Contributions to Protection from *Streptococcus pneumoniae* Infection Using Monovalent Recombinant Protein Vaccine Candidates PcpA, PhtD and PlyD1 in an Infant Murine Model During Challenge

David Verhoeven, Sheldon Perry, and Michael E. Pichichero*

Rochester General Hospital Research Institute, Rochester General Hospital, Rochester NY 14621

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*Corresponding Author:

Michael E. Pichichero, M.D.
Rochester General Research Institute
Rochester General Hospital
1425 Portland Ave
Rochester NY 14621
(585) 922-2411
Michael.Pichichero@rochestergeneral.org
ABSTRACT

A vaccine consisting of several conserved proteins with different functions directing pathogenesis of pneumonia and sepsis would be preferred for protection against infection by *Streptococcus pneumoniae*. Infants will be the major population targeted for next generation pneumococcal vaccines. Here, we investigated the potential efficacy provided by from three recombinant pneumococcal vaccine candidate proteins; pneumococcal histidine triad D (PhtD), detoxified pneumolysin (PlyD1), and pneumococcal choline-binding protein A (PcpA), toward reducing pneumonia and sepsis in an infant mouse vaccine model. We found vaccination with PhtD and PcpA provided high IgG antibody titers after vaccination in infant mice, similar to adult mice comparators. PlyD1-specific total IgG was significantly lower in infant mice with minimal boosting with the second and third vaccinations. Similar isotypes of IgG for PhtD and PlyD1 were generated in infant compared to adult mice. Although lower total specific IgG was elicited to all three proteins in infant compared to adult mice, the infant mice were protected from bacteremic pneumonia and sepsis mortality (PlyD1) and had lower lung bacterial burdens (PcpA and PhtD) after challenge. The observed immune responses coupled with bacterial reductions elicited by each of the monovalent proteins support further testing in human infant clinical trials.
INTRODUCTION

Infants will be the major population targeted for next generation pneumococcal vaccines. Therefore, using an infant animal model to study vaccine candidates provides value for determining the efficacy of potential candidate pediatric vaccines for protection from disease. A vaccine that elicits antibodies to several conserved proteins with different functions during pathogenesis of \textit{Streptococcus pneumoniae} (Spn) would be desirable to mitigate against vaccine-induced selection of strains expressing variations in the selected proteins (1, 2). Here, we studied three recombinant pneumococcal vaccine candidate proteins, pneumococcal histidine triad D (PhtD), detoxified pneumolysin (PlyD1), and pneumococcal choline-binding protein A (PcpA), to determine their role in protection against pneumococcal pneumonia and sepsis in an infant mouse vaccine model. We sought here to further define the individual contributions to the observed protection. By including parallel studies in adult mice we also sought to determine similarities and differences in immune responses elicited in infant and adult mice after vaccination. PhtD is a well-conserved surface protein and a member of the Pht protein family characterized as having a histidine triad motif and is regulated by extracellular zinc concentration (1-3). In adult animal models, PhtD have been studied extensively against sepsis, pneumonia and colonization with protection levels highly bacterial strain dependent and in many cases did not correlate with antibody titers (1, 2, 4-6). A two subunit, PhtD and dPly (detoxified pneumolysin), vaccine protected rhesus macaques from pneumonia that also led to better survival after challenge (7). Human antibodies to PhtD are reported to be functional in an adult murine passive protection sepsis model and a Phase I exploratory study of PhtD vaccine was shown to be safe and immunogenic in human adults (8). Natural colonization, as well as infection by \textit{Spn}, can lead
to antibodies directed against PhtD but antibody levels to PhtD have not correlated with protection against disease (4, 9-11). A study of natural plasma antibodies against PhtD showed reduced adhesion of *Spn* to lung epithelial cell *in vitro* (12) but it is not known whether a PhtD protein vaccination would offer similar protective antibodies in the lungs.

Pneumolysin (Ply) is a highly conserved, membrane pore-forming protein located in the cytoplasm but released into the media during autolysis (13). Ply is a major virulence factor that exerts cytotoxic on epithelial cells and immune cells (13). Human antibodies to Ply can be detected in colonized or convalescent humans and these antibodies can proved passive protection in challenged adult mice (14). However, due to its hemolytic activity, Ply needs to be detoxified for vaccination studies either genetically or chemically. Vaccines using chemically modified Ply to inactivate its hemolytic function have shown some level of protection in animal studies (5, 15-18) thus demonstrating that neutralization of Ply by antibodies may provide some protection against pneumonia and bacteremia. Recent development of a highly detoxified genetic mutant of Ply (PlyD1) has shown limited protection in mice against challenge with *Spn* and lung injury (17). Phase I studies have demonstrated that a highly detoxified genetic mutant of Ply (PlyD1) is safe and immunogenic in adults (19). Natural *Spn* colonization leads to lower Ply-specific plasma IgG levels in infants and young children compared to other *Spn* proteins or older children (9). Therefore, the concentration of total specific IgG and function generated to Ply after vaccination in infants would be important to study further to better understand the efficacy of a potential trivalent vaccine containing this component.

Pneumococcal choline-binding protein A (PcpA) is distinct from another pneumococcal choline-binding protein named CbpA or PspC (20). The *pcpA* gene is conserved among different *Spn* strains examined (21) and the PcpA protein is surface exposed (21) under the control of
extracellular manganese concentrations (22). PcpA is not required for colonization of the murine nasopharynx (21, 22). Human antibodies to PcpA have been detected in infants and children with pneumococcal bacteremia or pneumonia (23). In monovalent vaccines containing PcpA, some level of protection from pneumonia was found and a delay in morbidity after sepsis challenge was determined in an adult mouse model (21). The mechanism of this protection may be antibody mediated as interference to adhesion from antibodies derived from colonized human hosts can block binding to lung derived cell lines (12). A Phase I study of PcpA in combination with PhtD showed that the bivalent vaccine was safe and immunogenic in human adults (24).

In the current study, we examined the protection provided by monovalent vaccination with recombinant PcpA, PhtD and PlyD1 (designed from serotype 6B) from challenge with serotype 6A. This study is novel as it is the first to describe the efficacy of these proteins in an infant model of vaccination and to provide direct comparisons of immune responses between infant and adult mice in a model system. Moreover, determining protection for each of these components in an anticipated trivalent vaccine is critical for future efficacy clinical trials in infants and children.
METHODS and MATERIALS

Animals - 6-week old male and female C57BL/6 mice were purchased from NCI and housed in a SPF BSLII murine facility at Rochester General Hospital Research Institute (RGHRI) using microisolator housing. C57BL/6 infant mice were obtained by breeding at RGHRI. All procedures were approved by the IACUC at RGH. Vaccination experiments were performed using five mice per group with two or three separate replicates vaccinated at different times as stated in the figure legends.

Vaccinations - Recombinant PcpA, PhtD and PlyD1 proteins, constructed from a serotype 6B Spn strain, were obtained from Sanofi Pasteur. Protein doses, as previously described as optimal in BALB/c mice (25) and determined to be optimal in our preliminary work in C57BL/6 mice, were combined with aluminum hydroxide (Alum) as an adjuvant. Unvaccinated controls received Alum alone. Injections were performed using 25ul into both of the caudal muscles of the hind legs with three vaccinations in an accelerated weekly schedule, due to the rapid aging of infant mice (Fig 1).

Spn Challenge - Spn BG3722, a serotype 6A, was obtained from Sanofi Pasteur and expanded from streaked plates Todd Hewitt Broth (THB) with 1% yeast extract (Difco). Expanded Spn (OD$_{600}$ =0.6) was centrifuged, resuspended to 1x10$^8$ cfu/ml in THB with 10% glycerol, and frozen at -80$^\circ$ C. On the day of challenge, Spn was thawed and grown in THB with 1% yeast extract at 37$^\circ$ C with 5% CO$_2$ until mid-log phase (OD$_{600}$ = 0.6). Bacteria were then centrifuged and washed twice with PBS and resuspended at 25 x10$^6$ cfu/ml in PBS with dilution plating to confirm CFUs. Mice were anesthetized with Isoflurane and 40ul of bacterial challenge stock (1x10$^6$ CFU, 2LD$_{50}$) was instilled into the nasal passages of each mouse.
Antibody Assays- Serum was obtained after each vaccination by tail bleeding (Fig 1) and after
Spn challenge by cardiac puncture after euthanasia. Recombinant proteins, obtained from Sanofi
Pasteur, were plated on Immunlon II ELISA plates (ThermoFisher, Hampton NH) at 0.5 μg per
well overnight at 4°C. Plates were then blocked with nonfat milk and sera, as well as reference
serum, (obtained from vaccinated mice with known antibody concentrations) were assayed with
a secondary rabbit anti-mouse AP (Jackson Immuno, West Grove PA) at 1:10,000 dilution.
Calculation of total IgG for each serum was calculated from a standardized curve and reference
serum. End-point titers for total IgG or IgG1, IgG2a, IgG2b, IgG2c, and IgG3 were performed
for each antigen with specific donkey secondary antibodies AP (Jackson Immuno) at 1:10,000
dilution.
Lung Bacterial Counts- CFUs were determined by dilution plating from clarified lung extracts,
(48 hours post-challenge) processed by mortar and pestle and resuspended in one ml of PBS,
onto TSA II plates containing gentamicin (BD Biosciences, San Jose CA). Plates were incubated
overnight at 37°C.
Antibodies and Reagents- Fluorochrome-conjugated anti-CD3, CD11b, CD11c, GR-1, CD45,
F4/80, and MHC Iα/Iβ were obtained from BioLegend (San Diego, CA).
Flow cytometry- Lungs (mice perfused by PBS) from 48 hours post-challenged infected mice
underwent collagenase digestion (1 hour at 37°C in CDTI media containing 1mg/ml
collagenase, 1mg/ml DNase, 1mg/ml Trypsin inhibitor) and passed through a 100μm cell strainer
and washed 2 times with PBS. This was followed by lysis with ACK solution (5 minutes at
room temperature) to remove RBCs. Cells were then washed 2 times with PBS. Remaining
cells were surface stained with fluorochrome-conjugated antibodies, fixed, and acquired using an
LSRII flow cytometer (BD Biosciences) with a minimum acquisition of 300,000 events.
Analysis of acquisition events was accomplished using FlowJo software (Tree Star, Portland OR) with gating using live amine dye (Invitrogen, Carlsbad CA) and double discrimination with CD3+ dump gating.

**Histology** - Lungs were obtained from mice 48 hours post infection after vascular perfusion with PBS. The lobes were clamped at the bronchioles and perfused with 4% Buffered Formalin. 5μm sections were cut and H&E stained (AML labs). Image files were processed with Adobe Photoshop (San Jose, CA) with auto levels selected to automatically adjust brightness, color balance, and contrast for all images. Influenza infection leads to significant infection of the large left lobes (data not shown) and therefore we assumed equal distribution of *Spn* into the large left lobes since inoculation techniques were similar.

**Absolute counts** - Lungs were processed as previously stated using CDTI and processed over Ficoll gradient. Cell pellets containing macrophages and granulocytes were lysed with ACK as previously stated. Cell counts were obtained using a cell counter (Biorad, Hercules CA) and absolute numbers determined by calculation from data obtained by flow cytometric analysis of macrophage (GR1+, F4/80+) percentages.

**Bacterial Adhesion Assay** - For monovalent PlyD1 studies, *Spn* BG3722 was grown in Todd Hewitt Broth/1% yeast extract. For PhtD and PcpA studies, *Spn* was grown in Todd Hewitt Broth/1% yeast extract that was previously chelated with Chelex beads (Sigma) overnight with shaking and then supplemented with 1μM MgCl₂, 1μM MgSO₄, and 1μM CaCl₂. In all studies, bacteria were grown to an OD₆₀₀ of 0.6, washed extensively with PBS followed by staining of 10⁸ bacteria in 1mg/ml of FITC (Sigma) at 4⁰ C for 1 hour and then washed again with PBS. Approximately, 10⁶ *Spn* cells were incubated with antibodies from vaccinated mice that was diluted 1:10 in DMEM 10% media, with or without prior chelation and supplementation (as
described above), for 1 hour at 37⁰ C in the presence of 1μg/ml guinea pig complement (Fisher). Bacteria were transferred onto primary lung epithelial (Type II) or endothelial cells (Cell Biosystems), previously grown to 95% confluency in 8 well chamber slides, and incubated for 5 hours. Cells were washed extensively 3 times with PBS and then fixed for 5 minutes in cold acetone and air dried. Cells were rehydrated in PBS before mounting in Vectashield with Dapi. Bacterial adherence was imaged with an Axioshop (Zeiss) with filters for FITC and Dapi. Quantification was performed by manually counting the number of positive cells per 1mm² of cells in an average of 10 fields for each animal.

**PlyD Toxicity assay** - *Spn* BG7322 was grown in THB with 1% yeast extract. FITC labeled or unlabeled *Spn* was neutralized as mentioned above with antibodies generated in infant and adult mice vaccinated with PlyD1 vaccine. *Spn* was then added to primary epithelial and primary endothelial lung cells and incubated at 37⁰ C for 5 hours. Cells were washed 3 times with PBS and Sytox Orange (Invitrogen) was added at 0.1 μl/ml in PBS and incubated for 5 minutes followed by washing 3 times with PBS and then fixation, mounting, and imaging as mentioned above. Quantification was performed similar to adherence assay mentioned previously.

**Statistics** - Data was analyzed by two-tailed Student T-tests or ANOVA. Results with a p < 0.05 were considered significant. Data was analyzed by Prism software (Graph Pad, La Jolla CA).
RESULTS

Vaccination dosage optimization

In preliminary experiments, an optimal vaccine dose of each individual protein, PcpA (0.2 μg), PhtD (0.9 μg), and PlyD1 (5 μg), in infant and adult C57BL/6 mice was established by measuring antibody responses at 4 weeks post-tertiary vaccination (data not shown). We investigated a range of antigen dosages around those previously determined optimal in CB mice (personal communication from Sanofi Pasteur) and found the optimal dose (maximal IgG titers) to be similar, but not identical, in infant and adult mice for all three proteins. Alum was included as an adjuvant in all experiments described here because it is approved for use in humans and clinical trials and is included in current clinical trials with these proteins (19, 24).

Discordant antibody generation in infant and adult mice

Since protection from \textit{Spn} bacteremia has been correlated with seroconversion to surface proteins PcpA and PhtD (11), we assessed the total specific serum IgG concentrations for PcpA, PhtD and PlyD1 in monovalent injections at 7 days post-primary and secondary vaccinations, and 4 weeks post-tertiary vaccination (Fig 2A). We found comparable levels of IgG in infant and adult mice vaccinated with PcpA and PhtD, and both showed a similar increase trend after each vaccination. PlyD1-specific total IgG was significantly lower in infant mice with minimal boosting with the second and third vaccinations, a finding similar to that seen in young children who elicit only low IgG responses to Ply (native form of pneumolysin) during natural exposure (26).

We next assessed the IgG isotype responses (end-point titers) in the mice since isotype antibodies direct different functions (27). We used IgG2a as a negative control as C57BL/6 mice don’t make this isotype and no IgG2a antibody was detected for any of the antigens (Fig 2B).
Both infant and adult mice showed higher levels of IgG1 than other IgG isotypes (Fig 2B) for all antigens and were highest in adult versus infant mice. The antibody responses to PcpA in adult mice were significantly higher for IgG3 and IgG1 than in infant mice. Similar titers of IgG2b but no detectable titers of IgG2c were measured. PhtD vaccinated infant and adult mice had a non-significant difference in the IgG2b, IgG2c, and IgG3 titers but adult mice showed significantly higher IgG1 titers in adult mice. Antibody responses to PlyD1 were similar in both infant and adult mice in IgG2b and IgG2c but showed a trend of higher IgG1 and IgG3 titers in adults.

Given that IgG1 end point titers were the highest IgG isotype among all the *Spn* antigens, suggesting that IgG1 was the major induced antibody, there were then greater differences between infant and adult mice vaccinated with PcpA or PhtD as compared to PlyD1.

**Analysis of bacterial lung load, bacteremia and lung histopathology of mice vaccinated with monovalent PcpA, PhtD or PlyD1 and then challenged**

We assessed the protective capacity of each protein by measuring the bacterial counts in the lungs of challenged infant and adult mice. Vaccination with PcpA led to a 2-log reduction in lung burden 48 hours after challenge in infant mice and a 2.3 log reduction in adult mice (Fig 3A). PhtD vaccination led to a smaller but significant reduction (0.83 log) in lung bacterial counts in both infant and adult mice (Fig 3B). Vaccination with PlyD1 showed no reduction in lung bacteria in adult or infant mice (Fig. 3C). However, when we analyzed for bacteria in the blood, only PlyD1 vaccinated mice demonstrated a statistically significant reduction in bacteremia in both infant and adult mice (Fig 3D) suggesting that immune responses elicited by PlyD1 vaccination prevents transmigration of bacteria from the lungs and into the blood stream.

We compared the degree of lung histopathology in vaccinated infant and adult mice 48 hours post-challenge as shown in Figure 4. We found that vaccination with PcpA and PhtD led
to similar levels of lung histopathology in both infants (Fig 4 C-D) and adults (data not shown) as compared to unvaccinated infected control mice (Fig 4B). In agreement with a previous study (17), vaccination with PlyD1 led to a significant reduction in lung histopathology in infant (Fig 4E) and adult mice (Fig 4F) that appeared very similar to uninfected controls (Fig 4A). Using a histopathologic scoring system with higher numbers reflecting greater lung tissue damage (17), we found that PlyD1 vaccinated infant and adult mice had the best histopathologic scores after challenge (Fig 4G).

We tested survival after challenge and found that each vaccinated group had increased survival times that were better for PhtD and PlyD vaccinated mice (Fig 4H-J). For all vaccinees, adults had greater survival times than infants expect for PcpA vaccinated mice. Interestingly, we found 30% survival of PlyD1 vaccinated adult mice.

Antibodies containing both PcpA and PhtD reduce Spn adherence to lung epithelial cells

To assess a potential protective role in preventing Spn adherence to lung epithelium, we performed an in vitro adherence reduction assay using serum obtained from vaccinated mice (Fig 5A-F). We found abundant adherence of Spn to lung epithelial cells in the absence of Spn specific antibodies (Fig 5B). We found moderately lower adherence with antibodies directed to PcpA (Fig 5C-D). In contrast we found much lower adherence with antibodies to PhtD (Fig 5E-F) in vaccinated infant and adult mice that might correlate with better survival rates (Fig 4I).

Quantification of Spn in the presence of absence of antibodies raised in vaccinated mice show that vaccination with PhtD in both infant and adult mice significantly reduced the number of bacteria binding to cultured primary airway epithelial cells (Fig 5G).
To determine whether antibodies generated to PlyD1 could protect epithelial and endothelial damage from the toxicity of native Ply and thus prevent potential translocation of *Spn* into the lung vasculature, we performed an *in vitro* neutralization assay using primary lung cells (Fig 5H-I). We found that primary lung cells incubated with antibodies derived from PlyD1 vaccinated infant and adult mice had significantly less cell damage after 5 hours of incubation in the presence of *Spn*. Quantification of the number of damaged epithelial cells cultured with or without antibodies raised in PlyD vaccinated mice in the presence of *Spn* clearly demonstrate a significant reduction in cell damage (Fig 5J).

**Increased macrophage infiltration after challenge**

We examined the cell types infiltrating the lungs of post-challenge vaccinated mice by immunohistochemistry and found no difference in the staining (number or distribution) between infant and adult mice (data not shown). We found no significant differences in the percentages of pulmonary macrophages (CD45+GR-1lo/int, F4/80+, I_A/L_E+, CD11blo) or inflammatory monocytes (CD45+GR-1hi, CD11bhi, IA/I_Elo) for any of the post-challenged vaccinated mice as compared to unvaccinated infected controls. Figure 6A shows a representative flow dot graph of pulmonary macrophages from the lungs of PhtD vaccinated and challenged mice. When compared to uninfected controls, we observed increases in pulmonary macrophages and inflammatory monocytes in both infected controls and in PcpA and PhtD vaccinated and challenged mice. PlyD1 challenged mice showed only a slight increase in macrophages as compared to uninfected control (Fig 6B). Within each of the different groups, we observed similar levels in the number of infiltrating macrophages in both the infant and adult mice after challenge (Fig 6B). We did not observe a difference between vaccinated and unvaccinated
infected controls with respect to polymorphnuclear cells (GR1+F4/80-MHCII-) at 48 hours post-
infection (data not shown).
DISCUSSION

A multi-component protein-based vaccine that protects from all *S. pneumoniae* (Spn) strains would be a significant advance (28). Here we investigated the protective contributions of three *S. pneumoniae* proteins, PcpA, PhtD and PlyD1, as candidate vaccine antigens in an infant murine challenge model and we made direct comparisons of immune responses in infant and adult mice. We tested the protection afforded by the monovalent vaccines against a type 6A challenge strain, as this serotype was reproducibly lethal in C57BL/6 mice. The genetic background of the mice is important in studying pneumococci pathogenesis as CBA mice tend to be very susceptible to *S. pneumoniae* lethality, BALB/c mice are fairly resistant, and C57BL/6 mice in between, and the degree of granulocyte activation by *S. pneumoniae* dictates the level of subsequent pathogenesis (29, 30). We utilized the challenge strain BG3722 due to its reproducible lethality in C57BL/6 mice. We considered testing serotype 6B in the animal model to initially explore immune responses to identical amino acids as the vaccine antigens but this serotype is not lethal in C57BL/6 mice. Therefore, we chose a strain that has significant pathology in B6 mice and has been used by Sanofi Pasteur for their companion studies using these proteins.

Using monovalent vaccines, infant and adult mice showed similar increases in IgG antibody specific titer to the vaccinated antigens except for PlyD1 where infant mice elicited minimal titers. Subtyping of IgG specific antibody responses demonstrated a dominance of IgG1 antibodies. The dominance in IgG1 antibodies implies a strong Th2 or Tfh CD4 T-cell memory response. An accelerated vaccine schedule of primary and two boost vaccinations separated by one week was used in this study due to the rapid aging of the infant mice. Interestingly, the isotypes of IgG generated toward PcpA differed between infants and adults where infants failed to generate IgG3 after vaccination and neither infant nor adult mice generated an IgG2c...
detectable response. The somewhat higher titers of IgG2c in PhtD or PlyD1 vaccinated adult mice may indicate that Th1 memory development is more developed in adult mice as suggested by other studies (Verhoeven unpublished data). Th1 CD4 T-cell memory can influence the quality of the innate immune response and therefore the activity of innate cells could be altered by the higher potential memory in adult mice (31). Importantly, IgG1 and IgG2b and IgG3 have been shown to equally protective to phosphocholine antigens suggesting the broad class of isotypes elicited to these proteins could be additive for protection (32).

Lung bacterial loads were reduced with monovalent vaccines in both infant and adult mice. Mice given a lethal dose of Spn four weeks after vaccination with monovalent PcpA vaccine showed ~2 log reduction in bacterial lung load while PhtD vaccinated mice showed ~ 0.5 log reductions, and PlyD1 vaccinated mice showed no reduction. Our results are in agreement with previous studies that showed protection from Spn challenge after vaccination with PcpA and PhtD in adult mice (2, 21, 33). Contrary to bacterial lung load, PlyD1 showed the highest reduction in bacteremia compared to the other two monovalent vaccines. Ply expression is required for high levels of bacteremia (34), in part suggested by its ability to cooperate in degrading C3b on the bacterial surface (35), and we observed significantly lower levels of bacteremia in mice vaccinated with PlyD1. It has already been reported that PlyD1 vaccination prevents lung histopathology after lethal challenge by limiting lesion and inflammation in adult mice (14, 17, 36). We observed a reduction in bacteremia in PlyD1 vaccinated infant and adult mice that may result from better protection of lung epithelium from the toxic effects of Ply and subsequent reduction of bacteria from dissemination into the capillaries that line the epithelial barrier in the alveolar space. In fact, Ply is necessary for establishment of acute sepsis and high titer replication in the blood (34, 37). Of interest, even with lower antibody titers directed to...
PlyD1 in infants compared to adults, tissue histopathology was limited suggesting that low titers of antibody could still neutralize the damage of lung epithelial cells caused by Ply. The difference in antibody titers between total specific and isotypes most likely is due to the differences in precision between the two assays with higher precision in total specific IgG assays.

We focused on early antibody-mediated protective responses. For our analysis of absolute counts, we have taken into account the total cellularity in the lung of each mouse. However, we found no significant differences in absolute counts between PcpA, PhtD, and infected controls and imply the lungs of these mice might have been equally inflamed signaling for strong poly/mononuclear recruitment. What effects these vaccines have on activity would be something for future studies if a reliable protein antigen OPA is developed. The lower recruitment of mononuclear cells in Ply vaccinated mice may be due to significantly lower inflammation or protection by resident alveolar phagocytic function. Our assay conditions did not detect intracellular versus extracellular bacteria and thus it is possible the bacteria we detected may derive in part from phagocytized Spn. We intend to follow up these studies with additional characterization of these mechanisms.

We postulate that the levels of protection, albeit limited, afforded by each vaccine antigen may derive from two key aspects. First, vaccination with PlyD1 leads to neutralization of Ply that in turn limits damage to lung epithelium that allows Spn entry into the vasculature and initiation of bacteremia and lethal sepsis as stated previously. Neutralizing Ply could also affect the level of virulence by limiting Ply-mediated complement depletion (38), prevention of phagocytosis by autolysis (39), and reduction in polymorphonuclear cell anti-bacterial properties (40). However, neutralizing Ply could reduce bacteremia (37) but the observed death in our
PlyD1 vaccinated mice may indicate a reduction of bacteremia without a reduction in sepsis. Second, antibodies to PcpA and PhtD prevent binding of Spn to airway epithelium and here we observed prevention of binding to primary epithelial cells for the first time. Previous studies have demonstrated a correlation between the levels of antibodies to PcpA (in conjunction with Ply) and reduction in pneumonia (23). Antibodies directed to PcpA and PhtD also appear to correlate with a reduction in bacteremia (11). We observed some reduction in bacteremia (trends) with vaccinations with these two antigens. In contrast to another study (21), we did not observe complete protection with PcpA vaccination alone, indicating a multicomponent vaccine may be necessary. Importantly, however, we contend that prevention of Spn binding to epithelial cells may allow for higher mucociliary clearance, an important protective response for Spn (41), as observed with the reduced attachment and lower lung burdens. We did not observe an increase in macrophages/monocytes or polymorphonuclear cells in PcpA or PhtD vaccinated mice over infected controls. However, we did not determine whether vaccination with either of these antigens could affect their opsonophagocytic activity or if protection by these antigens is related more to mucociliary rates through reduction in attachment. PlyD1 vaccination to prevent epithelial cell death along with vaccine antigens to prevent attachment could protect mucociliary potential of the lungs. Our data suggest that vaccines with PhtD or PcpA alone not will be able to prevent significant cellular recruitment to the lungs and pneumonia. Our data suggest that vaccines that also contain PlyD1 will protect against pneumonia by reducing the level of inflammation in the lungs in conjunction with reductions of bacteria due to antibodies to PhtD and PcpA. These two mechanisms are targets for future studies especially if a reliable assay for protein antigen opsonophagocytosis can be developed.
In summary, we have shown that vaccination with monovalent protein based vaccines containing PcpA, PhtD and PlyD1 can contribute to the level of bacterial reductions in both infant and adult mice after a lethal dose heterologous challenge. This study highlights the interplay necessary between vaccine-induced antibodies for direct neutralization of toxic virulence factors to prevent epithelial damage and bacteremia and recruitment of innate cells to the lungs. We are actively pursuing additional antibody based assays to characterize the protection and in future studies hope to isolate and transfer these antibodies and observe effects on protection from challenge. Future studies to explore additional mechanisms of protection afforded by these vaccine proteins in infant mice are warranted.
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FIGURE LEGEND

Figure 1. Infant mice were injected at times shown with either monovalent vaccines containing PcpA, PhtD, or PlyD1. Injections were into both hind leg caudal muscles (25ul per muscle). Four weeks after the third vaccination, mice were challenged by intranasal instillation with Spn.

Figure 2. Total specific and isotype antibody titers elicited with monovalent vaccines.

Infant and adult mice were vaccinated using monovalent formulations of PcpA (0.2ug), PhtD (0.9ug), or PlyD1 (5ug). (A) Total antigen-specific antibody responses were determined at 7 days post vaccine 1 and 2, and 4 weeks post vaccine 3. (n=5 each for 3 independent experiments, mean +/- SD). (B) End-point titers for antigen specific antibody isotypes were determined 4 weeks after tertiary vaccine. (n=8 each group, mean +/- SD). *p<0.05 **p<0.001

Figure 3. Vaccination with monovalent antigens shows enhanced bacterial clearance. (A)

We assessed bacterial lung burdens in (A) PcpA, (B) PhtD, or (C) PlyD1 vaccinated infant and adult mice. (n=5 for 3 independent experiments, mean +/- SE) (D) Bacterial counts per ml of blood were assessed 48 hours after challenge. (n=5 each group for 2 independent experiments, mean +/- SE) p-values are as follows *p<0.001 **p=0.05 unless exact value is stated.

Figure 4. Vaccination with PlyD protects infants and adults from histopathology. We assessed lung tissue histology in (A) uninfected (B) or infected mice as controls (infant mouse lung tissue shown) 48 hours after infection. Representative histology is shown for 48 hours post-challenge for (C) PcpA vaccinated infants, (D) PhtD vaccinated infants, (E) PlyD1 vaccinated infants, and (F) PlyD1 vaccinated adults. Each slide at 20x magnification. (G) Average histopathologic scores for each lung section. Controls compile both infant and adults that had
similar histopathological scores. (n=5 per group for 2 independent experiments, mean+/−SD).

(H-J) Survival curves for mice vaccinated with PcpA, PhtD, and PlyD1 respectively (n=5 for each group for 3 independent experiments). *p<0.05

**Figure 5. Vaccination leads to a reduction in Spn adherence to primary lung epithelial cells.** (A-F) FITC labeled Spn was incubated with serum from vaccinated (PcpA or PhtD monovalent or mixed antibodies) or unvaccinated mice and with guinea pig complement for 1 hour prior to adding to C57BL/6 derived primary lung epithelial cells. Spn/No antibody control contained non-specific mouse serum. Green=Spn. (G) Quantification of number of Spn binding to 1mm² of cultured cells was performed for antibodies generated from vaccinated mice (n=4 each group). (H-M) Spn was incubated with serum from vaccinated or unvaccinated mice and with complement for 1 hour prior to adding to C57BL/6 derived primary lung epithelial cells. Cells were then incubated for 30 minutes with Sytox orange, which stains membrane damaged cells. PlyD1 monovalent vaccination leads to protection of lung epithelial cells from Spn mediated damage. (n=4 each from randomly chosen frozen serum for 2 independent experiments). Magnification is 20X. (N) Number of Sytox+ cells were counted per 1mm² from mice vaccinated with PlyD or sham vaccine (n=3 each group) * p-value<0.001.

**Figure 6. Increased recruitment of macrophages after infection.** (A) Flow cytometry detection of lung cell types infiltrating the lungs at 48 hours post-infection (n=5 per group for two independent experiments). Representative pulmonary macrophage flow dot plots are shown. (B) Absolute counts of macrophages in lungs 48 hours after challenge or in uninfected controls (mean+/−SD). *p<0.001 between PlyD1 vaccinated infants/adults and all other vaccinated or infected control groups.


## Vaccination and Challenge Schedule

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<td>Inject</td>
<td>Inject</td>
<td>Inject</td>
<td>Bleed</td>
</tr>
<tr>
<td></td>
<td>Bleed</td>
<td>Bleed</td>
<td>Bleed</td>
<td>Infect</td>
</tr>
</tbody>
</table>

*Figure 1*
Figure 3

Bacterial Counts Lungs

Bacterial Counts/ml Blood

Control-I
Control-A
Infant
Adult

PlyD1
PcpA
PhtD

p<0.01
Figure 4

A Uninfected
B Infected Control
C PcpA Vaccine
D PhtD Vaccine
E PlyD1 Vaccine
F PlyD1 Vaccine

G

Infants

Uninf UnVacc PcpA PhtD PlyD1

Adults

Uninf UnVacc PcpA PhtD PlyD1

H

% Survival

Days Post-Infection

PcpA

I

% Survival

Days Post-Infection

PhtD

J

% Survival

Days Post-Infection

PlyD1
Figure 5

**Spn**1 mm² of Epithelial Cells

- Controls
- PhtD
- PcpA
- PlyD

**Sytox+ cells/1 mm² of Epithelial Cells**

- Neg.
- Pos.

**Infant** vs **Adult**

- PhtD Adult
- PcpA Adult
- No Spn

- Adult PlyD1
- No Spn
- FITC Spn
Figure 6

A

GR-1+

F4/80+

Uninfected

Infected

Control

Adult

Infant

PhtD

B

Absolute Counts (macrophages)

Uninfected

Infected

Control

PhtD

PhtD

GR-1+

F4/80+