Serotype-Independent Protection Against Invasive Pneumococcal Infections Conferred by Live Vaccine With lgt Deletion

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Streptococcus pneumoniae is the most common respiratory bacterial pathogen among cases of community-acquired infection in young children, older adults, and individuals with underlying medical conditions. Although capsular polysaccharide-based pneumococcal vaccines have contributed to significant decrease in invasive pneumococcal infections, these vaccines have some limitations, including limited serotype coverage, lack of effective mucosal antibody responses, and high costs. In this study, we investigated the safety and immunogenicity of a live, whole-cell pneumococcal vaccine constructed by deleting the gene for prolipoprotein diacylglycerol transferase (lgt) from the encapsulated pneumococcal strain TIGR4 (TIGR4/Delta lgt) for protection against heterologous pneumococcal strains. Pneumococcal strain TIGR4 was successfully attenuated by deletion of lgt, resulting in the loss of inflammatory activity and virulence. TIGR4/Delta lgt colonized the nasopharynx long enough to induce strong mucosal IgA and IgG2b-dominant systemic antibody responses that were cross-reactive to heterologous pneumococcal serotypes. Finally, intranasal immunization with TIGR4/Delta lgt provided serotype-independent protection against pneumococcal challenge in mice.

Taken together, our results suggest that TIGR4/Delta lgt is an avirulent and attractive broad-spectrum pneumococcal vaccine candidate. More broadly, we assert that modulation of such “master” metabolic genes represents an emerging strategy for developing more effective vaccines against numerous infectious agents.

Keywords: Streptococcus pneumoniae, Lgt, lipoprotein, live attenuated vaccine, mucosal immunity

INTRODUCTION

Streptococcus pneumoniae (Sp, also known as pneumococcus) is the most common bacterial pathogen causing human diseases such as otitis media, pneumonia, and life-threatening invasive pneumococcal diseases, including meningitis and sepsis. It is responsible for an estimated 900,000 cases of pneumonia, 40,000 cases of invasive pneumococcal disease, and 4,000 associated deaths
annually in the U.S. alone (1). *Sp* asymptptomatically colonizes
the nasopharynx of ~60% of the population and disseminates
to cause invasive disease under immunocompromised conditions
and co-infection with respiratory viral infections (2). Thus,
immunization against pneumococcus is strongly recommended
for populations at risk, including young children, the elderly,
and patients with underlying medical conditions (3). A mucosal
protective immune response against pneumococcus may be the
most effective strategy for preventing opportunistic and invasive
infections by eliminating early pneumococcal colonization of the
nasopharynx (4, 5).

Currently, two types of licensed pneumococcal vaccines are
available, both of which are based on the generation of antibodies
against pneumococcal capsular polysaccharides (6). A
23-valent pneumococcal PS vaccine (PPV23), composed of 23
different capsular polysaccharide from commonly encountered
serotypes, is widely recommended for older adults but not for
young children due to its low immunogenicity in infants (7). A
protein-conjugated capsular polysaccharide vaccine (PCV)
was initially developed to provide protection against the seven
most prevalent serotypes causing invasive pneumococcal disease
in young children. This was later expanded to include 10
or 13 of the most invasive pneumococcal serotypes and is
highly immunogenic in both adults and infants (8). A dramatic
decrease in the burden of invasive pneumococcal diseases
has been reported in many countries in which PCVs are in
widespread use (9, 10). However, both vaccines are complex and
costly to manufacture, making them inaccessible for low-income
populations. Additionally, these serotype-based vaccines have
significant limitations in serotype coverage, resulting in serotypes
being increasingly replaced by non-vaccine serotypes or the
emergence of new serotypes and non-typeable pneumococcal
strains that cause invasive pneumococcal disease (11–13).
Although the overall incidence of invasive pneumococcal disease
has declined by 47% as a result of the introduction of PCVs,
the prevalence of non-vaccine serotypes (21, 23B, 33F, and 35F)
has significantly increased among asymptomatic carriers and
invasive pneumococcal diseases in countries in which a PCV is
used nationwide (3, 14). In addition, both PPVs and PCVs have
recently been reported to produce diminished mucosal immune
responses, especially in the production of immunoglobulin A
(IgA), a predominant Ig isotype at the respiratory surface
commonly colonized by pneumococcus (15). Due to these
limitations, inactivated and live whole-cell-based vaccines have
been extensively studied as cost-effective and broad-spectrum
vaccine candidates at the clinical and pre-clinical levels (16–19).
Also, a low cost of production and an ease of storage and handling
of whole cell vaccines compared to sophisticated alternative
vaccines, such as PCV and PPV, would facilitate the widespread
use of them in the developing and undeveloped countries, where
the vaccination for pneumococcus is greatly limited. Likewise,
the development of inactivated whole-cell-based vaccines for
pneumococcus has been ongoing in both clinical and preclinical
trials since 1911, and recent meta-analyses have affirmed their
efficacy (18, 20).

As another approach for developing whole-cell-based
vaccines, live attenuated vaccines are advantageous, as they
enhance humoral and mucosal immune responses following
a single-dose vaccination, in contrast to inactivated whole-
cell-based vaccines (4, 21). However, the main obstacle to
the development of live attenuated vaccines is achieving
satisfactory attenuation without compromising immunogenicity.
To date, most live attenuated pneumococcal vaccine strains
have been constructed in a capsule-negative background and
carry additional mutations in important virulence genes,
such as pspA, ply, and lytA (21, 22). However, these live
attenuated vaccines may be suboptimally immunogenic
due to the deletion of several virulence genes that also
induce protective immune responses. To overcome such
drawbacks, central regulatory or component “master” genes
have been used as alternative targets for live attenuated
vaccine development, since their deletion may downregulate,
but not necessarily silence the expression of many virulence
and metabolic genes (19, 23). For example, a pneumococcal
mutant strain deficient in the gene fitsY, which encodes
a central component of the signal recognition particle
(SRP) pathway responsible for delivering membrane and
secretory proteins, was highly protective against multiple
serotypes in mouse models of pneumococcal otitis media
and invasive pneumococcal disease by bolstering IgG2 serum
responses (21).

Lipoprotein diacylglycerol transferase (Lgt) is a highly
conserved, commitment-step enzyme that catalyzes
diacylglycerol attachment to an N-terminal cysteine residue
in a consensus peptide sequence known as a pre-prolipoprotein
on the membrane (24, 25). It is essential for growth in
most Gram-negative bacteria but not in *Streptococcus* spp.
or *Staphylococcus* spp. (26). Lipoproteins are membrane-
anchoring proteins, which locates beneath peptidoglycan layer
in Gram-positive bacteria, with a variety of physiological
and pathogenic functions in bacteria, including roles in cell
division, conjugation, nutrient acquisition, adhesion, invasion,
and immune evasion. In addition, the lipoprotein lipid moiety
significantly contributes to the activation of Toll-like receptor
2 (TLR2)-mediated host innate immune responses (27–29).
Following the diacylation of pre-prolipoproteins by Lgt, the
N-terminal signal peptide is cleaved off by lipoprotein signal
peptidase II (Lsp), resulting in a mature lipoprotein (25, 30, 31).
Deletion of *lgt* in Gram-positive bacteria can strongly attenuate
virulence, resulting in improper lipoprotein anchoring in the
cytoplasmic membrane (32–36). A previous study also showed
that a pneumococcal mutant strain deficient in *lgt* exhibited
reduced TLR2-mediated inflammatory responses in vitro
and diminished invasiveness in vivo (37). Since *lgt*-mutant
pneumococcal strains exhibit greatly diminished virulence while
still expressing many surface antigens, we hypothesized that a
novel intranasal vaccine based on an *lgt*-mutant strain would
be a promising and effective pneumococcal live attenuated
vaccines candidate.

In this study, we aimed to develop an encapsulated
pneumococcal TIGR4 strain deficient in *lgt* (TIGR4Δ*lgt*)
and evaluate its efficacy in protecting against
infection from heterologous pneumococcal strains
in mice.
MATERIALS AND METHODS

Reagents
All antibodies, purified proteins, antibiotics, and other reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). The mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit was purchased from eBioscience (San Diego, CA, USA). An anti-PsaA monoclonal antibody was kindly provided by Prof. Nahm (University of Alabama at Birmingham, AL, USA).

Bacterial Strains and Generation of Supernatants and Crude Extracts
The bacteria and plasmids used in this study are listed in Supplement Table 1. Streptococcus pneumoniae (Sp) strain TIGR4 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Sp serotypes 2 (D39), 3 (wu2), 6B, 9V, 19F, and 23F were kindly provided by Prof. Nahm (University of Alabama at Birmingham). Pneumococcal strains were grown in Todd-Hewitt broth (THB; Difco, Franklin Lakes, NJ, USA) supplemented with 0.5% yeast extract (Difco). At mid-log phase [optical density at 600 nm (OD600) of 0.3–0.4], bacteria were pelleted by centrifugation, and culture supernatants were filtered through a 0.22-μm membrane filter (Merck Millipore, Billerica, MA, USA). Bacterial pellets were resuspended in phosphate-buffered saline (PBS; Lonza, Basel, Switzerland).

Western Blotting Analysis
Bacterial culture supernatants were loaded and separated on Bis-Tris Bolt sodium dodecyl sulfate polyacrylamide gels (Invitrogen, Carlsbad, CA, USA), followed by transfer to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% dry skim milk (Bio-Rad) in PBS/0.05% Tween-20 (PBS-T) and then incubated with anti-PsaA monoclonal antibody (Xir-126) or anti-phosphocholine monoclonal antibody (TEPC-15, Sigma-Aldrich). The membrane was then washed with PBS-T and incubated with a horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Southern Biotech, Birmingham, AL, USA). Membrane-bound peroxidase was detected with 3,3′,5,5′-tetramethylbenzidine substrate solution (Thermo-Fisher Scientific, Waltham, MA, USA).

Construction of lgt-Deficient TIGR Strain
Primers used in this study are listed in Supplement Table 2. A gene replacement cassette was constructed by cloning the chromosomal regions flanking lgt and inserting them upstream and downstream of the kan gene in pK326, as described previously (38). A 1,023-bp upstream segment and a 930-bp downstream segment were amplified using paired primers (LGT-UpF/LGT-UpR and LGT-DnF/LGT-DnR, respectively). Amplified fragments were then cloned sequentially into the pK326 plasmid, resulting in the pKO-lgt plasmid, which was then introduced into TIGR4 by natural transformation, as previously described (39). In brief, a culture of TIGR4 was diluted 100-fold in fresh transformation THB supplemented with 2% bovine serum albumin (BSA; Sigma-Aldrich), 0.1% CaCl2 (Sigma-Aldrich), and 200 ng/ml competence-stimulating peptide. After incubation at 37°C for 14 min, cells were mixed with ~5 μg of pKO-lgt, followed by an additional 2 h of incubation at 37°C. Transformed cells were then plated onto blood agar plates containing 300 μg/ml kanamycin (Sigma-Aldrich) for selection of the TIGRΔlgt strain.

Inactivation of lgt-Deficient TIGR Strain
Harvested TIGRΔlgt (1 × 1010 CFU/ml) were irradiated using a 60-Co-gamma irradiator (point source AECL, IR-79, MDS Nordion International Co., Ottawa, ON, Canada) at the Advanced Radiation Technology Institute of Korea Atomic Energy Research Institute (Jeonju, Korea) with absorbed dose of 5 kGy for 1 h at room temperature.

Mammalian Cells and Culture Conditions
Mouse RAW264.7 cells were obtained from ATCC. RAW264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (Cellgro Mediatech, Herndon, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO2.

Measurement of Nitrite and TNF-α
Thioglycollate-elicited peritoneal cells were collected from wild-type (WT), ΔTLR2, and ΔTLR4 male C57BL/6 mice at 6 weeks of age provided kindly from Prof. Suzanne Michalek (University of Alabama at Birmingham). Mouse primary peritoneal cells or RAW264.7 cells were dispensed into 96-well plates (SPL, Suwon, Korea) at a density of 2 × 105 cells/ml and stimulated with TIGR4 or TIGR4Δlgt for 12 h. For nitrite measurement, 100 μl of culture supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at RT for 5 min. The optical density was then measured at a wavelength of 540 nm using a Victor X3 light plate reader (Perkin-Elmer, Waltham, MA, USA). NaNO2 solution was used to generate the standard curve. The amount of TNF-α in the cell culture supernatant was determined with a commercially available sandwich-type ELISA (eBioscience) according to the manufacturer's protocol.

Purification of Lipoteichoic Acid (LTA)
Pneumococcal LTA was prepared using organic solvent extraction, octyl-Sepharose, and ion-exchange chromatography, as previously described (40). Levels of endotoxin contamination were determined using the QCL-1000 quantitative chromogenic Limulus Amoebocyte Lysate (LAL) assay (Bio-Whittaker, Walkersville, MD, USA) according to the manufacturer’s instructions.

Microarray Analysis
To examine the effect of the loss of lipoprotein biosynthesis on global gene expression in Sp, microarray analysis was performed to compare transcript levels between WT TIGR4 and TIGR4Δlgt, as described previously (41). Total bacterial RNA was isolated from cells grown statically to mid-log phase (OD600 = ~0.35) using an SV total RNA isolation kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. A total of 5 kGy for 1 h at room temperature.
75 μg RNA with 3 μg random hexamer dissolved in 29.5 μl nuclease free-water was denatured at 65°C for 10 min and then placed on ice. After adding 6 μl of 0.1 M diithiothreitol, 12 μl first-strand buffer, 1.5 μl dNTP mix (25 mM dATP, 25 mM dGTP, 25 mM dCTP, 10 mM dTTP), 4 μl Superscript II® reverse transcriptase (Invitrogen), 2 μl RNasin (Promega), and 4 μl of either Cy3- or Cy5-conjugated dTTP (Amersham Biosciences, Buckinghamshire, UK), the labeling mixture (60 μl) was incubated at 42°C for 2 h and supplemented with 2 μl Superscript II at the end of the first hour. Each probe was then denatured with 10 μl of 1 M NaOH and neutralized with 10 μl of 1 M HCl, followed by purification with a PCR purification kit (Qiagen, Venlo, Netherlands). It was then concentrated with a vacuum drier. Each probe separately labeled with Cy3 or Cy5 was resuspended in 20 μl distilled water. Whole-genome TIGR4 oligonucleotide microarray chips were obtained from the J. Craig Venter Institute (Rockville, MD, USA). The array consisted of 70-mer oligonucleotide probes, representing 2060 TIGR4 open-reading frames (ORFs) and 457 ORFs from strains G54 and R6. Each oligonucleotide probe sequence was spotted at least five times on the array. All microarray experiments were performed by eBiogen (Seoul, Korea). In brief, equal volumes of labeled probes (20 μl each) from the TIGR4 and TIGR4Δgt strains were mixed with 40 μl of 2× hybridization solution consisting of 50% formamide, 10× saline-sodium citrate, and 0.2% SDS and denatured by boiling for 5 min. Probes were simultaneously hybridized to a chip overnight at 42°C in a hybridization chamber (Corning, Corning, NY, USA) submerged in water. Scans were performed with a Scan Array 5,000 laser scanner using ScanArray 2.1 software (Packard BioChip Technologies, Billerica, MA, USA). Signal intensity was quantified using QuantArray 3.0 software (Packard BioChip Technologies). The statistical significance of differential expression relative to that in the control was assessed using a paired t-test and Bayesian analysis, as Bayesian estimates of within-treatment variation tend to reduce the rate of false positives (42). The threshold for a significant change in gene expression was set at P < 0.001. Of the genes passing this threshold, only those with a Bayesian confidence value (B-value) above zero were regarded as being significantly affected by Δgt mutation. B-values were computed using Cyber-T (cybert.microarray.ics.uci.edu).

Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNAs were isolated from log-phase cultures of WT TIGR4 and TIGR4Δgt using an RNeasy® mini kit (Qiagen) according to the manufacturer’s instructions. For real-time PCR analysis, cDNA was synthesized from 1 μg total purified RNA using a Primerscript 1st strand cDNA synthesis Kit (Takara Bio Inc., Kyoto, Japan) following the manufacturer’s instructions. Primers for various genes were designed using Primer Express v2.0 software and are listed in Supplement Table 2. qRT-PCR amplification was performed with SYBR Premix Ex Taq (Takara Bio Inc.) on a Bio-Rad CFX Real-Time System. PCR reactions were performed with one cycle of 95°C for 5 min; 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s; and one cycle of 72°C for 10 min. Relative quantification of gene expression was determined by the comparative threshold (ΔΔCt) method. The 16S rRNA gene (rrsH) was used as a control to normalize the expression levels of target genes.

Animal Experiments

All animal experiments conducted in this study were approved by the Committee on the Use and Care of Animals at the Korea Atomic Energy Research Institute (KAERI) and performed according to accepted veterinary standards. Six-week-old male C57BL/6 mice were purchased from Orient Bio Inc. and were intranasally (i.n.) vaccinated twice at 14-day intervals with live or killed TIGR4Δgt. At 10 days after the second vaccination, blood was collected, and antibodies specific to pneumococcus were measured by ELISA. To examine the protective effect of TIGR4Δgt vaccination, mice were i.n. infected with pneumococcal strains TIGR4, wu2, or D39 at 10 days after the second vaccination. Mouse survival was monitored for 10 days after infection. Blood, nasal washes, and lungs were collected at the time of death or at the end of the experiment for bacterial counting. Tissues were homogenized in 1.5 ml PBS and passed through a 40-μm mesh strainer, and bacterial numbers were then counted.

Measurement of Mouse Immunoglobulin Levels

Mouse blood samples were taken every 2 weeks. Serum was isolated via centrifugation and stored at −80°C until use. Pneumococcal strains were cultured in THY (THB with 2% yeast extract) and harvested at mid-log phase. The absorbance of the pneumococcal pellet was adjusted to an OD560 of 0.1 by dilution with PBS. Then, 96-well immunoplates (SPL) were coated with 100 μl pneumococcal suspension and incubated overnight at 4°C to allow adhesion of bacterial cells. The plates were then washed five times with PBS-T, followed by blocking with 1% BSA in PBS for 1 h at RT. After blocking, diluted serum was added to each well and incubated at RT for 1 h, and unbound antibodies were removed by washing with PBS-T. Appropriate dilutions of goat anti-mouse IgG-HRP (Sigma-Aldrich), goat anti-mouse IgG-HRP (Southern-Biotech), or goat anti-mouse IgM-HRP (Southern-Biotech) were added to wells and incubated for 30 min at RT. After washing the plates five times with PBS-T, 100 μl TMB substrate reagent (BD Biosciences, Franklin Lakes, NJ, USA) was added. When colors developed, 50 μl of 2 N H2SO4 was added, and the absorbance was measured at 450 nm using a Victor X3 light plate reader (Perkin-Elmer).

Hematoxylin and Eosin (H&E) Staining

Following i.n. inoculation of WT TIGR4 or TIGR4Δgt, mouse lungs were harvested at 24 h after inoculation. The left lung of each mouse was fixed with 4% paraformaldehyde prior to paraffin embedding to preserve the pulmonary architecture. Paraffin-embedded tissues were then cut into 4-μm-thick sections and stained with H&E to observe histopathological changes. Tissue images were captured with a light microscope (Eclipse CI-L; Nikon Corp., Tokyo, Japan) and processed using i-Solution Lite software (iMTechnology, Vancouver, BC, Canada).
FIGURE 1 | Biochemical and Immunological Characteristics of TIGR4Δlgt. (A) Expression of PsaA protein in cell lysates and culture supernatants from wild-type (WT) and Δlgt-deficient (Δlgt) TIGR4 strains. (B,C) Nitric oxide (B) and TNF-α (C) production in culture supernatants of RAW264.7 cells treated with WT or Δlgt strain for 12 h. (D) TNF-α production in the culture supernatants of peritoneal cells from wild-type (WT), TLR2-deficient (ΔTLR2), and TLR4-deficient (ΔTLR4) mice infected with WT or Δlgt strain for 12 h. Data in (B,C), and (D) represent mean values ± S.D. of triplicate results. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to the WT treatment group. (E) LTA expression levels in 3-fold serial dilutions of culture supernatants from WT (W) or Δlgt (M) strains. (F) Quantification of LTA isolated from the pellets of WT and Δlgt strains. *P < 0.05 compared to the WT group.

Data Analysis

Data are expressed as mean ± standard deviation (SD). Data in bar graphs and bacterial numbers between groups were compared by unpaired t-tests. Mouse survival was analyzed by Kaplan–Meier analysis. The significance of differences was assessed by log-rank test using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Differences with P-values < 0.05 were considered statistically significant.

RESULTS

Biological Characteristics of lgt-Deficient Pneumococcus

To investigate its potential clinical use as a pneumococcal live attenuated vaccine, an lgt-deficient pneumococcal strain was constructed from the pneumococcal strain TIGR4 (TIGR4Δlgt). Its biological properties were then examined. First, to verify whether lipoprotein biosynthesis was abrogated in TIGR4Δlgt, the localization of PsaA, a major lipoprotein, was examined in cell lysates and culture supernatants (Figure 1A). As expected, the expression of PsaA was detected in the cell lysate of wild-type (WT) TIGR4, while PsaA accumulated in the culture supernatant of TIGR4Δlgt, indicating its improper anchoring in the membrane. Of note, TIGR4Δlgt grew well in nutrient-rich medium, similar to its parent strain (data not shown).

Since pneumococcus can produce over 32 families of lipoproteins and more than 88 lipoprotein-related proteins (43), it was possible that the lgt-deficient mutant would exhibit impairment of cellular functions associated with transporting and synthesizing membrane components. To determine whether the pneumococcal transcriptome was altered by lgt deletion, gene expression microarray analysis was performed. In TIGR4Δlgt, 164 genes were upregulated, and 161 genes were downregulated compared to their expression levels in the TIGR4 parent strain (P < 0.01). Among these, the mRNA expression levels of previously identified vaccine targets, such as choline binding proteins, lipoproteins, and LPxTG motif cell wall-anchoring proteins were further analyzed (Tables 1, 2). This revealed that, of 18 selected vaccine targets, expression levels of potD, vgcl, clpP, and htrK were downregulated but not significantly so in TIGR4Δlgt, while the expression of the other genes was similar to or significantly higher than that in TIGR4.

Lipoproteins are known to be potential inflammatory stimulants via the activation of TLR2. Various Gram-positive bacteria deficient in lgt have shown significant loss or complete abolition of inflammation-stimulating activity (29, 37). To determine the inflammation-stimulating activity of TIGR4Δlgt, mouse RAW264.7 cells were infected with either lgt.
### TABLE 1  | mRNA expression of candidate vaccine genes in wild-type TIGR4 and TIGR4Δlgt pneumococci.

| Locus tag | Gene   | Ratio (Δlgt/WT) | P-value | Description                                      | qPCR | References |
|-----------|--------|-----------------|---------|--------------------------------------------------|------|------------|
| SP1386    | potD   | 0.675           | 0.074   | Spermidine/putrescine ABC transporter             | ND   | (44)       |
| SP0189    | vgcL   | 0.717           | 0.090   | Conserved hypothetical ABC transporter            | ND   | (45)       |
| SP0746    | ctpP   | 0.949           | 0.058   | ABC transporter, ATP-binding protein              | ND   | (46)       |
| SP2239    | trnK   | 1.240           | 0.065   | 6-Phosphogluconate dehydrogenase                 | ND   | (49)       |
| SP0966    | pavA   | 1.305           | 0.0230  | Adherence and virulence protein A                 | 1.00 | (50)       |
| SP1128    | eno    | 1.335           | 0.036   | Enolase                                           | ND   | (53)       |
| SP0499    | pgkK   | 1.639           | 0.0132  | Phosphoglycerate kinase                           | 1.54 | (51)       |
| SP0121    | gapA   | 1.674           | 0.007   | Glycerol 3-phosphate dehydrogenase                | 1.61 | (52)       |
| SP0314    | hyl    | 1.725           | 0.042   | Hyaluronidase                                     | 1.61 | (53)       |
| SP2185    | glyP   | 2.850           | 0.031   | Glycerol uptake facilitator protein               | 4.05 | (54)       |
| SP1004    | phtE   | 3.049           | 0.005   | Conserved hypothetical protein                    | 1.58 | (55)       |
| SP1923    | ply    | 3.541           | 0.005   | Pneumolysin                                      | 3.45 | (55)       |
| SP1175    | phpB   | 4.227           | 0.036   | Conserved domain protein                          | 1.67 | (56)       |
| SP1174    | phpA   | 6.641           | 0.009   | Conserved domain protein                          | 1.44 | (56)       |
| SP1003    | phtD   | 7.383           | 0.036   | Conserved hypothetical protein                    | 2.29 | (55)       |
| SP1687    | nanB   | 8.947           | 0.005   | Neuraminidase B                                  | 3.11 | (57)       |

### TABLE 2  | Expression of choline-binding protein members and cell wall-anchoring protein genes in wild-type TIGR4 and TIGR4Δlgt pneumococci.

| Locus tag | Gene   | Ratio (Δlgt/WT) | P-value | Description                                      | qPCR |
|-----------|--------|-----------------|---------|--------------------------------------------------|------|
| SP0390    | cbpG   | 0.790           | 0.036   | Choline-binding protein G                         | 1.24 |
| SP0391    | cbpF   | 0.764           | 0.017   | Choline-binding protein F                         | 1.01 |
| SP2201    | cbpD   | 0.848           | 0.030   | Choline-binding protein D                         | 0.96 |
| SP1937    | lytA   | 0.959           | 0.030   | Autolysin                                         | 1.31 |
| SP0930    | cbpE   | 0.922           | 0.033   | Choline-binding protein E                         | 1.41 |
| SP0667    | cbpL   | 1.145           | 0.011   | Choline binding protein L                         | 1.01 |
| SP0966    | lytC   | 1.253           | 0.124   | Lysozyme                                         | 1.12 |
| SP0378    | cbpJ   | 1.225           | 0.004   | Choline-binding protein J                         | 1.45 |
| SP2190    | cbpA   | 1.220           | 0.006   | Choline-binding protein A                         | 1.53 |
| SP0377    | cbpC   | 1.162           | 0.242   | Choline-binding protein C                         | 1.02 |
| SP1573    | lytB   | 1.461           | 0.093   | Endo-beta-N-acetylglucosaminidase                 | 1.49 |
| SP0117    | pspA   | 1.096           | 0.566   | Pneumococcal surface protein A                    | 1.22 |
| SP2136    | pcpA   | 2.060           | 0.040   | Choline-binding protein PcpA                      | 1.38 |
| SP0069    | cbpI   | 5.329           | 0.045   | Choline-binding protein I                         | 8.48 |

For TIGR4 or TIGR4Δlgt for 12 h, and the production of nitrite and TNF-α was then examined (Figures 1B,C). TIGR4 treatment significantly upregulated levels of nitrite and TNF-α expression in a dose-dependent manner. By contrast, nitrite, and TNF-α production was not induced in cells infected with TIGR4Δlgt, indicating that TIGR4Δlgt failed to produce...
mature functional lipoproteins known to be responsible for stimulating inflammation. To examine whether the reduction in the inflammation-stimulating activity of TIGR4Δlgt was due to the loss of TLR2-stimulating activity, mouse peritoneal macrophages isolated from wild-type (WT), TLR2-deficient (ΔTLR2), or TLR4-deficient (ΔTLR4) mice were treated with TIGR4 or TIGR4Δlgt, and the level of TNF-α production was measured (Figure 1D). As expected, TIGR4 treatment induced TNF-α production in cells from WT and ΔTLR4 mice but not in cells from ΔTLR2 mice. However, TIGR4Δlgt treatment produced no or significantly lower levels of TNF-α in cells from all three groups, suggesting that pneumococcus-induced expression of functional TLR2 agonists is dependent on Lgt and that TIGR4Δlgt is likely defective in the production of TLR2 ligands.

Previous studies have suggested that LTA is another dominant mature functional lipoprotein known to be responsible for stimulating inflammation. To examine whether the reduction in the inflammation-stimulating activity of TIGR4Δlgt was due to the loss of TLR2-stimulating activity, mouse peritoneal macrophages isolated from wild-type (WT), TLR2-deficient (ΔTLR2), or TLR4-deficient (ΔTLR4) mice were treated with TIGR4 or TIGR4Δlgt, and the level of TNF-α production was measured (Figure 1D). As expected, TIGR4 treatment induced TNF-α production in cells from WT and ΔTLR4 mice but not in cells from ΔTLR2 mice. However, TIGR4Δlgt treatment produced no or significantly lower levels of TNF-α in cells from all three groups, suggesting that pneumococcus-induced expression of functional TLR2 agonists is dependent on Lgt and that TIGR4Δlgt is likely defective in the production of TLR2 ligands.

To examine the role of lgt in colonization, mice were i.n. inoculated with either TIGR4 or TIGR4Δlgt [10⁷ colony forming units (CFU)], and the number of bacteria colonizing the nasopharynx was measured in nasal washes at 24, 48, and 72 h post-infection (hpi) (Figures 2A–C). A significant reduction in the number of colonized bacteria was observed in TIGR4Δlgt-inoculated mice compared to that in TIGR4-inoculated mice. However, TIGR4Δlgt effectively colonized the nasopharynx for more than 3 days. No viable TIGR4Δlgt was detected at 14 days post-infection (dpi) (data not shown).

To determine the invasiveness of TIGR4Δlgt, mice were i.n. inoculated with TIGR4 (10⁵ CFU) or TIGR4Δlgt (10⁵, 10⁶, or 10⁷ CFU), and mouse survival was recorded for 24 days. As shown in Figure 2D, all mice infected with TIGR4Δlgt...
Induced by TIGR4

Protective Humoral Immune Responses

We next evaluated the immunogenicity of live TIGR4/Delta1 and red blood cell leakage (enhanced neutrophil infiltration, loss of alveolar architecture, microbiological analysis, lungs from mice infected with TIGR4 and data not shown). Consistent with findings from survival into the bloodstream was observed for up to 14 days (Figure 2F). TIGR4 was successfully attenuated by deleting lgt. No significant pathological change was found in the lungs of Delta1-modest number of TIGR4 inoculated mice (Figure 2E). The bacterial load in the lungs was significantly reduced CFU. However, all mice inoculated with 10^5 CFU of wild-type TIGR4 survived for more than 24 days, even at infection levels of 10^7 CFU. However, all mice inoculated with 10^5 CFU of TIGR4 died by 9 dpi. No significant weight loss or noticeable disease symptoms were observed in mice inoculated with TIGR4/Delta1, suggesting a lack of systemic toxicity (data not shown). Next, the dissemination of bacteria into the lungs and blood was measured at 3 dpi with TIGR4 or TIGR4/Delta1 (10^7 CFU). The bacterial load in the lungs was significantly reduced in TIGR4/Delta1-inoculated mice compared to that in TIGR4-inoculated mice (Figure 2E). No dissemination of TIGR4/Delta1 into the bloodstream was observed for up to 14 days (Figure 2F and data not shown). Consistent with findings from survival and microbiological analysis, lungs from mice infected with TIGR4 showed marked pathological changes in inflammation, including enhanced neutrophil infiltration, loss of alveolar architecture, and red blood cell leakage (Figure 2G). However, although a modest number of TIGR4/Delta1 was disseminated into the lungs, no significant pathological change was found in the lungs of TIGR4/Delta1-infected mice (Figure 2G). These data suggest that TIGR4 was successfully attenuated by deleting lgt but that TIGR4/Delta1 can still colonize the nasopharynx.

**Protective Humoral Immune Responses Induced by TIGR4/Delta1**

We next evaluated the immunogenicity of live TIGR4/Delta1 (live Delta1) and compared it with that of inactivated TIGR4/Delta1 (killed Delta1). Mice were i.n. immunized with either live or killed Delta1 twice at 14-day intervals (days 0 and 14). Pneumococcal-specific immunoglobulin levels in the serum and bronchoalveolar lavage fluid (BALF) were then measured at 10 days after the second immunization. As shown in Figures 3A,B, both live and killed Delta1 vaccines induced significantly higher serum levels of pneumococcal-specific IgM (Sp-specific IgM) and Sp-specific IgG than inoculation with PBS (control group). Moreover, Sp-specific IgM levels in the sera of live Delta1-immunized mice were significantly higher than those in the sera of mice immunized with killed Delta1. However, no differences in Sp-specific IgG levels were found between the live and killed Delta1 immunization groups. In addition, significant induction of Sp-specific IgA was found in BALF from both the live and killed Delta1-immunized groups (Figure 3C), indicating that both live and killed Delta1 vaccines effectively induced mucosal immune responses.

Distinct IgG subclasses have different efficacies in terms of their functions, such as serum bactericidal activity and opsonophagocytosis. Therefore, IgG subclass distributions may contribute to the protective effects of vaccination. Despite the fact that similar levels of serum IgG were elicited by live and killed Delta1, IgG subclass analysis showed markedly different IgG subclass patterns between the two immunization groups (Figure 3D). Specifically, IgG1 (44%) was predominant, followed by IgG2b (25%), IgG3 (16%), and IgG2a (15%), in the sera of mice immunized with killed Delta1, whereas a strong shift to an IgG2b...
(65%) antibody response was observed in mice immunized with live $\Delta lgt$.

Next, we determined the immunological efficacy of the live vs. killed $\Delta lgt$ vaccines by measuring their protective effects against detrimental challenge with TIGR4. Mice immunized with either live or killed $\Delta lgt$ were i.n. challenged with WT TIGR4 ($10^7$ CFU), and mouse survival was recorded 10 days after the second immunization. As shown in Figure 3E, 90% of control mice (PBS-immunized) died by 5 dpi, whereas 60% of mice immunized with killed $\Delta lgt$ and 100% of mice immunized with live $\Delta lgt$ survived for more than 6 dpi. Taken together, these findings demonstrate that live $\Delta lgt$ vaccination induces high levels of IgM and IgG2-prone humoral immune responses, providing protection against detrimental challenge with its parent strain, TIGR4, in mice.

**Serotype-Independent Protection Conferred by TIGR4$\Delta lgt$ Vaccination**

To overcome the major limitations of currently available pneumococcal vaccines, vaccines should ideally be designed to provide serotype-independent, broad-spectrum protection against heterologous pneumococcal serotypes. Therefore, we assessed the cross-reactivity of the antibodies induced by TIGR4$\Delta lgt$ to different pneumococcal serotypes. As shown in Figures 4A–F, the IgM and IgG antibodies induced by TIGR4$\Delta lgt$ showed capsular serotype-independent reactivities to heterologous pneumococcal serotypes (STs), including ST2 (D39), ST3 (wu2), ST6B, ST9V, ST19F, and ST23F. In addition, we investigated production of the antibodies against conserved pneumococcal surface antigens such as LytA, an autolysin, and pneumococcal surface protein PspA, a component of transport system. TIGR4$\Delta lgt$ immunization produced antibodies against LytA and PspA as well as antibodies against purified cell wall PS (Figures 4G,H).

Next, we investigated whether TIGR4$\Delta lgt$ vaccination provided cross-protection against different serotypes. Mice were i.n. immunized with TIGR4$\Delta lgt$ twice at 14-day intervals (day 0 and day 14) and challenged with a lethal dose of wu2 (Figure 5A) or D39 (Figure 5B) at 10 days after the second immunization. All mice challenged with wu2 without immunization (control) died by 7 dpi (0% survival). However, immunization with TIGR4$\Delta lgt$ resulted in complete protection.
against wu2 challenge (100% survival) (Figure 5A). Similarly, D39 challenge resulted in 100% mortality for the unimmunized group by 7 dpi, while 75% of mice immunized with TIGR4Δlgt survived for more than 10 dpi, indicating serotype-independent protection offered by TIGR4Δlgt vaccination (Figure 5B). Bacterial loads in the nasopharynx, lungs, and blood of mice were also significantly reduced by TIGR4Δlgt vaccination following detrimental challenge with either wu2 (Figures 5C–E) or D39 (Figures 5F–H).

**DISCUSSION**

After adding PCVs to the immunization schedule for the pediatric population, the incidence of invasive pneumococcal diseases in both vaccinated children and unvaccinated individuals of all ages has been significantly reduced (9, 10). However, non-vaccine serotypes and new serotypes have emerged as major causes of pneumococcal diseases (11, 12, 61). To overcome these limitations, the development of innovative vaccines including protein-based vaccines, inactivated whole-cell-based vaccines, and live attenuated vaccines capable of covering all or most pneumococcal serotypes is actively being pursued (16, 19, 20, 62). Among these vaccines, live attenuated vaccines offer several advantages as cross-protective, broad-spectrum pneumococcal vaccines, such as the effective induction of both memory and cell-mediated immune responses against common and important pneumococcal antigens (21). Despite these benefits, a degree of unpredictability raises safety and stability concerns regarding the widespread adaptation of live attenuated vaccines (63). For example, Bacillus Calmette-Guerin (BCG) vaccination for tuberculosis can result in severe local inflammation or disseminated infection (64). In the current study, we developed a live attenuated vaccine for pneumococcal infections by deleting the lgt gene in the encapsulated pneumococcal strain TIGR4 (TIGR4Δlgt), resulting in significant attenuation of invasiveness.
and inflammation-activating abilities. However, TIGR4Δlgt was able to colonize the mouse nasopharynx long enough to elicit mucosal antibody responses and IgG2b-dominant, Th1-biased systemic immune responses that were cross-reactive to heterologous pneumococcal serotypes. Moreover, the live TIGR4Δlgt vaccine provided better protection than the killed whole-cell-based vaccine against subsequent detrimental challenge with the parental WT (TIGR4) strain.

Lipoproteins comprise the largest group of bacterial surface proteins and contribute significantly to bacterial adaptation to environmental changes, uptake of nutrients, and adherence to host membranes during infection (24). The importance of bacterial lipoproteins in TLR2-mediated immune recognition and pro-inflammatory responses has been extensively investigated using lgt-deficient pathogens (27, 65, 66). Both the present and previous studies showed that lgt-deficient pneumococci cannot activate TLR2-dependent cellular responses in macrophages. Although TLR2 responses are required for stimulating the innate and adaptive immune responses (e.g., Th1, Th2, Th17, Treg) of the host (76), they may contribute to the enhanced protection induced by live pneumococci (77). The importance of TLR2 responses against various lethal pneumococcal strains. Despite such prolonged colonization, TIGR4Δlgt did not result in significant inflammation or dissemination into the lungs or bloodstream, even at a multiplicity of infection >1,000 times the median lethal dose of the parental strain. Such localized colonization indicates the highly successful attenuation of invasiveness, thus ensuring the great potential of TIGR4Δlgt as a vaccine candidate.

Since airway is a major route of pneumococcal infection, alveolar macrophages play a role on defense against pneumococcus. Although the level of IgA, which is a major Ig class in the airway, was not significantly different in BALF between killed and live TIGR4Δlgt vaccination, live TIGR4Δlgt immunization more effectively removed pneumococcus from the alveolar airway. These findings may suggest that alveolar macrophage-mediated phagocytosis may play roles on enhanced protection observed in live TIGR4Δlgt immunization. Although TIGR4Δlgt failed to produce mature lipoproteins functioning as adhesins, our microarray data indicated that most cell surface-anchoring proteins (e.g., choline-binding proteins) were expressed at levels similar to that in the parental TIGR4 strain, likely facilitating sufficient colonization. Thus, prolonged colonization of TIGR4Δlgt may elicit cross-reactive, pneumococcal-specific IgG and IgM production and, therefore, more efficient Th1-biased antibody responses, providing improved protective immunity against multiple serotypes.

In conclusion, this study demonstrated that the deletion of a single pneumococcal “master” gene, lgt, results in the creation of a live attenuated vaccine with attenuated virulence and invasive ability. Furthermore, this study showed that TIGR4Δlgt has the potential to induce broad-spectrum, serotype-independent, protective immunity against life-threatening pneumococcal infections. Since conventional serotype-based vaccines have limitations, including a lack of protection against non-vaccine serotypes, the need for multiple doses and adjuvants, and high cost, the findings of this study may be useful in the development of inexpensive, but highly efficacious, vaccines against pneumococcal infections. In a broader sense, the modulation of major metabolic regulators represents an emerging strategy in the design of immunologic agents.

ETHICS STATEMENT

All animal experiments conducted in this study were approved by the Committee on the Use and Care of Animals at the...
Korea Atomic Energy Research Institute (KAERI) and performed according to accepted veterinary standards.

**AUTHOR CONTRIBUTIONS**

JL and HS conceived the study. A-YJ, KA, and HS designed the experiments. A-YJ, KA, YZ, HJ-J, JZ, and HS performed the experiments. A-YJ, KA, and HS analyzed and/or interpreted the data and contributed to discussion of the results, followed by writing and reviewing the manuscript. SH, HG, SL, JS, JL, and HS provided critical comments and contributed to discussion of the results, followed by writing and reviewing the manuscript.

**REFERENCES**

1. Morrill HJ, Caffrey AR, Noh E, LalPlante KL. Epidemiology of pneumococcal disease in a national cohort of older adults. *Infect Dis Ther.* (2014) 3:19–33. doi: 10.1007/s40121-014-0025-y
2. Henriques-Normark B, Tuomanen EI. The pneumococcus: epidemiology, microbiology, and pathogenesis. *Cold Spring Harb Perspect Med.* (2013) 3:a010215. doi: 10.1101/cshperspect.a010215
3. Savulescu C, Krizova P, Lepoutre A, Mereckiene J, Vestrheim DF, Ciruela P, et al. Effect of high-valency pneumococcal conjugate vaccines on invasive pneumococcal disease in children in SpIDnet countries: an observational multicentre study. *Lancet Respir. Med.* (2017) 5:648–56. doi: 10.1016/S2213-2600(17)30110-8
4. Xu X, Wang H, Liu Y, Wang Y, Zeng L, Wu K, et al. Mucosal immunization with the live attenuated vaccine SPY1 induces humoral and Th2-Th17-regulatory T cell cellular immunity and protects against pneumococcal infection. *Infect Immun.* (2015) 83:90–100. doi: 10.1128/IAI.02334-14
5. Trzcinski K, Thompson CM, Srivastava A, Basset A, Malley R, Lipsitch M. Protection against nasopharyngeal colonization by *Streptococcus pneumoniae* is mediated by antigen-specific CD4+ T cells. *Infect Immun.* (2008) 76:2678–84. doi: 10.1128/IAI.00141-08
6. Wuorimaa T, Kayhty H. Current state of pneumococcal vaccines. *Sand J Immunol.* (2002) 56:111–29. doi: 10.1046/j.1365-3033.2002.01124.x
7. Vila-Corcoles A, Ochoa-Gondar O, Hospital I, Ansa X, Vilanova A, Rodriguez T, et al. Protective effects of the 23-valent pneumococcal polysaccharide vaccine in the elderly population: the EVAN-65 study. *Clin Infect Dis.* (2006) 43:860–8. doi: 10.1086/507340
8. Esposito S, Principi N. Impacts of the 13-valent pneumococcal conjugate vaccine in children. *J Immunol Res.* (2015) 2015:591580. doi: 10.1155/2015/591580
9. Corcoran M, Vickers I, Mereckiene J, Murchan S, Cotter S, Fitzgerald M, et al. The epidemiology of invasive pneumococcal disease in older adults in the post-PCV era. Has there been a herd effect? *Epidemiol. Infect.* (2017) 145:2390–9. doi: 10.1017/S0950268817001194
10. Conklin L, Loo JD, Kirk J, Fleming-Dutra KE, Deloria Knoll M, Park DE, et al. Systematic review of the effect of pneumococcal conjugate vaccine dosing schedules on vaccine-type invasive pneumococcal disease among young children. *Pediatr Infect Dis J.* (2014) 33 (Suppl. 2):S109–18. doi: 10.1097/INF.0000000000000778
11. Haasdorff WP, Van Dyke MK, Van Effelterre T. Serotype replacement after pneumococcal vaccination. *Lancet.* (2012) 379:1387–8; author reply 8–9. doi: 10.1016/S0140-6736(12)60589-3
12. Spratt BG, Greenwood BM. Prevention of pneumococcal disease by vaccination: does serotype replacement matter? *Lancet.* (2000) 356:1210–1. doi: 10.1016/S0140-6736(00)02779-3
13. Gladstone RA, Devine V, Jones J, Cleary D, Jefferies JM, Bentley SD, et al. Pre-vaccine serotype composition within a lineage signposts its serotype replacement - a carriage study over 7 years following pneumococcal conjugate vaccine use in the UK. *Microbiom.* (2017) 3:e000119. doi: 10.1099/mgen.0.000119
14. Gladstone RA, Jeffries JM, Tocheva AS, Beard KR, Garley D, Chong WW, et al. Five winters of pneumococcal serotype replacement in UK carriage following PCV introduction. *Vaccine.* (2015) 33:2015–21. doi: 10.1016/j.vaccine.2015.03.012
15. Lynch JM, Briles DE, Metzger DW. Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12. *Infect Immun.* (2003) 71:4780–8. doi: 10.1128/IAI.71.8.4780-4788.2003
16. Moftit KL, Malley R, Lu YJ. Identification of protective pneumococcal TH17 antigens from the soluble fraction of a killed whole cell vaccine. *PLoS ONE.* (2012) 7:e34345. doi: 10.1371/journal.pone.0034345
17. Moftit KL, Yadav P, Weibeberger DM, Anderson PW, Malley R. Broad antibody and T cell reactivity induced by a pneumococcal whole-cell vaccine. *Vaccine.* (2012) 30:4316–22. doi: 10.1016/j.vaccine.2012.01.034
18. Chien YW, Klugman KP, Morens DM. Efficacy of whole-cell killed bacterial vaccines in preventing pneumonia and death during the 1918 influenza pandemic. *J Infect Dis.* (2010) 202:1639–48. doi: 10.1086/657144
19. Rosch JW, Iverson AR, Humann J, Mann B, Gao G, Vogel P, et al. A live-attenuated pneumococcal vaccine elicits CD4+ T-cell dependent class switching and provides serotype independent protection against acute otitis media. *EMBO Mol Med.* (2014) 6:141–54. doi: 10.1002/emmm.201202150
20. Moftit KL, Malley R. Next generation pneumococcal vaccines. *Curr Opin Immunol.* (2011) 23:407–13. doi: 10.1016/j.coi.2011.04.002
21. Rosch JW. Promises and pitfalls of live attenuated pneumococcal vaccines. *Hum Vaccin Immunother.* (2014) 10:3000–3. doi: 10.4161/21645515.2014.970496
22. Roche AM, King SJ, Weiser JN. Live attenuated *Streptococcus pneumoniae* strains induce serotype-independent mucosal and systemic protection in mice. *Infect Immun.* (2007) 75:2469–75. doi: 10.1128/IAI.01972-06
23. Kim EH, Chot SI, Kwon MK, Tran TD, Park SS, Lee KJ, et al. *Streptococcus pneumoniae pep27* mutant as a live vaccine for serotype-independent protection in mice. *Vaccine.* (2012) 30:2008–19. doi: 10.1016/j.vaccine.2011.11.073
24. Kovacs-Simon A, Tibball RW, Michell SL. Lipoproteins of bacterial pathogens. *Infect Immun.* (2011) 79:548–61. doi: 10.1128/IAI.00682-10
25. Kohler S, Voss E, Gomez Mejia A, Brown JS, Hammerschmidt S. Pneumococcal lipoproteins involved in bacterial fitness, virulence, and immune evasion. *FEBS Lett.* (2016) 590:3820–39. doi: 10.1002/1873-3468.12352
26. Tavitian SV, Park JT. Lipoprotein of gram-negative bacteria is essential for growth and division. *Nature.* (1976) 263:323–6. doi: 10.1038/263323a0
27. Smitha D, Rosas FR, Gruber W, Wortham C, Kehry MR, Mond JJ. Bacterial lipoproteins may substitute for cytokines in the humoral immune response to T cell-independent type II antigens. *J Immunol.* (1995) 155:5882–9.
28. Hashimoto M, Tawaratsumida K, Kariya H, Kiyohara A, Suda Y, Krikae F, et al. Not lipoteichoic acid but lipoproteins appear to be the dominant FUNDING

This work was supported by the Nuclear R&D Program of the Ministry of Science and ICT (SL), National Research Foundation of Korea under Grants NRF-2017M2A2A602020925 and 2018K2A2A6060328 to HS and 2015R1D1A1A01059338 to JL, and the Ministry of Food and Drug Administration under Grant 18172MFD5253 to JS.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01212/full#supplementary-material
immunobiologically active compounds in Staphylococcus aureus. J Immunol. (2006) 177:3162–9. doi: 10.4049/jimmunol.177.5.3162

29. Kretschmer D, Hanzelmann D, Peschel A. Lipoprotein immunoproteomics question the potential of Staphylococcus aureus TLR2 agonists as vaccine antigens. Proteomics. (2016) 16:2603–4. doi: 10.1002/pmic.201605351

30. Mao G, Zhao Y, Kuong X, Li Z, Zhang Y, Wang X, et al. Crystal structure of E. coli lipoprotein diacylglycerol transferase. Nat Commun. (2016) 7:10198. doi: 10.1038/ncomms10198

31. Zuckert WR. Secretion of bacterial lipoproteins: through the cytoplasmic membrane, the periplasm and beyond. Biochim Biophys Acta. (2014) 1843:1509–16. doi: 10.1016/j.bbamer.2014.04.022

32. Baumann G, Karst U, Gerstel B, Loesnner M, Wehland J, Jansch L. Inactivation of lgt allows systematic characterization of lipoproteins from Listeria monocytogenes. J Bacteriol. (2007) 189:313–24. doi: 10.1128/JB.00976-06

33. Arimoto T, Igarashi T. Role of prolipoprotein diacylglycerol transferase (Lgt) and lipoprotein-specific signal peptide II (LspA) in localization and physiological function of lipoprotein Msme in Streptococcus mutans. Oral Microbiol Immunol. (2008) 23:515–9. doi: 10.1111/j.1399-302X.2008.00455.x

34. Bray BA, Sutcliffe JC, Harrington DJ. Impact of lgt mutation on lipoprotein biosynthesis and in vitro phenotypes of Streptococcus agalactiae. Microbiology. (2009) 155 (Pt 5):1461–8. doi: 10.1099/mic.0.025213-0

35. Wichgers Schreur PJ, Rebel JM, Smits MA, van Putten JP, Smith HE. Lgt processing is an essential step in Streptococcus suis lipoprotein mediated innate immune activation. PLoS ONE. (2011) 6:e22299. doi: 10.1371/journal.pone.002299

36. Pailler J, Aucher W, Pires M, Buddelmeijer J. Phosphatidylglycerol-lipoprotein diacylglycerol transferase (Lgt) of Escherichia coli has seven transmembrane segments, and its essential residues are embedded in the membrane. J Bacteriol. (2012) 194:2142–51. doi: 10.1128/JB.00641-11

37. Tomlinson G, Chimalapati S, Pollard T, Lapp T, Cohen J, Camberlein E, et al. AdcAII of Streptococcus pneumoniae highl...
66. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol. (2002) 169:10–4. doi: 10.4049/jimmunol.169.1.10

67. Barra NG, Gillgrass A, Ashkar AA. Effective control of viral infections by the adaptive immune system requires assistance from innate immunity. Expert Rev Vaccines. (2010) 9:1143–7. doi: 10.1586/erv.10.119

68. Iwasaki A, Medzhitov R. Control of adaptive immunity by the innate immune system. Nat Immunol. (2015) 16:343–53. doi: 10.1038/nii.3123

69. Wang J, Roderiguez G, Norcross MA. Control of adaptive immune responses by Staphylococcus aureus through IL-10, PD-1, and TLR2. Sci Rep. (2012) 2:606. doi: 10.1038/srep00606

70. Michaelsen TE, Kolberg J, Aase A, Herstad TK, Hoiby EA. The four mouse IgG isotypes differ extensively in bactericidal and opsonophagocytic activity when reacting with the P1.16 epitope on the outer membrane PorA protein of Neisseria meningitidis. Scand J Immunol. (2004) 59:34–9. doi: 10.1111/j.0300-9475.2004.01362.x

71. Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV. FcgammaRIV: a novel FcR with distinct IgG subclass specificity. Immunity. (2005) 23:41–51. doi: 10.1016/j.immuni.2005.05.010

72. Collins AM. IgG subclass co-expression brings harmony to the quartet model of murine IgG function. Immunol Cell Biol. (2016) 94:949–54. doi: 10.1038/icb.2016.65

73. Koppe U, Suttrop N, Opitz B. Recognition of Streptococcus pneumoniae by the innate immune system. Cell Microbiol. (2012) 14:460–6. doi: 10.1111/j.1462-5822.2011.01746.x

74. Rijneveld AW, Lauw FN, Schultz MJ, Florquin S, Te Velde AA, Speelman P, et al. The role of interferon-gamma in murine pneumococcal pneumonia. J Infect Dis. (2002) 185:91–7. doi: 10.1086/338122

75. Mureithi MW, Finn A, Ota MO, Zhang Q, Davenport V, Mitchell TJ, et al. T cell memory responseto pneumococcal protein antigens in an area of high pneumococcal carriage and disease. J Infect Dis. (2009) 200:783–93. doi: 10.1086/605023

76. Cohen JM, Khandavilli S, Camberlein E, Hyams C, Baxendale HE, Brown JS. Protective contributions against invasive Streptococcus pneumoniae pneumonia of antibody and Th17-cell responses to nasopharyngeal colonisation. PLoS ONE. (2011) 6:e25558. doi: 10.1371/journal.pone.0025558

77. Ferreira DM, Neill DR, Bangert M, Gritzfeld JF, Green N, Wright AK, et al. Controlled human infection and rechallenge with Streptococcus pneumoniae reveals the protective efficacy of carriage in healthy adults. Am J Respir Crit Care Med. (2013) 187:855–64. doi: 10.1164/rccm.201212-2277OC

78. Magee AD, Yother J. Requirement for capsule in colonization by Streptococcus pneumoniae. Infect Immun. (2001) 69:3755–61. doi: 10.1128/IAI.69.6.3755-3761.2001

79. Ogunniyi AD, LeMessurier KS, Graham RM, Watt JM, Briles DE, Stroher UH, et al. Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of Streptococcus pneumoniae D39 in a mouse model. Infect Immun. (2007) 75:1843–51. doi: 10.1128/IAI.01384-06

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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