Heterologous prime-boost vaccination against tuberculosis with recombinant Sendai virus and DNA vaccines

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Received: 25 March 2019 / Revised: 22 August 2019 / Accepted: 5 October 2019 / Published online: 30 November 2019
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Abstract

In an earlier study, a novel Sendai virus–vectored anti-tuberculosis vaccine encoding Ag85A and Ag85B (SeV85AB) was constructed and shown to elicit antigen-specific T cell responses and protection against *Mycobacterium tuberculosis* (*Mtb*) infection in a murine model. In this study, we evaluate whether the immune responses induced by this novel vaccine might be elevated by a recombinant DNA vaccine expressing the same antigen in a heterologous prime-boost vaccination strategy. The results showed that both SeV85AB prime-DNA boost (SeV85AB-DNA) and DNA prime-SeV85AB boost (DNA-SeV85AB) vaccination strategies significantly enhanced the antigen-specific T cell responses induced by the separate vaccines. The SeV85AB-DNA immunization regimen induced higher levels of recall T cell responses after *Mtb* infection and conferred better immune protection compared with DNA-SeV85AB or a single immunization. Collectively, our study lends strong evidence that a DNA vaccine boost might be included in a novel SeV85AB immunization strategy designed to enhance the immune protection against *Mtb*.

Key messages

- A heterologous prime-boost regimen with a novel recombinant SeV85AB and a DNA vaccine increase the T cell responses above those from a single vaccine.
- The heterologous prime-boost regimen provided protection against *Mtb* infection.
- The DNA vaccine might be included in a novel SeV85AB immunization strategy designed to enhance the immune protection against *Mtb*.

Keywords Tuberculosis · Sendai virus · DNA vaccine · Prime-boost · T cell responses

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Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00109-019-01844-3>) contains supplementary material, which is available to authorized users.

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Introduction

Tuberculosis (TB) is the leading cause of death due to a single infectious disease in the world [1]. The only licensed anti-TB vaccine, Bacillus Calmette-Guérin (BCG), which is an attenuated form of *Mycobacterium bovis*, was introduced a century ago. Although BCG shows efficacy against severe forms of meningitis and miliary TB in newborns and children, its protective efficacy against pulmonary TB in adults is inconsistent (0–80%) [2]. Therefore, a more effective vaccine would substantially accelerate global TB control.

Protection against infection by intracellular pathogens such as *Mycobacterium tuberculosis* (*Mtb*) requires the induction of robust T cell-mediated immune responses [3, 4]. However, *Mtb* has evolved ways to manipulate and modulate human immune responses to facilitate its survival and transmission. *Mtb* manipulates the interaction between innate and adaptive immunity to escape from immune killing during the early events after infection [5, 6]. It is also suggested that activated, antigen-specific T cells induced by dominant antigens are required for essential elements of the *Mtb* life cycle [7]. Thus, anti-TB vaccine strategies that use inactivated or live attenuated *Mtb* might contain antigens that mediated immune escape, leading to inappropriate immune responses against *Mtb* infection. In contrast, recombinant vectored vaccines, which encode the immune dominant antigens essential for the life cycle of *Mtb*, are able to induce potent protective antigen-specific immune responses and to optimize the immune protection primed by BCG [8, 9]. Both viral- and DNA-based recombinant vaccines are potential candidates to induce potent cellular immunity.

Recombinant viral-vectored vaccines are able to transiently express high levels of the antigens to induce potent adaptive immunity with support from inflammatory responses elicited by an adjuvant effect of the delivery vehicle [10]. Among such vectors, poxvirus, adenovirus, and parainfluenza virus are the most widely used. Sendai virus (SeV) is also attractive as a potential vector. It has characteristics in common with other viral vectors, such as low pathogenicity, a powerful capacity for gene expression, the ability to induce robust cytotoxic T lymphocyte responses, a wide host range, and so on [11]. Also, as a respiratory transmissible virus, SeV-based vaccines can elicit potent mucosal immunity [12–14]. One of the potential drawbacks of viral-based vaccines is that the response to the vectored antigens may be dampened by pre-existing or de novo adaptive immune responses to the vector itself [15]. Therefore, an advantage with using SeV is that pre-existing levels of anti-SeV neutralizing antibodies are very low; SeV does not infect humans and the low anti-SeV antibody levels do not block the ability of recombinant SeV vaccine to induce antigen-specific T cell immunity [16]. In addition, as a strong inducer of innate immunity, an SeV-derived RNA agonist of RIG-I could be used as adjuvant to enhance vaccine-induced

immune responses [17]. Consistent with this background, we have shown that a single immunization with SeV85AB, expressing immune dominant antigens Ag85A and Ag85B of *Mtb*, elicits antigen-specific CD4⁺ and CD8⁺ T cell responses [18], and enhances BCG-induced protection against TB in a prime-boost model [19].

Anticipating that only a single vaccination might not be adequate to provide full protection, we here investigate the potential of adding a booster vaccination approach. Considering that repeated immunizations with the same vectored vaccine (homologous boosting) might generate strong response against the vector backbone; herein, we combined SeV85AB vaccination with a recombinant DNA vaccine encoding the same antigens in a heterologous prime-boost model. DNA vaccines are based on bacterial plasmids that encode the sequence of target antigens that are expressed under a strong eukaryotic promoter to yield high levels of transgene expression [20]. DNA vaccines enable expressed antigens to be presented by both MHC class I and class II complexes, thereby enabling stimulation of both CD4⁺ and CD8⁺ T cells and humoral immunity [21]. They have been proved successful in several animal models in treating and preventing cancer, autoimmunity, and infectious diseases [22]. We have described several DNA-vectored vaccines conferring immune protection against *Mtb* infection [23–25]. Here, the prime-boost approach combines the advantage of DNA vaccines to raise focused immune responses against the encoding antigens in the absence of interference from vector immunogenicity, and the advantage of viral-vectored vaccines to greatly expand the immune memory due to the increased capacity to express candidate antigens in vivo and to elicit innate immunity [26]. In our earlier studies, we showed that a vaccinia virus-vectored vaccine significantly boosted the quantity and quality of T cell responses primed by DNA vaccine through a MyD88-Akt-mTOR signaling pathway [27, 28].

The order in which the priming vaccine and the boosting vaccine were given has in some instances been shown to affect the outcome [29–31] and in others not to do so [32, 33]. Accordingly, in this study, we sought to evaluate not only whether the immune responses induced by the novel SeV85AB vaccine might be elevated by DNA vaccine but also whether the order of delivery made a difference. We found that both SeV85AB prime-DNA boost (SeV85AB-DNA) and DNA prime-SeV85AB boost (DNA-SeV85AB) vaccination strategies significantly enhanced the antigen-specific T cell responses induced by either of the vaccines alone, both in the spleen and lung. Compared with either DNA-SeV85AB boost or a single vaccination, the SeV85AB-DNA-vaccinated mice induced higher levels of recall CD4⁺ and CD8⁺ T cell responses after *Mtb* infection and gave better immune protection. Therefore, our study supports the concept that a DNA vaccine boost might be included in a novel SeV85AB immunization strategy to protect against TB.

Materials and methods

Preparation of vaccines

The vector pVAX1 was used as the DNA vaccine vector. The SeV used in this study was the F gene-defective strain. A recombinant chimeric gene *fbpA/B* comprising full-length fibronectin-binding protein A gene (*fbpA*) into which a fragment encoding the 125–282 amino acids of *fbpB* had been inserted [18] was introduced into and expressed by both vaccines. The DNA vaccine encoding the same antigen was purified with Qiagen columns (EndoFree Plasmid Giga kit) and eluted in pyrogen-free deionized water. SeV85AB was propagated in LLCMK₂ cells.

Mice and immunization

All animal experiment protocols were approved by the Institutional Animal Care and Use Committee and were performed according to the guidelines of the Laboratory Animal Ethical Board of Shanghai Public Health Clinical Center. Specific pathogen-free BALB/c female mice, aged 6–8 weeks, were randomly divided into groups. For DNA vaccination, two doses of 100 µg DNA vaccine were injected intramuscularly (*i.m.*) in each hind leg in a volume of 100 µL at 2-week intervals, and SeV85AB was administered intranasally (*i.n.*) with a dose of 1×10^7 CIU (cell infectious units) in a volume of 20 µL. PBS was used as negative control. For BCG control in the challenge infection study, Danish strain was injected subcutaneously (*s.c.*) with 1×10^6 colony forming units (CFU) in 100 µL PBS around the hind legs. For the evaluation of primary T cell immune responses, 4 weeks after the final inoculation the mice were sacrificed and the lungs and spleens were aseptically removed for assaying of the antigen-specific cellular immune responses. For the evaluation of recall immune responses after infection, the mice were challenged by the introduction of the *Mtb* virulent strain H37Rv into the lungs 4 weeks after the final immunization and maintained in an ABSL-3 animal facility. Five weeks later, the animals were sacrificed, and the lungs were sampled.

Lymphocytes isolation

For the isolation of single lung cells, the lungs were sterilely removed and gently minced with scissors. Tissue pieces were digested for 30 min at 37 °C with 10 U of DNase I (Thermo) and 1 mg/mL of collagenase IV (Invitrogen) in R10 medium (RPMI-1640 medium containing 10% fetal bovine serum and 1% Penicillin & Streptomycin), the digest was passed through a 70-µm cell strainer (Fisher Scientific) by gently squashing with a syringe plunger. Red blood cells were lysed with lysis buffer. For the isolation of single splenocytes, the spleens were mechanically disrupted, single cells were filtered through

mesh gauze and then subjected to red blood cell lysis. The lung lymphocytes and splenocytes were washed twice and re-suspended in R10 medium.

Enzyme-linked immunospot assay

An interferon (IFN)-γ ELISPOT assay was performed according to the kit instructions (BD Biosciences). Briefly, 96-well plates were coated with capture antibody at 4 °C overnight. The plates were washed and blocked with R10 medium for 2 h at room temperature. The isolated single lung cells or splenocytes were added at 2×10^5 cells/well with the pool of Ag85AB peptides (5 µg/mL each). PMA (50 ng/mL, Sigma) plus ionomycin (1 µg/mL, Sigma) was used as the stimulation in positive controls and cells incubated with R10 medium were used as negative control. The cells were stimulated in a 5% CO₂ and humidified incubator at 37 °C for 20 h. The stimulated cells were aspirated and washed with deionized water and PBST (PBS containing 0.05% Tween-20), and then incubated with detection antibody for 2 h. After washing with PBST, the plates were incubated with streptavidin-HRP for an additional 1 h. After washing with PBST and PBS, AEC substrate solution was added and incubated for 30 min before rinsing with water. The plates were air-dried and analyzed with an Immunospot Reader (Chamspot III, Beijing Sage Creation Science).

Intracellular staining and flow cytometry analysis

The isolated single lung cells or splenocytes were stimulated with the pool of Ag85AB peptides (5 µg/mL each) in 96-well plates in a volume of 200 µL at 37 °C for 1 h, and then 1 µL/mL monensin and brefeldin A (BD Biosciences) were added and the cells were incubated for an additional 5 h. PMA (50 ng/mL) plus ionomycin (1 µg/mL) stimulation was used as positive control and cells incubated with R10 medium were used as negative control. After stimulation, cells were washed with PBS containing 2% fetal bovine serum and then incubated with a mixture of surface antibodies for 30 min at 4 °C. After washing, the cells were fixed and permeated with fix/perm buffer for 20 min at 4 °C (BD Bioscience). After washing, the cells were incubated with antibodies against intracellular cytokines for another 30 min at 4 °C. Then the cells were re-suspended for flow cytometry analysis (LSRFortessa, BD Biosciences), at least 50,000 T cells were screened.

Peptides and antibodies

The peptide pool used as the antigen-specific stimulant contained four dominant epitope peptides derived from Ag85A: MPVGGQSSF, 70–78aa; YAGAMSGSL, 145–152aa (CD8 epitopes); TFLTSELPQWLQANRHVKPT, 99–118aa (CD4 epitopes); GLSMAASSALTL, 124–125aa (both CD4

and CD8 epitopes) and four from Ag85B: IYAGSLSAL, 144–152aa; ALLDPSQGMGPSLIG, 151–165aa (CD8 epitopes); GPSSDPAWERNDPTQQIP, 181–198aa (both CD4 and CD8 epitopes); HSWYEWGAQLNAMKGDLDQ, 262–279aa (CD4 epitopes). They were synthesized by GL Biochem (Shanghai, China) with 95% purity.

The following antibodies were used in this study: CD3-eFluor 450 (clone 17A2), CD4-APC (clone GK1.5), IFN- γ -APC-eFluor780 (clone XMG1.2), IL-2-PE (clone JES6-5H4), and TNF- α -PE-Cyanine 7 (clone MP6-XT22). All were from eBioscience.

Challenge with virulent *Mtb* H37Rv strain

The murine model of *Mtb* infection was previously described [18]. The mice were infected with ~ 50 CFU per lung. The mycobacterial burden was determined by plating homogenates of the spleen and lung onto Middlebrook 7H11 agar plates supplemented with 10% OADC and antibiotic mixture (4 μ g/mL amphotericin, 50 μ g/mL carbenicillin, 40 U/mL polymyxin B, and 2 μ g/mL trimethoprim).

Statistical analysis

The statistical analysis was performed using GraphPad Prism 6.0 software. One-way ANOVA was used to analyze the statistical significance for comparison of multiple groups, and two-way ANOVA was used for the grouped analysis. The value of $p < 0.05$ was considered significant.

Results

A heterologous prime-boost regimen increased the IFN- γ responses induced by a single vaccine

To determine whether the combination of DNA and SeV85AB vaccine, as well as the order of delivery, could enhance the antigen-specific T cell immune responses induced by either vaccine separately, SeV85AB-DNA and DNA-SeV85AB immunization regimens were used as shown in Fig. 1A. DNA boost significantly enhanced the immune responses primed by SeV85AB: Ag85AB peptide stimulation revealed in a higher level of IFN- γ -secreting cells in the SeV85AB-DNA group compared with the SeV85AB group, both in the spleen (Fig. 1B and C, upper panel) and in the lung (Fig. 1C, lower panel). In contrast, the DNA primed responses were scarcely boosted by SeV85AB in the spleen (Fig. 1B and C, upper panel) but were significantly boosted in the lung (Fig. 1C, lower panel). From another perspective, the positive priming effect of both SeV85AB and DNA85AB were confirmed by showing that the IFN- γ responses in the lung were significantly higher in

Fig. 1 Heterologous prime-boost regimens increased the IFN- γ responses induced by a single vaccine. **(a)** Vaccination and detection schedule. Cellular immune responses to Ag85AB peptides were assayed 4 weeks after the final inoculation. **(b)** Representative T cell IFN- γ responses revealed by ELISPOT assay. Quantified responses from the spleen and lung **(c)** are shown as spot-forming units (SFUs). Values are expressed as mean $\pm$ SEM. Data are representative of 2 independent experiments with 4 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$

the SeV85AB-DNA group compared with the DNA group, and in the DNA-SeV85AB group compared with the SeV85AB group (Fig. 1C, lower panel). The IFN- γ response induced after SeV85AB-DNA was also higher than that after DNA-SeV85AB (Fig. 1C, lower panel). In summary, the heterologous prime-boost regimen increased the IFN- γ responses induced by either vaccine alone, and SeV85AB-DNA was a stronger inducer of the IFN- γ response than DNA-SeV85AB.

The heterologous prime-boost strategy increased poly-functional T cell responses induced by a single vectored vaccine

To further investigate the boost effect on the antigen-specific immune responses, we isolated splenocytes from the different immunization groups and stimulated them with the pool of Ag85AB peptides. Cells incubated with medium were used as negative control. ICS assessment of IFN- γ , IL-2 and TNF- α -secreting T cells were compared between groups. The gating strategy is shown in Fig. S1 and representative mono-positive dot plots are shown in Fig. 2A. Consistent with the ELISPOT assay, the frequency of cells producing IFN- γ was significantly enhanced in the SeV85AB-DNA group compared with a single vectored vaccine in both CD4⁺ (Fig. 2B, upper panel) and CD8⁺ T cells (Fig. 2B, lower panel), whereas the differences between the DNA-SeV85AB and single immunization groups did not reach significance in any of the T cell subsets (Fig. 2B). Compared with the SeV85AB group, the percentage of IL-2-producing cells in the CD4⁺ T cell subset was significantly higher in both the SeV85AB-DNA and the DNA-SeV85AB groups (Fig. 2B, upper

panel), whereas in the CD8⁺ T subset it was higher only in the DNA-SeV85AB group (Fig. 2B, lower panel). The TNF- α -secreting cells were only boosted in the SeV85AB-DNA group (Fig. 2B).

A poly-functionality analysis of the stimulated T cells showed that the SeV85AB-DNA regimen significantly enhanced the triple-positive, the dual-positive IFN- γ ⁺ TNF- α ⁺ and the mono-positive IFN- γ ⁺ or TNF- α ⁺ cells compared with either SeV85AB alone or DNA alone. The greatest increases were seen in the CD8⁺ cells (Fig. 3). In contrast, DNA-SeV85AB regimen typically caused only modest enhancements in CD8⁺ T cells compared with the single vaccines (Fig. 3).

The heterologous prime-boost regimen increased poly-functional T cell responses in the lung compared with vaccination with a single vaccine

Representative ICS mono-positive dot plots of stimulated lung cells are shown in Fig. 4A. Consistent with the ELISPOT assay, the SeV85AB-DNA protocol significantly enhanced the frequency of both CD4⁺ (Fig. 4B, upper panel) and

Fig. 2 Flow cytometric analysis of cytokine production by splenocytes of vaccinated mice. Four weeks after the final inoculation, splenocytes were collected and stimulated with Ag85AB peptides in the presence of brefeldin A and monensin, followed by the analysis of cytokine production by ICS assay. CD4⁺ T cells were gated as CD3⁺CD4⁺ and CD8⁺ T cells were defined as CD3⁺CD8⁺. Representative flow cytometric plots of IFN- γ , IL-2, and TNF- α staining in CD4⁺ T cells are shown in (a) and the proportion of cells producing these cytokines among CD4⁺ and CD8⁺ T cells (b) are shown as histograms. Values are expressed as mean $\pm$ SEM. Data are representative of 2 independent experiments with 4 mice per group. * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001

Fig. 3 Characterization of poly-functional antigen-specific T cell responses in splenocytes of vaccinated mice. T cells secreting IFN- γ , IL-2, and TNF- α were distinguished as seven sub-populations based on the secretion of these three intracellular cytokines in any combination. The percentage of the seven sub-populations as components of the total CD4 $^{+}$ or CD8 $^{+}$ T cells (**a**) and the pie chart analysis (**b**) are shown. Significant differences in frequency of poly-functional T cell subsets are indicated. Values are expressed as mean $\pm$ SEM. Data are representative of 2 independent experiments with 4 mice per group. * $P < 0.05$ and ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, when compared as indicated

CD8 $^{+}$ T cells (Fig. 4B, lower panel) that secreted IFN- γ compared with either vaccine alone. The DNA-SeV85AB regimen also increased the frequency of the CD8 $^{+}$ cells compared with the SeV85AB-only but did not increase the CD4 $^{+}$ cell frequency. In addition, these data confirmed the ELISPOT data showing that SeV85AB-DNA is a better inducer of IFN- γ than DNA-SeV85AB regimen. Both prime-boost regimens failed to enhance IL-2 responses in the lung, whereas either the DNA or the SeV85AB boost enhanced TNF- α responses in CD8 $^{+}$ but not CD4 $^{+}$ T cells (Fig. 4B).

The poly-functionality analysis of responding lung T cells is shown in Fig. 5. CD4 $^{+}$ T cells showed little enhancement by either of the prime-boost regimens compared with the single vaccine, with the exception that the SeV85AB-DNA regimen significantly enhanced the triple-positive, and mono-positive IFN- γ^{+} T cells (Fig. 5A, upper panel) and the DNA-SeV85AB regimen enhanced the dual-positive IFN- γ^{+} IL-2 $^{+}$ CD4 $^{+}$ T cells. In contrast, the CD8 $^{+}$ T cells showed major effects of boosting with either regimen in enhancing both dual-positive IFN- γ^{+} TNF- α^{+} and mono-positive TNF- α^{+} CD8 $^{+}$ cells (Fig.

5A, lower panel). The SeV85AB-DNA regimen additionally boosted the IFN- γ mono-positive cells.

Protection against challenge infection was associated with a strong recall of the Ag85AB-specific IFN- γ responses induced by SeV85AB-DNA

To directly assess the protective efficacy of these two prime-boost regimens, we challenged vaccinated mice with *Mtb* and determined the recall immune responses and bacterial loads in the lung 5 weeks post-infection (Fig. 6A). IFN- γ ELISPOT assay showed that mice that received SeV85AB-DNA regimen had developed the strongest Ag85AB-specific IFN- γ responses in the lung compared with a single vectored vaccine (Fig. 6B). Although the lung bacterial loads were significantly lower in all the immunization groups than in the PBS control group, the SeV85AB-DNA regimen resulted in the most conspicuous reduction, which was similar to BCG vaccination (Fig. 6C). In addition, the spleen bacillary loads were significantly decreased compared with the PBS group in the prime-boost and BCG vaccination groups (Fig. 6C). Thus, SeV85AB-DNA regimen possibly was superior to DNA-SeV85AB in the immune protection against *Mtb* infection.

Discussion

In a large phase IIb efficacy clinical trial, MVA85A, a recombinant vaccinia virus-vectored TB vaccine, showed no additional protection against TB progression compared with a placebo arm among 4-to-6-month-old infants who received neonatal BCG vaccination [34]. Although disappointing, this is not unusual in the progress of vaccine development. Recently, there is a growing body of evidence that aerosol vaccination of TB vaccines is superior to systemic immunization in several models [35–37], and the superiority might be ascribed to aerosol vaccine-induced lung-resident memory T cells (T_{RM}) [18, 38–40]. Thus, pulmonary delivery of TB vaccines to the site of primary infection now can guide TB vaccine development and MVA85A is re-entering clinical trial as an aerosol vaccine.

Although MVA85A was disappointing in the clinical IIb trial, Ag85B was found to be recognized in mice by other T cell epitopes than Ag85A [41], and the immune protection induced by the fusion protein Ag85AB was superior than either Ag85A and Ag85B [42]. Both Ag85 and Ag85B are strongly immunogenic in experimental and natural mycobacterial infections in both induction of antibody synthesis and T cell-mediated responses [43, 44] and in protecting animal models such as mice, guinea pigs and cattle [45, 46]. Accordingly, we continued to use both antigens in the SeV85AB vaccine.

Fig. 4 Flow cytometric analysis of cytokine production by lymphocytes from the lungs of vaccinated mice. Four weeks after the final inoculation, lung cells were collected and stimulated with Ag85AB peptides. Representative flow cytometric plots of IFN- γ , IL-2, and TNF- α staining in CD4 $^{+}$ T cells are shown in (a) and summarized data of single cytokine-producing CD4 $^{+}$ and CD8 $^{+}$ T cells (b) are shown. Values are expressed as mean $\pm$ SEM. Data are representative of 2 independent experiments with 4 mice per group. * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001, when compared as indicated

The combination of DNA-vectored and virus-vectored vaccines have been frequently used in vaccine strategy designs and the order in which the priming vaccine and the boosting vaccine were given sometimes affected the protective efficacy [29–31]; herein, both DNA-SeV85AB and SeV85AB-DNA were tested here. Our data showed that SeV85AB was only a weak booster of antigen-specific T cell immune responses induced by DNA prime, reflected by the modest enhancement of cellular responses in the DNA-SeV85AB group compared with the DNA group. In contrast, the DNA vaccine was a good booster of SeV85AB, resulting in significantly higher levels of T cell immune responses in the SeV85AB-DNA group than the single SeV85AB vaccination group. As a result, the SeV85AB-DNA regimen gave a better immune protection against *Mtb* challenge infection compared with DNA-SeV85AB (Fig. 6C). The mechanisms underlying these observations remain to be explored. It may be that an initial pulmonary burst of abundant antigen from a viral vector that engages TLR9 [47] and is followed by protracted but low-level production of antigen from residual DNA vaccine [48, 49] could provide important features in this prime-boost strategy.

Although intranasal vaccination of SeV85AB induced high levels of lung T_{RM}, this cell subset was not determined in the present study since systematic immunization with DNA-vectored vaccine failed to induced potent lung T_{RM} responses in a preliminary experiment (data not shown). Mucosal vaccination with BCG and recombinant influenza-vectored vaccine have previously been proven to confer T_{RM}-mediated protection [38–40]. Thus, it could be anticipated that the combinations of these vaccines might establish a more potent lung T_{RM}-mediated immune protection. However, we did not measure antibody responses and cannot exclude an antibody-mediated contribution to protection based on our current observations.

We tested this DNA and viral vector combination here in a stand-alone prime-boost strategy in comparison with BCG. The favorable outcome suggests that this strategy might additionally be used to boost the immunity provided by BCG thus: a BCG-SeV85AB-DNA strategy. SeV85AB showed potential as a booster of BCG in our earlier study and mucosal vaccination with BCG and a recombinant influenza vectored vaccine have been shown to confer T_{RM}-mediated protection [38–40]. Numerous precedents for boosting BCG with either

Fig. 5 Characterization of poly-functional antigen-specific T cell responses in lymphocytes from lungs of vaccinated mice. T cells secreting IFN- γ , IL-2, and TNF- α were distinguished as seven sub-populations. Their percentage as components of the total CD4⁺ or CD8⁺ T cells (**a**) and the pie chart analysis (**b**) are shown. Significant differences in frequency of the poly-functional T cell subsets are indicated. Values are expressed as mean $\pm$ SEM. Data are representative of 2 independent experiments with 4 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, when compared as indicated

DNA or viral vectors have been established and a BCG-SeV85AB-DNA strategy might prove to be the most clinically useful in the context of the existing almost universal delivery of BCG to infants. This remains to be tested.

In our earlier study, we showed that the immune protection afforded by a BCG prime-SeV85AB boost regimen was associated with IL-2-mediated responses [19]. In this study, IL-2 was only modestly boosted by the SeV85AB-DNA regimen, and IFN- γ responses predominated instead. IFN- γ is indispensable for host resistance against *Mtb*; however, IFN- γ responses do not always correlate with immune protection. Herein, we showed that although SeV85AB-DNA induced higher levels of IFN- γ than the DNA-SeV85AB regimen, only modest immune protection was observed. Recently, Barber et al. found that although IFN- γ accounts for $\sim 30\%$ of the cumulative CD4⁺ T cell-mediated reduction in lung bacterial loads, increasing the per capita production of IFN- γ led to the early death of the host [50]. Their study suggested that although IFN- γ secretion ability is crucial in controlling *Mtb* infection, its over-expression might be detrimental to the immune balance. Hence, the optimum magnitude of IFN- γ response elicited by an anti-TB vaccine still

Fig. 6 Vaccine protective efficacy and Ag85AB-specific recall T cell responses against *Mtb* challenge. (**a**) Vaccination, infection and detection schedule. Vaccinated mice were challenged with virulent *Mtb* H37Rv. Five weeks post-infection, recall lung IFN- γ responses were determined by ELISPOT assay (**b**). The numbers of live bacteria in homogenates of the lungs were counted as CFU after 3-week incubation on 7H11 agar and transformed as log₁₀ (**c**). Values are expressed as mean $\pm$ SEM, each dot represents calculated CFU from a single mouse. * $P < 0.05$ and ** $P < 0.01$, when compared with PBS control or as indicated

needs to be determined. In addition, the modest enhancement of immune protection induced even by SeV85AB-DNA suggest that this regimen still needs to be optimized; changing the doses and inoculation times of DNA vaccination, or the time intervals between inoculations, might result in different levels of IFN- γ responses and different degrees of protection upon challenge.

In summary, since we showed here that the SeV85AB-DNA regimen significantly enhanced the T cell immune memory and protection against *Mtb* challenge infection, further studies of SeV85AB that include a BCG prime and other animal model are warranted before a clinical trial of safety in humans.

Funding information This work was supported by grants from Chinese National Mega Science and Technology Program on Infectious Diseases (2018ZX10731301, 2018ZX10302301), the National Natural and Science Foundation of China (31771004, 81873884, 81501365, 81601735, 81770011), and the Shanghai Science and Technology Commission (19XD1403100, 17ZR1423900).

Compliance with ethical standards

Conflict of interest X.Y.F., T.S., Z.D.H., and D.B.L. are co-inventors on an SeV85AB vaccine patent application.

All animal experiments were approved by the Institutional Animal Care and Use Committee and were performed according to the guidelines of the Laboratory Animal Ethical Board of Shanghai Public Health Clinical Center.

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