

Directed vaccination against pneumococcal disease

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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 be adapted easily 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.

virulence | *S. pneumoniae* | pneumococcal | biofilm | vaccine

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 reconsidered in this context because of the 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 at 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 the disruption of avirulent commensal colonization.

Streptococcus pneumoniae (the pneumococcus) is regarded as a major human pathogen and is the most common cause of community-acquired pneumonia, bacterial meningitis, bacteremia, and otitis media (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 >95% of children are colonized within the first few weeks or months of life by serotypes that are replaced sequentially as more serotypes are acquired (2–4). Interestingly, pneumococcal colonization is asymptomatic, and it is only upon external triggering (e.g., viral infection) that virulent *S. pneumoniae* subpopulations disseminate and cause disease (Fig. 1A) (5). The illnesses caused by this transition from carriage to disease result in a mortality rate of ~15–20% in adults with an even higher rate in elderly patients (2–4). Pediatric cases include >20 million yearly occurrences, primarily middle ear infections, in the United States 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-limited countries, with an estimated 1 million children (11% of all deaths below age 5 y) succumbing to pneumococcal infection annually (8–11).

As introduced above, effectively treating pneumococcal disease is difficult because of the 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 by the emergence of antibiotic resistance. Notably, before the 1990s, most *S. pneumoniae* strains demonstrated universal sensitivity to penicillin (12). Today, however, 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 three or more drug classes (15–18), which creates substantial concerns about both the efficacy of current antibiotic regimens and the continual development of resistance. In addition, the formation of *S. pneumoniae* biofilm during colonization provides a barrier to effective antibiotic activity (19–23), thus limiting complete bacterial clearance and promoting the development of resistance (24, 25). Finally, and more importantly, even in the event of successful bacterial clearance with antibiotic treatment, there is a risk for recolonization by potentially more dangerous serotypes or alternative pathogens (e.g., *Staphylococcus aureus*), which are equally adept at biofilm formation and have effective mechanical tolerance of and high biological resistance to antibiotics (26–28).

Alternatively, two pneumococcal vaccine compositions are currently on the market in the United States: 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%

Significance

Pneumococcal disease represents a global health problem, especially for the young, the elderly, and the resource-limited. Disease progression begins with asymptomatic nasopharyngeal bacterial colonization before subsequent dissemination and disease (pneumonia, sepsis, and middle ear infection). Analysis of this transition from colonization to disease provided antigens that were tested in this study for directed vaccination against only the virulent subset of pneumococci. In so doing, a “smart” vaccine was sought that would address this disease broadly, effectively, and selectively.

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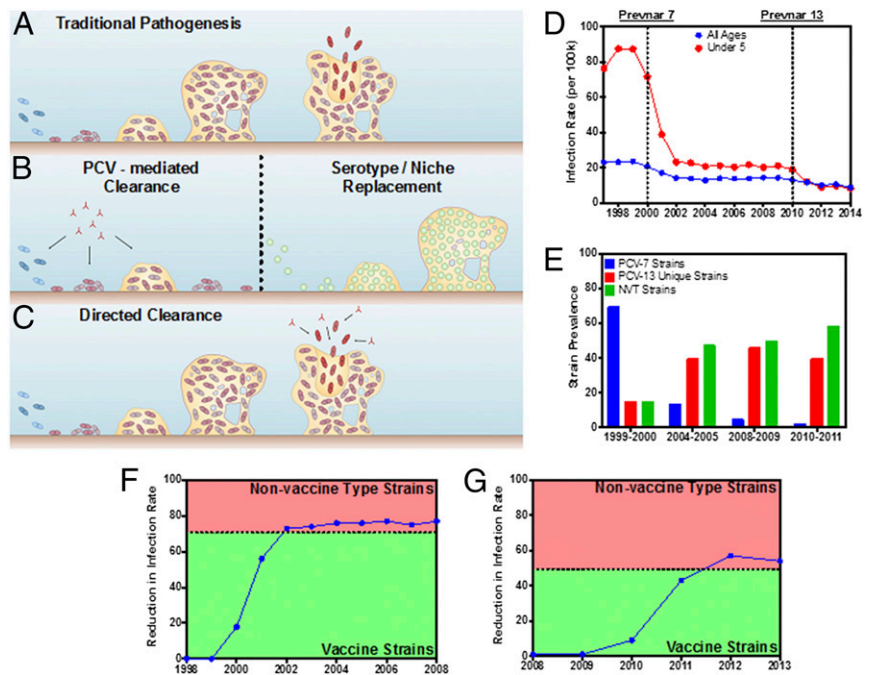
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Fig. 1. *S. pneumoniae* pathogenesis outcomes and infectious disease statistics in the United States (1998–2013). (A) *S. pneumoniae* colonizes the human nasopharynx and produces a bacterial biofilm with an accompanying extracellular matrix capable 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 conjugate vaccines (PCVs), such as the Prevnar family] mediate protection against certain bacterial serotypes by promoting clearance of pneumococci before biofilm establishment. Clearing all bacteria opens the niche to colonization by nonvaccine serotypes or other bacterial species. (C) The strategy featured in this work mediates clearance of only virulent biofilm-released bacteria while maintaining the presence of the preexisting biofilm. (D) The annual infection rate per 100,000 people for the total population (blue) and children under the age of 5 y (red), 1998–2013. The first years following the introduction of Prevnar 7 and 13 are marked with dotted lines. (E) Prevalence of infectious pneumococcal strains, 1998–2013 (29). Strains are grouped into those covered by Prevnar 7 (blue), those covered by Prevnar 13 (red), and non-vaccine types (NVT; green). (F) The reduction in the annual infection rate in children under the age of 5 y from 1998–2008 relative to 1998–1999. The dashed line corresponds to the division between Prevnar 7 vaccine and nonvaccine type strains in 1999–2000 (35). (G) The reduction in the annual infection rate in children under the age of 5 y, 2008–2013. The dashed line corresponds to the division between Prevnar 13 vaccine and nonvaccine type strains in 2008–2009 (35).



of invasive pneumococcal disease cases (29, 30). 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 (29, 30). However, current vaccination strategies have met with incomplete success because of (i) an inability to account for and include all current and future *S. pneumoniae* serotypes capable of establishing nasopharyngeal residence and (ii), analogous to antibiotic treatment, the displacement of the asymptomatic vaccine-type *S. pneumoniae* biofilms with nonvaccine serotypes and by organisms (such as methicillin-resistant *S. aureus* or *Haemophilus influenzae*) capable of equal or greater pathologies (Fig. 1B) (31–33).

Recognizing the need to develop a new generation of pneumococcal vaccines, we present a strategy to direct 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 on the fundamental insight that *S. pneumoniae* colonizes the nasopharynx as a biofilm and that disease progression occurs when 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, 34). 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) (35). Effectively, the propensity for serotype replacement, within or outside current treatment options, underscores the need to identify and use 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 significantly up-regulated 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 mouse protection assays, allowing a substantial increase in the number of strains tested in this study.

Initial protection then was investigated by immunizing mice with a range of promising antigen targets (Table S1) selected on the basis of (i) conserved sequence homology across *S. pneumoniae* strains (thus offering broad-coverage potential) and, critically, (ii) specific prominence in the virulent, biofilm-released bacterial population compared with 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 best-studied protein protective vaccine candidates and also is up-regulated during virulence transition (Fig. 2E) (36–38). Under these conditions, all antigens except DexB showed protection comparable to PspA, with two antigens, GlpO (an α -glycerophosphate oxidase) and PncO (a bacteriocin ABC transporter transmembrane protein), demonstrating promising individual protection surpassing that of PspA. Complete protection and effective bacterial reduction were conferred upon immunization with both antigens (Fig. 2F and G); thus, remaining analyses were conducted using these two antigens in combination.

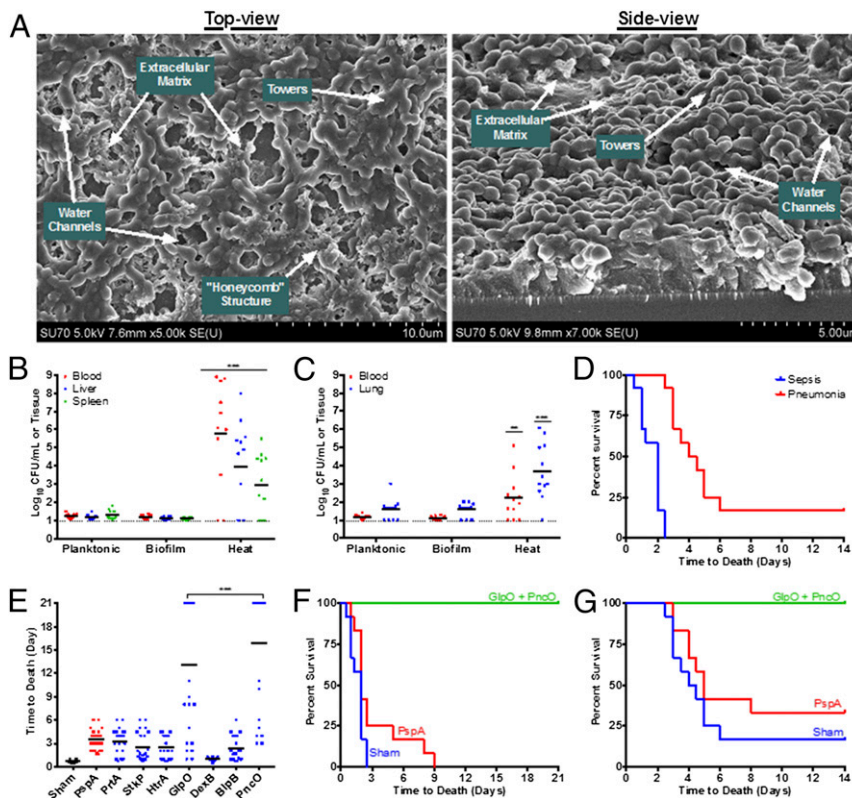


Fig. 2. Antigen identification and *S. pneumoniae* conditioning through an in vitro biofilm model. (A) *S. pneumoniae* were seeded on epithelial cells, and the biofilm structure was investigated using SEM. Visible in these images are the extracellular matrix, water channels, tower formations, and the honeycomb structure that pneumococci form with larger biofilms. (B and C) Mouse bacterial burden was determined after i.p. injections (sepsis model) (B) or aspiration with anesthesia (pneumonia model) (C) 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. The dotted line represents the limit of detection for bacterial counts. (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 die in any challenge model. (E–G) Mice were immunized with various antigens and challenged with biofilm heat-released EF3030 in sepsis (E and F) and pneumonia (G) models. *** $P < 0.001$, compared with planktonic and biofilm samples (B and C) and PspA (E).

Antigen Delivery Using Co-PoP Liposomal Surface Display. The codelivery of GlpO and PncO, both recombinantly produced with 6x histidine tags, was facilitated using a cobalt porphyrin-phospholipid (Co-PoP) liposomal carrier capable of surface-orienting and delivering multiple his-tagged peptide-based antigens (Fig. S1) (39). The liposomal device thus offers a unique vaccine formulation based on 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 the surface localization and presentation of hundreds of additional protein or peptide products.

Directed Response and Extended Coverage Provided by Combined Virulent Antigens. The directed nature of the antigens was tested in a series of experiments presented in Fig. 3A. Across different anatomical locations representative of bacterial colonization (nasopharynx) and displacement (nasopharynx lavage), disseminating pneumonia (lung), and invasive septicemia (blood), vaccinated and nonvaccinated mice were challenged with planktonic and in vitro biofilm-released *S. pneumoniae*, and bacterial clearance was monitored over time. In the absence of an external stimulus (e.g., viral infection), mice will remain colonized with planktonic D39 or EF3030 for 1–3 wk without infection of the lower respiratory

tract or the development of bacteremia. Biofilm-released bacteria demonstrate a colonization pattern similar to that of planktonic bacteria but also have the propensity to disseminate into secondary anatomical sites and cause disease. Thus, planktonic EF3030 and D39 cells provided a clearance baseline to compare bacterial loads using biofilm-released pneumococci challenge. Clearance of biofilm-released bacteria was mediated only in vaccinated mice; the bacterial load was increased significantly and was lethal in nonvaccinated mice. Interestingly, the rate of clearance of planktonic bacteria was unchanged despite vaccination. A comparison of bacterial burden across the planktonic and biofilm-released EF3030 clinical isolate is provided in Fig. 3B, emphasizing a directed vaccination strategy using GlpO and PncO.

However, the potential of *S. pneumoniae* for antigenic drift emphasizes the need for any new antigens to be general and effective across a wide range of challenge strains (Tables S2 and S3). To this end, the antigens were tested in mice infected with a range of *S. pneumoniae* strains chosen for the notable difficulty of protecting against these strains and for the variability in serotype and genetic background among the strains. Complete protection was provided for a panel of strains across both sepsis and pneumonia challenge models (Fig. 3B and C and Figs. S2 and S3). Ten additional strains were tested in protection experiments, in which the average time to death in treated mice ranged from 12–21 d as opposed to <3 d for controls (Fig. S4). 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

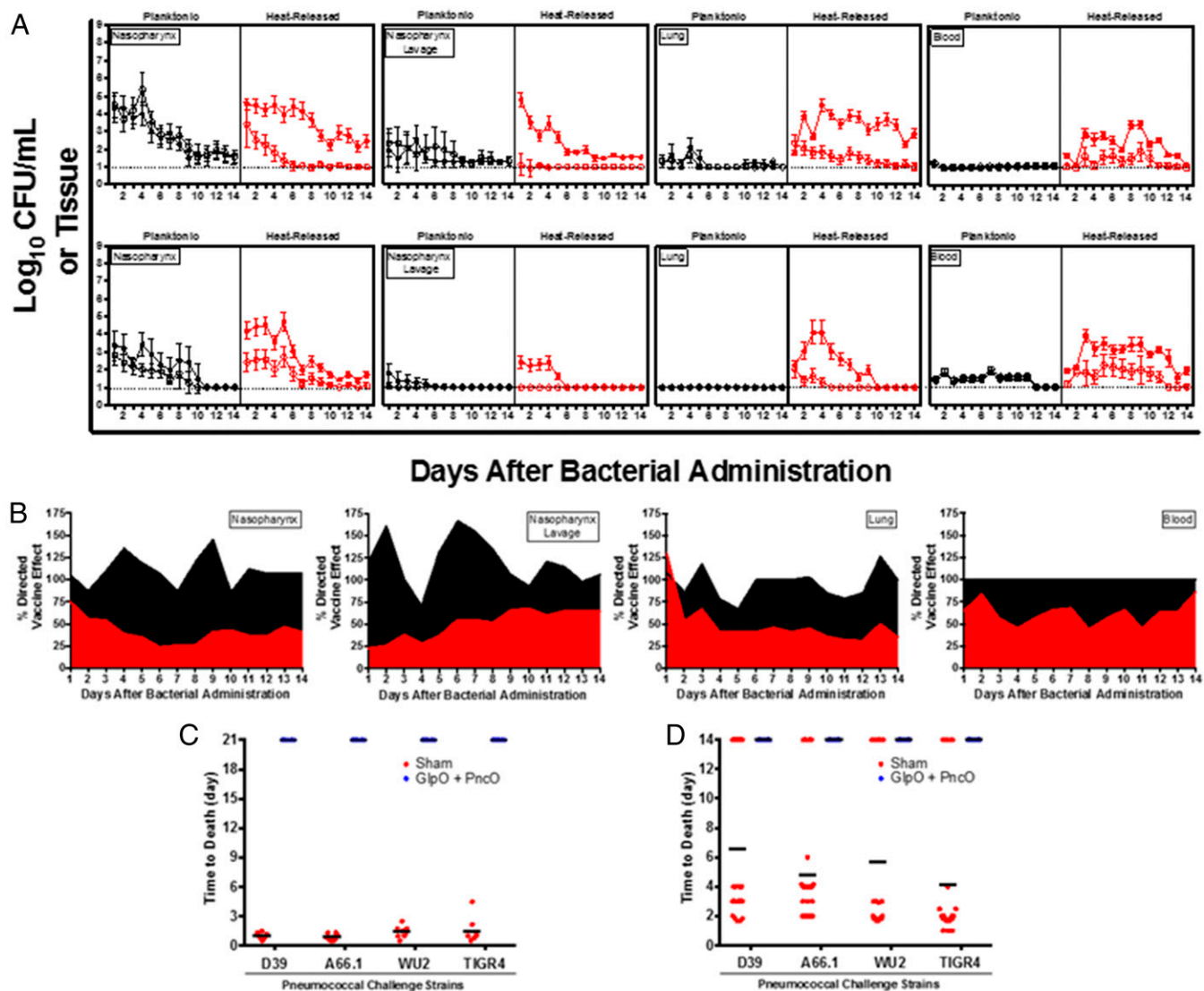


Fig. 3. Directed clearance of biofilm-released bacteria and protection against mouse-passaged challenge strains. (A) Bacterial burden at various anatomical sites was determined daily in unimmunized (filled circles) and GlpO + PncO immunized (open circles) mice. Mice were inoculated intranasally without anesthesia with planktonic or biofilm heat-released EF3030 (Upper) or D39 (Lower) bacteria. (B) Comparative analysis of the planktonic (black) and biofilm-released (red) EF3030 clinical isolate data from A. A value of 100% represents no difference between immunized and unimmunized subjects; values below 100% indicate a directed response. A full statistical comparison is presented in Table S5. (C and D) Protective capabilities of GlpO + PncO immunization were evaluated further in sepsis (C) and pneumonia (D) models with established mouse-passaged pneumococcal bacteria. Dotted lines represent the limit of detection for bacterial counts.

assessment. These challenge assays included strains of serotype 12, 15B, and 27 that are not covered by current vaccines and suggest that (i) this vaccine composition can offer protection against strains currently circulating and causing disease in the population and (ii) the methodology of *in vitro* biofilm release can be used to produce additional mouse-virulent bacterial populations (beyond those tested here) 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 identify and test new antigens continually 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 S4). The results therefore support the potential for widespread protection and resistance to antigenic drift.

Extension to an *in Vivo* Model of Virulence Progression. In a final set of experiments presented in Fig. 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 h 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, 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. 4 A and B). Of major importance, the nasopharyngeal burden in immunized and nonimmunized animals remained unchanged, suggesting that harmless and potentially beneficial commensal colonization was unaffected. This result further supports a

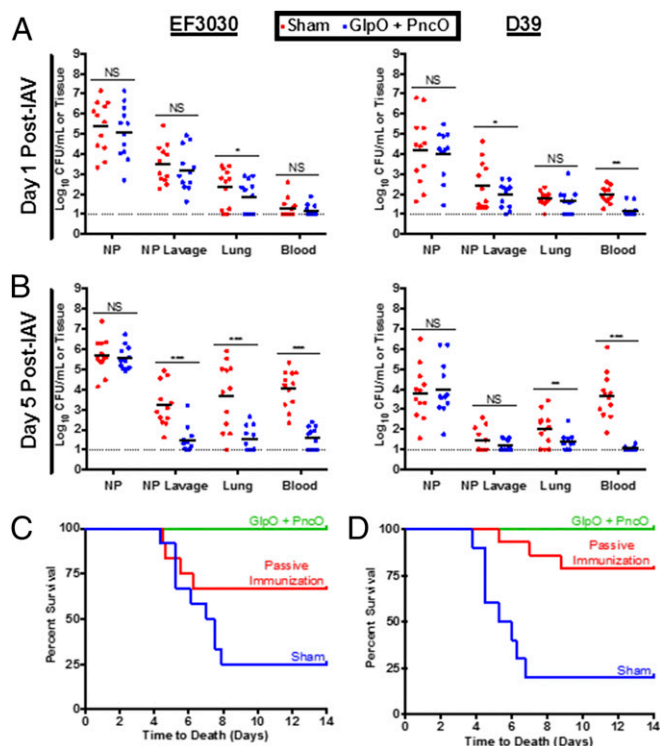


Fig. 4. Bacterial dissemination and time-to-death assessment of mice stably colonized with pneumococci and triggered with IAV. (A and B) Bacterial burden of EF3030 or D39 in unimmunized or GlpO + PncO immunized mice was measured at 1 (A) or 5 (B) d postinfection with IAV. The dotted line represents the limit of detection for bacterial counts. (C and D) Protective capabilities of traditionally or passively GlpO + PncO immunized mice against in vivo IAV-mediated bacterial release of EF3030 (C) or D39 (D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; NS, not significant.

paradigm shift in protection against accidental pathogens or those commensals that colonize asymptomatically 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 provided by the combination of GlpO and PncO using traditional immunization, whereas partial protection was accomplished via adoptive/passive vaccination strategies (Fig. 4 C and D and Fig. S4). The latter result indicates an important role of the humoral immune system arm in the mechanism driving the results obtained throughout this study and suggests that an additional T-cell response also contributes. Interestingly, these observations are in line with the recognition that priming of T_H17 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 characterize better the immunological underpinnings of the results presented. We also recognize the potential for immune response cross-talk, in that analogous antigens might be targeted in other commensal organisms. For the primary antigens tested 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, based upon homology analysis. 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 up-regulated versions of the antigens tested here. However, a more complete analysis of microbial content postvaccination would be required to

elucidate the impact on global microflora variation. This analysis would be a fruitful and interesting topic of future research.

Conclusions

In summary, the availability of tools derived from a better understanding of the mechanisms involved in pneumococcal biofilm establishment and transition to disease and of 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 protection against challenge and reduction in the 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 to growing concerns associated with current treatment options, including risks posed by emerging serotype- and niche-replacement pathogens. Importantly, the tools enabling the results of this work can be applied to identify new antigens continually and simultaneously to address the diversity and antigenic drift potential of *S. pneumoniae* and other pathogens that exhibit similar virulence progression.

Materials and Methods

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 (43). The protocols were approved by the Institutional Animal Care and Use Committee at the University at Buffalo, The State University of New York. All bacterial inoculations and treatments were performed under conditions designed to minimize any potential suffering of the animals.

Vaccine Formulation and Immunization. Antigen Co-PoP liposomal carrier vectors were generated as described previously (39). Each injection dose contained 25 μ g of monophosphoryl lipid A (MPLA) in liposomes comprising 1,2-dioleoyl-sn-glycero-3-phosphocholine:cholesterol:MPLA:Co-PoP at a molar ratio of 50:30:5:5. After liposomes were dissolved in chloroform in a test tube, the solvent was evaporated, and the film was further dried under vacuum overnight. Liposomes then were rehydrated with PBS and sonicated. The binding ability of recombinant antigens was evaluated by incubating 25 μ g of protein with 20 μ g of liposomes in 200 μ L PBS within a well of a 96-well plate. Fluorescence in the FRET channel (excitation: 430 nm, emission: 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 $^{\circ}$ 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 μ g).

Outbred 6-wk-old female CD-1 mice (Harlan Laboratories) were used in immunization experiments. Mice were immunized by s.c. injection (200 μ L). All samples contained PBS as the background solution, and final antigen (Table S1) doses ranged from 5–15 μ g. The sham vaccination control was the Co-PoP delivery device in PBS. When combined, PncO and GlpO (Table S4) were administered at 15 μ g each. After 14 d, 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 10 times and administered via i.p. injection (200 μ L).

Bacterial Preparation and Biofilm Release. The bacterial strains used in this study are listed in Tables S2 and S3 and were initially grown on Todd-Hewitt agar plates supplemented with 0.5% yeast extract and 5% (vol/vol) sheep blood and were incubated overnight at 37 $^{\circ}$ C. Single colonies were used to inoculate 5 mL of Todd-Hewitt broth containing 0.5% yeast extract and were incubated at 37 $^{\circ}$ C to an OD₆₀₀ of 0.6. At this point, mouse-passaged strains of *S. pneumoniae* (which display a virulent phenotype) were used for challenge studies after one washing with and resuspension 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 $^{\circ}$ C and 5% CO₂. After reaching 100% confluency, H292 cells were prefixed in 4% (wt/vol) buffered paraformaldehyde at 34 $^{\circ}$ C for 48 h followed by three washes with PBS. Pneumococci grown in

chemically defined bacterial growth medium (CDM) (JRH Biosciences) then were seeded onto fixed H292 cells, and the medium was changed every 12 h. Formed biofilms were exposed to heat (38.5 °C) for 4 h, and released cells were collected by centrifugation, washed and resuspended in PBS, and quantified by OD₆₀₀ measurement. Biofilm-associated cells were disrupted by gentle pipetting, collected by centrifugation, washed and resuspended in PBS, and quantified by OD₆₀₀ measurement.

Challenge Models. To induce sepsis or pneumonia, mice were administered i.p. or intranasally (with isoflurane), respectively, with 1×10^4 to 1×10^6 cfu of pneumococci strains (Tables S2 and S3). To induce colonization, mice were administered 1×10^6 cfu bacteria intranasally without isoflurane. To mimic influenza-induced pneumonia, pneumococci colonization (with biofilm-grown EF3030 or D39) was followed 48 h later by intranasal inoculation with 40 pfu of IAV in 50 μ L of 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 4 h for signs of morbidity (huddling, ruffled fur, lethargy,

and abdominal surface temperature). Mice found to be moribund were killed via CO₂ asphyxiation and cervical dislocation. When IAV addition was replaced with background PBS inoculation, mice remained colonized by *S. pneumoniae* strains D39 and EF3030 for 1–3 wk without lethargy, huddling, and ruffled fur (as observed with viral inoculation), 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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