Serotype-Independent Protection against Pneumococcal Infections Elicited by Intranasal Immunization with Ethanol-Killed Pneumococcal Strain, SPY1

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The 23-valent polysaccharide vaccine and the 7-valent pneumococcal conjugate vaccine are licensed vaccines that protect against pneumococcal infections worldwide. However, the incidence of pneumococcal diseases remains high in low-income countries. Whole-cell vaccines with high safety and strong immunogenicity may be a favorable choice. We previously obtained a capsule-deficient *Streptococcus pneumoniae* mutant named SPY1 derived from strain D39. As an attenuated live pneumococcal vaccine, intranasal immunization with SPY1 elicits broad serotype-independent protection against pneumococcal infection. In this study, for safety consideration, we inactivated SPY1 with 70% ethanol and intranasally immunized BALB/c mice with killed SPY1 plus cholera toxin adjuvant for four times. Results showed that intranasal immunization with inactivated SPY1 induced strong humoral and cellular immune responses. Intranasal immunization with inactivated SPY1 plus cholera toxin adjuvant elicited effective serotype-independent protection against the colonization of pneumococcal strains 19F and 4 as well as lethal infection of pneumococcal serotypes 2, 3, 14, and 6B. The protection rates provided by inactivated SPY1 against lethal pneumococcal infection were comparable to those of currently used polysaccharide vaccines. In addition, vaccine-specific B-cell and T-cell immune responses mediated the protection elicited by SPY1. In conclusion, the 70% ethanol-inactivated pneumococcal whole-cell vaccine SPY1 is a potentially safe and less complex vaccine strategy that offers broad protection against *S. pneumoniae*.

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Introduction

Streptococcus pneumoniae is a Gram-positive human pathogen that causes several diseases, including otitis media, pneumonia, bacteremia, and meningitis, particularly in high-risk groups, such as children, elderly, and immunocompromised people (Lynch and Zhanel, 2009). Pneumococcal disease causes approximately 826,000 deaths in children aged 1 to 59 months worldwide per year, of which 90% occurs in low-income or developing countries (O'Brien *et al.*, 2009; van der Poll and Opal, 2009). In addition, the worldwide continuous increase in aging population and increasing rates of multiple antibiotic resistance of *S. pneumoniae* emphasize the importance of preventing infective pneumococcal diseases (Braido *et al.*, 2008).

Vaccination is an effective strategy to prevent pneumococcal infection diseases. Commercially available vaccines against *S. pneumoniae* include polysaccharide vaccine and polysaccharide conjugate vaccine, which are based on capsular polysaccharide, the major determinant necessary for causing infection. The 23-valent polysaccharide vaccine (PPV23) contains the most common types of pneumococcal bacteria and induces T-cell-independent immune response; thus, PPV23 is not effective in children aged less than 2 years, who are the most vulnerable to pneumococcal infection (Greene *et al.*, 2006). Pneumococcal conjugate vaccines (PCV), such as PCV7, PCV10, and PCV13, elicit T-cell-dependent immune response and are effective in young children. However, PCVs reportedly induce selective pressure and gradual serotype replacement (Scott, 2007; Isaacman *et al.*, 2010). The efficacy of PCVs against the most frequent manifestations of infection, particularly mucosal infection (pneumonia and otitis media), is more limited than for invasive disease (Spratt and Greenwood, 2000; Toltzis and Jacobs, 2005). In addition, conjugate vaccines are complex and costly, making them inaccessible for populations in utmost need. Currently available vaccines induce systemic serum IgG rather than mucosal secretory IgA in the nasopharynx, where pneumococci colonize before causing subsequent invasive pneumococcal diseases (Kim *et al.*, 2012). To overcome these limitations, novel pneumococcal vaccines that not only induce mucosal immunity and systemic immunity to provide protection against colonization and its corresponding invasive diseases regard-

Table 1. Primers used in this study

Primer	Sequence (5′ → 3′)
SPD_1672 upstream P1 (UP- F)	TCCAAGGTTTCTGGGTCT
P2 (UP-R)	ATCAAACAAATTTTGGGCCCGGCATTGTTCCGTCCGTATT
SPD_1672 downstream P3 (DW-F)	AATTCTATGAGTCGCTGCCGACTTTGCCTGCTAATAGACCC
P4 (DW-R)	AAGATGAGCCCGATGA AG
*erm*F	CCGGGCCCAAAATTTGTTTGAT
*erm*R	AGTCGGCAGCGACTCATAGAAT

Note: The underline portions refer to the reverse complementary sequence of *erm*F and *erm*R respectively.

less of serotype but also provide herd immunity or indirect protection of nonvaccinated individuals must be developed (Käyhty *et al.*, 2006; Oliveira *et al.*, 2007).

Recent studies have attempted to develop different types of pneumococcal vaccines, such as protein vaccines, live attenuated vaccines, and whole-cell killed bacterial vaccines (Ogunniyi *et al.*, 2000; Roche *et al.*, 2007; Gong *et al.*, 2011). Among these vaccines, whole-cell killed bacterial vaccines are safe and retain major bacterial antigens possessing strong antigenicity and broad serotype coverage. Moreover, whole-cell killed bacterial vaccines are effective with potentially inexpensive and easily administered preparation, making them suitable for developing countries. Whole-cell killed pneumococcal vaccines were confirmed to confer protection (Hvalbye *et al.*, 1999). Recent studies have reported that whole-cell killed pneumococcal vaccines confer cross protection against colonization and invasive disease by capsulated pneumcocci when inoculated intranasally or subcutaneously (Malley *et al.*, 2001; Lu *et al.*, 2010).

Streptococcus pneumoniae strain SPY1 is a capsule-negative mutant derived from pneumococcus capsular serotype 2 strain in our previous study (Yin *et al.*, 2009). In this research, SPY1 was inactivated with 70% ethanol and protections elicited by intranasal immunization with killed SPY1 against colonization and lethal infection caused by different *S. pneumoniae* serotypes in mice were evaluated to investigate the potential of SPY1 as a whole-cell vaccine candidate. Furthermore, the underlying protection mechanism was initially investigated not only by measurement of antibody and cytokines but also by using active and passive protection models.

Materials and Methods

Animals

BALB/c mice and BALB/c nude mice (4 to 6 weeks, female) were purchased from Chongqing Medical University, Chongqing, China. The mice were kept under specific pathogen-free conditions at the animal facilities of Chongqing Medical University during the time of the experiments. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee's guidelines of Chongqing Medical University.

Bacterial strains

Streptococcus pneumoniae strains D39 (NCTC 7466, sero-

type 2) and R6 were obtained from the National Collection of Type Cultures (NCTC, UK). *S. pneumoniae* strain TIGR4 (ATCC BAA-334, serotype 4) was obtained from the American Type Culture Collection (ATCC, USA). *S. pneumoniae* clinical isolates CMCC 31693 (serotype 19F), CMCC 31207 (serotype 6B), CMCC 31614 (serotype 14), and CMCC 31203 (serotype 3) were obtained from the National Center for Medical Culture Collections (CMCC, China). All *S. pneumoniae* strains were grown in Columbia sheep blood agar plates or in C plus Y medium.

Acquisition of SPY1 vaccine strain

SPY1 was obtained after transformation to construct the *SPD_1672* deletion mutant according to the method previously described (Yan *et al.*, 2012). *SPD_1672* was deleted by an in-frame replacement by the *erm* cassette gene. The primers used for *SPD_1672* deletion are listed in Table 1. SPY1 was approved to be stable over 200 generation consecutive passages (Yin *et al.*, 2009).

Vaccine preparation

The preparation of 70% ethanol-killed whole-cell vaccine was performed as previously described (Lu *et al.*, 2010). SPY1 was grown at 37°C in C plus Y medium to approximately 2×10^8 CFU/ml. Further steps were performed at 4°C. The cells were collected by centrifugation, washed twice with lactated Ringer's solution (LR) (102 mM NaCl, 28 mM $NaC_3H_5O_3$, 1.5 mM $CaCl_2$, and 4 mM KCl), and then resuspended in LR to an A_{600} of 10. Ethanol was added to 70% (vol/vol) gradually within 15 min. The suspension was stirred for 55 min, and the cells were pelleted again, washed twice, resuspended in LR, cultured to ascertain sterility, and then stored at -80°C in single-use aliquots.

Active and passive immunization

The final vaccine mixture for routine immunization contained 1×10^8 CFU of 70% ethanol-inactivated SPY1 and 1 μg of cholera toxin adjuvant (CT, Sigma-Aldrich, USA) per 20 μl dose. Groups of BALB/c mice were anesthetized with diethyl ether and received vaccines or adjuvant alone intranasally for four times at 1-week interval. The positive control group comprised mice that received intraperitoneal injection of PPV23 (Chengdu Institute of Biological Products, China) for three times (5 μg per serotype) at 1-week interval. One week after the last immunization, blood was obtained by retro-orbital puncture, and serum samples were separated by centrifugation and stored at -80°C until use.

Passive-adoptive transfer was performed as previously described (Feunou *et al.*, 2010). Briefly, 200 µl of serum from immunized or control mice was intraperitoneally transferred to naïve BALB/c mice, and approximately 1×10^8 whole spleen cells from immunized or control mice were intraperitoneally transferred to naïve BALB/c nude mice. The mice were intraperitoneally infected with 1×10^3 CFU of D39 after 24 h, and survival rates were observed for 21 d.

The level of anti-pneumococcal specific antibodies

The expression of capsular polysaccharide was studied by whole-cell ELISA as previously described (Lu *et al.*, 2010). Washed SPY1 was diluted with coating buffer (0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$) to a final optical density of 0.1 and fixed by overnight incubation at 4°C in 96-well plates. After blocking in Phosphate-buffered saline (PBS) (2.7 mM KCl, 137 mM NaCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.0)/Tween 20 (0.1%) with 3% bovine serum albumin (Sigma), dilutions of type 2 capsular polysaccharide antisera (SSI, Denmark, REF 16745) were added and incubated at 37°C for 1 h. The plates were washed, and relevant secondary antibodies (Santa Cruz Biotechnology, USA) were added and incubated at 37°C for 45 min. The plates were washed and developed with tetramethylbenzidine as the substrate, and the absorbance at 450 nm was recorded.

Sera vaccine-specific total IgG and IgG subclasses IgG1, IgG2a, IgG2b, and IgG3, in immunized mice were determined by whole-cell ELISA using 70% ethanol-killed coated SPY1 and diluted SPY1-immunized mouse serum. Nasal washes and bronchoalveolar lavage fluids (BALFs) were also collected from intranasally immunized mice and then stored at -80°C until analysis. ELISA measurements of IgA in nasal washes and IgA in BALFs were carried out to determine serum IgG levels as previously described (Lu *et al.*, 2010).

Determination of cytokines in mouse serum

One week after the final immunization, cellular suspensions of splenocytes were obtained by passing spleens from immunized or control mice through 70 µm cell strainers. After washing and removal of red blood cells by hemolysis, the cells were plated in 24-well tissue culture plates at a concentration of 2×10^6 cells/well in 1 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Hyclone, USA). Concanavalin A (5 mg/ml, Sigma) as positive control and 70% ethanol-inactivated SPY1 (equivalent to 10^7 CFU/ml) were added, respectively. The supernatants were collected at 24, 48, 72, and 96 h poststimulation following centrifugation and then stored at -80°C until analysis. The levels of interferon gamma (IFN-γ), interleukin-4 (IL-4), interleukin-17A (IL-17A), and interleukin-10 (IL-10) in the culture supernatants were detected by ELISA kits (Biolegend, USA) according to the manufacturer's recommendations. The samples were diluted when required.

Opsonophagocytosis assay

The opsonophagocytosis assay was performed with differentiated HL-60 cells (promyelotic leukemia cells, CCL240; American Type Culture Collection, USA) (Romero-Steiner *et al.*, 1997). TIGR4 was grown on Columbia sheep blood agar plates overnight and then diluted to 50 CFU/µl in opsonophagocytosis buffer (Hanks' buffer with Ca^{2+}, Mg^{2+}, and 0.1% gelatin). Approximately 20 µl (1000 CFU) of TIGR4 was added to 20 µl of mouse serum in 96-well plates, followed by incubation at 37°C in 5% CO_2 atmosphere for 15 min. Subsequently, 10 µl of sterile baby rabbit serum as complement source and 40 µl of differentiated HL-60 cells were sequentially added. The plate was incubated at 37°C for 45 min with horizontal shaking (220 rpm), and a 5 µl aliquot from each well was plated. Killing was defined as the percent reduction CFUs in wells containing cocultures of phagocytes and bacteria compared with wells containing bacteria only.

Experimental infection

In the nasopharyngeal colonization model, eight mice per group were anesthetized and challenged with approximately 1×10^8 CFU of strain 19F (CMCC 31693) and strain 4 at 2 weeks following the last vaccination. At 3 d postchallenge, the mice were sacrificed, and nasal washes (approximately 0.1 ml) were harvested. The lungs were removed, washed in sterile PBS, and then homogenized. The nasal washes and lung homogenates were serially diluted with sterile PBS, and 100 µl aliquots of the final dilutions were plated on Columbia sheep blood agar plates. The number of colonies was counted after incubation overnight at 37°C in a 5% CO_2 atmosphere.

In the pneumococcal lethal intranasal challenge model, 12 mice per group were anesthetized and then inoculated intranasally with 30 µl of bacterial suspension containing *S. pneumoniae* strains D39 (7×10^7 CFU), CMCC 31614 (4×10^8 CFU), CMCC 31207 (8×10^8 CFU), or CMCC 31203 (4×10^8 CFU). The survival of each mouse was monitored for 21 d.

Statistical analysis

Data were analyzed using two-tailed unpaired Student's *t* test or one or two-way ANOVA, as appropriate. Colonization density was compared by Mann-Whitney U test. Survival studies were analyzed with log-rank test. Statistical analysis was performed with the aid of Graph Pad Prism (Prism 5; Graphpad Software, USA). Statistical significance was considered at $P<0.05$.

Results

In vitro characterization of *S. pneumoniae* SPY1

Strain SPY1 formed smaller bacterial colonies on Columbia sheep blood agar plate. The transmission electron micrographs showed that SPY1 had a significantly smaller capsular polysaccharide layer compared with strain D39 (Fig. 1A). As a negative control, strain R6 had no detectable capsule detected. This strain is a stable unencapsulated experimental strain derived from serotype 2 pneumococcal D39 (Kadioglu and Andrew, 2005). The results of ELISA with type 2 capsular polysaccharide antisera verified that the expression of capsular polysaccharide by SPY1 was significantly less than that of D39 and was the same as that of R6 (Fig. 1B).
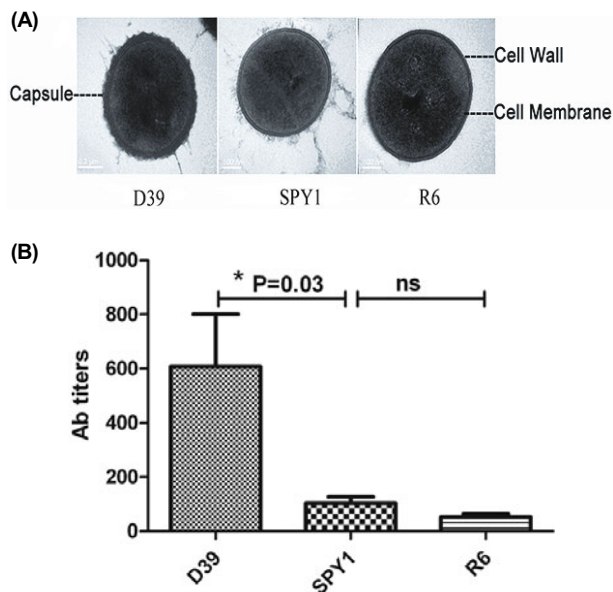
(A)

Capsule

Cell Wall

Cell Membrane

D39　　　SPY1　　　R6

(B)

Fig. 1. Significantly less capsular polysaccharide of SPY1. (A) Transmission electron micrograph analysis of D39 (left panel), SPY1 (middle panel), and R6 (right panel). The scale bar represents 0.2 μm (left panel) or 0.1 μm (middle panel and right panel). (B) Expression of capsular polysaccharide tested by ELISA with type 2 capsular polysaccharide antisera. * means $P<0.05$ compared with the control group.

Humoral immune response elicited by intranasal immunization with SPY1

Intranasal immunization with inactivated SPY1 in combination with CT adjuvant induced not only high levels of IgG in serum but also SPY1-specific IgA in nasal washes and BALFs; by contrast, no antibody response was detected in the CT group (Fig. 2A and 2B). This result implies the direct activation of mucosal and systemic humoral immune responses caused by SPY1. IgG subtypes were determined to assess the types of immune responses. IgG1 and IgG2b responses were predominantly induced, indicating a Th2-based vaccine-specific humoral immune response by intranasal vaccination with SPY1 (Fig. 2C).

S. pneumoniae SPY1-specific cellular immune response

Moreover, cytokine responses induced by intranasal immunization with SPY1 were determined to investigate the vaccine-specific cellular immune response. The levels of IFN-γ, IL-17A, IL-4, and IL-10 in the culture supernatants of mouse splenocytes were determined. The levels of IL-4 and IL-17A secreted by splenocytes from the SPY1-immunized mice were significantly higher than those from the control mice, peaking at 72 h poststimulation (Fig. 3B and 3C). The level of IL-10 from the immunized mice was also significantly higher than that from the control mice (Fig. 3D). However, compared with the adjuvant control mice, the SPY1-immunized mice demonstrated no vaccine-specific IFN-γ stimulation (Fig. 3A).

Protection against pneumococcal colonization by immunization with SPY1

The efficacy of vaccination with SPY1 against pneumococcal colonization was evaluated with two heterologous strains. The immunized mice were intranasally challenged with 1 ×
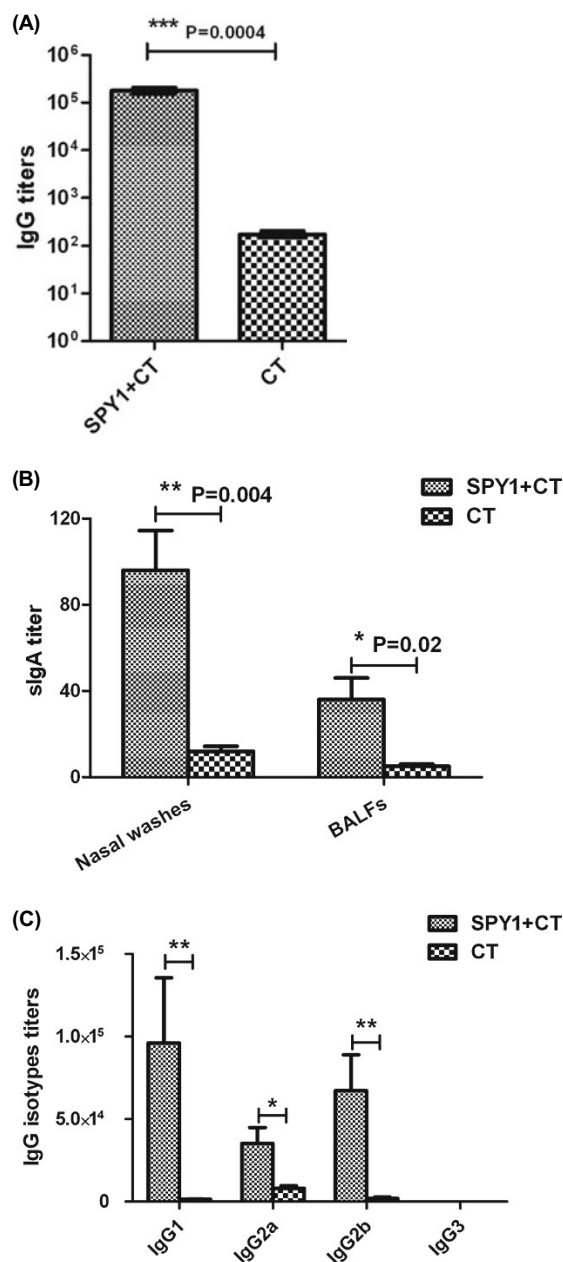
(A)

(B)

(C)

Fig. 2. Effect of mucosal immunization with 70% ethanol-inactivated SPY1 on antibody responses in mice. (A) Vaccine-specific IgG responses in serum, with the CT adjuvant group as negative control. (B) Vaccine-specific IgA responses in nasal washes and BALFs, with the respective CT adjuvant group as negative control. (C) IgG isotypes in serum samples after immunization, with the respective CT adjuvant group as negative control. The antibody titers were expressed as the reciprocal of the highest serum samples dilution, giving 2.1-fold of the background absorbance at 450 nm. * means $P<0.05$, ** means $P<0.01$, and *** means $P<0.001$, compared with the CT control group.

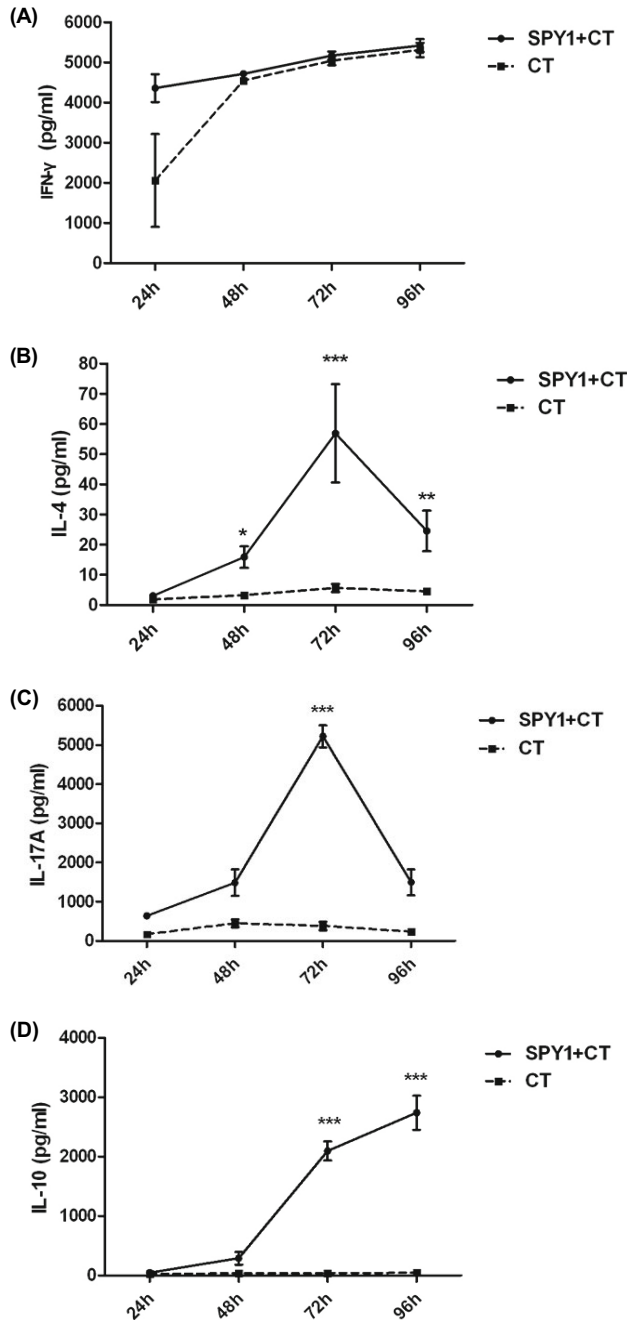**Fig. 3.** **Effect of intranasal immunization with 70% ethanol-inactivated SPY1 on cellular immune responses in mice.** Splenocytes were harvested from SPY1 immunized (solid line) or CT adjuvant control mice (dashed line) for the detection of (A) IFN-γ, (B) IL-4, (C) IL-17A, and (D) IL-10 levels by ELISA. Values are represented as Mean ± SEM. * means $P<0.05$, ** means $P<0.01$, and *** means $P<0.001$, compared with the CT control group.

10^8 CFU of either serotype strain 19F (CMCC 31693) or serotype strain 4 (TIGR4), and bacterial loads in nasal washes and lung homogenates were determined. Intranasal immunization with inactivated SPY1 drastically reduced bacterial loads in the nasopharynx (Fig. 4A) and lungs (Fig. 4B).
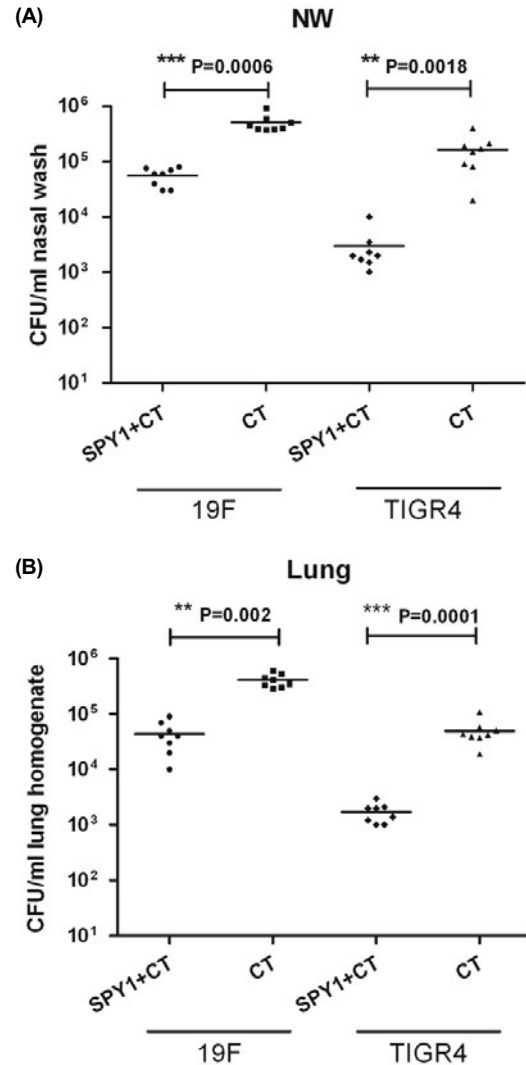

Fig. 4. **Effect of intranasal immunization with SPY1 in pneumococcal colonization model.** Groups of BABL/c mice were immunized with SPY1 plus CT or CT control alone and intranasally challenged with *S. pneumoniae* strains 19F (1×10^8 CFU) or 4 (1×10^8 CFU) at 2 weeks after the final immunization. Bacterial loads in (A) nasal washes and (B) lung homogenates were determined at 3 d postinfection. Each point represents one mouse, and each horizontal line represents the median CFU in each group. ** means $P< 0.01$ and *** means $P<0.001$, compared with the CT control group.

Protection against pneumococcal lethal challenge by immunization with SPY1

Two weeks after the final immunization, the mice were challenged intranasally with a lethal dose of pneumococcal serotype 2 (D39), serotype 14 (CMCC 31614), serotype 3 (CMCC 31203), or serotype 6B (CMCC 31207), and survival was monitored for 21 d. Approximately 83.3%, 78.5%, 73.3%, and 75% of the immunized mice survived from D39 (Fig. 5A), CMCC 31614 (Fig. 5B), CMCC 31203 (Fig. 5C), and CMCC 31207 (Fig. 5D) infections, respectively. All control mice died after D39 challenge, and only 20%, 25%, and 30% control mice survived from CMCC 31614, CMCC 31203, and CMCC 31207 infections, respectively. In addition, the
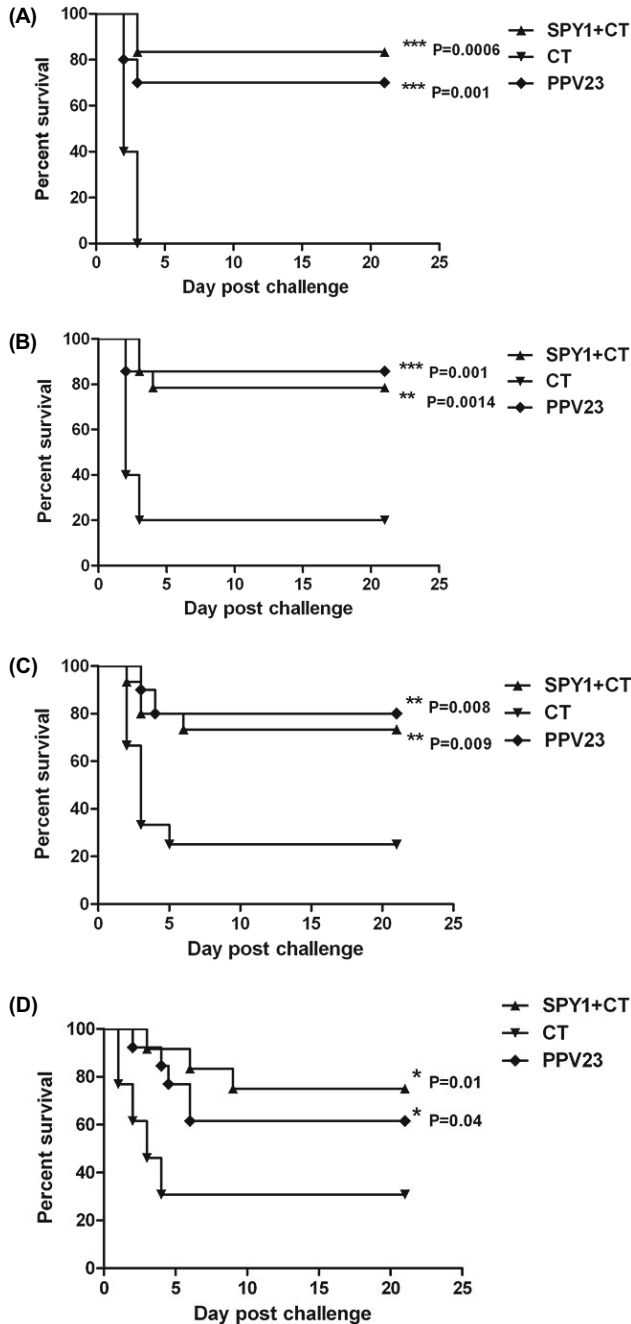
Fig. 5. Intranasal immunization with SPY1 induces effective protection against lethal *S. pneumoniae* infection. BABL/c mice were immunized with SPY1 plus CT (*n*=12) or CT control (*n*=12) and then intranasally challenged with *S. pneumoniae* strains (A) D39 (7×10^7 CFU), (B) serotype 14 (CMCC 31614, 4×10^8 CFU), (C) serotype 3 (CMCC 31203, 4×10^8 CFU) or (D) serotype 6B (CMCC 31207, 8×10^8 CFU). Mice vaccinated intraperitoneally with PPV23 served as positive control. The survival of each mouse was monitored for 21 d. * means $P<0.05$, ** means $P<0.01$, and *** means $P<0.001$, compared with the CT control group.

protection efficacy of SPY1 immunization was comparable with that of PPV23 vaccination, which conferred 70%, 85%, 80%, and 60% of mouse survival against the D39, CMCC 31614, CMCC 31203, and CMCC 31207 strains, respectively.

Roles of B- and T-cells in protection induced by vaccine SPY1

The contributions of T cells and antibodies in the protection conferred by SPY1 against lethal D39 infection were evaluated using transfer experiments. Serum (200 µl) from immunized mice or the adjuvant control mice were intraperitoneally transferred to groups of eight naïve BALB/c mice and whole spleen cells (1×10^8) from immunized mice or the adjuvant control mice were intraperitoneally transferred to groups of eight naïve BALB/c nude mice. At 24 h after transfer, the mice were intraperitoneally infected with 1×10^3 CFU of *S. pneumoniae* D39, and survival rates were observed. Prior to transfer, we verified that all naïve BALB/c mice and nude mice died in 2 d after intraperitoneal infection with 1×10^3 CFU of *S. pneumoniae* D39 (Fig. 6). As shown in Fig. 6A, the transfer of 200 µl serum from the immunized mice conferred strong protection, as evidenced by the higher survival rate and longer survival time after the challenge. The mean survival time of the adjuvant control mice was similar to that of the infected control mice, suggesting that no protection was achieved with serum transfer from the adjuvant control mice. The transfer of spleen cells from the SPY1-immunized mice conferred a significant level of protection to the naïve BALB/c nude mice (T-cell function deficient) against infection with *S. pneumoniae* D39. This result was evidenced by the significant increase in survival time compared with the nontransferred mice or naïve
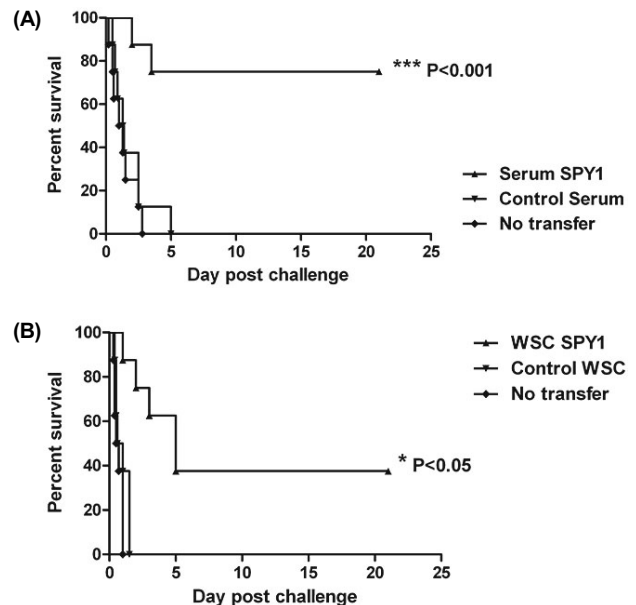

Fig. 6. Transfer of protection by spleen cells and serum from 70% ethanol-inactivated SPY1-immunized mice. (A) Groups of BALB/c mice (*n*=8) were intraperitoneally injected with 200 µl of pooled serum, and (B) BALB/c nude mice were intraperitoneally injected with approximately 1×10^8 of pooled whole spleen cells (WSC) from SPY1-immunized mice. The respective control mice were injected with 200 µl of pooled sera or 1×10^8 of pooled whole spleen cells from adjuvant control mice. At 24 h after injection, the mice were infected intraperitoneally with 1×10^3 CFU of D39, and survival rates were observed for 21 d. * means $P<0.05$ and *** means $P<0.001$ compared with the CT control group.
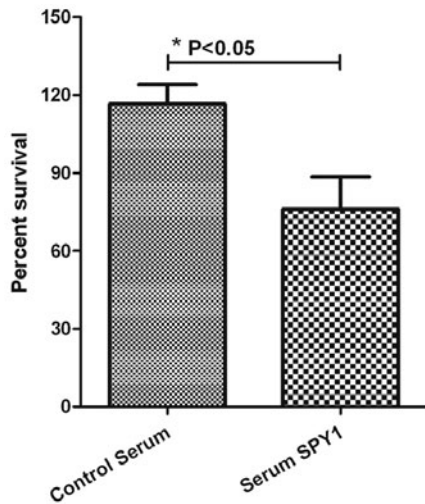
Fig. 7. *In vitro* **survival of** *S. pneumoniae* **TIGR4 in the presence of serum from SPY1-immunized mice.** 1000 CFU of *S. pneumoniae* TIGR4 was incubated at 37 °C in a 5% CO_2 atmosphere for 15 min in the presence of 20 µl of serum from 70% ethanol-inactivated SPY1-immunized or adjuvant control mice. Then, 10 µl of sterile baby rabbit serum and 40 µl of differentiated HL-60 cells were sequentially added. The plate was incubated at 37°C for 45 min with horizontal shaking (220 rpm), and 5 µl of aliquot from each well was plated. Killing was defined as the percent reduction CFUs in wells containing cocultures of phagocytes and bacteria compared with wells just containing bacteria. Results are presented as percent survival relative to that of a PBS control (in the absence of serum) for three mice per group and are representative of three independent experiments. * means *P*<0.05 compared with the CT control group.

mice that received spleen cells from adjuvant control donors (Fig. 6B). These data demonstrate that B and T cells are involved in the 70% ethanol-inactivated SPY1-mediated protection.

Serum from 70% ethanol-inactivated SPY1-vaccinated mice can enhance opsonophagocytosis of pneumococcal TIGR4 in vitro

The success of serum from SPY1-immunized mice to protect naïve mice against pneumococcal infection suggests that vaccine-specific antibodies can enhance the opsonophagocytosis of *S. pneumoniae* by neutrophils, which are the leading immune defense against *S. pneumoniae* (Romero-Steiner *et al.*, 1997). We conducted the opsonophagocytosis to compare the survival of pneumococcal TIGR4 after incubation with or without serum from adjuvant control or SPY1-immunized mice. As shown in Fig. 7, in the presence of SPY1-specific serum, fewer bacteria survived under the function of neutrophils compared with the control group. This result suggests that SPY1-specific antibodies can enhance the opsonophagocytosis of *S. pneumoniae*.

Discussion

Streptococcus pneumoniae expresses at least 93 structurally unique capsular polysaccharides. However, the currently used pneumococcal polysaccharide and polysaccharide conjugate vaccine are effective only against limited pneumo-

coccal serotypes. Thus, finding a novel pneumococcal vaccine that could elicit broad serotype-independent protection against pneumococci is desirable. Our previous research showed that pneumococcal serotype 2 mutant strain SPY1 as a live attenuated vaccine can provide effective serotype-independent protection but with deficiencies in safety. Therefore, in this study, SPY1 was inactivated by 70% ethanol to abolish the potential virulence. Then, the protective ability and related immune mechanism of SPY1 were evaluated.

Traditional vaccine inactivation methods such as heat, formalin, or UV radiation have some disadvantages. Treatment with UV radiation cannot guarantee thorough inactivation of live bacteria, thereby causing concealed safety problem. Heat and formalin treatments could degenerate bacterial surface proteins, thereby seriously destroying the antigenicity and immunogenicity of vaccines (Furuya *et al.*, 2010). In the present study, the whole-cell vaccine SPY1 was treated with 70% (vol/vol) ethanol at 4°C, retaining intact surface antigens as much as possible and producing more immunogenic antigens (Lu *et al.*, 2010). The protective effect of SPY1 killed by heat was also not satisfactory (data not shown). In the present study, killed SPY1 was delivered via intranasal route in combination with CT adjuvant, which can effectively induce mucosal and systemic immune responses and thus provide comprehensive protection (Malley *et al.*, 2004). In addition, easily administered preparation and non-sterile injection facility are required for the delivery of the vaccine, thereby providing convenience and suggesting suitability for application in developing countries (Black *et al.*, 2000).

Asymptomatic colonization on the upper respiratory tract is not only an initial and vital stage for *S. pneumoniae* to cause invasive infections (Ring *et al.*, 1998; Bogaert *et al.*, 2004) but also an essential element for horizontal transmission within the community (Austrain, 1986). Therefore, vaccines that can eliminate the nasopharyngeal colonization are assumed to contribute to the protection against invasive infection and trigger herd immunity in the population. The two immune factors, activated Th17 cellular immune response and vaccine-specific secretory-IgA (sIgA) antibodies, closely influence *S. pneumoniae* nasopharyngeal colonization (Zhang *et al.*, 2009; Fukuyama *et al.*, 2010). In agreement with their results, we observed vaccine-specific sIgA and increased IL-17A in the killed SPY1-immunized mice. Furthermore, we demonstrated that nasal and lung colonization of pneumococcal strains 19F and TIGR4 in killed SPY1-vaccinated mice were significantly reduced.

Humoral immune response is an important immune factor in pneumococcal vaccine protection (Dagan *et al.*, 2002). In the present research, intranasal immunization with 70% ethanol-killed SPY1 in combination with CT adjuvant induced high levels of systemic IgG and secretory IgA in nasal washes and BALFs in BALB/c mice. This result indicates the activation of humoral immune responses by vaccination. The protection provided by the passive immunization of vaccinated serum and vaccine-specific serum enhanced opsonophagocytosis of pneumococcal TIGR4 *in vitro* confirming the vital function of humoral immune response in protection elicited by SPY1 vaccination. We also observed the activation of T-cell immune response in immunized mice,

as evidenced by the result that the transfer of spleen cells from the vaccinated mice protected naïve BALB/c nude mice against pneumococcal D39 infection. The significantly increased vaccine-specific IL17A and IL-4 in the spleen cells of immunized mice indicated that the activation of Th17 and Th2 immune responses was stimulated by SPY1. The increase in anti-inflammatory cytokine IL-10 indicated the activation of regulatory T-cells, which were recently reported to control susceptibility to invasive pneumococcal pneumonia in mice. In addition, strong T regulatory cell responses are correlated with pneumococcal infection resistance (Pido-Lopez *et al.*, 2011; Neill *et al.*, 2012). IL-10 could also promote B-cell survival, proliferation, and antibody production (Saraiva and O'Garra, 2010). Overall, intranasal immunization with SPY1 induced a combined humoral and cellular immune response that provides protection against colonization and lethal infection of different pneumococcal strains.

Aside from the homologous virulent strain D39, heterologous *S. pneumoniae* serotype isolates (serotypes 19F, 4, 14, 6B, and 3), which are prevalent in China (Liu *et al.*, 2008), were used in the challenge study to further investigate the protection induced by SPY1. As an unencapsulated pneumococcal strain, SPY1 induced mucosal and systemic immune responses independent of capsular polysaccharide and thus elicited efficient serotype-independent cross protection.

Several studies reported on unencapsulated and avirulent pneumococcal strains as vaccine candidates. Intraperitoneal immunization with heat-inactivated and lyophilized R36A (an unencapsulated derivative of D39) elicited humoral immune response and protected 70% of immunized mice against invasive infection of pneumococcal strain WU2 (serotype 3) (Mi *et al.*, 2000). However, there has been no data about protection of R36A against pneumococcal colonization. Rx1AL⁻ is an autolysin (LytA)-negative mutant of Rx1 (capsule-negative mutant derived from D39, Malley *et al.*, 2001). The protection of Rx1AL⁻ was only evaluated in pneumococcal strain 6B colonization model in mice (Malley *et al.*, 2001, 2004; Lu *et al.*, 2010). SPY1, by comparison, can elicit cross-protection against not only colonization of pneumococcal strains 19F and 4, but also invasive infection of pneumococcal strains 2, 3, 6B and 14 in mice. In addition, the protection induced by SPY1 is mediated by humoral and cellular immune responses. We observed no protection against colonization and invasive infection when 70% ethanol-inactivated R6 was used as a pneumococcal vaccine (data not shown).

The protective effects of Rx1AL⁻ and EHpep27 against pneumococcal colonization were better than those of inactivated SPY1. This finding can be attributed to different types of mice that were used. C57BL/6 mice and CD1 mice were used to evaluate the protective effects of Rx1AL⁻ and EHpep27, respectively. Mice differ in their susceptibility and resistance to pneumococcal infection (Kadioglu and Andrew, 2005). In addition, the virulence of pneumococcal strains used in the colonization model in this study is different from that used in the studies mentioned above. In the absence of a capsule surrounding SPY1 during vaccination, several pneumococcal surface antigens, such as pneumolysin, choline-binding proteins, pneumococcal surface antigen A (Kadioglu

et al., 2008), and other potential protein vaccine candidates (Oliveira *et al.*, 2010; Wu *et al.*, 2010; Gong *et al.*, 2011), were exposed to the host, probably being responsible for the protective immunity.

In conclusion, intranasal immunization with 70% ethanol-inactivated SPY1 plus CT adjuvant can induce strong mucosal and systemic humoral immune response and cellular immune responses. These responses may be the basis for the sufficient serotype-independent protection against pneumococcal colonization and lethal pneumococcal infection. Overall, the 70% ethanol-inactivated unencapsulated pneumococcal strain SPY1 is a safe and effective *S. pneumoniae* whole-cell vaccine candidate.

Acknowledgements

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