



# Immunization with pneumococcal elongation factor Tu enhances serotype-independent protection against *Streptococcus pneumoniae* infection



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## ABSTRACT

Vaccination is an effective strategy to prevent pneumococcal diseases. Currently, licensed vaccines include the pneumococcal polysaccharide vaccine (PPSV) and the pneumococcal conjugate vaccine (PCV), which target some of the most common of the 94 serotypes of *S. pneumoniae* based on their capsular composition. However, it has been reported that PPSV is not effective in children aged less than 2 years old and PCV induces serotype replacement, which means that the pneumococcal population has changed following widespread introduction of these vaccines, and the non-vaccine serotypes have increased in being the cause of invasive pneumococcal disease. Therefore, it is important that there is development of novel pneumococcal vaccines to either replace or complement current polysaccharide-based vaccines. Our previous study suggested that *S. pneumoniae* releases elongation factor Tu (EF-Tu) through autolysis followed by the induction of proinflammatory cytokines in macrophages via toll-like receptor 4, that may contribute to the development of pneumococcal diseases. In this study, we investigated the expression of EF-Tu in various *S. pneumoniae* strains and whether EF-Tu could be an antigen candidate for serotype-independent vaccine against pneumococcal infection. Western blotting and flow cytometry analysis revealed that EF-Tu is a common factor expressed on the surface of all pneumococcal strains tested, as well as intracellularly. In addition, we demonstrate that immunization with recombinant (r) EF-Tu induced the production of inflammatory cytokines and the IgG1 and IgG2a antibodies in mice, and increased the CD4<sup>+</sup> T-cells proportion in splenocytes. We also reveal that anti-EF-Tu serum increased the phagocytic activity of mouse peritoneal macrophages against *S. pneumoniae* infection, independent of their serotypes. Finally, our results indicate that mice immunized with rEF-Tu were significantly and non-specifically protected against lethal challenges with *S. pneumoniae* serotypes (2 and 15A). Therefore, pneumococcal EF-Tu could be an antigen candidate for the serotype-independent vaccine against pneumococcal infection.

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## 1. Introduction

*Streptococcus pneumoniae*, also known as pneumococcus, is a gram-positive diplococcus and a major human pathogen. This bac-

terium asymptotically colonizes the upper respiratory airway and causes common clinical syndromes, such as otitis media, sinusitis, bronchitis, and empyema, or even severe life-threatening diseases, including pneumonia, meningitis, and septicemia [1,2]. Pneumococcal infections have led to significant morbidity and mortality worldwide, especially in children under 5 years old and adults over 65 years old in developing countries [3]. The primary treatment of pneumococcal diseases involves the use of effective antibiotics. However, with the widespread availability and overuse of antibiotics, drug-resistant strains of

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pneumococci have become increasingly prevalent around the world [4]. Our previous data suggested that around 40% of *S. pneumoniae* isolates from pneumococcal diseases were resistant to benzylpenicillin in Japan. In addition,  $\geq 80\%$  of *S. pneumoniae* isolates were resistant to macrolides [5]. Therefore, basic research is still necessary to identify additional therapeutic targets to prevent pneumococcal pneumonia.

Vaccination is an effective strategy to prevent pneumococcal diseases. Currently, licensed vaccines include the pneumococcal polysaccharide vaccine (PPSV) and the pneumococcal conjugate vaccine (PCV), which target some of the most common of the 94 serotypes of *S. pneumoniae* based on capsular composition. The 23-valent PPSV induces T-cell-independent immune response in adults, but PPSV is not effective in children aged less than 2 years old [6]. PCVs, such as the 7-, 10-, and 13-valent ones, elicit T-cell-dependent immune response and are effective in young children. However, PCVs reportedly induce serotype replacement, which means that the pneumococcal population has changed following widespread introduction of these vaccines, and the non-vaccine serotypes have increased in being the cause of invasive pneumococcal disease [7,8]. Therefore, many researches have focused on the development of novel pneumococcal vaccines to either replace or complement current polysaccharide-based vaccines [9]. In particular, a number of pneumococcal protein antigens have been studied as vaccine candidates against pneumococcal infection [10,11].

Our previous study revealed that *S. pneumoniae* releases elongation factor Tu (EF-Tu) through autolysis followed by the induction of proinflammatory cytokines in macrophages via toll-like receptor 4, that may contribute to the development of pneumococcal diseases [12]. EF-Tu is one of the most abundant and conserved bacterial proteins found in different species of bacteria and fungi, including pathogenic and non-pathogenic organisms [13–15]. It belongs to the group of G-proteins and plays a crucial role in the elongation cycle of translation as a universal carrier of aminoacyl-tRNA from the cytosol of the cell to the A site of the ribosome [16,17]. Moreover, EF-Tu is a component of the bacterial membrane cytoskeleton involved in adhesion of several pathogenic bacteria to the host cells [18]. In *S. pneumoniae*, it has been reported that EF-Tu is localized on the cell surface and may increase the pathogenicity through the complement inhibition and degradation of extracellular matrix [19].

In this study, we investigated the expression of EF-Tu in various *S. pneumoniae* strains. In addition, we also analyzed whether EF-Tu induces immune responses in mice and could be an antigen candidate for the development of serotype-independent vaccine against pneumococcal infection.

## 2. Materials and methods

### 2.1. Bacterial strains

*S. pneumoniae* strain D39 (NCTC 7466, serotype 2) was purchased from the National Collection of Type Cultures (Salisbury, UK). Other *S. pneumoniae* strains NP3, NP7F, NP10A, NP12F, NP15A, and NP19A (serotypes 3, 7F, 10A, 12F, 15A, and 19A, respectively) were clinically isolated from patients with invasive pneumococcal disease, at Niigata University Medical and Dental Hospital. All strains were grown in tryptic soy broth (TSB; Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C under aerobic conditions. Optical densities (OD) of bacterial cultures were analyzed using miniphoto518R (TAITEC, Saitama, Japan) for determination of bacterial growth.

### 2.2. Animals

Male 6–8-week-old BALB/c mice (Nihon CLEA, Tokyo, Japan) were maintained under standard conditions in accordance with our institutional guidelines. All animal experiments were approved by the Institutional Animal Care and Use Committee of Niigata University (SA00002).

### 2.3. Expression and purification of pneumococcal EF-Tu

Recombinant (r) EF-Tu was expressed in *Brevibacillus choshinensis*, using the *Brevibacillus* secretory expression system (TaKaRa, Shiga, Japan) according to the manufacturer's instructions [20,21]. Briefly, chromosomal DNA from *S. pneumoniae* strain D39 was extracted and purified using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA). The forward primer (5'-GATGACGATGACAAAATGGCAAAGAAAAATACGA-3') and reverse primer (5'-CATCCTGTTAAGCTTTTAAAGCTTCGATTCTGTAA-3') were used to amplify the *tuf* gene (Ref. Seq. WP\_001040724.1) by polymerase chain reaction (PCR). The primers were designed with 15-base 5' overhangs that were homologous to pBIC expression vector, and with sequences (20 bases) for amplifying the target gene at the 3' ends. The resulting PCR fragments were mixed with the vector and transformed into *Brevibacillus* competent cells. Cells were grown in medium supplemented with 50 µg/mL neomycin (Thermo Fisher scientific, Waltham, MA, USA) for selection, after which rEF-Tu with an N-terminal His<sub>6</sub>-tag was expressed in the culture supernatant and purified using an Ni-nitrilotriacetic acid column (Qiagen, Hilden, Germany). The concentration of protein was measured by Bicinchoninic acid protein assay kit (Thermo Fisher Scientific). The amount of lipopolysaccharides (LPS) in 1 µg of purified protein was determined to be <2 pg, as measured using an LPS-detection kit (GenScript, Piscataway Township, NJ, USA).

### 2.4. Production of polyclonal antibodies against rEF-Tu

Polyclonal antibodies against pneumococcal EF-Tu were generated by Eurofins Genomics K.K. (Tokyo, Japan). Briefly, a rabbit was immunized intracutaneously with 200 µg of rEF-Tu emulsified with an equal volume of Freund's complete adjuvant. Two weeks later, booster immunizations were conducted using the same amount of rEF-Tu protein emulsified with incomplete Freund's adjuvant. After another two weeks, second booster immunizations were conducted. Ten days after the second booster immunizations, rabbits were sacrificed and sera were extracted from the blood that was collected by cardiac puncture. Immunoglobulin G (IgG) antibody was purified using protein A column (Thermo Fisher Scientific).

### 2.5. Western blot analysis

*S. pneumoniae* strains were inoculated into TSB and incubated at 37 °C until they reached early stationary phase (OD<sub>600</sub> = 0.2). Thereafter, culture supernatants were removed by centrifugation at 3000 ×g for 10 min. The bacterial pellets were resuspended in 2% sodium dodecyl sulfate (SDS) sample buffer and homogenized with a MagNA Lyser instrument (Roche Diagnostics, Basel, Switzerland), using 0.1 mm silica beads in a 2-mL tube (MP Biomedicals, Santa Ana, CA, USA). The whole-cell lysate mixed with SDS-sample buffer was heated at 95 °C for 3 min, then separated by SDS-PAGE using 12% gels (Bio-Rad Laboratories, Hercules, CA, USA), and later transferred to polyvinylidene difluoride membranes (Merck Millipore, Billerica, MA, USA). The membranes were incubated with blocking reagent (Nacalai Tesque Kyoto, Japan) to block nonspecific binding, and probed with rabbit anti-EF-Tu

antibody and anti-GAPDH antibody diluted in Tris-buffered saline containing 0.05% Tween 20 (TaKaRa). The membrane was then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (Cell Signaling Technology, Danvers, MA, USA) in Tris-buffered saline containing 0.05% Tween 20. The membrane was treated with HRP substrates (GE Healthcare, Little Chalfont, UK) and analyzed by a chemiluminescence detector (Fujifilm, Tokyo, Japan). Antibody against pneumococcal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and rGAPDH were generated as described previously [12].

### 2.6. Detection of EF-Tu on pneumococcal surface

*S. pneumoniae* strains were grown until they reached early stationary phase and fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS), followed by incubation of the cells in a blocking solution (Thermo Fisher Scientific) for 30 min. For detection of EF-Tu on the surface, bacterial cells were stained with rabbit anti-EF-Tu antibody or nonimmune control antibody in the blocking solution. After 1 h of incubation at 4 °C, the secondary AlexaFluor 488-conjugated goat anti-rabbit IgG antibody (Thermo Fisher Scientific) in blocking buffer was added, followed by 1 h of incubation in the dark. Cells were then analyzed using NovoCyte flow cytometer with NovoExpress software (ACEA Biosciences, San Diego, CA, USA) and confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

### 2.7. Immunization of mice

Mice were divided into three groups and subcutaneously injected with either 50 µg of rEF-Tu mixed with an equal volume of Inject Alum Adjuvant (Thermo Fisher Scientific), or with adjuvant only, or with sterile PBS only. All the groups were immunized three times each at 0, 2, and 4 weeks. Blood samples in each group was collected at 1 day and 1 week after the first immunization for cytokine measurement, and again obtained at 1 week after the last immunization for antibodies evaluation. The serum was extracted from the blood and stored at –20 °C until further use.

### 2.8. Measurement of cytokines

The levels of interleukin-6 (IL-6), tumor necrosis factor alpha (TNF $\alpha$ ), and interferon gamma (IFN- $\gamma$ ) in the sera of mice were measured using enzyme-linked immunosorbent assay (ELISA) kit (Biolegend, San Diego, CA, USA) according to the manufacturer's protocol.

### 2.9. Determination of antibodies against rEF-Tu

The levels of specific antibodies against rEF-Tu in mouse sera were determined by ELISA at 1 week after the last immunization. The 96-well plates (Becton Dickinson) were coated with 100 ng rEF-Tu diluted in PBS and incubated overnight at 4 °C. After blocking with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature, the mouse sera that were serially diluted from the initial 100-fold dilution in PBS were added and incubated for 2 h. HRP-conjugated rat anti-mouse IgG1, IgG2a, or IgE (SouthernBiotech, AL, USA) were used as the secondary antibody to detect the bound primary antibodies. Finally, the reaction was developed for 20 min with tetramethylbenzidine (Biolegend, San Diego, CA, USA) and stopped with 2 M H<sub>2</sub>SO<sub>4</sub>. OD<sub>450</sub> was determined in each well using a Multiskan FC microplate reader (Thermo Fisher Scientific). The reciprocal of the highest dilution with OD<sub>450</sub>  $\geq$  0.100 was considered as the antibody titer.

### 2.10. Flow cytometry analysis of splenocytes

The proportion of T-cell subsets with CD4 and CD8 molecules in splenocytes of mice was analyzed using flow cytometry technique at 1 week after the last immunization. The spleen was placed in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Wako Pure Chemical Industries, Osaka, Japan) at 4 °C. After homogenization of spleen tissues, cells were harvested through a 40 µm cell strainer (Funakoshi, Tokyo, Japan) and resuspended in ammonium-chloride-potassium lysing buffer (Lonza, Basel, Switzerland) for 10 min at room temperature to lyse the red blood cells. The remaining cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Thereafter, cells were washed with PBS and stained with allophycocyanin (APC)-conjugated rat anti-CD3, phycoerythrin (PE)-conjugated rat anti-CD4, fluorescein isothiocyanate (FITC)-conjugated rat anti-CD8a, or isotype control antibody (Thermo Fisher Scientific) in PBS containing 1% BSA for 1 h at 4 °C in the dark. After washing with PBS, cells were analyzed using NovoCyte flow cytometer with NovoExpress software (ACEA Biosciences). The lymphocytes were determined and gated by their forward and side scatter. The CD3<sup>+</sup> T-cells were then further identified and divided by the expression of CD4 and CD8.

### 2.11. In vitro analysis of cytokine production in CD4<sup>+</sup> T-cells against *S. pneumoniae*

Splenocytes were prepared from mice at 1 week after last immunization described as above. Then, CD4<sup>+</sup> T-cells were isolated by negative selection using EasySep mouse T cell isolation kit (Stem Cell Technologies, Vancouver, BC, Canada) in accordance with the manufacturer's guideline. The cells were seeded onto 24-well plate at a concentration of  $2.0 \times 10^6$  cells/mL in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Japan Bio Serum, Hiroshima, Japan). Then CD4<sup>+</sup> T-cells were stimulated with heat-killed *S. pneumoniae* strain D39, which was inactivated by heating at 60 °C for 1 h. After 24 h, the levels of IL-6, TNF $\alpha$ , IFN- $\gamma$ , and IL-17 in the cell culture supernatants were determined by using ELISA kit (Biolegend) according to the manufacturer's protocol.

### 2.12. Phagocytic activity assay

Mice were injected intraperitoneally with 4 mL of 4% thioglycolate medium (Becton Dickinson). Four days later, peritoneal macrophages were isolated by peritoneal lavage using 10 mL sterile PBS. The cells ( $1.0 \times 10^5$  cells/200 µL) were seeded onto a 96-well plate and cultured in RPMI 1640 with incubation at 37 °C in 95% air and 5% CO<sub>2</sub>. After 2 h, cells were washed with RPMI 1640 to remove non-adherent cells, hereafter referred to as peritoneal macrophages.

*S. pneumoniae* strains were grown until they reached early stationary phase. Approximately  $5.0 \times 10^6$  colony forming units (CFU) per 200 µL of these strains were pretreated with RPMI 1640 containing 10% sera from mice immunized with PBS or rEF-Tu for 1 h at 37 °C. These *S. pneumoniae* strains were added to the peritoneal macrophages and incubated for 2 h at 37 °C in 95% air and 5% CO<sub>2</sub>. This was followed by washing with PBS to remove extracellular nonadherent bacteria and 1 h treatment with antibiotics [200 µg/mL gentamicin, and 20 U/mL penicillin (Wako Pure Chemical Industries)] to eliminate residual or extracellular adherent bacteria. The macrophages were then washed twice and lysed in distilled water. Viable counts of phagocytized *S. pneumoniae* were determined by plating serial dilutions of macrophage lysates on sheep-blood agar plates (Becton Dickinson). The percentages of captured *S. pneumoniae* were calculated compared with the number of control *S. pneumoniae* culture incubated in RPMI 1640 with-

out the macrophages. To elucidate intracellular bacterial killing, the peritoneal macrophages were infected with *S. pneumoniae* strain D39 ( $5.0 \times 10^6$  CFU), which was pretreated with sera from mice immunized with PBS or rEF-Tu, for 2 h followed by washing and antibiotic treatment. Then, cells were re-incubated in RPMI 1640, which contains neither *S. pneumoniae* nor antibiotics. Then, intercellular bacterial killing ratio after phagocytosis was analyzed in the same method at each time point.

### 2.13. Protective efficacy of immunization with rEF-Tu

Mice were intraperitoneally infected with *S. pneumoniae* strains D39 and NP15A (serotype 2 and 15A;  $1.0 \times 10^7$  CFU in 100  $\mu$ L PBS) at 1 week after the last immunization, and then survival was monitored every 12 h. For the comparison, mice were subcutaneously immunized with PPSV (Pneumovax NP; MSD K.K., Tokyo, Japan) as described elsewhere [22,23], and infected with *S. pneumoniae* strains in the same manner at 1 week after the immunization.

### 2.14. Statistical analysis

Data were analyzed statistically by Student's T-test, one-way analysis of variance with Dunnett's multiple-comparison test, and one-way analysis of variance with Tukey's multiple-comparison test. The Kaplan-Meier survival curve was analyzed using the log-rank test equivalent to the Mantel-Haenszel test, with the Bonferroni correction for multiple comparisons. All statistical analyses were performed using Graph Pad Prism Software version 7.03 (GraphPad Software, La Jolla, CA, USA).

## 3. Results

### 3.1. EF-Tu is a common factor expressed in various pneumococci

Initially, we investigated whether EF-Tu is expressed in various pneumococci. Western blot analysis showed that EF-Tu was detected from whole-cell lysates of all pneumococcal strains (Fig. 1A). In addition, EF-Tu was observed on the surface of *S. pneumoniae* strains by flow cytometry analysis (Fig. 1B). Immunofluorescence analysis also revealed EF-Tu expression on the surface of *S. pneumoniae* strain D39 (Fig. 1C). These findings suggest that EF-Tu is a general factor expressed on the surface of various pneumococcal strains, as well as intracellularly.

### 3.2. rEF-Tu immunization induces cytokines production

Innate immune responses are essential for the induction of adaptive immunity after vaccinations [24]. Fig. 2 shows that subcutaneous injection of rEF-Tu transiently increased IL-6 and TNF $\alpha$  levels in sera at 1 day after rEF-Tu injection. However, the levels of IL-6 and TNF $\alpha$  decreased almost to the control levels at 1 week after rEF-Tu injection, suggesting that rEF-Tu did not cause chronic inflammation. On the other hand, the IFN- $\gamma$  level was quickly increased at 1 day after injection of rEF-Tu (Fig. 2). Furthermore, increased serum IFN- $\gamma$  level was observed at least until 4 weeks after the injection (Fig. S1). There was no change in cytokines production in the PBS or the adjuvant group between 1 day and 1 week after injection.

### 3.3. Immunization of mice with rEF-Tu induces specific antibodies

The levels of specific antibodies against rEF-Tu in sera were measured by ELISA at 1 week after the last immunization. As shown in Fig. 3, higher levels of IgG1 and IgG2a antibodies were detected in the sera of mice immunized with rEF-Tu as compared

with the PBS and the adjuvant groups. However, specific IgE antibody production was not induced by sensitization with rEF-Tu. Taken together, these results indicate that immunization with rEF-Tu was able to induce the humoral immune responses without allergic reaction in mice.

### 3.4. rEF-Tu immunization increases CD4<sup>+</sup> T-cells proportion in splenocytes

Table 1 shows that the proportion of CD4<sup>+</sup> T-cell was increased significantly in the spleen lymphocytes of mice immunized with rEF-Tu at 1 week after the last immunization compared with the control groups. In contrast, the proportion of CD8<sup>+</sup> T-cell was decreased significantly in the rEF-Tu immunized group. These findings suggest that immunization with rEF-Tu promoted differentiation of lymphocytes into CD4<sup>+</sup> T-cells. In addition, these CD4<sup>+</sup> T-cells from mice immunized with rEF-Tu induced the production of cytokines (IL-6, TNF $\alpha$ , IFN- $\gamma$ , and IL-17) against heat-killed *S. pneumoniae* strain D39 (Fig. 4). These results suggested that immunization with rEF-Tu enhances immune responses in CD4<sup>+</sup> T-cells against *S. pneumoniae*.

### 3.5. Pretreatment of pneumococcus with anti-EF-Tu serum increases phagocytic activity of peritoneal macrophages

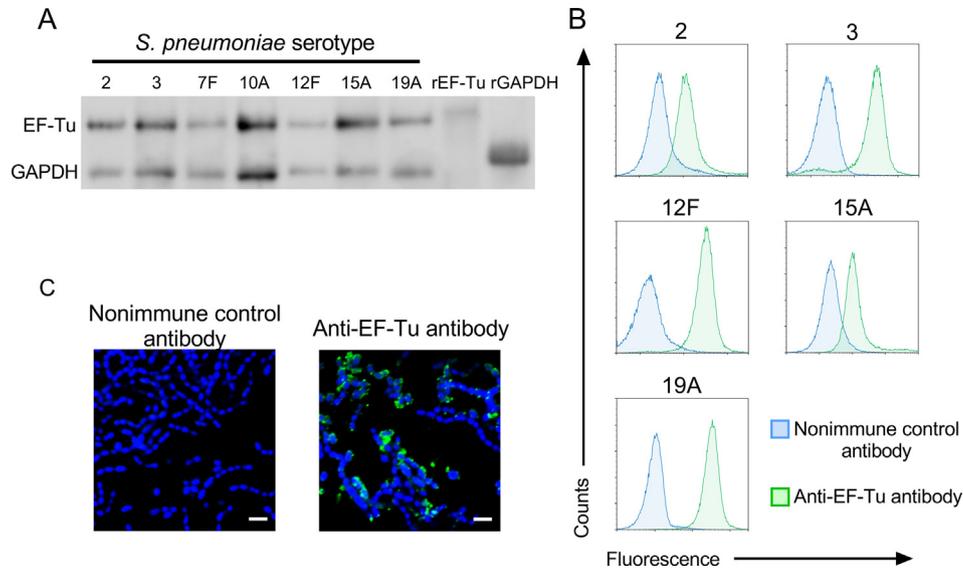
We investigated the effect of sera from mice immunized with rEF-Tu on phagocytic activity of peritoneal macrophages against *S. pneumoniae* strains. Fig. 5A shows that the relative phagocytosis was increased in all *S. pneumoniae* strains pretreated using anti-EF-Tu serum compared with control serum from mice immunized with PBS. These results indicate that anti-EF-Tu serum increases the phagocytic activity in mouse peritoneal macrophages against *S. pneumoniae* infection independent of its serotypes. In addition, pretreatment of *S. pneumoniae* with serum from rEF-Tu-immunized mice significantly decreased the ratio of live intercellular bacteria compared with control serum after 120 min (Fig. 5B). These findings suggest that treatment with anti-EF-Tu serum increased not only phagocytic activity, but also intracellular bacterial killing after phagocytosis in macrophages.

### 3.6. Immunization with rEF-Tu enhances protection against pneumococcal infection

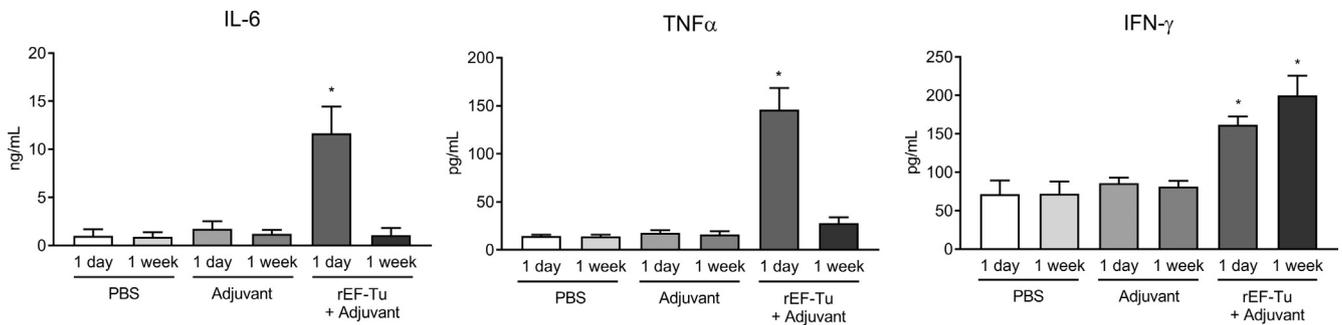
Finally, we evaluated the protective efficacy of immunization with rEF-Tu against pneumococcal infection using *S. pneumoniae* strains D39 (serotype 2; included in PPSV) and NP15A (serotype 15A; not included in PPSV). Fig. 6 illustrates the cumulative survival of mice after intraperitoneal infection with each strain. Immunization with PPSV significantly prolonged the survival of mice inoculated with *S. pneumoniae* strain D39, whereas PPSV was not effective against *S. pneumoniae* strain NP15A. However, immunization with rEF-Tu significantly decreased the mortality of mice compared to that in the PBS control group, against both the *S. pneumoniae* strains D39 and NP15A. These data indicate that immunization with rEF-Tu induces the protection against pneumococcal infection from not only the serotype contained in PPSV, but also from the serotype not contained in PPSV.

## 4. Discussion

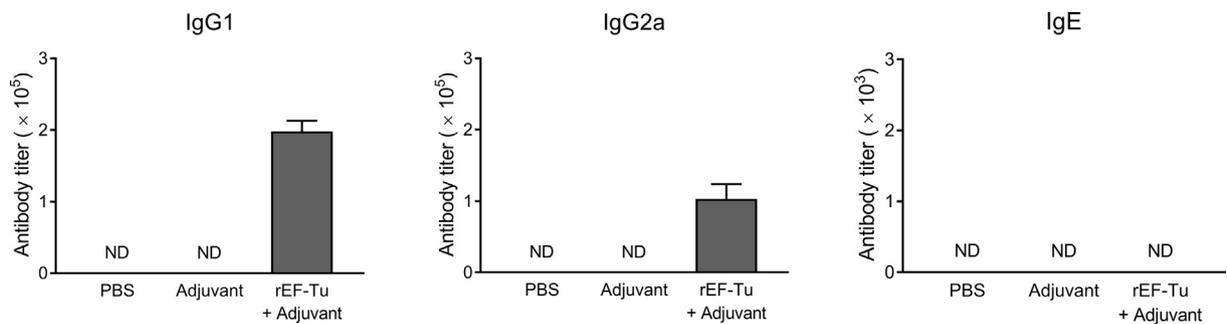
Immunization with well-conserved protein antigens could provide protection across all pneumococcal serotypes and be an alternative to conventional polysaccharide-based vaccines. In recent years, some protein-based pneumococcal vaccines have been developed and evaluated in clinical trials after demonstration of



**Fig. 1.** EF-Tu is expressed on the surface of various pneumococcal strains. (A) EF-Tu from whole-cell lysates of various pneumococcal strains D39, NP3, NP7F, NP10A, NP12F, NP15A, and NP19A (serotypes 2, 3, 7F, 10A, 12F, 15A, and 19A, respectively) was detected by Western blot analysis using anti-EF-Tu antibody. (B) EF-Tu on the surface of various pneumococcal serotypes was observed by flow cytometry analysis using nonimmune control antibody (blue) and anti-EF-Tu antibody (green). (C) Representative fluorescence-microscopy images of *S. pneumoniae* strain D39 stained for DNA (DAPI; blue) and EF-Tu (green). Scale bar: 5  $\mu$ m.



**Fig. 2.** rEF-Tu immunization induced the production of cytokines. The concentrations of IL-6, TNF $\alpha$ , and IFN- $\gamma$  in mouse sera were determined by ELISA at 1 day and 1 week after first injection. Data are shown as the mean  $\pm$  SD (n = 4 per group). The group means were compared using one-way analysis of variance with Dunnett's multiple-comparisons test compared with PBS group. \* $p$  < 0.05 was considered statistically significant.



**Fig. 3.** Specific antibodies were induced by rEF-Tu immunization. The levels of specific IgG1, IgG2a, and IgE antibodies against rEF-Tu in mouse sera were determined by ELISA at 1 week after the last immunization. The mouse sera serially diluted from the initial 100-fold dilution in PBS, were analyzed and the reciprocal of the highest dilution with OD<sub>450</sub>  $\geq$  0.100 was considered as the antibody titer. Data are shown as the mean  $\pm$  SD (n = 4 per group). ND: not-detected.

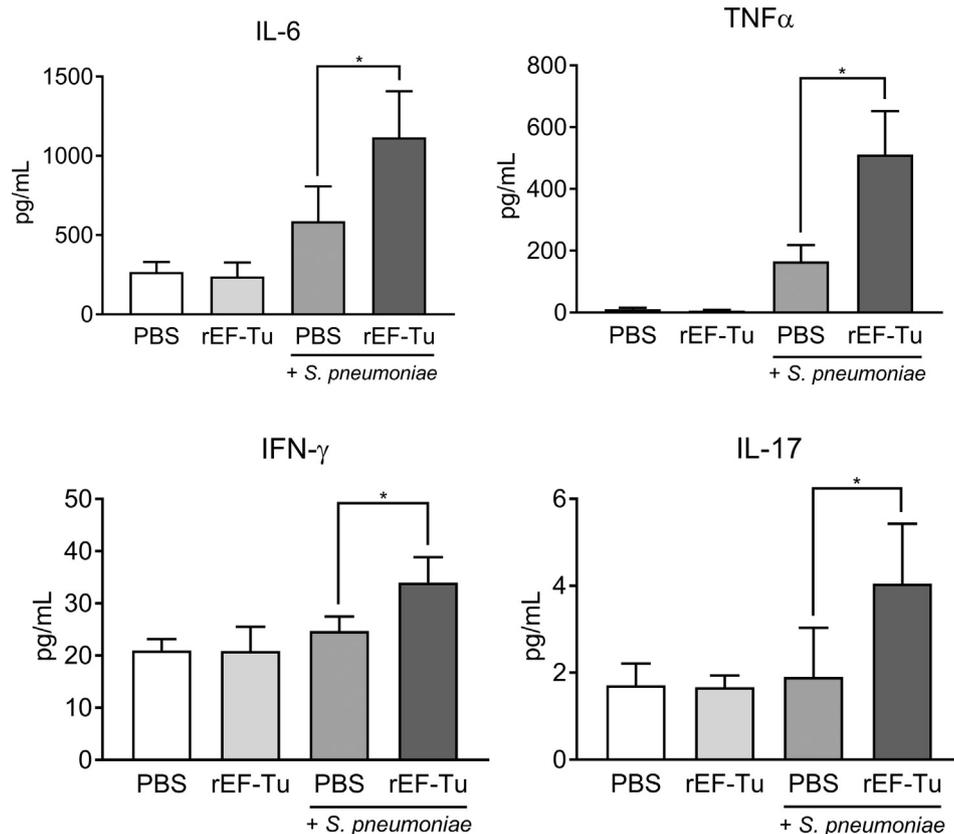
their protection against pneumococcal disease in animal models [25,26]. To date, one of the best studied protein antigens is the pneumococcal surface protein A (PspA), a surface expressed choline-binding protein that contributes to immune evasion by inhibiting the deposition of the complement on the bacterial cell surface [27]. Another well-studied pneumococcal protein is pneumolysin (Ply), a cholesterol-dependent pore-forming cytolytic and

pneumococcal virulence factor [28,29]. It has been shown that immunization with PspA and Ply induced the protection against multiple pneumococcal serotypes in a murine model [30,31]. In this study, we showed that EF-Tu is expressed in various strains of *S. pneumoniae*. In addition, immunization with rEF-Tu activates innate immune response including cytokines and antibodies production in mice.

**Table 1**  
Flow cytometry analysis of the proportion in T lymphocyte subsets.

	PBS		Adjuvant		rEF-Tu + Adjuvant	
	%	±SD	%	±SD	%	±SD
CD4 <sup>+</sup>	64.29	0.31	64.46	0.46	66.28 <sup>*</sup>	0.85
CD8 <sup>+</sup>	30.64	1.07	30.12	0.65	28.03 <sup>*</sup>	0.70

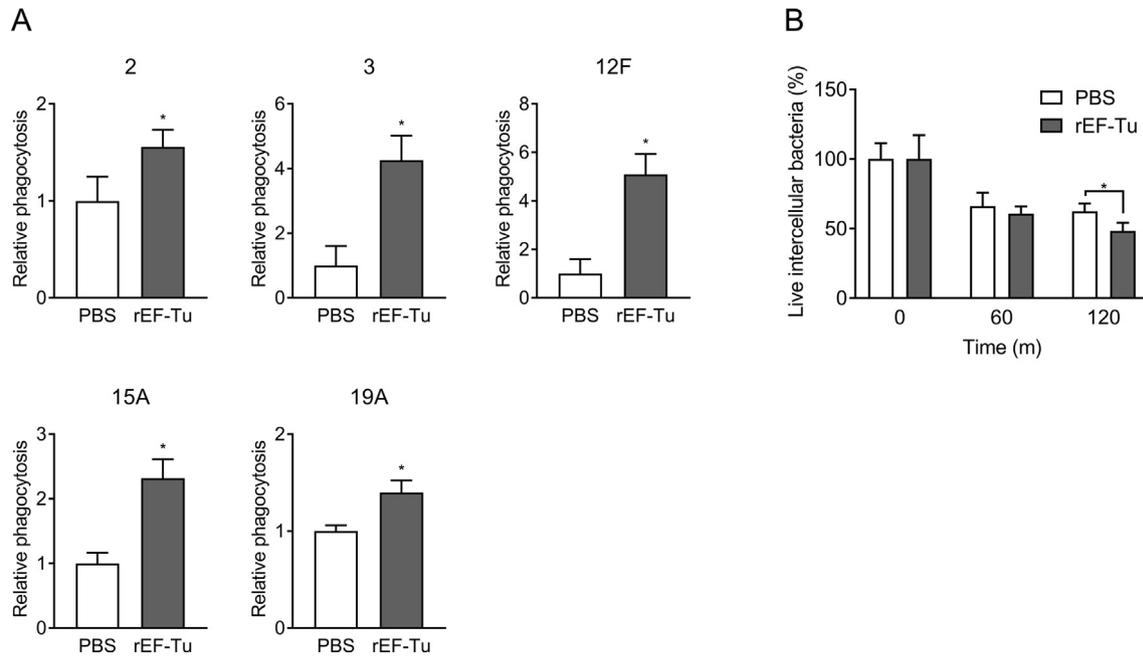
The proportion of T-cell subsets with CD4 and CD8 molecules in splenocytes of mice was analyzed using flow cytometry technique at 1 week after the last immunization. Data are shown as the mean ± SD (n = 4 per group). The group means were compared using one-way analysis of variance with Turkey's multiple-comparisons test. <sup>\*</sup>p < 0.05 was considered statistically significant.



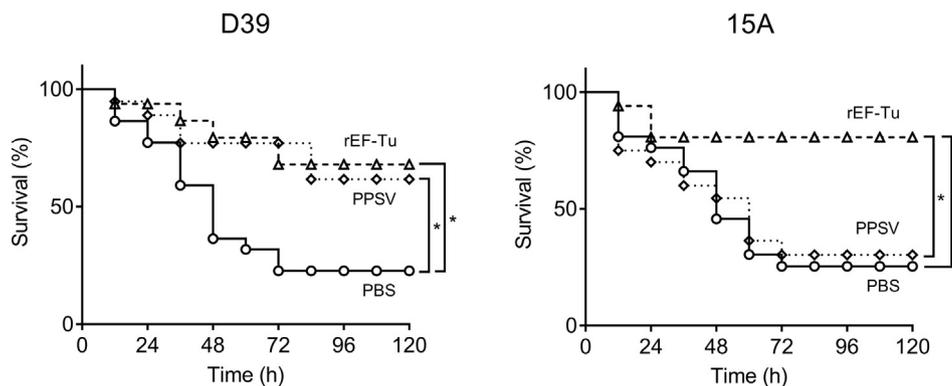
**Fig. 4.** CD4<sup>+</sup> T-cells produced cytokines against *S. pneumoniae* *in vitro*. CD4<sup>+</sup> T-cells ( $2.0 \times 10^6$  cells/mL) from mice immunized PBS or rEF-Tu were stimulated with heat-killed *S. pneumoniae* strain D39, which was inactivated by heating at 60 °C for 1 h. After 24 h, the levels of IL-6, TNFα, IFN-γ, and IL-17 in the cell culture supernatants were determined by ELISA. Data are shown as the mean ± SD (n = 4 per group). The group means were compared using one-way analysis of variance with Turkey's multiple-comparisons. <sup>\*</sup>p < 0.05 was considered statistically significant.

The activation of innate immunity with inflammatory responses by vaccines is indispensable for immunogenicity. However, the enhanced production of inflammatory cytokines may cause immunotoxicity [32]. Therefore, immunogenicity assessment is an important issue for ensuring the safety and efficacy of vaccines. In this work, we demonstrated that injection of pneumococcal rEF-Tu temporarily enhanced the production of cytokines including IL-6, TNFα, and IFN-γ. Interestingly, we found that the serum level of IFN-γ was further increased one week after immunization with rEF-Tu. IFN-γ regulates activity of macrophages, monocytes, and natural killer cells; it also induces the expression of major histocompatibility complex class II, and increases T helper (Th) 1 cell maturation [33]. IFN-γ is important for host defense through its ability to activate neutrophils and other innate immune cells in pneumococcal infection. It has been reported that clearance of *S. pneumoniae* after intratracheal instillation is impaired in mice deficient in IFN-γ [34]. However, overexpression of IFN-γ enhances the pathogenesis of meningitis by *S. pneumoniae* [35]. Therefore, it is important to identify the exact role of IFN-γ mediated by rEF-Tu immunization in pneumococcal infection.

Production of antibodies in serum against pneumococcal proteins, as with all currently licensed vaccines, is anticipated to provide protection from *S. pneumoniae* infection [36]. We observed that immunization with rEF-Tu was able to induce specific IgG1 and IgG2a antibodies in serum, suggesting that rEF-Tu could elicit a humoral immune response in mice. It is known that CD4<sup>+</sup> Th1 cells induce B-cells to proliferate and produce antibodies such as IgG1, while CD4<sup>+</sup> Th2 cells stimulate B-cells to produce IgG2a antibody in mice [37]. We revealed that immunization with pneumococcal rEF-Tu enhances both Th1 and Th2 responses in mice. IgG antibody is considered one of the most important antibody class in host defense against pneumococcal disease. Specific IgG antibody confers protection by opsonization, which facilitates pneumococcal phagocytosis by leukocytes through interaction with IgG receptors [38,39]. Our findings suggest that anti-rEF-Tu serum mediates opsonization of *S. pneumoniae* and activates phagocytosis followed by intracellular bacterial killing in mouse peritoneal macrophages. In this study, IgA antibody was not detected in serum after subcutaneous immunization with rEF-Tu (data not shown). Secretory IgA is the major mediator of mucosal immunity



**Fig. 5.** Pretreatment with anti-EF-Tu serum increased phagocytic activity of peritoneal macrophages. (A) The phagocytic activity against pneumococcal strains D39, NP3, NP12F, NP15A, and NP19A (serotypes 2, 3, 12F, 15A, and 19A, respectively) pretreated with sera from mice immunized with PBS or rEF-Tu, was measured using mouse peritoneal macrophages. Results were presented as the fold change relative to the phagocytic activity of the group immunized with PBS, which were assigned an average value of 1. (B) Intercellular bacterial killing after phagocytosis was analyzed using *S. pneumoniae* strain D39. The ratio of live intracellular bacteria at each time point was calculated compared with the number of live bacteria at 0 min of each group. Data are shown as the mean  $\pm$  SD ( $n = 4$  per group) and compared using Student's T-test. \* $p < 0.05$  was considered statistically significant.



**Fig. 6.** Survival curve of mice after lethal challenge infection with *S. pneumoniae* strains D39 and NP15A. Mice were intraperitoneally infected with *S. pneumoniae* strains D39 and NP15A (serotype 2 and 15A;  $1.0 \times 10^7$  cells in 100  $\mu$ L PBS) at 1 week after the last immunization, and then survival was monitored every 12 h ( $n = 18$ –20 per group). The Kaplan-Meier survival curve was analyzed using the log-rank test equivalent to the Mantel-Haenszel test, with the Bonferroni correction for multiple comparisons. \* $p < 0.008$  was considered statistically significant.

and an important component of host defenses that control invasive pneumococcal infections [40,41]. Since *S. pneumoniae* primarily invades via mucosal tissues in the respiratory tract, vaccines that induce local immunity are highly desirable. Therefore, further study is required to ascertain whether intranasal immunization with rEF-Tu induces local and systemic protection against *S. pneumoniae* infection.

It has been shown that antigen-specific CD4<sup>+</sup> T-cells reduce pneumococcal colonization [42,43]. Moreover, an effective T-cell response has been associated with protection from invasive pneumococcal disease and chronic obstructive pulmonary disease caused by *S. pneumoniae* [44]. In the present study, we found that immunization with rEF-Tu increased the proportion of CD4<sup>+</sup> T-cells in mouse splenocytes and these CD4<sup>+</sup> T-cells induced the production of cytokines against *S. pneumoniae*, suggesting that CD4<sup>+</sup> T-cell-mediated immune responses were elicited by rEF-Tu

immunization. In addition, we revealed that IL-17 production was increased against *S. pneumoniae* in CD4<sup>+</sup> T-cells from mice immunized with rEF-Tu. It has been suggested that Th17, which is one of the IL-17 producing cells, plays an important role against nasopharyngeal colonization of *S. pneumoniae* [45]. Therefore, further research is still needed to explore the differentiation of Th subsets in CD4<sup>+</sup> T-cells induced by rEF-Tu immunization and identify their exact functions in pneumococcal infection.

The lethal challenge experiments across pneumococcal serotypes (2 and 15A) confirmed the protective limits of PPSV. Pneumococcal serotype 15A is not included in current conjugate vaccines, hence serotype 15A infections and colonization increased in many countries after the introduction of these vaccines [46–48]. In addition, it has been reported that multidrug-resistant pneumococcal serotype 15A was isolated from different areas of the world [49,50]. Our results indicated that mice immunized with rEF-Tu

were significantly and non-specifically protected against lethal challenges with *S. pneumoniae* serotypes (2 and 15A). Therefore, EF-Tu could be an antigen candidate for the serotype-independent vaccine against pneumococcal infection.

## 5. Conclusions

In the present study, we demonstrated that systemic immune responses were enhanced by subcutaneous injection of pneumococcal rEF-Tu. In addition, serotype-independent protection against invasive pneumococcal infection was achieved by immunization with rEF-Tu, thus indicating that EF-Tu could be an efficient novel vaccine strategy against *S. pneumoniae*. Moreover, EF-Tu is one of the highly conserved and ubiquitously expressed factor among not only *S. pneumoniae*, but also other pathogenic bacteria (e.g. BLAST analysis shows that EF-Tu of *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* have about 97%, 76%, and 70% homology with EF-Tu of *S. pneumoniae*, respectively). Hence, immunization with pneumococcal EF-Tu might induce cross-reactive protection for another bacterial infection [51,52].

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## Conflicts of interest

None of the authors have conflicts of interest associated with this study to report.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2018.11.015>.

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