL of dose, compared to a control of adjuvant immunization alone. All immunizations for female and male BALB/c mice (The Jackson Laboratory) were done intraperitoneally on day 0. Two

booster doses were given on days 14 and 28 respectively. Sera from the mice were obtained on days 14, 28, and 35 post immunizations as well as on day 42 prior to the challenge.

Sepsis Challenge.

Mid-log-phase ATCC 6308 cultures were washed with sterile PBS and diluted to a concentration of 5×10^3 CFU/mL. Groups of 4 unanesthetized female and male BALB/c mice (The Jackson Laboratory) were injected intraperitoneally (i.p.) with 50 CFU. Animals were monitored every 8 h for signs of moribundity.

Nasal Colonization/Challenge.

An intranasal colonization was performed as described previously [21]. Briefly, groups of 10 female mice were anesthetized with isoflurane and inoculated with 5×10^3 CFU bacteria by applying 5 μ L to the opening of each nare by pipette for a total of 10 μ L per mouse. The number of bacteria administered was determined immediately afterward by serial dilution plating on Trypticase soy agar plates with 5% sheep's blood (TSAB). Mice were monitored every 8 h and upon reaching a moribund state, mice were sacrificed to obtain lung and blood samples. An endpoint at day 6 was selected to collect lung and blood samples from mice that had not reached moribund state.

Enzyme-linked immunosorbent assay (ELISA).

ELISA plates (384 well; Thermo Scientific) were coated with 1×10^5 heat-killed pneumococcus serotype 8 bacterial cells (ATCC 6308) per well in 20 μ L of sterile phosphate-buffered saline (PBS) overnight at 4 °C. Plates were manually washed 3 times with PBS plus 0.1% Tween (PBS-T) 20. After 1 h of blocking at room temperature with 50 μ L per well of 1% bovine serum albumin (BSA) in PBS, wells were incubated for 2 h with 20 μ L of a 2-fold dilution series of individual mouse sera (from 1:200 to 1:6400) diluted in PBS-T per well. Plates were washed and then incubated for 2 h at room temperature with a 1:2000 dilution of goat anti-mouse IgG-HRP (horseradish peroxidase) (BioLegend). Plates were developed with a 3,3',5,5'-tetramethylbenzidine substrate (TMB; BioLegend), and development was halted with 2 N sulfuric acid. Optical densities were determined at 450 nm with a microplate reader (Synergy H1, Bio-Tek). IgG subclasses were detected by HRP-conjugated anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (dilution 1:10,000, Abcam) and TMB substrate as described above. For IgM, a 1:2000 dilution of alkaline-phosphatase-linked human-adsorbed goat anti-mouse IgM was used for secondary detection, developed using 20 μ L of p-nitro-phenylphosphate (PNPP) (2 mg/mL) dissolved in 100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl₂, pH 9.5 at 37 °C, and analyzed at a wavelength of 405 nm.

Opsonophagocytic Killing Assay (OPKA).

An opsonophagocytic killing assay was performed as previously described [9,18,22]. ATCC 6308 strain was incubated in triplicate wells in a 96-well round-bottom plate for 1 h at 37 °C with the indicated sera samples (5 μ L serum/50 μ L total reaction volume/well) in opsonization buffer B (OBB; sterile 1x PBS with Ca²⁺ + Mg²⁺ + 0.1% gelatin, and 5% heat-inactivated Hyclone FetalClone I Serum). Cells of the human promyelocytic leukemia cell line HL-60 (ATCC) were cultured in RPMI with 10% heat-inactivated Hyclone

FetalClone I Serum and 1% L-glutamine. HL-60 cells were differentiated using 0.6 % N,N-dimethylformamide (DMF) for 3 days before performing the OPKA, harvested, and resuspended in OBB. Baby rabbit complement (Pel-Freez) was added to HL-60 cells at a 1:5 final volume. The HL-60 – complement mixture was added to the bacteria at 1×10^5 cells/well. The final reaction mixture was incubated at 37 °C for 1 h with shaking. The reactions were stopped by incubating the samples on ice for approximately 20 min. Then, 10 μ L of each reaction mixture (triplicate) was diluted to a final volume of 50 μ L and plated on tryptic soy agar plates with 5% sheep's blood (TSAB). Plates were incubated overnight at 30 °C and counted the next day. The percentage of bacterial killing was calculated as each sample replicate normalized to the mean value obtained for the control samples.

4. Results

Serotype 8 bioconjugate vaccine production and analytical characterization.

Previously, we developed a first of its kind bioconjugation system for generating pneumococcal glycoconjugate vaccines using a new conjugating enzyme, PglS [14]. More recently, we refined the system and determined the minimal sequon sufficient for PglS dependent glycosylation. In this system, PglS transfers a pneumococcal capsular polysaccharide to a genetically deactivated exotoxin A protein from *Pseudomonas aeruginosa* (EPA) engineered to contain a 23 amino acid sequon (also known as a GlycoTag) between residues Ala489–Arg490 [23]. This construct, referred to as EPA^{iGTcc}, served as an efficient acceptor protein for PglS-dependent glycosylation with the pneumococcal type 8 capsular polysaccharide (CPS8).

As the next step, here we produced the CPS8-EPA^{iGTcc} bioconjugate in a fed-batch bioreactor system and subsequently purified and characterized it via Coomassie staining, western blotting and intact mass spectrometry. As seen in Fig. 1A, the CPS8-EPA^{iGTcc} bioconjugate migrated predominately between the 100 and 150 kDa markers, displaying a modal laddering pattern typical for bioconjugate vaccines. Each band on the Coomassie image represents a distinct glycoform population of increasing polysaccharide mass as the EPA^{iGTcc} carrier protein has only a single site of glycosylation per protein molecule. The theoretical mass of the unglycosylated carrier protein is 69,414.92 Da and was visually absent in the CPS8-EPA^{iGTcc} bioconjugate preparation as determined by SDS-PAGE analysis. Western blot analysis using reference anti-CPS8 antisera from Statens Serum Institut confirmed the seroreactive of the CPS8 portion of the bioconjugate, which co-localized with the anti-protein signal (Fig. 1B-1D).

The repeating unit of the type 8 polysaccharide consists of $\rightarrow 4$ - β -D-GlcP-(1 \rightarrow 4)- β -D-GlcP-(1 \rightarrow 4)- α -D-GlcP-(1 \rightarrow 4)- α -D-GalP-(1 \rightarrow), with an approximate mass of 662 Daltons [24]. Intact mass spectrometry analysis of the CPS8-EPA^{iGTcc} glycoconjugate demonstrated glycoforms differing in mass by an average of 662.28 Da, consistent with the CPS8 repeat structure (Fig. 2A and B). The MS1 spectrum confirmed the presence of EPA^{iGTcc} glycosylated with CPS8 repeat units ranging from nine (total mass of 75,373.86 Da) to eighteen (total mass of 81,334.44 Da). Polysaccharide to protein ratios were calculated using the summed ion intensities for each glycoform as previously described [18] and determined to be 11.8% (Table 1). Intact mass spectrometry revealed minimal

unglycosylated EPA^{iGTcc} carrier protein (4.3% of the total observed population) present in the purified CPS8-EPA^{iGTcc} preparation.

Effective immune response following minimal dosage of CPS8-EPA^{iGTcc}.

To investigate the CPS8-EPA^{iGTcc} bioconjugate's dose efficiency, immunization groups of four female or male BALB/c mice were vaccinated intraperitoneally in two-week intervals in a prime-boost-boost regimen using one of three doses of the CPS8-EPA^{iGTcc} bioconjugate mixed with alum adjuvant (0.2 µg, 1 µg, or 2 µg serotype 8 polysaccharide) or adjuvant alone as a control. For brevity, we refer to these dose groups as low (0.2 µg CPS8), medium (1 µg CPS8), or high (2 µg CPS8). Sera were collected on days 14, 28, and 35 and assessed for humoral responses against serotype 8 heat-killed bacteria by ELISA. IgM and IgG titers for individual mice in all immunized groups showed a significant increase in CPS8 IgGs compared to control and no significant changes between groups regarding dosage amount or biological sex (Fig. 3A, B). We further characterized the mouse immune response by IgG subclasses and observed a predominantly IgG1 response among all immunized groups (Fig. 3C). These results indicate that the CPS8-EPA^{iGTcc} bioconjugate induces a predominantly IgG1 humoral response and the preclinical factors of biological sex or dosage volume are not significantly impacting vaccine efficacy.

CPS8-EPA^{iGTcc} Protects Mice from Type 8 Pneumococcal Challenges.

To assess the protective capacity of antibodies induced by CPS8-EPA^{iGTcc}, we first conducted an *in vitro* bacterial opsonophagocytic killing assay (OPKA) against Spn type 8 with all dosage groups. Spn type 8 cultures were incubated with sera from each immunization group and subsequently treated with complement and differentiated HL-60 phagocytes. We observed >65% bacterial killing among all groups (Fig. 4A). Like the IgG titers, there were no significant differences between dosages or biological sex. This indicates that CPS8-EPA^{iGTcc} can effectively induce production of functional opsonizing antibodies that can target Spn type 8 for phagocytotic clearance, even at the lowest vaccine dose tested.

To further assess protection conferred by the CPS8-EPA^{iGTcc} bioconjugate, we challenged each immunization group on day 42 with Spn type 8 intraperitoneally (I.P.). All mice that received bioconjugate immunizations regardless of dosage were protected from infection, while control mice injected with Alum succumbed to the infection within 18 h (Fig. 4B). We also assessed the bioconjugate vaccine in an intranasal colonization/infection model to better represent the natural mode of pneumococcal transmission. Because the low dose (0.2 µg CPS8) of the vaccine conferred protection by I.P., groups of 10 mice were either immunized with the low dose or Alum control by the same prime-boost-boost regimen and subsequently challenged on day 42 with $\sim 5 \times 10^3$ CFU Spn as an LD₅₀ [21]. We observed a 100% survival in the immunized group compared to a 30% survival rate in the control group (Fig. 5A). Bacterial load in the lungs and blood showed $>10^9$ bacterial CFU in non-immunized mice while bacterium was undetectable among all individual mice that were immunized (Fig. 5B). Taken together, these data demonstrate effective protection by the CPS8-EPA^{iGTcc} bioconjugate vaccine in multiple challenge models.

5. Discussion

Bioconjugation via different bacterial glycosylation OTase pathways has become a highly promising approach for expanding current conjugate vaccine coverage or reducing commercial manufacturing costs for conjugate vaccine production. While PglB and PglL have been established for many years, the recent discovery of the PglS glycosylation system has unlocked access to potential polysaccharide-protein *in vivo* conjugations that were previously inaccessible with PglB and PglL; namely, bioconjugation of polysaccharides that have a glucose residue at the reducing end to engineered carrier proteins. In this study we demonstrate that CPS8 bioconjugates containing only a single site of glycosylation internal of the EPA coding sequence are highly immunogenic, elicit serotype specific CPS8 IgM and IgG antibody responses, and provide protection from *S. pneumoniae* serotype 8 infection and colonization.

Upon administration, glycoconjugate vaccines induce T cell-mediated humoral immune response evidenced by IgM-to-IgG class switching and immunological memory [10]. Using BALB/c mice for our immunizations, we observed a strong IgM and IgG response among all groups irrespective of the biological sex of the mice or administered dose for the doses tested. The profile of IgG isotype in response to the vaccine was predominantly IgG1. These results are in congruence with multiple other conjugate vaccine studies that show IgG1 subclass as >80% of total IgG response in mice [25,26] or a strong increase in IgG1 in young children [27]. The CPS8-EPA^{iGTcc} bioconjugate vaccine also conferred strong protection in murine sepsis and nasal infection models. All vaccinated groups survived the intraperitoneal (I.P.) or the intranasal (I.N.) infection with the bacterium, while unvaccinated mice succumbed at approximately 18 h (I.P.) and 48 h (I.N.) post infection, demonstrating the efficacy of the vaccine.

Interestingly, the lowest dose tested (0.2 µg polysaccharide) of the CPS8-EPA^{iGTcc} bioconjugate vaccine elicited similar immune responses (immunogenicity and protective efficacy) in all experiments when compared to the medium and higher doses. During the manuscript review, a 20 valent pneumococcal conjugate vaccine (PCV20) prepared using conventional chemical approaches was FDA approved. PCV20 contains a serotype 8 conjugate with 2.2 µg of the serotype 8 polysaccharide per dose. While the exact polysaccharide to protein ratio is not available for the serotype 8 conjugate, PCV20 contains a total of 46.2 µg of polysaccharide to 51 µg CRM₁₉₇ resulting in an overall ratio of 0.905. It is reasonable to assume that the serotype 8 conjugate has a similar polysaccharide to protein ratio as the overall drug product. The CPS8-EPA^{iGTcc} bioconjugate employed for these studies has a polysaccharide to protein ratio markedly lower at 0.118, which is expected as the carrier protein contains only one polysaccharide strand per protein molecule. Given that CPS8-EPA^{iGTcc} bioconjugate tested contains a single site of glycosylation per protein molecule, it is notable that such low vaccine doses (0.2 µg polysaccharide) elicit potent immune responses. Bioconjugates consist of non-derivatized polysaccharides conjugated at their reducing end to the carrier protein, which is hypothesized to resemble the natural polysaccharide antigen as presented by the invading pathogen [28]. Conventionally prepared glycoconjugate vaccines on the other hand are generated through random linkages between activated polysaccharides to carrier proteins, which can alter immunogenicity by creating,

altering, or destroying epitopes [29,30]. It is tempting to speculate that the CPS8-EPA^{iGTcc} bioconjugate vaccine may not require as much polysaccharide concentration to elicit robust serotype specific IgG immune responses as the CPS8 polysaccharide is presented in its fully native conformation; however, additional studies on interrogating B and T cell responses to bioconjugate vaccines as compared to conventionally prepared glycoconjugates are required to decipher these mechanisms. PCV20 is now commercially available, and our future work will focus on the development of a 20+ valent bioconjugate vaccine for potential benchmarking studies comparing chemically prepared conjugates to bioconjugate vaccines.

The increasing need for low-cost alternatives for vaccine production to provide access to low-income countries makes bioconjugate pipelines an extraordinarily valuable commodity. Compounded with the notion that bioconjugate vaccines could offer non-inferior or potentially superior immunogenicity over conventionally prepared vaccines further increases the value of the bioconjugation platforms for vaccine production. The CPS8-EPA^{iGTcc} bioconjugate vaccine is the first product in a line of future pneumococcal conjugates that has made its way stepwise to demonstrate the potential for ease of manufacturing, straightforward purification processes, and strong protection through this promising approach.

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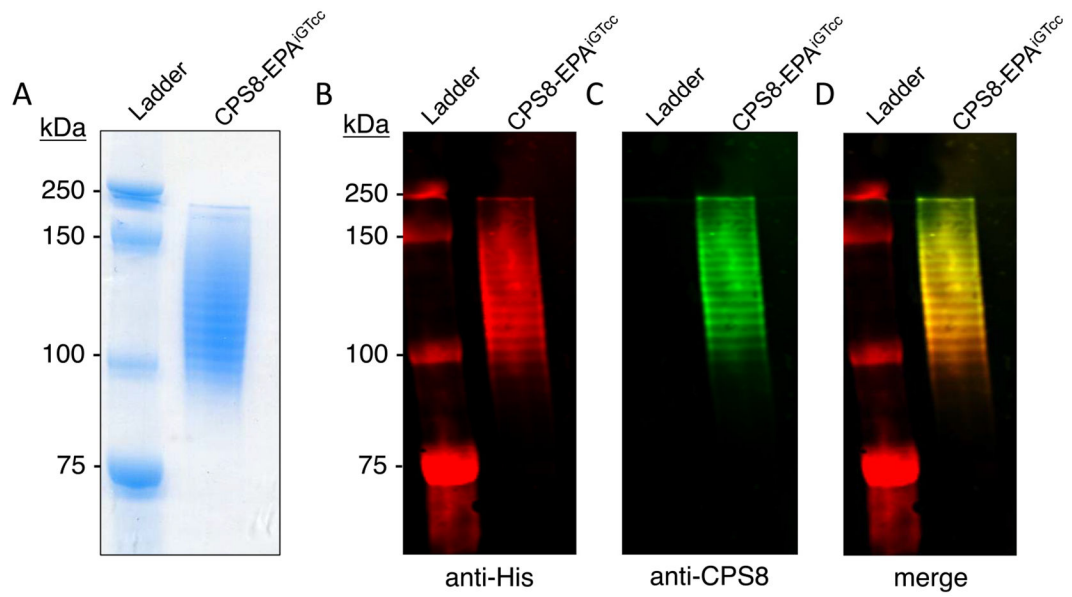


Fig. 1. CPS8-EPA^{iGTcc} bioconjugate vaccine. (A) Coomassie blue-stained image of purified CPS8-EPA^{iGTcc} bioconjugate. Each lane was loaded with 5 μ g of bioconjugate based on total protein concentration. (B-D) Western blots were probed with a monoclonal anti-His antibody and polyclonal anti-CPS8 antisera from Statens Serum Institut. Each lane was loaded 0.5 μ g of bioconjugate based on total protein concentration.

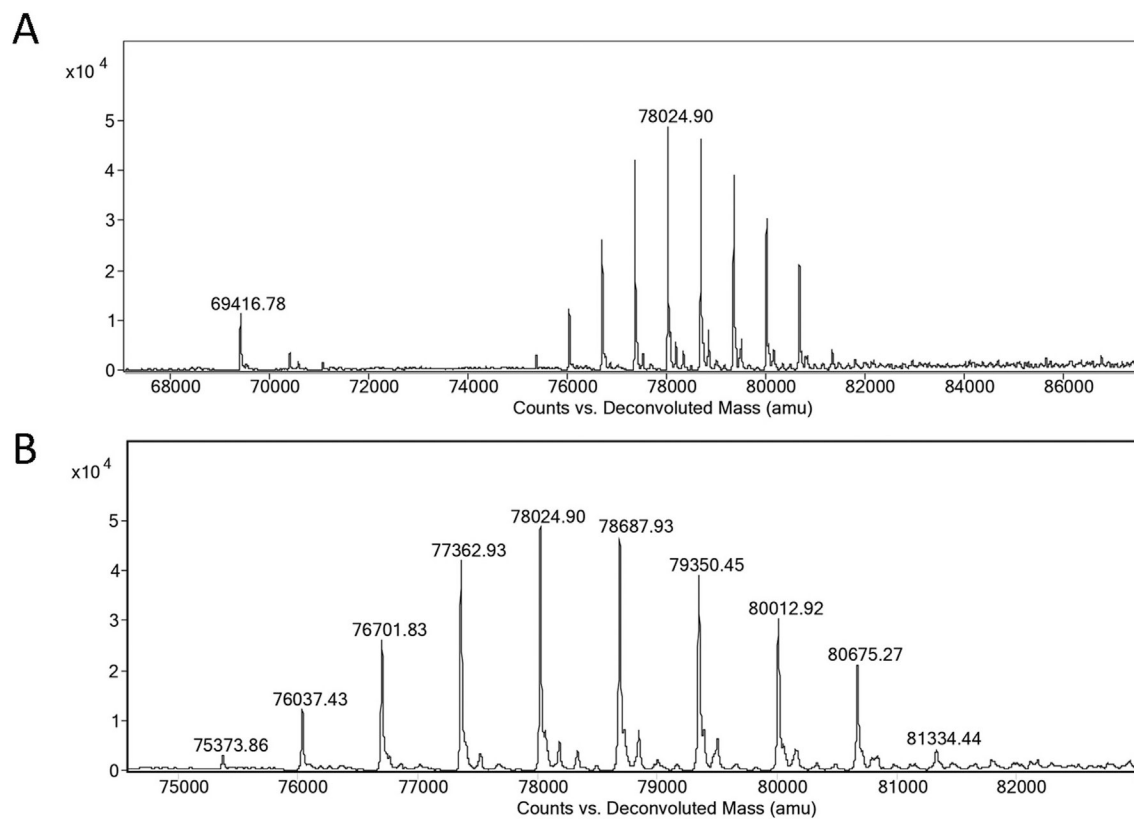


Fig. 2. Quadrupole-time of flight-mass spectrometry of the intact CPS8-EPA^{iGTcc} bioconjugate. (A) Multiple glycoforms, consisting of the EPA^{iGTcc} carrier protein containing nine to eighteen CPS8 repeat units, were observed in the MS1 spectrum. The unglycosylated EPA^{iGTcc} carrier protein has a theoretical mass of 69,414.92 and an observed mass of 69,416.78 on the MS1 spectrum. (Corresponding to a mass accuracy of <50 ppm). (B) Zoomed in view of the mass range spanning 75,000 amu to 82,000 amu showing the observed mass for each CPS8-EPA^{iGTcc} glycoform.

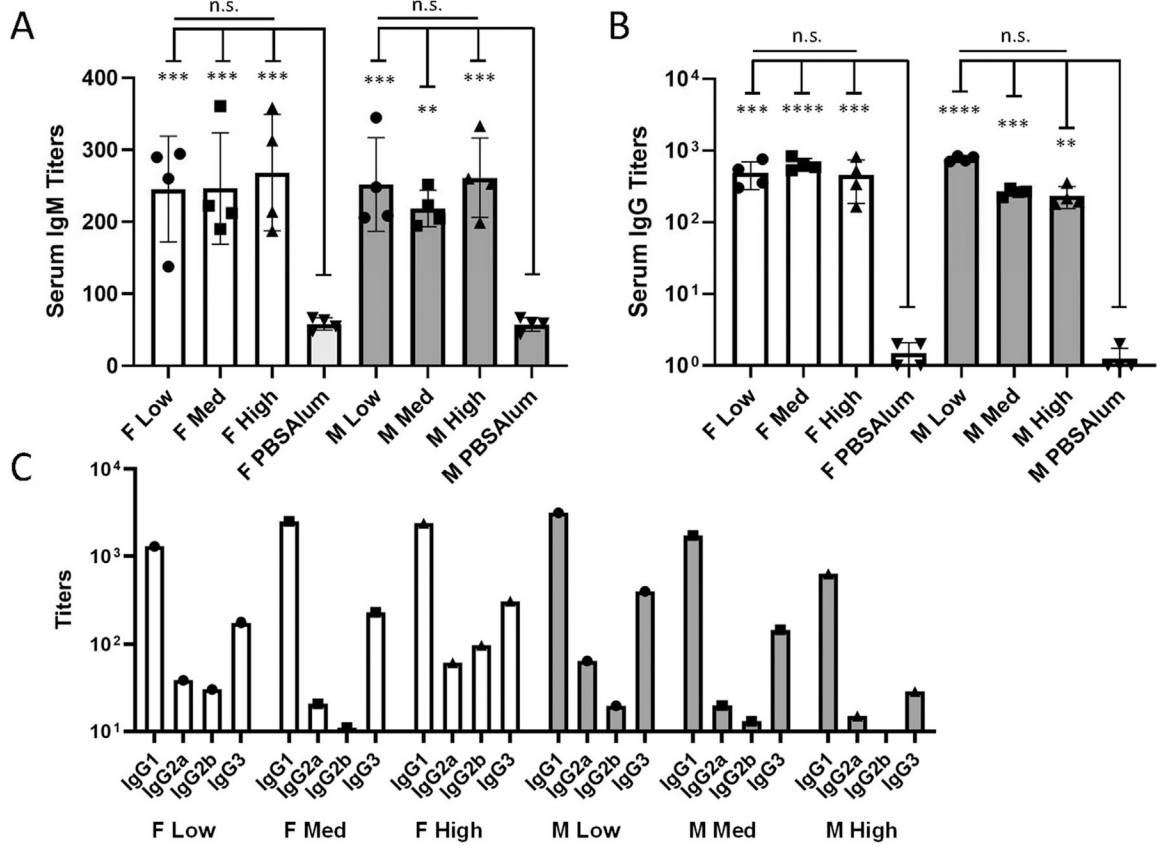


Fig. 3. Comparison of IgG levels in relation to biological sex and different dosages of CPS8-EPA^{iGTcc}. BALB/c mice were immunized three times (Days 0, 14 and 28) with different dosages of CPS8-EPA^{iGTcc} per group (n = 4). Individual mouse serum from Day 35 bleeds were tested against heat killed Spn type 8 bacteria by ELISA for (A) IgM, (B) IgG, and (C) IgG isotype-specific responses. Serum titers are reported as the reciprocal dilution that results in an OD of 0.5. Values represent the mean ± standard deviation (SD) of IgM or IgG titers. ****p < 0.0001, ***p < 0.001, **p < 0.01, and n.s. were determined using one-way analysis of variance (ANOVA) with Dunnett’s multiple comparisons test. For IgG isotypes, values were normalized against the adjuvant injection control.

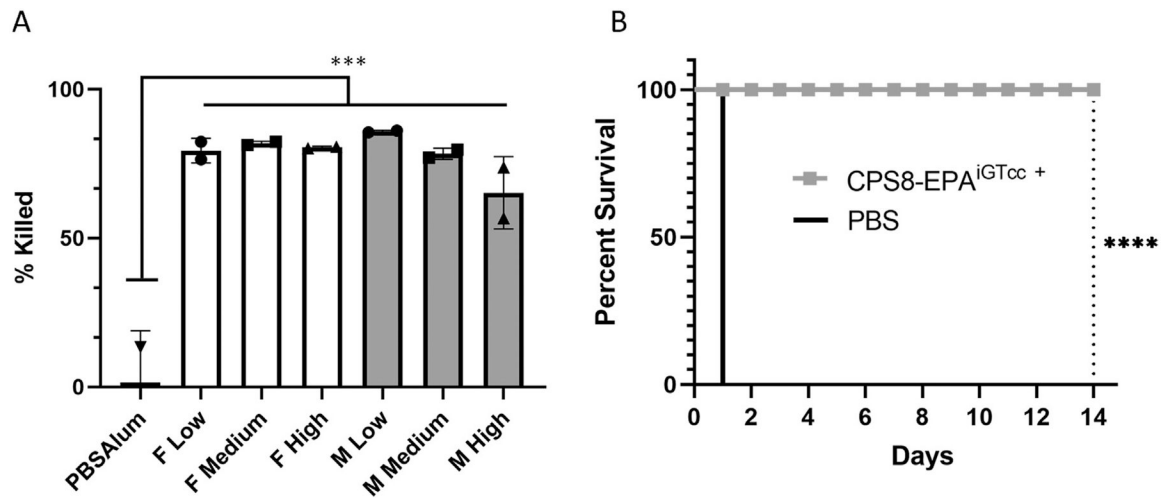


Fig. 4. Protective ability of CPS8-EPA^{iGTcc}. (A) Functional test of anti-CPS8 antibodies in serum using opsonophagocytic killing assay (OPKA), displaying percent killing of bacterial cells after incubation of HL-60 cells in the presence of serum and complement. (B) BALB/c male and female mice are protected when given CPS8-EPA^{iGTcc} low (0.2 μ g polysaccharide), medium (1 μ g polysaccharide), or high (2 μ g polysaccharide) dosages from type 8 pneumococcal infection. All groups of mice (n = 4) were infected through intraperitoneal injection of 50 log-phase virulent type 8 *S. pneumoniae* cells (ATCC strain 6308). “CPS8-EPA^{iGTcc} + Alum⁺” represents 6 groups of male or female mice with dosages of 0.2, 1, or 2 μ g polysaccharide. Values in OPKA represent the mean \pm SD of the CFU killed. *** $p < 0.001$ was determined using one-way analysis of variance (ANOVA) with Dunnett’s multiple comparisons test. For the survival assay, **** $p < 0.0001$ was determined using a Wilcoxon rank-sum T-test.

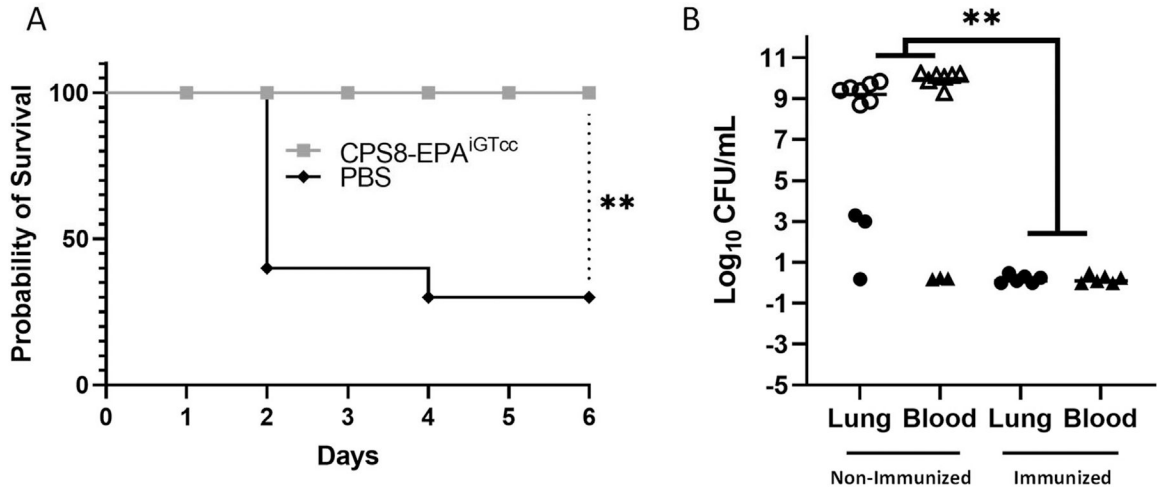


Fig. 5. Protective ability of CPS8-EPA^{iGTcc}. (A) BALB/c female mice are protected when given CPS8-EPA^{iGTcc} low (0.2 µg polysaccharide) dose from type 8 pneumococcal nasal infection. Both groups of mice (n = 10) were infected through intranasal injection of 5 × 10³ log-phase virulent type 8 *S. pneumoniae* cells (ATCC strain 6308) in 10 µL sterile PBS. (B) Lung and blood samples were acquired from moribund mice or at day 6 after the intranasal challenge. Solid shapes represent relatively healthy mice, while hollowed-out shapes indicate moribund mice. Serial dilutions were made in sterile PBS, plated, and counted for CFUs. **p < 0.01 and *p < 0.05 were determined by one-sample *t* test.

Table 1CPS8-EPA^{iGTcc} Bioconjugate Glycoform Characterizations.

Bioconjugate	Repeat Unit (RU) Range	Predominant Glycoform	Polysaccharide – Protein Ratio
CPS8-EPA ^{iGTcc}	9–18	13 RU	0.118

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