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MICROBIAL IMMUNITY AND VACCINES

A Cross-Reactive Protein Vaccine Combined with PCV-13 Prevents *Streptococcus pneumoniae*- and *Haemophilus influenzae*-Mediated Acute Otitis Media

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ABSTRACT Acute otitis media is one of the most common childhood infections worldwide. Currently licensed vaccines against the common otopathogen *Streptococcus pneumoniae* target the bacterial capsular polysaccharide and confer no protection against nonencapsulated strains or capsular types outside vaccine coverage. Mucosal infections such as acute otitis media remain prevalent, even those caused by vaccine-covered serotypes. Here, we report that a protein-based vaccine, a fusion construct of epitopes of CbpA to pneumolysin toxoid, confers effective protection against pneumococcal acute otitis media for non-PCV-13 serotypes and enhances protection for PCV-13 serotypes when coadministered with PCV-13. Having cross-reactive epitopes, the fusion protein also induces potent antibody responses against nontypeable *Haemophilus influenzae* and *S. pneumoniae*, engendering protection against acute otitis media caused by emerging unencapsulated otopathogens. These data suggest that augmenting capsule-based vaccination with conserved, cross-reactive protein-based vaccines broadens and enhances protection against acute otitis media.

KEYWORDS *Haemophilus influenzae*, *Streptococcus pneumoniae*, otitis media, vaccines

Encapsulated bacteria such as *Streptococcus pneumoniae* (pneumococcus) and *Haemophilus influenzae* are significant mucosal pathogens that can cause invasive disease, especially in young children (1, 2). As part of the normal respiratory flora, both species are frequent colonizers of the nasal passages in healthy individuals and can persist asymptomatically for prolonged periods without progressing to disease (3). However, when these organisms translocate to the lungs or middle ear, they can cause pneumonia and acute otitis media (AOM), respectively.

AOM is the most frequently diagnosed infection of children and is the most common reason for prescribing antibiotics to children in the United States (4). Despite prolonged exposure to broad-spectrum antibiotics, a high percentage of children who experience acute otitis media will have frequent recurrences of infection (5). The most common bacterial pathogens responsible for AOM are *H. influenzae*, *S. pneumoniae*, and *Moraxella catarrhalis* (6). When multiple infectious agents are present, the subsequent risk for developing acute otitis media is higher than that of carrying any individual pathogen (7). Both pneumococcus and *H. influenzae* have a propensity to form biofilms during colonization and otitis media, which are then inherently difficult to clear by antibiotics (8). These factors contribute to the continued high incidence of pediatric AOM.

Conjugate vaccines based upon capsular antigens have greatly reduced the incidence of invasive disease by pneumococcus (9) and *H. influenzae* type b (10) in children and adults. However, colonization with nonvaccine serotypes in the case of pneumococci and predominance of nonencapsulated *H. influenzae* (NTHi) have resulted in these

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pathogens continuing to be a significant medical burden, predominantly for infections of the mucosa. This is observed in the incidence of both community-acquired pneumonia (9, 11) and acute otitis media (12), which both occur frequently despite widespread use of currently licensed vaccines.

The initial pneumococcal polysaccharide conjugate vaccine (PCV-7) effectively reduced the overall incidence of invasive disease (13) and partially reduced otitis media (14). Expanding vaccine coverage from 7 to 13 serotypes (PCV-13), including serotypes frequently isolated from pneumococcal AOM, has further decreased pneumococcal AOM incidence, although significant disease burden persists (15). An alternative strategy to expand coverage beyond pneumococcus employed capsules from 10 serotypes conjugated to a surface-exposed lipoprotein of *H. influenzae* (PHiD-CV) (16). This strategy also has somewhat decreased AOM incidence by both pneumococcus and NTHi (17). However, conjugate vaccination does not decrease recurrent pneumococcal AOM (18), and acute otitis media remains a significant health burden (19). This phenomenon is not fully explained by serotype replacement to non-vaccine-type strains, as even vaccine serotypes continue to be isolated from pneumococcal AOM cases in vaccinated populations (16, 20). This issue could be due to poor mucosal antibody production or low expression of capsular antigen by colonizing pneumococci (21). The recent discovery of active enzymatic removal of capsular polysaccharide at the mucosal surface may also help *S. pneumoniae* evade anticapsular antibodies (22).

The incidence of both mucosal and invasive pneumococcal disease decreases beyond early childhood, a phenomenon thought to be the result of accumulating protein antigen exposure leading to building broad protective antibody-mediated immunity (23). Inclusion of protein-based antigens to supplement currently licensed capsule-based vaccines may be a viable strategy for reducing the incidence of mucosal infections, particularly in young children, in a serotype-independent manner. Inclusion of protein-based antigens that cross-react with multiple bacterial pathogens might further extend protection. One candidate is the pneumococcal choline binding protein A (CbpA) (24), also named pneumococcal surface protein C (PspC) (25). Vaccination with recombinant CbpA elicits antibodies that are cross-reactive against *H. influenzae* serogroup b and most strains of pneumococci (26). CbpA is a pneumococcal adhesin with domains targeted to the nasopharyngeal mucosa (YLN) and the blood-brain barrier (NEEK). In this study, we examined whether coadministering the commercially available capsular vaccine PCV-13 with a pneumococcal fusion protein (YLN) composed of two epitopes of CbpA fused to the termini of a pneumolysin toxoid would broaden protection against AOM beyond PCV-13 serotypes and/or affect PCV-13-induced antibody titers in a murine model.

RESULTS

We initially sought to determine whether coadministering a fusion protein vaccine with polysaccharide conjugate vaccine PCV-13 would significantly enhance the levels of IgG against whole pneumococci. YLN is a fusion protein in which the adhesin domains of CbpA (YPT and NEEK) have been fused to an L460D toxoid of pneumolysin (26, 27). Administering either PCV-13 or YLN induced a potent antibody response against serotype 4, 19F, and 7F pneumococci (Fig. 1a to c), all of which are included in the PCV-13 vaccine. PCV-13 vaccination did not elicit significant titers against a serotype 2 strain, which is not included in the PCV-13 vaccine. However, the YLN vaccine induced a robust antibody response against this strain (Fig. 1d). Coadministering YLN with PCV-13 significantly ($P < 0.05$ by Mann-Whitney test) elevated serum antibody titers against pneumococci of all 3 vaccine serotypes (4, 7F, and 19F) and nonvaccine serotype 2 beyond that elicited by PCV-13 alone, as measured by whole-cell enzyme-linked immunosorbent assay (ELISA). This result suggests that YLN vaccination induces antibodies recognizing antigens common to multiple serotypes of pneumococcus, including those not in the current PCV-13 vaccine, and that adding protein vaccine elevates the overall antibody response when administered with PCV-13.

We next sought to ascertain whether YLN alone or in combination with PCV-13

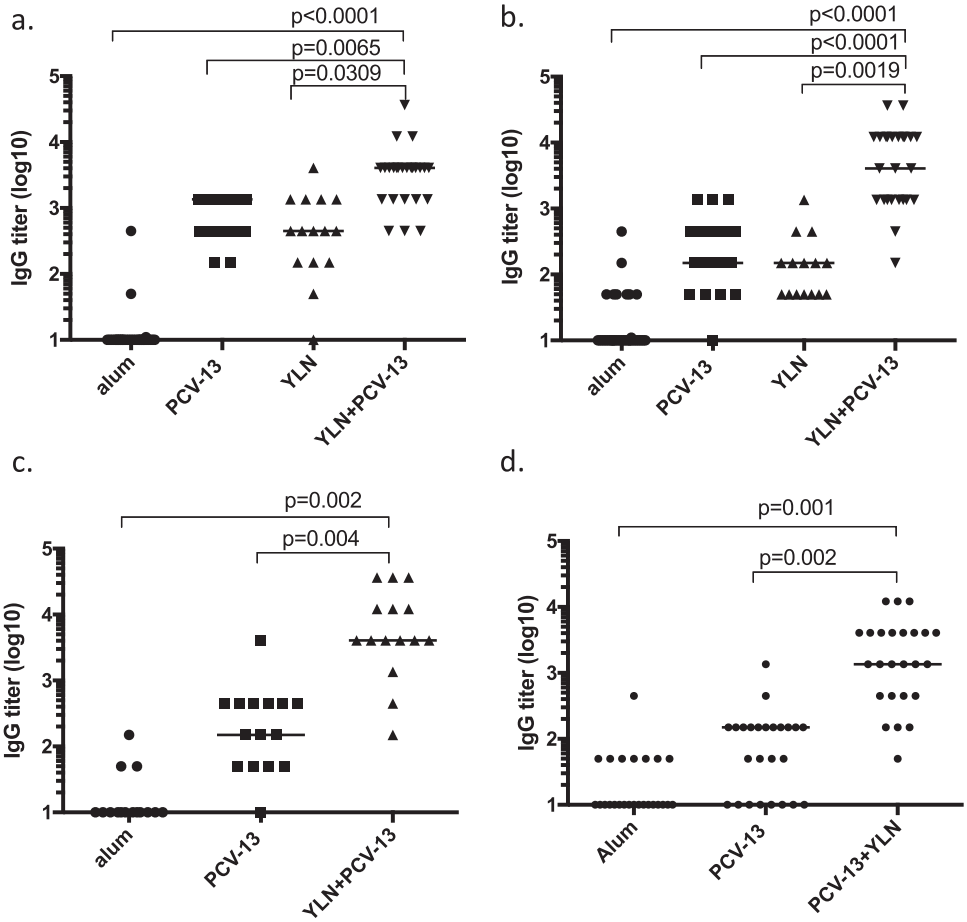

FIG 1 Protein-based vaccines enhance IgG antibody responses against *S. pneumoniae*. ELISA-based measurements of cross-reactive IgG from vaccinated mice receiving alum (vehicle control), PCV-13, YLN, or PCV-13 with YLN. Sera from vaccinated animals were tested against whole-cell lysates of serotype 4 (a), 19F (b), 7F (c), or 2 (d) pneumococcal strains. Each data point represents titers from an individual animal. Groups were different by ANOVA, with *P* values of <0.0001 for serotypes 4, 19F, and 2 and a *P* value of 0.0002 for serotype 7F. Indicated pairwise comparisons were made by Mann-Whitney testing. *P* values are indicated, with a *P* value of <0.05 being considered significant. Horizontal bars indicate the medians for each group.

would engender protection against pneumococcal acute otitis media. We used luminescent derivatives of serotypes 19F (19F/x) and 7F (7F/x), both of which translocate from the nasal passages into the middle ear, resulting in acute otitis media in murine models (28). The capsule types 7F and 19F are serotypes included in the PCV-13 vaccine but are still frequently isolated from patients with AOM, even in the post-PCV-13 era (29, 30).

At 24 h after challenge with the 19F/x strain, significant reductions in overall titers were observed in response to the PCV-13 vaccination administered alone or with YLN vaccination compared to titers for alum-vaccinated mice. While YLN vaccination alone did not confer significant protection against AOM, vaccination with both YLN and PCV-13 enhanced protection beyond that of PCV-13 alone (Fig. 2a). This was shown in the overall incidence of AOM, with the total number of animals with at least one ear testing positive for bacteria and the percentage of ears having positive cultures being significantly reduced only in the PCV-13/YLN dual-vaccinated animals (Fig. 2b and c). These data were further supported with measurements of bioluminescent intensity in the ears after challenge, with the dual-vaccinated group having the lowest level of bacterium-containing ears by IVIS imaging (Fig. 2d to g). No significant alterations in initial colonization density were observed in response to any of the vaccine groups compared to levels for PCV-13 alone at this early 24-h time point (see Fig. S1 in the

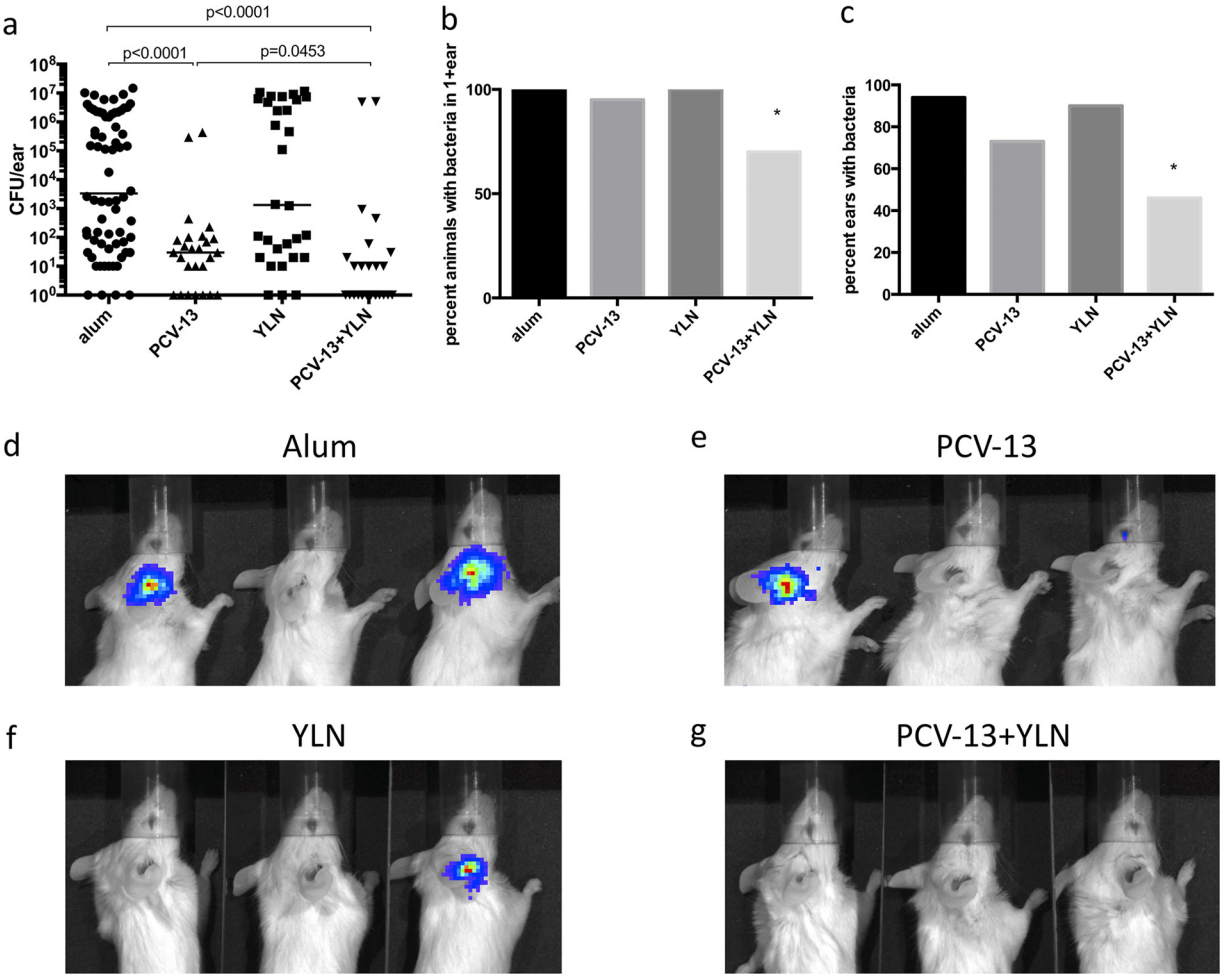

FIG 2 Impact of vaccine interventions on serotype 19F-mediated acute otitis media. (a) Vaccination with either PCV-13 or PCV-13 with YLN significantly reduced bacterial burden of serotype 19F *S. pneumoniae* in the middle ear 24 h postchallenge. Each data point represents titers from an individual animal. Groups were significantly different by ANOVA at a *P* value of 0.0011. Indicated pairwise comparisons were made by Mann-Whitney testing, with a *P* value of <0.05 being considered significant. Horizontal bars indicate the medians for each group. (b and c) Percentages of animals with bacteria recovered from the ears (b) or the percentage of bacterium-positive ears (c) indicates that PCV-13 supplemented with YLN significantly reduced the overall burden of AOM. Comparison was made by chi-square test. *, $P < 0.05$ compared to alum-vaccinated mice. Representative Xenogen images of alum (d)-, PCV-13 (e)-, YLN (f)-, and PCV-13 with YLN (g)-vaccinated animals.

supplemental material). These data indicate that vaccination with YLN in conjunction with PCV-13 enhances protection against AOM by a serotype 19F strain independent of colonization density.

We next tested the strain specificity of the protection engendered by the PCV-13/YLN vaccine combination by testing an additional strain of pneumococcus, a luminescent derivative of a serotype 7F isolate, 7F/x, in our model of AOM. The PCV-13-vaccinated group had significantly lower overall bacterial titers in the middle ear, with the protective effect being significantly enhanced upon addition of the YLN vaccine (Fig. 3a). Although vaccination with PCV-13 lowered the overall bacterial burden in the ears, the incidence of AOM on a per-animal basis was lowered only in the PCV-13/YLN dual-vaccination group (Fig. 3b). These data were further supported by the results of bioluminescent imaging of the ears, in which the greatest reduction in ears testing positive was observed in the PCV-13/YLN dual-vaccination group (Fig. 3c to e). Coadministration of YLN and PCV-13 significantly reduced 7F/x nasal titers below levels seen in alum-vaccinated mice but not below levels in PCV-13-vaccinated animals. These data indicate that adding YLN to the PCV-13 vaccine can reduce the incidence of AOM against homologous challenge strains.

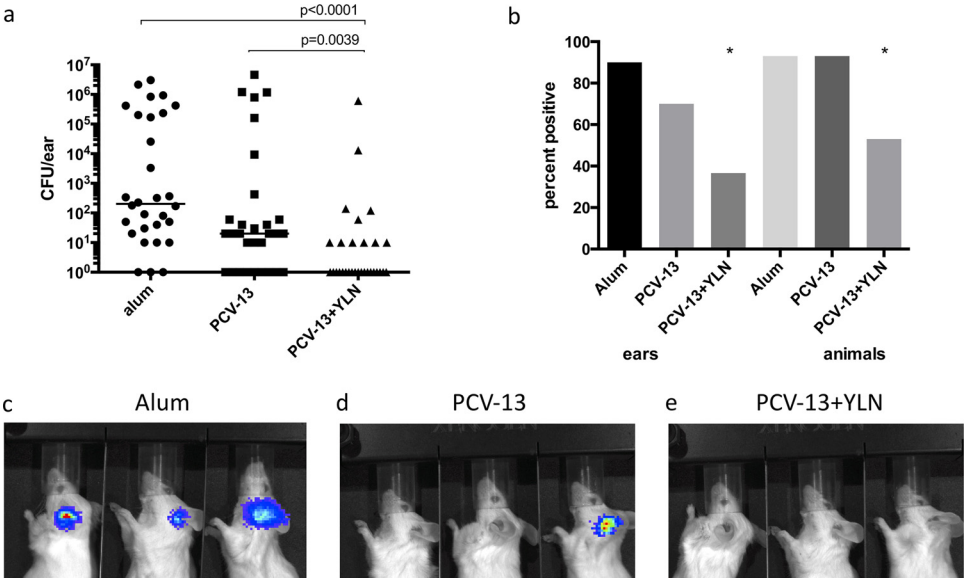

FIG 3 Impact of vaccine interventions on serotype 7F-mediated acute otitis media. (a) Twenty-four hours post-challenge, 7F *S. pneumoniae*-inoculated animals that were vaccinated with either PCV-13 or PCV-13 with YLN had a significantly lower bacterial burden in the middle ear than did those that received other vaccinations. Each data point represents titers from an individual animal/ear. Groups were not significantly different by ANOVA, with a *P* value of 0.2226. Indicated pairwise comparisons were made by Mann-Whitney testing, with a *P* value of <0.05 being considered significant. Horizontal bars indicate the median for each group. (b) The percentage of animals with bacteria recovered from the ears or the percentage of bacterium-positive ears indicate that PCV-13 supplemented with YLN significantly reduced the overall burden of AOM. Comparison was made by chi-square test. *, $P < 0.05$ compared to alum-vaccinated mice. (c and d) Representative Xenogen images of animals vaccinated with alum (c), PCV-13 (d), or PCV-13 with YLN (e).

Although vaccines targeting the polysaccharide capsule are effective against homologous capsule types, one challenge in the field is the prevalence of nonencapsulated pneumococci that retain the capacity to cause infections at mucosal sites. Such isolates are associated with outbreaks of conjunctivitis and are significant contributors to AOM infections (31). These nonencapsulated strains also frequently carry multiple antibiotic resistance genes that can be transferred to susceptible isolates by horizontal transfer (31). Vaccination with YLN, either alone or with PCV-13, resulted in high IgG antibody titers against the nonencapsulated strain MNZ67 (Fig. 4a). Vaccination with YLN, either alone or with PCV-13, conferred protection against AOM caused by MNZ67, reducing bacterial titers in the middle ear (Fig. 4b). This protection was operative even in the absence of reduced nasal burden (Fig. S1C).

Numerous toxoid versions of pneumolysin have been proposed as vaccine antigens, both as fusion proteins and as components of multivalent vaccines (27, 32–41). Therefore, we next sought to determine whether a different pneumolysin toxoid fused to fragments of CbpA would confer a degree of protection similar to that of YLN vaccination. To this end, additional animals were vaccinated with a different fusion, YDN. This construct fuses the same domains of CbpA, YPT, and NEEK to a Δ6D385N pneumolysin toxoid. As observed with YLN, adding YDN to the PCV-13 vaccination regimen significantly enhanced total IgG responses against whole-cell lysates of both serotype 4 and 19F strains (Fig. 5a and b). The YDN vaccine alone did not confer significant protection against a serotype 19F challenge in an AOM model. The PCV-13 and PCV-13/YDN groups demonstrated significant and similar lower bacterial burdens in the ears following challenge than those of other vaccinated groups (Fig. 5c), observations confirmed by bioluminescence measurements of the middle ear. These data indicate that the YLN construct provides protection superior to that of YDN against pneumococcal acute otitis media.

One intriguing aspect that makes including specific CbpA epitopes in fusion protein vaccines particularly attractive is the structural similarity that is shared between mul-

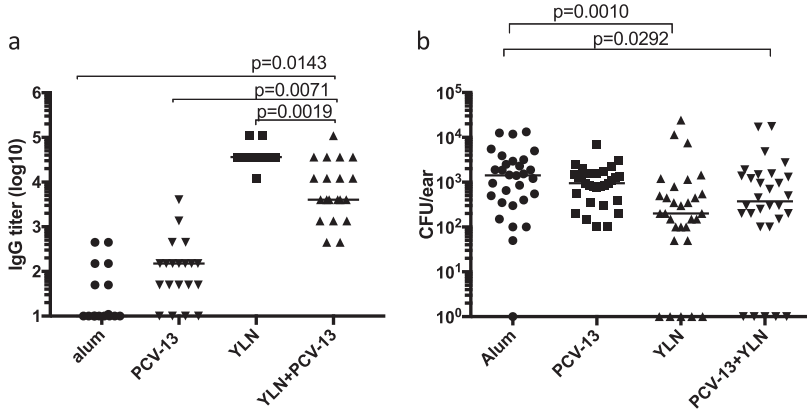

FIG 4 YLN vaccination induces potent antibody responses and protection against nonencapsulated *S. pneumoniae* AOM. Shown are ELISA-based measurements of cross-reactive IgG from vaccinated mice receiving alum (vehicle control), PCV-13, YLN, or PCV-13 with YLN. (a) Sera from vaccinated animals were tested against whole-cell lysates or nonencapsulated strain MNZ67. Each data point represents titers from an individual animal/ear. Groups were significant by ANOVA at *P* values less than 0.0001. Indicated pairwise comparisons were done by Mann-Whitney test. *P* values are indicated, and a *P* value of <0.05 was considered significant. (b) Twenty-four hours postchallenge, nonencapsulated *S. pneumoniae*-inoculated animals that were vaccinated with either PCV-13 or PCV-13 with YLN had a significantly lower bacterial burden in the middle ear than did those that received other vaccinations. Groups were not significantly different by ANOVA, with a *P* value of 0.5669. Indicated pairwise comparisons were made by Mann-Whitney testing, with a *P* value of <0.05 being considered significant. Horizontal bars indicate the medians for each group.

tiple etiological agents of meningitis, including OmpP2 of *H. influenzae* serotype b (26). Such cross-reactive epitopes can confer protection against the translocation of multiple bacterial pathogens across the blood-brain barrier (26). NTHi is not a major cause of meningitis, yet these nonencapsulated pathogens encode OmpP2 (42), which is cross-reactive to CbpA. Therefore, we next sought to determine whether vaccination with either YLN or YDN would induce cross-reactive antibodies against NTHi and whether these responses would be sufficient to mediate protection against NTHi-mediated AOM. Vaccination with YDN and YLN, either alone or with PCV-13, induced potent IgG responses against NTHi, as measured by whole-cell ELISA (Fig. 6a). Vaccination with YDN, either alone or with PCV-13, conferred significant reductions in the overall bacterial burden of NTHi in the middle ears at 24 h postchallenge, reducing the overall incidence of AOM in infected animals from 100% in the alum control group to 50% in the PCV-13/YDN vaccine groups (Fig. 6b). These data indicate that fusion protein-based vaccines with cross-species reactivity are an attractive strategy for targeting multiple otopathogens simultaneously.

DISCUSSION

Here, we show that coadministering a protein vaccine that is cross-reactive against pneumococcus and NTHi with commercially available PCV-13, currently in use to prevent invasive disease by *S. pneumoniae*, significantly reduces AOM incidence and bacterial burden in the middle ear in a murine model. This vaccination strategy generates cross-reactive antibodies recognizing multiple bacterial otitis pathogens and reduces AOM caused by leading pneumococcal serotypes and nontypeable *H. influenzae*.

Our model recapitulates natural infection, with nasal acquisition followed by dissemination to the ear. We used clinical isolates in challenges, demonstrating efficacy against clinically relevant otitis-causing strains of multiple pneumococcal serotypes, including nontypeable strains, and against NTHi, the leading causes of bacterial AOM. Even with this intervention, we were unable to completely eliminate otitis in our model. However, our definition was strict, with any recoverable bacterial burden in the ear being deemed positive for otitis. Any reduction in bacterial load induced by vaccine protection may be clinically relevant, because fewer bacteria and bacterial products will

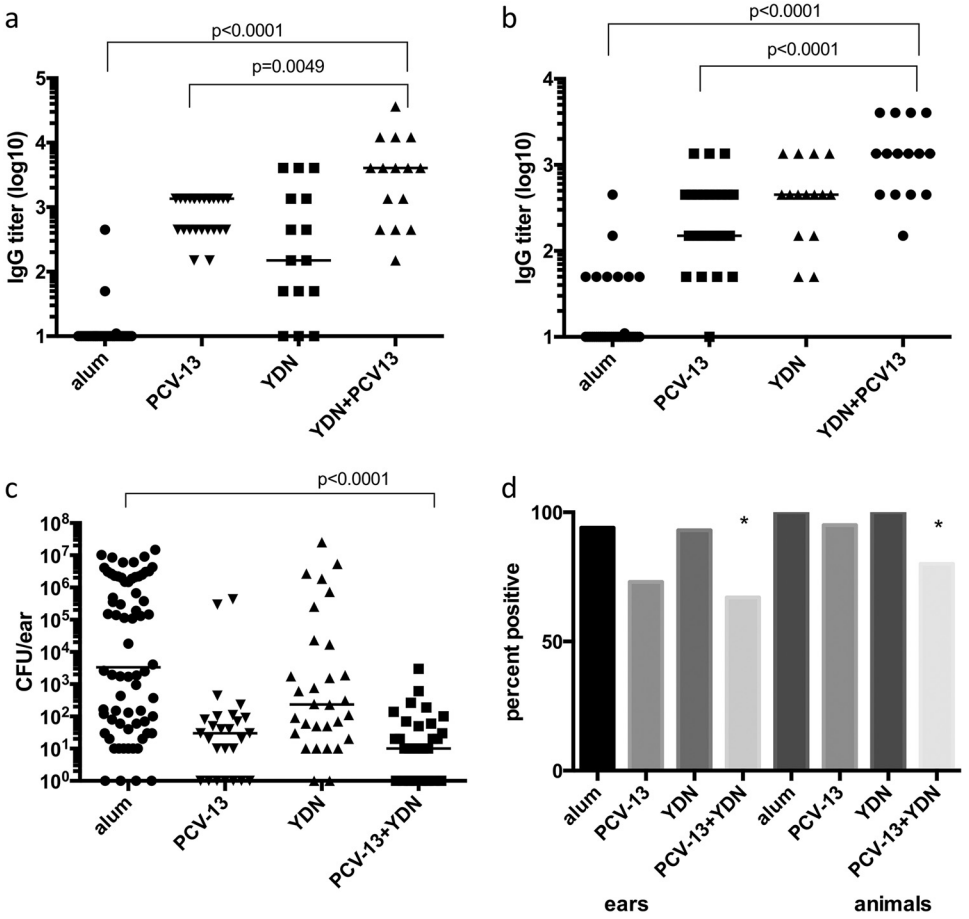

FIG 5 YDN vaccination antibody responses and protective capacity against *S. pneumoniae* AOM. Shown are ELISA-based measurements of cross-reactive IgG from vaccinated mice receiving alum (vehicle control), PCV-13, YDN, or PCV-13 and YDN simultaneously. (a and b) Sera from vaccinated animals were tested against whole-cell lysates from serotype 4 (a) and 19F (b) strains. Groups were significant by ANOVA at *P* values less than 0.0001. Indicated pairwise comparisons were done by Mann-Whitney testing. *P* values are indicated, and a *P* value of <0.05 was considered significant. Each data point represents titers from an individual animal. (c) Vaccination with either PCV-13 or PCV-13 with YDN significantly reduced bacterial burden of *S. pneumoniae* in the middle ear 24 h postchallenge. Groups were significantly different by ANOVA at a *P* value of 0.0475. Indicated pairwise comparisons were made by Mann-Whitney testing, with a *P* value of <0.05 being considered significant. Horizontal bars indicate the medians for each group. (d) Percentage of animals with bacteria recovered from the ears or the percentage of bacterium-positive ears indicates that PCV-13 supplemented with YDN significantly reduced the overall burden of AOM. Comparison was made by chi-square test. *, $P < 0.05$ compared to alum-vaccinated mice.

be present to directly damage the ear tissue or stimulate immune-mediated tissue damage. While the serum antibody titers were significantly improved by combination vaccination, the reduction in bacterial burden was less robust. This could be due to antibodies that recognize the bacterial surface but do not prevent bacterial translocation into the ear. Additionally, serum antibody levels may not accurately reflect mucosal antibodies present in the nasopharynx or ears. Mucosal administration of the vaccine may improve the mucosal antibody responses and provide improved protection at these tissues.

Pneumolysin toxoids are an attractive vaccine candidate, because this factor is highly conserved in pneumococci (43) and tissue damage resulting from the action of the toxin is responsible for many disease symptoms (44). Additionally, immunity against pneumolysin is raised during natural infection (45), suggesting that it is available to the immune system during infection. Pneumolysin is especially relevant in protection against otitis, as it can directly cause cochlear damage and hearing loss in animal models (46), suggesting that a neutralizing antibody could protect against this sequela

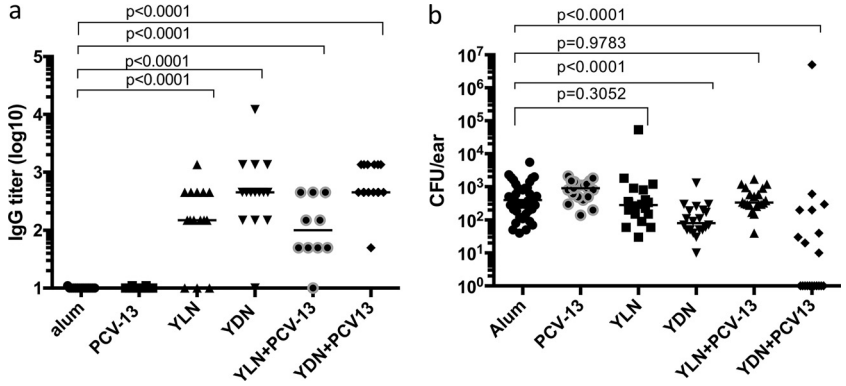

FIG 6 Efficacy of YLN and YDN against NTHi AOM. (a) ELISA-based measurements of cross-reactive IgG from vaccinated mice receiving alum (vehicle control), PCV-13, YDN, YLN, or PCV-13 with YDN/YLN. Sera from vaccinated animals were tested against whole-cell lysates from NTHi, with each point being the serum from an individual animal. Groups were not significantly different by ANOVA, with a *P* value of 0.0766. Indicated pairwise comparisons were done by Mann-Whitney testing. *P* values are indicated, and a *P* value of <0.05 was considered significant. (b) Ear tissues were harvested 72 h postinfection with NTHi, and bacterial burdens were enumerated. Each data point represents titers from an individual ear. Horizontal bars indicate the medians for each group. Groups were not significantly different by ANOVA, with a *P* value of 0.3125. Indicated pairwise comparisons were made by Mann-Whitney testing, with a *P* value of <0.05 being considered significant.

of AOM. Additionally, levels of antipneumolysin antibody have been correlated with reduced incidence of pneumococcal AOM in children (45).

Highly conserved surface proteins of the pneumococcus are also attractive vaccine candidates, although the diverse pangenome of pneumococcus (43), especially that of the nontypeable strains (47), can result in a diverse repertoire of surface proteins among strains (43). Therefore, multivalent vaccines may have the greatest potential to provide the broadest protection against multiple serotypes and nonencapsulated pneumococci. We observed the best protection from AOM in our animals that were vaccinated with both capsule polysaccharide conjugate vaccine and protein vaccine, suggesting that responses against both capsular antigen and protein antigens are important for protection against AOM by encapsulated pneumococcal strains. Even when the capsule antigen was not present in PCV-13 (as in serotype 2 strain D39, nontypeable strain MNZ67, and NTHi), antibody titers were higher in animals vaccinated with the combination of PCV-13 and the fusion protein rather than the fusion protein alone, suggesting that the capsular antigens or the CRM_{197} carrier protein which promotes polysaccharide immunogenicity in infants is enhancing the response against the fusion peptide. We propose that future pneumococcal conjugate vaccines should consider the benefit of the "neglected valency" (48) of the protein carrier in conjugate vaccines. The utilization of cross-reactive protein antigens, such as CbpA-Ply fusions, in conjunction with existing capsular vaccines may broaden protection against not only pneumococcus but also other AOM pathogens, such as NTHi.

MATERIALS AND METHODS

Bacterial strains. Pneumococcal strains were grown on solid tryptic soy agar (TSA) (GranCult; EMD Millipore) medium supplemented with 3% sheep blood (Lampire Biologicals) and 20 μg/ml neomycin at 37°C in a 5% CO_2 atmosphere. Overnight growth was directly inoculated into liquid media (CY) (49) and grown under static conditions until reaching mid-log phase (optical density at 620 nm [OD_{620}] of 0.400). Bacteria were centrifuged and resuspended in phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PBS) (HyClone-GE) at the desired concentration for mouse infections (see below) or whole-cell ELISA (see below). The pneumococcal strains used were TIGR4 (serotype 4), D39 (serotype 2), 19F/x and 7F/x (luminescent derivatives of otitis media isolates of *S. pneumoniae* serotypes 19F and 7F, respectively) (50), and MNZ67 (a nontypeable pneumococcal isolate) (51).

Nontypeable *H. influenzae* 86-028NP, originally isolated from a patient with chronic otitis media, was grown on solid chocolate agar medium supplemented with 11,000 U/liter bacitracin (Remel) at 37°C in a 5% CO_2 atmosphere. Overnight growth was directly inoculated into brain heart infusion broth (BD) supplemented with 0.2% yeast extract (BD), 10 μg/ml NAD^+ (Sigma, St. Louis, MO), and 10 μg/ml hemin

(Sigma, St. Louis, MO) and then grown with aeration until the OD_{600} reached 0.160. Bacteria were centrifuged and resuspended in PBS at the desired concentration for mouse infections.

Fusion protein vaccines. YLN and YDN are fusions of YPT and NEEK peptides of CbpA to the termini of pneumolysin toxoids L460D (27) and Δ6D385N (26, 27) and were prepared as His-tagged proteins (as described in references 26 and 27).

Animal vaccine/challenge. All experiments involving animals were performed with prior approval of and in accordance with guidelines of the St. Jude Institutional Animal Care and Use Committee. The St. Jude laboratory animal facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. Laboratory animals are maintained in accordance with the applicable portions of the Animal Welfare Act and the guidelines prescribed in the DHHS publication *Guide for the Care and Use of Laboratory Animals* (52).

Female 7-week-old BALB/c mice were intraperitoneally vaccinated with a 1:50 human dose (10 μl vaccine in 90 μl PBS) of PCV-13 (Prevnar13; Pfizer), 10 μg YLN or YDN protein with 130 μg alum (alhydrogel; Brenntag Biosector), or 130 μg alum alone in a volume of 100 μl. At week 3, mice were administered booster doses of the initial vaccine. At week 4, mice were bled by the retro-orbital route to determine serum antibody response. At week 5, mice were anesthetized with 2.5% isoflurane and intranasally infected with 1×10^5 to 2.5×10^5 CFU of 19F/x, 5×10^5 CFU of 7F/x, 1×10^6 CFU of MNZ67, or 1×10^7 CFU of NTHi in a volume of 100 μl sterile PBS. Twenty-four hours postinfection (hpi), mice infected with pneumococcus strain 19F/x or 7F/x were imaged via IVIS (PerkinElmer) to visualize otitis media. Nasal passages and ears were harvested from pneumococcus-infected mice 24 hpi and from NTHi-infected mice 72 hpi and then homogenized in 0.5 ml sterile PBS and serially diluted, and CFU were enumerated by counting the number of viable colonies of bacteria. To reduce the limit of detection for bacterial burden in the ears, 100 μl was also plated directly from the homogenate for CFU enumeration. Ears with 0 CFU were assigned a value of 1 for graphing and calculations. For selection, pneumococci were grown on TSA plates supplemented with 3% sheep blood in the presence of 20 μg/ml neomycin, and NTHi organisms were grown on chocolate agar with 11,000 U/liter bacitracin.

Whole-cell ELISA. Each well of a 96-well high-binding ELISA plate (430341; Nunc) was coated with 10^6 CFU of bacteria in carbonate-bicarbonate buffer reconstituted from a tablet (C3041; Sigma). Bacteria were pelleted at the bottom of the plate by centrifugation, the supernatant was removed, and the plates were air dried overnight before being blocked with 10% heat-inactivated fetal bovine serum (FBS) in PBS for 2 h. Serum from vaccinated mice was serially diluted 1:2 from a 1:50 starting dilution in 10% FBS in PBS, added to the wells, and incubated for 1 h at room temperature. Plates were washed 5 times with Tris-buffered saline (TBS). The secondary antibody (anti-mouse; 1030-04; Southern Biotech) was diluted 1:2,000 in blocking buffer and incubated in the wells for 1 h at room temperature. Plates were washed 5 times with TBS. Substrate (P7998; Sigma) was added for 30 min, and the OD_{405} was read in a 96-well plate reader. Results are reported as the inverse of the last dilution with a reading above background level.

Statistical analysis. Prism 6 (GraphPad) was used for all calculations, with the respective statistical tests indicated in the respective figure legends. For bacterial burden and ELISA titers, analysis of variance (ANOVA) was utilized to compare variation between groups followed by *post hoc* Mann-Whitney test to compare individual groups. For measures of relative incidence of disease, comparisons were made by chi-square test. For all statistical tests, a P value of <0.05 for experimental groups compared to corresponding alum controls was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00253-19.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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E.T. is a patent holder on pneumococcal protein vaccines described in the manuscript.

H.M.R., J.W.R., and E.T. designed the experiments. H.M.R., A.P., A.I., and B.M. performed the experiments. J.W.R., H.M.R., and B.M. interpreted the data. J.W.R., H.M.R., and E.T. wrote the manuscript. H.M.R., B.M., A.P., A.I., E.T., and J.W.R. edited and approved the final version of the manuscript.

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