Construction of an inactivated typhoid vaccine candidate expressing *Escherichia coli* heat-labile enterotoxin B subunit and evaluation of its immunogenicity in a murine model

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**Abstract**

**Purpose.** Typhoid fever caused by *Salmonella enterica* serovar Typhi has contributed to the global public health burden, particularly in developing countries. In this study, an *S*. Typhi ghost was developed and its capacity as a vaccine candidate against typhoid fever was assessed.

**Methodology.** An *asd* plasmid pJHL187 harbouring a ghost cassette comprising the PhiX 174 Elysis gene tightly controlled under the convergent promotor system was transformed into an *asd* gene-deleted mutant *S*. Typhi strain (STG). The eltB gene encoding the *E*. coli heat-labile enterotoxin (LTB) protein was subcloned into a foreign antigen delivery cassette of pJHL187 to increase mucosal immunity.

**Results.** The stringent repression and expression of the lethal E lysis gene in the system allowed stable production of the ghost strain and secretion of LTB, which was confirmed by immune blot analysis. The level of IgG and sIgA was significantly increased in the mice subcutaneously immunized with STG-LTB compared to the non-immunized mice (*P*<0.05). The CD3+CD4+ T cell subpopulation was augmented in the immunized group (*P*<0.05) and showed the increment of immunomodulatory cytokines IL-2, IL-6, IL-12, IL-17 and IFN-γ in *in vitro* restimulated splenocytes isolated from the inoculated mice. The serum bactericidal activity of antibodies generated in the rabbits injected with STG-LTB was proved by the elimination of approximately 87.5% of wild-type *S*. Typhi in the presence of exogenous complement.

**Conclusion.** The results demonstrated that the STG-LTB ghost effectively enhanced the immunological responses, meaning that STG-LTB is potentially available as a vaccine candidate against typhoid fever.

**INTRODUCTION**

Bacterial ghosts (BGs) were developed as a novel non-living vaccine platform using the DX174 lysis E gene [1]. Transmembrane tunnels are formed by the transcription of the lysis gene in Gram-negative bacteria. Subsequently, the contents are expelled during lysis, but intact bacterial surface envelope structures remain [2, 3]. Bacterial ghosts containing the preserved antigenic conformational epitopes have induced efficient immune responses against target antigens [3]. In the context of the safety and practicality of producing ghost vaccines by the expression of a lethal lysis gene E under inducible promoters, several Gram-negative bacteria have been successfully prepared for BGs for the development of a human vaccine against *Escherichia coli* O157: H7, *Vibrio cholera*, *Helicobacter pylori* and *Chlamydia trachomatis* [4–8].

Typhoid fever caused by *Salmonella enterica* serovar Typhi presents a global health burden, especially for immunocompromised residents in developing countries with inadequate sanitation [9, 10]. *S*. Typhi, a human-restricted pathogen, is estimated to cause approximately 33 million cases of typhoid fever and half a million deaths annually worldwide [11]. Treatment with antibiotics is limited due to the high incidence of multidrug-resistant *S*. Typhi in endemic areas [12, 13]. Two licensed typhoid vaccines, the parenteral Vi polysaccharide (Vi) (Typherixor Typhim Vi) and oral live attenuated Ty21a (Vivotif), are currently available and recommended for use. Despite the presence of these approved
vaccines, vaccines have primarily been applied for travellers to the countries, rather than residents living in the endemic areas, due to the limited health budgets in these areas, resulting in an inappropriate control of typhoid fever [14]. Based on a database from the last 3 years, a recent systemic review reported that Vi vaccine based on typhoid Vi antigen and Ty21a vaccine conferred 30 to 70 % protective efficacy [15]. Furthermore, the Vi-based vaccine has a lack of booster effect because of the failure to mount T cell-related immune responses [16, 17]. Thus, the introduction of a new-concept vaccine may be needed to reduce costs and improve the immunogenicity and storability of the existing vaccines in endemic areas.

In the early stage of vaccine development against typhoid fever, parenteral phenol-preserved or heated-inactivated whole-cell vaccines were introduced. However, their continuing use was hampered by severe side-effects caused by the overt reactogenicity [18]. In contrast, for the ghost vaccine system, Mader et al. indicated that empty bacterial ghosts preserve all of the surface antigenic determinants, containing the same entire surface antigenic determinants as their living counterparts, including lipopolysaccharide, capsular and flagella, etc. [19]. In addition, the research group also revealed that both E. coli O26:B6 and Salmonella Typhimurium ghosts induced no marked pyrogenic activity in rabbits immunized with relatively high doses (250 ng per kg) of the ghost cells [19, 20]. Thus, Bs could replace the use of live attenuated or polysaccharide capsule vaccines against typhoid fever [21] and might efficiently induce immunogenicity against these intact antigenic components.

This strategy also embodies the concept of the incorporation of a foreign immuno-stimulatory protein, such as E. coli heat-labile enterotoxin B unit protein (LTB), into the envelope of bacterial ghosts, using its capacity to deliver a foreign recombinant antigen [3]. E. coli LTB is nontoxic, but elicits powerful mucosal and cellular immune responses [22]. This study describes the construction of a Salmonella Typhi ghost (STG) expressing LTB protein (STG-LTB) to exploit mucosal and cellular immune responses in immunized mice and then compare their immunogenicity in a murine model. To evaluate the functional antibodies generated by the immunization, which eliminates the pathogen circulated in the host, serum bactericidal assay was performed. This study is the first report of the introduction of the ΦX174 lysis gene E into S. Typhi and preparation for an inactivated vaccine candidate against typhoid fever.

METHODS

Bacterial strains, plasmids, and primers

The bacterial strains, plasmids and primers used in the current study are listed in Table 1. Strains carrying ghost plasmids were incubated in the presence of 0.2 % L-arabinose. All bacterial strains were stored at −80 °C in Luria–Bertani (LB) broth containing 20 % glycerol.

Salmonella Typhi ghost construction

Salmonella enterica serotype Typhi Ty21a strain JOL1497, which carries no plasmid, was used to construct the ghost strain. The aspartate β-semialdehyde dehydrogenase (asd) gene-deleted mutant of S. Typhi Ty21a strain JOL1498 originated from JOL1497 and was prepared using allelic exchange as previously described [23]. The JOL1498 was transformed with pJHL187-LTB by means of electroporation and the resultant strains were designated S. Typhi JOL1502. The cassette system for the construction of ghosts comprised the ΦX 174 E lysis gene stringently controlled under the two face to face promoters that are a sense thermo-sensitive promoter with a cI857 regulatory component and an antisense ParaBAD promoter repressed by araC component in the absence of arabinose [24]. The unnecessary transcription of the E gene from the sense thermo-sensitive promoter at 27 °C can be compromised with an antisense RNA simultaneously expressed by the ParaBAD promoter in the presence of arabinose, contributing to the production of a high-yield ghost at 27 °C [25]. The temperature increased to 42 °C and the removal of arabinose in the ghost cell culture induced E gene-mediated lysis to inactivate the cells. For the heterologous antigen delivery system, the outer-membrane protein A signal sequence was placed to secrete LTB protein to the periplasmic area. A single colony of JOL1502 was cultivated in 50 ml of nutrient broth (NB) with 0.2 % L-arabinose at 27 °C until the mid-logarithmic phase. The cells were collected, washed twice with NB and resuspended in 50 ml of NB without 0.2 % L-arabinose at 42 °C in a shaking incubator at 200 r.p.m. to induce the expression of lysis gene E. After 48 h, the bacterial cells were harvested via centrifugation for 10 min at 4000 r.p.m. and resuspended in 1 ml of sterile phosphate-buffered saline (PBS). To remove the remaining risk of its use as a vaccine strain, chemically induced lysis mediated by sodium hydroxide (NaOH) treatment was applied to prepare the ghost vaccine strain. The concentration of sodium hydroxide was determined using the minimum inhibitory concentration for effective lysis as previously described [26]. We added 0.25 mg ml⁻¹ NaOH onto the culture of the lysed ghost bacterial cell mediated by the E gene for 24 h, and incubated for 5 min at 37 °C to completely inactivate the lysed cells [26], which were subsequently washed three times with sterile PBS. Complete lysis of the ghost cells was verified by counting the number of viable cells and measuring the optical density (OD; at 600 nm wavelength). The final cell pellets were resuspended in autoclaved PBS and stored at −20 °C until use. For morphological visualization of the lysed JOL1502 ghosts, scanning electron microscopy (SEM) was performed as previously described [27].

Immunoblotting

The LTB protein secreted in JOL1502 was confirmed by Western blot analysis. The bacterial ghost samples and polyclonal LTB-specific rabbit anti-serum were generated as described in a previous study [28]. Briefly, JOL1502 ghost cells were sonicated and centrifuged at 13 000 r.p.m. for
PBS and 100 µl of a suspension containing 1 and B were subcutaneously vaccinated with 100 µl of sterile randomly assigned into two groups. The mice in groups A and B were twice injected subcutaneously with 100 µl of PBS were sampled from the mice. For immunological analysis, the collected pellets were boiled at 95 °C for 30 min. The collected pellet was resuspended in Tris-Sarkosyl buffer (20 mM Tris containing 1% sarkosyl, pH 8.6). A similar procedure was used to prepare the bacterial ghost treated with NaOH. Unlysed JOL1502 was applied as the negative control. The collected pellets were boiled at 95 °C for 10 min and then loaded into sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gels. Following the electrophoretic transfer of the separated protein onto polyvinylidene fluoride membranes (Millipore, Temecula, CA, USA), the immunoblot analysis was performed as previously described [29].

**Animal experiments**

A total of 20 female BALB/C mice at 5 weeks of age were randomly assigned into two groups. The mice in groups A and B were subcutaneously vaccinated with 100 µl of sterile PBS and 100 µl of a suspension containing 1×10⁸ of colony-forming units (c.f.u.) of JOL1502, respectively, at weeks 0 and 2. At weeks 0, 2, 4 and 6 post-immunization (p.i.), sera were obtained from all groups by centrifuging blood collected from the orbital sinus of the mice, and vaginal washes with PBS were sampled from the mice. For immunological analysis using the primed splenocytes, an additional 10 mice (5 mice from each group) were inoculated with JOL1502 ghost and their capacity to mediate serum antibodies raised in the rabbits immunized with JOL1502 ghost at a multiplicity of infection (m.o.i.) of 10. The mRNA expression levels of the cytokines were determined by cycle threshold (ΔCₜ) values calculated based on the internal standard, β-actin. The fold changes of the mRNA levels were presented as 2⁻ΔΔCₜ compared to those of the non-immunized group [31]. The primer pairs of the mouse cytokines were obtained in a previous study [32]. Simultaneously, to detect the protein level of IL-4 and IFN-γ secreted in the primed splenocytes during the stimulation with the JOL1502 ghost, the cytokines were quantified in the cell supernatants using the mouse IL-4 ELISA Ready-SET-Go and mouse IFN-gamma ELISA Ready-Set-Go kits (eBioscience, San Diego, CA, USA) following the manufacturer’s instructions.

**Assessment of systemic and T cell-associated immune responses**

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tr>
<td><strong>Bacterial strains</strong></td>
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<td>Lab stock</td>
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<tr>
<td>E. coli JOL232</td>
<td>F’ lacZYA-argF endA1 recA1 hsdR17 deoR thi-1 glnV44 grfA96 relA1 ΔasdA4</td>
<td></td>
</tr>
<tr>
<td>S. Typhi JOL380</td>
<td>S. Typhi wild-type from human</td>
<td>Lab stock</td>
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<tr>
<td>JOL1497</td>
<td>S. Typhi Ty21a</td>
<td>Lab stock</td>
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<tr>
<td>JOL1498</td>
<td>Δasd, a derivative of S. Typhi Ty21a</td>
<td>This study</td>
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<tr>
<td>JOL1502</td>
<td>JOL1498 containing pJHL187-eltB</td>
<td>This study</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pJHL187</td>
<td>asd vector, p15A ori plasmid carrying ss ompA/His6, multiple cloning site, cI857/PR promoter, araC PₐraBAD, phiX174</td>
<td>[28]</td>
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<tr>
<td>pJHL187-eltB</td>
<td>pJHL187 harbouring eltB gene</td>
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The presence of S. Typhi-specific immunoglobulin G (IgG) and secretory immunoglobulin A (sIgA) antibodies in sera and vaginal washes, respectively, following the immunization was determined by indirect enzyme-linked immunosorbent assay (ELISA) as previously described [29]. Outer membrane (OMP) protein extracted from the wild-type S. Typhi (ST) strain JOL380 was used as the coating antigen (500 ng per well). For the measurement of T cell increment in the vaccinated mice, the mice were sacrificed and their spleens aseptically isolated at week 2 p.i., and single-cell suspensions were seeded into the 96-well cell-culture plate. Fluorescence-activated cell-sorting (FACS) staining and analysis was performed to assess the change of the CD3+ and CD4+CD45+ T cell subpopulations in the splenocytes isolated from the immunized mice, following the previously published protocol [30].

**Detection of immunomodulatory cytokines**

To measure the immunomodulatory cytokine profiles induced by the immunization, the expression level of the cytokine genes containing IL-2, IL-4, IL-6, IL-12, IL-17, IL-23 and IFN-γ were detected by reverse transcription/polymerase chain reaction (RT-PCR) [30]. The genes were amplified in the 1×10⁸ of the splenocytes from immunized or non-immunized mice, which were stimulated with the JOL1502 ghost at a multiplicity of infection (m.o.i.) of 10. The mRNA expression levels of the cytokines were determined by cycle threshold (ΔCₜ) values calculated based on the internal standard, β-actin. The fold changes of the mRNA levels were presented as 2⁻ΔΔCₜ compared to those of the non-immunized group [31]. The primer pairs of the mouse cytokines were obtained in a previous study [32]. Simultaneously, to detect the protein level of IL-4 and IFN-γ secreted in the primed splenocytes during the stimulation with the JOL1502 ghost, the cytokines were quantified in the cell supernatants using the mouse IL-4 ELISA Ready-SET-Go and mouse IFN-gamma ELISA Ready-Set-Go kits (eBioscience, San Diego, CA, USA) following the manufacturer’s instructions.

**Serum bactericidal activity assay**

A serum bactericidal activity (SBA) assay was performed in serum antibodies raised in the rabbits immunized with JOL1502 ghost and their capacity to mediate in vitro complement-dependent elimination of a virulent S. Typhi strain was determined. The growth inhibition of S. Typhi by serum was examined as previously described with a slight modification [33]. Briefly, two New Zealand White rabbits were twice injected subcutaneously with 1×10⁸ of the lysed JOL1502 at 2-week intervals. Blood was collected at weeks...
0, 4 and 6. The serum was heat-inactivated at 56 °C for 30 min to block the complement alternative pathway. Subsequently, serum sampled from the rabbits at weeks 0, 4 and 6 were diluted with PBS as follows – 1:1, 1:50 and 1:100 – to determine the optimal serum concentration. Baby rabbit complement (Sigma-Aldrich, St Louis, MO, USA) was used as the exogenous complement source. The JOL380 strain was grown at 37 °C to the mid-logarithmic phase in LB medium overnight, and the cultured bacterial cells were diluted to 10^8 c.f.u./10 µl in PBS. The suspension was mixed with 10 µl of the optimized concentration (1:1) of the serum and 12.5% final concentration of baby rabbit complement, and incubated at 37 °C for 1 h. The number of bacterial cells surviving at the end of the period was quantified as the exogenous complement source. The JOL380 strain complement (Sigma-Aldrich, St Louis, MO, USA) was used to determine the optimal serum concentration. Baby rabbit serum and 12.5% final concentration of baby rabbit complement.

Statistical analysis
Statistical analyses were carried out using GraphPad Prism (GraphPad Software, CA, USA). The statistical differences among the immunized and non-immunized groups were evaluated using the non-parametric Mann–Whitney U test. P values <0.05 were considered to be significant.

RESULTS
Construction and production of STG-LTB
An asd^+ ghost plasmid, pJHL187, carrying the ϕX174 E lysis gene cassette and heterologous antigen delivery systems was employed to construct the S. Typhi ghost strain. An asd-deleted S. Typhi JOL1498 was transformed with pJHL187-LTB by electroporation, and transformants containing the plasmid were selected on BGA without DAP. For the production of BGs from the resultant strain, JOL1502, the lysis mediated by the E gene, was activated during the mid-log growing phase by an increase of temperature to 42 °C, resulting in thermal inactivation of the cI857 repressor protein in the absence of l-arabinose in the cultures. The ghost-cell cultures contained 0 to 10 c.f.u. per ml after 48 h of the lysis process (Fig. 1), resulting in the accomplishment of 99.99% lysis efficiency. For the final construction of STG-LTB, the cells were harvested after 24 h of lysis induced by the E gene, and subsequently 0.25 mg ml⁻¹ NaOH was added to the culture to ensure the complete inactivation of JOL1502. This mixture was incubated at 37 °C for 5 min. No viable colonies were recovered after this process. An electron microscopic analysis of S. Typhi ghosts was carried out to evaluate the morphological changes in the ghost strains. The transmembrane tunnels induced by E gene-mediated lysis were observed on the surface of JOL1502 cells (Fig. 2b, arrowheads). The tunnels where cytoplasmic contents were expelled might result in partially disrupted ghost cell envelopes. By contrast, the unlysed JOL1502 showed no phenotypic alteration and retained an intact form of the cells (Fig. 2a).

Expression of LTB protein in S. Typhi ghosts
Western blot analysis was performed to confirm the secretion of LTB protein in JOL1502. The unlysed JOL1502 was used as a negative control. The expected size fusion protein size (approximately 27 kDa) was detected (Fig. 3; lane B). The protein size for the LTB expression from STG-LTB JOL1502 treated with NaOH was also detected (Fig. 3; lane C), while the control did not show the prominent corresponding protein size (Fig. 3; lane A).

Systemic antibody responses in immunized mice
After the STG-LTB inoculation to the mice, noticeable side-effects, such as hair election, depression, low appetite and diarrhoea were not observed. The markedly raised titres of antibodies specific to the ST OMP compared to those of the mock
immunized group during the observation period \((P<0.05)\) (Fig. 4a, b). The mice receiving a secondary dose showed the peak IgG response at week 2 after the second immunization (i.e. week 4 p.i.), while the greatest increase of sIgA titre was observed at week 2 p.i. after the first immunization. In addition, the mice immunized with JOL1502 produced a significantly increased level of LTB-specific IgG in comparison to the control mice at weeks 2 and 4 \((P<0.05)\) (Fig. 4c).

**T cell-related immune responses**

The relative alteration of the surface markers expressed in splenic T cells was examined to assess the magnitude of the immuno-stimulatory effects elicited by STG-LTB. T lymphocyte subsets in the spleens of the mice immunized with JOL1502 were analysed by flow cytometry. The percentage of CD3\(^+\) and CD3\(^+\)CD4\(^+\) T cell subpopulations was elevated in the mice immunized with JOL1502 compared to the non-immunized mice (Fig. 5). In particular, the CD3\(^+\)CD4\(^+\) T lymphocytes in the vaccinated mice showed an approximately twofold increase by comparison to those of the control mice at week 2 p.i. (Fig. 5b).

**Detection of cytokines**

To evaluate the expression levels of the cytokines regulating defence against infection, the number of immunomodulatory cytokine mRNA copies was analysed in the in vitro restimulated splenic lymphocytes by performing RT-PCR. The immunization with STG-LTB JOL1502 induced a marked augmentation of the relative fold values for IL-2, IL-6, IL-12, IL-17 and IFN-\(\gamma\) in the immunized mice compared to the non-immunized mice \((P<0.05)\) (Fig. 6). In particular, the IL-6 cytokine gene associated with the induction of surface IgA-positive B cells [34] increased by approximately 600-fold in the immunized mice. Further, the expression of IFN-\(\gamma\) mRNA increased by 6.81±2.91-fold in the mice immunized with JOL1502 ghost cells, while IL-4 mRNA was downregulated in both the immunized and the non-immunized mice (Fig. 6). For the cytokines measured in the protein level, the IFN-\(\gamma\) cytokine detected in the cell supernatant following the stimulation also increased significantly, whereas the production of IL-4 protein was not elevated in the immunized mice compared to the non-immunized mice (Fig. 7). This comparison may imply that the expression levels of cytokine mRNA tend to be proportional to the level of the cytokine proteins detected.

**Serum bactericidal activity of STG-LTB**

The in vitro SBA of STG-LTB contributing to in vitro clearance of the virulent S. Typhi JOL380 was determined by the magnitude of the functional antibodies (Abs) derived from the rabbits immunized with STG-LTB, collected at weeks 0, 4 and 6. The serum Abs produced in the immunized rabbits showed a marked increment of SBA compared to those of the non-immunized controls at weeks 4 and 6 p.i. \((P<0.05)\).
(Fig. 8). In particular, at week 6 p.i. the serum Abs exhibited approximately 87.5±1.25 % SBA against the virulent S. Typhi strain, which indicated that the serum Abs generated in the mice inoculated with STG-LTB could recognize and eliminate a virulent S. Typhi strain in vivo.

**DISCUSSION**

The aim of this study was to construct a *Salmonella* Typhi ghost expressing LTB and determine its immunogenicity in a murine model to develop a vaccine candidate against typhoid fever. To prepare for the final stage of STG-LTB construction, chemical treatment was incorporated into the ghost production process. NaOH has been found to hydrolyze the cell envelope [35]. STG-LTB inactivated by the *E* gene for 24 h was added to 0.25 mg ml⁻¹ of NaOH and incubated for 5 min. This consequently exhibited a complete form of ghost, and no living cells were observed in the culture of the final STG-LTB. Quantitative inactivation of the JOL1502 ghost was accomplished through the process and a fusion LTB protein of the expected size was detected via immunoblot analysis (Fig. 3; lanes B and C). These findings supported that the components on the cell surface are not altered by this minimal concentration of NaOH and can function as antigenic determinants.

Another benefit from application of the convergent promoter elements to STG production was the stable expression of the *eltB* gene. In this study, the plasmid pJHL187 carrying the ghost cassette possessed a signal peptide of the *ompA* protein, a major outer-membrane protein of *E. coli*, which had sufficient capacity to direct the secretion of the fusion protein across the cytoplasmic membranes of bacteria [36]. The *eltB* gene clone was fused with the *ompA* and the resultant fusion protein was secreted and maintained with STG cells, triggered by expression of the ApR promoter. The expected size of the fusion protein expressed in JOL1502 was confirmed by immunoblot analysis (Fig. 2). The pentameric B subunit of *E. coli* LTB is nontoxic and has been applied as a potent mucosal immunogen and immunoadjuvant towards co-administered antigens. LTB has a high affinity for glycolipid and glycol protein receptors on
intestinal epithelial cells [37, 38]. The LTB binds to the receptors that stimulate a mucosal surface and consequently the uptake of antigens coupled with LTB across the mucosa was simultaneously enhanced [39]. In the current study, we observed significant increases of IgG specific to the ST OMP or LTB specific to the ST OMP in the mice immunized with STG-LTB compared to the non-immunized mice during the experimental period (Fig. 4). In general, it is recommended that the mucosal adjuvant be injected via an oral route, since Peyer’s patches, a component of gut-associated lymphoid tissues functioning as a mucosal inductive site, were stimulated with the ingested vaccine strain [40]. Since subcutaneous administration was applied for the mice immunization to maximize the immunogenicity of the non-living ghost vaccine candidates in this study [41], the LTB expressed in JOL1502 ghosts could reach the mucosal surfaces, resulting in its binding to the glycolipid and glycol protein receptors and a marked increase of mucosal IgA in the immunized mice compared to the non-immunized mice (Fig. 4b).

In general, the cytokines generated in the immunized mice can characterize the profile of the immune responses elicited in the animals. Antigen-specific IFN-γ and IL-2 cytokines

Fig. 6. Fold change of cytokine gene expression levels for in vitro restimulated splenocytes of the mice immunized with JOL1502 (n=5) in comparison to the non-immunized mice. β-actin was used as the normalizing internal control. The mRNA expression of the detected genes was measured by qRT-PCR and the error bars show sd. * indicates statistically significant changes when compared to non-immunized mice. NI, non-immunized mouse.

Fig. 7. IFN-γ and IL-4 protein detected in the cell supernatants secreted from the primed splenocyte following in vitro stimulation. (A) Non-immunized mice. (B) Immunized mice with JOL1502.

Fig. 8. Serum bactericidal activity (SBA) against a wild-type S. Typhi. Sera were collected from the rabbits immunized with STG-LTB JOL1502 at weeks 0, 4 and 6. SBA was measured by the extent to which the virulent S. Typhi could survive in the presence of the collected serum and complement. The error bars indicate sd. **, P<0.05.
produced by Th1 responses promote phagocytosis and upregulate bacterial elimination [42]. Thus, CD4+ T cells, which are differentiated into Th1 cells when activated by antigens, are responsible for acquired immunity against S. Typhi for induction of the clearance of the bacterial infection in the late phase of infection [43]. S. Typhi penetrates the mucosal barrier of a host, and subsequently migrates to the mesenteric lymph node after the ingestion of an infective dose of S. Typhi via contaminated food or drink. Through blood vessels, bacteria are translocated to organs, including the spleen and liver, where they replicate [44]. Considering the invasive traits of the pathogen, an efficient vaccine candidate against typhoid fever can induce cell-mediated immune responses (CMI) to prevent systemic dissemination of the pathogen into the host. In this study, the STG-LTB increased the percentage of the CD3+ and CD4+ T cell subpopulation in the immunized mice. In addition, increased numbers of CD4+ T cells (Fig. 5) coincided with the levels of IFN-γ mRNA expression observed in the immunized mice (Fig. 6). However, interestingly, the cytokines associated with T cell differentiation, such as IL-2, IL-6 and IL-12 [45], and Th-17-derived cytokines, including IL-17 [46], were significantly upregulated in the in vitro stimulated splenocytes of the mice injected with JOL1502 ghost compared to those of the non-immunized mice (Fig. 6). These results imply that STG-LTB may be recognized and processed by antigen-presenting cells (APCs) to elicit the sequential secretion of immunomodulatory cytokines. The capacity of STG to mature APCs was not elucidated in the current study, thus, further study will be needed to understand its immunomodulatory property in the adaptive immune system acquired by STG-LTB.

Ab generation also plays a crucial role in protecting against typhoid fever through complement-dependent bactericidal activity in a cell-independent manner [47]. Thus, the SBA is one of the tests that can determine the functional property of a serum antibody for the licensure of bacterial vaccines, and it has been considered to be acceptable evidence of the protective efficacy of vaccines against bacterial disease [48, 49]. We attempted to determine the sensitivity of the virulent S. Typhi to the bactericidal effect of the serum Abs elicited in the immunized rabbits. The serum Abs elicited by STG-LTB inoculation at week 6 were able to mediate approximately 87.5% of the wild-type S. Typhi strain in the presence of the complement (Fig. 8). A significant increase of the bactericidal effect demonstrated that the bactericidal potential of the Abs was generated in the immunized animal. Given the data presented, it seems that the surface antigenic determinants derived from the ghost cells are responsible for functional Ab induction in the host.

In the present study, we demonstrated that the constructed asd-deleted S. Typhi Ty21a mutant ghosts expressing LTB showed potential as a vaccine candidate against typhoid fever, especially with respect their ability to markedly raise the humoral immune response, including efficient production of functional Abs and establishment of T cell-related immunity. Collectively, these findings suggest that the strategy for a typhoid vaccine based on a recombinant bacterial ghost system needs to be taken into account in the design of future vaccines against typhoid fever.

**Funding information**

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MISP) (no. 2013R1A1A069486).

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

The animal-specific pathogen-free BALB/c SPF inbred mice experimental procedures were approved (CBNU2015-00085) by the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care and Korean Animal Protection Law, 2007, article 13 (Experiments with Animals).

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