Molecular characterization of pneumococcal surface protein K, a potential pneumococcal vaccine antigen

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ABSTRACT
The pneumococcal capsule is indispensable for pathogenesis in systemic infections; however, many pneumococcal diseases, including conjunctivitis, otitis media, and some systemic infections in immunocompromised patients, are caused by nonencapsulated Streptococcus pneumoniae (NESp). Null capsule clade 1 (NCC1), found in group 2 NESp, expresses pneumococcal surface protein K (PspK) and is becoming prevalent among pneumococcal organisms owing to the widespread use of pneumococcal conjugate vaccines. Despite its clinical importance, the molecular mechanisms underlying the prevalence of NCC1 have not been fully elucidated. Here, we investigated the role of the R3 domain of PspK in the epithelial cell adherence of NCC1. We found that the R3 domain of PspK mediated NCC1 adherence via its direct interaction with the epithelial surface protein annexin A2. Additionally, neutralization with purified recombinant PspK-R3 or rabbit anti-UD:R3 IgG inhibited binding of NESp to lung epithelial cells in vitro. Immunization with the ‘repeat’ domain of PspK-R3 or PspK-UD:R3 effectively elicited mucosal and systemic immune responses against PspK-R3 and provided protection against nasopharyngeal, lung, and middle ear colonization of NESp in mice. Additionally, we found that rabbit anti-UD:R3 IgG bound to PspC-R1 of the encapsulated TIGR4 strain and that UD:R3 immunization provided protection against nasopharyngeal and lung colonization of TIGR4 and deaths by TIGR4 and D39 in mice. Further studies using 68 pneumococcal clinical isolates showed that 79% of clinical isolates showed cross-reactivity to rabbit anti-UD:R3 IgG. About 87% of serotypes in the 13-valent pneumococcal conjugate vaccine (PCV) and 68% of non-vaccine serotypes were positive for cross-reactivity with rabbit anti-UD:R3 IgG. Thus, the R3 domain of PspK may be an effective vaccine candidate for both NESp and encapsulated Sp.

KEYWORDS
annexin A2; pneumococcal surface protein K; Streptococcus pneumoniae; vaccine

Introduction
The wide use of pneumococcal conjugate vaccine (PCV) had led to dramatic decreases in the prevalence of invasive pneumococcal disease and in nasopharyngeal colonization rates of multiple vaccine serotypes. However, infection and nasopharyngeal colonization by non-vaccine serotypes and nonencapsulated Streptococcus pneumoniae (NESp) have increased worldwide. Although NESp strains are generally considered less virulent than encapsulated strains, NESp strains have been repeatedly isolated from patients with various pneumococcal diseases, including conjunctivitis, acute otitis media (AOM), acute rhinosinusitis, and systemic infections (albeit rarely, and mostly in immunocompromised patients).

NESp strains were originally divided into 2 distinct groups (group 1 and group 2) based on the structure of the capsular polysaccharide synthesis (CPS) gene locus. In group 1 NESp strains, the CPS gene locus encodes non-functional cps genes owing to point mutations, insertions, or deletions. In group 2 NESp strains, all cps genes are replaced with virulence proteins, such as aliB-like homologs or pneumococcal surface protein K (PspK). In recent studies, group 2 NESp strains were subdivided into 3 null capsule clades (NCCs) based on which genes were located within the CPS locus. NCC1 strains express a novel cell wall anchoring protein, PspK, whereas NCC2 and NCC3 strains express one or 2 aliB-
like homologs. Among these strains, NCC1 strains are clinically important because of their high prevalence in patients with conjunctivitis, AOM, and acute rhinosinusitis, particularly in young children. Moreover, these strains have been shown to exhibit increased antibiotic resistance.\textsuperscript{16-19}

The prototype of PspK is a structurally well-characterized cell wall anchoring protein. Its N-terminal and C-terminal ends encode a conserved secretion signal motif and a LPxTG motif for secreting and anchoring on the peptidoglycan layer, respectively.\textsuperscript{20} Previous studies have shown that PspK mediates the binding of NESp to human lung epithelial cells and nasopharyngeal colonization in mice.\textsuperscript{21} In addition, the expression of PspK in NCC1 has been shown to be critical for the development of AOM in a pneumococcal AOM model in chinchillas.\textsuperscript{19,21} Because bacterial adherence to mammalian cells is often mediated by specific interactions between bacterial adhesins and mammalian cell surface receptors in many bacterial infections,\textsuperscript{22-25} the specificity of NCC1 adherence to epithelial cells may also be mediated through the interaction of PspK with the receptor protein(s) on the surface of epithelial cells. However, no reports have described the detailed molecular mechanisms underlying the binding/interaction of PspK to epithelial cells.

Accordingly, with the aim of improving our understanding of the molecular basis of PspK-mediated adherence and how this process affects NESp colonization, we sought to identify the critical domain(s) of PspK and the epithelial protein(s) involved in adherence of NCC1 to lung epithelial cells. In addition, we investigated whether this defined domain of PspK could be used as a protein vaccine for preventing nasopharyngeal colonization and subsequent infection by NESp in mice.

**Results**

**PspK mediated MNZ11b adherence to mammalian epithelial cells and was required for the clearance of intra-epithelial pneumococcus**

Pneumococcal infection is initiated by adherence of pneumococci to the host cells and, in certain cases, is followed by cellular invasion and subsequent trans-epithelial dissemination. Since PspK replaces the \textit{cps} gene, which is important for the adherence of pneumococci to host cells, \textit{in vitro} adherence and invasion of wild-type (WT) MNZ11b and its isogenic \textit{pspK}-deficient mutant (\textit{ΔpspK}) was assessed in A549 human alveolar epithelial cells to elucidate whether PspK expression affected the virulence of NCC1 through mediating the interaction with host cells. As shown in Fig. 1A, adherence of MNZ11b to the epithelial cells was decreased by up to 49.5% ± 7.2% in PspK-deficient cells compared with that in WT MNZ11b (0.47×10^6 CFU in Δ\textit{pspK} vs. 0.91×10^6 CFU in MNZ11b). Interestingly, despite the significant reduction in adherence to the host cells, intra-epithelial growth of Δ\textit{pspK} was markedly enhanced compared with that of WT MNZ11b (1.80×10^8 CFU in Δ\textit{pspK} vs. 4.65×10^4 CFU in MNZ11b) (Fig. 1B). These data suggest that PspK mediates MNZ11b adherence to epithelial cells, and it is required for the effective bacterial effects against pneumococcus in epithelial cells.

To confirm the role of PspK expression in adherence to epithelial cells and invasion into epithelial cells, we next assessed the effects of neutralizing antibodies against PspK-UD:R3 on adherence to and invasion into A549 cells (Fig. 1C and 1D). In control studies, co-incubation of either pneumococcal strain with normal rabbit control IgG had little or no effect on the adherence to and the invasion into A549 cells. However, co-incubation of MNZ11b with rabbit anti-UD:R3 IgG markedly inhibited adherence to A549 cells (0.84×10^6 CFU in rabbit control IgG vs. 0.16×10^6 CFU in rabbit anti-UD:R3 IgG) and enhanced invasion of MNZ11b into A549 cells (1.50×10^6 CFU in rabbit control IgG vs. 3.65×10^6 CFU in rabbit anti-UD:R3 IgG). In addition, pre-treatment with rabbit anti-UD:R3 IgG had no effect on the adherence and invasion of Δ\textit{pspK}. These results indicated that MNZ11b adherence to the host epithelial cells was mediated by the surface expression of PspK protein and that its interaction with host receptors may be required for optimal anti-pneumococcal activities of epithelial cells.

**Identification of the domain of PspK responsible for binding with A549 cells**

Although several pneumococcal adhesin molecules and corresponding mammalian binding receptors have been identified, the molecular mechanism underlying PspK binding to epithelial cells has not been fully elucidated. To improve our understanding of the structural determinants present within PspK, bioinformatic analysis was performed to identify predicted binding sites. Using the NCBI Conserved Domain Database (CDD) search system\textsuperscript{26} and PSI-BLAST analysis, bioinformatic analysis of the predicted amino acid sequence of PspK identified an unknown domain (UD) at AA31–187, the ‘repeat’ domain (R3) at AA187–279, EEA KRKA repeats at AA280–577, and the cell-wall anchoring domain (LPXTG) at AA563–7 (Fig. 2A). The R3 domain exhibits 61% amino acid identity to the R1 domain of PspC and 19% amino acid identity to the lactoferrin-binding domain of PspA (Fig. 2A). Structure prediction analysis using PHYRE,\textsuperscript{27} Swiss-Model,\textsuperscript{28} and HHPRED\textsuperscript{29}
Figure 1. PspK-mediated MNZ11b adherence to and invasion in human alveolar epithelial cells. ((A)& B) Adherence (A) and invasion (B) of WT MNZ11b or its isogenic ΔpspK mutant to A549 cells following infection at an MOI of 10 were assessed. ((C) & D) The effects of neutralization with rabbit anti-UD:R3 IgG on the adherence (C) and invasion (D) of WT and ΔpspK to A549 cells were assessed. Values are expressed as the total cell-associated CFU recovered compared with that of the original inoculum. Experiments were performed in triplicate and repeated at least twice. A representative experiment is shown. Data were analyzed by Student’s t tests. *, p < 0.05 compared with MNZ11b in (A) and (B) and PBS in (C) and (D).

Figure 2. Identification of the PspK domain mediating MNZ11b binding to A549 cells. (A) Domain organization of PspK and its homologs. The domains of PspK were compared with those of its closest homologs, PspC and PspA, using the NCBI BLAST program. Levels of amino acid identity between regions are indicated. (B) Alignment of the truncated form of PspK for purification. P: proline-rich region; R1, R2, and R3: ‘repeat’ domains; CBD: choline-binding domain; UD: undefined domain; BR: binding region; 6×His: 6 histidine tag. (C) Effects of pre-incubation with recombinant PspK domain fragments (UD, R3, or UD:R3) on MNZ11b binding to A549 cells. Values are expressed as the total cell-associated CFU recovered compared with that of the original inoculum. Experiments were performed in triplicate and repeated at least twice. A representative experiment is shown. Data were analyzed by Student’s t tests. *, p < 0.05 compared with PBS.
algorithms also identified the R3 of PspK as having structural similarity to the R1 of PspC (HHPred; 100% probability, e = 9.6e-38; Fig. S1).

To identify the domain involved in MNZ11b adherence to human lung epithelial cells, 3 truncated domain fragments of PspK (R3, UD, and UD:R3) were constructed and purified (Fig. 2B). A549 cells were pre-incubated with purified recombinant UD, R3, or UD:R3 domain proteins, and MNZ11b adherence to A549 cells was then assessed. As shown in Fig. 2C, UD domain pre-treatment did not inhibit MNZ11b adherence to A549 cells, whereas both R3 and UD:R3 pre-treatments significantly inhibited MNZ11b adherence to A549 cells. These findings indicated that the R3 domain of PspK was involved in MNZ11b adherence to A549 cells.

**Identification of a mammalian binding receptor for PspK**

Next, to identify the mammalian binding receptor for R3, far western blotting with PspK-R3 was conducted against A549 membrane proteins (Fig. 3A). Although A549 membrane extracts contained numerous proteins, ranging in mass from 5 to 300 kDa, FLAG-tagged R3 (FLAGR3) bound only a small number of proteins, and high levels of binding were observed for 3 proteins of molecular weights (MWs) 50, 35, and 30 kDa. To characterize these binding proteins, LC/LTQ-Orbitrap mass spectrometry (MS) was performed. From this analysis, the 50-kDa protein was identified as cytokeratin 18 (MW 47.3 kDa), the 35 kDa protein was identified as annexin A2 (MW 38.5 kDa), and the 30 kDa protein was identified as prohibitin 2 (32.3 kDa).

Among these 3 identified mammalian proteins, cytokeratin 18 and annexin A2 are known to play important roles during bacterial infections in epithelial cells. Therefore, we further investigated whether cytokeratin 18 and/or annexin A2 were involved in binding of PspK-R3 to epithelial cells by performing enzyme-linked immunosorbent assay (ELISA)-based binding assays. As shown in Fig. 3B,
FLAGR3 showed significant binding activity to immobilized purified annexin A2, whereas no binding was found for the mixture of keratins isolated from human epidermis. In addition, to confirm whether FLAGR3 interaction with annexin A2 is specific, we assessed the binding of the different concentration of FLAGR3 with annexin A2. As shown in Fig. 3C, FLAGR3 showed significant binding to annexin A2, which is increased in direct proportion to the amount of protein applied. However, in control studies with immobilized casein blocking reagent, no significant binding by FLAGR3 was detected. Since we found the specific binding activity between R3 domain of PspK and epithelial annexin A2 in vitro, we, next, assessed if this phenomenon occurs in vivo in live bacteria. As shown in Fig. 3D, annexin A2 bound to WT MNZ11b, and it was significantly inhibited by the loss of PspK in ΔpspK. These data suggest that annexin A2 may function as a mammalian epithelial binding receptor for PspK, and the annexin A2 binding domain of PspK is indeed located within the R3 domain (AA187–279).

The role of PspK expression in MNZ11b infection in epithelial cells in vitro and in mice in vivo

To investigate the in vivo role of PspK-mediated binding of MNZ11b to the host cells in the colonization and pathogenesis of pneumococcal pneumonia, we assessed the nasopharyngeal colonization and

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**Figure 4.** Role of PspK expression in pneumococcal colonization and invasion. ((A)& B) Mice were inoculated with the indicated numbers of WT MNZ11b or ΔpspK cells intranasally (i.n.), and the numbers of colonized bacteria in the nasopharyngeal tract (A) and lungs (B) at 15 h postinfection (hpi) were counted. ((C)& D) Mice were i.n. inoculated with 10⁵ CFU of WT or ΔpspK, and the numbers of colonized bacteria in the nasopharyngeal tract (C) and lungs (D) at 15, 36, and 64 h after inoculation were analyzed. The horizontal lines denote the median number of bacteria in each group of 5 mice. (E) Histopathology of representative lungs from mice infected with WT or ΔpspK (H&E staining). * p < 0.05 compared with WT.
pulmonary growth of MNZ11b and ΔpspK in CD-1 mice. CD-1 mice were intranasally (i.n.) inoculated with either MNZ11b or ΔpspK strains at various titers, and nasal washes were collected 24 h after challenge. As shown in Fig. 4A, inoculation with MNZ11b led to significant, dose-dependent nasopharyngeal colonization. In contrast, the colonization of ΔpspK was significantly lower than that of MNZ11b. Higher levels of nasopharyngeal colonization persisted over time up to 64 hours postinfection (hpi) in MNZ11b-inoculated mice, whereas the colonization level of ΔpspK was dramatically reduced in a time-dependent manner (Fig. 4C).

We also determined the colonization and growth level of pneumococci in the lungs of mice. As shown in Fig. 4B and 4D, MNZ11b exhibited significantly higher lung colonization at 15 hpi than ΔpspK; however, both strains were cleared before 36 hpi. Additional histological analysis of the lungs of mice showed more severe inflammatory lesions, including enhanced neutrophil infiltration, loss of alveolar architecture, and RBC leakage in the lungs of MNZ11b-inoculated mice compared with those of ΔpspK-inoculated mice (Fig. 4E). Consistent with the histological findings of the lungs, bronchoalveolar lavage (BAL) fluid from mice inoculated with MNZ11b showed significantly higher levels of inflammatory cytokines and chemokines than that of mice infected with ΔpspK (Fig. S2).

**Protective effects of PspK-R3 immunization on MNZ11b colonization**

Because we identified R3 domain of PspK as an important domain responsible for the binding of MNZ11b to A549 cells, probably through direct interaction with cell surface annexin A2, we next examined the effects of R3 immunization on the nasopharyngeal colonization of MNZ11b. CD-1 mice were immunized i.n. or intraperitoneally (i.p.) with either recombinants R3 consisting of the residues AA187–
279 or UD:R3 consisting of the residues AA31–279 (Fig. 2A), followed by challenge with MNZ11b. Both nasal colonization of MNZ11b and bacterial load of MNZ11b in the lungs were not significantly affected by systemic immunization via i.p. injection with either R3 or UD:R3 (Fig. 5A and 5B). However, i.n. immunization of both R3 and UD:R3 significantly inhibited not only nasal colonization of MNZ11b but also bacterial load in the lungs (Fig. 5A and 5B). To elucidate the molecular mechanisms underlying PspK-R3 immunization-mediated protection against nasal colonization in mice, R3-specific IgG, IgM, and IgA antibodies were measured at 7 d post-immunization (Fig. 5C–E). Both i.n. and i.p. immunization of R3 or UD:R3 significantly enhanced the levels of IgG and IgM. In mice immunized by i.p. injection, no difference on the immune responses against R3 or UD:R3 was found. However, i.n. immunization of UD:R3 showed greatly enhanced production of both IgG and IgM compared with those in R3-immunized mice (Fig. 5C and 5D). No measurable IgA response in serum was detected in neither i.n. nor i.p. immunization groups (Fig. 5E). Because both immunization methods elicited comparable systemic immune responses but i.p. immunization was not sufficiently effective for protection against mucosal colonization of and invasion by MNZ11b, mucosal immune response following i.n. immunization was assessed using BAL fluid (Fig. 5F). Immunization with either R3 or UD:R3 elicited significantly higher levels of mucosal IgA and IgG specific to R3, with UD:R3 showing a much greater increase than R3 (Fig. 5F). These data indicated that both R3 and UD:R3 effectively elicited humoral immune responses and that UD:R3 was a better antigen for induction of mucosal immune responses than R3. During nasopharyngeal colonization and invasion by MNZ11b, mucosal immune responses play a critical role in protection against MNZ11b colonization and invasion. Taken together, these data suggested that i.n. immunization with UD:R3 or R3 was a good strategy to protect against nasal colonization and lung invasion of NCC1 in mice.

**Effects of PspK-R3 immunization on MNZ11b-induced AOM in mice**

Unlike the remarkable effects of PCV on the reduction in invasive pneumococcal disease (IPD), the effects of PCV on protection against the induction of AOM are still unclear.37 Because nasopharyngeal colonization of Sp is a major risk factor for developing AOM and recurrent OM and because PspK-R3 immunization has been found to be effective for protection against nasopharyngeal colonization of MNZ11b, we next examined whether immunization with R3 could also provide protection against AOM with MNZ11b. B6 mice were immunized i.n. with R3, followed by transtympanic inoculation with MNZ11b. After 1–3 days, bullae were dissected from the skulls of the mice. The presence of serous and/or mucoid effusion was recorded under a surgical microscope as the presence of OM pathology, and bacterial numbers in the bullae were counted from middle ear cavity washes. As shown in Table 3, all MNZ11b inoculated ears (8/8 ears; 100%) showed middle ear effusion on day 1, and 3 of 6 ears (50%) were still positive for the middle ear effusion on days 2 and 3. However, only 2 of 8 ears (25%) of R3 immunized mice were positive for middle ear effusion on day 1, and this number was further reduced to 1 of 6 (16.6%) on day 2 and no effusion positive ears (0%) on day 3. In addition, bacteria numbers in the ears of unimmunized control mice were significantly higher than those in the ears of R3 immunized mice (Fig. 6). These data clearly indicated that i.n. immunization of R3 not only provided protection against nasopharyngeal colonization and invasion into lungs but also inhibited development of AOM in response to MNZ11b inoculation in mice.

**Cross-protection of encapsulated pneumococci by R3 immunization**

Because the R3 domain shares 61% amino acid sequence identity with the R1 domain of PspC expressed by most pneumococci, we next evaluated the cross-reactivity of rabbit anti-UD:R3 IgG to purified R1 protein cloned...
from the TIGR4 strain (serotype 4) using ELISA-based binding assays. As shown in Fig. 7A, rabbit anti-UD:R3 IgG also bound to the immobilized R1 domain of PspC. Based on these findings, we then determined the binding capacity of rabbit anti-UD:R3 IgG to intact TIGR4 using flow cytometry. As summarized in Table 4, among 68 clinical isolates tested, 54 isolates (79%) showed cross-reactivity to rabbit anti-UD:R3 IgG. About 87% (20 out of 23) of PCV13 serotypes and 69% (18 out of 26) of non-vaccine serotypes were found to be cross-reactive to rabbit anti-UD:R3 IgG.

Because we found that rabbit anti-UD:R3 IgG was cross-reactive with PspK-negative encapsulated pneumococci, we next examined whether UD:R3 immunization could also provide protection against infection with PspK-negative encapsulated pneumococci. Mice immunized with UD:R3 were challenged with encapsulated TIGR4 (serotype 4), and nasal washes and lungs were collected to measure the nasopharyngeal colonization and bacterial loads in the lung by

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Figure 7. Cross-protection against pneumococcal infection by immunization with UD:R3. (A) Rabbit anti-UD:R3 IgG diluted in PBS was incubated in 96-well plates containing 1μg/mL of immobilized purified R1, UD:R3, UD, or R3. The binding activity of rabbit anti-UD:R3 IgG to R1 was compared with reactivity to UD:R3, UD, and R3. (B) TIGR4 or MNZ11 was incubated with rabbit anti-UD:R3 IgG, followed by staining with FITC-conjugated goat anti-rabbit IgG, and FITC-positive pneumococci were analyzed by flow cytometry. (C)&D) Mice were intranasally (i.n.) immunized with 10 μg of UD:R3 with 1 μg of cholera toxin, followed by challenge with 10^5 CFU of TIGR4. At 15 h postinfection (hpi), the numbers of bacteria in the nasopharynx (C) and lungs (D) were counted. ((E)&F) Mice were i.n. immunized with 10 μg of UD:R3 with 1 μg of cholera toxin, followed by challenge with 10^5 CFU of TIGR4 (E) or D39 (F). Survival of mice were monitored for 14 d. *p < 0.01.
TIGR4. As shown in Fig. 7C and 7D, i.n. immunization with UD:R3 significantly inhibited nasopharyngeal colonization by TIGR4 and bacterial loads in the lungs compared with those of cholera toxin-inoculated control mice. In addition, mice immunized with UD:R3 were challenged with encapsulated TIGR4 (serotype 4) or D39 (serotype 2), and survival was monitored for 14 d (Fig. 7E and 7F). Fourteen days after challenges, all cholera toxin-inoculated control mice (PBS: n = 5) were died, but 80% (WU2) or 60% (D39) of UD:R3 immunized mice were survived, respectively. Taken together, these data indicated that immunization with UD: R3 antigen not only provided effective protection against nasopharyngeal colonization of and invasion by PspK-positive NESp but also provided cross-protection against PspK-negative encapsulated pneumococci.

Discussion

The widespread use of PCV has led to a significant decrease in the colonization and subsequent development of invasive and non-invasive pneumococcal diseases by various serotypes. However, at the same time, concomitant increases in the colonization of NESp and pneumococcal infections with NESp have been reported. Increased colonization of NESp in the nasopharynx is thought to be a major risk factor for AOM, adenoiditis, pneumonia, and bacteremia. Although NESp does not produce polysaccharide capsule, novel protein PspK, which is expressed by NESp, enhances the adherence of NESp to respiratory epithelial cells and may facilitate nasopharyngeal colonization of NESp. Additional studies have shown that PspK functions as a critical pathogenic factor in the development of pneumococcal infections by NESp, such as AOM and chronic adenoiditis. Despite its important role in the pathogenesis of these infections, the underlying molecular mechanisms by which PspK contributes to the pathogenesis of NESp infections have not been clearly defined.

In this study, we found that PspK could promote the adherence of NESp to lung epithelial cells through direct interacts with the epithelial cell surface protein annexin A2. Binding of PspK to annexin A2 was restricted to the R3 domain (AA187–279), further indicating that this was a specific process and was likely to be an essential step for initiating colonization of NESp in the nasopharynx. Annexin A2 is a well-known membrane protein involved in a variety of actin-driven membrane processes, such as phagocytosis, endocytosis, and membrane ruffling and is known to mediate the efficient binding of *Salmonella Typhimurium*, enteropathogenic *Escherichia coli* (EPEC), and *Mycoplasma pneumoniae* toxin to epithelial cells. The predicted amino acid sequence of the R3 domain of PspK has significant homology to the R1 and R2 domains of PspC, a cell wall-associated choline-binding protein. PspC has been shown to bind factor H, vitronectin, and slgA to protect against complement-mediated killing and to enhance its adherence and infection in vitro and in vivo. Interestingly, despite its high homology to the R3 domain, the purified R1 domain of PspC did not bind to annexin A2 (data not shown), indicating that R3 may bind annexin A2 through a distinct mechanism.

Previous studies have shown that although PspK is required for the attachment of NESp to epithelial cells, intracellular invasion of NESp is not affected by PspK deficiency. In our study, however, we found that anti-pneumococcal clearance activity of epithelial cells was significantly downregulated in the cells infected with ΔpspK and that neutralization of PspK binding to annexin A2 using rabbit anti-UD-R3 IgG also significantly inhibited epithelial anti-pneumococcal activities against NESp. These findings suggested that recognition of PspK, likely the R3 domain, by annexin A2 was critical for developing or activating anti-pneumococcal immune responses and subsequent clearance of intra-epithelial pneumococci in epithelial cells. Annexin A2 activation by bacterial antigens is known to modulate Toll-like receptor (TLR) and kinase signaling pathways, which are also known to be activated by pneumococcus in epithelial cells. Thus, further studies of PspK-annexin A2-mediated intracellular signaling pathways are required to improve our understanding of the role of this interaction in the pathogenesis of pneumococcal infections in epithelial cells. Although the mechanisms and downstream signaling pathways involved in this interaction were not fully elucidated in this study, our results provided the first evidence of a direct interaction between gram-positive bacteria and annexin A2 during the process of bacterial adherence and invasion.

Among many pathogenic factors, the polysaccharide capsule is known to be essential for the colonization of pneumococcus in the respiratory tract. Therefore, immunization with PCVs decreases the burden of pneumococcal colonization and invasive diseases caused by the vaccine serotypes. However, emerging independent vaccine serotypes, including NESps, and serotype replacement in the nasopharynx following PCV introduction led us to focus on development of a new generation of protein-based pneumococcal vaccines conferring broad serotype coverage. Previous report suggested that intranasal immunization of the part of PspA protein of pneumococcus was shown to be very effective for preventing encapsulated pneumococcal colonization and infection however, effects against non-encapsulated pneumococci has not been studied. Because nasopharyngeal colonization of pneumococcus is a
prerequisite for pneumococcal infections and because PspK is critical for the nasopharyngeal colonization of NESp, the efficacy of PspK for developing pneumococcal vaccine has been investigated. In this study, the authors found that i.n. immunization with recombinant PspK elicited good protection against nasopharyngeal colonization of not only NESp but the encapsulated strain in mice. In the present study, however, we identified the R3 domain of PspK as the structure responsible for binding to epithelial cells via cell surface annexin A2, and immunization with the R3 or UD:R3 fragment effectively elicited an immune response against nasopharyngeal colonization and subsequent development of AOM and lung infection.

AOM is one of the most common reasons for doctor’s visits and for the use of oral antibiotics in children; however, the development of effective vaccine(s) for OM in children has been a major challenge. Three pathogens, *S. pneumoniae*, *Haemophilus influenzae* (*H. influenzae*), and *Moraxella catarrhalis* (*M. catarrhalis*), are responsible for almost 97% of OM episode. Currently available *H. influenzae* vaccine, *H. influenza* type b conjugate vaccine (Hib), had minimal effect on the incidence of AOM, and no vaccine is available for *M. catarrhalis*. Use of protein conjugated pneumococcal vaccine was extraordinary effective for preventing invasive pneumococcal disease but found to have only limited effect against AOM with about 6% of reduction. This phenomenon was explained by increased in AOM with non-vaccine pneumococcal serotypes and AOM with *H. influenzae*. Therefore, the use of newly developed PCV with more pneumococcal serotypes and conjugated with *H. influenzae* protein D is expected to be more effective against AOM, but it has not been studied yet. Although pneumococcal infection is one of the most frequent causes of AOM, the effectiveness of available pneumococcal vaccines for AOM is not sufficient. Recently, many studies worldwide have reported the increased occurrence of NESp-induced AOM. Therefore, based on this view, our current findings are may provide exciting insights into potential therapeutic strategies for management of this condition.

In summary, our findings demonstrated that NESp expressed the novel protein PspK, which could bind directly to epithelial annexin A2 via its R3 domain, and R3-mediated activation of annexin A2 was required for developing epithelial anti-pneumococcal defense responses. In addition, immunization with R3 or UD:R3 of PspK elicited effective humoral and mucosal immune responses against nasopharyngeal colonization and subsequent development of AOM and pulmonary infection in mice. Furthermore, rabbit anti-UD:R3 IgG bound to the R1 domain of PspC, and thus, immunization with R3 provided protection against PspK-negative encapsulated pneumococcus. Collectively, our findings identified R3 as a novel vaccine candidate for universal pneumococcal vaccine development.

**Materials and methods**

**Reagents**

Purified annexin A2 was obtained from Abcam (Cambridge, MA, USA). All antibodies used, purified keratin, antibiotics, and other reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Bacterial strains and growth conditions**

The bacteria and plasmid DNA used in this study are listed in Table 1. All 68 pneumococcal clinical isolates were collected from Ewha Womans University Mokdong Hospital, Seoul, Korea from 2011 to 2013, and their serotypes were determined by multibead serotyping assays, as described previously by the Bacterial Respiratory Pathogen Reference Laboratory (www.vaccine.uab.edu). Pneumococcal strains were grown in Todd-Hewitt broth (Difco, Franklin Lakes, NJ, USA) supplemented with 0.5% yeast extract (Difco). All pneumococcal mutant strains grew comparably well in vitro (data not shown).

**Table 1. Bacterial strains and plasmid DNAs.**

| Strain or plasmid | Genotype or description | Source |
|-------------------|------------------------|--------|
| *Escherichia coli* | DH5α | F− r− m− 800m•lacZΔM15 | Gibco BRL Novagen |
|                   | BL21 (DE3) | Expression host, inducible T7 RNA polymerase |
| *Streptococcus pneumoniae* | MNZ11b | NESp NCC1, PspK*Δ* |
|                   | MNZ11bΔpspK | ΔpspK |
|                   | TIGR4 | Serotype IV |
| Plasmid | pET28FLAG | Expression vector with FLAG-tag, Kan* |
| pET28FLAG-R3 | Vector for expression of PspC174-287 |
| pET28FLAG-R2 | Vector for expression of PspC327-442 | This study |
| pET28FLAG-R3 | Vector for expression of PspK187-279 | This study |
| pET28FLAG-UD | Vector for expression of PspK131-187 | This study |
| pET28FLAG-UD-R3 | Vector for expression of PspK21-279 | This study |
E. coli strains were grown at 37°C under aeration in Luria broth (LB; Difco), and appropriate concentrations of antibiotics for mutant strains were added to the medium, as required.

**Cloning and expression of PspK fragments**

Genomic DNA was isolated from the pneumococcal MNZ11b strain using a Wizard Genomic DNA purification kit (Promega, Madison, WI, USA), according to the manufacturer’s instructions. Polymerase chain reaction (PCR) primers used for the cloning of PspK and PspC fragments in this study are listed in Table 2. PCR products were purified, digested, and then ligated into pET28-FLAG to express FLAG-tagged versions of PspK and PspC truncates, as follows: PspC-R1 (AA174–287; R1), PspC-R2 (AA327–442; R2), PspK-UD (AA31–187; UD), PspK-UD:R3 (AA31–279; UD:R3), and PspK-R3 (AA187–279; R3). The plasmids encoding FLAG-tagged truncates were then introduced into E. coli BL21(DE3) for overexpression, and recombinant proteins were purified using an Ni-NTA purification system (Promega), according to the manufacturer’s instructions.

**Isolation of membrane proteins from A549 cells**

Membrane proteins from A549 human lung epithelial cells were isolated with a Pierce Cell Surface Protein Isolation Kit (Pierce Biotechnology, Rockford, IL, USA) following the manufacturer’s protocol. Briefly, A549 cells were grown to 90–95% confluence in T75 tissue culture flasks. Cells were labeled with Sulfo-SS-Biotin for 30 min at 4°C, and the reaction was then stopped by adding Quenching Solution. Cells from 4 flasks were harvested into 50-mL conical tubes, and cell pellets were transferred to 1.5-mL microcentrifuge tubes for lysis with a mild lysis buffer. Cell lysates were then collected and bound to NeutrAvidin Agarose. The agarose-bound proteins were released by incubating with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 50 mM dithiothreitol (DTT).

**Far western blotting and LC/LTQ-Orbitrap MS**

Far western blot method, which is widely used for identifying a specific binding partner of specific target proteins, has been used as described previously. The membrane protein extracts of A549 cells were separated by electrophoresis using 2 NuPAGE Bis-Tris PAGE gels (Invitrogen, Carlsbad, CA, USA), followed by staining with Coomassie blue staining solution (Biosesang, Korea) or transfer to nitrocellulose membranes (Pierce Biotechnology). The membranes were treated with a casein-based blocking solution (Sigma-Aldrich) at room temperature and then incubated for 1 h with FLAG-R3 (5 μg/mL) suspended in phosphate-buffered saline (PBS) with 0.05% tween 20 (PBS-T). The membranes were then washed 3 times for 15 min in PBS-T, and bound probe proteins were detected with mouse anti-FLAG monoclonal antibodies (Sigma-Aldrich). Multiple identified lanes on the stained gels were excised and then digested and fragmented using trypsin. The peptide

### Table 2. PCR primers.

| Primers | Sequences | Description |
|---------|-----------|-------------|
| Z3001   | TCTCGAGTTTATTAGCTTG | PspK-R3   |
| Z5001   | AGGCTCGAGAATACGAGCCTGAG | PspK-UD   |
| Z3002   | TCTCGAGTTTATTAGCTTG | PspP-R2   |
| Z5002   | AGGCTCGAGAGAGTACGAGCCTGAG | PspP-R1   |
| Z3011   | TCTCGAGCTTCTTACTCTGCTTTCAG | PspP-UD   |
| Z5011   | AGGCTCGAGAGAGTACGAGCCTGAG | PspP-R1   |
| Z3012   | TCTCGAGCTTCTTACTCTGCTTTCAG | PspP-R2   |

**Note.** Effusion-positive ear/ear inoculated with MNZ11b

### Table 3. Presence of middle ear effusion.

| Day     | PBS     | PsP-KR3 |
|---------|---------|---------|
| Day 1   | 8/8 (100%) | 2/8 (25%) |
| Day 2   | 3/6 (50%)  | 1/6 (16.6%) |
| Day 3   | 3/6 (50%)  | 0/6 (0%)  |

**Note.** Effusion-positive ear/ear inoculated with MNZ11b
sequences of fragmented proteins were analyzed using LC/LTQ-Orbitrap MS (Thermo-Fisher, Waltham, MA, USA).

Analysis of PspK-R3 binding to immobilized annexin A2 and keratin by ELISA

Purified annexin A2 or keratin (0.1 µg/well) was immobilized in 96-well microtiter plates (SPL, Seoul, South Korea) by overnight incubation at 4°C. A casein-based blocking solution (Sigma-Aldrich) was used as a control for non-specific binding. The wells were washed twice with PBS (Intron, Suwon, South Korea) and blocked with 300 µL of a casein-based blocking solution (Sigma-Aldrich) for 1 h at room temperature.56,59 The plates were washed 3 times with PBS-T, and FLAG-R3 (1 µg/well) in PBS-T was added. The plates were then incubated for 1 h at 37°C. Unbound protein was removed by washing with PBS-T, and the plates were incubated with mouse anti-FLAG antibodies diluted 1:4000 in PBS-T for 1 h at 37°C. Wells were washed and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG diluted 1:5000 in PBS-T for 1 h at 37°C. The plates were then washed 5 times with PBS-T, and 100 µL of TMB substrate reagent (BD Biosciences, Franklin Lakes, NJ, USA) was added. When the colors developed, 50 µL of 2 N H₂SO₄ was added, and the absorbance was measured at 450 nm using a Victor X3 light plate reader (Perkin-Elmer, Waltham, MA, USA).

Binding of MNZ11b to immobilized annexin A2

MNZ11b and its isogenic mutant (ΔpspK) was harvested at A₆⁰₀ = 0.5–0.8 by centrifugation and adjusted to a concentration of 10⁸ CFU/ml in PBS. Annexin A2 (10 µg) was immobilized in 96-well microtiter plates as described above, and then incubated with 100 µL of MNZ11b or ΔpspK suspension for 30 min at 37°C. The wells were then washed to remove unbound bacteria, and the ratio of bound bacteria was determined by staining with crystal violet (0.5% v/v), as described previously.23

Mammalian cells and mammalian cell adherence and invasion assay

Human lung type II epithelial cells (A549 cells; ATCC CCL-185) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Big Cabin, OK, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mM l-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO₂ humidified incubator. Bacterial adherence and intra-epithelial invasion assays were performed as described previously.23 In brief, bacteria were grown to mid-log phase and then added to confluent A549 monolayers at a multiplicity of infection (MOI) of 10. After 30 min of incubation, monolayers were washed 6 times with PBS to remove non-adherent bacteria and then lysed. Lysates were then plated on blood agar plates (BAPs) to enumerate the adhered bacteria. For intra-epithelial invasion assays, at 60 min after infection with bacteria, monolayers were treated with gentamycin (200 µg/mL) to remove adhered bacteria for 60 min. After washing 3 times with PBS, monolayers were then lysed and plated on BAPs to enumerate the intracellular bacteria. For inhibition assays, bacteria were pre-treated either with rabbit anti-UD:R3 IgG or rabbit control IgG for 30 min, and bacteria adherence and invasion to epithelial monolayer were measured as described above. In addition, UD, R3, or UD:R3 proteins were pre-treated to epithelial monolayer for 30 min followed by inoculating with NESp, and adherence and invasion of NESp were measured as described above. Adhered and intracellular bacteria were calculated as [recovered CFU/initial inoculum CFU] × 100%.

Mice and animal experiments

All animal experiments were approved by the Committee on the Use and Care of Animals at Korea Atomic Energy Research Institute (KAERI) and were performed according to accepted veterinary standards. Outbred 6- to 8-week-old male CD-1 and inbred 6-week-old male C57BL/6 (B6) mice were purchased from Orient Bio. (Suwon, Korea). Mouse models of nasopharyngeal colonization and AOM have been described previously.21,60 For immunization with recombinant R3 or UD:R3 fragments, mice were immunized twice at 10-day intervals either via the i.n. or i.p. route. For i.n. immunization, mice were i.n. inoculated with 10 µg of R3 or UD:R3 with 1 µg cholera toxin in 50 µL PBS. For i.p. immunization, mice were i.p. inoculated with 10 µg of R3 or UD:R3 in 100 µL of 2% aluminum hydroxide gel (Invivogen, San Diego, CA, USA). At 5 d after the second immunization, mice were challenged with pneumococcus either via the i.n. or transtympanic membrane (t.t.m.) route for nasopharyngeal colonization or AOM, respectively. Fifteen hours after challenge, nasal washes were collected from i.n. challenged mice with 20 µL PBS from each naris, and lungs were dissected and homogenized by plunging in 1.5 mL PBS through a 40-µm mesh strainer. At 1, 2, and 3 d after t.t.m. challenge, bullae of mice were dissected from the skull, and the middle ear cavity was
washed 20 times with 10 μL PBS. Samples were pooled, and bacterial cell numbers were counted by culturing serially diluted nasal washes, lung homogenates, or bullae washes on BAPs. Blood was collected from the tail vein, and PspK-R3-specific IgG, IgM, and IgA were measured by ELISA. BAL fluid was collected from the lungs of mice, and PspK-specific IgA and IgG were measured by ELISA. Formalin-fixed lungs were embedded in paraffin and stained with hematoxylin and eosin (H&E), as described previously.22

**Multiplex immunoassay using luminex**

At 15 hpi, BAL was conducted with 200 μL PBS through the trachea, and the concentrations of cytokines and chemokines in BAL fluid were measured using ProcartaPlex Mouse Cytokine & Chemokine Panel 20-plex kits (eBioscience, San Diego, CA, USA) with a Luminex 200 system according to the manufacturer’s recommendations.

**Flow cytometry assay**

Rabbit anti-UD:R3 IgG was produced from 2 New Zealand white rabbits immunized with recombinant UD:R3 protein. Flow cytometry was used to detect PspK or PspK homolog expression on the surface of pneumococcal clinical isolates, as described previously.42 Briefly, pneumococci were incubated with rabbit anti-UD:R3 IgG at a dilution of 1:100 for 30 min at 4°C. After washing, bound antibodies were detected with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC; Sigma-Aldrich). Stained pneumococci were analyzed using a FACSCalibur (BD Biosciences). If over 50% of gated pneumococci were positive to rabbit anti-UD:R3 IgG, it was considered as a positive strain to rabbit anti-UD:R3 IgG. Bacteria stained with the secondary antibody alone were used as a negative control.

**Data analysis**

Data expressed as means ± standard deviations were compared for statistical significance by paired or unpaired t tests. Differences with p values of less than 0.05 were considered significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Funding**

This study was supported by the Nuclear R&D Program of the Ministry of Science, ICT, & Future Planning (MSIP), Republic of Korea (to S.L.), intramural research promotion grants from Ewha Womans University School of Medicine (to J.H.L.), an RP-grant 2014 from the School of Medicine, Ewha Womans University (to K.H.K.), National Research Foundation of Korea (grant no. NRF-2013R1A1A2007513 to K.H.K., NRF-2015R1A1A1059338 to J.H.L., 2012R1A1A205675 to I.H.P.), and the Ministry of Food and Drug Safety (grant no. 15172MFDS164 to K.H.K.).

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**References**

[1] Zhu F, Hu Y, Li J, Ye Q, Young MM, Jr., Zhou X, Chen Z, Yan B, Liang JZ, Gruber WC, et al. Immunogenicity and safety of 13-valent pneumococcal conjugate vaccine compared with 7-valent pneumococcal conjugate vaccine among healthy infants in China. Pediatr Infect Dis J 2016; 35(9):999-1010; PMID:27254028; https://doi.org/10.1097/INF.000000000001248

[2] Welte T. Pneumococcal conjugate vaccine–equally effective for everyone? Dtsch Arztebl Int 2016; 113(9):137-8. PMID:26987461

[3] van Werkhoven CH, Hollingsworth RC, Huijts SM, Bolkenbaas M, Webber C, Patterson S, Sanders EA, Bonten MJ. Pneumococcal conjugate vaccine herd effects on non-invasive pneumococcal pneumonia in elderly. Vaccine 2016; 34(28):3275-82; PMID:27171754; https://doi.org/10.1016/j.vaccine.2016.05.002

[4] Valente C, Hinds J, Gould KA, Pinto FR, de Lencastre H, Saléo R. Impact of the 13-valent pneumococcal conjugate vaccine on Streptococcus pneumoniae multiple serotype carriage. Vaccine 2016; 34(34):4072-8; PMID:27325351; https://doi.org/10.1016/j.vaccine.2016.06.017

[5] Grant LR, Hammitt LL, O’Brien SE, Jacobs MR, Donaldson C, Weatherholtz RC, Reid R, Santosham M, O’Brien KL. Impact of the 13-valent pneumococcal conjugate vaccine on pneumococcal carriage among American Indians. Pediatr Infect Dis J 2016; 35(8):907-14; PMID:27171679; https://doi.org/10.1097/INF.0000000000001207

[6] Singleton RJ, Hennessy TW, Bulkow LR, Hammitt LL, Zulz T, Hurlburt DA, Butler JC, Rudolph K, Parkinson A. Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA 2007; 297(16):1784-92; PMID:17456820; https://doi.org/10.1001/jama.297.16.1784

[7] Pelton SI, Huot H, Finkelstein JA, Bishop CJ, Hsu KK, Kel-lenberg J, Huang SS, Goldstein R, Hanage WP. Emergence of 19A as virulent and multidrug resistant Pneumococcus in Massachusetts following universal immunization of infants with pneumococcal conjugate vaccine. Pediatr Infect Dis J 2007; 26(6):668-72; PMID:17529860; https://doi.org/10.1097/INF.0b013e31830d9ca

[8] Hicks LA, Harrison LH, Flannery B, Hadler JL, Schaffner W, Craig AS, Jackson D, Thomas A, Beall B, Lynfield R, et al. Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the
United States during the era of widespread PCV7 vaccination, 1998–2004. J Infect Dis 2007; 196(9):1346-54; PMID:17922399; https://doi.org/10.1086/521626

[9] Brueggemann AB, Pai R, Crook DW, Beall B. Vaccine escape recombinants emerge after pneumococcal vaccination in the United States. PLoS Pathog 2007; 3(11): e168; PMID:18020702; https://doi.org/10.1371.journal.ppat.0030168

[10] Keller LE, Robinson DA, McDaniel LS. Nonencapsulated Streptococcus pneumoniae: emergence and pathogenesis. MBio 2016; 7(2):e01792; PMID:27006456; https://doi.org/10.1128/mBio.01792-15

[11] Hilty M, Wüthrich D, Salter SJ, Engel H, Campbell S, Sá-Leão R, de Lancastre H, Hermans P, Sadowy E, Turner P, et al. Global phylogenomic analysis of nonencapsulated Streptococcus pneumoniae reveals a deep-branching classical lineage that is distinct from multiple sporadic lineages. Genome Biol Evol 2014; 6:3281-94; PMID:25480686; https://doi.org/10.1093/gbe/evu263

[12] Norcross EW, Tullos NA, Taylor SD, Sanders ME, Marquart ME. Assessment of Streptococcus pneumoniae capsule in conjunctivitis and keratitis in vivo. Neuraminidase activity increases in nonencapsulated pneumococci following conjunctival infection. Curr Eye Res 2010; 35(9):787-98; PMID:20795860; https://doi.org/10.3109/02713683.2010.492462

[13] Hathaway LJ, Stutzmann Meier P, Battig P, Aebi S, Mülemann K. A homologue of aliB is found in the capsule region of nonencapsulated Streptococcus pneumoniae. J Bacteriol 2004; 186(12):3721-9; PMID:15175285; https://doi.org/10.1128/JB.186.12.3721-3729.2004

[14] Schaffner TO, Hinds J, Stutzmann Meier P, Aebi S, Mülemann K, Hilty M, Hathaway LJ. A point mutation in cpsE renders Streptococcus pneumoniae nonencapsulated and enhances its growth, adherence and competence. BMC Microbiol 2014; 14:210; PMID:25163487; https://doi.org/10.1186/s12866-014-0210-x

[15] Advisory Committee on Immunization Practices. Pneumococcal polysaccharide vaccine. MMWR 1989; 38:64-8; PMID:2492366

[16] Dixit C, Keller LE, Bradshaw JL, Robinson DA, Swiatlo E, McDaniel LS. Nonencapsulated Streptococcus pneumoniae as a cause of chronic adenoiditis. IDCases 2016; 4:56-8; PMID:27144125; https://doi.org/10.1016/j.idcr.2016.04.001

[17] Murrah KA, Pang B, Richardson S, Perez A, Reimche J, King L, Wren J, Swords WE. Nonencapsulated Streptococcus pneumoniae causes otitis media during single-species infection and during polymicrobial infection with nontypeable Haemophilus influenzae. Pathog Dis 2015; 73(5):ftu011 PMID:26014114; PMID:26014114; https://doi.org/10.1093/tempsd/ftu011

[18] Keller LE, Luo X, Thornton JA, Seo KS, Moon BY, Robinson DA, McDaniel LS. Immunization with pneumococcal surface protein K of nonencapsulated Streptococcus pneumoniae provides protection in a mouse model of colonization. Clin Vaccine Immunol 2015; 22:1146-53; PMID:26311246; https://doi.org/10.1128/CVI.00456-15

[19] Keller LE, Friley J, Dixit C, Nahm MH, McDaniel LS. Nonencapsulated Streptococcus pneumoniae cause acute otitis media in the chinchilla that is enhanced by pneumococcal surface protein K. Open Forum Infect Dis 2014; 1(2):ofu037; PMID:25734113; https://doi.org/10.1093/ofid/ofu037

[20] Lofling J, Vimberg V, Battig P, Henriques-Normark B. Cellular interactions by LPxTG-anchored pneumococcal adhesins and their streptococcal homologues. Cell Microbiol 2011; 13(2):186-97; PMID:21199258; https://doi.org/10.1111/j.1462-5822.2010.01560.x

[21] Keller LE, Jones CV, Thornton JA, Sanders ME, Swiatlo E, Nahm MH, Park IH, McDaniel LS. PspK of Streptococcus pneumoniae increases adherence to epithelial cells and enhances nasopharyngeal colonization. Infect Immun 2013; 81(1):173-81; PMID:23115034; https://doi.org/10.1128/IAI.00755-12

[22] Seo HS, Mu R, Kim BJ, Doran KS, Sullam PM. Binding of glycoprotein Srr1 of Streptococcus agalactiae to fibrinogen promotes attachment to brain endothelium and the development of meningitis. PLoS Pathog 2012; 8(10): e1002947; PMID:23055927; https://doi.org/10.1371/journal.ppat.1002947

[23] Wang NY, Patras KA, Seo HS, Cavaco CK, Rosler B, Neely MN, Sullam PM, Doran KS. Group B streptococcal serine-rich repeat proteins promote interaction with fibrinogen and vaginal colonization. J Infect Dis 2014; 210(6):982-91; PMID:24620021; https://doi.org/10.1093/infdis/jiu151

[24] Sillanpää J, Nallapareddy SR, Houston J, Ganesh VK, Bourgogne A, Singh KV, Murray BE, Höök M. A family of fibrinogen-binding MSCRAMMs from Enterococcus faecalis. Microbiology 2009; 155(Pt 7):2390-400; PMID:19389755; https://doi.org/10.1099/mic.0.027821-0

[25] Ganesh VK, Barbhuiya EM, Deivanayagam CC, Le B, Anderson AS, Matsuka YV, Lin SL, Foster TJ, Narayana SV, Höök M. Structural and biochemical characterization of Staphylococcus aureus clumping factor B/ligand interactions. J Biol Chem 2011; 286(29):25963-72; PMID:21543319; https://doi.org/10.1074/jbc.M110.217414

[26] Marchler-Bauer A, Panchenko AR, Shoemaker BA, Thiessen PA, Geer LY, Bryant SH. CDD: a database of domain architectures and their streptococcal homologues. Cell Microbiol 2011; 13(2):186-97; PMID:21199258; https://doi.org/10.1111/j.1462-5822.2010.01560.x

[27] Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 2006; 22(2):195-201; PMID:16301204; https://doi.org/10.1093/bioinformatics/btj770

[28] Biegert A, Mayer C, Remmert M, Soding J, Lupas AN. The MPI Bioinformatics Toolkit for protein sequence analysis. Nucleic Acids Res 2006; 34:W335-9; PMID:16845021; https://doi.org/10.1093/nar/gkl217

[29] Harada T, Ogawa Y, Eguchi M, Shi F, Sato M, Uchida K, Nakayama H, Shimoji Y. Erysipelothrix rhusiopathiae exploits cytokeratin 18-positive epithelial cells of porcine tonsillar crypts as an invasion gateway. Vet Immunol Immunopathol 2013; 153(3-4):260-6; PMID:23601839; https://doi.org/10.1016/j.vetimm.2013.03.013
[31] Tam C, Mun JJ, Evans DJ, Fleiszig SM. Cytokeratins mediate epithelial innate defense through their antimicrobial properties. J Clin Invest 2012; 122(10):3665-77; PMID:23060638; https://doi.org/10.1172/JCI64416

[32] Batchelor M, Guignot J, Patel A, Cummings N, Cleary J, Knutton S, Holden DW, Connerton I, Frankel G. Involvement of the intermediate filament protein cytokeratin-18 in actin pedestal formation during EPEC infection. EMBO Rep 2004; 5(1):104-10; PMID:14710194; https://doi.org/10.1038/sj.emboj.7400038

[33] Jolly C, Winfree S, Hansen B, Steele-Mortimer O. The Annexin A2/p11 complex is required for efficient invasion of Salmonella typhimurium in epithelial cells. Cell Microbiol 2014; 16(1):64-77; PMID:23931152; https://doi.org/10.1111/cmi.12180

[34] Somarajan SR, Al-Asadi F, Ramasamy K, Pandranjki L, Baseman JB, Kannan TR. Annexin A2 mediates Mycoplasma pneumoniae community-acquired respiratory distress syndrome toxin binding to eukaryotic cells. MBio 2014; 5(4):e01497-14; PMID:25139904; https://doi.org/10.1128/mBio.01497-14

[35] Zhang S, Yu M, Guo Q, Li R, Li G, Tan S, Li X, Wei Y, Wu M. Annexin A2 binds to endosomes and negatively regulates TLR4-triggered inflammatory responses via the TRAM-TRIF pathway. Sci Rep 2015; 5:15859; PMID:26527544; https://doi.org/10.1038/srep15859

[36] Li R, Tan S, Yu M, Junct MC, Zhang S, Wu M. Annexin A2 regulates autophagy in Pseudomonas aeruginosa infection through the Akt1-mTOR-ULK1/2 signaling pathway. J Immunol 2015; 195(8):3901-11; PMID:26371245; https://doi.org/10.4049/jimmunol.1500967

[37] Fortanier AG, Venekamp RP, Boonacker CW, Hak E, Schilder AG, Sanders EA, Damaoisiaux RA. Pneumococcal conjugate vaccines for preventing otitis media. Cochrane Database Syst Rev 2014; 4:CD001480.

[38] Ref K. Ing J, Knutto S, Hulten KG. Characterization of nontypeable and atypical Streptococcus pneumoniae pediatric isolates from 1994 to 2010. J Clin Microbiol 2012; 50(4):1326-30; PMID:22238440; https://doi.org/10.1128/JCM.05182-11

[39] Park IH, Gono KA, Sherwood LK, Nahm MH, Beall B. Population-based analysis of invasive nontypeable pneumococci reveals that most have defective capsule synthesis genes. PLoS One 2014; 9(5):e92845; PMID:24831650; https://doi.org/10.1371/journal.pone.0092845

[40] Lacapa R, Bliss SJ, Laurzeler-Hinton F, Eagle KJ, McGinty DJ, Parkinson AJ, Santosham M, Craig MJ, O’Brien KL. Changing epidemiology of invasive pneumococcal disease among White Mountain Apache persons in the era of the pneumococcal conjugate vaccine. Clin Infect Dis 2008; 47(4):476-84; PMID:18627249; https://doi.org/10.1086/590001

[41] Okade H, Funatsu T, Eto M, Furuya Y, Mizunaga S, Nomura N, Mitsuyama J, Yamagishi Y, Mikamo H. Impact of the pneumococcal conjugate vaccine on serotype distribution and susceptibility trends of pediatric non-invasive Streptococcus pneumoniae isolates in Tokai, Japan over a 5-year period. J Infect Chemother 2014; 20(7):423-8; PMID:24802765; https://doi.org/10.1016/j.jiac.2014.03.010

[42] Park IH, Kim KH, Andrade AL, Briles DE, McDaniel LS, Nahm MH. Nontypeable pneumococci can be divided into multiple cps types, including one type expressing the novel gene pspK. MBio 2012; 3(3):e00035-12; PMID:22532557; https://doi.org/10.1128/mBio.00035-12

[43] Hajjar KA. The biology of annexin A2: from vascular fibrinolysis to innate immunity. Trans Am Clin Climatol Assoc 2015; 126:144-55. PMID:26330668

[44] Briles DE, Hollingshead SK, Slatio E, Brooks-Walter A, Slatia I, Virolainen A, McDaniel LS, Benton KA, White P, Prellner K, et al. PspA and PspC: their potential for use as pneumococcal vaccines. MicrobDrug Resist in press 1997; 3:401-08.

[45] Voss S, Hallstrom T, Saleh M, Burchhardt G, Pribyl T, Singh B, Riesbeck K, Zipfel PF, Hammerschmidt S. The choline-binding protein PspC of Streptococcus pneumoniae interacts with the C-terminal heparin-binding domain of vitronectin. J Biol Chem 2013; 288:15614-27; PMID:23603906; https://doi.org/10.1074/jbc.M112.443507

[46] Dave S, Brooks-Walter A, Pangburn MK, McDaniel LS. PspC, a pneumococcal surface protein, binds human factor H. Infect Immun 2001; 69:3435-7; PMID:11292770; https://doi.org/10.1128/IAI.69.5.3435-3437.2001

[47] Yuste J, Khandavilli S, Ansari N, Muttardi K, Ismail L, Baseman JB. Annexin A2 mediates membrane fusion and atypical inflammatory responses via the Akt1-mTOR-ULK1/2 signaling pathway. J Immunol 2012; 47(4):e00035-12; PMID:22532557; https://doi.org/10.1128/IAI.69.5.3435-3437.2001

[48] Felix F, Gomes GA, Cabral GA, Cordeiro JR, Tomita S. The in vivo and in vitro Roles of PspC on complement-mediated immunity to Streptococcus pneumoniae vary with strain background and capsular serotype. Infect Immun 2010; 78:283-92; PMID:19884335; https://doi.org/10.1128/IAI.69.5.3435-3437.2001

[49] Yuste J, Khandavilli S, Ansari N, Muttardi K, Ismail L, Hyams C, Weiser J, Mitchell T, Brown JS. The effects of PspC on complement-mediated immunity to Streptococcus pneumoniae, in binding human secretory IgA and factor H. J Immunol 2004; 173:471-7; PMID:15210807; https://doi.org/10.4049/jimmunol.173.1.471

[50] Shin SG KS, Lim JH. The in vivo and in vitro Roles of Epithelial Pattern Recognition Receptors in Pneumococcal Infections. J Bacteriol Virol 2014; 44:121-32; https://doi.org/10.1128/JBV.2014.44.121

[51] Lim JH, Jono H, Komatsu K, Woo CH, Lee J, Miyata M, Matsuno T, Xu X, Huang Y, Zhang W, et al. CYLD negatively regulates transforming growth factor-beta-signalling via deubiquitinating Akt. Nat Commun 2012; 3:771; PMID:22491319; https://doi.org/10.1038/ncomms1776

[52] Kuipers K, Daleker-Schermerhorn MH, Jong WS, ten Hagen-Jongman CM, van Opzeeland F, Simonetti E, Lurink J, de Jonge MI. Salmonella outer membrane vesicles displaying high densities of pneumococcal antigen at the surface offer protection against colonization. Vaccine 2015; 33:2022-9; PMID:25776921; https://doi.org/10.1016/j.vaccine.2015.03.010

[53] Felix S, Gomes GA, Cabral GA, Cordeiro JR, Tomita S. The role of new vaccines in the prevention of otitis media. Braz J Otorhinolaryngol 2008; 74:613-6; https://doi.org/10.1016/S1808-8694(15)30612-1

[54] American Academy of Pediatrics Subcommittee on Management of Acute Otitis Media. Diagnosis and management of acute otitis media. Pediatrics 2004; 113:1451-65; PMID:15121972; https://doi.org/10.1542/peds.113.5.1451
[54] Agrawal A, Murphy TF. *Haemophilus influenzae* infections in the *H. influenzae* type b conjugate vaccine era. J Clin Microbiol 2011; 49:3728-32; PMID:21900515; https://doi.org/10.1128/JCM.05476-11

[55] Yu J, Lin J, Benjamin WH, Jr., Waites KB, Lee CH, Nahm MH. Rapid multiplex assay for serotyping pneumococci with monoclonal and polyclonal antibodies. J Clin Microbiol 2005; 43:156-62; PMID:15634965; https://doi.org/10.1128/JCM.43.1.156-162.2005

[56] Seo HS, Xiong YQ, Mitchell J, Seepersaud R, Bayer AS, Sullam PM. Bacteriophage lysin mediates the binding of *Streptococcus mitis* to human platelets through interaction with fibrinogen. PLoS Pathog 2010; 6:e1001047; PMID:20714354; https://doi.org/10.1371/journal.ppat.1001047

[57] Sanchez CJ, Shivshankar P, Stol K, Trakhtenbroit S, Sullam PM, Sauer K, et al. The pneumococcal serine-rich repeat protein is an intra-species bacterial adhesin that promotes bacterial aggregation in vivo and in biofilms. PLoS Pathog 2010; 6:e1001044; PMID:20714350; https://doi.org/10.1371/journal.ppat.1001044

[58] Takamatsu D, Bensing BA, Cheng H, Jarvis GA, Siboo IR, Lopez JA, Griffiss JM, Sullam PM. Binding of the *Streptococcus gordonii* surface glycoproteins GspB and Hsa to specific carbohydrate structures on platelet membrane glycoprotein Ibalpha. Mol Microbiol 2005; 58:380-92; PMID:16194227; https://doi.org/10.1111/j.1365-2958.2005.04830.x

[59] Seo HS, Sullam PM. Characterization of the fibrinogen binding domain of bacteriophage lysin from *Streptococcus mitis*. Infect Immun 2011; 79:3518-26; PMID:21690235; https://doi.org/10.1128/IAI.05088-11

[60] Lee J KK, Lee BC, Lim JH, Jono H, Xu H, Kai H, Zhang ZJ, Yan C, Li JD. Phosphodiesterase 4B mediates extracellular signal-regulated kinase-dependent up-regulation of mucin MUC5AC protein by *Streptococcus pneumoniae* by inhibiting cAMP-protein kinase A-dependent MKP-1 phosphatase pathway. J Biol Chem 2012; 287:22799-811.

[61] Trzcinski K, Thompson CM, Lipsitch M. Construction of otherwise isogenic serotype 6B, 7F, 14, and 19F capsular variants of *Streptococcus pneumoniae* strain TIGR4. Appl Environ Microbiol 2003; 69:7364-70; PMID:14660386; https://doi.org/10.1128/AEM.69.12.7364-7370.2003