HHS Public Access

Author manuscript

Vaccine. Author manuscript; available in PMC 2021 June 28.

Published in final edited form as:

Vaccine. 2021 March 12; 39(11): 1652–1660. doi:10.1016/j.vaccine.2020.04.064.

Preclinical in vitro and in vivo profile of a highly-attenuated, broadly efficacious pneumolysin genetic toxoid

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Abstract

Pneumolysin is a highly conserved, cholesterol-dependent cytolysin that is an important *Streptococcus pneumoniae* virulence factor and an attractive target for vaccine development. To attenuate pneumolysin toxicity, a genetic toxoid was constructed with two amino acid changes, G293S and L460D, termed PLY-D, that reduced cytolytic activity > 125,000-fold. In mice, PLY-D elicited high anti-PLY IgG antibody titers that neutralized the cytolytic activity of the wild-type toxin in vitro. To evaluate the protective efficacy of PLY-D, mice were immunized intramuscularly and then challenged intranasally with a lethal dose of 28 clinical isolates of *S. pneumoniae* originating from different geographical locations, disease states (i.e. bacteremia, pneumonia), or body sites (i.e. sputum, blood). PLY-D immunization conferred significant protection from challenge with 17 of 20 serotypes (85%) and 22 of 28 strains (79%). Further, we demonstrated that immunization with PLY-D provided statistically significant improvement in survival against challenge with serotype 4 and 18C strains compared to mice immunized with a pneumococcal conjugate vaccine Prevnar 13® (PCV13). Co-administration of PLY–D and PCV13 conferred greater protection against challenge with a serotype 6B strain than immunization with either vaccine alone. These data indicate that PLY-D is a broadly protective antigen with the potential to serve as a serotype-independent vaccine against invasive pneumococcal disease either alone or in combination with PCVs.

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Declaration of Competing Interest

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

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2020.04.064.

Keywords

Streptococcus pneumoniae; Serotype-independent pneumococcal; vaccine; Universal pneumococcal vaccine; Pneumolysin; Serotype replacement; Mouse model

1. Introduction

Streptococcus pneumoniae is an encapsulated Gram-positive human pathogen that is a significant cause of global morbidity and mortality[1]. *S. pneumoniae* is a leading cause of pneumonia, meningitis, and septicemia[2] especially in vulnerable populations, including children under 5 years of age and adults over 65 years of age[1,2]. There are three widely available commercial vaccines to combat *S. pneumoniae* disease, Synflorix® (GSK), Prevnar 13® (Pfizer), and Pneumovax® (Merck), however, they target a limited subset (10-, 13- and 23-valent, respectively) of the more than 90 *S. pneumoniae* polysaccharide capsular serotypes. The polysaccharide antigens in Synflorix® and Prevnar 13® are conjugated to a protein carrier to improve immunogenicity in infants and children, while the polysaccharide antigens in Pneumovax® are unconjugated and only approved for persons >2 years old.

While the introduction of pneumococcal conjugate vaccines (PCVs), like Synflorix® (PCV10) and Prevnar 13® (PCV13), have profoundly reduced the incidence of invasive pneumococcal disease (IPD), there are increasing concerns about sustaining this level of protection[3,4]. PCVs are inherently limited by their ability to only protect against the serotypes included in the formulation, and there has been a documented increase in incidence of disease caused by non-vaccine serotypes (NVTs)[4,5]. After the introduction of PCV13, the proportion of childhood IPD cases attributed to NVTs grew to 42% worldwide with a notably steeper increase of 72% in Europe[6]. This has prompted manufacturers to increase the valency of PCVs to cover these emerging serotypes, which adds to an already expensive and complex manufacturing process[7]. Toward this end, Merck and Pfizer have recently completed Phase 3 clinical trials with their 15 and 20-valent PCVs, respectively [8,9].

The rapid evolution of serotype prevalence in pneumococcal disease and inherent limitations and expense of PCVs warrants the development of a less expensive, serotype-independent pneumococcal vaccine. A promising target for *S. pneumoniae* vaccine development is pneumolysin (PLY), a cholesterol-dependent cytolysin (CDC) that plays crucial roles in the pathogenesis of *S. pneumoniae* [10]. The cytolytic and proinflammatory activities of PLY enable the disruption of the respiratory epithelium and subsequent pneumococcal invasion[11]. PLY has also been shown to facilitate myocardial invasion and lesion formation which likely contributes to the increased incidence of severe cardiac events following bacteremic pneumococcal pneumonia[12]. Furthermore, PLY has been shown to facilitate *S. pneumoniae* transmission by increasing bacterial shedding and promoting bacterial survival outside of the host [13]. A vaccine targeting PLY, therefore, may not only confer protection against acute pneumonia, but may also decrease the long-term sequelae associated with IPD as well as prevent spread of bacteria from host to host.

To develop a serotype-independent vaccine candidate, Matrivax is utilizing a genetic PLY toxoid (PLY-D), that contains two highly attenuating amino acid changes (G239S and L460D). The amino acid changes were selected using a structure-based approach in the laboratory of Dr. Rodney Tweten (University of Oklahoma Health Sciences Center, Oklahoma City, OK). Since CDCs, like PLY, rapidly and non-stochastically assemble into a pre-pore complex following cholesterol binding, which may obscure neutralizing epitopes[14], locking the PLY toxoid in a soluble pre-oligomerization conformation through genetic mutation may severely attenuate cytolytic activity while still allowing for production of functional antibodies that neutralize PLY cytolytic activity [10]. The L460D mutation was engineered into PLY-D to prevent cholesterol binding [15] resulting in a soluble protein. This attenuation renders the epitopes involved in membrane interaction and oligomerization accessible to the immune system enabling the generation of antibodies that inhibit cytolytic activity. The ability of PLY to oligomerize was further minimized by the G293S mutation which 'locks' the mutant protein in a soluble, monomeric, pre-oligomerization conformation [16]. G293 is one of a conserved di-glycine pair in the CDCs, which is involved in monomer-monomer interaction to form a pre-pore complex [17].

Here, we describe the immunogenicity profile and protective capacity of PLY-D in a murine lethal intranasal (IN) infection model, both as a stand-alone vaccine antigen and in combination with PCV13. PLY-D elicited high titer antibody responses that provided significant protection against 17 of 20 different serotypes and improved the protective capacity of PCV13. Collectively, the data presented here indicate that PLY-D is an effective, serotype-independent vaccine candidate that broadens protection against pneumococcal disease.

2. Results

2.1. PLY-D is well-tolerated, highly immunogenic, and elicits neutralizing antibodies at a wide range of dose levels in mice

PLY-D used for immunization and challenge experiments was expressed and purified from recombinant *Escherichia coli*. The gene encoding PLY-D was cloned to contain an amino terminal 6-histidine tag and purified using a metal affinity resin. The resulting purified material, when separated by SDS-PAGE, contained a single protein band that migrated at a molecular weight consistent with PLY-D containing a 6-His tag (Supplemental Fig S1) that was detected by an antibody specific for PLY^{WT} (data not shown). The endotoxin contamination of the purified PLY-D was 0.15 EU/mg of protein. When assessed in an in vitro hemolysis assay for residual cytolytic activity, a >125,000–fold reduction in the cytolytic activity of PLY-D relative to PLY^{WT} was observed (data not shown).

Before determining the protective capacity of PLY-D in an murine lethal IN infection model, PLY-D was assessed in a series of dose-response murine studies to determine optimal immunogenicity. In these studies, PLY-D was shown to require an adjuvant to elicit high anti-PLY IgG antibody titers in mice immunized intramuscularly (IM) with PLY-D adjuvanted with aluminum hydroxide (Alhydrogel®) eliciting higher anti-PLY IgG titers than PLY-D adjuvanted with aluminum phosphate (data not shown). To determine the optimal dose level of PLY-D, groups of mice were immunized IM three times at a biweekly

interval with 2, 10, 25 and 100 μg of PLY-D doses adjuvanted with Alhydrogel®. No adverse effects (i.e. redness/edema at the injection site or overall poor body condition) were observed with any of the dosing regimens. All dose levels of PLY-D elicited robust anti-PLY IgG antibody responses that ranged from 685,935 to 1,341,600 reciprocal geometric mean titers (GMT) (Table 1). The PLY-specific IgG antibody response did not increase between the 2 μg to 10 ug dose regimens, but did significantly increase from 10 μg to 25 μg. At the 100 μg dose, anti-PLY IgG titers were lower than that observed with the 2 and 10 μg dose regimens. The functionality of the antisera from the dose study was evaluated in an in vitro hemolytic assay to quantify neutralization of PLY cytolytic activity. All dose levels induced equivalent anti-hemolytic or neutralizing antibody titers (Table 1). Since all dose levels were highly immunogenic and generated equivalent functional antibody titers, subsequent murine studies used a 2 μg dose vaccination regimen.

2.2. PLY-D confers significant protection against 79% of all strains and 85% of S. pneumoniae serotypes evaluated in a murine lethal IN challenge model

To evaluate the protective capacity of PLY-D in a murine lethal IN challenge model, groups of mice were immunized using a biweekly dosing regimen (Days 0, 14, and 28) with 2 μg PLY-D adjuvanted with Alhydrogel® or PBS control. Two weeks following the third immunization, anti-PLY IgG antibody responses were determined and the mice challenged IN with an array of highly virulent *S. pneumoniae* clinical isolates (Table 2). Collectively, 28 different *S. pneumoniae* strains were evaluated spanning 20 different serotypes (both PCV13 and emerging serotypes). These strains originated from various geographical locations and were isolated from different body sites and different pneumococcal disease states (i.e. bacteremia, pneumonia, carriage). The challenge dose used for each serotype was determined from murine median lethal dose (LD_{50}) studies (data not shown). A lethal infectious dose was achieved for all the strains except the serotype 23B strain 6362, which was not virulent in this model (Supplemental Fig S2). Most strains had an LD_{50} challenge dose level between 1×10^7 to 1×10^8 colony forming units (CFU) except for strains of serotypes 1, 3 and 8 which had LD_{50} values below 1×10^6 CFU. Two dose levels of bacteria were used to challenge groups of immunized mice, the lowest dose of bacteria that elicited 100% lethality from the preliminary LD_{50} studies (1X dose) and a two-fold dilution of that amount of bacteria (0.5X).

The anti-PLY IgG GMT of PLY-D immunized mice in these studies ranged from 500,000 to 3,200,000 (Table 3) and following IN challenge, PLY-D immunized mice were significantly protected against 17 of the 20 *S. pneumoniae* serotypes tested (85%) and 22 out of 28 different strains (78.5%) (Table 3 and Fig. 2A). Table 3 shows the geometric mean time to death and percent survival of mice challenged with 19 of the serotypes. Representative survival curves of clinical isolates that showed statistically significant survival compared to controls are shown in Supplemental Fig S3. The immunogenicity and protection data from mice immunized with PLY-D and challenged with *S. pneumonia*e serotype 4 is excluded from Table 3 and is shown separately in Fig. 1A and B, due to the weekly, rather than biweekly, immunization schedule for that particular challenge study. PLY-D immunization did not confer significant protection against the serotype 1 strain and the majority of the serotype 3 strains. These strains, however, were highly virulent and even the 0.5X challenge

dose level resulted in 100% lethality (Supplemental Fig S4). The serotype 23B strain was also not included as a protected serotype although, as mentioned above, this strain was not virulent in this infection model even at the 1X dose level. Serotype 22F was scored as a protected serotype since PLY-D-immunized mice demonstrated significantly increased survival time following challenge with two out of three tested strains (Supplemental Fig S5). Collectively, these data show that PLY-D immunization conferred a broad breadth of protection against a variety of clinical isolates of pneumococcal serotypes and strains.

2.3. PLY-D immunization confers significantly improved protection against challenge with S. pneumoniae serotypes 4 and 18C compared to PCV13 immunization

To compare the protective capacity of PLY-D to that of a polysaccharide conjugate vaccine, mice were immunized three times at a weekly interval with PLY-D or PCV13 and then challenged IN with a lethal dose of serotypes 4 and 18C *S. pneumoniae* strains TIGR4 and ML-1, respectively. The weekly immunization regimen resulted in an ~ 32-fold lower anti-PLY IgG antibody response than was observed with the biweekly immunization regimen with anti-PLY IgG antibody titers of 16,141 to 52,817 for groups challenged with serotype 4 (Fig. 1B) and anti-PLY IgG antibody titers of 26,782 to 100,900 for groups challenged with serotype 18C (Fig. 1D).

Following challenge with serotype 4 and serotype 18C strains, PLY-D-immunized mice showed increased percent survival and prolonged survival time compared to PCV13-immunized mice (Fig. 1A and 1C). About 50% of PLY-D-immunized mice survived following IN challenge with serotype 4 compared to only 20% survival with PCV13-immunized mice (Fig. 1A). The geometric mean time to death following serotype 4 challenge was 138.8 hours post-infection (hpi) for PLY-D immunized mice, a 22-fold increase in time to death from naïve mice (6.0 hpi) or PCV13-immunized mice (5.7 hpi). Following challenge with serotype 18C, PLY-D immunized mice showed a prolonged survival time, but not a statistically significant increase in percent survival compared to PCV13-immunized mice or naïve mice (Fig. 1C). PLY-D immunized mice had a geometric mean time to death of 25.7 hpi, compared to 18.3 hpi and 16.5 hpi for PCV13 and naïve mice, respectively.

Although minimal protection was observed in PCV13 immunized mice, pneumococcal polysaccharide (PPS)-specfic antibody responses to pneumococcal polysaccharides PPS 4 and 18C were observed (Supplemental Table S2). However, the antibody titers were low which may have contributed to the lack of protection seen with the PCV. Interestingly, mice immunized with PLY-D on a biweekly regimen and challenge with the serotype 18C strain showed higher anti-PLY IgG titers rates of survival (100%) and time to death (128 hpi) (Table 2), than the mice immunized on a weekly regimen indicating that a certain level of anti-PLY antibody must be achieved to provide significant protection.

2.4. Co-administration of PLY-D and PCV13 confers enhanced protection against challenge with S. pneumoniae serotype 6B

To determine if PLY-D could act synergistically with PCV13 to protect mice from lethal IN infection, groups of mice were immunized with PLY-D or PCV13 alone or in combination

and subsequently challenged with *S. pneumoniae* serotype 6B strain P084. Following a three-dose biweekly vaccine regimen, mice immunized with PLY-D alone had anti- PLY IgG antibody GMTs of 1,040,000 to 1,820,000, while mice co-immunized with PLY-D and PCV13 had slightly lower GMTs of 523,000 to 1,060,000. Not surprisingly, immunization with PCV13 alone resulted in no anti- PLY IgG titer (Fig. 2B) and immunization with PLY-D alone did not generate an anti-6B IgG antibody titer (Fig. 2C). However, co-administration of PLY-D and PCV13 resulted in a 4-fold increase in anti-6B IgG antibody titers compared to the anti-6B IgG antibody titer induced by immunization with PCV13 alone (Fig. 2C).

When challenged IN with the serotype 6B strain, 40% of the mice immunized with PLY-D in combination with PCV13 survived compared to 0% with all other groups (Fig. 2A). Additionally, co-administration of PLY-D and PCV13 significantly prolonged the geometric mean time to death to 85 hpi compared to 32 and 31 hpi for the PCV13 and naive groups, respectively. Although immunization with PCV13 alone did not significantly prolong survival compared to naive mice, a significant increase in time to death was observed in mice immunized with PLY-D alone compared to naïve mice (Fig. 2).

3. Discussion

PLY is a major virulence factor of *S. pneumoniae* and a potential target for a non-serotype dependent vaccine to prevent pneumococcal disease. Unlike the current commercially available vaccines against *S. pneumoniae* that target the highly variable cell surface capsular polysaccharide, PLY is a highly conserved protein antigen that is produced by nearly all clinical isolates [18]. Although PLY has long been recognized as an important virulence determinant for *S. pneumoniae*, only recently has PLY been developed and clinically evaluated as a vaccine candidate. In an early study exploring the protective efficacy of a PLY-based vaccine, Alexander, *et al.* demonstrated that a genetic PLY toxoid was able to protect mice against 9 different serotypes of *S. pneumoniae* in murine IN and intraperitoneal (IP) challenge models [19]. This genetically engineered toxoid carried a W433F mutation affecting channel formation but still retained about 0.05% of wild-type toxin cytolytic activity [20]. More recently, both Sanofi and GlaxoSmithKline (GSK) have evaluated other genetically and chemically attenuated PLY toxoids, respectively, in clinical studies. Both PLY toxoids were proven safe and immunogenic in healthy adults [21–23], However, GSK demonstrated in two Phase 2 clinical trials in healthy infants that the addition of formalin-inactivated PLY toxoid (dPly) and pneumococcal histidine triad protein D (PhtD) to PCV10 and PCV13 did not significantly impact nasal colonization, acute otitis media [24], and more recently acute lower respiratory tract infections in infants [25]. These results are inconsistent with what has been observed in animals where PLY has been shown to play a more substantial role in these disease models [11,26–30]. Another potential reason for lack of efficacy in the GSK studies was that the dPLY was formalin inactivated, which is non-specific, and therefore epitopes that are critical for eliciting PLY neutralizing antibodies may have been modified rendering them conformationally altered or inaccessible to B-cells. Although dPly elicited high anti-PLY IgG titers in adults and infants, no data were presented on the ability of the antibodies to neutralize PLY activities [22,24,31]. Regardless for the reason for lack of efficacy of dPLY in clinical studies, these data suggest that the animal

models for pneumococcal disease may not accurately reflect the role of specific virulence factors played in human disease and further research is necessary to improve our understanding.

The genetic inactivation of cytolytic activity in PLY-D was devised using a structure-based, protein modeling approach, changing amino acids that inhibit cholesterol binding (L460D) and prevent oligomerization (G293S). These mutations reduced cytolytic activity >125,000-fold while locking the protein in a soluble pre-pore conformation and preserving protein structure. When used to immunize mice, PLY-D elicited high titer anti-PLY IgG antibodies that inhibited cytolytic activity of wild-type PLY in an in vitro hemolytic assay. Although higher anti-PLY IgG antibody titers were observed with a 25 μg dose regimen than 2 and 10 μg dose regimens, a corresponding increase in the anti-hemolytic titers was not observed. This indicates that increases in antibody titers does not directly correlate with generation of higher levels of functional antibodies. A similar observation was made by Sanofi in the Phase 1 clinical testing of their genetic toxoid [21].

In addition to eliciting antibodies that inhibit PLY cytolytic activity in vitro, PLY-D also conferred broad protective efficacy in a murine IN infection model. When challenged with 28 different *S. pneumoniae* clinical isolates that encompassed 20 serotypes, PLY-D immunized mice were significantly protected from 22 of the strains and 17 serotypes including both PCV13 and emerging serotypes. PLY-D did not confer protection against the serotype 1, 3, and 23B strains. While the serotype 23B strain was not virulent in this mouse model, the inability of PLY-D to confer protect against challenge with serotype 1 and 3 strains could be due to the high level of virulence of these serotypes in this model. For these strains, even infectious dose levels that were 10 to 100-fold lower than the other serotypes resulted in 100% lethality. Thus it is possible that PLY-D would confer protection against serotype 1 and 3 strains if the mice were challenged with a lower dose of bacteria. Furthermore, the serotype 1 strain was subsequently shown to produce a non-hemolytic variant of PLY (data not shown) and thus PLY may not play as important of a role in infection with this strain. While we scored PLY-D as not protective against the serotype 23B strain due to its lack of virulence in this model, it is possible that immunization with PLY-D would protect mice against a more invasive serotype 23B strain.

When the protective capacity of PLY-D was compared directly to the current polysaccharide based vaccine PCV13, immunization with PLY-D conferred statistically significant improved survival following challenge of mice with serotype 4 and 18C strains. In these studies, the mice were immunized weekly rather than biweekly which resulting in a ~32-fold reduction in anti-PLY IgG titers. With the serotype 18C strain challenge, this reduction in antibody titer resulted in less survival and a earlier time-to-death than mice immunized on a biweekly regimen indicating that a threshold level of anti-PLY antibodies needs to be achieved to confer protection. Although anti-PLY IgG titers from the efficacy studies varied from 500,000 to 3,200,000, there did not seem to be a correlation between the anti-PLY IgG titers and the degree of protection. This observation suggest that, while PLY is a critical virulence factor for most strains and serotypes, overall virulence of each strain in this model is likely due to a combination of virulence factors.

Although PLY-D provided equivalent if not better protection than PCV13 in this murine model of pneumonia/septicemia, PLY's limited role in nasopharygeal colonization [28,30] suggests that it may not induce the herd protection observed with vaccines that target the polysaccharide capsule. However, a vaccine combining PLY-D with a PCV could provide improved serotype coverage and protection against early and later stages of pneumococcal disease. When mice were co-administered PLY-D and PCV13, there was an increase in survival following challenge with a serotype 6B strain compared to mice immunized with either vaccine alone. While in this study immunization with PCV13 alone did not impact survival or time to death compared to unimmunized mice, a slight increase in time to death was observed in mice immunized with PLY-D alone. Interestingly, mice co-immunized with PLY-D and PCV13, showed a 4-fold increase in anti-PPS6B IgG antibody response compared to PCV13 alone. It is unclear whether this increase in anti-PPS6B titers or the combination of the anti-PLY and anti-capsular antibodies resulted in the improved survival of mice immunized with both antigens. The data do indicate, however, that PLY-D can act synergistically with a PCV to protect against lethal IN infection.

In summary, the continued health burden of *S. pneumoniae* despite efficacious pneumococcal vaccines and antibiotics warrants the serious consideration of serotype-independent vaccine candidates. The World Health Organization estimated that in 2005, pneumococcal infection took the lives of 1.6 million people [32] and in the United States in 2004 *S. pneumoniae* infections were associated with direct medical costs of $3.5 billion [33]. Serotype replacement and the high manufacturing cost of PCVs emphasize the need for a more universal, less expensive vaccine candidate to reduce the global burden of pneumococcal disease. The results from the present study show that PLY-D can provide broad serotype independent protection and can act synergistically with a PCV to protect against invasive disease.

4. Materials and methods

4.1. PLY-D purification and bacterial strain preparation

PLY-D was provided by Dr. Rodney Tweten and purified using the same procedure as described for histidine-tagged intermedilysin (ILY) in Soltani C. E. et al [34]. Pneumococcal clinical isolates used in IN challenge studies were provided by Dr. Steve Pelton (Boston University Medical Center, Boston, MA). Strain designations and sources are described in Table 2. Briefly, pneumococcal strains were grown at 37 °C in Todd-Hewitt broth supplemented with yeast extract (THY) until mid-log phase (OD_{600}: 0.4–0.55). Cultures were centrifuged at 5000 × *g* for 10 min to pellet the bacteria. The bacterial pellet was resuspended in phosphate-buffered saline (PBS) to a pre-determined concentration chosen via murine LD_{50} studies (data not shown). The suspensions were stored on ice until ready for IN instillation. Bacterial suspensions were plated before and after instillation and averaged to determine the CFU/mL used for IN challenge (Supplemental Table S1).

4.2. Murine immunization and pneumococcal challenge

PLY-D immunization and *S. pneumoniae* IN challenge experiments were performed both at Matrivax Research and Development (Boston, MA) and at the University of North Texas

(Fort Worth, TX). The methods for animal experimentation were approved by and conducted according to the Boston University Medical Center and the University of North Texas Institutional Care and Use Committees (IACUC). Unless otherwise noted, groups of 10 three- to five-week-old female BALB/c mice (Charles River Laboratory, Wilmington, MA) were immunized intramuscularly (IM) three times at a biweekly interval with 2 μg PLY-D adjuvanted with 50 μg Alhydrogel® (Aluminum hydroxide; Al–OH) (Brenntag Biosector, Ballerup, Denmark) or PBS as a control. A PBS control group that was formulated with Alhydrogel® was included in the dose–response murine study shown in Table 1. Formulation of PBS with or without Alhydrogel® did not appear to affect baseline responses in either ELISA or functional assays. Two weeks following the third immunization (Day 42), mice were infected by IN instillation with one of two dose levels (designated 1X or 0.5X) of the *S. pneumoniae* strain determined from preliminary LD_{50} studies (data not shown). The first challenge dose level (1X) was the fewest number of bacteria that resulted in 100% lethality, usually > 1×10^8 CFU/mL, and the second dose level, 0.5X contained half of the bacteria of the 1X lethal dose. The 1X challenge dose level ranged from 1.6×10^6 CFU to 6.3×10^8 CFU and the 0.5X challenge dose level ranged from 6.9×10^5 CFU to 2.5×10^8 CFU (For all challenge doses see Supplemental Table S1). The survival of mice following challenge was analyzed using a Log-rank Mantel-Cox test for determining statistical significance. For experiments comparing multiple treatment groups (such as PLY-D, PCV13, and PBS-immunized groups) a Holm-Bonferroni correction for multiple comparisons was performed on the survival analyses. A result was considered significant when the P-value was <0.05 ($P < 0.05$) for single-treatment experiments, or if the P-value was less than the α determined by the Holm-Bonferroni method.

4.3. PLY-specific IgG antibody ELISA

Serum samples were collected from mice two weeks following the final immunization to determine anti-PLY IgG antibody titers by ELISA. Immulon 96 well ELISA plates (VWR) were coated with a 0.05 μg/well of wild-type PLY (PLY^{WT}) in PBS and incubated at 4 °C overnight. Plates were washed and then blocked the following day with PBS containing 0.05% tween (PBST) and 3% bovine serum albumin (BSA) (Sigma-aldrich) at 25 °C for 2 h. Serum samples diluted in PBST containing 1.5% BSA were then added and incubated overnight at 4 °C. The next day plates were incubated with biotinylated anti-mouse IgG (Sigma) diluted in 1.5% BSA PBST for 2 h at 25 °C. Plates were washed and then streptavidin-AP (BD Biosciences) diluted in 1.5% BSA PBST was added to each well and incubated for 1 h at 25 °C. Plates were washed and *para*-nitrophenyl phosphate (pNPP) solution (Sigma) was added to each well and incubated for 30 min at 25 °C in the dark. Absorbance of each well was measured at a wavelength of 405 nm on a plate reader (Molecular Devices). The endpoint titer cutoff is defined as the A_{405} value that is two standard deviations above the mean of the negative control serum sample [35]. Negative control mouse serum and a positive reference control PLY antiserum were included on each plate to validate assay performance. Where indicated, the individual endpoint titers for each mouse serum sample in an experimental group were used to calculate the geometric mean titer (GMT). For pooled mouse serum samples, the antibody titer was reported as the reciprocal antibody titer as determined by ELISA. GMTs were analyzed for statistical significance using a Mann-Whitney U Test when comparing two groups and a One-way

ANOVA with a Tukey's test for multiple comparisons for analyzing multiple groups. A result was considered significant when $P < 0.05$.

4.4. Pneumococcal polysaccharide 6B (PPS6B)-Specific IgG antibody ELISA

Serum samples from groups of immunized mice were analyzed for antigen-specific IgG by ELISA adapted from methods described preciously by Khan *et al.* [36]. For pneumococcal polysaccharide (PPS) specific ELISAs, immune sera were serially diluted in PBS-0.05% Tween 20 containing 10 μg/ml pneumococcal cell wall polysaccharides, CWPS Multi, (MiraVista Diagnostics/Statens Serum Institut) which is a mixture of CWPS1 and CWPS2. CWPS-adsorbed immune sera were added to Immulon 4HB microtiter plates (VWR International) coated 16–20 h with 100 μL per well of 5 μg/mL pneumococcal polysaccharide antigen diluted in PBS. Following incubation for approximately 16 h at 4 °C with immune serum, wells were washed 4 times with PBS-0.05% Tween 20 and then incubated with 100 μL/well of biotinylated goat anti-mouse gamma-chain specific IgG (Sigma) followed by addition of 100 μL/well of streptavidin conjugated to alkaline phosphatase (BD Biosciences). Plates were developed by the addition of 100 μL per well of 1 mg/ml PNPP in 0.2 M Tris buffer (Sigmafast tablets from Sigma) and quantitated by reading absorbance at 405 nm. Data are reported as reciprocal geometric mean titer (GMT), with the endpoint titer cutoff calculated as the absorbance value that is two standard deviations above the mean of the negative control.

4.5. Anti-Hemolysis assay

The anti-hemolysis assay tested the PLY-neutralizing ability of antibodies from serum samples collected from mice immunized with PLY-D on a biweekly or weekly schedule. Rabbit red blood cells (RRBC) were washed and diluted to 10% in PBS. In a 96-well flat-bottomed plate sera samples were serially diluted 2-fold in PBS to a final volume of 75 μL. Pre-diluted serum samples from each well were transferred to a 96-well flat-bottomed half-area plate and incubated at a 1:1 ratio with PLY-WT (100 ng/mL final concentration) at room temperature for 20 min. After the sera was incubated with PLY-WT, 50 μL from each well was transferred to a round-bottomed plate and 50 μL of the 10% RBCs solution was added. The plate was incubated at room temperature for 1 h, then centrifuged for 5 min at 2,000 relative centrifugal force (rcf), and 50 μL of supernatant was transferred from each well to a 96-well flat-bottomed half-area plate. The absorbance of each well was read at 540 nm using a plate reader. The endpoint titer cutoff was defined by the 2-fold standard deviations below the average absorbance of the positive control (PLY-WT).

5. Author summary

S. pneumoniae is a leading cause of global morbidity and mortality despite the availability of effective antibiotics and vaccines. While the current pneumococcal conjugate vaccines (PCVs) are highly efficacious, they only protect against a subset of *S. pneumoniae* serotypes and are complex and expensive to manufacture thereby limiting their availability to less developed countries. Since the widespread introduction of PCVs, the incidence of non-PCV serotypes in colonization and pneumococcal disease has increased requiring PCV manufacturers to expand the number of serotypes covered by the vaccines in order to

maintain pace with the evolving serotype prevalence. The limitations and expense of PCVs has renewed interest in the development of non-serotype dependent or ‘universal’ vaccines to combat the continued threat of pneumococcal disease. A promising vaccine target is the highly-conserved pneumococcal toxin, pneumolysin (PLY), which plays a major role in mediating disease symptoms associated with pneumococcal pneumonia and invasive disease. Here, we describe the protective capacity of a highly attenuated genetic PLY toxoid (PLY-D) in a murine intranasal lethal infection model. In this model, PLY-D immunization conferred protection against challenge with 17 of 20 serotypes tested (85% efficacy), including both PCV and emerging serotypes. Additionally, PLY-D conferred better protection against challenge with serotype 4 and 18C strains than PCV13. Furthermore, immunization with PLY-D co-administered with PCV13 enhanced protection against challenge with serotype 6B strain compared to immunization with either vaccine antigen alone. Together, these data indicate that PLY-D is broadly protective and could potentially serve as a serotype-independent vaccine for preventing the global burden of pneumococcal disease either alone or in combination with a PCV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Jia He and Richard Wilson for their assistance with conducting animal studies, sample and data analysis, and helpful discussions.

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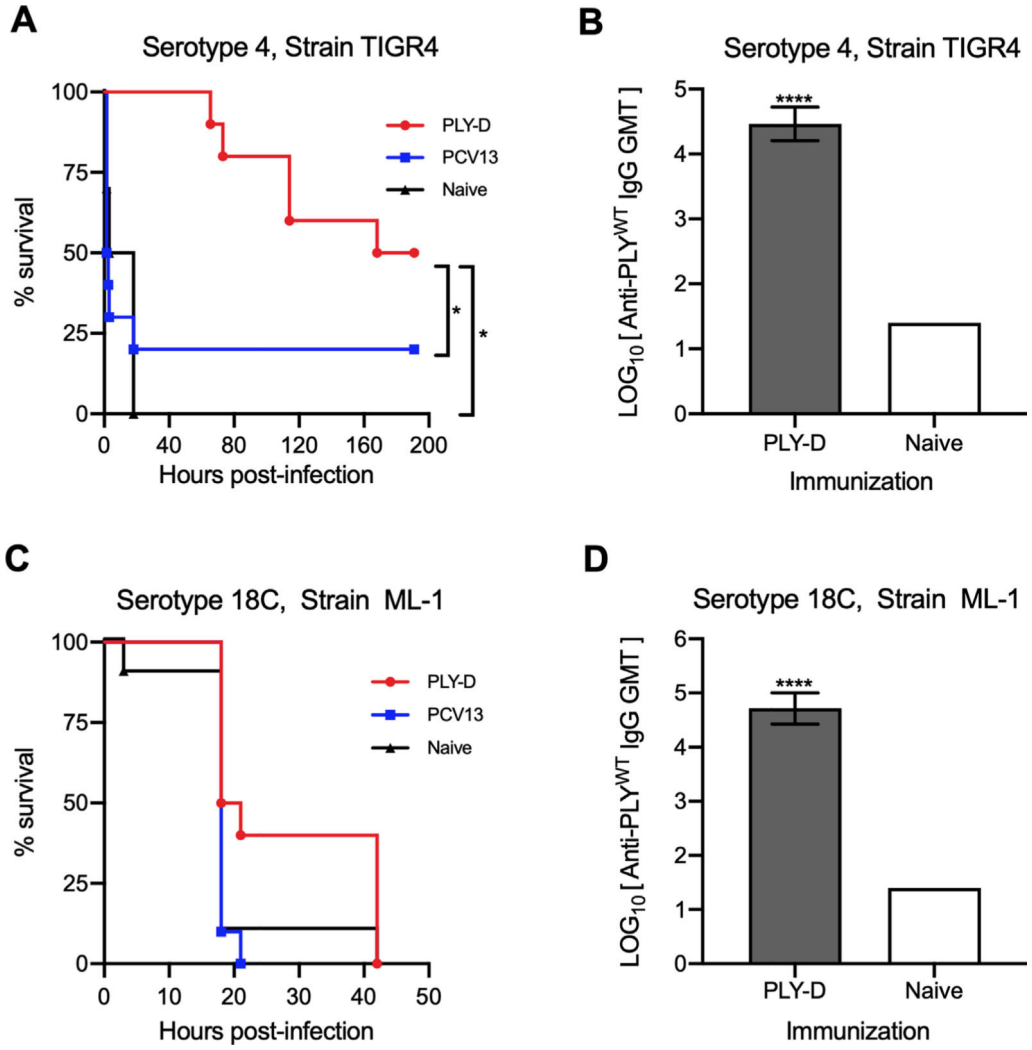
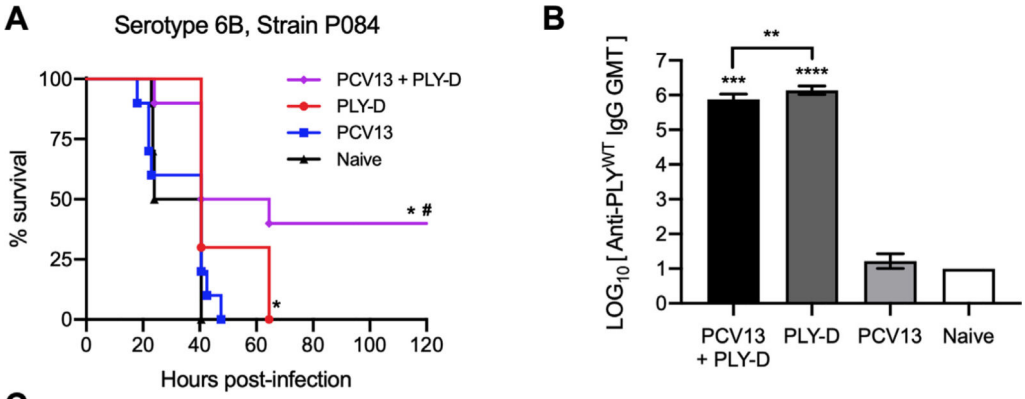


Fig. 1.

Weekly immunization with PLY-D improves survival following challenge with *S. pneumoniae* serotypes 4 or 18C compared to immunization with PCV13. Groups of 10 mice were immunized IM three times at a weekly interval with 2 μg PLY-D adjuvanted with Alhydrogel® (Al-OH), PCV13 (~0.2 μg each polysaccharide), or PBS control (naïve). Following the final immunization, mice were challenged by IN instillation of either 1.4×10^9 CFU of *S. pneumoniae* serotype 4, strain TIGR4 **(A)** or 4×10^8 CFU serotype 18C, strain ML-1 **(C)** and monitored for survival and time to morbidity or death. Prior to challenge, anti-PLY IgG antibody responses were determined for each individual mouse by ELISA **(B and D)**. *S. pneumoniae* challenge results are reported as percent survival and analyzed using a Log-rank Mantel-cox test with a Holm-Bonferroni correction for multiple comparisons; where (*) represents a significant increase in percent survival. **(A and C)**. ELISA data is shown as the logarithm of the anti-PLY IgG GMT with the 95% confidence interval. Data was analyzed using a Mann-Whitney U Test; (****) indicates a *P*-value < 0.0001 relative to naive **(B and D)**.



C

Test Articles Administered	Adjuvant	Anti-6B IgG Titer (Day 42)
0.2 µg PCV13 + 2 µg PLY-D	Al-PO_4 + 50 µg Al-OH	51,200
2 µg PLY-D	50 µg Al-OH	25
0.2 µg PCV13	Al-PO_4	12,800
PBS	None	25

Fig. 2.

Biweekly immunization with PLY-D admixed with PCV13 boosts survival following challenge with *S. pneumoniae* serotype 6B. Groups of 10 mice were immunized IM three times at a biweekly interval with 2 µg PLY-D adjuvanted with Alhydrogel® (Al-OH), PCV13 (~0.2 µg each polysaccharide), PCV13 admixed with PLY-D, or PBS control (naïve). **(A)** Two weeks following the final immunization, mice were challenged IN with 3×10^8 CFU of *S. pneumoniae* serotype 6B, strain P084 and monitored for survival and time to death. Results are reported as percent survival and analyzed using a Log-rank Mantel-cox test with a Holm-Bonferroni correction for multiple comparisons; where (*) represents a significant increase in percent survival from naïve and (#) represents a significant increase in percent survival compared to PCV13 alone. **(B)** Prior to challenge, anti- PLY IgG antibody responses of each mouse were determined by ELISA. Data is shown as the logarithm of anti-PLY IgG GMT with the 95% confidence interval. ELISA data was analyzed using a One-Way ANOVA with a Tukey's Test for multiple comparisons, (**) indicates a *P*-value of 0.001 to 0.01, (***) a *P*-value of 0.0001 to 0.001, and (****) a *P*-value of < 0.0001. **(C)** Anti-6B IgG antibody responses of each group were assayed by ELISA two weeks following the third and final immunization.

Table 1

Summary of anti-PLY IgG antibody responses and preliminary anti-hemolytic titers following three biweekly immunizations.

Test Articles Administered with 50 μg Al-OH [a]	Anti-PLY IgG GMT (Day 42)[a]	Anti-Hemolytic Titer (Day 42)[a]
2 μg PLY-D	844,485	600
10 μg PLY-D	844,485	600
25 μg PLY-D	1,341,600 *	600
100 μg PLY-D	685,935	600
PBS	10	150

(Abbreviations: PBS: Phosphate-buffered saline)

* IgG titer from 25 μg dose regimen is significantly different than titers from 2 μg, 10 μg, and 100 μg dose regimens (p value < 0.05 (One way ANOVA)).

[a] Groups of 10 mice were administered test articles intramuscularly three times at a biweekly interval (Days 0, 14, and 28). Sera were assayed for anti-PLY IgG antibody titers two weeks following the third and final immunization. All groups were formulated with Alhydrogel® (Al-OH).

Table 2

Characterization of clinical isolates of 20 serotypes/28 strains of *S. pneumoniae*.

Serotype	Strain	PCV13 Serotype	Source
1	5267	Yes	Clinic (CA)
3	09AR0038	Yes	Clinic-Blood
	14AR0032	Yes	Clinic-Pneumonia
	11AR0031	Yes	Clinic-Bacteremia
	CH2439	Yes	Clinic-Carriage
4	TIGR4	Yes	Lab
5	5577	Yes	Clinic (CA)
6A	06AR0213	Yes	Clinic-Blood
6B	P084	Yes	Clinic (Dom. Rep.)
8	P014b	No	Clinic (Dom. Rep.)
11A	LE4018	No	Clinic-Nasopharynx
	P073	No	Clinic (Dom. Rep.)
14	645	Yes	Clinic
15A	6343	No	Clinic (CA)
15B/C	MIB02–102	No	Clinic-Nasopharynx
18C	ML-1	Yes	Lab
19F	TW14	Yes	Clinic-Sputum
	17AR0017	Yes	Clinic-IPD (MA)
	17AR0030	Yes	Clinic-IPD (MA)
19A	FG23	Yes	Clinic-Nasopharynx
22F	06AR0298	No	Clinic-Blood
	17AR0025	No	Clinic-IPD
	CNPCX1037	No	Clinic-Carriage
23F	P098	Yes	Clinic (Dom. Rep.)
23A	609	No	Clinic (Finland)
23B	6362	No	Clinic (CA)
33F	07AR0098	No	Clinic-Blood
35B	229	No	Clinic (Finland)

Table 3

Protective capacity of PLY-D against intranasal challenge with pneumococcal clinical isolates.

Serotype[a]	Day 42 Anti-PLY IgG Titer[a]	Percent Mouse Survival (Time to Death)[c]			
		1X *S. pneumoniae* Infectious Dose[b]		0.5X *S. pneumoniae* Infectious Dose[b]	
		PLY-D Immunized	PBS	PLY-D Immunized	PBS
1	1,280,000	0% (51 hpi))	0% (47 hpi)	0% (53 hpi)	0% (52 hpi)
3	1,026,043	0% (42 hpi) *	0% (39 hpi)	0% (54 hpi) **	0% (42 hpi)
	1,280,000	0% (45 hpi)	0% (40 hpi)	0% (62 hpi)	0% (64 hpi)
	3,200,000	0% (58 hpi)	0% (57 hpi)	0% (67 hpi)	0% (63 hpi)
	2,560,000	0% (62 hpi)	10% (66 hpi)	0% (79 hpi)	10% (86 hpi)
5	1,280,000	100% (240 hpi) ****	0% (71 hpi)	100% (240 hpi)	100% (240 hpi)
6A	565,386	0% (58 hpi) *	0% (39 hpi)	100% (142 hpi) ****	0% (50 hpi)
6B	1,280,000	0% (35 hpi) ****	0% (20 hpi)	60% (166 hpi) ****	0% (36 hpi)
8	2,560,000	0% (46 hpi) *	0% (43 hpi)	0% (57 hpi)	0 (50 hpi)
11A	985,092	0% (30 hpi) **	0% (22 hpi)	90% (154 hpi) ***	10% (49 hpi)
	720,000	20% (54 hpi) ****	0% (17 hpi)	90% (149 hpi) **	30% (68 hpi)
14	836,807	100% (168 hpi) ****	10% (81 hpi)	100% (168 hpi)	100% (168 hpi)
15A	1,200,000	40% (153 hpi) ****	0% (19 hpi)	100% (240 hpi) ****	0% (51 hpi)
15B/C	723,598	80% (198 hpi) ****	0% (20 hpi)	100% (264 hpi) **	30% (78 hpi)
18C	721,406	90% (128 hpi) ****	0% (26 hpi)	NA	NA
19F	627,830	10% (43 hpi)	10% (40 hpi)	100% (216 hpi) **	30% (82 hpi)
	2,560,000	0% (31 hpi) ***	0% (19 hpi)	20% (73 hpi) ***	0% (31 hpi)
	3,200,000	20% (77 hpi) ***	0% (24 hpi)	70% (144 hpi) **	20% (55 hpi)
19A	507,230	10% (58 hpi) ****	0% (18 hpi)	100% (168 hpi) **	40% (70 hpi)
22F	866,050	0% (45 hpi)	0% (52 hpi)	80% (222 hpi)	70% (218 hpi)
	1,280,000	40% (95 hpi) ****	0% (23 hpi)	90% (227 hpi) ***	17% (54 hpi)
	1,200,000	78% (132 hpi) **	10% (60 hpi)	100% (168 hpi)	75% (121 hpi)

Serotype[a]	Day 42 Anti-PLY IgG Titer[a]	Percent Mouse Survival (Time to Death)[c]			
		1X *S. pneumoniae* Infectious Dose[b]		0.5X *S. pneumoniae* Infectious Dose[b]	
		PLY-D Immunized	PBS	PLY-D Immunized	PBS
23F	600,000	50% (110 hpi) *	10% (55 hpi)	100% (168 hpi)	100% (168 hpi)
23A	960,000	90% (157 hpi) *	30% (105 hpi)	100% (168 hpi)	90% (162 hpi)
23B	1,200,000	100% (168 hpi)	90% (144 hpi)	100% (168 hpi)	100% (168 hpi)
33F	760,490	0% (47 hpi) ***	0% (25 hpi)	100% (168 hpi) **	40% (96 hpi)
35B	1,280,000	22% (82 hpi) **	0% (30 hpi)	90% (148 hpi)	100% (168 hpi)

* p value 0.05–0.01

** p value 0.01–0.001

*** p value 0.001–0.0001

**** p value < 0.0001; log-rank Mantel-Cox Test.

[a] Anti-PLY antibody titers induced by IM administration of PLY-D at a biweekly interval (Days 0, 14, and 28). Sera was assayed by ELISA at Day 42, two weeks following the third and final immunization. The anti-PLY IgG titer was determined by using endpoint titer cutoff method.

[b] Challenge dose of clinical isolates were determined by LD_{50}. 1X dose was the highest dose from LD50 study that caused near 100% lethality. The 0.5X dose was a 1:1 dilution of the 1X dose.

[c] The average time to death presented in hours post infection (hpi).