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Immunization with attenuated non-transformable pneumococcal *pep27* and *comD* mutant provides serotype-independent protection against pneumococcal infection

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ABSTRACT

Streptococcus pneumoniae is a well-known pathogenic bacterium with a high mortality rate. Currently, a 23-valent pneumococcal polysaccharide vaccine (PPV23) and protein-conjugate vaccines (PCVs) are available on the market. However, both of these vaccines have limitations; specifically, PPV23 produces weak antibody responses in children younger than 2 years and PCVs only partially protect against secondary infection. Previously, we showed serotype-nonspecific protection by *Δpep27* vaccine, but the reversion of *Δpep27* to the wild type serotype during immunization cannot be excluded. To ensure the safety of the *Δpep27* vaccine, *comD*, an important protein that activates competence, was inactivated, and the transformability of the double mutant (*Δpep27ΔcomD*) was determined. The transformation ability of this double mutant was successfully abolished. *Δpep27ΔcomD* immunization significantly increased the survival time after heterologous challenge(s), and diminished colonization levels independent of serotype, including a non-typeable strain (NCC1). Moreover, the double mutant was found to be highly safe in both normal and immunocompromised mice. In conclusion, this pneumococcal *Δpep27ΔcomD* vaccine appears to be a highly feasible and safe vaccine to prevent various types of pneumococcal infections.

1. Introduction

Streptococcus pneumoniae is a human pathogen, which accounts for significant global morbidity and mortality. It causes a variety of diseases from mild infections such as sinusitis, conjunctivitis, and otitis media, to life-threatening diseases, such as pneumonia, bacteremia, and meningitis [1–3]. It accounts for approximately 1 million annual deaths, generally in children younger than 5 years and in the elderly [4].

The increasing problem of pneumococcal disease is partially due to the frequency at which this bacterium is gaining resistance to several antimicrobials and the quick spread of these exceedingly resistant strains worldwide [5]. Vaccination is considered an effective method for the prevention of pneumococcal disease. Frequently, the 23-valent pneumococcal polysaccharide vaccine (PPV23) and protein-conjugate vaccines (PCVs) are recommended to protect against pneumococcal diseases [6]. Although conjugate vaccines are very effective, their high expense makes them unaffordable in developing countries. In addition, the existing vaccines can induce serum IgG only against a restricted number of capsular serotypes, and are unable to induce secretory IgA in the intranasal mucosa, where pneumococci reside as a niche in the initial phase of disease onset [7]. However, after the introduction of a pneumococcal vaccine in the population, the serotypes included in the vaccine begin to be substituted by other serotypes [8]. Thus, there is an urgent requirement to develop a vaccine capable of conferring serotype-independent protection at the colonization stage.

Mucosal vaccines are known to induce the secretion of mucosal IgA and systemic IgG, which can provide protection against both the early colonization and subsequent invasive diseases [9,10]. However, the adjuvants used during mucosal vaccination do have some adverse effects [11]. The mucosal vaccination strategy does not involve the use of needles that can spread diseases if shared during the time of immunization, and thus can provide extensive immunization [12]. Although, prevention of transmission may not benefit the individual but is important in herd immunity, but we are still unaware of any specific formulations that provide better herd immunity. Therefore, an ideal pneumococcal vaccine must exhibit sufficient potency to provide serotype-independent mucosal and systemic immunity, and it must impede initial intranasal colonization along with subsequent invasive diseases [13–15].

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There is an intensive global focus on strategies to develop alternative pneumococcal vaccine strategies that address the weaknesses of current approaches, without lessening the efficacy. One such tactic uses whole cell vaccines that are reported to provide serotype-independent protection [16,17]. Our previous study indicated that deletion of the *pep27* gene (Δ*pep27*) rendered the pneumococcus resistant to lysis and incapable of invading the lungs, blood, and brain [18]. In addition, we showed that adjuvant-free intranasal Δ*pep27* immunization could provide long-lasting protection against heterologous strains [18]. A recent study also showed that immunization with *Δpep27* can confer protection against the influenza virus and secondary pneumococcal infections [19].

As the competence for transformation in *S. pneumoniae* is well-established [20], an attenuated *Δpep27* mutant may revert back to the wild type (WT) strain via transformation. Earlier studies had shown that mutation in *comD*, one of the genes of the Com operon, blocks transformation [21]. Thus, in the present study, *comD* was mutated in the *Δpep27* background to abrogate reversion with the aim to determine whether the *Δpep27ΔcomD* vaccine can complement the limitations of the previously reported *Δpep27* vaccine, without compromising its efficacy. Here, safety, survival, colonization studies demonstrate that *Δpep27ΔcomD* immunization can provide protection against heterologous strains, suggesting that *Δpep27ΔcomD* is a safe and effective vaccine against pneumococci.

2. Material and methods

2.1. Bacterial strains

The bacterial strains that were used are listed below (Table 1). Each strain of *S. pneumoniae* was cultured in Todd-Hewitt broth with 0.5% yeast extract (THY; Difco Laboratories Inc., Detroit, MI, USA) at 37 °C.

2.2. Construction of the TLΔpep27ΔcomD::ermB mutant

To construct the non-transformable *Δpep27* mutant (TL*Δpep27ΔcomD::ermB* mutant), an *erm*B cassette was inserted to disrupt expression of the *comD* gene (GeneBank: ABJ53801.1) in the TH*Δpep27* mutant (Fig. 1) [22]. The *ermB* cassette was amplified with prs3 and prs4 primers [22]. The left fragment containing parts of both the *comD* gene and *ermB* cassette was amplified from the D39 genome using the primers *ermB::comD*-1 and *ermB::comD*-2 (Table 2). The right fragment containing parts of both the *comD* gene and *ermB* cassette was amplified from the D39 genome using the primers *ermB::comD*-3 and *ermB::comD*-4 (Table 2). The left fragment, *ermB* cassette, and right fragment were used for triple-joining PCR using the primers *ermB::comD*-1 and *ermB::comD*-4 (Table 2). The triple PCR program was carried out as follows: denaturation step (95 °C for 2 min); amplification steps (denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 2 min 30 s) for 30 cycles; final extension step (72 °C for 5 min); and hold at 4 °C. All PCR amplifications were performed using nPFU-Forte DNA Polymerase (Enzymnomics, Seoul, Korea). The PCR products were then purified using a PCR Purification Kit (Cosmogenetech, Seoul, Korea). The competence of *S. pneumoniae* was controlled by a supplementation of competence signal peptide 1 [23]. Subsequently, the TH*Δpep27* strain was transformed with the purified DNA to obtain a double TL*Δpep27ΔcomD::ermB* mutant, which has *ermB* integrated into the genome via homologous recombination. The transformants were selected by 2.5 μg/ml erythromycin (Sigma-Aldrich, St. Louis, MO, USA). The presence of the *ermB* cassette in each colony was confirmed by PCR with the prs3 and prs4 primers. Concomitantly, to confirm the disruption of the *comD* gene in the mutant, total RNA from the bacteria was isolated using a hot-phenol method [23] and RT-PCR was performed using the *comD* and *pep27* primer pairs (Table 2).

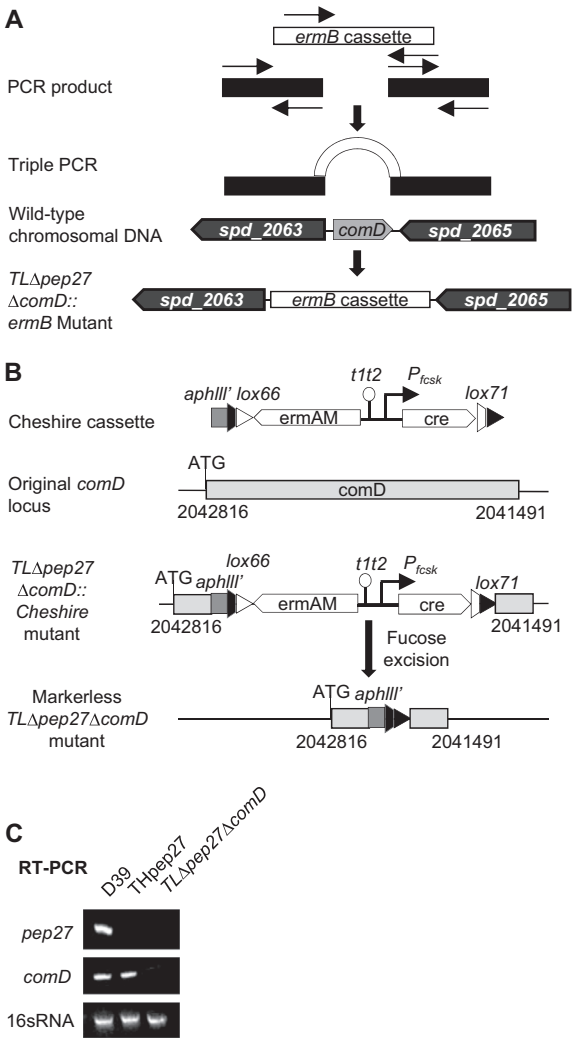

Fig. 1. *Δpep27ΔcomD* mutant construction. The *comD* gene was removed by insertion and orientation of the *ermB* cassette (A) or Cheshire cassette (B) between spd_2063 and spd_2065. The representative nucleotide sequence numbers in the D39WT genome are shown. Subsequently, the TH*Δpep27* mutant was transformed with purified DNA to obtain a double TL*Δpep27ΔcomD::ermB* or TL*Δpep27ΔcomD::Cheshire* mutant, which has the *ermB* or Cheshire cassette integrated into the genome via homologous recombination. Total RNA from the bacteria was isolated and mRNA was analyzed by RT-PCR (C).

Table 1
Pneumococcal strains used in this study.

Strain (Characteristic)	Reference
D39 (2)	[49]
ST180 (3)	Samsung Medical Center
NCCP10225 (4)	Korea Centers for Disease Control & Prevention
BG7322 (6B)	[22]
KUI (10A)	Korea University Hospital
ST83 (15)	Samsung Medical Center
ST320 (19A)	Samsung Medical Center
ST4467 (19F)	Samsung Medical Center
ST880 (23F)	Samsung Medical Center
NCC1 (Non-typeable)	[50]
ST1160 (Non-typeable)	Samsung Medical Center
THpep27 (D39 *Δpep27:: Cheshire ermB* Em^r)	[22]

Table 2
Primers used in this study.

Name	Sequence (5′-3′)	Reference
TL*Δpep27ΔcomD::ermB* mutant		
prs3	*ccg ggc cca aaa ttt gtt tga t*	[51]
prs4	*agt cgg cag cga ctc ata gaa t*	[51]
ermB::comD-1	*tgt act gcc ttc cat ctc tg*	This study
ermB::comD-2	*att cta tga gtc gct gcc gac tgc atg cag atg gca att gac*	This study
ermB::comD-3	*atc aaa caa att ttg ggc ccg ggc cat ctg cat gct gac aag*	This study
ermB::comD-4	*acg gtc gca ggt tcg aat cc*	This study
RT-PCR		
comD-RT-F	*acc aaa ctt cgt aaa gct ga*	This study
comD-RT-R	*atg gca att gac agt ggt aa*	This study
pep27-RT-F	*gct ttc agt tgt ttt ctt tg*	[18]
pep27-RT-R	*gtc tgt aag taa ctg atc ac*	[18]
16S-RT-F	*ccc ctt atg acct gg gct aca*	[18]
16S-RT-R	*cgg ctt gcg act cgt tgt*	[18]
Markerless TL*Δpep27ΔcomD* mutant		
Cheshire-F	*tgg ctt acc gtt cgt ata g*	[22]
Cheshire-R	*tcg ata ccg ttc gta taa tgt*	[22]
Cheshire::*comD*-1	*tgt act gcc ttc cat ctc tg*	This study
Cheshire::*comD*-2	*cta tac gaa cgg taa gcc aga tgg caa ttg aca gtg gta*	This study
Cheshire::*comD*-3	*aca tta tac gaa cgg tat cga aag gat aaa ggt agt cct cgt*	This study
Cheshire::*comD*-4	*acg gtc gca ggt tcg aat cc*	This study

2.3. Construction of a markerless double TL*Δpep27ΔcomD* mutant

To construct a markerless double TL*Δpep27ΔcomD* mutant, a Cheshire cassette was inserted to disrupt expression of the *comD* gene (GeneBank: ABJ53801.1) in the TH*Δpep27* mutant (Fig. 2) [23]. A Cheshire cassette (GenBank: FJ981645) carrying the erythromycin-resistance marker (*ermAM*), which can be used as a temporary marker for selection [23], was kindly provided by Dr. Donald Morrison (University of Illinois at Chicago). The cassette was amplified using the primers Cheshire-F and Cheshire-R (Table 2) [23]. The two arms were flanked by the *S. pneumoniae* TH*Δpep27* mutant DNA genome as follows: the left arm was initiated at the *comD* start codon and contained a part of the Cheshire cassette, using the primers Cheshire::*comD*-1 and Cheshire::*comD*-2; the right arm, which contained a part of the Cheshire cassette and ended at the *comD* stop codon, using the primers Cheshire::comD-3 and Cheshire::*comD*-4 (Table 2). The left fragment, Cheshire cassette, and right fragment were used for a triple-joining PCR using the primers Cheshire::*comD*-1 and Cheshire::*comD*-4 (Table 2). The triple PCR program was carried out as follows: denaturation step (95 °C for 2 min); amplification steps (denaturation at 95 °C for 30 s, annealing at 55 °C for 60 s, extension at 72 °C for 4 min 15 s) for 30 cycles; final extension step (72 °C for 5 min); and hold at 4 °C. All PCR amplifications were performed using nPFU-Forte DNA Polymerase (Enzymnomics). The PCR products were then purified using a PCR Purification Kit (Cosmogenetech). The competence of the *S. pneumoniae* was controlled by a supplementation of competence signal peptide 1 [24]. The Cheshire cassette was integrated into the genome via homologous recombination. Specifically, the TH*Δpep27* mutant was transformed with the purified DNA to obtain a double TL*Δpep27Δϖ comD*::Cheshire mutant, which has the Cheshire cassette integrated into the genome via homologous recombination. The transformants were selected by 0.1 μg/ml erythromycin (Sigma-Aldrich). The presence of the cassette in each colony was confirmed by PCR with the Cheshire-F and Cheshire-R primer pairs (Table 2). The selected colony was then confirmed by sequencing (Cosmogenetech).

The Cheshire cassette excision was then induced by adding 1.0% L-fucose (Sigma-Aldrich). The fucose-treated cultures were serially

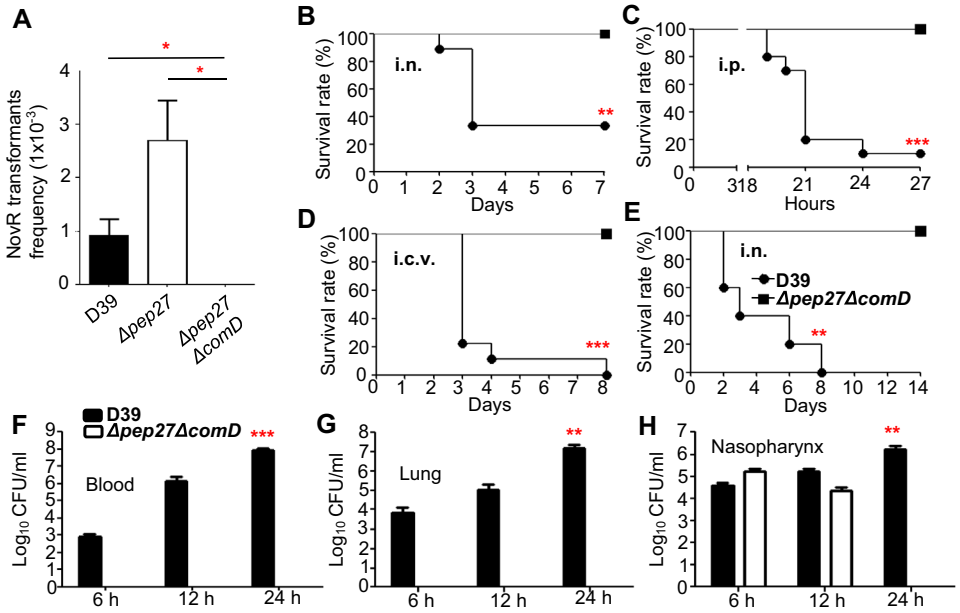

Fig. 2. Abrogation of transformability in Δ*pep27*Δ*comD* mutant attenuates *in vivo* virulence of Δ*pep27*Δ*comD*. Transformation competence was determined in the presence of CSP-1 for 15 min, 200 ng of 5MC donor DNA was added, and again incubated at 37 °C for 90 min. Subsequently, novobiocin-resistant transformants were quantified by enumeration of colony number on THY agar (A). Group of 10 mice were infected *i.n.* (B), *i.p.* (C), or *i.c.v.* (D) with 0.6 – 1 × 10^4 CFU of D39 or its isogenic Δ*pep27*Δ*comD* mutant. SCID mice were infected *i.n.* with 1 × 10^4 CFU of D39 or its isogenic Δ*pep27*Δ*comD* mutant (E). The survival was recorded daily (B, D, and E) or hourly (C). Group of 4 mice were infected *i.n.* with 1 – 3 × 10^8 CFU of D39 or its isogenic Δ*pep27*Δ*comD* mutant. Then, the blood, lungs, and nasopharyngeal tissues were collected 6 (F), 12 (G), and 24 h (H) post-infection. Viable cell numbers were determined after serial dilution and plating on THY agar. Significant differences in transformation and colonization experiments were analyzed by unpaired *t* tests and survival was analyzed by Mantel-Cox test (*p < 0.05, **p < 0.01, ***p < 0.001 compared with D39 infected group).

diluted and plated on THY agar plates to obtain single colonies. The presence of the Cheshire cassette was confirmed in each colony using PCR amplification with the Cheshire-F and Cheshire-R primers (Table 2). In parallel, the non-Cheshire colony candidates were streaked on THY blood agar plates in the presence or absence of 0.1 μg/ml erythromycin. A colony that was unable to grow on the THY agar plate containing 0.1 μg/ml erythromycin was subsequently selected from the non-erythromycin THY blood agar plate. Finally, to confirm the disruption of the *comD* gene in the mutant, total RNA from the bacteria was isolated using the hot-phenol method [25] and RT-PCR was performed using the *comD* and *pep27* primers (Table 2).

2.4. Transformation assay

Complete transformation medium was prepared from THY broth by the addition of 0.1 mM $CaCl_2$. The culture was incubated at 37 °C until the $O.D_{550}$ reached approximately 0.05–0.1 and was then exposed to 250 ng/ml of competence stimulating peptide 1 (CSP-1) for 15 min followed by the addition of 200 ng of 5MC donor DNA. The transformation mixture was then incubated at 37 °C for 1.5 h. The culture was plated on THY agar overlaid with 10 μg/ml of novobiocin for transformant selection. Then, the plates were incubated overnight at 37 °C in an atmosphere of 95% air-5% CO_2. The number of transformed colonies were then counted and divided by the total number of colonies for normalization.

2.5. DNA uptake assay

DNA uptake assay was performed using a Cy3-tagged fluorescent DNA as described previously [18]. Once competence was induced, 200 ng of non-fluorescent control DNA and fluorescent tagged DNA were independently added to the cultures of WT and *Δpep27ΔcomD* strains. Transformation was carried out at 37 °C for 30 min in a 5%–CO2 incubator. Bacterial pellets were collected after centrifugation and non-specific fluorescence was removed by washing with PBS. Bacterial pellets were resuspended in sterile PBS and fluorescence was measured using Multimode Plate Reader (PerkinElmer, USA).

2.6. In vivo safety test

A group of 10 CD1 (Orient Bio Inc., Korea) and SCID mice (Charles River Laboratories, Japan) were infected via intranasal (*i.n.*), intraperitoneal (*i.p.*), or intracerebroventricular (*i.c.v.*) routes with D39 or its isogenic Δ*pep27*Δ*comD* strain. The survival was recorded daily.

For confirmation of rapid clearance of pneumococcal Δ*pep27*Δ*comD*, a group of 4 mice were infected *i.n.* with D39 or Δ*pep27*Δ*comD*. The mice were euthanized 6, 12, and 24 h after infection, and the blood, lungs, and nasopharyngeal tissues were harvested and homogenized on ice in 1 ml of phosphate buffered saline (PBS) (except for blood) with a homogenizer (PRO Scientific Inc., Oxford, CT, USA, Model 200 Double insulated). Then, the homogenized tissues were serially diluted, and plated on THY agar supplemented with 5% defibrinated sheep blood (MB cell, USA). Gentamicin (10 μg/ml; for D39; Sigma Aldrich) or 2.5 μg/ml erythromycin (for pneumococcal Δ*pep27*Δ*comD*; Sigma Aldrich) were added to the agar for selection. Subsequently, the plates were incubated for approximately 18 h at 37 °C in an atmosphere of 95% air-5% CO_2, after which the colonies were counted and averaged between replicates.

2.7. Immunization experiment

Four-week-old mice (CD1, male) were purchased from Koatech (Pyeongtaek, Korea). To test the effect of the vaccine, the mice were anesthetized with ketamine-xylazine mixtures (10 ml of ketamine, 2.5 ml of xylazine, and 12.5 ml of PBS) and immunized once a week for 4 weeks with the isogenic Δ*pep27*Δ*comD*. All animal experiments were in accordance with the guidelines of the Korean Animal Protection Law.

2.8. Determination of IgG antibody titer

The immune sera were collected by retro-orbital bleeding of the mice 6 days after every immunization. On the first day, a 96-well immunoplate was coated with D39 whole cells overnight and then washed three times with phosphate-buffered saline Tween buffer (PBST, PBS with 0.05% Tween 20). After one hour of blocking with blocking buffer that contained 1% bovine serum albumin (BSA; Sigma Aldrich), the collected immune sera were added to the plate and incubated 2 h at room temperature. Then, an anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP, 1:3000 dilution; GeneTex, USA) was used as the secondary antibody. After treating with ECL buffer, the IgG titer was measured by using a Molecular Device microplate reader at a wavelength of 450 nm.

2.9. Survival study

CD1 mice were anesthetized with a ketamine-xylazine mixture and challenged *i.n.* with D39 (type 2) or BG7322 (6B), one week after the last pneumococcal Δ*pep27*Δ*comD* immunization. The survival of the mice was observed from the day after infection until 15 days post-challenge.

2.10. Colonization studies

Seven days and 2 months after the fourth immunization, the CD1 mice were challenged with D39 (type 2), BG7322 (type 6B), or NCC1 (Non-typeable) after anesthesia. After 24 h, the mice were euthanized and their blood, nasal washes, and lungs were collected. Each collected sample was then diluted and plated on THY agar. The number of colonies was counted after an overnight incubation.

2.11. Cytokine assays

Blood, bronchoalveolar lavage fluid (BALF), and splenocytes were collected from the CD1 mice on the seventh day after the last immunization. Then, the levels of interleukin (IL)-17, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-4, and IL-10 in each sample were analyzed using enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, San Diego, CA, USA) following the manufacturer's instructions.

2.12. Statistics

The experimental data are shown as means ± standard deviations. The survival analysis was performed using a Log-rank test (Mantel-Cox), ELISA and colonization values were compared using unpaired *t* tests. The differences were considered significant at $P < 0.05$.

3. Results

3.1. Transformation abrogation of *Δpep27* mutant attenuates virulence in vivo

Although the virulence of Δ*pep27* was previously shown to be impaired and the *pep27* mutant could protect the host against pneumococci *in vivo* [18], transformation of this strain to the virulent WT strain cannot be excluded. To avoid this reversion,

comD, an essential gene for transformation [21,26], was deleted to make the Δ*pep27*Δ*comD* strain, and then its transformation ability was assessed. The resulting Δ*pep27*Δ*comD* strain failed to form any detectable transformants, whereas its parental strains, D39, and Δ*pep27*, were successfully transformed (Fig. 2A). This result indicates that the transformation ability of the Δ*pep27* strain was successfully abolished by the *comD* deletion. Consistently, DNA uptake assay revealed that Δ*pep27*Δ*comD* strain was unable to endorse exogenous DNA (Supplementary Fig. 1).

To determine virulence attenuation of the Δ*pep27*Δ*comD* strain, mice were infected with the Δ*pep27*Δ*comD* strain via three different routes. Mice infected with the Δ*pep27*Δ*comD* strain successfully survived whereas mice infected with the D39 WT strain showed higher mortality (Fig. 2B, C, D). Moreover, Δ*pep27*Δ*comD* infection was not lethal to immunocompromised SCID mice (Fig. 2E). It was previously reported that the *pep27* mutant was not detected in blood after *i.n.* infection [18]. In order to assess the invasiveness of Δ*pep27*Δ*comD* into other tissues, bacterial loads in the blood, lung, and nasopharynx were determined. The results demonstrated that no Δ*pep27*Δ*comD* was detected in the blood and lung, whereas D39 WT showed substantially increased numbers (Fig. 2F, G). In the nasopharynx, the double mutant was detected, but it was cleared rapidly within 24 h, whereas the significantly higher bacterial load of D39 WT persisted (Fig. 2H). Δ*pep27*Δ*comD* did not kill any mice by *i.p.* and *i.n.* routes, similar to that previously shown for the Δ*pep27* strain [18]. These results indicate that the Δ*pep27*Δ*comD* strain is safe enough to be used as an attenuated vaccine.

3.2. Δpep27ΔcomD immunization elicits IgG and provides protection

IgG is a major antibody that is produced after immunizing with pneumococcal vaccines such as polysaccharide or conjugate vaccines [27]. It is produced by memory B cells and confers protection by strongly binding to pneumococcal polysaccharide capsule [28]. Our results revealed that Δ*pep27*Δ*comD* immunization *i.n.* significantly elevated IgG titer against D39 whole cells, which increased more than 50-fold between the first and fourth immunization (Fig. 3A). Consistently, the Δ*pep27*Δ*comD* immunization elicited PspA-specific IgG (Fig. 3B).

To determine whether Δ*pep27*Δ*comD* immunization could provide protection from *S. pneumoniae* infections, the mice were immunized *i.n.* once a week for 4 weeks, and were then challenged *i.n.* with D39 or 6B one week after the last immunization. The data show that immunization significantly increased the survival rate compared to that of the control group, regardless of the challenge serotype (i.e., either type 2 (D39) or 6B) (Fig. 3C, D).

3.3. Δpep27ΔcomD immunization impairs colonization serotype-independently

To determine whether the increased mouse survival by immunization is due to impaired colonization, viable cell numbers were determined 24 h post-challenge. When type 2 (D39), 6B, or non-typeable NCC1 was used for infection, the Δ*pep27*Δ*comD* immunization inhibited colonization levels significantly in the blood, nasal wash, and lung homogenates (Fig. 4A, B, C).

3.4. Δpep27ΔcomD immunization elicits cytokine expression

Secretary IgA antibody is induced by vaccination and protects against many pathogenic microorganisms, which invade the host mucosal surface, by triggering T helper cells (T_H cell), especially T_H1 (IFN-γ and TNF-α) and T_H2 (IL-4 and IL-10). Furthermore, T_H17 (IL-17) is essential for reducing the duration of colonization [29]. Therefore, we investigated whether these cytokines are

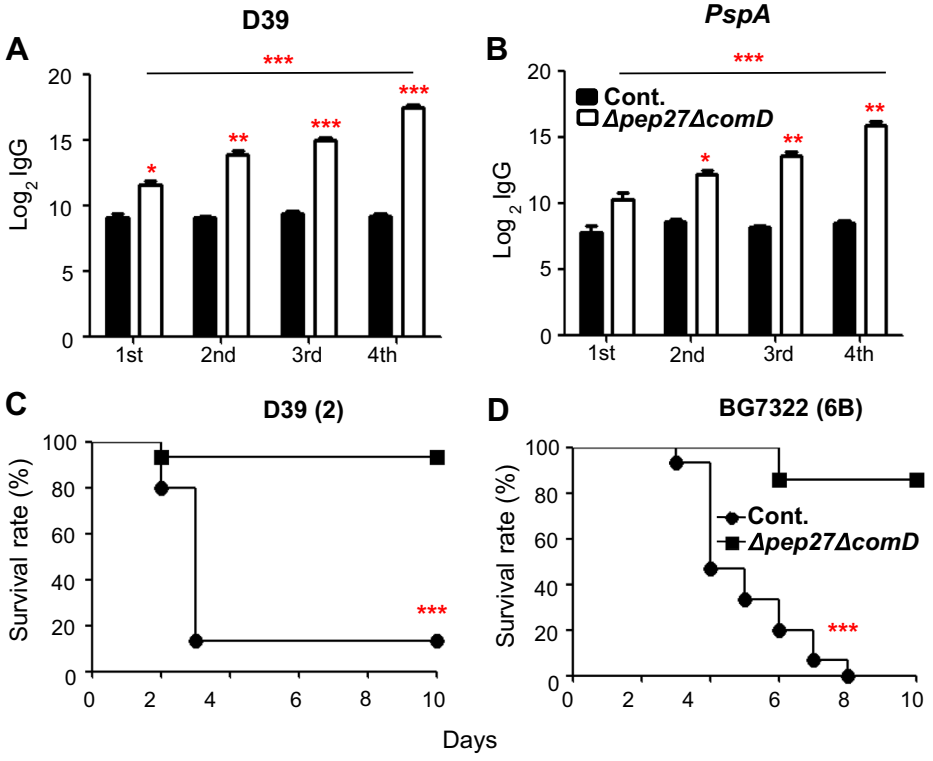

Fig. 3. Increase in IgG titer by pneumococcal Δ*pep27*Δ*comD* immunization, and Δ*pep27*Δ*comD* immunization provides serotype-independent protection . A group of 5 mice were immunized with 1×10^8 CFU of Δ*pep27*Δ*comD* weekly for 4 times. Six days after every immunization, the immune sera were collected from retro-orbital bleeding. The IgG titer against D39 (A) and PspA (B) was determined by ELISA. Immunized mice (n = 15/group) were challenged with 7×10^7 CFU of D39 (C) or 3×10^8 CFU of BG7322 (type 6B) (D) 7 days after last immunization. Subsequently, the survival was recorded daily. Significant differences in antibody titer were analyzed by unpaired *t* tests and survival was analyzed by Mantel-Cox test (*p < 0.05, **p < 0.01, ***p < 0.001 compared with D39 infected group).

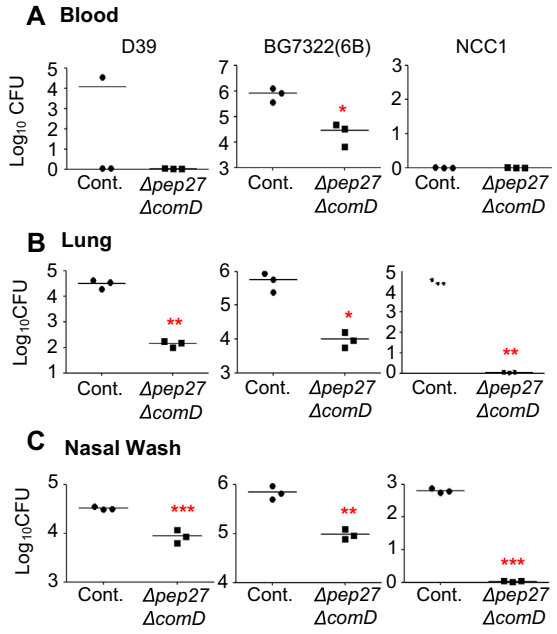

Fig. 4. Serotype-independent inhibition of colonization by Δ*pep27*Δ*comD* immunization. A group of 3 mice were immunized with 1×10^8 CFU of Δ*pep27*Δ*comD* 4 times over 4 weeks. Each group was challenged with 1×10^8 CFU of D39 (A), 3×10^8 CFU of BG7322 (6B) (B), or 1×10^8 CFU of non-typeable NCC1 (C). Samples of blood, lung homogenates, and nasal washes were used for viable bacterial counts. Significant differences were determined by unpaired *t* tests (*p < 0.05, **p < 0.01, ***p < 0.001 compared with control group).

elicited by Δ*pep27*Δ*comD* immunization. After immunization, the expression levels of these cytokines were detected from BALF and splenocytes by ELISA. The results show that IL-17, TNF-α, IFN-γ, IL-10, and IL-4 were significantly increased in all samples except for IL-4 in splenocytes (Fig. 5A, B).

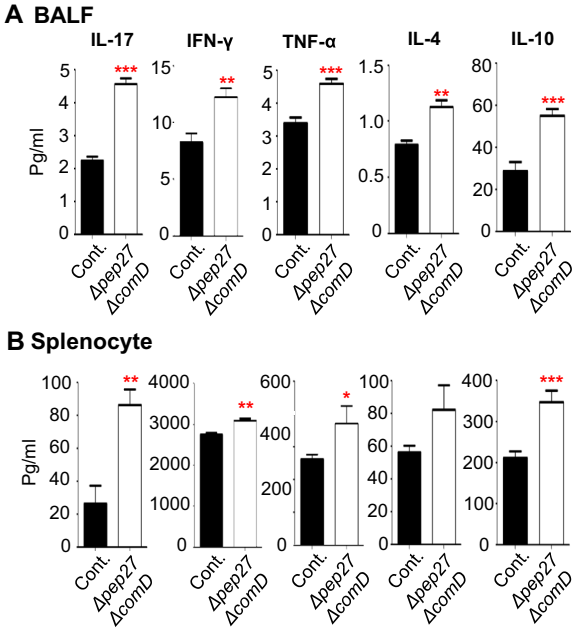

Fig. 5. Increased cytokine expression by Δ*pep27*Δ*comD* immunization. A group of 6 mice were immunized with 1×10^8 CFU of Δ*pep27*Δ*comD* once a week for 4 weeks, and euthanized 1 week after the final immunization. Then, BALF (A) and splenocytes (B) were collected from each group of mice. The production of IL-17, TNF-α, IFN-γ, IL-4, and IL-10 were analyzed using ELISA kits. Significant differences were determined by unpaired *t* tests (*p < 0.05, **p < 0.01, ***p < 0.001 compared with control group).

3.5. Δpep27ΔcomD immunization provides long-term immunity against lethal challenge

The resultant mucosal immunity after vaccine administration to the mucosal surface plays an important part in the durability of protective immunity [30,31]. In addition, extended antibody production can confer protection against the encapsulated bacteria for a longer period of time. Therefore, we investigated whether Δ*pep27*Δ*comD* immunization could elicit persistent IgG titer. After Δ*pep27*Δ*comD* immunization, the immune sera were collected 2 months after the last immunization and the IgG titer was measured by ELISA. The results show that Δ*pep27*Δ*comD* immunization could elicit long-lived mucosa-specific IgG titer against both D39 whole cells and PspA protein (Fig. 6A, B).

Following these data, we determined whether Δ*pep27*Δ*comD* immunization has an effect on clearance of bacteria even after an extended period. The mice were challenged intranasally with D39, 6B, or NCC1 2 months after the last immunization, and the viable numbers of bacteria in the lungs were determined. Δ*pep27*Δ*comD* immunization decreased bacterial CFU in the type 2 D39 and non-typeable NCC1 challenged mice significantly (Fig. 6C, E), but the decreases were not significant in the type 6B challenged group (Fig. 6 D).

4. Discussion

Although antimicrobial therapies and conjugate vaccines are available, pneumococci continue to cause a broad spectrum of diseases. The conjugate vaccines can confer protection against serotypes that are mostly responsible for invasive diseases. However, the worldwide health problem of pneumococcal diseases has been augmented by the rapid spread of multidrug resistant strains [32], and this problem is being exacerbated by the limitations of current vaccines, including their high prices, serotype-specific modes of protection, and partial serotype coverages [33]. To overcome these unmet medical needs, serotype-independent pneumococcal vaccines need to be developed urgently.

Live attenuated pneumococcus might be a potent vaccine, as it can facilitate reaction to pneumococcal proteins in a serotype-independent manner. For example, Moffitt *et al.* reported the induction of wide-ranging antibody and T cell reactivity by the combined introduction of a pneumococcal whole-cell vaccine and adjuvant [34]. Additionally, a strain with mutations in three different virulence factors (SPY1) or a heat-killed Rx1 strain (RM200) was used as a mucosal vaccine, and both were reported to induce cytokines, IgG and IgA antibodies, and phagocytic activity, but only in the presence of adjuvants [9,35]. Moreover, immunization with a heat-killed pneumococcal vaccine of capsular serotype 4 without any adjuvant caused considerably less bacterial burden in the blood following challenge, but reduced intranasal IgG or IgA antibody concentrations were recorded compared to those in adjuvant-treated mice [36]. Our previous report already showed that Δ*pep27* immunization without adjuvant offered a wide range of protection, and secretion of mucosal antibodies and cytokines was induced. This ultimately resulted in neutrophil activation and weakening of early colonization potential, which promoted clearance. Thus, we previously demonstrated a substantial improvement in mucosal vaccine development.

Although they are exceedingly effective, there are some limitations to using live attenuated vaccines. A major concern is the threat of reversion to a more virulent strain of the bacteria being targeted. The possibility of a secondary mutation always exists, which might lead to a more transmittable and virulent form of the bacteria. Thus, steps must be taken to diminish the risk of reversion before making the vaccine commercially available. Pneumococcal genetic alteration by natural transformation takes place

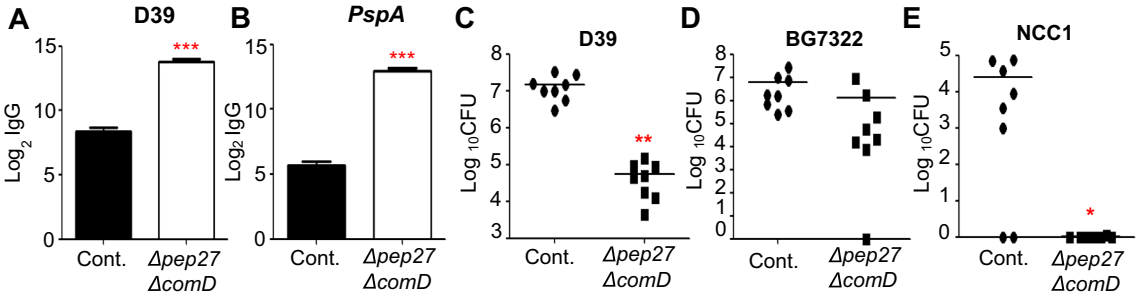

Fig. 6. Long-term immunity against lethal challenge induced by Δ*pep27*Δ*comD* immunization. A group of 6 mice were immunized with 1×10^8 CFU of Δ*pep27*Δ*comD* weekly for 4 weeks. After 2 months of housekeeping, IgG titer against D39 (A) and PspA (B) was determined in serum by ELISA assay. A group of 8 mice were immunized with 1×10^8 CFU of Δ*pep27*Δ*comD* once a week over 4 weeks. Each group of mice was challenged with 1×10^8 CFU of D39 (C), 7×10^8 CFU of BG7322 (D), or 3×10^8 CFU of non-typeable NCC1 (E) 2 months after the last immunization. Samples of lung homogenates were used to determine viable bacterial counts. Significant differences in antibody titers and colonization numbers were determined by unpaired *t* tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control group).

in a cell density-dependent manner and is regulated by the well-characterized *com* locus [21]. The *com* locus consists of three genes, *comC*, *comD*, and *comE*, which encode a CSP, histidine kinase, and response regulator, respectively [21,37,38]. It has been reported that mutation in the *comD* gene renders the pneumococcus incapable of transformation [21]. Thus, in the present study, we introduced a mutation in the *comD* gene of the previously reported Δ*pep27* live vaccine in order to minimize the risk of reversion via genetic transformation, without compromising its potential as an ideal mucosal vaccine.

The transformation potential of this newly constructed *Δpep27*Δ*comD* live vaccine was compared with that of the Δ*pep27* and wild type D39 strains in order to determine the feasibility of reversion. We found that the transformation ability of pneumococcal Δ*pep27*Δ*comD* was decreased significantly compared to that of the D39 WT and Δ*pep27* mutant (Fig. 2A and Supplementary Fig. 1). The Δ*pep27*Δ*comD* vaccine showed significantly attenuated virulence compared to that of D39 WT, as validated by the survival of the mice irrespective of the administration route (Fig. 2B, C, D). This double-mutant strain exhibited its potential as a vaccine even in immunocompromised mice by producing significantly reduced mortality after infection compared to that after infection with the WT strain (Fig. 2E). Moreover, this double mutant was rapidly cleared and was not detected in the blood and lungs within 6 h of infection (Fig. 2F, G); it was completely removed from the nasopharynx 24 h after infection (Fig. 2H), suggesting a reduced ability of this mutant to invade tissues. This rapid bacterial removal could promote macrophage apoptosis and consequently protect the host against pneumococcal infection [39].

Roche *et al.* already reported that a two-dose regimen of a single *cps* mutant promoted mucosal protection and could be used without adjuvant [10]. IgG antibody produced as a result of intranasal immunization of a *cps* mutant was reported to confer protection against lethal challenges [40]. Accordingly, our double mutant vaccine also enabled increased IgG titer with each immunization (Fig. 3A, B) and provided protection after virulent strain challenges (Fig. 3C, D).

Alterations in nasopharyngeal colonization frequency of particular serotypes are incidentally associated with epidemiologic variations in invasive pneumococcal disease as a result of the invasive potential of each serotype [41]. Therefore, the effect of vaccines on this alteration in nasopharyngeal burden is vital for herd immunity [42]. A previous report showed that the currently available PCV13 vaccine can confer protection only against the included serotypes and there would be emergence of serotype replacement, i.e., colonization by non-vaccine types ([43]. In contrast, immunization with the Δ*pep27* vaccine can significantly impair bacterial viability in various tissues after challenge with different pneumococcal serotypes [44]. Consistent with this previous findings, immunization with our newly constructed Δ*pep27*Δ*comD* vaccine significantly decreased bacterial burden in the blood, lungs, and nasal wash after challenge with serotypes 2, 6B, and non-typeable NCC1, 24 h post-infection (Fig. 4A, B, C). These results indicated that Δ*pep27*Δ*comD* immunization was capable of conferring serotype-independent protection, even against the non-typeable pneumococcal strains.

It is a well-known fact that recruitment of T cells occurs in the lungs in the course of pulmonary infection [16]. Th1 and Th2 cells cooperatively partake in the secretory IgA response, which plays various roles in mucosal defenses [45]. Whole cell vaccine immunization can provide protection against pneumococcal colonization via the cytokine IL-17 generated by Th17 cells [46]. In the current study, *i.n.* immunization with Δ*pep27*Δ*comD* induced augmented levels of Th1 (TNF-α and IFN-γ), Th2 (IL-4, IL-10), and Th17 (IL-17) cytokine secretion in BALF compared with that in the non-immunized group (Fig. 5A). The cytokine secretion profile of the splenocytes followed the same pattern except that there was no significant difference in the level of IL-4 secretion after double-mutant immunization compared to that of the non-immunized mice (Fig. 5B), reestablishing the fact that Δ*pep27* immunization shows preferential induction of Th1 cells [44]. Our results suggest that the significant decrease in pneumococcal colonization as a result of Δ*pep27*Δ*comD* immunization was associated with elevated levels of Th1/17-dependent cytokine production.

The resultant mucosal immunity after vaccine administration to mucosal surfaces plays a vital part in prolonged protective immunity [31]. Our results showed that Δ*pep27*Δ*comD* immunization can provide long-lasting protection, with elevated IgG levels (Fig. 6A, B) and decreased bacterial loads following challenges with type 2 and non-typeable NCC1 pneumococcal strains, up to 2 months post-immunization (Fig. 6C, E). However, this double-mutant immunization did not provide long-term protection when challenged with the type 6B strain (Fig. 6D). Further studies regarding whether the double-mutant immunization could protect against type 6B in human subjects may offer insights into the mechanism by a live attenuated *S. pneumoniae* mucosal vaccine. Also further studies warrant the chance to explore regulatory changes inside the pneumococcal strain used in challenge in response to the host environment and moreover the outcome of competition initiated by the challenge pneumococcal strain with other nasopharyngeal commensals [47,48]. However, the absence of long-term immunity provided by commercial pneumococcal vaccines strongly necessitates the usage of our double-mutant vaccine.

In conclusion, the pneumococcal Δ*pep27*Δ*comD* mutant is a highly feasible, inexpensive mucosal vaccine that abrogates the

transformability of the Δ*pep27* vaccine without compromising its protective effect.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.11.027.

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