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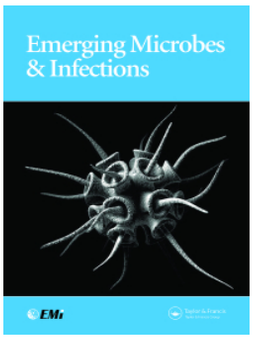
Publisher's version / Version de l'éditeur:
https://doi.org/10.1080/22221751.2023.2272656
Emerging Microbes & Infections, 12, 2, 2023-10-26



Emerging Microbes & Infections

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/temi20

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To cite this article: Fang-Feng Chiu, Ling-Ling Tu, Wangxue Chen, Hongyan Zhou, Bing-Sin Liu, Shih-Jen Liu & Chih-Hsiang Leng (2023) A broad-spectrum pneumococcal vaccine induces mucosal immunity and protects against lethal *Streptococcus pneumoniae* challenge, Emerging Microbes & Infections, 12:2, 2272656, DOI: 10.1080/22221751.2023.2272656

To link to this article: https://doi.org/10.1080/22221751.2023.2272656

Emerging Microbes & Infections
2023, VOL. 12, 2272656 (11 pages)
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A broad-spectrum pneumococcal vaccine induces mucosal immunity and protects against lethal *Streptococcus pneumoniae* challenge

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ABSTRACT

Pneumococcal disease is a major threat to public health globally, impacting individuals across all age groups, particularly infants and elderly individuals. The use of current vaccines has led to unintended consequences, including serotype replacement, leading to a need for a new approach to combat pneumococcal disease. A promising solution is the development of a broad-spectrum pneumococcal vaccine. In this study, we present the development of a broad-spectrum protein-based pneumococcal vaccine that contains three pneumococcal virulence factors: rlipo-PsaA (lipidated form), rPspAΔC (truncated form), and rPspCΔC (truncated form). Intranasal immunization with rlipo-PsaA, rPspAΔC, and rPspCΔC (LAAC) resulted in significantly higher IgG titres than those induced by administration of nonlipidated rPsaA, rPspAΔC, and rPspCΔC (AAC). Furthermore, LAAC immunization induced the production of higher IgA titres in vaginal washes, feces, and sera in mice, indicating that LAAC can induce systemic mucosal immunity. In addition, administration of LAAC also induced Th1/Th17-biased immune responses and promoted opsonic phagocytosis of *Streptococcus pneumoniae* strains of various serotypes, implying that the immunogenicity of LAAC immunization provides a protective effect against pneumococcal infection. Importantly, challenge data showed that the LAAC-immunized mice had a reduced bacterial load not only for several serotypes of the 13-valent conjugate pneumococcal vaccine (PCV13) but also for selected non-PCV13 serotypes. Consistently, LAAC immunization increased the survival rate of mice after bacterial challenge with both PCV13 and non-PCV13 serotypes. In conclusion, our protein-based pneumococcal vaccine provides protective effects against a broad spectrum of *Streptococcus pneumoniae* serotypes.

ARTICLE HISTORY Received 13 July 2023; Revised 11 September 2023; Accepted 15 October 2023

Keywords Pneumococcal vaccine; lipoprotein; opsonophagocytosis; *Streptococcus pneumoniae*; mucosal immunity

Introduction

Pneumonia is a leading cause of death in children under five years old worldwide and was responsible for 15% of all deaths in this age group in 2017. Among the different types of pneumonia in children, *Streptococcus pneumoniae* (*S. pneumoniae*) bacterial pneumonia is the most common [1]. In addition, pneumonia is a significant cause of morbidity and mortality in elderly individuals [2]. Currently, there are two types of vaccines available for preventing pneumococcal infections in humans: the pneumococcal conjugate vaccine (PCV) and the pneumococcal polysaccharide vaccine (PPV). The World Health Organization recommends incorporating the PCV into the routine infant immunization schedule worldwide [3]. Currently, the composition of the PCV administered (PCV13, PCV15, and PCV20) varies depending on an individual's age and medical history [4]. While there are approximately 100 serotypes of *S. pneumoniae*, only a limited number of them are available in current pneumococcal vaccines [5, 6]. However, the use of current vaccines in immunization programmes has led to a rising incidence of infection by nonvaccine serotypes [6, 7]. The replacement of vaccine serotypes with nonvaccine serotypes is a significant public health issue, highlighting the critical and unmet medical need for the development of broad-spectrum pneumococcal vaccines.

In addition to polysaccharide-based pneumococcal vaccines, there is significant interest in developing novel vaccines using pneumococcal virulence proteins due to the highly conserved nature of these factors in

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Supplemental data for this article can be accessed http://doi.org/10.1080/22221751.2023.2272656.

most pneumococcal strains. These proteins offer the potential for serotype-independent protection against pneumococcal disease [8, 9]. However, selecting suitable antigens for vaccine development is crucial, and promising vaccine candidates may be identified from among pathogenesis-related proteins including those in the pneumococcal histidine triad protein (Pht) family, pneumococcal surface protein A (PspA), pneumococcal choline-binding protein A (PcpA), or virulence factors such as pneumolysin [10]. PlyD1, a detoxified pneumolysin, in combination with other antigens and PhtD formulated with other compositions have been investigated in phase 2 clinical studies. However, the results indicate that these formulations do not offer any additional protection against infant pneumococcal nasopharyngeal carriage [11, 12]. Therefore, in addition to selecting antigens, ensuring sufficient pulmonary mucosal immunity is induced and that Th1/Th17-biased immune responses are promoted by utilizing adjuvants or immunopotentiators may play a critical role in the prevention of pneumococcal infection [13, 14].

In our previous studies, we observed that recombinant lipoproteins containing intrinsic toll-like 2 (TLR2) activity enhanced both humoral and cellular immunity against infectious diseases [15, 16]. Additionally, these lipoproteins also displayed promising antitumour activity [17, 18]. Considering the findings from other research groups, which suggested that TLR2 signalling could improve IgA antibody responses [19, 20], we further explored the possibility of this approach to enhance mucosal immunity against pneumococcal infection. In this report, we have developed a pneumococcal vaccine candidate that combines lipidated PsaA with nonlipidated truncated PspA and PspC. Pneumococcal surface adhesin A (PsaA) is a lipoprotein found in *S. pneumoniae* isolates of nearly all serotypes [21]. Intranasal immunization with PspA along with an adjuvant has been shown to protect mice from pneumococcal infections [22], and PspC has been found to provide protection against *S. pneumoniae* challenge [23]. To demonstrate the broad-spectrum protection offered by our vaccine candidate, we compared its protective effects against PCV13 serotypes and non-PCV13 serotypes.

Results

Production and characterization of the recombinant pneumococcal proteins

The coding sequences of rPspAΔC (deletion of the choline-binding domain), rPspCΔC (deletion of the choline-binding domain), rPsaA (without the N-terminal lipidation signal sequence) and rlipo-PsaA were cloned into pET-22b, and expression was controlled by the T7 promoter. Each of these recombinant proteins contained a His Tag of six histidines at their C-termini. These constructs are schematically depicted in Figure 1A. The $PspA_{1\text{-}300}$, $PspC_{1\text{-}449}$ and $PsaA_{21\text{-}309}$ gene plasmids were transformed into the *Escherichia coli* strain BL21star (DE3) and expressed following induction with isopropyl β-D-thiogalactoside (IPTG). The rPspAΔC, rPspCΔC, and rPsaA proteins were purified from the clarified cell lysate using immobilized metal affinity chromatography (IMAC). The recombinant proteins were analyzed by SDS–PAGE and detected with anti-His Tag antibodies (Figure 1B and C). The apparent molecular masses of rPsaA, rPspAΔC, and rPspCΔC were 38, 60, and 70 kDa, respectively. The full-length PsaA gene was transformed into the *E. coli* strain C43(DE3) to produce the lipoprotein recombinant lipidated PsaA (rlipo-PsaA). Purified rlipo-PsaA was also analyzed by SDS-PAGE and immunoblotting, with an apparent molecular mass of 33 kDa (Figure 1B and C).

The tryptic fragments of rlipo-PsaA were analyzed using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Approximately 70% of the tryptic fragments from rlipo-PsaA were detected, indicating a significant match with rlipo-PsaA tryptic fragments (Figure S1A). Subsequently, we measured the N-terminal fragments of rlipo-PsaA, whose structure is N-acyl-S-diacylglycerol cysteine-Ala-Ser-Gly-Lys. Previously, we discovered that the molecular mass of N-acyl-S-diacylglycerol cysteine is 904.71 Da [24]. By adding the theoretical molecular mass of -Ala-Ser-Gly-Lys, which is 361.40 Da, we therefore determined that the theoretical molecular mass of N-acyl-S-diacylglycerol cysteine-Ala-Ser-Gly-Lys is 1266.11 Da. We used mass spectrometry to analyze the N-terminal fragments of rlipo-PsaA and identified the major peak at m/z 1265.976 (Figure S1B). The mass error is 106 ppm. Based on this, we identified that the N-terminal sequence of rlipo-PsaA is lipidated. To accurately reflect the immune responses of purified antigens, lipopolysaccharide (LPS) was removed from all recombinant proteins during purification (less than 30 EU/mg each protein).

Activation of murine bone marrow-derived dendritic cells by rlipo-PsaA

Since the activation of antigen-presenting cells plays a crucial role in enhancing immune responses, we investigated the capability of lipidated rlipo-PsaA to activate bone marrow-derived dendritic cells (BMDCs). BMDCs were stimulated with either rlipo-PsaA or rPsaA recombinant antigens, and the secretion of IL-12p70, IL-12p40, IL-6, and TNF-α was measured. To eliminate the impact of residual LPS in the recombinant proteins, polymyxin B (PMB) was used to block the effect of LPS. The results showed that rlipo-PsaA effectively stimulated the secretion of IL-12p70, IL-12p40, IL-6, and TNF-α from BMDCs, while rPsaA

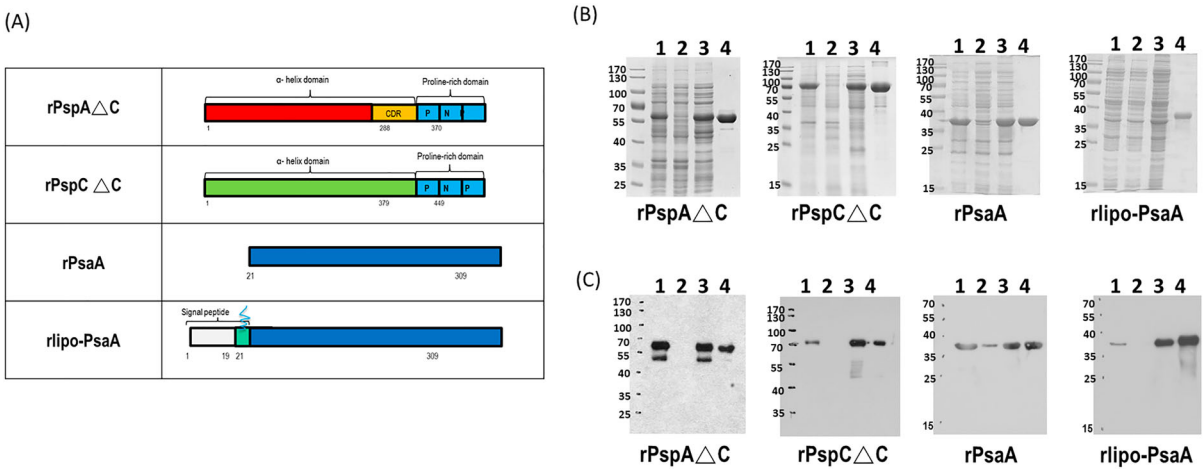

Figure 1. Construction and production of rPspAΔC, rPspCΔC, rPsaA, and rlipo-PsaA. (A) The coding sequences of rPspAΔC, (deletion of the choline-binding domain), rPspCΔC (deletion of the choline-binding domain), rPsaA (without the N-terminal lipidation signal sequence) and rlipo-PsaA (full-length containing the N-terminal lipidation signal sequence) were cloned into pET-22b, and expression was controlled by the T7 promoter. The respective amino acid sequence positions for rPspAΔC, rPspCΔC, rPsaA, and rlipo-PsaA are also provided. (B) The proteins rPspAΔC, rPspCΔC, rPsaA, and rlipo-PsaA were produced in *E. coli* by inducing cultures with isopropyl β-D-thiogalactoside (IPTG). The production of each protein was monitored using 12%, 15%, 15%, and 15% reducing SDS-PAGE, respectively, followed by staining with Coomassie Blue. The gel analysis revealed the following lanes: Lane 1 - IPTG induction, Lane 2 - non-IPTG induction, Lane 3 - protein extraction fraction, and Lane 4 - purified protein for each recombinant protein. (C) The protein of interest was subjected to immunoblotting analysis using anti-His Tag antibodies. The resulting blot showed distinct bands in the following lanes: Lane 1 - IPTG induction, Lane 2 - non-IPTG induction, Lane 3 - protein extraction fraction, and Lane 4 - purified protein for each recombinant protein.

did not. Additionally, PMB was used to block the potential impact of residual LPS in the recombinant proteins but did not affect the activation by rlipo-PsaA (Figure 2). These results demonstrate that rlipo-PsaA has intrinsic adjuvant activity that can activate BMDCs, indicating that rlipo-PsaA can enhance immune responses.

Induction of humoral immunity in mice by the vaccine candidate

Our previous research has indicated that antibody titres can be increased through immunization with lipoproteins without the use of adjuvants [25,26]. In this study, we aimed to determine whether the enhancement of antibody titres could also be observed when nonlipidated pneumococcal antigens were mixed with lipoproteins. We designed a pneumococcal vaccine candidate that comprised rlipo-PsaA, rPspAΔC, and rPspCΔC (LAAC). We used a mixture of nonlipidated proteins, rPsaA, rPspAΔC, and rPspCΔC (AAC), as a comparison. Two doses of the pneumococcal vaccine candidate (10 μg of each protein) were administered intranasally at a two-week interval. The antibody titres against rPsaA, rPspAΔC, and rPspCΔC were then measured. Our results showed that the antibody titres in the LAAC group were significantly higher than those in the AAC group (Figure 3A, B, and C). This result suggests that the antibody titres against nonlipidated antigens can be increased when the antigens are mixed with lipoprotein. The antibody isotypes were subsequently examined to assess the Th1 and Th2 immune responses. The results showed that LAAC immunization elicited the production of high levels of both IgG1 and IgG2a antibodies, whereas AAC immunization yielded minimal IgG1 levels and no IgG2a antibodies (Figure 3D). These results imply that LAAC immunization potentially promotes Th1-dominant immune reactions. Further details regarding T-cell responses are provided in a forthcoming section.

The significant role of mucosal IgA in guarding against pneumococcal infection and *S. pneumoniae* colonization led us to investigate the production of mucosal IgA [27,28]. To determine the ability of LAAC immunization to induce mucosal IgA production, the levels of specific IgA antibodies were analyzed in serum, vaginal wash, and fecal samples collected from immunized mice. The results demonstrated that compared to AAC immunization, LAAC immunization resulted in a more significant increase in IgA antibody levels against PsaA, rPspAΔC, and rPspCΔC in all three types of samples (serum, vaginal wash, and feces) (Figure 4). These findings suggest that the LAAC immunization approach effectively stimulates systemic production of mucosal-specific IgA, which may contribute to reducing the risk of pneumococcal infection and *S. pneumoniae* colonization.

Stimulation of cellular immunity in mice

To further validate that the LAAC vaccine candidate triggers Th1/Th17-biased immune responses, we conducted an experiment to analyze the cytokine profiles.

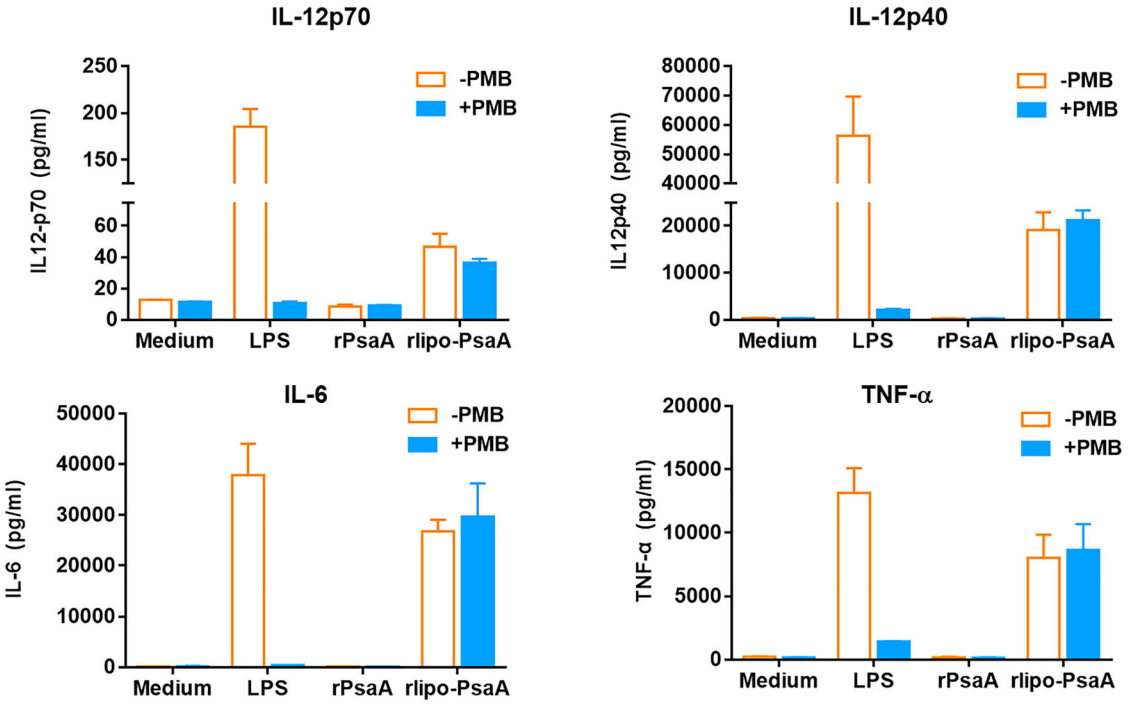

Figure 2. Activation of bone marrow-derived dendritic cells by rlipo-PsaA. Bone marrow-derived dendritic cells (5×10^5/ml cells) were stimulated with medium, LPS (0.1 μg/ml), rPsaA (50 nM), or rlipo-psaA (50 nM) in the presence or absence of 20 μg/ml polymyxin B (PMB). After a 24-hr incubation, the supernatants were harvested and analyzed for IL-12p70, IL-12p40, IL-6 and TNF-α production by ELISA. Significance was determined using Student's t test. The data represent the mean ± standard deviation of triplicate samples.

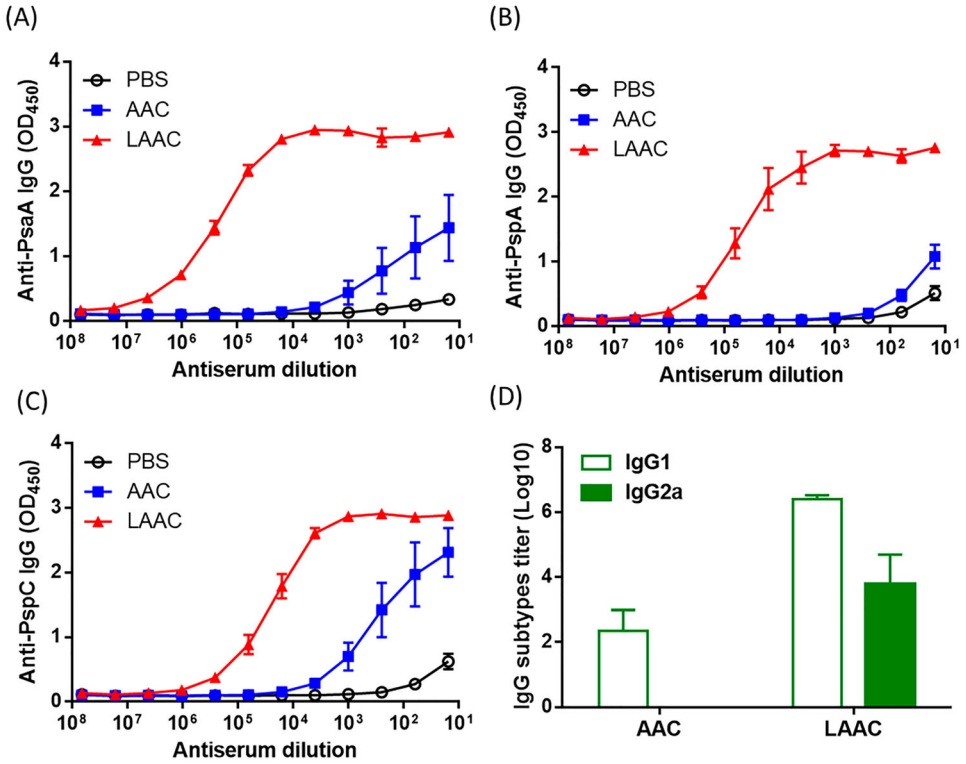

Figure 3. Immunization with the vaccine candidate LAAC boosted antigen-specific antibody production and induced Th1-biased immune responses. Groups of mice, each consisting of 6 individuals, were subjected to different administrations: rlipo-PsaA/rPspAΔC/rPspCΔC (LAAC), rPsaA/rPspAΔC/rPspCΔC (AAC), and PBS. Serum samples were collected from each mouse group one week after the final immunization. The collected sera were then diluted and added to plates coated with the corresponding recombinant proteins. The resulting titration curves are shown for rPspAΔC (A), rPspCΔC (B), and rPsaA (C). As a control, mice were injected with PBS alone. The specific antibody titres were significantly higher in the LAAC immunized mice. In addition, the sera were diluted with rPsaA protein to analyze the B-cell subtype. The IgG2a/IgG1 titres indicated a Th1-biased immune response in the LAAC group, as indicated in (D).

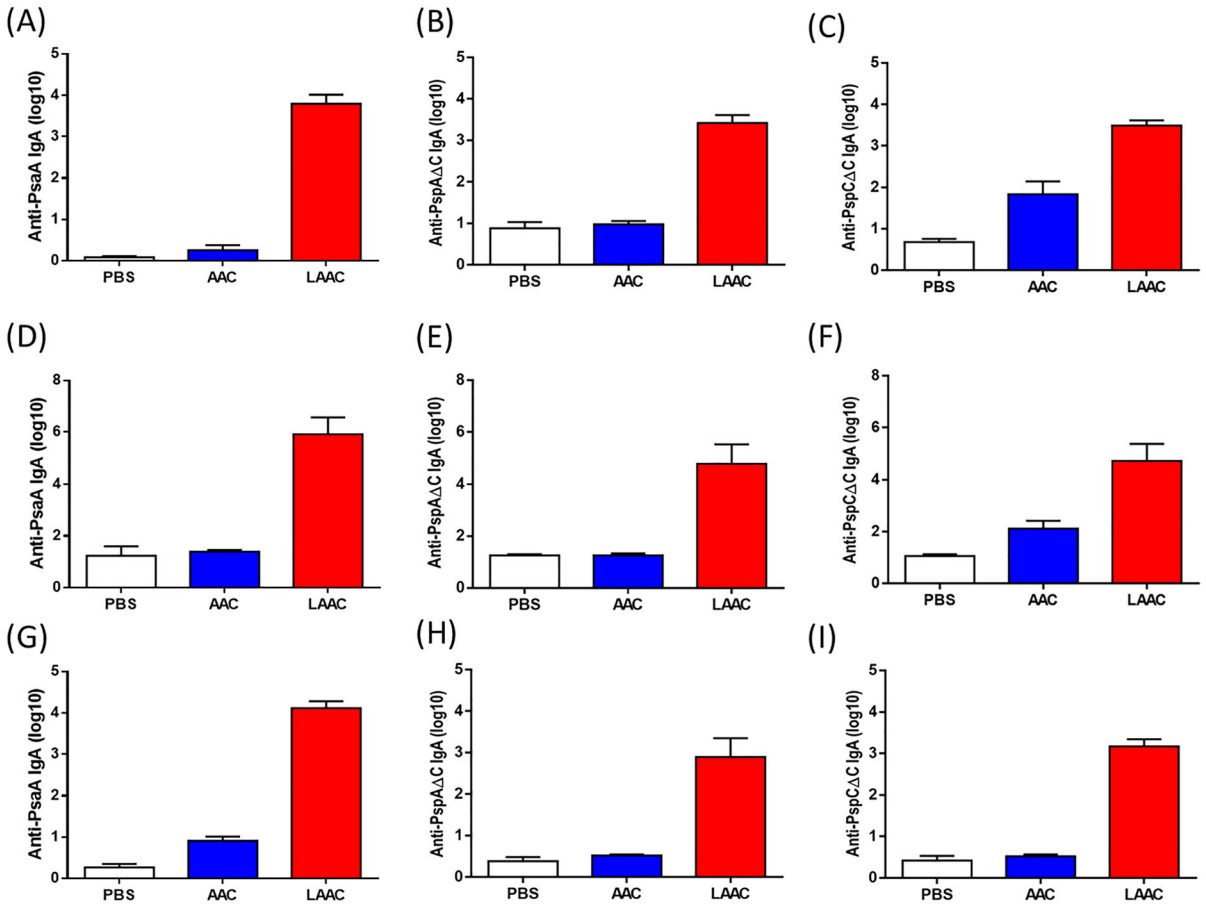

Figure 4. Immunization with the vaccine candidate LAAC enhanced systemic mucosal IgA antibody production. Systemic mucosal immunity was induced by the administration of LAAC. A total of six mice were intranasally (IN) administered PBS, AAC, or LAAC. Each mouse received 30 µg of protein three times at two-week intervals. Vaginal wash, fecal, and serum samples were collected prior to the infection challenge. The vaginal wash was obtained by flushing with 50 µl of PBS. Fresh fecal pellets were collected and stored at −20°C until analysis. Fecal suspensions (10%) were prepared and diluted in PBS containing 0.04% sodium azide and 2% FBS. The samples were centrifuged at 13,000 rpm for 10 min, and the resulting supernatants were used for analysis. Specific IgA antibodies against rPsaA, rPspAΔC, and rPspCΔC were measured in the vaginal wash (A, B, C), feces (D, E, F), and serum (H, I, J) of the mice. The data are presented as the mean ± standard deviation of the samples (n = 6).

Mice were given two intranasal immunizations with either LAAC or AAC at three-week intervals. Seven days after the final immunization, splenocytes were collected and stimulated with three different antigens (rPsaA, rPspAΔC, and rPspCΔC) for three days. Analysis of the cytokine profile was then performed on the collected supernatant using a multiplex immunoassay for 36 cytokines. The results showed that the secreted levels of IFN-γ, a Th1-related cytokine, were several hundred to thousand times higher in the LAAC-immunized mice than in the AAC-immunized mice (Figure 5A). The same trend was observed for other Th1-related cytokines, such as IL-12p70, IL-18, TNF-α, and GM-CSF (Figure S2). In addition, the secreted levels of IL-17, a Th17-related cytokine, were also several hundred to thousand times higher in the LAAC-immunized mice than in the AAC-immunized mice (Figure 5B). Other Th17-related cytokines, such as IL-1β, IL-6, and IL-22, also showed a similar pattern (Figure S3). However, the secreted levels of IL-5 were similar between the two groups of mice (Figure 5C). These findings show that administration of LAAC induces a Th1/Th17-biased immune response, which improves resistance against bacterial infections [29].

Generation of functional immunity against S. pneumoniae infection in mice

The opsonic phagocytosis assay simulates the *in vivo* mechanism of antibody-mediated protection and provides a more accurate representation of the protective effect of vaccine-generated antibodies [30]. To assess the effectiveness of vaccines, opsonic phagocytosis assays were performed using various serotypes of *S. pneumoniae*. The sera from mice immunized with LAAC or AAC were mixed with fluorescence-labeled *S. pneumoniae* for one hour, and then human polymorphonuclear neutrophils and complement were added to enable phagocytosis of the bacteria. As shown in Figure 6, it is evident that the sera obtained from mice immunized with LAAC produced a remarkable increase in phagocytosis of *S. pneumoniae* serotypes 23F, 2 (strain D39), and 15B compared to the

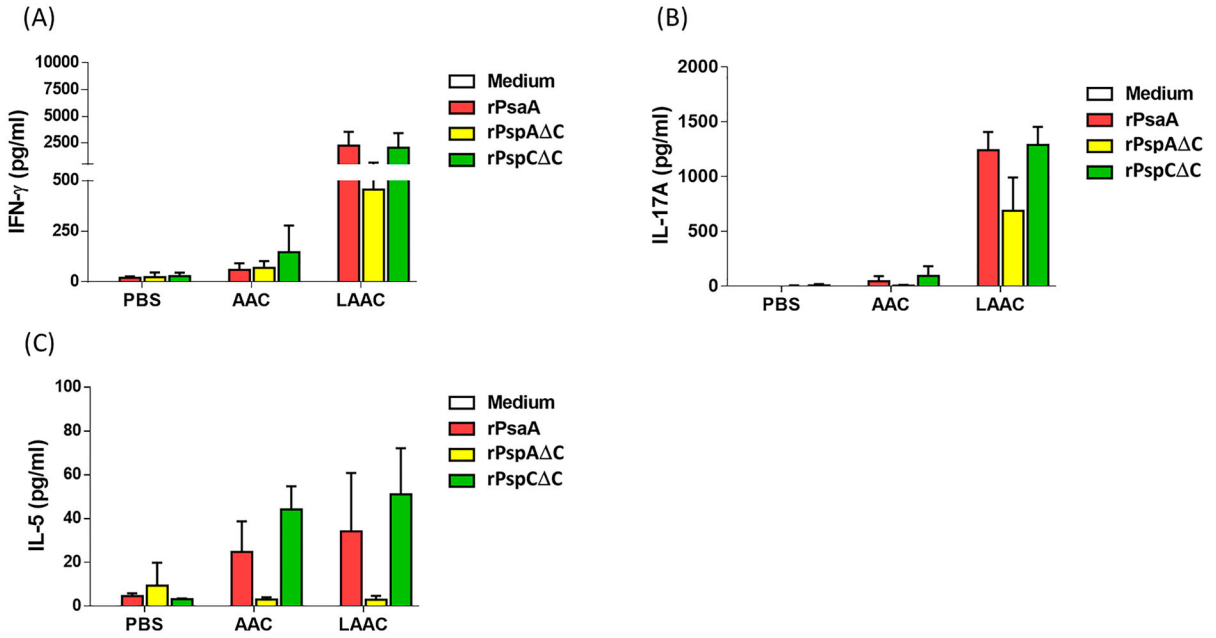

Figure 5. Immunization with the vaccine candidate LAAC induced Th1/Th17-biased immune responses. Lymphocytes were isolated from the spleen of immunized CD1 mice and cultured in 96-well plates at a density of 2×10^5 cells per well. Subsequently, these cells were stimulated with rPsaA, rPspAΔC, or rPspCΔC at a concentration of 10 μg/ml for 72 h. The resulting data represent the mean ± standard deviation of triplicate samples. Supernatants were collected, and the levels of various cytokines, including IFN-g, IL-12p70, IL-5, IL-6, TNF-α, GM-CSF, IL-18, IL-17A, and IL-22, were assessed using ProcartaPlex immunoassays. The cytokines associated with Th1, Th17, and Th2 responses are indicated as (A), (B), and (C), respectively. The reported data reflect the mean value ± standard deviation of three samples (n = 3).

sera obtained from mice immunized with AAC. The phagocytosis rates were significantly higher in the LAAC group than in the AAC group, with 85% versus 8% for serotype 23F, 40% versus 18% for serotype 2, and 12% versus 5% for serotype 15B. The results indicate that LAAC immunization results in the production of functional antibodies that enhance opsonic phagocytosis.

The pneumococcal conjugate vaccine PCV13 was employed as a benchmark to assess the protective abilities of the vaccine candidate *in vivo*. Concurrently, we conducted validation assessments for the hemolysis pattern and confirmed bacterial counts at each challenge study. Mice were administered two doses of PCV13 before being intratracheally challenged with either PCV13 serotypes (6A, 19A, and 23F) or non-PCV13 serotypes (2 and 35B) of *S. pneumoniae*. A bronchoalveolar lavage fluid (BALF) sample was examined to determine the bacterial load 24 h after the challenge. The LAAC group showed decreased bacterial levels for both PCV13 serotypes and non-PCV13 serotypes, while the AAC group did not. Moreover, in the PCV13 vaccine group, there was no significant decrease in bacterial load observed in

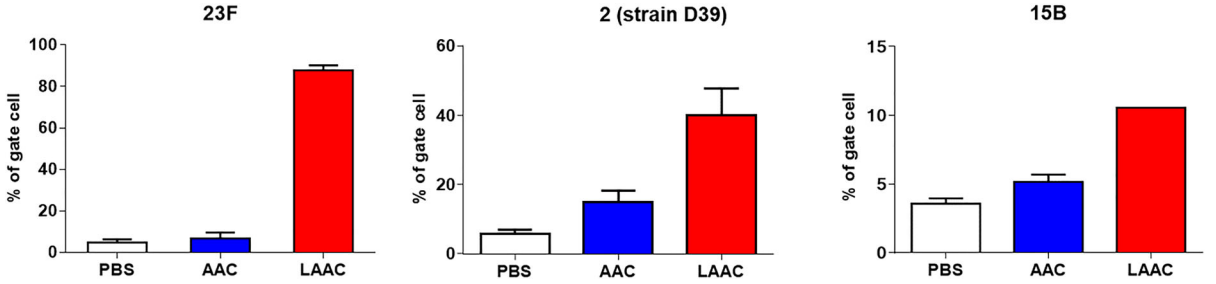

Figure 6. Immunization with the vaccine candidate LAAC induced higher opsonic phagocytosis activity. Six mice were subjected to intranasal injection of PBS, LAAC or AAC, which was repeated three times. One week later after the final immunization, serum samples were collected from each group. To assess the opsonic reaction, 5×10^6 CFU of *S. pneumoniae* serotypes 23F, 2 (strain D39), or 15B in 10% serum were immobilized at room temperature. Subsequently, freshly isolated polymorphonuclear neutrophils (PMNs) were introduced to the plate for the phagocytosis of bacteria opsonized with antibody and 1% human complement. Following a 30-minute phagocytosis reaction at 37°C, the reaction complexes were stained intracellularly using anti-IgG-Fc-FITC. The percentage of phagocytic cells positive for FITC was determined using flow cytometer analysis (BD Biosciences). Flow cytometry evaluations were conducted on at least 10,000 gated events. The results are presented as the mean ± standard deviation of the samples (n = 6).

the BALF from mice challenged with both PCV13 serotypes and non-PCV13 serotypes of *S. pneumoniae* (Figure 7A).

In addition to BALF samples, the bacterial load in serum samples was also checked after challenge with *S. pneumoniae*. Similar results were obtained, with the LAAC group exhibiting a decreased bacterial load in serum samples, while the AAC group did not (Figure 7B). Notably, the PCV13 immunization group showed a decrease in bacterial load in serum samples from mice challenged with the PCV13 serotypes but not in those challenged with the non-PCV13 serotypes (Figure 7B). The results suggest that anti-pneumococcal polysaccharide antibodies alone may not be sufficient to decrease bacterial levels in the BALF. Cellular immunity involving Th17 CD4+ T cells or CD8 + mucosa-associated invariant T (MAIT) cells may be important in defense.

Protective efficacy of the vaccine candidate

To evaluate the effectiveness of vaccine candidates against *S. pneumoniae*, we utilized mouse models and challenged them with serotype 6A (PCV13 serotype) and serotype 2 (non-PCV13 serotype). In our preliminary study involving serotype 6A, we found that the survival study following intrathecal challenge was unable to observe the significant differences. Consequently, for our survival study using serotype 6A, we intravenously infected the mice. Mice were intranasally immunized twice at a two-week interval and intratracheally challenged with *S. pneumoniae* two weeks after the final immunization. The data revealed that the survival rate in the control group was 20% after being challenged with a fatal dose of serotype 6A, whereas the survival rates in the PCV13, LAAC, and AAC immunization groups were 80%, 70%, and 50%, respectively, at 26 days after PCV13 serotype challenge (Figure 8A). Accordingly, the survival rate in the LAAC immunization group was 100% but less than 30% in the PCV13 and AAC immunization groups at 7 days after non-PCV13 serotype challenge (Figure 8B). These results indicated that LAAC immunization can protect mice from lethal challenge with *S. pneumoniae* serotypes 6A and 2 and protect against not only PCV13 serotypes but also non-PCV13 serotypes.

To further assess the protective efficacy of our vaccine candidate in the elderly population compared to that of PCV13, we immunized aged mice (over one year old) with PCV13 or LAAC and challenged them with *S. pneumoniae* serotype 6A. The control group showed no survival beyond 9 days following *S. pneumoniae* challenge, and the survival rate was almost the same (~30%) in both the PCV13 and LAAC immunization groups after *S. pneumoniae* serotype 6A challenge (Figure 8C). These results suggest that the LAAC vaccine candidate can induce protection in aged mice as effectively as PCV13.

Discussion

Enhancing pulmonary mucosal immunity through mucosal vaccination is believed to be the key to preventing pneumococcal infections [14]. Therefore, the identification of safe and effective mucosal adjuvants is crucial for the development of mucosal vaccines [31]. The successful utilization of lipoproteins in human vaccines, such as Trumenba®, an FDA-approved vaccine for meningococcal group B [32], indicates that lipoproteins have a proven safety record compared to untested adjuvants. Additionally, our study demonstrated that intranasal administration of LAAC can enhance mucosal immune responses. Coadministration of lipoprotein and nonlipidated antigens through the intranasal route can stimulate mucosal immunity without the need for additional adjuvants, as shown in Figure 4. This approach is straightforward, minimizes safety concerns, and boosts systemic mucosal immunity, making it a promising strategy for the development of mucosal vaccines.

Various studies have reported that the immune response to *S. pneumoniae* linked to protection by pneumococcal proteins is dependent on CD4+ T cells rather than antibodies [33]. In particular, the Th17 and Th1 arms are necessary for the development of the protective immune response, whereas the Th2 response is negligible [34,35]. Furthermore, Th17 responses have been suggested to mediate cross-protection against pneumococcal carriage [36]. In the present study, LAAC immunization induced antibody isotype bias toward Th1 (Figure 3D) and promoted immune responses toward the Th17 and Th1 arms (Figure 5, S2 and S3). Consequently, these findings indicate that our vaccine candidate is promising for the development of a broad-spectrum pneumococcal vaccine.

Age-related disparities play a crucial role in the development of a comprehensive pneumococcal vaccine. Due to weakened immune systems and an age-related decline in immune function, older individuals are more vulnerable to severe pneumococcal infections. A recent investigation involving US Medicare beneficiaries aged 65 and older revealed that PCV13 use could potentially reduce the likelihood of pneumonia-related hospitalizations [37]. In comparison to PCV13, our vaccine candidate demonstrated a similar outcome in an animal model (Figure 8C). However, further study is needed because the vaccine may require optimization at a different dose to achieve the desired immune response.

The maturation of TLR2-dependent antigen-presenting cells activates naive CD4+ T cells, leading to their differentiation into effector cells [38]. In animal models of pneumococcal infection, TLR2 has been

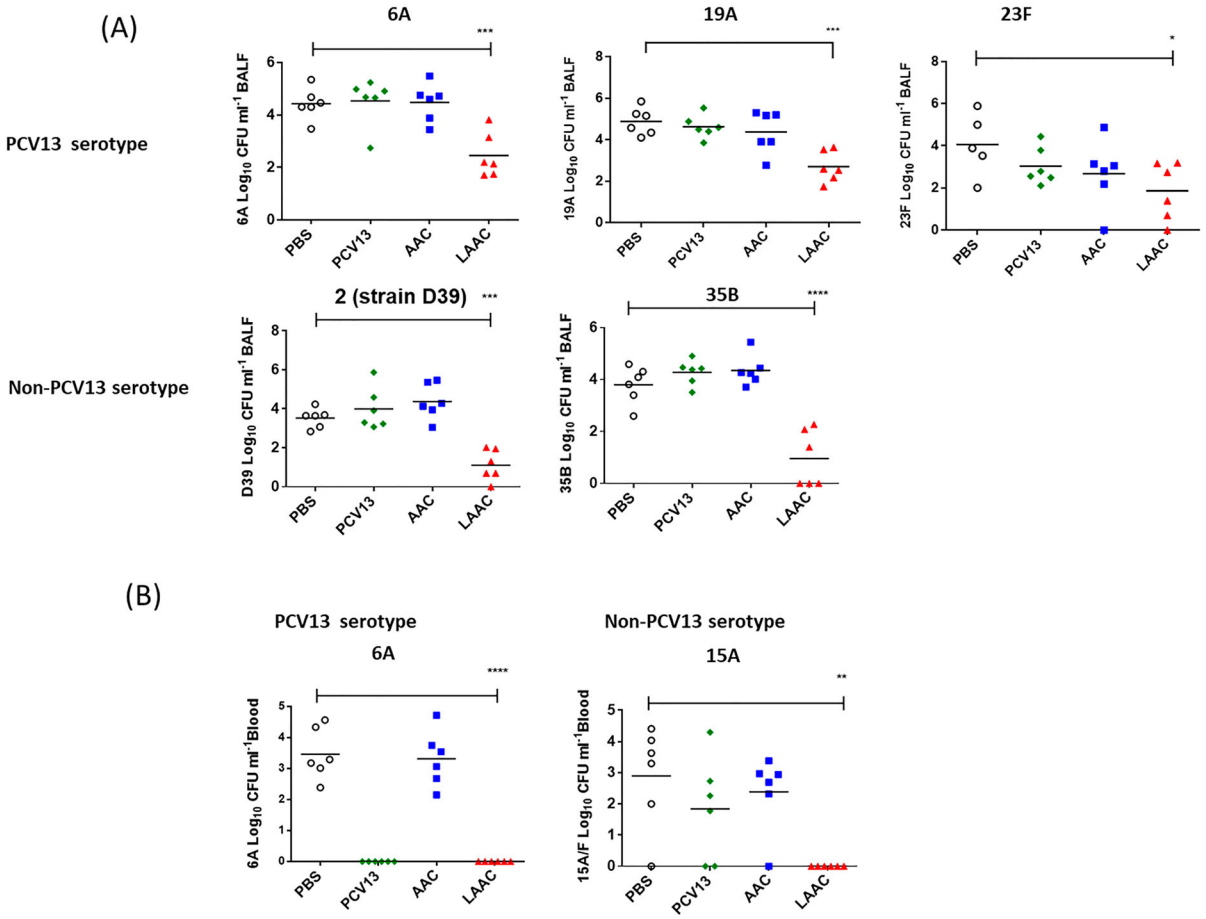

Figure 7. Immunization with the vaccine candidate LAAC efficiently prevented bacterial colonization and bacteremia. Female CD1 mice, aged 6–8 weeks, were immunized intranasally with PBS, LAAC or AAC. The immunizations were repeated three times at two-week intervals. As a control group, the mice received an intraperitoneal injection of 100 µL of PCV-13, which corresponds to 1/10th of the human dose. Following immunization, the mice were intratracheally infected with *S. pneumoniae* serotypes 6A, 19A, and 23F (included in PCV13) or non-PCV13 serotypes 2 (strain D39), 35B, and 15A. Samples were collected at 24 h post-infection and assessed using tryptic soy agar (TSA) with 5% sheep blood by plate counting. The bacterial load of serotypes 6A, 19A, 23F, 2 (strain D39), and 35B in bronchoalveolar lavage fluid (BALF) is presented in (A), while the results for serotypes 6A and 15A in the blood are shown in (B). The data are presented as the mean ± standard deviation of the samples (n = 6).

shown to be critical for bacterial clearance in meningitis [39] and colonization [40]. A lipoprotein-based subunit vaccine containing SP_2108 and SP_0148 has been shown to reduce the colonization of *S. pneumoniae* through activation of TLR2 [41]. These findings suggest that activation of the TLR2 signalling pathway is an effective way to clear pneumococcal colonization. Previously, we established a platform technology for the production of recombinant lipoproteins in an *E. coli* system. These lipoproteins can activate the immune system via TLR2 [15]. In this report, we utilized this platform to produce rlipo-PsaA, a key antigen for TLR2 activation, and demonstrated the effectiveness of our vaccine candidate in protecting against pneumococcal infection. However, further study is necessary to fully understand the precise mechanism of action underlying these effects.

Subunit vaccines offer several advantages, including increased safety and reduced incidence of adverse reactions. They focus on selected antigens, simplifying manufacturing procedures by excluding complete pathogens. However, a common challenge with subunit vaccines is the need for adjuvants to enhance immunogenicity. This reliance on adjuvants can complicate regulatory approval or incur significant costs from external suppliers. In this regard, our approach presents a promising solution. By incorporating inherent adjuvanticity within the vaccine itself, the need for additional adjuvants is eliminated. This approach has the potential to not only lower the expenses associated with acquiring adjuvants but also alleviate potential constraints in the regulatory approval process.

A noteworthy observation is that PCV13-immunized mice tend to have higher bacterial titres than LAAC-immunized mice, even when infected with the specific vaccine serotypes (6A, 19A, 23F). Figure 4 and a relevant reference [42] suggest that induced IgA may play a role in reducing bacterial titres in BALF. Furthermore, PCV13 stimulates the production of antibodies, notably IgG, which circulate in the

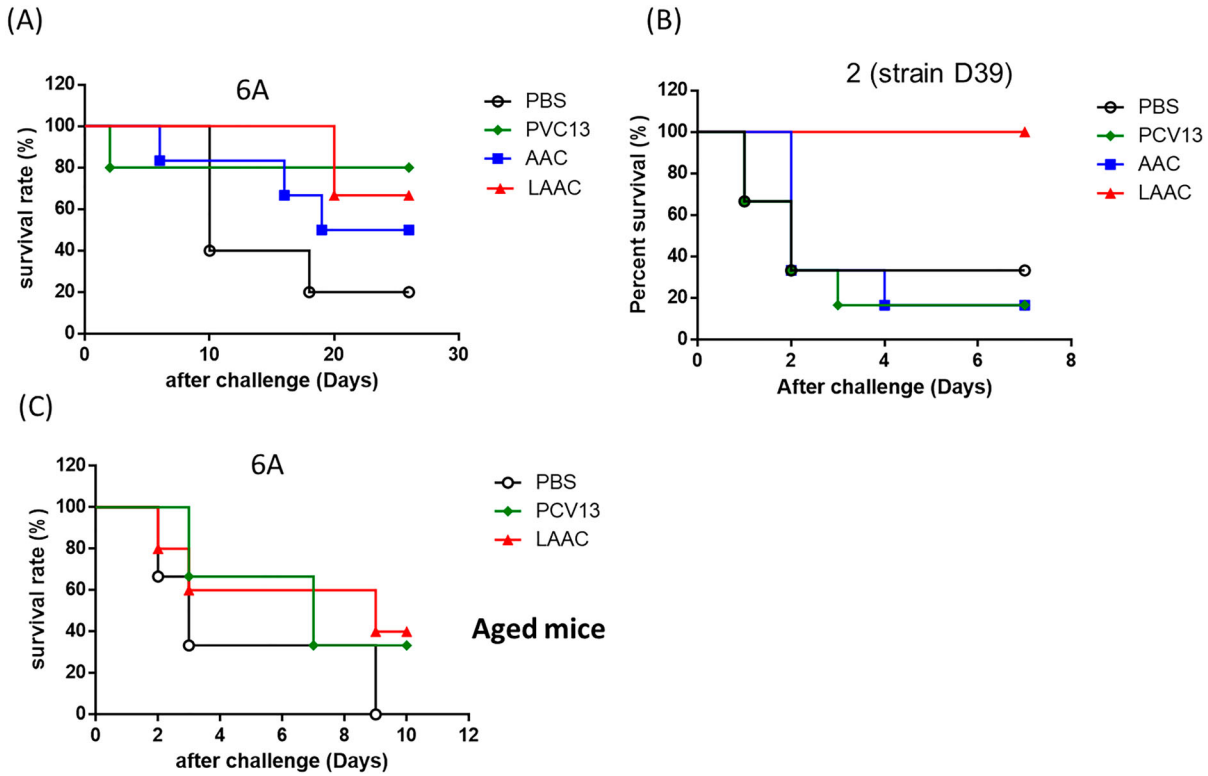

Figure 8. Protective effects of the vaccine candidate LAAC after immunization in a bacterial challenge model. Female CD1 mice, aged 6–8 weeks or 24–30 months, were immunized intranasally with PBS, LAAC or AAC. The immunizations were repeated three times at two-week intervals. As a control group, the mice received an intraperitoneal injection of 100 µL of PCV13, representing 1/10th of the human dose. Subsequently, adult mice were intravenously infected with 2×10^6 CFU of *S. pneumoniae* PCV13 serotype 6A (A), and mice were intratracheally infected with 2×10^6 CFU of *S. pneumoniae* non-PCV13 serotype 2 (strain D39) (B). Additionally, aged mice received an intravenous infection of 2×10^7 CFU of *S. pneumoniae* serotype 6A, as indicated in (C).ss

bloodstream and offer protection against the corresponding serotypes of *S. pneumoniae*. This phenomenon is also observed in Figure 7 (B) left panel, where samples collected from the blood show comparable bacterial titres between PCV13 and LAAC-immunized mice. Consequently, in BALF samples, PCV13-immunized mice exhibit higher bacterial titres than LAAC-immunized mice when infected with the specific vaccine serotypes, whereas in blood samples, both PCV13 and LAAC-immunized mice display similar bacterial titres.

The presence of more than 90 serotypes of pneumococcal bacteria poses a continuous challenge despite the effectiveness of current pneumococcal vaccines. To address this challenge, the development of a broad-spectrum vaccine is crucial to minimize serotype replacement and offer comprehensive protection. This report presents promising outcomes from animal models, demonstrating strong immune responses and protection against the selected PCV13 serotypes and non-PCV13 serotypes of *S. pneumoniae*. However, it is essential to conduct human trials to further investigate these findings and evaluate the vaccine's effectiveness, safety profile, and potential adverse events. With ongoing progress, this vaccine candidate has significant potential to contribute greatly to the prevention and control of pneumococcal disease at a population level.

Acknowledgments

This study was funded by grants from the National Health Research Institutes of Taiwan to S.-J.L. (IV-110-PP-14) and C.-H.L. (IV-111-GP-03), by a collaboration project grant 000 under the National Research Council Canada (NRC) and Ministry of Science and Technology (MOST), Taiwan, and to a grant to W.C. from the Vaccines and Immunotherapeutics Programme (NRC).

We express our gratitude to Dr. Kristopher Siu from the National Health Research Institutes, Taiwan, and Dr. Tsai-Ling Yang Lauderdale, Dr. Shu-Chen Kuo, and Dr. Ying-Chi Huang from the Taiwan Surveillance of Antimicrobial Resistance programme, National Health Research Institutes, Taiwan, for generously providing us with the *S. pneumoniae* serotypes and the necessary cultivation conditions.

Funding

This work was supported by National Health Research Institutes: [grant no IV-110-PP-14 & IV-111-GP-03]; National Research Council Canada: [grant no collaboration project grant 000].

Authors' contributions

F.-F.C. and L.-L.T. carried out the protein production and animal experiments, performed the challenge assays, and analyzed the results. B.-S.L. and H.Z. designed and performed the bacterial experiments

and the immune assessments. W.C., S.-J.L., and C.-H.L. designed experiments, conducted all experiments, analyzed results, wrote the manuscript and provided financial support.

Disclosure statement

W.C. and C.-H.L. are inventors on patent applications (WO2017096486; US10406221B2; TW201731866A) filed by the NRC and NHRI, and S.-J.L. and C.-H.L. are inventors on patent applications (US8426163B2; I376385; US8771990B2) filed by the NHRI.

No additional potential conflict of interest was reported by the authors.

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