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# Immunization with Pneumococcal Surface Protein K of Nonencapsulated *Streptococcus pneumoniae* Provides Protection in a Mouse Model of Colonization

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**Current vaccinations are effective against encapsulated strains of *Streptococcus pneumoniae*, but they do not protect against nonencapsulated *Streptococcus pneumoniae* (NESp), which is increasing in colonization and incidence of pneumococcal disease. Vaccination with pneumococcal proteins has been assessed for its ability to protect against pneumococcal disease, but several of these proteins are not expressed by NESp. Pneumococcal surface protein K (PspK), an NESp virulence factor, has not been assessed for immunogenic potential or host modulatory effects. Mammalian cytokine expression was determined in an *in vivo* mouse model and in an *in vitro* cell culture system. Systemic and mucosal mouse immunization studies were performed to determine the immunogenic potential of PspK. Murine serum and saliva were collected to quantitate specific antibody isotype responses and the ability of antibody and various proteins to inhibit epithelial cell adhesion. Host cytokine response was not reduced by PspK. NESp was able to colonize the mouse nasopharynx as effectively as encapsulated pneumococci. Systemic and mucosal immunization provided protection from colonization by PspK-positive (PspK$^+$) NESp. Anti-PspK antibodies were recovered from immunized mice and significantly reduced the ability of NESp to adhere to human epithelial cells. A protein-based pneumococcal vaccine is needed to provide broad protection against encapsulated and nonencapsulated pneumococci in an era of increasing antibiotic resistance and vaccine escape mutants. We demonstrate that PspK may serve as an NESp target for next-generation pneumococcal vaccines. Immunization with PspK protected against pneumococcal colonization, which is requisite for pneumococcal disease.**

Vaccination has been the single most effective means of preventing death by infectious organisms (1). *Streptococcus pneumoniae* (pneumococcus) is the etiological agent of several human diseases such as pneumonia, sinusitis, otitis media (OM), meningitis, and septicemia (2). The prevalence of invasive pneumococcal disease (IPD) was significantly reduced after the introduction of currently licensed pneumococcal vaccines (3). Pneumococcal vaccines target specific pneumococcal polysaccharide serotypes, 23 in Pneumovax (Pneumovax 23 [PPSV23]; Merck, Whitehouse Station, NJ, USA) and 13 in Prevnar (Prevnar 13 [PCV13]; Pfizer [formerly Wyeth Pharmaceuticals], New York, NY, USA). With over 90 known antigenically distinct pneumococcal serotypes, there is a significant deficit in vaccine coverage of the serological diversity expressed by the species (4). This coverage gap is widened by the increase in nonencapsulated *Streptococcus pneumoniae* (NESp) carriage since the introduction of the pneumococcal conjugate vaccine (PCV) (5). Pneumococcal disease is predicated by carriage, and NESp is associated with cases of OM and conjunctivitis (6–8). NESp cannot be protected against by current vaccine formulations due to the lack of the capsular polysaccharide. Vaccination with a pneumococcal protein antigen can provide broader pneumococcal protection and be more cost-effective to produce, but a suitable candidate that covers the majority of pneumococci has yet to be developed. While numerous protein-based candidates have been tested, such as PspA, PspC, and PcpA, they have been found to be effective to various degrees based on pneumococcal strain (9–12). Combinations of proteins have been found to be more effective and to have broader coverage (13, 14). NESp does not contain the aforementioned proteins, increasing the need for a protein target effective against NESp (15, 16).

Pneumococcal surface proteins are potential targets for immunization due to accessibility and the function of the protein during colonization. Pneumococcal surface proteins are classified by means of surface attachment and include choline binding proteins (CBPs), LPxTG binding proteins, lipoproteins, and nonclassical surface proteins (17, 18). Some of the most well characterized surface proteins are CBP and LPxTG binding proteins (17). These proteins are immunogenic and aid in colonization. Colonization is requisite for pneumococcal disease in encapsulated and nonencapsulated strains (2, 17). Pneumococcal surface protein K (PspK), an LPxTG-anchored surface protein, has been shown to be necessary for colonization in a subset (null capsule clade I) of NESp and plays a role in virulence during experimental OM (19–

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21). The role of PspK in colonization makes it a potential vaccine target. While we have previously demonstrated an increase in epithelial cell adherence due to PspK, it is unknown if there are other effects PspK exerts during colonization (20).

Pneumococcal surface protein C (PspC), a CBP, shares some sequence identity with PspK (21). PspC has been reported to aid in epithelial cell adhesion, recruitment of immune factors, and regulation of specific cytokines (22, 23). All of these functions are important for initial colonization and persistence in the nasopharynx. The pneumococcus must attach to epithelial cells to effectively colonize the nasopharynx. Once established, the pneumococcal population must persist and survive against the host innate immune response that is triggered by pathogen-associated molecular patterns (PAMPs), such as peptidoglycan and lipoteichoic acid (24–27). Activation of the host innate immune response through PAMP recognition is intended to clear bacteria from the nasopharynx through stimulating inflammation and the recruitment of leukocytes (27, 28).

The pneumococcus has evolved methods to downregulate some of these responses. PspC has been shown to downregulate the chemokine interleukin-8 (IL-8) and the macrophage inflammatory protein 2 (MIP-2), which could aid in maintaining a commensal state (23). While it has been shown that PspK does not reduce complement deposition as PspC does, PspK may have an anti-inflammatory effect (20, 29). Once an encapsulated pneumococcal population establishes in the nasopharynx, it can persist for weeks to months with the ultimate clearance of the strain from an antibody-mediated response (30, 31).

How NESp colonize and persist in the nasopharynx is unknown. PspK may perform functions in colonization unrelated to epithelial cell adherence. We examined the ability of NESp to effectively colonize and persist within the mouse nasopharynx along with the effects of PspK on host cytokine responses. Additionally, the potential of PspK as an NESp vaccine target was examined. We demonstrated that NESp can establish long-term colonization, but PspK does not modulate host inflammatory responses. Also, PspK immunization is able to induce significant protection against colonization.

**TABLE 1** Cytokine response of PspK$^+$ NESp compared to that of an isogenic PspK mutant

| Cytokine/ chemokine[a] | MNZ11 (PspK$^+$) pg/ml (mean ± SE) | MNZ1131 (PspK$^-$) pg/ml (mean ± SE) | *P* value |
|---|---|---|---|
| Eotaxin | 15.85 ± 3.98 | 24.35 ± 2.9 | 0.12 |
| G-CSF | 454.4 ± 95.79 | 407.7 ± 166.04 | 0.80 |
| GM-CSF | 36.6 ± 8.09 | 20.05 ± 3.42 | 0.10 |
| IFN-γ | 66.00 ± 9.63 | 31.65 ± 11.22 | 0.053 |
| IL-1α | 82.7 ± 23.26 | 75.55 ± 28.14 | 0.35 |
| M-CSF | 81.15 ± 28.95 | 44.6 ± 6.55 | 0.25 |
| IL-1β | 22.15 ± 5.58 | 9.15 ± 1.59 | 0.055 |
| IL-2 | 31.7 ± 9.25 | 39.00 ± 17.96 | 0.73 |
| IL-3 | 59.00 ± 4.19 | 40.8 ± 8.13 | 0.08 |
| IL-4 | 27.6 ± 5.66 | 29.3 ± 8.41 | 0.87 |
| IL-5 | 23.2 ± 4.44 | 32.15 ± 10.21 | 0.44 |
| IL-6 | 67.05 ± 16.76 | 26.40 ± 8.47 | 0.06 |
| IL-7 | 41.85 ± 12.10 | 31.6 ± 5.67 | 0.46 |
| IL-9 | 31.25 ± 9.93 | 26.2 ± 6.33 | 0.68 |
| IL-10 | 25.5 ± 3.13 | 16.7 ± 5.1 | 0.18 |
| IL-12 (p40) | 64.95 ± 20.35 | 42.75 ± 18.17 | 0.44 |
| IL-13 | 69.1 ± 7.82 | 54.00 ± 24.63 | 0.58 |
| IL-15 | 37.25 ± 8.11 | 39.95 ± 16.23 | 0.89 |
| IL-17 | 31 ± 6.92 | 29.00 ± 6.45 | 0.84 |
| IP-10 | 28.65 ± 8.18 | 57.55 ± 19.23 | 0.21 |
| MIP-2 | 20.45 ± 4.27 | 32.65 ± 6.63 | 0.16 |
| KC | 312.15 ± 86.04 | 159.65 ± 48.28 | 0.16 |
| LIF | 36.45 ± 10.31 | 28.9 ± 9.55 | 0.61 |
| LIX | 238.25 ± 93.3 | 115.85 ± 19.71 | 0.24 |
| MCP-1 | 32.15 ± 10.6 | 40.75 ± 13.01 | 0.62 |
| MIP-1α | 34.4 ± 9.78 | 19.2 ± 2.64 | 0.17 |
| MIP-1β | 48.15 ± 5.99 | 38.8 ± 10.24 | 0.45 |
| MIG | 12.7 ± 5.17 | 32.45 ± 7.17 | 0.056 |
| RANTES | 97.1 ± 26.55 | 44.8 ± 5.95 | 0.09 |
| TNF-α | 38.00 ± 6.47 | 11.3 ± 5.57 | 0.014 |
| IL-12 (p70) | 41.25 ± 18.09 | 39.5 ± 6.72 | 0.93 |
| VEGF | 494.85 ± 85.3 | 295.1 ± 106.23 | 0.18 |

[a] G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; IP, interferon-induced protein; VEGF, vascular endothelial growth factor.

## MATERIALS AND METHODS

**Bacterial strains.** Bacterial strains were grown at 37°C in 5% $CO_2$ on sheep blood agar (BA) or in Todd-Hewitt broth with 0.5% yeast extract (THY). Strains used in the current study include *S. pneumoniae* EF3030 (serotype 19F), D39 (serotype 2), PspK-negative (PspK$^-$) strain MNZ85 (NESp), PspK-positive (PspK$^+$) strain MNZ67 (NESp), PspK$^+$ strain MNZ11 (NESp), and PspK$^-$ MNZ1131 (mutant of MNZ11) (21, 32, 33). All MNZ strains are carriage isolates from a study of Korean daycare centers, while strain EF3030 (19F) was used because it is a known strain that colonizes and does not disseminate into systemic infections. Isogenic mutant, MNZ1131, was produced by allelic replacement as described previously (21).

**Cloning and expression of recombinant PspK.** Recombinant PspK (rPspK) was isolated for initial immunization studies as described previously using the B-PER 6×His fusion protein purification kit (ThermoFisher Scientific) (20). PspC-LXS234 (amino acids [aa] 1 to 455) used for mucosal immunization was isolated as previously described (34). PspA for immunization was isolated through His-tagged protein fusion as previously described (35).

To express PspK in a Gram-positive background for tagless purification, a gene encoding *pspK* was fused to a gene harboring promoter and signal peptide sequences of the *hla* gene from *Staphylococcus aureus* using overlapping PCR. Briefly, PCR was performed to amplify a gene encoding a part of *pspK* (from nucleotide 32 to 502, excluding a signal peptide sequence at the N terminus and a LPxTG motif at the C terminus) from the MNZ11 chromosomal DNA template using a forward PspK primer (5′-AATCCTGTCGCTAATGCCGAGATGGCATCGACAGCA-3′; the primer sequence overlapping the *hla* gene is underlined), a reverse PspK primer (5′-GCGCGGATCCTTATTCTTGTTTACCTTTTTTTGCAGC-3′; the BamHI site is underlined), and Platinum Pfx DNA Polymerase (Life Technologies). PCR was performed to amplify a gene harboring a promoter and a signal peptide sequence of the *hla* gene from the *S. aureus* chromosomal DNA template using a forward Hla primer (5′-GCGCGAATTCCATTAGAAGCTAACCTATACTC-3′; the EcoRI site is underlined) and a reverse Hla primer (5′-GGCATTAGCGACAGGATTCATT-3′). Overlapping PCR was performed to join the two amplification products above, using the two PCR amplification products as templates and a forward Hla primer and a reverse PspK primer. The resulting PCR product was digested with EcoRI and BamHI restriction enzymes, ligated into pMK4 vector, and cloned in *Escherichia coli* DH5α. A cloned plasmid was purified and transformed into *S. aureus* RN4220. The cloned *S. aureus* strain was grown in brain heart infusion (BHI) overnight at 37°C. The culture supernatant was harvested by centrifugation and concentrated using an Amicon Ultra-15 centrifugal filter unit (EMD Millipore). Concentrated supernatant was equilibrated with ammonium sulfate (35% [vol/vol]). Linear ammonium sulfate gradient (35% to 0%) hydrophobic

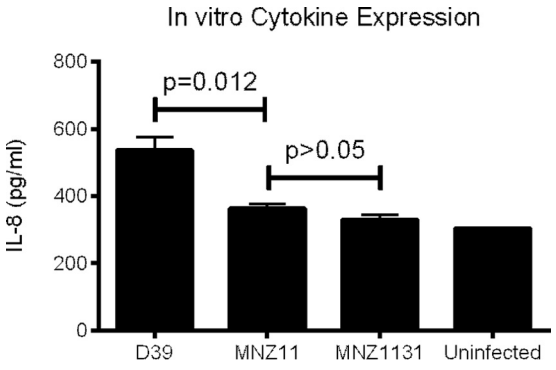


**FIG 1** Epithelial cell expression of IL-8 after pneumococcal stimulation. IL-8 secretion from Detroit 562 human pharyngeal epithelial cells was stimulated by encapsulated strain D39 (serotype 2) as well as MNZ11 ($PspK^+$) and MNZ1131 ($PspK^-$). Epithelial cells were challenged with $1 \times 10^7$ pneumococci, and 4 h postchallenge, supernatants were collected and assayed by ELISA. Results are from two independent experiments done in triplicate and expressed as mean ± standard error (SE).

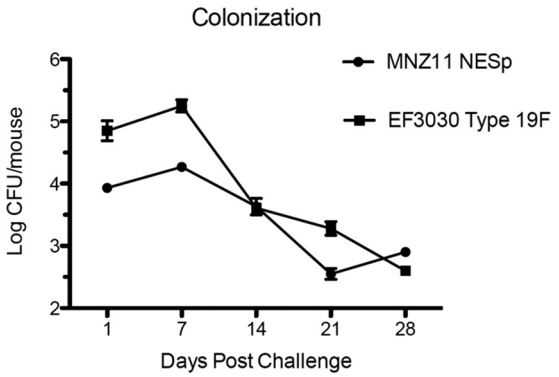


**FIG 2** Mouse nasopharyngeal colonization. Mice were challenged with $1 \times 10^7$ CFU of pneumococci. Colonization of EF3030 (serotype 19F) and MNZ11 ($PspK^+$) was assessed weekly from a minimum of three mice per strain. Results are expressed as mean ± SE.

interaction chromatography was performed using an Octyl-Sepharose column and AKTA pure (GE Healthcare).

**Mouse nasopharyngeal colonization.** Six- to eight-week-old C57/BL6 mice were used for colonization studies. Mice were lightly anesthetized with isoflurane for easier handling. Colonization was achieved through slow administration to the outer nares of 10 μl of Ringer's lactate solution containing $1 \times 10^7$ CFU, which is inhaled by the mouse. The inoculum dose was chosen to allow for consistent colonization so differences observed during colonization would be directly related to the ability to colonize. At times, indicated mice were sacrificed, and the nasopharynx was washed with 200 μl of Ringer's lactate solution. This was followed by homogenization of mouse nasal tissue to collect surface-associated bacteria that were not obtained in the wash. Nasal tissue was obtained by decapitation of euthanized mice, denuding of the skull, bisection of the skull behind the eyes, and transverse sectioning of the rostral portion. The nasal passage was then excised with forceps and homogenized in 200 μl phosphate-buffered saline (PBS). Samples were plated on BA with 5 μg/ml gentamicin, and the number of CFU of colonizing bacteria was determined. The amount of total colonizing bacteria was obtained through adding bacteria recovered from the nasal wash and the nasal tissue combined. All strains were tested in at least two separate experiments. Studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center.

**Milliplex cytokine assay.** Production of 32 cytokines and chemokines was analyzed using the quantitative, multiplexed Milliplex multi-analyte panels (MAP) assay according to the manufacturer's protocols (Millipore, Billerica, MA, USA). Five mice per strain were colonized with either MNZ11 or the isogenic PspK mutant MNZ1131 as described above, and nasal washes were collected 2 days postchallenge for cytokine analysis. To avoid the effect of interassay variation, only samples that were analyzed on the same plate were compared statistically. The lower detection limit was 4 pg/ml for all analytes, and intra-assay variability was <10%. Data were collected and analyzed using a Luminex 200 instrument running xPONENT software version 3.1 (Luminex Corporation, Austin, TX).

***In vitro* cytokine detection.** Chemokine (C-X-C motif) ligand 8 (CXCL8/IL-8) release was measured in cell culture supernatants by specific enzyme-linked immunosorbent assay (ELISA) (R&D Systems, United Kingdom). Human pharyngeal epithelial cell line Detroit 562 was grown to ~95% confluence in 6-well plates. Cells were incubated with $1 \times 10^7$ CFU, and supernatants were collected 4 h postchallenge and assayed by ELISA. Values represent results of two independent experiments done in triplicate.

**Immunization.** Mice were subinguinally immunized with 100 μl of 10 μg recombinant protein suspended in PBS and combined with Imject Alum (Pierce) according to the manufacturer's protocol three times at 1-week intervals. Mice were colonized as described above 3 weeks following the last boost. Mucosal immunizations were administered to anesthetized mice through application to mouse nares of 14 μl of PBS, which contained 10 μg recombinant protein with 4 μg of cholera toxin β subunit (CTB) (Sigma-Aldrich, St. Louis, MO). Mice were immunized weekly for 3 weeks. The mice were rested for 3 weeks after the boost and then challenged intranasally. Serum was collected through retro orbital bleeding, and mouse saliva was collected by an intraperitoneal injection of 0.1 mg of pilocarpine-HCl (Sigma-Aldrich) in 100 μl of PBS (36).

Levels of PspK-specific antibodies were calculated using standard ELISA protocol compared to the standard curve of known mouse IgG concentration. In brief, 0.3 μg rPspK was coated on 96-well enzyme immunoassay/radioimmunoprecipitation assay (EIA/RIA) plates (Costar) overnight. Plates were washed and serum was serially diluted followed by specific antibody detection through mouse Ig-specific antibody conjugated to alkaline phosphatase.

**Epithelial cell adhesion assay.** The epithelial cell adhesion assay was performed as previously described on human pharyngeal epithelial cell line Detroit 562 (20). Inhibition of adhesion was determined by incubating epithelial cells with 10 μg rPspK in 1 ml media for 30 min before addition of bacteria. The inoculum also contained 10 μg/ml of rPspK. Anti-PspK and secretory IgA (sIgA) inhibitory effect was determined by 30-min preincubation of inoculums with serum collected from systemically immunized mice at a 1:200 dilution factor or 10 μg/ml of sIgA.

**Statistical analysis.** Statistical analysis was performed using Student's *t* test on the InStat program (Prism GraphPad) to determine the statistical difference for immunization and epithelial cell adhesion experiments.

## RESULTS

**Mammalian cytokine response to NESp expressing PspK.** Nasal washes of mice colonized for 48 h with either wild-type MNZ11 ($PspK^+$) or isogenic PspK mutant MNZ1131 were analyzed for cytokine levels using a Milliplex cytokine assay, which quantitates 32 different cytokines in a multiplex reaction. Based on cytokine levels from five mice for each pneumococcal strain tested, there was no statistically significant difference between levels of any of the tested cytokines, with the exception of inflammatory cytokine tumor necrosis factor alpha (TNF-α) (Table 1). Forty-eight hours postchallenge was utilized for analysis to allow sufficient time for surface-associated communities to form but to retain a comparable number of colonizing bacteria. Unfortunately, there were significantly fewer MNZ1131 than MNZ11 (log CFU 3.08 ± 0.12 compared to log CFU 4.01 ± 0.16; $P = 0.011$) obtained from the

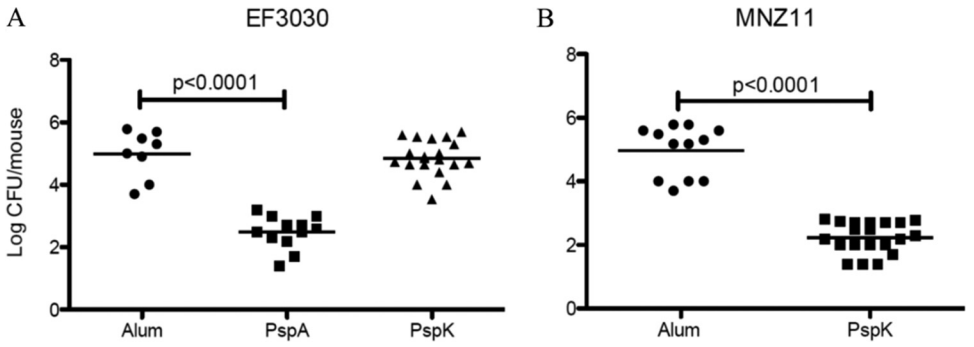


**FIG 3** Systemic immunization with rPspK provides protection from colonization by PspK$^+$ NESp. Groups of mice were immunized weekly for 3 weeks with recombinant protein and rested 3 weeks before intranasal challenge. Bacterial burden was assessed 5 days postchallenge. (A) Immunization with rPspA was able to reduce nasopharyngeal colonization by EF3030 (PspA$^+$), but immunization with rPspK had no effect on EF3030 colonization compared to that of mock-immunized mice. (B) Immunization with rPspK was able to significantly reduce MNZ11 (PspK$^+$) colonization compared to that of mock-immunized mice.

nasal washes, explaining why increased levels of TNF-α were observed with MNZ11 colonization. Other proinflammatroy cytokines, IL-1β, IL-6, and gamma interferon (IFN-γ), also trended toward increased expression during MNZ11 colonization (Table 1). Since human IL-8 had previously been demonstrated to be modulated by PspC (23), we tested the expression of human IL-8 in an *in vitro* assay after 4 h of stimulation. Based on analysis of IL-8 levels through an ELISA, there was no significant difference detected between PspK$^+$ MNZ11 and PspK mutant MNZ1131 (Fig. 1).

**Persistent pneumococcal colonization.** The ability to persist in the mouse nasopharynx was assessed by colonization with either MNZ11 (PspK$^+$) or EF3030 (serotype 19F). There were recoverable bacteria at all times tested for MNZ11 and EF3030 (Fig. 2). While there were significantly more encapsulated bacteria recovered at earlier time points, the general trend of colonization was the same between strains. There was an initial increase in recovered bacteria from day 1 to day 7 followed by decreasing amounts of bacteria to 28 days (Fig. 2). Additionally, persistent colonization by MNZ11 appears to depend on PspK as MNZ1131 (PspK$^-$) is cleared from the nasopharynx after 5 days (21).

**Murine PspK immunization.** Systemic immunization with recombinant protein produced significant protection from colonization in strains expressing PspA for EF3030 and PspK for

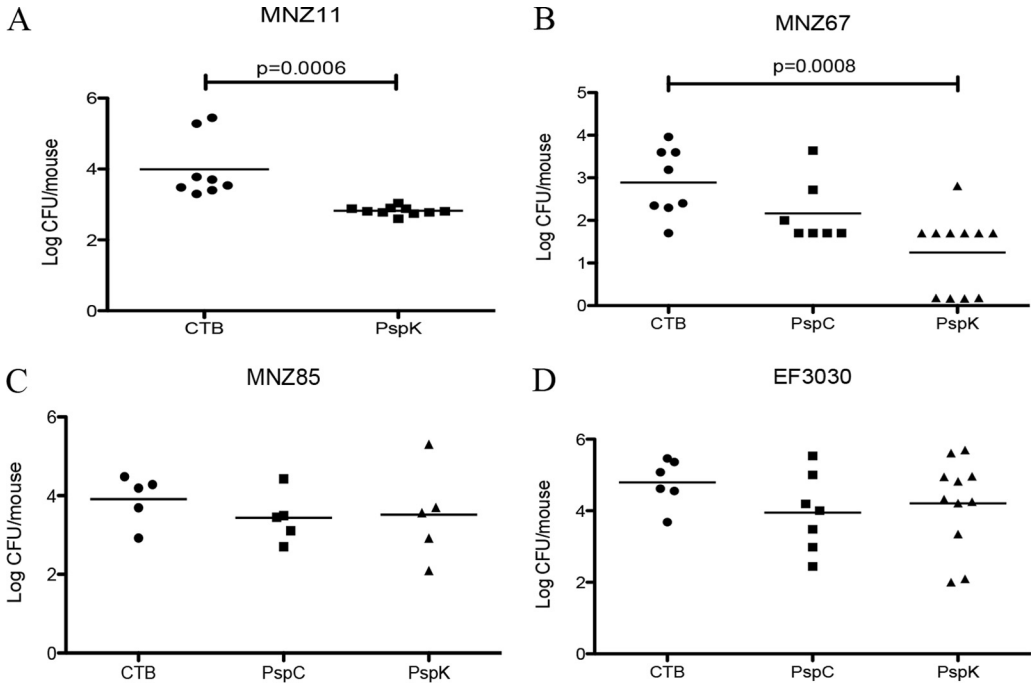


**FIG 4** Mucosal immunization with rPspK provides protection from colonization by PspK$^+$ NESp. Mice were immunized intranasally with recombinant protein and CTB as an adjuvant. Mice received weekly immunizations for 3 weeks, rested 3 weeks, and then were challenged intranasally. (A) A significant reduction in bacterial colonization was observed when mice were mucosally immunized with rPspK and challenged with MNZ11 (PspK$^+$). (B) The same trend is observed when mice were challenged with MNZ67 (PspK$^+$), but protection from rPspC immunization was not observed. (C) No reduction in bacterial colonization was observed when mice were immunized with either rPspC or rPspK and challenged with MNZ85 (PspK$^-$). (D) Mice challenged with EF3030 had no significant reduction in bacteria recovered when immunized with either rPspC or rPspK compared to control.

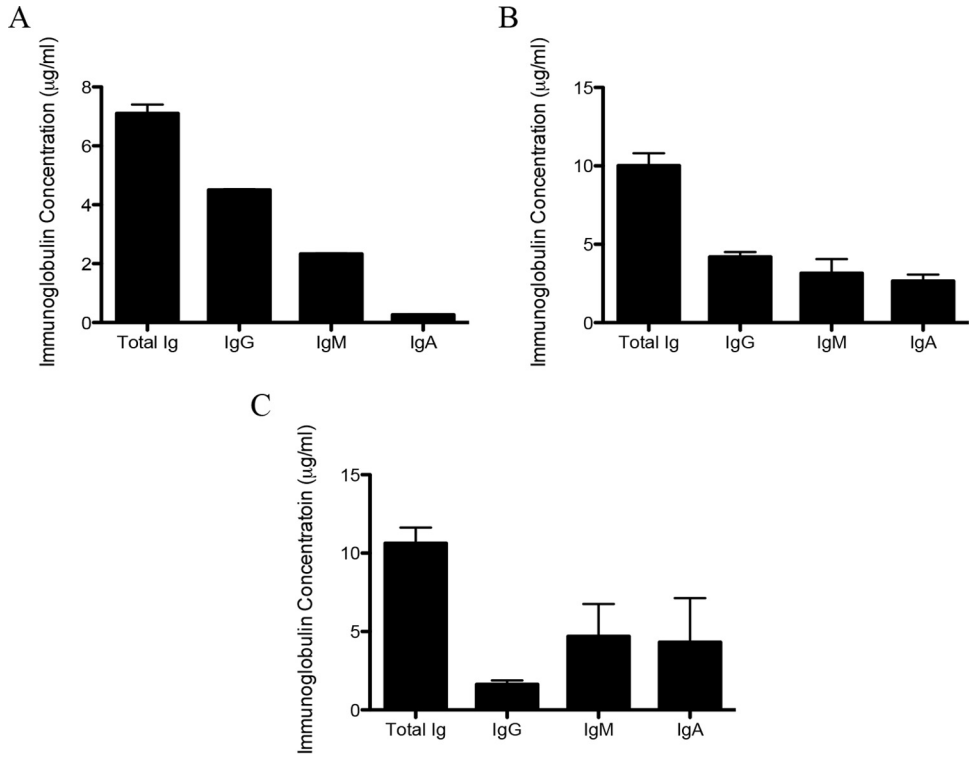


**FIG 5** Antibody isotype response to rPspK immunization in mice. (A) Systemic immunization with rPspK led to PspK-specific antibody response. ELISA was used to determined PspK-specific serum IgG, IgM, and IgA levels. (B) Mucosal immunization with rPspK led to PspK-specific antibodies in mouse serum. (C) Mucosal immunization with rPspK led to PspK-specific antibody response in murine saliva. All samples were collected 3 weeks after the last immunization from a minimum of three mice. Anti-PspK antibodies were assayed in duplicate for each sample by ELISA. The results are expressed as the mean ± SE.

MNZ11. While complete protection from colonization was not obtained, there was a significant decrease from 4.98 ± 0.27 to 2.48 ± 0.15 ($P < 0.0001$) in EF3030 recovered from the mouse nasopharynx 5 days postchallenge (Fig. 3A). Immunization of mice with rPspK followed by subsequent challenge with EF3030 had no effect on recovered bacteria, 4.98 ± 0.27 to 4.85 ± 0.13 (Fig. 3A). No effect on EF3030 colonization was achieved by rPspK immunization, but there was a significant decrease from 4.97 ± 0.23 to 2.23 ± 0.11 ($P < 0.0001$) in recovered MNZ11 ($PspK^+$) (Fig. 3B).

Mucosal immunization with rPspK induced protection from colonization by $PspK^+$ strains MNZ11 and MNZ67. Compared to mock-immunized mice, MNZ11 was carried at higher densities, 3.99 ± 0.31, than that in rPspK-immunized mice, 2.82 ± 0.04 ($P = 0.0006$) (Fig. 4A). The same degree of protection was observed when mice were challenged with another $PspK^+$ strain (MNZ67, mock immunized, 2.89 ± 0.28, compared to immunized, 1.25 ± 0.27) ($P = 0.0008$) (Fig. 4B). Carriage of MNZ85, a $PspK^-$ NESp strain, was not significantly reduced when immunized with PspC or PspK compared to that of mock immunization, 3.57 ± 0.26 and 3.58 ± 0.50 compared to 3.88 ± 0.24 (Fig. 4C). There was no effect of rPspK immunization on nasopharyngeal carriage when mice are challenged with EF3030 (19F), 4.79 ± 0.27 compared to 4.21 ± 0.38 (Fig. 4D). Immunization with rPspC did not significantly reduced murine colonization when challenged with either MNZ67, 2.17 ± 0.28 compared to 2.89 ± 0.28 (Fig. 4B), or EF3030, 3.95 ± 0.41 compared to 4.79 ± 0.27 (Fig. 4D).

**PspK immunization isotype response.** Serum from mice either systemically or mucosally immunized and saliva from mucosally immunized mice were collected and PspK-specific antibody isotypes were determined. PspK-specific antibodies of all isotypes tested, IgG, IgM, and IgA, were isolated from all samples. Mice systemically immunized with rPspK contained 4.5 μg/ml, 2.33 μg/ml, and 0.265 μg/ml of each isotype, respectively (Fig. 5A). Mice mucosally immunized with rPspK contained each isotype tested in mouse serum (Fig. 5B), while antibodies in saliva of each isotype are shown in Fig. 5C.

**Inhibition of epithelial cell adhesion.** We examined the ability of proteins or antibodies to inhibit epithelial cell adhesion of NESp based on the presence of PspK, in a $PspK^+$ strain (MNZ11), a strain naturally lacking PspK (MNZ85), or a PspK deletion mutant (MNZ1131). Epithelial cell adhesion of MNZ11 was significantly reduced when incubated with an anti-PspK antibody from mice, rPspK, and sIgA from human colostrum. Compared to control epithelial cell adhesion of MNZ11 set at 100%, there were 32.22%, 52.22%, and 59.62% reductions in adhesion, respectively ($P = 0.01$, $P = 0.0035$, $P = 0.01$, respectively) (Fig. 6A). No significant difference was seen in the ability of either MNZ85 or MNZ1131 to adhere to epithelial cells under any of the conditions tested (Fig. 6B and C).

## DISCUSSION

$PspK^+$ NESp persistently colonized the mouse nasopharynx, and immunization with PspK induced protective antibodies. We did not detect significant reduction of host inflammatory responses

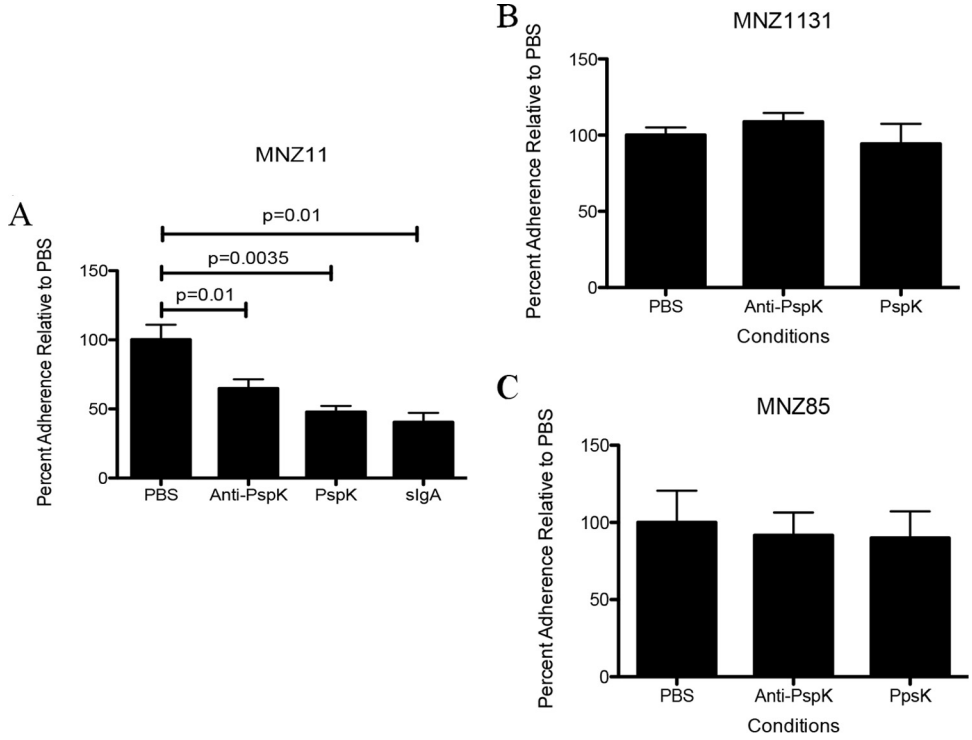


**FIG 6** Epithelial cell adherence reduced by antibodies. (A) Significant inhibition of epithelial cell adhesion by MNZ11 (PspK$^+$) occurred when cells were incubated with either anti-PspK antibodies, rPspK, or sIgA. (B) Epithelial cell adhesion of PspK mutant MNZ1131 was not affected by any of the conditions tested. (C) Epithelial cell adhesion by NESp strain MNZ85, which naturally lacks PspK, was not affected by any of the conditions tested.

by PspK$^+$ NESp. It is important to understand the role of PspK in colonization, as it is the only known NESp-specific virulence factor. NESp strains are increasing in prevalence, in nasopharyngeal colonization, and in disease (5, 6, 37). Nasopharyngeal colonization is requisite for pneumococcal disease, and the ability to persist in the nasopharynx increases the potential for a colonizing strain to invade sterile sites and cause disease. During initial colonization, there are numerous host factors the pneumococcus must evade (38, 39).

PspK contains an R1 and R2 region similar to PspC that comprises ~15% of the *pspK* gene. PspC has been reported to aid in adhesion, recruitment of immune factors, and regulation of specific cytokines (22, 23). All of these functions are important for initial colonization along with persisting in the nasopharynx. The pneumococcal capsule has been reported to be required for effective colonization (40, 41). It has been shown to reduce clearance from the nasopharynx by hindering agglutination in the host mucous. Expression levels of the capsule vary during colonization, which allows for attachment mediated by surface proteins (41). The mechanisms that NESp use to colonize the human nasopharynx are not fully understood (5, 21).

Due to localized sequence identity of PspK with PspC, we hypothesized that PspK may modulate host inflammatory responses. However, we were unable to detect any significant reduction in inflammatory response between a PspK$^+$ NESp or a PspK isogenic mutant during mouse colonization (Table 1). While inflammatory response was not reduced, an increase in TNF-α was observed, probably due to increased amount of bacteria recovered from the nasopharynx. Increased trends in other inflammatory cytokines were also observed. Despite this, downstream signaling pathways may be inhibited by PspK-induced signaling because migration inhibition factor (MIG), eotaxin, and MIP-2 were trending toward reduced levels. Interestingly, while IFN-γ trends toward higher levels with MNZ11 (PspK$^+$), MIG, whose production is stimulated by IFN-γ, trends toward lower expression levels when PspK is present (Table 1). The presence of PspC has been shown to downregulate IL-8 production in an *in vitro* model system (23). We failed to detect PspK modulation of IL-8 using a similar approach. Since the examined strains of NESp lack PspC, some means for regulating the host immune response would be important for persistent colonization. Therefore, NESp must use another mechanism, such as potentially increased biofilm formation, which acapsular strains are known to make in excess compared to their encapsulated counterparts (42–44).

We determined that an NESp strain expressing PspK was able to persistently colonize the mouse nasopharynx. During colonization, the host is exposed to the bacterial surface, allowing for specific antibody responses. Thus, we determined if antibodies to PspK would protect against colonization. Whether we immunized systemically or mucosally, we were able to induce the production of protective antibodies in mice that were specific to PspK (Fig. 3 and 4). Given the localized sequence identity between PspC and PspK, we hypothesized that protection from PspK$^+$ strain colonization may be induced by PspC immunization. We chose to focus on mucosal immunization, as it would induce the strongest response against colonizing strains. Colonization by NESp was not reduced by PspC immunization. Structural differences between PspC and PspK may limit exposure to homologous regions, resulting in protective epitopes being differentially exposed between proteins. We also determined the concentration of anti-PspK an-

tibodies and the isotypes produced against PspK. We found that the relative concentrations of different isotypes of antibodies produced against PspK were similar to what has been seen for other pneumococcal surface proteins (45–47).

The identification of novel targets for immunization against *S. pneumoniae* is necessary due to emerging serotypes and increasing numbers of NESp isolates (5, 48, 49). The unique characteristics of strains of NESp, which seem to have a different surface structure than encapsulated strains based on sequence analysis and recent reports, require vaccine targets that may be specific for NESp (7, 15, 16). PspK has been shown to be a surface-exposed colonization factor and, more recently, a virulence factor for OM, making it an ideal candidate for vaccination (19, 20). While PspK may be included in next-generation pneumococcal vaccines, PspK will not protect against all NESp since not all strains have this antigen. Serum from mice immunized with PspK was able to reduce epithelial cell adherence of strains expressing PspK indicating that initial host interaction is disrupted by immunization. Reducing colonization by limiting epithelial cell attachment prevents invasive and noninvasive pneumococcal disease. Also, we found that anti-PspK antibodies do not reduce epithelial cell adhesion of NESp strains that lack PspK. The NESp strains that naturally lack PspK have been shown to cause OM at a lower frequency than $PspK^+$ stains but have also been isolated from invasive infections (19, 37). This further increases the need for future research into NESp-specific vaccines or a general pneumococcal vaccine that is effective against encapsulated and NESp strains.

NESp strains are an increasing proportion of the pneumococcal population. With the implementation of widespread PCV use in underdeveloped countries, there is an even greater chance that NESp may become more common within the human population. The pneumococcus, once a major cause of morbidity and mortality worldwide, has been well contained within developed countries due to prolific PCV use. This highly adaptive bacterial species now harbors antibiotic resistance to most antibiotics and can once again become a significant pathogen in the developed world due to our inability to effectively treat and now effectively vaccinate against certain pneumococcal strains.

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