An attenuated Salmonella-vectorized vaccine elicits protective immunity against Mycobacterium tuberculosis

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Abstract

There is a need to develop protective vaccines against tuberculosis (TB) that elicit full immune responses including mucosal immunity. Here, a live attenuated Salmonella typhimurium aroA SL7207 vector TB vaccine, namely SL(E6-85B), harboring the Mycobacterium tuberculosis (M. tb) H37Rv ESAT6-Ag85B fusion gene was developed. The experimental data demonstrated that this SL(E6-85B) vaccine, or when it is combined with BCG vaccination, induced the strongest TB Ag-specific mucosal, humoral, and cellular immune responses comprised of increased proliferation of T cells, IFN-gamma expression, granzyme B production, as well as the greatest IFN-gamma production of effector-memory T (T EM) or effector CD8+ T cell responses and exerted high protective efficacy in mice against virulent M. tb H37Rv challenge compared to the other vaccinated groups (mice immunized with SL(Ag85B), a DNA vaccine or BCG only). This strategy may represent a novel promising mucosal vaccine candidate for the prevention of TB which are inexpensive to produce, efficacious, and able to be given orally rather than by injection.

1. Introduction

Tuberculosis (TB) remains a major global health problem, and there is an increased incidence of drug-resistant TB cases among human immunodeficiency virus (HIV)-infected patient populations [1–3]. It is estimated that throughout the world, one out of three individuals are infected with TB, and that six people die from TB every minute [1]. This high rate of mortality has persisted despite the availability of an attenuated strain vaccine of bacillus Calmette-Guérin (BCG) for more than 50 years. BCG only decreases childhood TB and gives little protection against adult lung tuberculosis; the protection in adults varies from zero to 80% [4,5]. These data indicate that the BCG vaccine lacks the immunogenicity required to generate protective immunity in the immunized adult populations. Therefore, the development of safe, efficacious and mucosal TB vaccines that can confer potent protection in the respiratory tract mucosa remains a major challenge to TB vaccinologists.

Comparative genomic analyses have identified more than 100 coding sequences that are missing from BCG but are present in Mycobacterium tuberculosis (M. tb) [6], including the 6-kDa early secreted antigenic target antigen (Ag) (ESAT6), which has been shown to be a protective Ag in several animal studies [7,8]. Recent reports describing new vaccines against TB have implicated various target Ags that can be used to immunize against M. tb. These include Ag85B (Ag85B) and ESAT6, which are important secreted proteins in the short-term culture filtrate protein (CFP) of M. tb [9–11] and are involved in inducing multiprotective immunity. These Ags are considered to be immunodominant and promising vaccine candidates against M. tb challenge. Vaccination with the Ag85B-ESAT6 fusion protein in adjuvant has been shown to induce greater protective immunity than an unfused mixture of these two proteins [12]. Vaccination with a DNA vaccine expressing a recombinant ESAT6-Ag85B fusion protein reduced the number of colony-forming units (CFUs) of M. tb in mouse lungs [13,14]. Although several different vaccine constructions have been developed, including subunit vaccines and DNA vaccines, the level of protection that they induce has not exceeded the levels conferred by BCG vaccination. Attenuated Salmonella typhimurium strains, such as the aroA strain SL7207, hold a prominent position among live bacterial vectors [15–18].
and as live vectors to deliver heterologous Ags, such as HIV Gag, gp120 and Ag85A, which can stimulate mucosal, humoral and cellular immune responses after animals are immunized via mucosal surfaces [21,22]. However attenuated Salmonella vector TB vaccine that carrying ESA6 and Ag85B of virulent M. tb H37Rv strain has not been reported as yet.

Oral vaccination has shown promising results in the augmentation of mucosal immunity and the combat of pathogens at the site of respiratory tract infection. In the present study, two recombinant, orally delivered and attenuated S. typhimurium vaccine strains, SL(85B) and SL(E6-85B), that harbor the M. tb H37Rv Ag85B gene and the ESA6-Ag85B fusion gene carried by a plasmid pVAX1, respectively, were constructed. Comparisons and analysis of the immune responses and protective efficacy of the SL(85B) and SL(E6-85B) vaccines, the DNA vaccine pVAX1-Ag85B (pV-85B), the BCG vaccine and combinations of these vaccines against M. tb were carried out.

2. Materials and methods

2.1. Strains, plasmids and animal protocol

M. tb, H37Rv (strain ATCC 93009) and bacillus Calmette-Guérin (BCG) were purchased from Beijing Biological Product Institute [23]. The bacteria were maintained on Lowenstein-Jensen (L-J) medium and were harvested while in log phase growth. Bacilli were washed in PBS with 0.05% Tween 80 and triturated uniformly before use. The cell culture conditions of the recombinant Salmonella strains reported in this paper are listed in Table 1. BALB/c mice (from the Animal Laboratory Center, Wuhan University) of either sex were used at 5–6 weeks of age. All bacterial cultures and animal experiments were carried out in the Animal Biosafety Level 3 Laboratory (ABSL-III) of the Wuhan University School of Medicine. The murine bacterial challenge protocols were performed in compliance with all guidelines and were approved by the Institutional Animal Care and Use Committee of Wuhan University.

2.2. Construction of eukaryotic and prokaryotic expression plasmids encoding Ag85B and ESA6-Ag85B

The entire Ag85B gene sequence from M. tb H37Rv chromosomal DNA was amplified by PCR and subcloned into the prokaryotic expression vector pGEX-KG (Pharmacia, Uppsala, Sweden) and the eukaryotic expression vector pVAX1 (Invitrogen, USA). The primer sequences were (forward) 5′-GCAAGCTTATGACAGACAGCCGTGAA-3′ and (reverse) 5′-TTCTAGTTGCAAGATCCCTGAGCT-3′. ESA6 was digested with HindIII and BamHI and ligated to the eukaryotic expression plasmid pVAX1-Ag85B, which was also digested with HindIII and BamHI to yield the eukaryotic expression plasmid pVAX1-ESA6-Ag85B. The plasmids pGEX-Ag85B, pVAX1-Ag85B (also called pV-85B) and pVAX1-ESA6-Ag85B (also called pV-E6-85B) were confirmed by DNA sequence analysis.

2.3. Construction of attenuated S. typhimurium recombinant vaccine strains carrying eukaryotic expression plasmids encoding Ag85B and ESA6-Ag85B

The eukaryotic expression plasmids pV-85B and pV-E6-85B were electrotransformed into the attenuated S. typhimurium strain aroA SL7207 [24] via the S. typhimurium-modifying strain LB5000 (r m-). The attenuated Salmonella recombinant vaccine strains SL(85B) and SL(E6-85B) were generated and confirmed by PCR and plasmid DNA restriction enzyme digestion analysis.

2.4. Purification of recombinant Ag85B protein and preparation of polyclonal antibody against Ag85B

The plasmid pGEX-Ag85B was transformed into Escherichia coli BL21(DE3)[pLysS] (Invitrogen). The GST-Ag85B fusion protein was overexpressed in E. coli BL21(DE3)[pLysS] after the induction of IPTG. The expressed GST-Ag85B protein was purified by Glutathione sepharose 4B (Amersham Biosciences). Similarly, the GST protein was overexpressed in E. coli BL21(DE3)[pLysS] transformed with pGEX-KG, and purified by Glutathione sepharose 4B. The purified GST-Ag85B, and GST proteins were determined by SDS-PAGE and Western Blot analysis. Anti-GST and anti-Ag85B polyclonal antibodies were prepared by immunized rabbit with the purified GST or Ag85B proteins, respectively.

2.5. SDS-PAGE and immunoblotting

Purified GST-Ag85B and Ag85B proteins were separated on a 10% denaturing polyacrylamide gel by electrophoresis. The proteins were transferred from the gel to nitrocellulose membranes (Bio-Rad) via Western blotting. The nitrocellulose membranes were blocked with 5% skim milk at 4 °C overnight and reacted with rabbit anti-GST serum (1:10,000) for 2 h at room temperature. After reacting with the primary antibody, the blots were washed three times with TBST (0.5 M NaCl–0.02 M Tris [pH 7.5], 0.05% Tween 20) and incubated for 1 h at room temperature with alkaline phosphatase conjugated anti-rabbit IgG (1:10,000) (Promega). After three washes with TBST, the reaction mixture was developed by incubation with nitroblue tetrazolium chloride and BCIP (5-bromo-4-chloro-3-indolyl-1-phosphate) at room temperature.

2.6. Immunization

One hundred and sixty 7–8-week-old female BALB/C mice were divided into eight groups (20 mice per group). Three groups of...
mice were orogastrically (o.g.) immunized with the attenuated *Salmonella* vaccine strains SL(85B) or SL(E6-85B), or the parental bacterium strain SL7207 by placing 100 μl of vaccine suspension containing 10^7 CFU into the lower esophagus using a gavage needle on days 0, 15 and 30. Another two DNA vaccination groups of mice that were preinjected with 10 μl 2% lidocaine were intramuscularly immunized with 100 μg plasmid DNA pV-85B or the empty vector control pVAX1 per mouse on days 0, 15 and 30 by gene gun with an Electric Square Porator (Scientz biotechnology), basically according to previous method [25]. All DNA preparations were produced using endotoxin-free purification columns (Qiagen). The relative amount (percentage) of supercoiled isoform in each vaccine sample was determined using densitometric analysis and reference plasmid preparations for calibration. The BCG control group was vaccinated with 5 × 10^6 CFU of BCG subcutaneously (s.c.) and the combined vaccination group was immunized with BCG and SL(E6-85B) to assess the cumulative action of these two vaccines. A negative control group received equal volumes of subcutaneously administered phosphate-buffered PBS.

2.7. Measurement of antibody levels by ELISA

The anti-Ag85B IgG or IgA antibody was detected 2 weeks after each immunization using an ELISA method. Briefly, ELISA plates were coated with recombinant Ag85B protein (5 μg/well) overnight at 4°C. Free binding sites were blocked by 1% bovine serum albumin-PBS. Individual serum or tissues samples from immunized mice were analyzed in threefold dilutions. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (DAKO) and HRP-conjugated rabbit anti-mouse IgG1, IgG2a (Promega) or IgA (DAKO) were added and incubation proceeded for 1 h at 37°C. The plate was then developed with substrate buffer. After 30 min of incubation at room temperature, the reaction was stopped by adding 0.5 mol/L H_2SO_4 and absorbance was measured at 450 nm using a microplate reader. Titters are shown as the sample dilution resulting in an OD_450 equal to twice the mean background of the assay.

2.8. ELISPOT

Two weeks after the final immunization, mice were sacrificed. IFN-γ and IL-4-producing cells from splenocytes of immunized or control mice were quantified using a cytokine-specific enzyme-linked immunospot assay (ELISPOT) kit (U-Cytech). Ninety-six-well nitrocellulose-backed plates were coated with 100 μg of anti-mouse IFN-γ McAb or anti-IL-4 McAb overnight at 4°C, then washed with phosphate-buffered saline (PBS) and blocked for 1 h with 5% BSA. The splenocytes (2 × 10^6/well) were stimulated with Ag85B (5 μg/ml) or RPMI-1640 alone (negative control) in triplicate wells at 37°C for 16 h. Then the cells were washed with PBS and incubated with biotinylated rabbit anti-IFN-γ or anti-IL-4 antibodies for 1 h at room temperature, followed by adding a streptavidin-HRP conjugate. Spots representing individual cytokine-producing cells were visualized using the HRP substrate and spots were then counted using an immunospot image analyzer.

2.9. Intracellular cytokine analysis

Splenocytes from the immunized mice were harvested 2 weeks after the third immunization and cultured in 6-well plates (5 × 10^6 cells/well). Then cells were stimulated with Ag85B protein (5 mg/ml) or ConA (10 μg/ml) at 37°C in 5% CO_2 for 72 h. ConA was used as a positive control. 1 μg/ml monensin (ebioscience), an inhibitor of intracellular protein transport, was added for 4 h to block cytokines releases at 37°C in a 5% (v/v) CO_2 atmosphere. After 4 h of incubation, CD4^+ and CD8^+ T cells were purified from the splenocytes using the BD™ IMag Mouse CD4^+ and CD8^+ T lymphocyte enrichment set-DM and the BD™ IMagnet (BD Biosciences Pharmingen, USA) via negative selection. The purified CD4^+ or CD8^+ T cells were then fixed in 4% paraformaldehyde in PBS at RT for 15 min, permeabilized and stained with PE- or FITC-conjugated anti-mouse IFN-γ or IL-4 mAbs (ebioscience) at 37°C for 30 min according to the manufacturer's instructions. Productions of IFN-γ or IL-4 were analyzed using a Beckman Coulter EPICS ALTRA II flow cytometer. Isotype-matched negative controls consisted of PE- or FITC-conjugated rat IgG1.

2.10. Measurements of CD8^+CCR7^- T cell responses

Two weeks after the final immunization, splenocytes from immunized mice were incubated with or without 5 μg of Ag85B protein at 37°C for 4 h, and were then stained with PE-Cy5 anti-CCR7, and PE-anti-CD8 antibodies at 37°C for 40 min. The CD8^+CCR7^- T cells were detected by flow cytometric analysis. For analysis of the intracellular IFN-γ-producing CD8^+CCR7^- T cells, the cells were then fixed and permeabilized according to the manufacturer's instructions, and stained with FITC-IFN-γ at 37°C for 40 min. Intracellular IFN-γ-producing CD8^+CCR7^- T cells were examined by flow cytometry.

2.11. Detection of granzyme B (GrB) expression of CD8^+ T cells

Two weeks after the final immunization, splenocytes of each mouse were cultured in 6-well plates (5 × 10^6/well), and then stimulated with Ag85B protein (5 mg/ml) for 6 h. The cells were then stained with FITC-conjugated rat anti-mouse CD8, PE-cy5-conjugated anti-mouse CD3 and PE-conjugated GrB antibodies (Caltag laboratories, USA) and analyzed by flow cytometry.

2.12. Proliferation of T cells

Splenocytes from the immunized mice were harvested 2 weeks after the third immunization and cultured in 6-well plates (5 × 10^6 cells/well). Cells were labeled with 5 μM of CFSE (Sigma) for 10 min at room temperature, then stimulated with 5 mg/ml Ag85B protein for 4 days. After 4 days of incubation, CD4^+ and CD8^+ T cells were purified from the splenocytes using the BD™ IMag Mouse CD4^+ and CD8^+ T lymphocyte enrichment set-DM and the BD™ IMagnet (BD Biosciences Pharmingen, USA) via negative selection. The purified cells were assayed by flow cytometry.

2.13. Experimental infections

Two weeks after the final immunization, mice were divided into eight groups (six mice in each group) and were infected i.v. via the lateral tail vein or intranasally (i.n.) with an inoculum of 5 × 10^6 CFU of *M. tb* H37Rv suspended in 0.1 ml PBS [23]. These mice were sacrificed 9 weeks after challenge. Mouse lung tissues were perfused with Ziehl-Neelsen acid-fast stain or with hematoxylin and eosin (H&E) reagent and were evaluated by light microscopy.

2.14. Statistical analysis

Statistical analysis was performed using Mann–Whitney's U-test and the Kruskal–Wallis test. Values are expressed as the mean ± SD. A 95% confidence limit was considered to be significant (p < 0.05).
3. Results

3.1. Construction of recombinant Salmonella vaccine strains harboring the Ag85B gene or the ESAT6-Ag85B fusion gene

To construct the recombinant plasmids, the DNA fragment of the entire Ag85B gene sequence from *M. tb* H37Rv was amplified and cloned into the prokaryotic expression vector pGEX-KG (Fig. 1A, Lane 2) or the eukaryotic expression vector pVAX-1 (Fig. 1A, Lane 4). The DNA fragment of the entire EAST6 gene sequence was amplified from *M. tb* H37Rv and then inserted into pVAX1-Ag85B to yield the recombinant plasmid pV-E6-85B (Fig. 1A, Lane 5). Restriction enzyme digestion analysis (Fig. 1A) showed that the relative molecular mass (Mr) of each inserted DNA fragment was identical to the value predicted. The recombinant *Salmonella* vaccine strain harboring the Ag85B gene, termed SL(Ag85B), was generated after the pVAX1-Ag85B (pV-E6-85B) plasmid was transformed into the attenuated *Salmonella* strain (SL7207) (Table 1). Using similar approaches, the other recombinant *Salmonella* vaccine strain SL(E6-85B) harboring the ESAT6-Ag85B fusion gene was generated (Table 1).

The recombinant prokaryotic expression plasmid pGEX-KG-Ag85B was transformed into *E. coli* BL21(DE3). GST-Ag85B fusion protein expression was induced by IPTG and was then identified by SDS-PAGE (Fig. 1B) and western blotting (Fig. 1C).

We examined the proteins expression of Ag85B and ESAT6-Ag85B in the different tissues of immunized mice after oral administration of recombinant *Salmonella* vaccine strains SL(85B) and SL(E6-85B) to mice. Western blot analysis showed protein bands of approximately 28 kDa (corresponding to the molecular mass (MW) of Ag85B protein) and 34 kDa (corresponding to the MW of ESAT6-Ag85B protein) in the muscle, spleen, intestines and lungs (Fig. 1D) and the bands are absent in the control animals (vaccinated with PBS or with the parental SL7202 vector alone). These results demonstrated that the ESAT6-Ag85B and Ag85B proteins were successfully expressed by the vaccine strains in the immunized mice. We have demonstrated the stability and proteins expressions of vaccine strains after several in vitro passages.

3.2. Recombinant Salmonella vector TB vaccine SL(E6-85B) predominantly elicited IgG2a subclass antibody responses and induced higher titers of Ag-specific IgG than BCG

To determine the antibody responses against the recombinant *Salmonella* strains, BALB/c mice were randomly divided into eight groups and immunized orogastrically with the recombinant vaccine strains. Every 2 weeks after the immunization, levels of Ag85B-specific IgG in the sera of immunized mice were determined via ELISA. Titers of Ag85B-specific IgG increased in each immunized group as the number of immunizations increased (Fig. 2A). After the third immunization, all vaccine groups that were immunized with the DNA vaccine, recombinant *Salmonella* vaccine strains or BCG developed higher levels of IgG antibody production compared
Fig. 2. Determination of Ag85B-specific IgG and SIgA responses of immunized mice. (A) Total serum Ag85B-specific IgG responses of immunized mice. Mice were injected i.m. with DNA vaccine pV-85B or o.g. with SL(85B) or SL(E6-85B). The immunization was performed on days 0, 15, and 30, and Ag85B-specific IgG titers were assayed by ELISA 2 weeks after each immunization. Six mice were used in each group. Data points represent means ± SEM. *p < 0.05, vs. parental vector control groups. (B) Ag85B-specific IgG1, **p < 0.01 vs. PBS group and parental vector control groups. (C) Ag85B-specific IgG2a. *p < 0.01 vs. PBS group and parental vector control groups.

to the parental strain (SL7207), the empty vector group (pVAX-1) and the non-immunized mice (PBS controls) (Fig. 2A, *p < 0.05). Additionally, the antibody titers were similar in the SL(E6-85B) group, the BCG group and the SL(E6-85B) and BCG combination group. These three groups induced the strongest IgG antibody responses among all groups.

To further observe IgG antibody subclass responses, the IgG1 and IgG2a antibody titers against Ag85B protein were also determined (Fig. 2B and C). Results from the ELISA analysis showed that significantly higher levels of IgG1 and IgG2a were induced by the SL(85B) and SL(E6-85B) vaccines compared to the parental strain SL7207 vaccine or the DNA vaccine pV-85B (Fig. 2B and C, *p < 0.05, **p < 0.01). Consistent with the IgG production, there were higher levels of IgG2a and IgG1 in the sera from the SL(E6-85B) group compared to the SL(85B) group. The combined immunization of SL(E6-85B) and BCG triggered the highest IgG2a production among

Fig. 3. Ag85B-specific IFN-γ- and IL-4-producing assay. Each group of mice (n = 6) was immunized three times, and cytokine production was assayed by ELISPOT 2 weeks after the final immunization. (A) IFN-γ-producing cells per 10^6 splenocytes. *p < 0.05, **p < 0.01 vs. PBS group and parental vector control groups. (B) The illustrated data shown for IFN-γ-producing cells are representative of six separate experiments. (C) Intracellular IFN-γ productions in CD8+ T cells by flow cytometry analysis. The illustrated data shown for intracellular IFN-γ productions in CD8+ T cells and CD4+ T cells represent the mean ± SEM (D), and the data are from six independent experiments. *p < 0.05 vs. PBS group, SL7207 group, pV-E6-85B, and SL(85B) groups.
Table 2

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>pVAX-1</th>
<th>pV-85B</th>
<th>SL7207</th>
<th>SL(85B)</th>
<th>SL(E6-85B)</th>
<th>BCG</th>
<th>BCG+SL(E6-85B)</th>
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<tbody>
<tr>
<td>Stomach</td>
<td>41 ± 4.2</td>
<td>90 ± 7.2</td>
<td>98 ± 6.9</td>
<td>81 ± 6.1</td>
<td>281 ± 18.6</td>
<td>305 ± 19.5(^a)</td>
<td>292 ± 17</td>
<td>312 ± 20.8</td>
</tr>
<tr>
<td>Intestine</td>
<td>42 ± 3.9</td>
<td>140 ± 11</td>
<td>185 ± 16.5</td>
<td>321 ± 22.4</td>
<td>770 ± 47.6</td>
<td>952 ± 68(^b)</td>
<td>684 ± 3</td>
<td>1210 ± 67.2</td>
</tr>
<tr>
<td>Serum</td>
<td>12 ± 3.6</td>
<td>18 ± 2.9</td>
<td>21 ± 4.7</td>
<td>17 ± 6.2</td>
<td>39 ± 5.8</td>
<td>92 ± 6(^a)</td>
<td>50 ± 5.2</td>
<td>109 ± 7.0</td>
</tr>
<tr>
<td>Lung</td>
<td>16 ± 2.9</td>
<td>16 ± 3.2</td>
<td>16 ± 4.2</td>
<td>16 ± 4.8</td>
<td>16 ± 3.1</td>
<td>32 ± 2.7(^a)</td>
<td>32 ± 3.4</td>
<td>32 ± 5.7</td>
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\(^a\) p < 0.05 vs. the DNA vaccine and the parental vector control groups.
\(^b\) p < 0.05 vs. BCG, DNA vaccine and the parental vector control groups.

all groups. This IgG2a production was almost five to six times greater than the IgG1 production. The presence of IgG2a antibodies in mice is indicative of a T helper (Th1)-biased response since the Th1 cytokines are necessary for this isotype shift in B cells. Our results demonstrate that immunization with SL(E6-85B) or BCG induced a greater IgG2a Th1-type response than the DNA vaccine.

The live recombinant Salmonella vaccines have the unique advantage of being able to adhere to and invade epithelial cells to deliver the heterologous genes, thereby inducing stronger mucosal immune responses [17]. To observe the mucosal immune responses against recombinant Salmonella vaccine strains, titers of Ag85B-specific mucosal secretory IgA (SIgA) antibodies from the stomach, intestine and lung tissues of immunized mice were determined by ELISA. Mice immunized with SL(E6-85B) elicited much higher levels of gastric, intestinal and lung SIgA and serum IgA than BCG, the DNA vaccine and the parental vector control groups (Table 2). The intestinal SIgA production in mice immunized with the recombinant Salmonella vaccine strain SL(E6-85B) combined with BCG was most increased compared to the SIgA production in the stomach and lung.

3.3. SL(E6-85B) significantly induced IFN-γ production

IFN-γ secretion is indicative of a Th1-biased response, while IL-4 production is indicative of a Th2-biased response. Using the ELISPOT technique, the production of IFN-γ and IL-4 by splenocytes was detected for all immunized mouse groups. After stimulation with the Ag85B protein, the numbers of IFN-γ-producing cells in the SL(85B) group, the SL(E6-85B) group, the BCG group and the SL(E6-85B) combined with BCG group were significantly greater than the DNA vaccinated group (Fig. 3A and B). All of the vaccine groups had higher numbers of IFN-γ-producing cells than the parental SL7207 strain group, the parental vector pVAX1 group and the unimmunized PBS group (Fig. 3A, p < 0.05). However, there were no significant differences in the numbers of IL-4-producing cells among the recombinant vaccine groups, the parental strain group, the BCG group, and the unimmunized PBS group (data not shown). Our results indicated that recombinant Salmonella vaccines induced a Th1-biased immune response.

Intracellular cytokines IFN-γ and IL-4 productions of CD4+ and CD8+ T cells were further examined by flow cytometry. After stim-
ulation with the Ag85B protein, CD8+ T cells from the SL(E6-85B) group and the BCG group had significantly much higher intracellular IFN-γ productions compared to those from the SL(85B), SL7207 and pV-E6-85B groups (Fig. 3C and D, *p<0.05). Furthermore, the SL(E6-85B) combined with BCG vaccination group elicited the highest levels of IFN-γ of CD8+ T cells among all groups (Fig. 3C and D, *p<0.05). There was no significant production of IL-4 in both CD4+ and CD8+ T cells after stimulated with Ag85B protein (data not shown). Thus, our data indicated that recombinant Salmonella vaccine SL(E6-85B) induced a Th1-biased immune response.

CCR7−CD8+ T cells are defined as effector-memory T (TEM) or effector CD8+ T cells [26–29]. We found that treatment with SL(E6-85B) combined with BCG elicited the highest percentages of CCR7−CD8+ T cells among all groups (Fig. 4A). In addition, the SL(E6-85B) combined with BCG group contained the highest percentage of IFN-γCCR7−CD8+ T cells (16.9%) among all groups (Fig. 4B and C), illustrating that SL(E6-85B) combined with BCG treatment induced the most effector CD8+ T cells. We observed that the SL(E6-85B) group contained a greater percentage of CCR7−CD8+ cells (64%) in CD8+ T cells, and IFN-γCD8+CCR7− cells (9.8%) in CD8+CCR7− cells than the SL(85B) group (CCR7−CD8+ cells: 46.3%; IFN-γCCR7−CD8+ cells: 6.2%) (Fig. 4A–C). Thus, the ESAT6-Ag is more important for eliciting effector-memory or effector CD8+ T cell responses.

### Table 3
Comparisons of various vaccines-induced protection efficacy in mice infected with H37Rv through i.v. infection route.

<table>
<thead>
<tr>
<th>Groups</th>
<th>T50 (days)</th>
<th>Mortality (%) (50 days)</th>
<th>Mortality (%) (60 days)</th>
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<tbody>
<tr>
<td>PBS (n=6)</td>
<td>29</td>
<td>85</td>
<td>100</td>
</tr>
<tr>
<td>pVAX-1 (n=6)</td>
<td>31</td>
<td>70</td>
<td>83.3</td>
</tr>
<tr>
<td>pV-85B (n=6)</td>
<td>35</td>
<td>70</td>
<td>66.6</td>
</tr>
<tr>
<td>SL7207 (n=6)</td>
<td>41</td>
<td>50</td>
<td>50.0</td>
</tr>
<tr>
<td>SL(85B) (n=6)</td>
<td>&gt;61</td>
<td>33.3</td>
<td>33.3</td>
</tr>
<tr>
<td>SL(E6-85B) (n=6)</td>
<td>&gt;61</td>
<td>16.6</td>
<td>16.6</td>
</tr>
<tr>
<td>BCG (n=6)</td>
<td>&gt;61</td>
<td>16.6</td>
<td>16.6</td>
</tr>
<tr>
<td>SL(E6-85B)+BCG (n=6)</td>
<td>&gt;61</td>
<td>0</td>
<td>16.6</td>
</tr>
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T50: time when 50% mortality.

3.4. SL(E6-85B) significantly induced high levels of granzyme B expression of CD8+ T cells

Granzyme B expression of Ag-specific CD8+ and CD4+ T cells was examined via flow cytometric analysis. We observed that the SL(85B) group, the SL(E6-85B) group and the BCG group induced significantly higher levels of granzyme B expressions of CD8+ T cells compared to the DNA vaccine pV-85B, SL7207 and pVAX1 groups (Fig. 5A and B, *p<0.05). Furthermore, the SL(E6-85B) combined with BCG vaccination group produced the highest levels of granzyme B expression of CD8+ T cells.
Fig. 6. Proliferation of T cells. Splenocytes from the immunized mice were labeled with CFSE, then stimulated with Ag85B protein for 4 days. CD4+ and CD8+ T cells were then purified from the splenocytes. The stained CD4+ (A) and CD8+ T cells (B) were assayed by flow cytometry. *p < 0.05 vs. PBS group, SL7207 group and pV-E6-85B group.

granzyme B expression of both CD4+ and CD8+ T cells among all groups (Fig. 5B and C). The intracellular granzyme B production of CD8+ T cells was almost three times greater than those of CD4+ T cells (Fig. 5B and C). Protective immunity in tuberculosis is dependent on the coordinated release of cytolytic effector molecules (e.g. granzyme) from effector T cells and the subsequent granule-associated killing of infected target cells [30,31], which suggest granzyme B plays an important role during CD8+ T cell cytotoxicity against M. tb infection. The lysis of M. tb-infected cells by CD8+ T cells releases the pathogen into the extracellular environment where it can be taken up by freshly activated macrophages, which are better equipped for killing the M. tb. Thus, our results demonstrate that recombinant Salmonella vaccines can elicit strong CD8+ cytotoxic T cell responses against M. tb.

3.5. SL(E6-85B) significantly induced the proliferations of both CD4+ and CD8+ T cells

Since both T-helper 1 (Th1) and CD8+ T cells play important roles in effectively inhibiting M. tb infection [32–35], we further examined the proliferations of CD4+ and CD8+ T cells of the splenocytes from the immunized mice with CFSE-staining method. We found that SL(E6-85B) and BCG group significantly induced the proliferations of both CD4+ and CD8+ T cells compared to the SL(85B), SL7207

Fig. 7. M. tb colony-forming (CFU) assay. Two weeks after infection, mice were sacrificed, the numbers of M. tuberculosis CFU in lungs (A) and spleens (B) were counted. *p < 0.05, **p < 0.01 vs. PBS group, pV-85B group and parental vector control groups.
Acid-fast stain analyses. The immunized mice were sacrificed 9 weeks after challenge with virulent M. tb H37Rv via i.v. and i.n. route. The lung tissue sections were analyzed with Ziehl–Neelsen acid-fast stain.

3.6. SL(E6-85B) induced protective immunity against i.v. M. tb H37Rv infection

To evaluate protective responses after M. tb challenge, immunized mice were infected i.v. with M. tb H37Rv. The recombinant Salmonella vaccine strain groups – SL(E6-85B) and SL(85B) – and the BCG group had longer 50% death times (T50) and lower death rates mice compared to the DNA vaccine pV-85B group, the parental strain group and the non-immunized PBS group (Table 3). This indicated that the bacterial vaccines can induce stronger protective immunity against M. tb than the DNA vaccine. In addition, the mice immunized with SL(E6-85B) survived nearly as long as the mice immunized with BCG, while the SL(E6-85B) combined with BCG vaccination was the most effective because all mice in this group were alive at 50 days post-infection, while 16–85% of the mice in the other groups were dead (Table 3).

We also determined the M. tb CFU in the lungs and spleens of the infected mice (Figs. 7A and 8) and assessed the degree of lung pathology by histopathologic analysis (Fig. 9). The results of staining of acid-fast bacilli in the lung tissue sections of the mice infected by M. tb H37Rv via i.v. route (Fig. 8) and i.n. route (data not shown) also showed that the M. tb numbers in mice immunized with SL(E6-85B) were significantly reduced compared to SL(85B) and DNA vaccine pV-85B groups. The greatest decrease in CFU values of lung was detected in the SL(E6-85B) combined with BCG group (Figs. 7 and 8). Moreover, the H&E-stained sections showed that the alveolar tissue appeared to be more intact and only mild signs of alveolitis were apparent in the SL(85B) group, BCG group, and especially in the SL(E6-85B) group or SL(E6-85B) combined with BCG group (Fig. 9). Multiple coalescing granulomatous lesions involving a significant amount of lung tissue were apparent in the PBS group, the pVAX1 group and the SL7207 groups (Fig. 9), suggesting that the functional capability of the lungs had been severely compromised. The experimental data illustrated that mice immunized with recombinant Salmonella vaccine strain SL(E6-85B), or combined with BCG, resulted in better control of the bacterial growth, less inflammation and an improved overall host response.

4. Discussion

From an immunological point of view, M. tb and S. typhimurium survive in similar intracellular habitats and may share some common factors that influence Ag processing mechanisms, ultimately leading to CD8+ and CD4+ T cell responses [36]. The bacterial vector may mimic natural infection and interact with the mucosal, humoral and cellular compartments of the immune system [16–18]. Moreover, recombinant Salmonella vaccines are candidates for oral vaccines that have several advantages with regard to their application [20,21,24,37–39]. Therefore, we have constructed recombinant Salmonella vaccine strains and evaluated their efficacy.

In the present study, we generated two recombinant Salmonella vaccine strains, SL(85B) and SL(E6-85B), which harbor the M. tb Ag85B gene and the ESAT6-Ag85B fusion gene, respectively. Our data strongly suggest that a novel recombinant Salmonella vaccine strain – SL(E6-85B) – that harbors an ESAT6-Ag85B polyprotein gene can protect against M. tb infection. Mice vaccinated with SL(E6-85B) contained high titers of Ag85B-specific IgG in their serum and SIgA in their stomach, intestine and lung, indicating that vaccination with SL(E6-85B) induced both a local and a systemic immune response. Moreover, the SL(E6-85B) vaccine strain could induce high levels of Ag-specific IgG2a isotype antibody, IFN-γ production and granzyme B expression of CD8+ T cells, as well as enhanced T cells proliferations, demonstrating that SL(E6-85B) triggered a strong cellular immune response. Both Ag85B and ESAT6 proteins have been identified as immunogenic for human leukocyte Ag class I-restricted CD8+ T cells [34,40]. Therefore, the SL(E6-85B) vaccine strain, which not only expressed the ESAT6-Ag85B fusion protein, but also delivered the plasmid encoding ESAT6-Ag85B gene into the host cells, could induce high levels of IFN-γ and granzyme expression and improved Th1-type cellular responses, which play
an important role in the prevention of TB. Following i.v. or i.n. challenge with *M. tb*, survival studies, bacterial burden assessments and histopathologic analyses also indicated that immunization with the SL(E6-85B) vaccine strain induced significant protective immunity.

Weinrich Olsen et al. [14] reported that administration of a subunit vaccine based on fusion proteins of the immunodominant ESAT-6 and Ag85B in adjuvant could induce stronger immune responses than those induced by the single Ag85B protein. In our work, the SL(E6-85B) vaccine strain showed more efficient protection against *M. tb* infection compared to SL(85B), which is consistent with previous studies.

We observed that vaccination with the control vectors (pVAX1 and SL7207) substantially increased titters of both antigen-specific IgG (Fig. 2) and IgA (Table 2). In agreement with these observation, Table 3 also shows substantial protection was elicited by the SL7207 control vector. Several reasons may explain these observations. The control vectors (pVAX1 and SL7207) contain bacterial DNA, or LPS, etc. The bacterial DNA contains CpG structure which may activate TLR9, while bacterial LPS can bind and stimulate TLR4. The activated TLR4 and TLR9 could stimulate antigen presenting cells (APC), subsequently elicit immune responses of APC and lymphocytes in the immunized mice. Moreover, in some other work, the control DNA vector pVAX1 can also elicit low level antigen-specific immune response [41,42], which were consistent with our results.

Because *M. tb* entry most often occurs through the upper respiratory tract, which is followed by colonization and establishment of primary infection in the lungs, a TB vaccine should preferably elicit mucosal immunity both in the upper and lower airways in addition to eliciting a systemic immune response. Compared to DNA vaccine, recombinant Salmonella vaccine strains have two unique characteristics. First, bacterial vectors replicate within the host, and they may provide sustained exposure to the Ag, potentially augmenting the type and level of immune response. Second, *Salmonella* vectors can be used as oral vaccines, which can induce strong mucosal immune responses. In our work, the newly constructed recombinant *Salmonella* vaccine strain SL(E6-85B) could induce both local mucosal and systemic protective immune responses against *M. tb*. In particular, the SL(E6-85B) vaccine could elicit the highest Ag85B-specific IgA production not only in the stomach, intestine and lung, but also in the serum, compared to mice immunized with DNA vaccine and BCG.

In summary, our results demonstrate that the most effective recombinant *Salmonella* vaccine strain in our study, SL(E6-85B), would be a novel vaccine candidate for the prevention of TB. Furthermore, treatments combining vaccination with SL(E6-85B) and BCG against TB seem to be promising.

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