

Multivalent Pneumococcal Protein Vaccines Comprising Pneumolysin with Epitopes/Fragments of CbpA and/or PspA Elicit Strong and Broad Protection

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Immunization with the pneumococcal proteins pneumolysin (Ply), choline binding protein A (CbpA), or pneumococcal surface protein A (PspA) elicits protective responses against invasive pneumococcal disease in animal models. In this study, we used different mouse models to test the efficacy of a variety of multivalent protein-based vaccines that comprised various combinations of full-length or peptide regions of the immunogens Ply, CbpA, or PspA: Ply toxoid with the L460D substitution (referred to herein as L460D); L460D fused with protective peptide epitopes from CbpA (YPT-L460D-NEEK [YLN]); L460D fused with the CD2 peptide containing the proline-rich region (PRR) of PspA (CD2-L460D); a combination of L460D and H70 (L460D+H70), a slightly larger PspA-derived peptide containing the PRR and the SM1 region; H70+YLN; and other combinations. Each mouse was immunized either intraperitoneally (i.p.) or subcutaneously (s.c.) with three doses (at 2-week intervals) of the various antigen combinations in alum adjuvant and then challenged in mouse models featuring different infection routes with multiple *Streptococcus pneumoniae* strains. In the i.p. infection sepsis model, H70+YLN consistently provided significant protection against three different challenge strains (serotypes 1, 2, and 6A); the CD2+YLN and H70+L460D combinations also elicited significant protection. Protection against intravenous (i.v.) sepsis (type 3 and 6A challenge strains) was largely dependent on PspA-derived antigen components, and the most protection was elicited by H70 with or without L460D or YLN. In a type 4 intratracheal (i.t.) challenge model that results in progression to meningitis, antigen combinations that contained YLN elicited the strongest protection. Thus, the trivalent antigen combination of H70+YLN elicited the strongest and broadest protection in diverse pneumococcal challenge models.

Streptococcus pneumoniae (the pneumococcus) is responsible for almost 1 million deaths worldwide in children <5 years of age each year (1) and is a leading cause of both invasive pneumococcal diseases (IPD) (e.g., bacteremia, bacteremic pneumonia, and meningitis) and noninvasive diseases (e.g., nonbacteremic pneumonia, otitis media, and sinusitis) (2). Collectively, these conditions account for greater global morbidity and mortality than diseases caused by any other pathogen. Antipneumococcal vaccination strategies currently target the capsular polysaccharides, which are serologically varied, with >93 distinct serotypes identified to date (3). A 23-valent pneumococcal polysaccharide vaccine (PPV23) has been used in many countries for several decades; it provides strictly serotype-dependent protection but does not elicit immunological memory and shows poor immunogenicity in children <2 years, the age group with the highest incidence of IPD (4).

A 7-valent pneumococcal conjugate vaccine (PCV7) was licensed in 2000 to overcome the abovementioned shortcomings of PPV23. Unlike PPV23, PCV7 was highly protective in young children against IPD caused by the included serotypes. There was also a marked herd immunity effect due to its capacity to reduce nasopharyngeal carriage of vaccine-type pneumococci, thereby reducing the transmission of these serotypes to nonvaccinated individuals within the community (5). However, the declines in the incidences of IPD and nasopharyngeal colonization by vaccine-type pneumococci have been offset to various extents by increases in both carriage and disease due to nonvaccine serotypes (5–7).

This serotype replacement can result from two scenarios: first, unmasking of nonvaccine types already present in the nasopharynx in low numbers or elsewhere in the community, and second, by acquisition of a nonvaccine serotype capsule biosynthesis locus by a vaccine type strain through natural genetic transformation (3, 8). This second scenario may be of greater potential significance, as it enables highly transmissible, virulent, and often antibiotic-resistant clones to escape the PCV. More recently licensed PCVs have increased serotype coverage (10- and 13-valent) (9, 10), but this is at best only a stop-gap measure. The emergence of a high proportion of virulent strains outside the 13-valent vaccine coverage has recently been documented in the high-risk sickle cell disease population (11).

Received 7 May 2015 Returned for modification 8 June 2015

Accepted 28 July 2015

Accepted manuscript posted online 5 August 2015

Citation Chen A, Mann B, Gao G, Heath R, King J, Maissoneuve J, Alderson M, Tate A, Hollingshead SK, Tweten RK, Briles DE, Tuomanen EI, Paton JC. 2015. Multivalent pneumococcal protein vaccines comprising pneumolysin with epitopes/fragments of CbpA and/or PspA elicit strong and broad protection. *Clin Vaccine Immunol* 22:1079–1089. doi:10.1128/CI.00293-15.

Editor: D. L. Burns

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doi:10.1128/CI.00293-15

Pneumococcal protein-based vaccine formulations comprising combinations of conserved virulence proteins that function at different stages of pathogenesis offer an attractive alternative to PCVs. Three proteins stand out as the best candidate vaccine antigens, namely, pneumolysin (Ply), pneumococcal surface protein A (PspA), and choline binding protein A (CbpA) (also known as PspC). These proteins elicit some of the highest antibody titers in sera from humans exposed to pneumococci (12–15), and their functions in pathogenesis are well characterized, involving processes in all body compartments (16–22).

Ply is a cholesterol-binding toxin produced by virtually all strains of *S. pneumoniae*. It was one of the first virulence-related proteins identified in *S. pneumoniae*, and its cytotoxicity emanates from its capacity to insert into eukaryotic cell membranes and oligomerize to form large transmembrane pores, resulting in cell lysis (17). A number of immunogenic pneumolysoids generated by point mutations in the *ply* gene have reduced cytotoxicity and provide significant protection against different serotypes of *S. pneumoniae* at multiple stages of infection in various murine models. One such toxoid, PdB, has a single substitution (W433F) that reduces hemolytic activity by >99% (23), and it is one of the most well-studied antigens. It protects mice from diverse *S. pneumoniae* strains when used as either a single immunogen or in combination with other proteins, particularly PspA and CbpA (24–28). More recently, a triple mutant toxoid, PlyD1 (comprising T65C, G293C, and C428A mutations), with only 0.001% residual hemolytic activity, was also shown to elicit protection against pneumococcal infection and lung injury (29). A novel Ply toxoid devoid of detectable cytotoxicity contains an L460D substitution, which disrupts a threonine-leucine pair that comprises the cholesterol recognition motif (CRM) and is critical for cholesterol binding on target cells by several cholesterol-dependent cytotoxins (CDCs) (30). In GenBank, this CRM region is strictly conserved in approximately 50 different bacterial species that express a CDC. Due to its undetectable cytotoxicity, Ply L460D (referred to as L460D herein) is potentially a more suitable vaccine candidate than PdB.

PspA is one of the best-characterized members of the choline binding protein family. It is found on the surfaces of all pneumococci and has important roles in the pathogenesis of disease (31, 32). During invasive disease, it interferes with complement-dependent host defense and thereby reduces the deposition of complement on the cell surface; concomitantly, it impairs complement receptor-mediated clearance. PspA also blocks killing by host cationic bactericidal peptides (33) and interferes with phagocytosis by a complement-independent mechanism (34). The PspA protein consists of three main regions: the N-terminal alpha-helical domain, a proline-rich region (PRR), and a choline binding domain. Previous studies have focused on the N-terminal alpha-helical domain of PspA, which elicits protection against invasive disease (28, 35–37). However, the alpha-helical domain of PspA is variable in terms of its amino acid sequence and antigenic epitopes (38–41). Attention has recently shifted to the PRR, as this region is much more conserved and contains epitopes on the pneumococcal surface that are antibody accessible (42). The PRR has also been shown to elicit broader cross-reactive antibodies and cross-protection against pneumococcal infection (42, 43).

Another well-characterized pneumococcal choline binding protein is CbpA, which participates in pneumococcal adherence to host cells and helps pneumococci evade complement attack and

TABLE 1 *S. pneumoniae* challenge strains used in this study

Strain	Capsular type	Origin
D39	2	26
1861	1	55
P9	6A	This study
TIGR4	4	54
EF3030	19F	C. Svanborg
A66.1	3	This study
DBL6A	6B	This study

opsonophagocytosis. CbpA plays important roles in many stages of disease and elicits protection against pneumococcal infection in different animal models (20, 26, 27, 44–46). The N-terminal domain of CbpA binds to the secretory component of secretory immunoglobulin A (sIgA), C3, factor H, and the laminin receptor (47–52). This domain consists of two nearly identical repeat regions that each assemble into antiparallel helices (not-coiled coils), and the turns between the helices show extremely high conservation across most pneumococcal strains (49, 53). An RRNYPT sequence in the first turn binds to the epithelial polymeric immunoglobulin receptor (pIgR), and the EPRNEEK sequence in the second turn binds to the laminin receptor on the blood-brain barrier endothelium (52). Recently, a recombinant fusion protein consisting of these two turns fused to either terminus of L460D has been shown to elicit broad protection against multiple stages of pneumococcal infection (54).

The aim of the present study was to develop a multivalent and broadly efficacious pneumococcal protein vaccine via a thorough examination of the protective immunogenicity of the proteins L460D, CbpA, and PspA. Vaccine candidates consisted of either full-length or peptide derivatives of these proteins, recombinant fusion proteins comprising immunogenic regions from these proteins fused to the full length or portions of one of the other proteins, or these in various combinations. The resultant multivalent protein candidates were tested for protective efficacy in different animal models against a wide range of pneumococcal challenge strains to establish the optimal formulation of a multivalent vaccine.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *S. pneumoniae* serotype 4 bioluminescent strain TIGR4X was grown in chemically defined medium (C+Y) without aeration at 37°C in 5% CO₂ to an optical density at 620 nm (OD₆₂₀) of 0.4 to 0.5 (54). *S. pneumoniae* strains D39, 1861, and P9 were grown in serum broth to an OD₆₀₀ of 0.18, as previously described (55). *S. pneumoniae* strains DBL6A, A66.1, and EF3030 were grown in Todd-Hewitt broth to an OD₆₀₀ of 0.4, as previously described (42).

Construction and production of recombinant proteins. The features of the recombinant proteins and expression constructs used in this study are presented in Table 2. The PCR primers used for subcloning are listed in Table 3. All expression constructs were sequenced at the St. Jude Children's Research Hospital (SJCRH) Hartwell Center. All proteins were purified by the SJCRH Protein Production Facility and stored in 10 mM histidine buffer (pH 6.0) with 15% trehalose.

Pneumolysoid. The *ply* gene was first PCR amplified from strain D39 and then cloned into vector pQE30. The L460D substitution was then introduced by overlapping PCR, as previously described (56). The resulting plasmid, pQE30-L460D, was used as the template to obtain the L460D gene using primers PLYNDE and PLYSAC, and the L460D gene was subse-

TABLE 2 Features of recombinant proteins and expression constructs

Construct	Protein(s) expressed (aa)	Source strain(s)	Template for cloning	5' primer	3' primer
L460D	Pneumolysoid	D39	pQE30-L460D	PLYNDE	PLYSAC
CD2	PspA proline-rich region	AC094	gDNA	PI5-up-LIC	PI5-down-LIC
H70	PspA proline-rich region and SM1	Rx1	pIDTSMART-H70 ^a	H70NDE	H70SAC
pUAB055	PspA (1–302)	Rx1	gDNA	pspA1-F	pspA6-R
rCbpA	CbpA (175–443)	TIGR4	gDNA	A175NDE	A443SAC
YPT-L460D-NEEK (YLN)	Pneumolysoid and CbpA peptides	TIGR4 and D39	pET28-L460D	YPTNDE	NEEKSAC
CD2-L460D	PspA peptide and pneumolysoid	AC094 and D39	pCD2, pET28-L460D	CD2NDE	PLYSAC

^a Initial construct made by Integrated DNA Technologies.

quently cloned into vector pET28a (Novagen) (54). The recombinant plasmid pET28a-L460D was then transformed into *Escherichia coli* strain BL21(DE3) cells for protein production.

CbpA derivatives and fusion proteins. CbpA derivatives YPT and NEEK from TIGR4 were constructed as previously described (54). Briefly, YPT-L460D-NEEK (YLN) and L460D-NEEK were both constructed by incorporating the constrained CbpA sequences of YPT and NEEK into the primers used to amplify L460D. The construction of the YLN fusion protein was previously described (54). Each fragment was cloned into the vector pET28a (Novagen), and the desired constructs were verified by DNA sequencing.

PspA derivatives and fusion proteins. The construction of pUAB055, carrying genes encoding a truncated version of PspA containing the N-terminal region (amino acids [aa]1 to 302), was described previously (39). Briefly, a pUAB055 fragment was amplified by PCR from *S. pneumoniae* strain Rx1 using primers pspA1-F and pspA6-R and was then cloned into vector pCRII (Invitrogen) to form plasmid pCRII-pUAB055. The plasmid was digested with NcoI and XhoI to obtain the 918-bp pUAB055 fragment that was then cloned into the NcoI/XhoI sites of vector pET20b. The resulting plasmid was then transformed into the *E. coli* strain RosettaBlue (DE3) pLysS for protein production.

The construction of peptide CD2 was previously described (42). It contains the proline-rich region (PRR) of the PspA from *S. pneumoniae* strain AC94. Briefly, the PRR (315 bp) was PCR amplified using the primers PI5-up-LIC and PI5-down-LIC and cloned into vector pET-46Ek/LIC. This recombinant plasmid was then transformed into *E. coli* BL21 Star (DE3) cells for protein production.

Peptide H70 is slightly larger than CD2, comprising the PRR and an

SM1 peptide, which is naturally located upstream of the PRR in strain Rx1. The region containing the SM1 and PRR was PCR amplified from strain Rx1 using the primers EKLIC-BglII and EKLIC_SM1-ProR. The resulting PCR fragment was then cloned into vector pET46-Ek/LIC and verified by DNA sequence to confirm its insertion of the expected 393-bp fragment. This recombinant plasmid was then used as the template to obtain an H70 fragment using primers H70NDE and H70SAC, and it was subsequently cloned into vector pET28a (Novagen). The recombinant plasmid pET28a-H70 was then transformed into BL21 (DE3) cells for protein production.

Intraperitoneal sepsis model. All animal experiments were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (57) and the South Australian Animal Welfare Act 1985 (58) and were approved by the animal ethics committee of the University of Adelaide, South Australia, Australia. Outbred 4- to 6-week-old female CD1 (Swiss) mice were used in all intraperitoneal (i.p.) sepsis experiments. Each mouse was immunized i.p. three times with 100 µl of 0.5% saline containing 10 µg of each protein and 200 µg of alum adjuvant (Alhydrogel; InvivoGen) at 14-day intervals. Serum samples were collected from the mice by submandibular bleeding 1 week after the third immunization. A week later, the mice were challenged i.p. with strains D39 (2.5 × 10⁴ CFU, serotype 2), P9 (5 × 10⁴ CFU, serotype 6A), and 1861 (5 × 10² CFU, serotype 1). The infected mice were observed for up to 21 days to determine the number of days until they were moribund.

Meningitis/sepsis model. The experiments were approved by the St. Jude Institutional Animal Care and Use Committee and were carried out essentially as previously described (54). Briefly, 6- to 7-week-old female

TABLE 3 PCR primers used in this study

Primer	Sequence (5'–3')
PLYNDE	CGCGCGCGCATATGGCAAATAAAGCAGTAAATGAC
PLYSAC	GCGCGCGAGCTCTTACTAGTCATTTTCTACCTTATCC
YPTNDE	CGCGCGCGCATATGGCTTGTA AAAAAGCCGAGGATCAAAAAGAAGATCGCCGTA ACTACCCAA CCAATACTTACAAAACGCTTGAACCTGAAATGTGCTGAGGGTGGTGCAAATAAAGCAGTAAATG
NEEKSAC	CGCGCGGAGCTCCTATTTACATTGCTTAACTTTTTCTCGTTTCGAGGTTCCCTTAGCACACTCTTTGT CATTTTCTACCTTATCCTC
A175NDE	GCGGCCATATGCCAGGAGAAAAGGTAGCAG
A443SAC	GCGCGCGAGCTCTTATGGTTTTTCTTTAACTTTATC
CD2NDE	GCGGCCATATGGCCATGGCTGACCTTAAG
CD2F2	CGGTATGTGGATGGCAAATAAAGCAGTAAATG
CD2R1	CTGCTTTATTTGCCATCCACATACCGTTTTTCTTG
H70NDE	GCGGCCATATGTATTTAAAGAAGGGTTAG
H70SAC	GCGCGAGCTCCTAACCATTTTCTTGTTTCCACCCAG
PI5-up-LIC	GACGACGACAAGATGGCTGACCTTAAGAAAGC
PI5-down-LIC	GAGGAGAAGCCCGTTTTACCACATACCGTTTTCTTG
pspA1-F	GCCATGGAAGAATCTCCCGTAGCC
pspA6-R	CTCGAGTCTGGGGCTGGAGTTTC
EKLIC-BglII	GACGACGACAAGAGATCTTACTTTAAAGAAGGTTTAGAG
EKLIC_SM1-ProR	GAGGAGAAGCCCGGTCTAGGATCCCATACCGTTTTCTTGTTTCC

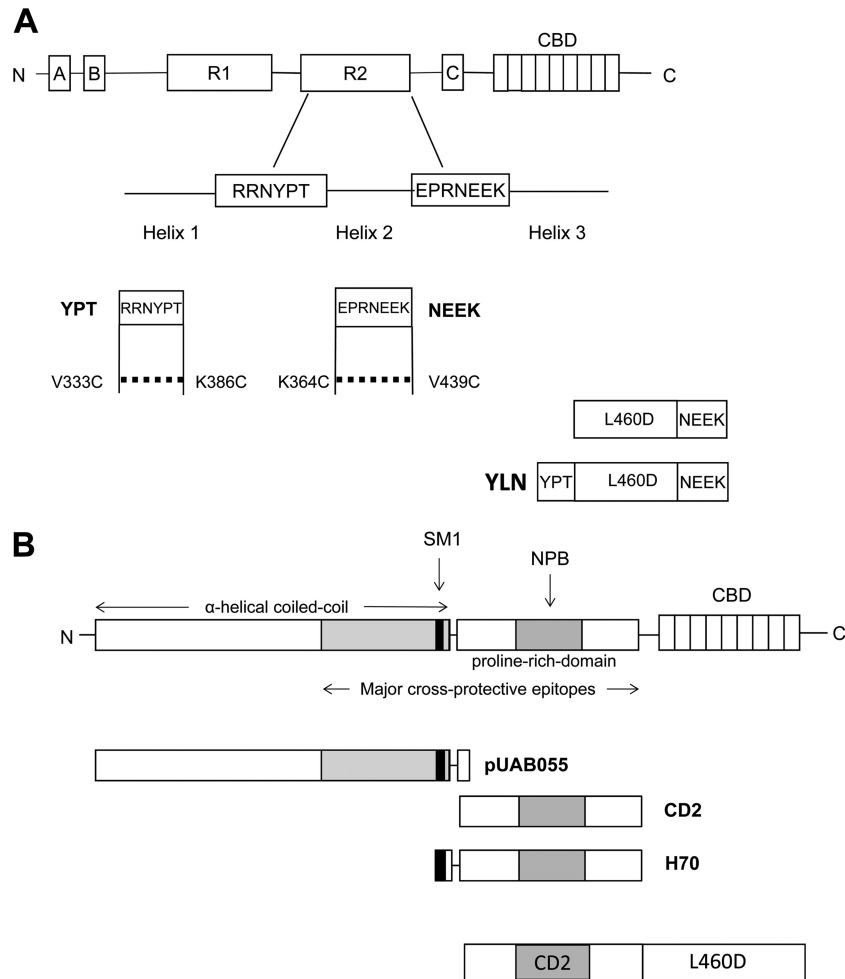


FIG 1 Schematic representation of CbpA and PspA derivatives used in vaccine constructs. (A) Full-length CbpA (top) and regions used to make each construct, YPT and NEEK (bottom). The dotted lines indicate disulfide bridges formed by dual substitutions made in YPT (V333C and K386C) and NEEK (K364C and V439C) to engender a constrained structure of native CbpA. Fusion constructs of peptides with L460D are shown. (B) Full-length PspA (top) and regions used to make each vaccine construct: pUAB055, CD2, H70, and CD2-L460D (bottom).

BALB/c mice were immunized i.p. with 10 μ g of protein containing 130 μ g of alum (Brenntag), and subsequent boosts were given at weeks 2 and 4. Serum samples were collected from the mice at week 5 for functional antibody analysis. The mice were challenged intratracheally (i.t.) with 1×10^7 CFU of TIGR4X (serotype 4) at week 7, and survival was monitored for 14 days. Meningitis was observed by physical signs and confirmed through Xenogen imaging and plating of cerebrospinal fluid samples. Nasal washes of the survivors were collected and plated on day 14 post-challenge.

Intravenous sepsis model and i.t. focal pneumonia models. These experiments were approved by the University of Alabama at Birmingham (UAB) Animal Care Committee. This intravenous (i.v.) sepsis model was carried out as previously described (42). Briefly, CBA/CaHNVrkxid/J (CBA/N) mice were immunized subcutaneously (s.c.) three times at 2-week intervals with 100 μ l containing 5 μ g of protein adjuvanted with alum. Three weeks after the last immunization, blood samples were taken from the mice for enzyme-linked immunosorbent assay (ELISA). The following day, the mice were infected i.v. with 4×10^3 CFU of strain A66.1 (serotype 3) or DBL6A (serotype 6B) diluted from a frozen infectious stock in lactated Ringer's solution containing 7.8% glycerol. The infected mice were observed for up to 21 days to determine the number of days until they were moribund.

In the i.t. focal pneumonia model, CBA/N mice were immunized as

described above, except for the challenge, for which mice were first anesthetized lightly with isoflurane and then challenged i.t. with 5×10^6 CFU of *S. pneumoniae* strain EF3030 (serotype 19F), as described previously (28). On day 5 postinfection, the mice were euthanized, and blood and lungs were harvested and plated for CFU recovery.

Measurement of antibody responses. Antigen-specific antibody responses were determined by ELISA using serum samples collected from immunized mice, as previously described (42, 59).

Hemolysis and anti-hemolytic activity assays. The hemolytic activity of pneumolysin and the capacity of murine serum to neutralize the hemolytic activity of wild-type Ply were assayed, as previously described (60).

Statistical analysis. Statistical analysis was carried out using GraphPad Prism 6. The Mann-Whitney U test (two-tailed) was used for all statistical comparisons carried out in this study (median survival, CFU recovery, and antibody levels). *P* values of <0.05 were considered statistically significant.

RESULTS

Construction of multivalent protein vaccine candidates. L460D, CbpA, PspA, and their various derivatives (Fig. 1) were tested to determine the optimum formulation of a multivalent vaccine. The immunogens were formulated as mixtures of one, two, or three

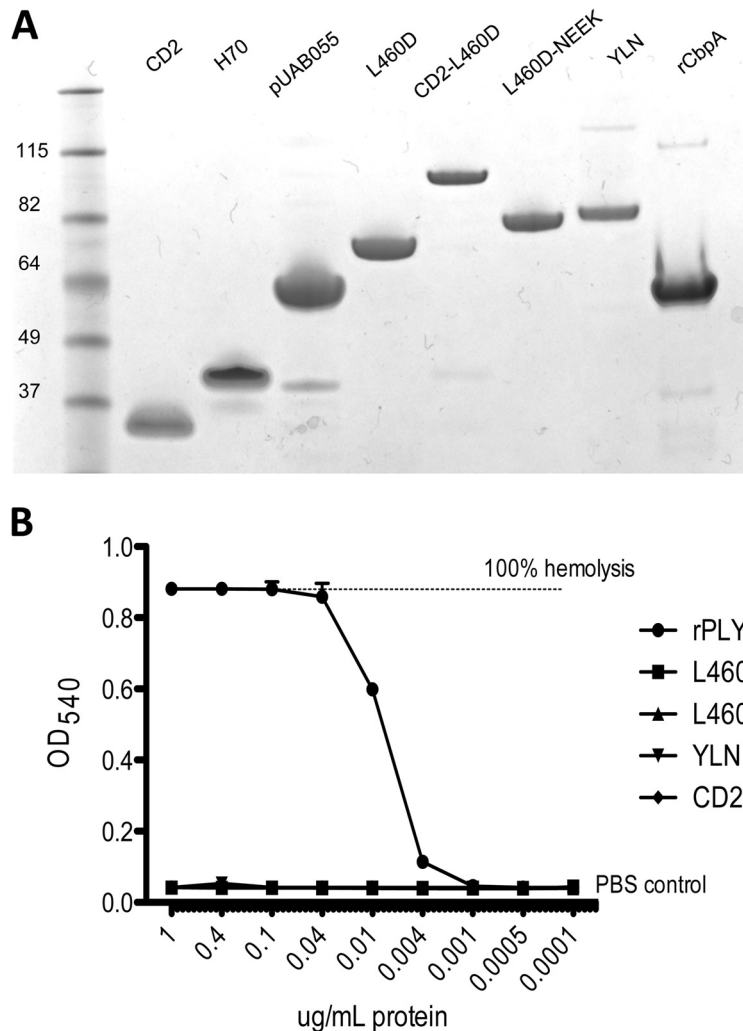


FIG 2 Properties of vaccine constructs. (A) SDS-PAGE analysis of purified recombinant proteins stained with Coomassie blue. A molecular size marker ladder is also shown. (B) *In vitro* hemolytic activity of L460D, L460D fusions, and wild-type Ply. The indicated dilutions of Ply and L460D constructs were incubated with 1% washed human erythrocytes for 30 min at 37°C. After centrifugation, hemoglobin release was quantitated by absorbance at 540 nm. Triton X-100- and PBS-treated erythrocytes were used as controls for 100% and 0% lysis, respectively.

proteins/peptides. Formulations that included L460D consisted of full-length L460D alone or as a fusion construct with the PspA peptide CD2 (CD2-L460D) or with either one or two constrained CbpA peptides (L460D-NEEK or YPT-L460D-NEEK [YLN] [54]). Derivatives of PspA were also mixed as separate proteins with L460D or YLN.

All of the constructs were purified at the St. Jude Protein Production Facility and then tested for purity, size, concentration, and contamination with endotoxin. Analysis by SDS-PAGE (Fig. 2A) showed that all proteins migrated according to their respective molecular sizes, except for CD2 and H70, which migrated more slowly than predicted: CD2 is 13 kDa but migrated at 25 kDa, and H70 is 15 kDa but migrated at 33 kDa. This anomalous electrophoretic mobility is likely due to their high proline content. The complete absence of hemolytic activity of purified L460D and its fusion derivatives was confirmed by hemolysis assay (Fig. 2B).

Immunogenicity of the multivalent protein vaccine candidates. Groups of 12 to 15 mice (BALB/c or CD1) were immunized intraperitoneally (i.p.) or subcutaneously (s.c.) with each of the

various antigens, singly or in combination, using alum adjuvant. Mice were injected three times at 2-week intervals (see Materials and Methods). A serum sample was then collected from each mouse 1 week after the final immunization for analysis of antibody responses. Antigen-specific IgG responses were measured by ELISA, using L460D, recombinant CbpA (rCbpA), or H70 as a coating antigen (Fig. 3). All vaccine formulations elicited strong antibody responses to L460D, with geometric mean (GM) anti-L460D IgG titers of >4,000 for all groups, compared to <200 for the alum placebo group (Fig. 3A).

Pneumolysin antibodies are expected to neutralize the wild-type toxin. Accordingly, the capacity of sera from mice immunized with the various L460D-containing formulations to neutralize the hemolytic activity of wild-type pneumolysin was also assessed (Fig. 3B). All antigens elicited significant toxin-neutralizing responses (GM antihemolytic titers, ~300, compared with approximately 20 for the alum placebo group). Analysis of CbpA-specific antibody levels also showed strong responses (GM ELISA titers, >3,000) for all groups immunized with antigens containing

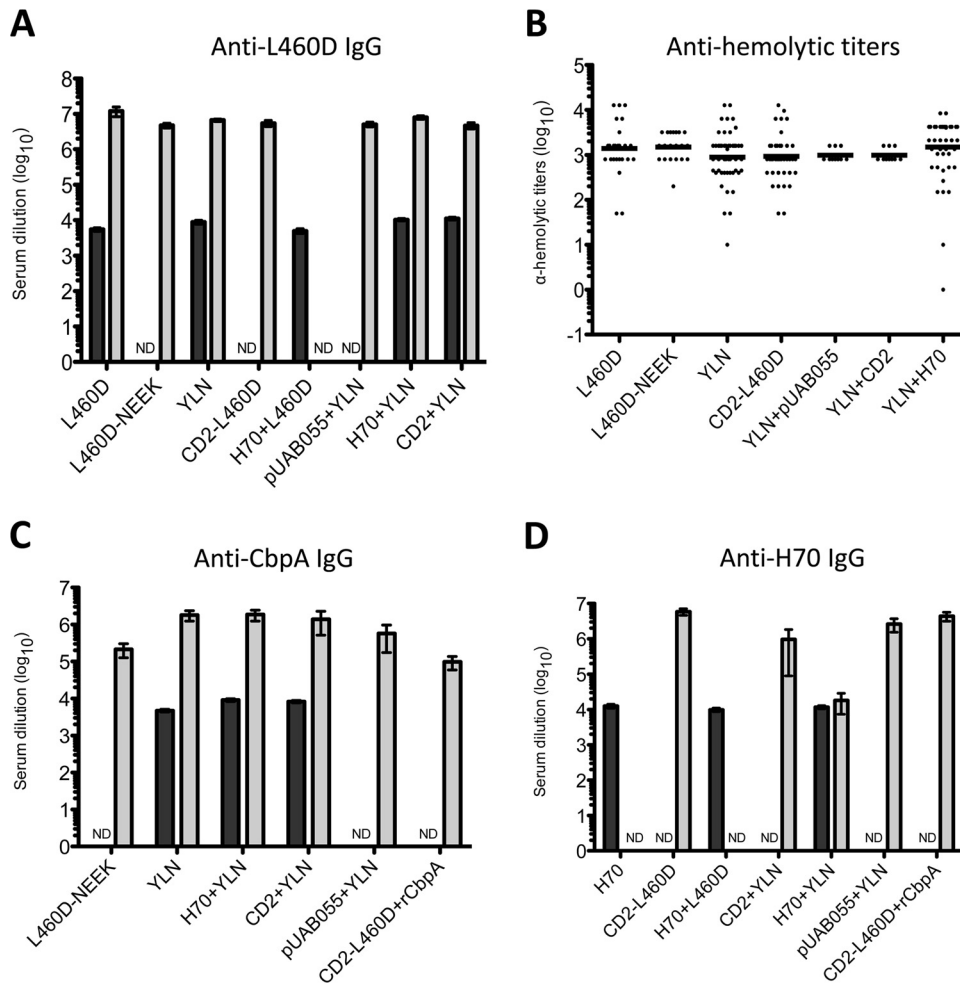


FIG 3 Immune responses in mice. (A, C, and D) Serum IgG responses to the various antigens were measured by ELISA using L460D (A), rCbpA (C), or H70 (D) as a coating antigen. BALB-C mice, gray bars; CD1 mice, black bars. The data are shown as the geometric mean (GM) titer, with the error bars denoting the 95% confidence intervals (CI). ND, not determined. (B) Functional activity of L460D antibodies elicited by various vaccine constructs was measured by the ability to neutralize 50% of the hemolytic activity of a standard dose of wild-type Ply. The data are shown as the GM antihemolytic titers. In this assay, the average hemolytic titer of the alum control group was 16.

YLN relative to the responses for the alum placebo group and antigen formulations lacking the CbpA-derived peptide epitopes (GM ELISA titers, <200) (Fig. 3C). However, in CD1 mice, both the H70-plus-YLN combination (H70+YLN) and CD2+YLN elicited significantly higher anti-CbpA levels than YLN alone ($P < 0.0001$ for both groups), although the biological significance of this numerically modest difference is uncertain. In CD1 mice, the three formulations containing H70 (H70, H70+L460D, and H70+YLN) elicited similarly strong IgG responses (GM ELISA titers, >10,000) compared with the response in the alum placebo group (GM titer, <200) (Fig. 3D). However, in BALB/c mice, anti-H70 responses to H70+YLN were lower than those elicited by the formulations containing either CD2 or pUAB055. Nevertheless, even for H70+YLN, the GM titer was >10,000, compared with <200 for the alum placebo.

Protective efficacy in sepsis models. The efficacy of the vaccine formulations was determined using two sepsis models: i.p. challenge with one of three strains, D39 (serotype 2), P9 (serotype 6A), or 1861 (serotype 1), or i.v. challenge with strain DBL6A or A66.1 (serotypes 6B and 3, respectively). Immunized mice were

challenged 2 weeks after the final immunization, and animals were monitored for survival for up to 21 days. In the i.p. challenge model, the H70+YLN, CD2+YLN, H70+L460D, YLN, and L460D vaccine groups all exhibited significant protection against two challenge strains, D39 and 1861 (Fig. 4A and B). However, only the trivalent H70+YLN group exhibited significant protection against strain P9, a clinical isolate that causes meningitis in approximately 50% of unprotected mice (Fig. 4C). In this experiment, any mice infected with P9 that displayed clinical signs of meningitis were promptly euthanized. The difference in protection seen between the trivalent H70+YLN and all the other groups, including the placebo control, was largely attributable to a lower rate of progression to meningitis, with only approximately 20% of mice immunized with H70+YLN showing symptoms, compared to approximately 50% for all other groups (results not shown). Peptides H70 and CD2 were not protective when given alone, but when each was combined with YLN, the level of protection increased significantly relative to that with YLN alone, indicating that the strongest protection is achieved by the combi-

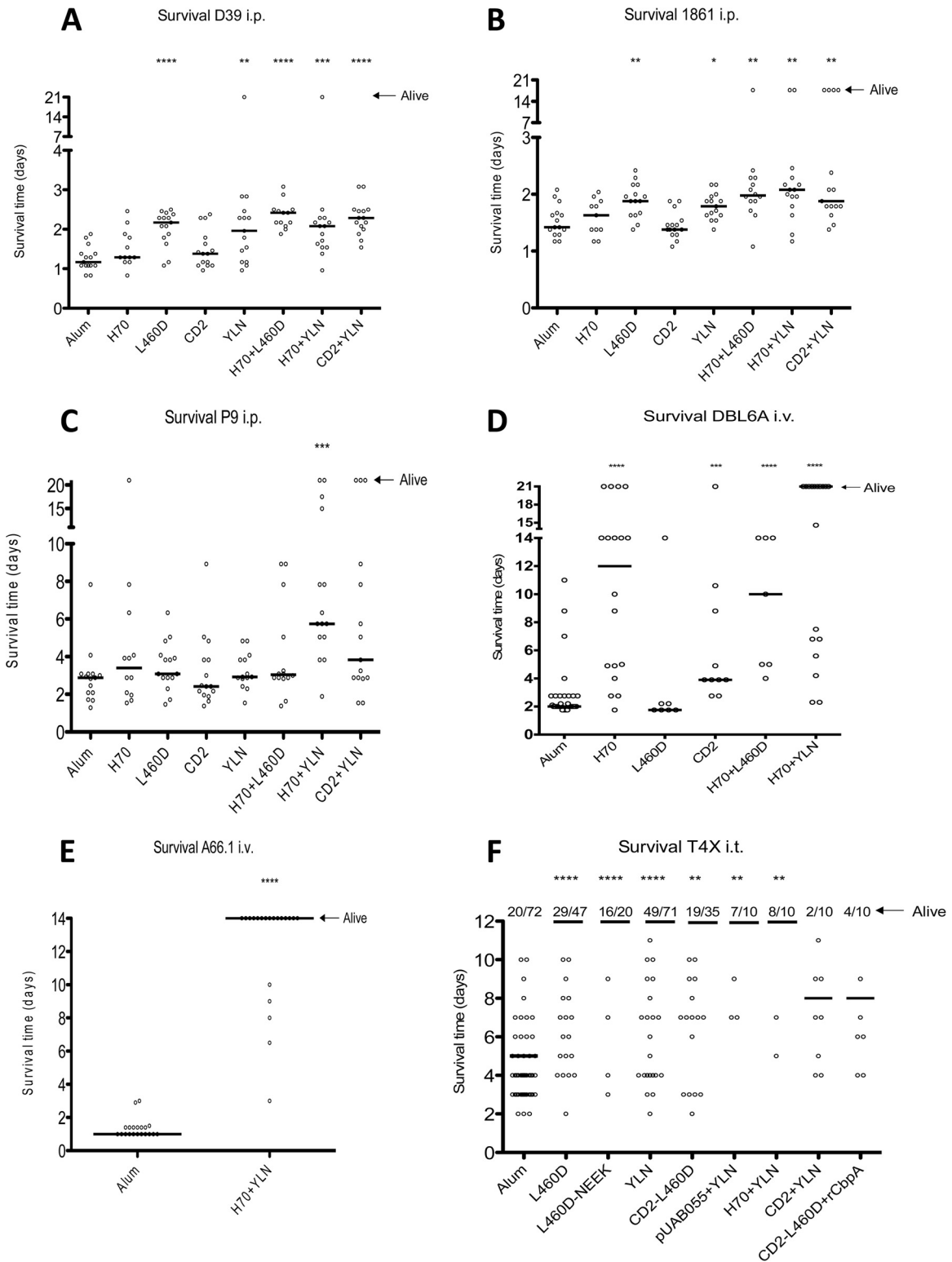


FIG 4 Survival time of vaccinated mice in three sepsis models. (A to C) Intraperitoneal sepsis model. Mice were challenged with strain D39 (A), 1861 (B), or P9 (C). The solid lines denote the median survival time for each group, and mice that survived to 21 days postinfection were plotted as alive. Significant protection compared to that with the alum control group is indicated in each panel by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). (D and E) Intravenous sepsis model. Mice were challenged with strain DBL6A (D) or A66.1 (E). The solid lines denote the median survival time for each group, and mice that survived to 21 days postinfection were plotted as alive. (F) Intratracheal sepsis/meningitis model. Mice were challenged with strain TIGR4X. The solid lines denote the median survival time for each group. The number of mice in each group and the number of mice alive at day 14 are depicted at the top of the panel.

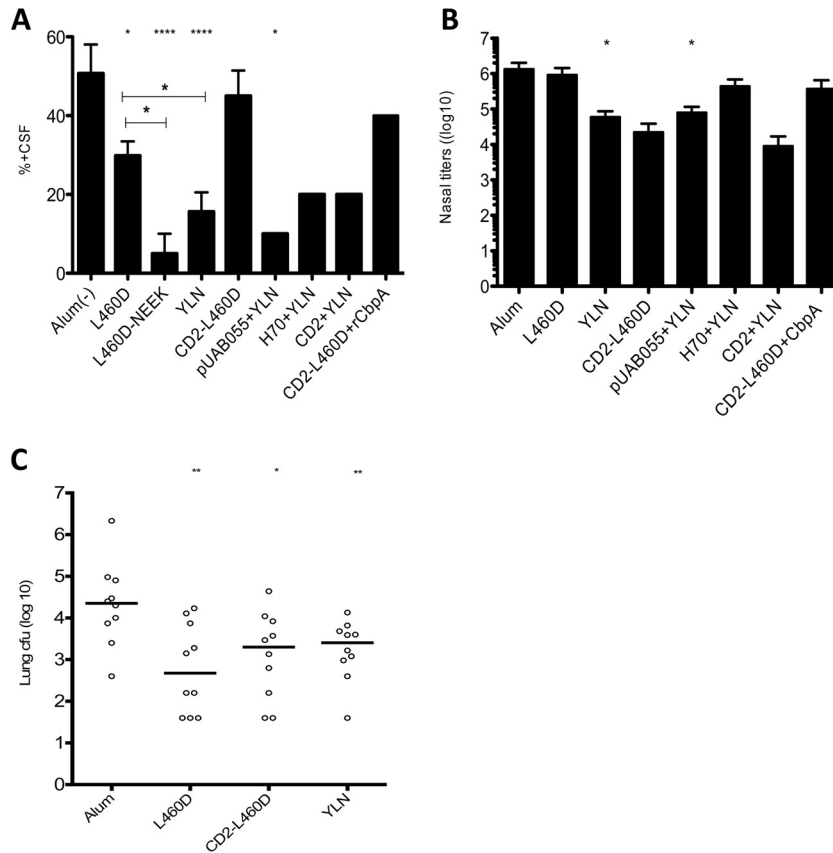


FIG 5 Organ-specific analysis of vaccine protection. (A) Percentage of mice infected with strain TIGR4X that developed meningitis in each vaccine group, as determined by positive cerebrospinal fluid (CSF) culture (mean \pm standard deviation). (B) Bacterial density in the nasopharynx of mice surviving to day 14 postinfection with TIGR4X (mean \pm standard deviation). (C) Focal pneumonia: Mice were challenged with EF3030, and lungs were harvested at day 5 for CFU recovery. The solid lines denote the group medians. For all graphs, significant differences relative to the alum control group are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$).

nation of pneumolysoid and immunogenic portions of PspA and CbpA.

Mice immunized with either H70 or CD2 exhibited significant protection against challenge with DBL6A in the i.v. sepsis model compared to that in the alum control group (Fig. 4D). H70 immunization elicited slightly superior protection to that with CD2, but this was not statistically significant. When protection following immunization with either L460D or H70 was compared with that for the H70+L460D group, significant protection relative to that in the alum placebo was seen for both H70 ($P < 0.0001$) and H70+L460D ($P < 0.0001$) but not for L460D alone (Fig. 4D). However, the combination of H70+YLN elicited the strongest protection against strain DBL6A ($P < 0.0001$) (Fig. 4D) and very strong protection against A66.1 ($P < 0.0001$) (Fig. 4E).

Multivalent protein vaccine candidates confer protection in a meningitis/sepsis infection model. When mice are infected i.t. with strain TIGR4X (serotype 4), disease rapidly progresses from infection of the upper and lower respiratory tract to sepsis and meningitis. The survival time was the longest in mice immunized with combinations of all three antigens, followed by L460D or YLN alone ($P < 0.0001$) (Fig. 4F). Other groups, such as CD2-L460D, YLN+H70, and YLN+pUAB055, elicited modestly higher protection than that in the alum group. The proportion of mice developing meningitis, indicated by the degree of CFU re-

covery in cerebrospinal fluid, was significantly lower in mice immunized with L460D alone than in the alum group ($P < 0.05$). Further significant reductions were seen in groups immunized with L460D-NEEK and YLN ($P < 0.05$ in both cases), and similar low levels of meningitis were observed in any of the other groups immunized with antigen combinations including YLN (Fig. 5A). The numbers of bacteria in the nasopharynx of survivors at day 14 indicated that only animals immunized with YLN or pUAB055+YLN showed a significant decrease in bacterial load (Fig. 5B).

Single-valent and multivalent protein vaccine candidates confer protection against pneumonia. In a focal pneumonia model (without subsequent progression to sepsis), mice were challenged i.t. with type 19F strain EF3030. After 5 days, mice were euthanized, and blood and lungs were harvested for analysis of CFU recovery. No CFU were recovered from blood of any group. Analysis of CFU recovery from the lungs revealed that immunization with L460D alone ($P = 0.0031$) or in combination in either the trivalent YLN ($P = 0.007$) or the divalent CD2-L460D ($P = 0.023$) constructs afforded significant protection compared to that in the alum control group (Fig. 5C).

DISCUSSION

This, to our knowledge, is one of the most comprehensive studies conducted to identify the best potential protein-based alternative

to the current polysaccharide-based pneumococcal vaccines. Here, we describe the testing of new vaccine formulations comprising multiple protein antigens in different challenge models. The protein antigens L460D, PspA, and CbpA used in this study are the best characterized pneumococcal proteins to date in terms of their roles in pathogenicity and protective efficacy. Furthermore, several derivatives of PspA have recently been shown to potentially confer protection with fewer safety concerns (42). It is therefore reasonable to test them in various combinations in order to identify the best formulation of combinations of these protein antigens. The protein antigens L460D, pUAB055, H70, CD2, and YLN were tested singly or in various combinations in models of sepsis, pneumonia, and meningitis using three different mouse backgrounds and six different challenge strains. All combinations were highly immunogenic, with no indication of interference. Fusion constructs elicited titers to individual component antigens that were equal or higher than those of single proteins. Antihemolytic titers against wild-type pneumolysin were equivalent, regardless of construct. Intraperitoneal and subcutaneous routes of immunization were also equivalent.

Pneumolysin has long been recognized as a critical virulence factor in pneumococci, capable of lysing cells by forming membrane pores and interacting with the immune system by activating complement and inducing inflammation (17, 61–63). More recently, pneumolysin has been shown to be involved in evading recognition by pattern recognition receptors in the innate immune system (64, 65). Thus, a pneumolysin toxoid, such as L460D, is a highly desirable vaccine component. To limit the number of separate proteins in the multicomponent product, L460D was used as a backbone for several fusion proteins. The addition of CbpA and PspA derivatives (YPT-L460D-NEEK [YLN] and CD2-L460D) expanded the anti-Ply-mediated protection to the pathogenic processes of adherence, penetration of the blood-brain barrier, and opsonization. In the case of pneumonia, PspA and L460D derivatives were important for protection, while CbpA components played a strong protective role against meningitis. The trivalent combinations CD2+YLN or H70+YLN elicited protection against all six serotypes tested in the challenge models.

A previous study characterized YLN in the i.t. meningitis model and found that immunization with this fusion protected against sepsis, meningitis, pneumonia, and otitis media (54). Passive protection was also examined; mice immunized with anti-YLN rabbit serum were also protected against these diseases. YLN was further shown to provide cross-protection against meningococcal and *Haemophilus* infection, as the sequence EPRNEEK in the NEEK peptide is able to bind to the same domain of the laminin receptor of the blood-brain barrier as *Haemophilus influenzae* and *Neisseria meningitidis* (52).

The PspA peptide CD2 has been examined in the i.v. sepsis model in a prior study (42). As mentioned above, CD2 contains the highly conserved proline-rich region of PspA present in all PspAs and almost all CbpAs. CD2 is advantageous over the N-terminal region of PspA for inclusion in a vaccine, as the CD2 region varies only slightly between PspA and CbpA and elicits cross-reactive antibodies to both PspA and CbpA. Immunization with CD2 or passive immunization with CD2 monoclonal antibodies elicited protection of mice against three challenge strains. Also, upon exposure of children to pneumococci, cross-reactive CD2 antibodies were detected, regardless of the PspA family of the in-

fecting strains (43). In this study, we have shown that in a comparison of H70 and CD2, H70 appeared to more consistently elicit stronger protection than CD2, either singly or in combination with YLN. CD2 and H70 both contain the proline-rich region of PspA, while H70 has the additional SM1 peptide. This SM1 peptide is located just upstream of the proline-rich region of PspA in strain Rx1. Human lactoferrin is known to kill many species of bacteria, including pneumococci, and PspA has been observed to specifically inhibit *in vitro* killing of pneumococci by apolactoferrin (the iron-free form of lactoferrin); this action is believed to be due to the SM1 peptide that blocks the binding of lactoferrin to PspA (66). Thus, the addition of the SM1 peptide in H70 is likely to have provided enhanced protection over CD2.

Taken together, these results clearly indicate that the trivalent H70+YLN combination is the optimal protein vaccine candidate tested here. It consistently demonstrated significantly broad and strong protection against multiple diseases, such as sepsis, pneumonia, and meningitis, caused by different strains/serotypes in different animal models. We also show that the ability of the L460D antibody to neutralize the cytolytic activity of the wild-type toxin Ply was not compromised by creating fusion constructs that minimize vaccine components.

ACKNOWLEDGMENTS

This work was supported by grants from PATH (to J.C.P., E.I.T., and D.E.B.). E.I.T. was also supported by NIAID R0127913, the Schafer Family Gift, and the American Lebanese Syrian Associated Charities. J.C.P. is a National Health and Medical Research Council of Australia (NHMRC) Senior Principal Research Fellow. D.E.B. and S.K.H. were also supported by NIH grant R01-AI021458. R.K.T. is supported by grant 1R01 AI037657 from the NIH National Institute of Allergy and Infectious Diseases.

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