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## **Directed Vaccination against Pneumococcal Disease**

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**Keywords:** virulence, *S. pneumoniae*, pneumococcal, biofilm, vaccine

**Abstract:**

Immunization strategies against commensal bacterial pathogens have long focused on eradicating asymptomatic carriage, as well as disease, resulting in changes in the colonizing microflora with unknown future consequences. Additionally, current vaccines are not easily adaptable to sequence diversity and immune evasion. Here, we present a “smart” vaccine that leverages our current understanding of disease transition from bacterial carriage to infection with the pneumococcus serving as a model organism. Using conserved surface proteins highly expressed during virulent transition, the vaccine mounts an immune response specifically against disease-causing bacterial populations without affecting carriage. Aided by a delivery technology capable of multivalent surface display, which can easily be adapted to a changing clinical picture, results include complete protection against the development of pneumonia and sepsis during animal challenge experiments with multiple, highly-variable, and clinically-relevant pneumococcal isolates. The approach thus offers a unique and dynamic treatment option readily adaptable to other commensal pathogens.

**Significance Statement:**

Pneumococcal disease represents a global health problem especially for the young, elderly, and resource-limited. Disease progression begins with asymptomatic nasopharyngeal bacterial colonization before subsequent dissemination and disease (pneumonia, sepsis, middle ear infection). Analysis of this transition provided antigens that were tested in this study for

directed vaccination against only the virulent sub-set of pneumococci. In so doing, a “smart” vaccine was sought to broadly, effectively, and selectively address this disease.

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## **Introduction**

Human-microbe interactions serve numerous symbiotic purposes. However, certain colonizing microorganisms have the capacity to become virulent and trigger disease. The two most common antimicrobial therapies, antibiotics and vaccines, must be re-considered in this context because of numerous pitfalls associated with traditional metrics of “success.”

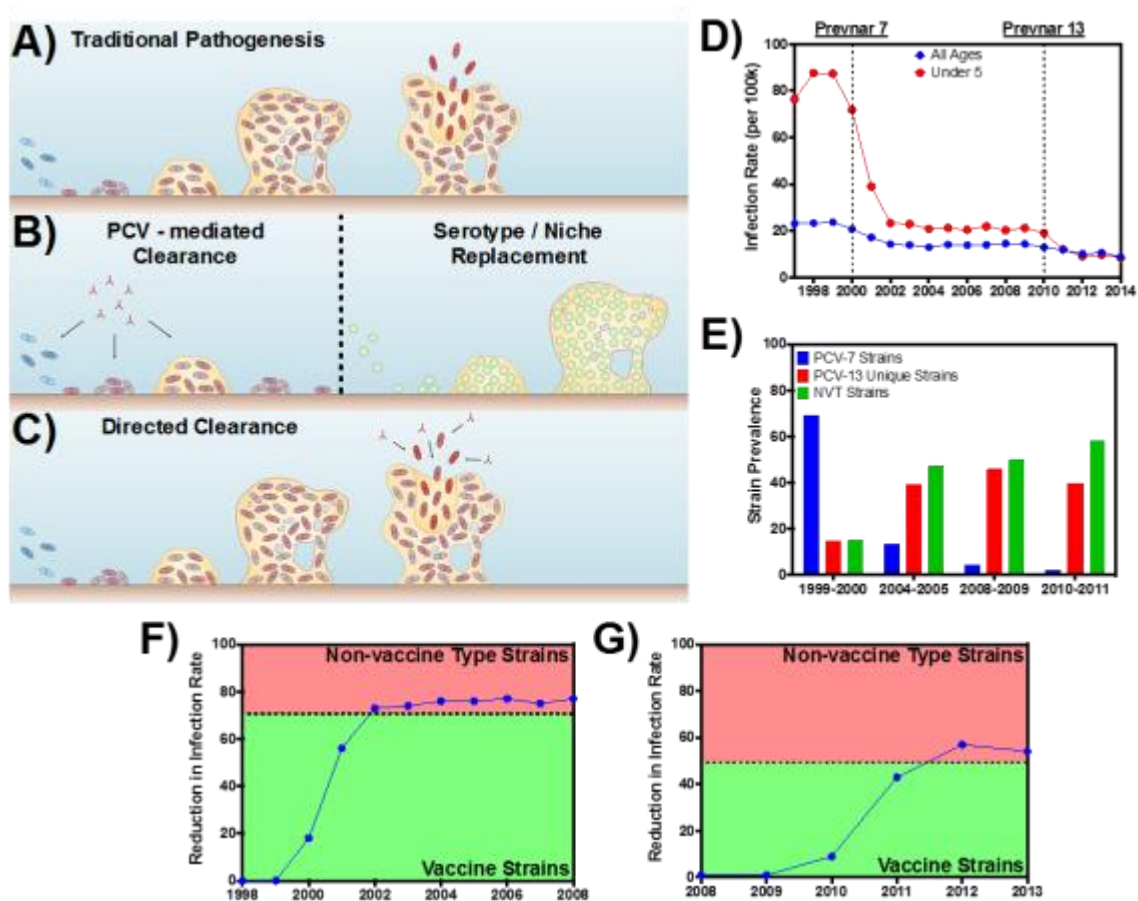
Specifically, we suggest that treatment must be directed at a disease progression state of a microbial population and not the population more generally. Doing so offers the potential to optimize treatment and reduce unintended pathological consequences. In this paper, we present such an approach in the context of pneumococcal disease, culminating in a “smart” vaccine that directs an immune response to virulent cell populations while minimizing disruption of avirulent commensal colonization.

*Streptococcus pneumoniae* (the pneumococcus) is regarded as a major human pathogen and the most common cause of community-acquired pneumonia (CAP), bacterial meningitis, bacteremia, and otitis media (OM)(1). In addition, *S. pneumoniae* has been implicated as an important cause of sinusitis, septic arthritis, osteomyelitis, peritonitis, and endocarditis(1). Regardless of clinical manifestation, infection is always preceded by colonization of the nasopharynx and greater than 95% of children will have been colonized within the first few weeks or months of life by serotypes that are sequentially replaced as more serotypes are acquired(2-4). Interestingly, pneumococcal colonization is asymptomatic, and it is only upon external triggering (via viral infection, for example) that virulent *S. pneumoniae* sub-populations

disseminate and cause disease (Fig. 1A)(5). The illnesses caused by this transition from carriage to disease result in an approximately 15-20% mortality rate in adults with an even higher rate in elderly patients(2-4). Pediatric cases include >20 million yearly occurrences in the U.S., primarily of middle ear infections, and account for the majority of emergency room admissions and associated antibiotic prescriptions, accruing billions of dollars in annual socioeconomic costs(6, 7). Invasive disease has a more devastating impact in resource-poor countries with an estimated one million children (11% of all deaths in children below age five) succumbing to pneumococcal infection annually(8-11).

As introduced above, effectively treating pneumococcal disease is difficult due to multiple populations of *S. pneumoniae* with different characteristics, including cells localized to a colonizing biofilm and cells triggered for dissemination and disease. Antibiotic treatment options have become limited due to the emergence of antibiotic resistance. Notably, prior to the 1990s, most *S. pneumoniae* strains demonstrated universal sensitivity to penicillin(12). However, today penicillin-resistance varies from 5-60% in various parts of the world(13, 14). Of particular concern is the increase in multidrug-resistant *S. pneumoniae* strains, demonstrating resistance to 3+ drug classes(15-18), which attaches substantial concerns to current antibiotic regimens regarding both efficacy and continual resistance development. In addition, *S. pneumoniae* biofilm formation during colonization provides a barrier to effective antibiotic activity(19-23), thus, limiting complete bacterial clearance and promoting resistance development(24, 25). Finally, and more importantly, even in the event of successful bacterial clearance with antibiotic treatment, there is a risk for re-colonization by potentially more dangerous serotypes or alternative pathogens (e.g., *Staphylococcus aureus*), which are equally adept at biofilm formation as well as effective mechanical tolerance and high biological

resistance to antibiotics(26-28).



**Figure 1.** *Streptococcus pneumoniae* pathogenesis outcomes and infectious disease statistics in the United States (1998-2013). (A) *S. pneumoniae* colonizes the human nasopharynx and produces a bacterial biofilm with an accompanying extracellular matrix capable of providing protection from external and host challenges. External triggers such as viral infection prompt the active release of virulent pneumococci that disseminate to secondary sites and cause disease. (B) Leading vaccination strategies (polysaccharide conjugates vaccines [PCVs], such as the Prevnar family) mediate protection against certain bacterial serotypes by promoting clearance of pneumococci prior to biofilm establishment. By clearing all bacteria, the niche will be replaced by non-vaccine serotypes or other bacterial species. (C) The strategy featured in this work

mediates clearance of only virulent biofilm-released bacteria while simultaneously maintaining the presence of the pre-existing biofilm. (D) Annual infection rate per 100,000 people for the total population (blue) and children under the age of five (red) from 1998-2013. The first year following introduction of Prevnar 7 and 13 are marked with dotted lines. (E) Prevalence of infectious pneumococcal strains from 1999 to 2011(29). Strains are grouped into those covered by Prevnar 7 (blue), those covered by Prevnar 13 (red), and non-vaccine types (NVT; green). (F) Reduction in annual infection rate in children under the age of five from 1998 to 2008 relative to 1998-1999. Dashed line corresponds to division between Prevnar 7 vaccine and non-vaccine type strains in 1999-2000(29). (G) Reduction in annual infection rate in children under the age of five from 2008 to 2013. Dashed line corresponds to division between Prevnar 13 vaccine and non-vaccine type strains in 2008-2009(29).

Alternatively, there are currently two pneumococcal vaccine compositions on the US market: the Prevnar® family (Pfizer) and Pneumovax® (Merck). Prevnar vaccines contain capsular polysaccharides conjugated to the diphtheria CRM197 protein. The most recent composition is Prevnar 13, which is designed to encompass 13 of the most common invasive serotypes of *S. pneumoniae* and provides protection against 74-88% of invasive pneumococcal disease cases(30, 31). Pneumovax is a pneumococcal polysaccharide vaccine, introduced in 1977, that since 1983 has provided protection against 23 serotypes of *S. pneumoniae* (PPSV23) with 56-75% efficacy overall(30, 31). However, efforts with current vaccination strategies have met with incomplete success due to 1) an inability to account for and include all current and future *S. pneumoniae* serotypes capable of establishing nasopharyngeal residence and 2) analogous to antibiotic treatment, the complete eradication and displacement of the asymptomatic vaccine-type *S.*

*pneumoniae* biofilms with non-vaccine serotypes and by organisms (such as methicillin-resistant *S. aureus* [MRSA] or *Haemophilus influenzae*) capable of equal or greater pathologies (Fig. 1B)(32-34).

Recognizing the need to develop a new generation of pneumococcal vaccines, the current work presents a strategy capable of providing directed protection against virulent biofilm-released *S. pneumoniae*, while retaining their stable nasopharyngeal commensalism (Fig. 1C). Specifically, vaccine candidates (i.e., antigens) were discovered by building upon the fundamental insight that *S. pneumoniae* colonizes the nasopharynx as a biofilm and that disease progression occurs as external triggers resulting from changes in the nasopharyngeal environment prompt escape from the asymptomatic biofilm of bacteria with a changed transcriptional profile associated with increased virulence(22, 23, 35). Although current vaccines have provided protection and expanded coverage over time (Fig. 1D), clinical data suggest the emergence of new serotypes that must be addressed in future vaccination efforts (Fig. 1E-G)(29). Effectively, the propensity for serotype replacement, within or outside of current treatment options, underscores the need to identify and utilize pneumococcal antigens capable of providing broad serotype coverage in a manner that will minimize asymptomatic biofilm disruption and the associated opportunities for niche replacement. One option in this regard is to target only those pneumococci triggered for virulent biofilm escape in response to changes in the nasopharyngeal environment (Fig. 1C).

## **Results and Discussion**

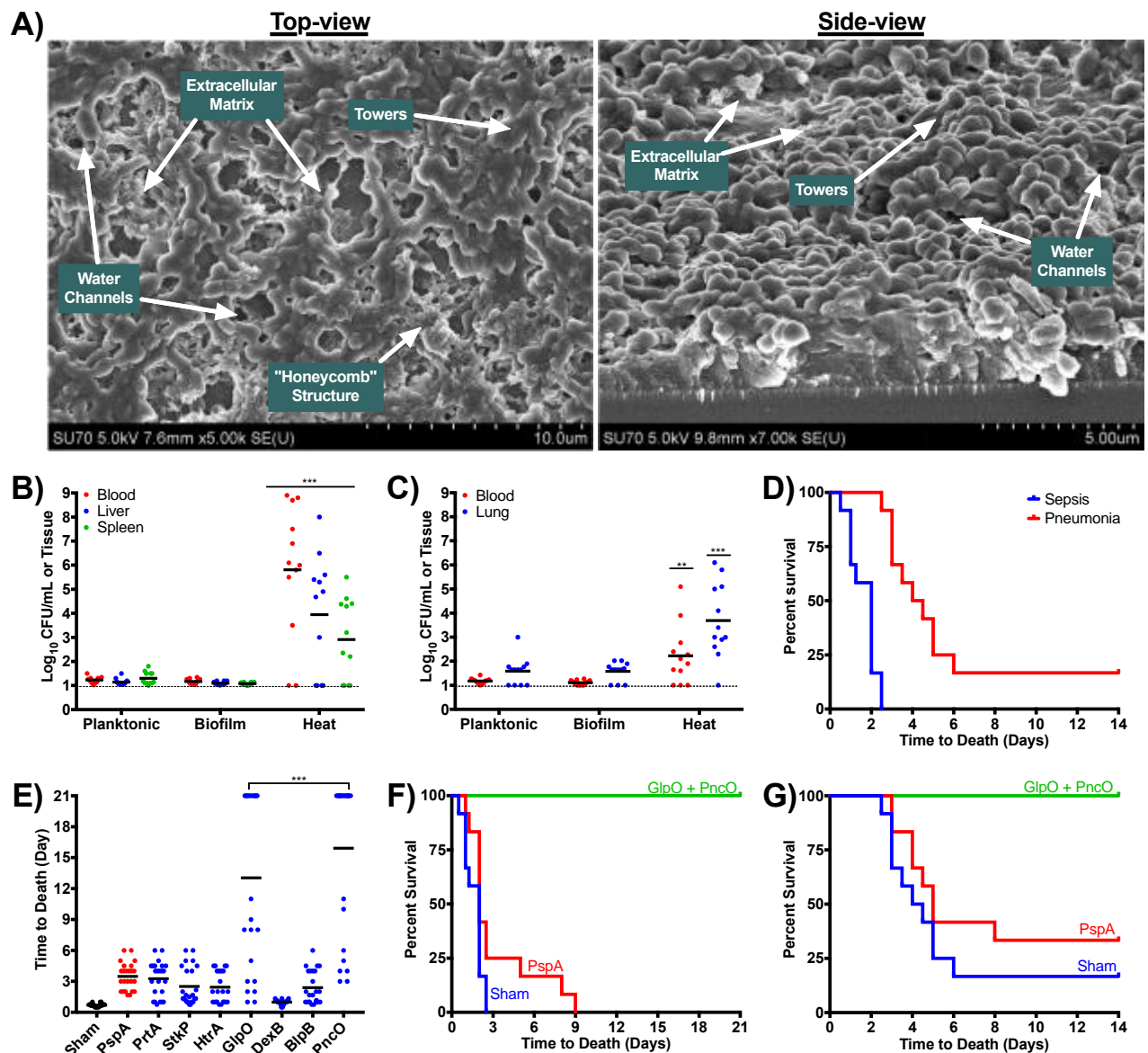
**Virulence-associated Antigens Selected From and Screened Against Biofilm-released, Clinically-conditioned *S. pneumoniae*.** Through the combination of an *in vitro* biofilm model (Fig. 2A) and transcriptional analysis of the bacterial populations comprising this model(23, 36),



antigens were identified that were specifically and significantly upregulated in biofilm-released pneumococci demonstrating increased virulence. In addition to providing target antigens, the biofilm model served another key purpose in this study. Namely, *S. pneumoniae* is a human pathogen that, with the exception of a few strains, cannot cause invasive disease in mice. Thus, the biofilm model was used to condition a clinical isolate of *S. pneumoniae* (EF3030, serotype 19F) to become lethally infectious. Specifically, EF3030 cells released from biofilms by increased temperature (38.5°C, mimicking fever) induced septicemia and death in mice and thus offered a clinically-relevant surrogate model of human pneumococcal disease (Fig. 2B-D). Broth grown (planktonic) EF3030 pneumococci or those mechanically isolated from biofilms had no such virulence. As such, the biofilm model enables clinically-conditioned *S. pneumoniae* strains to be subsequently assessed in mice protection assays, allowing a substantial increase in the number of strains tested in this study.

Initial protection was then investigated by immunizing with a range of promising antigen targets (Table S1) selected on the basis of 1) conserved sequence homology across *S. pneumoniae* strains (thus, offering broad coverage potential), 2) membrane association and/or outer surface accessibility, and, critically, 2) specific prominence in the virulent, biofilm-released bacterial population compared to asymptomatic biofilm pneumococci. Namely, antigen targets up-regulated in biofilm-released bacteria were prepared as recombinant proteins and tested for protection relative to the well-established *S. pneumoniae* surface protein antigen (PspA), which is one of the most studied protein protective vaccine candidates, and is also up-regulated during virulence transition(36-38) (Fig. 2E). To better compare efficacy of selected antigens with PspA, a bacterial challenge dose was chosen that resulted in incomplete protection with PspA vaccination. Under these conditions, all antigens except DexB showed protection that was at

least as good as PspA, with two antigens, GlpO (an  $\alpha$ -glycerophosphate oxidase) and PncO (a bacteriocin ABC transporter transmembrane protein) demonstrating promising individual protection, significantly superseding that of PspA. However, complete protection and effective bacterial reduction were conferred upon immunization with both antigens (Fig. 2F&G); [thus, remaining analyses were conducted using these two antigens in combination.](#)



**Figure 2.** Antigen identification and *S. pneumoniae* conditioning through an *in vitro* biofilm model. (A) *S. pneumoniae* were seeded on epithelial cells, and the biofilm structure was investigated using SEM. Visible in these images are the extracellular matrix, water channels, tower formations, and the “honeycomb” structure that pneumococci form with larger biofilms. Mouse bacterial burden was determined after (B) intraperitoneally injections (sepsis model) or (C) intranasal aspiration with anesthesia (pneumonia model) using broth grown (Planktonic), biofilm-associated (Biofilm), or biofilm heat-released (Heat) *S. pneumoniae* strain EF3030. Each dot in the graphs represents an individual mouse. (D) Time to death assessment of mice inoculated with biofilm heat-released bacteria; mice that were inoculated with either planktonic or biofilm-associated bacteria did not render death in any challenge model. Mice were immunized with various antigens and challenged with biofilm heat-released EF3030 in sepsis (E and F) and pneumonia (G) models. Dotted line represents limit of detection for bacterial counts. \*\*\* $P < 0.001$ , compared to Planktonic and Biofilm samples (B and C) and PspA (E).

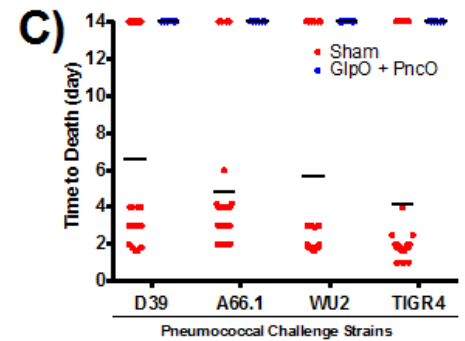
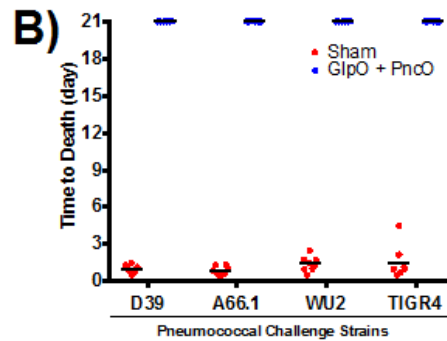
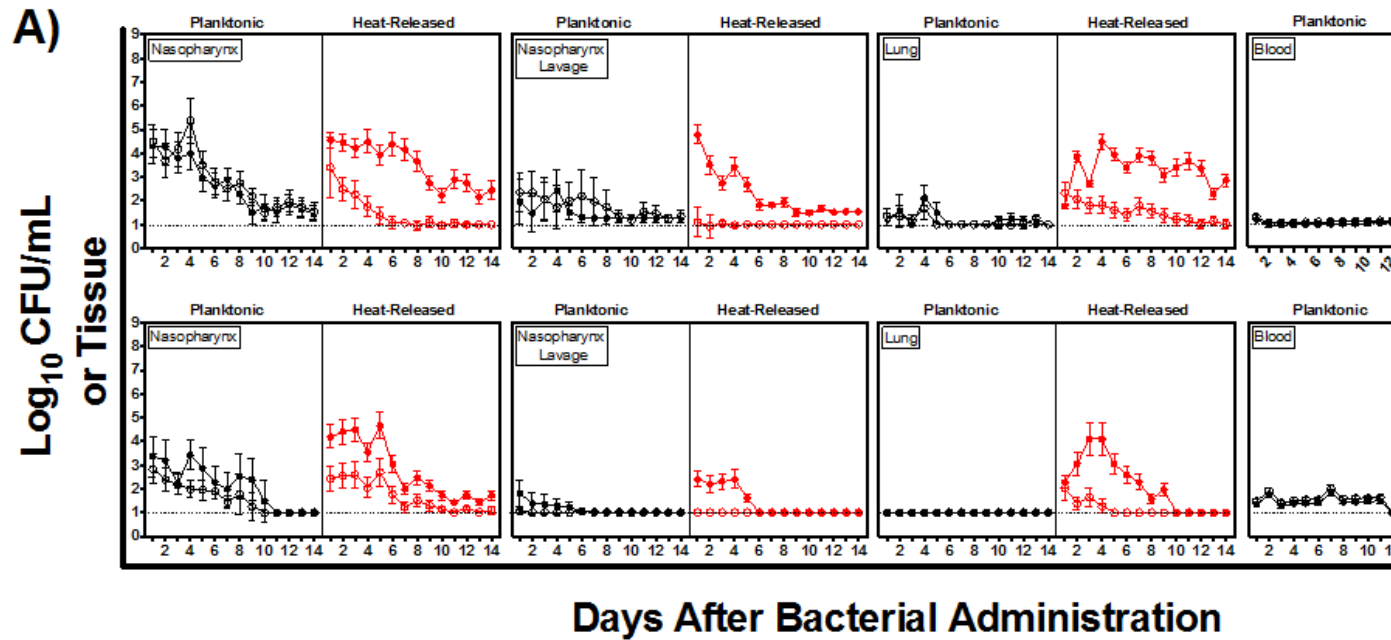
**Antigen Delivery Using Co-PoP Liposomal Surface Display.** The co-delivery of GlpO and PncO, both recombinantly produced with 6×histidine tags, was facilitated using a novel cobalt porphyrin-phospholipid (Co-PoP) liposomal carrier capable of surface-orienting and delivering multiple his-tagged peptide-based antigens(39)(Fig. S1). The liposomal device thus offers a unique vaccine formulation based upon a simple and stable antigen-carrier complex without the need for advanced chemical conjugation. Furthermore, the technology is well-aligned with the antigen discovery and production techniques offered by the aforementioned biofilm model and well-established recombinant protein production. Therefore, we adopted the technology here in the delivery of GlpO and PncO. Looking forward, the Co-PoP delivery platform offers even

more potential in the way of unprecedented valency of discovered antigens via surface localization and presentation of 100s of additional protein or peptide products.

**Directed Response and Extended Coverage Provided by Combined Virulent Antigens.** The directed nature of the new antigens was then tested in a series of experiments presented in Figure 3A. Across different anatomical locations representative of bacterial colonization (nasopharynx) and displacement (nasopharynx lavage), disseminating pneumonia (lung), and invasive septicemia (blood), vaccinated and non-vaccinated mice were challenged with less virulent (planktonic) and virulent (*in vitro* biofilm-released) *S. pneumoniae* and bacterial clearance monitored over time. In the absence of an external stimulus (e.g., viral infection), mice will remain colonized with planktonic D39 or EF3030 for 1 to 3 weeks without infection of the lower respiratory tract or the development of bacteremia. However, biofilm-released bacteria will demonstrate a similar colonization pattern as compared to avirulent bacteria but will also have the propensity to disseminate into secondary anatomical sites and cause disease. Thus, in this study, planktonic EF3030 and D39 cells provided a clearance baseline to compare reduced bacterial loads using virulent challenge. Clearance of biofilm-released bacteria was only mediated in vaccinated mice; whereas, the bacterial load was significantly increased and lethal in non-vaccinated mice. Interestingly, the rate of clearance of planktonic bacteria was unchanged despite vaccination. Thus, the data further support a directed vaccination strategy using GlpO and PncO.

However, the antigenic drift potential of *S. pneumoniae* emphasizes the need for any new antigens to be general and effective across a wide range of challenge strains (Table S2). To this end, the new antigens were tested in mice infected with a range of *S. pneumoniae* strains chosen for their notable difficulty to protect against and the variability between strains with regard to

serotype and genetic background. Importantly, like EF3030, several of the *S. pneumoniae* strains tested required virulent conditioning using the *in vitro* biofilm model, thus, emphasizing the importance of this tool in both antigen discovery and broad challenge assessment. Complete protection was provided for a panel of strains across both sepsis and pneumonia challenge models (Fig. 3B&C; Figs. S2&S3). Ten additional strains were tested in protection experiments with average time to death ranging from 12-21 days as opposed to <3 days for controls (Fig. S4). These challenge assays included strains of serotype 12, 15B, and 27 that are not covered by current vaccines and suggest that 1) strains currently circulating and causing disease in the population can be protected against with this vaccine composition and 2) the methodology of *in vitro* biofilm-release can be used to produce additional mouse-virulent bacterial populations useful for future vaccine protection screening. The combined results emphasize a degree of coverage not previously reported when using a protein-based antigen with the added potential to continually identify and test new antigens in response to disease variation over time. Finally, broad protection is supported by a sequence conservation analysis of the new antigens across *S. pneumoniae* serotypes (Table S3). The results therefore support widespread protection potential and resistance to antigenic drift.



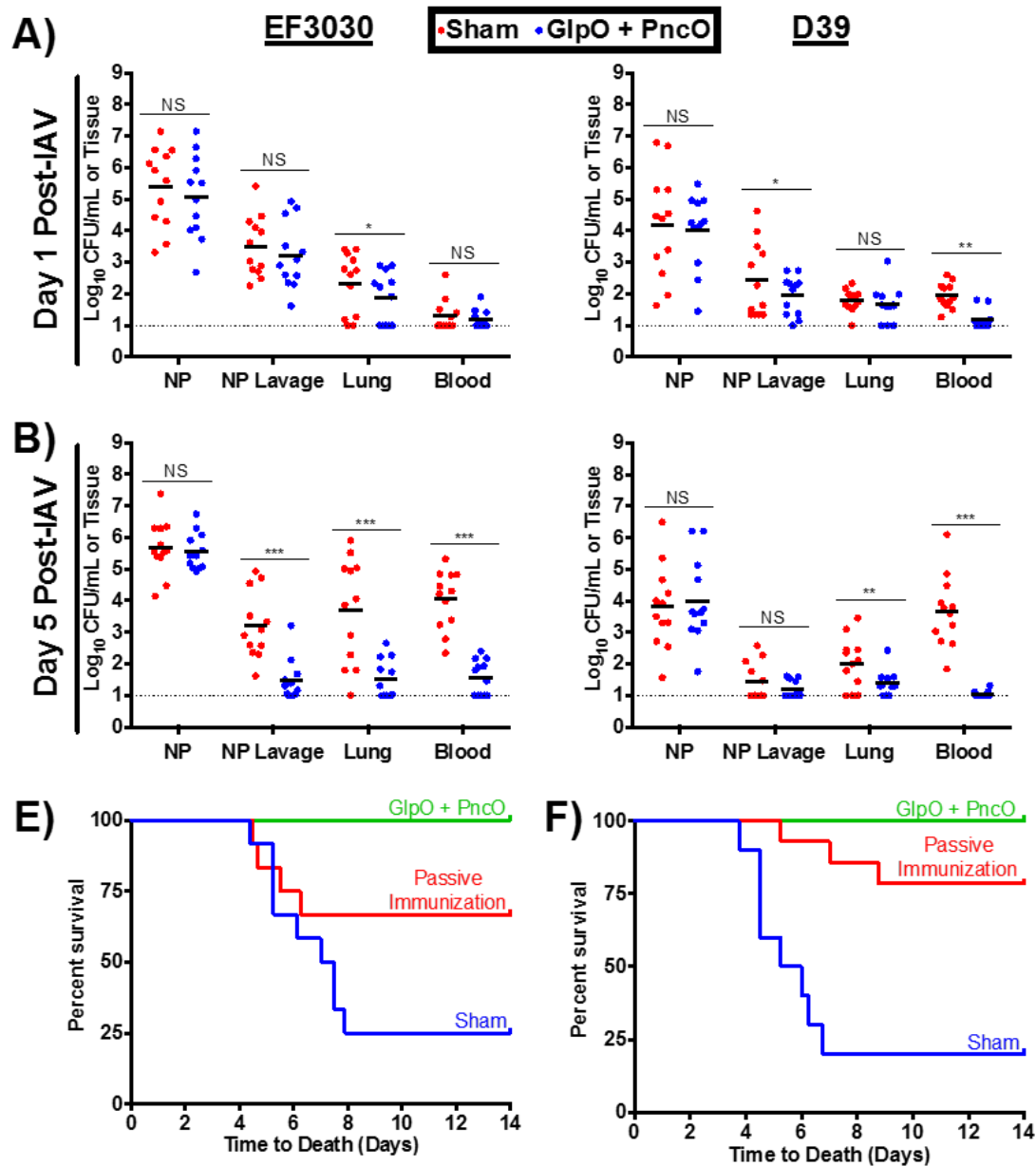
**Figure 3.** Directed clearance of biofilm-released bacteria and protection against mouse-passaged challenge strains. (A) Bacterial burden at various anatomical sites was determined daily in unimmunized (filled circles) and GlpO + PncO immunized (open circles) mice. Mice were inoculated intranasally without anesthesia with planktonic or heat-released EF3030 (top) or D39 (bottom) bacteria. Protective capabilities of GlpO + PncO immunization were further evaluated in sepsis (B) and pneumonia (C) models with established mouse-passaged pneumococcal bacteria. Dotted lines represents limit of detection for bacterial counts.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ .

**Extension to an *in vivo* Model of Virulence Progression.** In a final set of experiments presented in Figure 4, we explored protection in an *in vivo* model that mimics the clinical progression of pneumococcal disease onset. Epidemiological evidence suggests that pneumococcal disease is strongly associated with a concomitant infection with upper respiratory tract viruses, such as influenza A virus (IAV)(40, 41). Mice were infected intranasally with IAV 48 hours after colonization with *S. pneumoniae*, a protocol designed to mediate the release of virulent pneumococci from colonizing biofilms for subsequent dissemination to the lungs and blood. Mice vaccinated with GlpO and PncO displayed a limited spread of D39 and EF3030 *S. pneumoniae* strains in this clinically-relevant model system with the reduced onset of dissemination of virulent organisms indicated on day 1 post viral infection and significantly pronounced reduction in the lung and the blood on day 5 (Fig. 4A&B). Of major importance is the fact that the nasopharyngeal burden in immunized and non-immunized animals remained unchanged, suggesting that harmless and potentially beneficial commensal colonization was unaffected. This further supports a paradigm shift in protection against “accidental pathogens” or those commensals that colonize asymptotically but have the capacity to cause disease in response to inflammation or other external triggers.

When using this same *in vivo* model of IAV-induced pneumococcal disease, full protection against septicemia and death was conferred by the combination of GlpO and PncO using traditional immunization while partial protection was accomplished via adoptive/passive vaccination strategies (Figs. 4E&F and S4). The latter result indicates an important role of the humoral immune system arm in the mechanism driving the results obtained throughout this study, while simultaneously suggesting that an additional T cell response is also contributing.

Interestingly, these observations are in line with the recognition that priming of T<sub>H</sub>17 cells is an important factor mediating the clearance of pathogens at mucosal surfaces(42). However, a more mechanistic analysis of individual and combined antigens is required to better characterize the immunological underpinnings of the results presented. We also recognize the potential for immune response “cross-talk” in that analogous antigen targets might be targeted in other commensal organisms. For our major antigens in this study, GlpO is present in other Streptococcal species as well as in Enterococci, Lactococci and Lactobacilli. However, PncO appears to be restricted to pneumococci, at least based on homology searches. Similar to the pneumococci emphasis of this study, we would expect that cross reactivity with other members of the host microbial flora would be restricted to those populations that have upregulated versions of the antigens tested here. However, a more complete analysis of microbial content post-vaccination would be required to elucidate impact upon global microflora variation. This too is considered a fruitful and interesting topic of future research.





**Figure 4.** Bacterial dissemination and time to death assessment of mice stably colonized with pneumococci and triggered with influenza A virus (IAV). Bacterial burden of EF3030 or D39 in unimmunized or GlpO + PncO immunized mice was measured at 1 (A) or 5 (B) days post-infection with IAV. Protective capabilities of traditionally or passively GlpO + PncO immunized mice against *in vivo* IAV-mediated bacterial release of EF3030 (E) or D39 (F). Dotted line represents limit of detection for bacterial counts. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; NS: not

significant.

## **Conclusions**

In summary, the availability of new tools derived from a better understanding of the mechanisms involved in pneumococcal biofilm establishment and transition to disease, together with effective delivery technology, has enabled a directed and potent strategy to induce an antibody-mediated immune response against a disease-causing subset of a commensal microbial population. Specifically, antigens associated with biofilm-released, virulent pneumococci have demonstrated unequivocal challenge protection and reduction in bacterial burden of a range of clinically-relevant *S. pneumoniae* strains under varying disease conditions. The results offer a response to a globally-relevant disease and growing concerns associated with current treatment options including risks posed by emerging serotype- and niche-replacement pathogens. Importantly, the same tools enabling the results of this work can be applied to continually identify and simultaneously deliver new antigens as a means to address the diversity and antigenic drift potential of *S. pneumoniae* and other pathogens that exhibit similar virulence progression.

## **Materials and Methods**

**Vaccine Formulation and Immunization.** Antigen cobalt porphyrin–phospholipid (Co-PoP) liposomal carrier vectors were generated as described previously(39). Each injection dose contained 25 µg monophosphoryl lipid A (MPLA) in liposomes comprising DOPC:cholesterol:MPLA:Co-PoP at a molar ratio of 50:30:5:5. After dissolving liposomes in chloroform in a test tube, the solvent was evaporated and the film was further dried under vacuum overnight. Liposomes were then rehydrated with phosphate buffered saline (PBS) and sonicated. Binding ability of recombinant antigens was evaluated by incubating 25 µg of protein

with 20 µg of liposomes in 200 µL PBS within a well of a 96-well plate. Fluorescence in the FRET channel (ex: 430 nm, em: 525 nm) was measured periodically with a fluorescence microplate reader (Tecan Infinite II). Data were normalized to the FRET signal for protein without addition of liposomes. Once binding was confirmed, antigens were incubated at 4°C with Co-PoP liposomes overnight before animal injections. Dynamic light scattering was used to evaluate the particle diameter and zeta potential of liposomes containing three concentrations of PspA (0, 5, and 15 µg).

Outbred 6-week-old female CD-1 mice (Harlan Laboratories, Indianapolis, IN) were used in immunization experiments. Mice were immunized by intraperitoneal injection (i.p.; 200 µL), subcutaneous injection (s.q.; 200 µL), and intranasal aspiration (i.n.; 40 µL). All samples contained PBS as the background solution, and final antigen (Table S1) doses ranged from 5 to 15 µg. The sham vaccination control was the Co-PoP delivery device in PBS. When combined, PncO and GlpO (Table S3) were administered at 15 µg each. After 14 days, mice were boosted with the same formulations. At day 14 and day 28, serum samples were collected from the mice by retro-orbital bleeding. For passive immunizations, respective sera were diluted ten times and administered via i.p. injection (200 µL).

**Bacterial Preparation and Biofilm Release.** Bacterial strains used in this study are listed in Table S2 and were 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 with shaking to an OD<sub>600</sub> of 0.6. At this point, mouse passaged-strains of *S. pneumoniae* (which display a virulent phenotype) were used for challenge studies after washing one time with and resuspending in PBS (Fig. S5).

Other *S. pneumoniae* strains are clinical isolates that do not demonstrate a virulent phenotype in mice unless conditioned using an *in vitro* biofilm release model. Specifically, NCI-H292 epithelial cells were cultured in RPMI-1640 medium in T75 flasks at 37°C and 5% CO<sub>2</sub>. After reaching 100% confluency, H292 cells were prefixed in 4% buffered paraformaldehyde at 34°C for 48 hours followed by three washes with PBS. CDM-grown pneumococci were then seeded onto fixed H292 cells with change of media occurring every 12 hours. Formed biofilms were exposed to heat (38.5°C) for 4 hours and released cells were then collected by centrifugation, washed and resuspended in PBS, and quantified by OD<sub>600</sub> measurement. Biofilm associated cells were disrupted by gentle pipetting, collected by centrifugation, washed and resuspended in PBS, and quantified by OD<sub>600</sub> measurement.

**Challenge Models.** To induce sepsis or pneumonia, mice were administered i.p. or i.n. (with isoflurane), respectively, with  $1 \times 10^4$  to  $1 \times 10^6$  CFU of pneumococci strains (Table S2). To induce colonization, mice were administered  $1 \times 10^6$  CFU bacteria i.n. without isoflurane. To mimic influenza-induced pneumonia, pneumococci colonization (with biofilm-grown EF3030 or D39) was followed 48 hours later by intranasal inoculation with 40 plaque forming units of IAV in 50  $\mu$ L PBS. Mouse-adapted A/PR/8/34 (H1N1) (ATCC VR-95) was used, and viral titers were determined by plaque assays. Mice were 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<sub>2</sub> asphyxiation and cervical dislocation. When IAV addition was replaced with background PBS inoculation, mice remain colonized by *S. pneumoniae* strains D39 and EF3030 for 1 to 3 weeks without 1) lethargy, huddling, and ruffled fur (as was observed for viral inoculation) and 2) infection of the lower respiratory tract or the development of bacteremia.

All remaining experimental details are described in *SI Materials and Methods*.

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