Sendai Virus Mucosal Vaccination Establishes Lung-Resident Memory CD8 T Cell Immunity and Boosts BCG-Primed Protection against TB in Mice

Zhidong Hu,1,4,5 Ka-Wing Wong,1,4,5 Hui-Min Zhao,1 Han-Li Wen,2 Ping Ji,1 Hui Ma,1 Kang Wu,1 Shui-Hua Lu,1,4 Feng Li,1 Zhong-Ming Li,1 Tsugumine Shu,3 Jian-Qing Xu,1,4 Douglas B. Lowrie,1,4 and Xiao-Yong Fan1,2,4

1Shanghai Public Health Clinical Center, Key Laboratory of Medical Molecular Virology of MOE/MOH, Fudan University, 2901 Caolang Road, Shanghai 201508, China; 2School of Laboratory Medicine and Life Science, Wenzhou Medical University, Wenzhou 325035, China; 3ID Pharma, Ibaraki 300-2611, Japan; 4TB Center, Shanghai Emerging and Re-emerging Infectious Disease Institute, Shanghai 201508, China

INTRODUCTION

Tuberculosis (TB) is among the most deadly health threats to human-kind. Bacille Calmette-Guérin (BCG), which is an attenuated form of Mycobacterium bovis, was introduced nearly a century ago, but remains the only licensed vaccine against TB. It is delivered intradermally and usually protects children efficiently against miliary and meningeal TB,1 but the protective efficacy against pulmonary TB in adults has been found to vary from 0% to 80%.2 BCG is unable to induce effective CD8+ T cell responses, and this deficiency might be one of the factors accounting for the poor efficacy of BCG.3

Considering that Mtb is a mucosal pathogen that targets primarily the lungs, potent T cell immunity at this site is critical for protection.4-8 Hence, an ideal anti-TB vaccine should be able to elicit potent T cell responses in the lung and be safe when delivered intranasally. Although it is recognized that mucosal immunization by direct delivery of BCG into the respiratory tract might give superior protection,9,10 this can also induce a potentially harmful dose-dependent granulomatous infiltration.11 In addition, boosting BCG with additional doses of the same vaccine does not generally enhance protection against TB in humans and can promote pathology in mice.12,13 Consequently, the alternative of boosting through the airway mucosa by using various respiratory virus vectors has attracted attention.

Sendai virus (SeV) is attractive as an alternative vector. It is a negative sense, single-stranded, and non-integrating RNA virus of the family paramyxoviridae and is also known as murine parainfluenza virus type 1. It has low pathogenicity, powerful capacity for foreign gene expression, and wide host range.14 It elicits high levels of antigen-specific CD8+ T cell responses.15,16 Furthermore, being a respiratory transmissible virus, SeV provides a basis for vaccines that elicit potent antigen-specific mucosal immune responses.17-19 It has been well tolerated and immunogenic when used as a vector for a recombinant vaccine against human parainfluenza virus (hPIV), with which it has similarity in terms of sequence, structure, and antigenicity.22 Recently, recombinant vaccines based on a replication-deficient SeV vector have been developed against human immunodeficiency virus,15,18,19 influenza,20 and respiratory syncytial virus.21,22,23 There are several attractive features of SeV-based vaccines. First, intranasal (i.n.) administration is more immunogenic than intramuscular (i.m.) vaccination.25 Second, although pre-existing anti-viral immunity may hinder the use of virus-based vectors, pre-existing anti-SeV neutralizing antibodies remain at a low level in humans since SeV does not infect humans; this low anti-SeV background does not block the ability of recombinant SeV vaccine to induce antigen-specific immunity.4,26

Accumulating evidence has shown the protective role of CD8+ T cells in vaccine-induced immunity against Mycobacterium tuberculosis (Mtb) despite controversy over their role in natural immunity. However, the current vaccine BCG is unable to induce sufficient CD8+ T cell responses, especially in the lung. Sendai virus, a respiratory RNA virus, is here engineered firstly as a novel recombinant anti-TB vaccine (SeV85AB) that encodes Mtb immuno-dominant antigens, Ag85A and Ag85B. A single mucosal vaccination elicited potent antigen-specific T cell responses and a degree of protection against Mtb challenge similar to the effect of BCG in mice. Depletion of CD8+ T cells abrogated the protective immunity afforded by SeV85AB vaccination. Interestingly, only SeV85AB vaccination induced high levels of lung-resident memory CD8+ T (T RM) cells, and this led to a rapid and strong recall of antigen-specific CD8+ T cell responses against Mtb challenge. Furthermore, when used in a BCG prime-SeV85AB boost strategy, SeV85AB vaccine significantly enhanced protection above that seen after BCG vaccination alone. Our findings suggest that CD8+ T RM cells that arise in lungs responding to this mucosal vaccination might help to protect against TB, and SeV85AB holds notable promise to improve BCG’s protective efficacy in a prime-boost immunization regimen.

Received 31 October 2016; accepted 28 February 2017; http://dx.doi.org/10.1016/j.ymthe.2017.02.018.

These authors contributed equally to this work.

Correspondence: Xiao-Yong Fan, Shanghai Public Health Clinical Center, Fudan University, 2901 Caolang Road, Shanghai 201508, China.
E-mail: xyan008@fudan.edu.cn
for the weakness of BCG in a prime-boost model and resulted in markedly enhanced immune protection against Mtb challenge. Taken together, our evidence shows that the RNA-based vaccine SeV85AB confers tissue-resident memory CD8+ T cell responses (TRM) when delivered i.n. and holds notable promise to improve the protective efficacy of BCG in a prime-boost immunization regimen.

RESULTS

Construction and Characterization of SeV85AB
To harness SeV as an anti-TB vaccine, the fbpA/B chimeric gene was introduced into the SeV vector to construct the SeV85AB vaccine (Figure 1A). The expression of Ag85A/B chimeric protein was confirmed in the cell lysate from SeV85AB-infected LLC-MK2 cells by western blotting with mouse antiserum to Ag85A (Figure 1B).

Intranasal Immunization with SeV85AB Elicited Antigen-Specific CD4+ and CD8+ T Cell Responses in the Lung, Spleen, and Draining Lymph Nodes
To determine the immunogenicity of SeV85AB, we vaccinated mice i.n. with high or low titers of SeV85AB, or with an empty SeV vector as negative control, or vaccinated subcutaneously (s.c.) with BCG as positive control (Figure 2A). At 2 weeks and 8 weeks after vaccination, we measured the antigen-specific immune responses in the spleen, lung, and draining lymph nodes (DLN). At 2 weeks after immunization with a high titer dose of SeV85AB (SeV85ABhi, 1 × 10^7 CIU i.n.), an IFN-γ enzyme-linked immunospot (ELISPOT) assay of primary CD4+ and CD8+ T cells responses showed that the vaccine induced strong antigen-specific immune responses to Ag85AB peptides in the mediastinal LN (Figure 2B), while weak responses were seen in the lung (Figure 2C) and spleen (Figure 2D). At 8 weeks, the notable early Ag85B-specific CD8+ T cell responses in the lung that were induced by BCG vaccination alone had decreased (Figure 2C), whereas a higher proportion of Ag85AB-specific T cells had accumulated in the lung in SeV85ABhi-immunized mice (Figure 2C). Moreover, both CD4+ and CD8+ T cells showed stronger Ag85AB-specific responses in the lung, compared with cells from BCG-vaccinated mice (Figure 2C). In contrast, only BCG immunization induced robust immune responses in the subiliac LN (Figure 2E). Stimulation of the cells with a negative control peptide, i.e., TB10.3/420 (C2), containing an MHC-I-restricted epitope, did not give rise to an IFN-γ response (Figure S1), rendering unlikely a non-specific innate imprinting effect of the SeV vector. In addition, analysis of cell cytokine profiles by intracellular staining (the gating strategy is shown in Figure S2) showed that the single mucosal immunization with SeV85ABhi established a potential for significantly higher multi-functional CD8+ T cell responses than BCG vaccination did. This greater potential was seen at 8 weeks post-vaccination when the lung cells were stimulated with Ag85AB peptides (Figure 2F) in contrast to the similar CD4+ T cell responses between SeV85AB and BCG groups (Figure 2G).

Enhanced Cytotoxic Lymphocytes Were Induced by SeV85AB Immunization
We next used an in vivo killing assay to test whether the response to SeV85AB included development of CD8+ effector T cells with
cytotoxic function. Splenocytes from naive mice were prepared as targets by loading them with peptides of Ag85AB appropriate for CD8+ T cell antigen receptors then adoptively transferred together with non-loaded cells into immunized mice. The survival ratio of these cells in vaccinated mice was then tested (Figures 3A and 3B). BCG vaccination enhanced the in vivo cytotoxicity modestly, whereas cytotoxicity in both high and low titer SeV85AB-immunized groups was two times stronger than BCG (Figures 3C and 3D). Moreover, as expected, SeV85AB hi vaccination induced more cytotoxicity than SeV85AB low (Figures 3C and 3D). These findings showed that SeV85AB vaccination was more efficient than BCG in eliciting Ag85AB-specific cytotoxic CD8+ T cell responses.

Protection against Mtb Infection Was Associated with Strong Recall of Ag85AB-Specific CD8+ T Cell Responses

To directly assess the protective efficacy of SeV85AB, we challenged vaccinated mice with Mtb aerosol and determined the bacterial loads in the lung and spleen 5 weeks post infection (Figure 4A). Compared to SeV control treatments, both SeV85AB and BCG immunization resulted in a significant reduction in bacterial loads in lung (Figure 4B) and spleen (Figure 4C). When the recall T cell responses were assayed, IFN-γ ELISPOT results showed that mice that received SeV85AB hi vaccination had developed the strongest Ag85AB-specific responses in the lung at 5 weeks post-infection compared with the SeV and BCG controls (Figure 4D). Moreover, compared with SeV and BCG controls, SeV85AB vaccination primed for the induction of a higher percentage of lung CD8+ T cells that gave an Ag85AB-specific polyfunctional responses, and this was most notable for the phenotypes IL-2+TNF-α+ and IFN-γ+TNF-α+ at 5 weeks post infection (Figure 4E).

SeV85AB-Induced Protection Is Exclusively Mediated by CD8+ T Cells

Confirmation that CD8+ T cells made a major contribution to the SeV85AB-induced protection against Mtb was obtained by depleting these cells using YTS169.4 anti-CD8 monoclonal antibody (mAb) before and immediately after challenge infection (Figure 5A). The depletion of CD8+ T cells was verified by flow cytometry analysis at 2 days after the final treatment with anti-CD8 mAb (Figure S3A). Depletion of CD8+ T cells in the PBS control group had only a
mild effect on protective immunity, suggesting that the background of systemic protection was not mediated mainly by CD8+ T cells (Figures 5B and 5C). Depletion in the BCG group likewise had little effect, consistent with a minor contribution by CD8+ T cells to immunity induced by BCG vaccination.3 In contrast, CD8+ T cell depletion almost completely abolished the protective immunity induced by SeV85AB, resulting in significantly higher bacterial CFU loads in both lung and spleen (Figures 5B and 5C). Thus, our data showed that the protective immunity afforded by SeV85AB vaccination was mainly mediated by CD8+ T cells, which were otherwise deficient in responses to the mycobacterial infection.

Establishment of Lung-Resident Memory T Cells by SeV85AB Vaccination

TRM cells constitute a recently identified lymphocyte lineage that occupies tissues without recirculating and provides a first response against reencountered infections.29 We asked whether the mucosal vaccination with SeV85AB was able to establish local T_{RM} cells in the lung (Figure 6A) since this vaccine was delivered i.n. into the pulmonary mucosal surfaces. To address this question, fluorochrome-labeled CD45 antibody was intravenously (i.v.) injected 3 min before euthanasia to only label cells within lung vasculature, but not parenchyma.30 In combination with a panel of surface markers and a dump channel of lineage-specific markers (the gating strategy is shown in Figure S4A), Ag85A MHC I and MHC II tetramers were used to define antigen-specific CD8+ and CD4+ T cells, respectively. We found that 89.8% of the tetramer-binding CD8+ T cells in the lungs of BCG-vaccinated mice were labeled with the i.v. antibody (Figure 6B) and the percentage was significantly lower in SeV85AB-immunized animals (Figures 6B–6D). Thus, the SeV85AB vaccine was more efficient in inducing lung parenchyma-located memory CD8+ T cells (i.v.). This conclusion was

Figure 3. Enhanced In Vivo Cytolytic Activity of Antigen-Specific CD8+ T Cells Induced by SeV85AB Vaccination

(A) Immunization and detection schedule. Briefly, 8 weeks after immunization, splenocytes from naive mice were loaded with CD8 peptides or not, stained with PHK-26-PE-labeled cell marker, and then differentially stained with high or low levels of CFSE fluorescent marker, mixed, and adoptively transferred into the immunized mice (n = 6) as described in Materials and Methods. The percentage of specific cell lysis was then calculated by using the formula: 100 – [(100 × (% CFSEhi / % CFSElo) × (% CFSEhi in naive mouse / % CFSElo in naive mouse)]. (B and C) The gating strategy and representative profiles of CFSE-labeled cells are shown in (B) and (C). (D) Percentage specific killing of peptide-loaded cells. *p < 0.05, **p < 0.01, and ***p < 0.001, when compared with SeV control or as indicated.
further supported by the staining results using CD103 as a marker of lung resident CD8+ T cells (Figures 6E and 6F). In contrast, similar levels of labeling by the i.v. antibody (Figures S6B–S6D) and of CXCR3+KLRG1− subset establishment among antigen-specific CD4+ T cells (Figures S6E and S6F) were observed in all the vaccinated groups. These features are thought to be markers of lung parenchyma-located CD4+ T cells.32 Thus, our evidence clearly showed that SeV85AB immunization preferentially established lung-resident memory CD8+ T cells instead of CD4+ T cells, but BCG vaccination did not do this.

SeV85AB Boosted BCG-Induced Immune Protection

In view of the different vaccine-induced immunity of BCG and SeV85AB, we tested whether subsequent mucosal delivery of SeV85AB could improve the efficacy of BCG immunization (prime) in a prime-boost model (Figure 7A). Indeed, in comparison to vaccination with either BCG or SeV85AB alone, a boost with SeV85AB resulted in an immediate protection against Mtb challenge in the lung that was seen at 1 week post infection (Figure 7B). It also gave the largest reduction of CFU loads in the lung (Figure 7C) and spleen (Figure 7D) at 5 weeks post-infection and the least pulmonary granulomatous consolidation at that time (Figure 7E). Thus, the potent CD8+ T cell immune responses derived from TRM memory CD8+ T cells in the lung induced by SeV85AB mucosal vaccination appeared to compensate for the weak induction of this T cell subset by subcutaneous BCG immunization.

DISCUSSION

Although Th1 CD4+ T cells mediate immune protection against Mtb infection,33 CD8+ T cells also play a protective role in infection models in mice,34,35 cattle,36 and macaques.37 Furthermore, Mtb-specific CD8+ T cell responses have been found to contribute to modest or strong immune protection induced by several other novel anti-TB vaccines.38–40 Hence, an insufficient induction of these cells by BCG vaccination might underlie the vaccine’s inadequacies and boosting of these responses by novel vaccines might be a key vaccine strategy. Our findings here are consistent with this view, since the superior protective effects that we obtained with Sendai virus-based vaccine SeV85AB were accompanied by superior CD8+ T cell immunity including antigen-specific primary responses (Figure 2), enhanced cytotoxic lymphocytes (CTL) activity (Figure 3), definitive lung tissue-resident memory (Figure 6), and strong recall responses post infection (Figures 4D and 4E). Moreover, depletion of CD8+ T cells almost completely abolished the improvement (Figure 5).

We must be cautious in extrapolating these results in mice to predict future utility of the vaccine in humans. Although virus-based vaccines in general induce strong CD8+ T cell responses,41 the few that have entered clinical trial as anti-TB vaccine candidates have given limited evidence to support protective efficacy. A vaccinia virus-based vaccine (MVA85A) and two adenovirus vectored vaccines (Crucell Ad35 and AdAg85A) are the most advanced candidates. Although MVA85A was effective in boosting BCG immunization in a variety of Mtb animal challenge models,42–45 recent results of a phase IIb clinical trial indicated that MVA85A may not be effective at boosting BCG protection in infants.46 BCG is effective in establishing effector memory in the lungs which can give some protection to vaccinated
neonates for up to 10–15 years. However, this short-lived memory immunity wanes gradually over time, with the outcome that as adults, the vaccinees become as susceptible as people not receiving the vaccine. Accordingly, it is considered that boosting residual BCG-induced immunity in teenagers might be more efficient than in infants and hence that boosting in infancy may partly explain the failure of the MVA85A phase IIb clinical trial. However that may be, the failure of this leading new TB vaccine candidate emphasizes that novel vaccine platforms or optimized delivery systems are urgently needed.

The efficacy of our SeV-based vaccine seen here is consistent with a predictable importance of delivery of the vaccine directly into the lung. Since Mtb is transmitted primarily as an aerosol, it may be particularly important to elicit local immune responses at the pulmonary mucosal surfaces where Mtb first gains host entry. Indeed, mucosal delivery has been shown to be more immunogenic than parenteral immunization with several virus-based anti-TB vaccines, including MVA85A, AdAg85A, and SeV85AB here in a preliminary test (Figure S5). Hence, another potential shortcoming of the MVA85A phase IIb study is that the vaccine was delivered parenterally, whereas i.n. vaccine administration may have the best chance to elicit optimal anti-TB T cell responses.

Our evidence suggests that at least part of the mechanism of creating enhanced protection possibly may be the generation of tissue resident-memory T cells in the lung. T_{RM} cells respond rapidly to pathogen challenge in tissues independently of recruitment of T cells from the blood, thus they mediate the rapid protective immune response that is the hallmark of adaptive immune memory. However, one of the mechanisms by which Mtb escapes acquired immunity is by delaying initiation of the T cell responses and the recruitment of activated circulating T cells to the site of primary infection that is in the lung. Although we found that SeV85AB mucosal vaccination was similar to BCG in inducing the presence of more lung-resident parenchymal and vascular memory CD4+ T cells (Figure S4), the SeV-based vaccine was much more effective than BCG in seeding lung-resident CD8+ memory cells (Figures 6B–6D). We hypothesize that the increasing antigen-specific recalling CD8+ T cell responses seen after Mtb aerosol challenge of SeV85AB-vaccinated mice (Figures 4D and 4E) was likely to have been primed by these resident CD8+ memory cells in the lung (Figure 6). This role of lung-resident memory CD8+ T cells (CD103+) would be consistent with evidence that Mtb-specific CD4+ T cells from lung parenchyma (CXCR3+ KLRG1−) were highly proliferative and provided better immune protection than their blood counterpart (CXCR3+ KLRG1+) in adoptive transfer experiments. Because the activation of antigen-specific

Figure 5. Crucial Role of CD8+ T Cells in SeV85AB-Induced Protection against Mtb
(A) Immunization and infection schedule. Groups of mice were immunized as indicated and then 8 weeks later were treated or not treated with anti-CD8 mAb 1 day before and 1 day after aerosol challenge with virulent Mtb H37Rv. (B and C) Protective efficacy against Mtb infection. Numbers of live bacteria in homogenates of spleen (B) and lung, excluding right superior lobe (C), were counted as CFU after 3-week incubation on 7H11 agar and transformed as log_{10}. **p < 0.01 when compared with each other as indicated. ns indicates no significant difference. The data are derived from one experiment with six mice per group.
Figure 6. Establishment of Lung-Resident Memory of CD8+ T Cells by SeV85AB Vaccination

(A) Immunization and detection schedule. At 8 weeks after immunization, mice were i.v. injected with fluorochrome-labeled CD45 antibody 3 min before euthanasia, and their lung cells were assayed for resident memory CD8+ T cell content by tetramer and surface marker staining. (B) Representative gating of MHC I tetramer and CD45 i.v.− cells. (C and D) The percentages (C) and numbers (D) of i.v.− cells in CD3+Dump−tetramer+ cells are shown. The antibodies included in the dump channels were CD4, CD11b, CD14, CD16/32, and CD19. (E–G) The gating of CD103 (E), the percentage (F), and the summary data of total numbers (G) of CD103+CD8+ tetramer+ cells per lung are shown. The data are representative of two independent experiments with at least five mice per group. *p < 0.05 and **p < 0.01, when compared with each other as indicated.
CD8+ T cells induced by BCG vaccination is delayed and of low magnitude,3 the distinctive activation of recalling CD8+ T cell responses might underlie the ability of SeV85AB to boost the BCG-mediated protective immunity (Figure 7).

Although we did not optimize the dose size, it is encouraging that a single boost of i.n.-delivered SeV85AB significantly enhanced protective efficacy in the BCG-primed mice (Figure 7) and did so without incurring pathology. A recombinant vaccine expressing Ag85B that

---

**Figure 7. Protection Conferred by BCG Immunization Can Be Boosted by a Single Dose of Intranasal SeV85AB Vaccination**

(A) Immunization and infection schedule. BCG-primed mice were i.n. boosted with 1 x 10^7 CFU SeV85AB or not and 4 weeks later they were aerosol-infected with Mtb. The controls received PBS or SeV85AB only. (B–D) Bacterial burden in infected lung at 1 week (B) and in infected lung (C) and spleen (D) at 5 weeks post infection. The homogenates of lung, excluding right superior lobe, were plated for CFU counting. The enumerated CFUs are shown as log10. (E) Representative histological appearance of tissue sections from the right superior lobes of infected lung (H&E staining). (F) The percentage of granulomatous infiltration/consolidation was quantified by the Image Pro Plus program. *p < 0.05 and **p < 0.01, when compared with SeV control or as indicated.
was based on a similar virus, hPIV2, has also been used to elicit protection against *Mtb* infection in mice, but four i.n. administrations were required to achieve protection against *Mtb* that was better than BCG immunization. A hPIV3-vectored anti-TB vaccine expressing Ag85A has also shown substantial efficacy, although its protective mechanism still remains unknown and there is controversy over the safety of hPIV as a vaccine vehicle. Although we confirmed here that it is possible to boost BCG-mediated immunity in mice by a single dose of i.n.-delivered RNA vector, at present, it is unknown whether i.n. SeV85AB administration is also safe and well tolerated in humans. An i.n.-delivered SeV-based vaccine against hPIV has already been shown to be well tolerated and immunogenic in a phase 1 clinical trial. Additionally, intramuscular administration of a single dose of a recombinant SeV-based vector expressing human fibroblast growth factor-2 has been reported to be safe and well tolerated in humans. It also gave significant clinical improvements in patients with peripheral arterial disease. Given our promising results with i.n. SeV vaccination in mice here, further studies on i.n. SeV85AB administration in other animal models of TB are warranted before a clinical trial of safety in humans.

**MATERIALS AND METHODS**

**Construction of SeV85AB**

Ag85A/B chimeric DNA vaccine provided the antigen-coding sequence, a recombinant chimeric gene *fbpA/B* comprising full-length *fbpA* gene into which a fragment encoding the 125–282 amino acids of *fbpB* had been inserted. *fbpA/B* was amplified and ligated into pSeV(+18)/dp CDNA containing the F-gene-defective SeV genome. The ligated DNA pSeV(+)18 df*Ag85AB* DNA was then mixed with plasmids pGEM-N, pGEM-P, and pGEM-L that respectively contained the SeV NP, P/C, or L gene cloned downstream of the T7 promoter. The plasmid mixture was transfected into LLCMK2 cells that were infected with vaccinia virus vTF7-3 containing the phage T7 RNA polymerase gene. Cytoplasmic extracts were prepared from the transfected LLCMK2 cells for identification of Ag85AB expression by western blotting 2 hr after transfection, virus was recovered, cloned, and amplified to provide recombinant virus for vaccination.

**Animal and Immunization**

Specific pathogen-free (SPF) female BALB/c mice aged 6–8 weeks were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. and maintained under SPF conditions with food and water ad libitum until challenge. Infected mice were maintained in a biosafety level 3 (BSL-3) containment animal facility. 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.

Mice were either immunized i.n. with 2 × 10⁶ or 1 × 10⁷ cell infectious units (CIU) of SeV85AB in 20 μL PBS or vaccinated subcutaneously with 1 × 10⁶ colony forming units (CFU) of BCG Danish strain in 100 μL PBS around the hind legs. Empty SeV vector (1 × 10⁷ CIU in 20 μL PBS) was used as negative control. To assess primary T cell immune responses, immunized animals were sacrificed 2 and 8 weeks after vaccination and DLN, spleens, and lungs were aseptically removed. The 8-week-vaccinated mice were either aerosol challenged with virulent *Mtb* H37Rv or BCG Pasteur strain or boosted i.n. with 1 × 10⁷ CIU SeV85AB and then maintained in a biosafety level 2 or level 3 containment animal facility as appropriate. At 1 week or 5 weeks post-infection, the pooled right superior lobes of H37Rv challenged mice or the entire lungs of BCG Pasteur challenged mice was sampled for the determination of secondary T cell responses or H&E staining.

**Preparation of Splenocytes and Lung Single Cells**

Spleens from the sacrificed mice were mechanically disrupted and single splenocytes were filtered through mesh gauze. Red blood cells (RBC) were lysed with lysis buffer. Lungs were minced finely and then incubated at 37°C with 1 mg/mL of Collagenase IV (Invitrogen) and 10 U of DNase I (Thermo Fisher Scientific) in 10 mL of R10 medium (RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin and streptomycin) for 30 min. Then, collagenase-treated lung pieces were gently squashed and filtered through a 70 μm cell strainer (Thermo Fisher Scientific). Next, the cell suspension was centrifuged and subjected to RBC lysis.

**Peptides**

The peptides used in this study were synthesized by GL Biochem with 95% purity. In accord with previous reports, the peptides of Ag85A TFLTSLEPGWQLQANRHVKPT (amino acids 99–118) and MPVGGQSSF (aa 70–78), and YAGAMSGL (aa 145–152) were used as dominant epitopes for inducing CD4⁺ and CD8⁺ T cell responses, respectively, together with the Ag85A peptide GLMSAASSALT (aa 124–135). The peptides of Ag85B HSWEYWGQNLMKGDQ (aa 262–279), and IYAGSLSAL (aa 144–152) and ALLDSPQGMGPSLIG (aa 151–165) were used as dominant epitopes for inducing CD4⁺ and CD8⁺ T cell responses, respectively, along with the Ag85B peptide GPSSPAWERNPDPTQQIP (aa 181–198).

**Antibodies**

The antibodies used were: CD3-PB (clone 17A2), CD3-AF700 (clone 17A2), CD4-Percp Cy5.5 (clone RM4-4), CD44-APC Cy7 (clone IM7), and CD45-PB (clone 30-F11) from Biologend; CD4-Alexa Fluor 647 (clone RM4-5), CD8-FITC (clone 53-67), IFN-γ-APC-Cy7 (clone XMG1.2), and IL-2-PE (clone JES6-5H4) from BD Biosciences; and CD4-FITC (clone GK1.5), CD8a-Percp Cy5.5 (clone 53-67), CD11b-Percp Cy5.5 (clone M1/70), CD14-Percp Cy5.5 (clone SA2-8), CD16/32-Percp Cy5.5 (clone 93), CD19-Percp Cy5.5 (clone 1D3), CD103-PE (clone 2E7), KLRG1-PE Cy7 (clone 2F1), CXC3R-PE (clone CXC3R-173), and TNF-α-PE Cy7 (clone MP6-XT22) from eBioscience.

**IFN-γ Enzyme-Linked Immunospot Assay**

ELISPOT assays were performed according to the IFN-γ ELISPOT kit instructions (BD Biosciences), and the spot-forming cells (SFCs) were then counted.
Intracellular Cytokine Staining and Tetramer Staining

The freshly harvested splenocytes or lung cells were stimulated with the peptide pools of Ag85A and Ag85B (5 μg/mL) or PPD (10 μg/mL) in 96-well plates at 37°C and 5% CO2 for 1 hr or 12 hr, respectively. They were incubated for an additional 5 hr after adding 1 μL/mL Brefeldin A and Monensin (BD Biosciences). Then, cells were incubated on ice with a mixture of antibodies against surface markers for 30 min, followed by washing, fixation, and permeabilization with the fix/perm buffer (BD Biosciences). The fixed cells were then treated with antibodies against intracellular cytokines and incubated for another 30 min on ice, then analyzed by flow cytometry analysis with LSRFortessa (BD Biosciences).

For CD4+ T and CD8+ T cells tetramer staining, the Ag85A-derived peptide (LPGWLVQANRHKVPT) that was bound to the BALB/c major histocompatibility complex (MHC) class II allele I-Eα tetramer and conjugated with APC was provided by the NIH tetramer core facility (Order No. 12018), and the peptide (MPVGGQSST)61 that was conjugated with PE was obtained from QuantoBio Biotech. Cells were incubated with the peptide-bound tetramer for 30 min in the dark at room temperature with PE was obtained from QuantoBio Biotech. Cells were incubated with the peptide-bound tetramer for 30 min in the dark at room temperature and then stained with surface antibodies, before resuspension for flow cytometry analysis. A dump channel (CD4/CD8, CD11b, CD14, CD16/32, and CD19) was used to eliminate non-specifically staining cells gated on CD3+dump−CD4+CD44+ or CD3+ dump−CD8+CD44+, respectively.

In Vivo Cytolytic Assay

The in vivo cytolytic assay was as described elsewhere, but with minor modifications. Briefly, BALB/c splenocytes were loaded with 1 μM CD8 peptides or left untreated at 37°C for 1 hr, then stained with PHK-26-PE using Fluorescent Cell Linker Kit (MINI26, Sigma). After washing, peptide-loaded cells and unloaded cells were labeled with 5 μM or 0.8 μM CFSE, respectively. Then, these CFSE labeled cells (4 x 10^6 CFSEhi and CFSElo, respectively) were adoptively transferred into immunized mice. Killing of peptide-loaded cells was detected in the spleen at 4 hr after adoptive transfer by analyzing CFSE-labeled cells in the populations of PE+ splenocytes.

In Vivo Depletion of CD8+ T Cell Subset

The CD8+ T cells were in vivo depleted as described elsewhere. Briefly, anti-CD8 mAb (clone YTS169.4, BioXcell) was injected intraperitoneally (i.p.) at 0.25 mg/mouse on days −1 and 1 of Mtb infection. Depletion of T cells was confirmed by flow cytometry on day 3 of infection. Typically, about 99% of the CD8+ T cell subset was depleted. Considering YTS169.4 recognizes the α chain of the CD8 molecule and has been used as a stain for CD8+ myeloid dendritic cells (DCs), an effect of this antibody treatment on the stability of DC frequencies was precluded (Figure S3B).

Mycobacterial Challenge and Bacterial Counting

Mice were exposed to an aerosol of Mtb H37Rv or BCG Pasteur strain to deposit a low dose of ~100 CFU per lung by an inhalation exposure system (Glas-Col). The mycobacterial burden was determined by plating homogenates of spleen and lung onto Middlebrook 7H11 agar plates supplemented with 10% OADC and antibiotic mixture (40 U/mL polymyxin B, 4 μg/mL amphotericin, 50 μg/mL carbenicillin, and 2 μg/mL trimethoprim).

Histopathological Analysis

The right superior lobes of infected lung were fixed in formalin and embedded in paraffin. Then, the embedded lung lobes were sectioned in thickness of 5 μm, stained with H&E, and photographed using a Olympus CKX41 microscope (Olympus) fitted with an Olympus DP20 camera connected to a computer. The Image Pro Plus program (Media Cybernetics) was utilized to objectively assess the level of inflammation present in each image. The inflammatory areas stained a more intense purple than the non-inflammatory areas. The percentage of lung with granulomatous infiltration/consolidation, as so defined, was quantified averaging from three to five lung sections of each of the different groups of mice.

Statistical Analysis

The statistical analysis was performed using GraphPad Prism software. Statistical significance for comparison of multiple groups was determined using one-way ANOVA. For the grouped analysis, two-way ANOVA with multiple comparisons was used.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.ymthe.2017.02.018.

AUTHOR CONTRIBUTIONS

Conceived the project: X.-Y.F., T.S., and Z.-M.L. Designed experiments: X.-Y.F., Z.H., and T.S. Performed experiments: Z.H., K.-W.W., H.-L.W., P.J., H.M., K.-M.Z., and F.L. Analyzed data: Z.H., X.-Y.F., S.-H.L., D.B.L., and J.-Q.X. Wrote the manuscript: Z.H., X.-Y.F., S.-H.L., D.B.L., and J.-Q.X. Obtained funding: X.-Y.F. and Z.H. S.-H.L., T.S., J.-Q.X., and D.B.L. are senior authors. X.-Y.F., D.B.L., and K.-W.W. Obtained funding: X.-Y.F. and S.-H.L. X.-Y.F., S.-H.L., D.B.L., and J.-Q.X. Wrote the manuscript: Z.H., X.-Y.F., S.-H.L., D.B.L., and J.-Q.X. Analyzed data: Z.H., X.-Y.F., S.-H.L., D.B.L., and J.-Q.X. Obtained funding: X.-Y.F. and Z.H. S.-H.L., T.S., J.-Q.X., and D.B.L. are senior authors.

CONFLICTS OF INTEREST

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

ACKNOWLEDGMENTS

This work was supported by grants from the Chinese National Mega Science and Technology Program on Infectious Diseases (2013ZX10003007-003), National Natural Science Foundation of China (81273328 and 81501365), and Shanghai Science and Technology Commission (17ZS1423900 and 15ZS1434600). We appreciate Professors Guo-ping Zhao from Shanghai Institutes for Biological Sciences and Ying Wang from Shanghai Jiao Tong University for their valuable comments on the experiment design and data interpretation. We are grateful to NIH Tetramer Core Facility for generously providing Ag85A peptide-MHC II tetramers (Order No. 12018).

Molecular Therapy Vol. 25 No 5 May 2017 1231
REFERENCES

1. Rodrigues, L.C., Diwan, V.K., and Wheeler, J.G. (1993). Protective effect of BCG against tuberculous meningitis and miliary tuberculosis: a meta-analysis. Int. J. Epidemiol. 22, 1154–1158.

2. Colditz, G.A., Brewer, T.F., Berkey, C.S., Wilson, M.E., Burdick, E., Fineberg, H.V., and Mosteller, F. (1994). Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA 271, 698–702.

3. Ryan, A.A., Nambiar, J.K., Wozniak, T.M., Roediger, B., Shkolovskaya, E., Britton, W.J., Fazekas de St Groth, B., and Triccas, J.A. (2009). Antigen load governs the differential priming of CD8 T cells in response to the bacille Calmette Guerin vaccine or Mycobacterium tuberculosis infection. J. Immunol. 182, 7172–7177.

4. Goonetilleke, N.P., McShane, H., Hannan, C.M., Anderson, R.J., Brookes, R.H., and Hill, A.V. (2003). Enhanced immunogenicity and protective efficacy against Mycobacterium tuberculosis of bacille Calmette-Guérin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara. J. Immunol. 171, 1602–1609.

5. Beverley, P.C., Sridhar, S., Lalvani, A., and Tchilian, E.Z. (2014). Harnessing local and systemic immunity for vaccines against tuberculosis. Mucosal Immunol. 7, 20–26.

6. Diogo, G.R., and Reljic, R. (2014). Development of a new tuberculosis vaccine: is there value in the mucosal approach? Immunotherapy 6, 1001–1013.

7. Källenius, G., Pawlowski, A., Brandtzæg, P., and Svenson, S. (2007). Should a new tuberculosis vaccine be administered intranasally? Tuberculosis (Edinb.) 87, 257–266.

8. Belyakov, I.M., and Ahlers, J.D. (2009). What role does the route of immunization play in the generation of protective immunity against mucosal pathogens? J. Immunol. 183, 6883–6892.

9. Chen, L., Wang, J., Zgniaicz, A., and Xing, Z. (2004). Single intranasal mucosal Mycobacterium bovis BCG vaccination confers improved protection compared to subcutaneous vaccination against pulmonary tuberculosis. Infect. Immun. 72, 238–246.

10. Derrick, S.C., Kolibab, K., Yang, A., and Morris, S.L. (2014). Intranasal administration of Mycobacterium bovis BCG induces superior protection against aerosol infection with Mycobacterium tuberculosis in mice. Clin. Vaccine Immunol. 21, 1443–1451.

11. Tree, J.A., Williams, A., Clark, S., Hall, G., Marsh, P.D., and Ivanysz, I. (2004). Intranasal bacille Calmette-Guérin (BCG) vaccine dosage needs balancing between protection and lung pathology. Clin. Exp. Immunol. 138, 405–409.

12. Karonga Prevention Trial Group (1996). Randomised controlled trial of single BCG, and sequence-modified HIV-1 gag genes in a recombinant Sendai virus vector. Curr. HIV Res. 8, 199–206.

13. Cruz, A., Fraga, A.G., Fountain, J.J., Rangel-Moreno, J., Torrado, E., Saraiva, M., Pereira, D.R., Randall, T.D., Pedrosa, J., Cooper, A.M., and Castro, A.G. (2010). Boost strategies based on DNA, adenovirus, and Sendai virus vectors expressing the G glycoprotein of respiratory syncytial virus (RSV) elicits immune protection against RSV. J. Virol. 84, 6043–6047.

14. Jones, B.G., Sealy, R.E., Rudraraju, R., Traina-Dorge, V.L., Finneyfrock, B., Cook, A., Takimoto, T., Portner, A., and Hurwitz, J.L. (2012). Sendai virus-based RSV vaccine protects African green monkeys from RSV infection. Vaccine 30, 959–968.

15. Moriya, C., Horiba, S., Kurihara, K., Kamada, T., Takahara, Y., Inoue, M., Iida, A., Hara, H., Shu, T., Hasegawa, M., and Matano, T. (2011). Intranasal Sendai viral vector vaccination is more immunogenic than intramuscular under pre-existing anti-vector antibodies. Vaccine 29, 8557–8563.

16. Moriya, C., Horiba, S., Inoue, M., Iida, A., Hara, H., Shu, T., Hasegawa, M., and Matano, T. (2008). Antigen-specific T-cell induction by vaccination with a recombinant Sendai virus vector even in the presence of vector-specific neutralizing antibodies in rhesus macaques. Biophy. Biophys. Res. Commun. 371, 850–854.

17. Brooks, A.R., Harkins, R.N., Wang, P., Qian, H.S., Liu, P., and Rubanyi, G.M. (2004). Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. J. Gene Med. 6, 395–404.

18. Liang, Y., Wu, X., Zhang, J., Xiao, L., Yang, Y., Bai, X., Yu, Q., Li, Z., Bi, L., Li, N., and Wu, X. (2012). Immunogenicity and therapeutic effects of Ag85A/B chimeric DNA vaccine in mice infected with Mycobacterium tuberculosis. FEMS Immunol. Med. Microbiol. 66, 419–426.

19. Schenkel, J.M., and Masopust, D. (2014). Tissue-resident memory T cells. Immunity 41, 866–889.

20. Anderson, K.G., Mayer-Barber, K., Sung, H., Beura, L., James, B.R., Taylor, J.J., Qunaj, W.J., Fazekas de St Groth, B., and Triccas, J.A. (2009). Antigen load governs the differential priming of CD8 T cells in response to the bacille Calmette Guerin vaccine or Mycobacterium tuberculosis infection. J. Immunol. 182, 7172–7177.

21. Potent specific immune responses induced by prime-boost strategies based on DNA, adenovirus, and Sendai virus vectors expressing gag gene of Chinese HIV-1 subtype B. Vaccine 26, 6124–6131.

22. Duan, X., Yonemitsu, Y., Chou, B., Yoshida, K., Tanaka, S., Hasegawa, M., Tetzutani, K., Ishida, H., Himeno, K., and Hisaeda, H. (2009). Efficient protective immunity against Trypanosoma cruzi infection after nasal vaccination with recombinant Sendai virus vector expressing amastigote surface protein-2. Vaccine 27, 6154–6159.

23. Chen, L., Wang, J., Zgniaicz, A., and Xing, Z. (2004). Single intranasal mucosal Mycobacterium bovis BCG vaccination confers improved protection compared to subcutaneous vaccination against pulmonary tuberculosis. Infect. Immun. 72, 238–246.

24. Beura, L.K., and Masopust, D. (2014). SnapShot: resident memory T cells. Cell 157, 1488–1488.e1.

25. Sakai, S., Kaufman, K.D., Schenkel, J.M., McCerry, C.C., Mayer-Barber, K.D., Masopust, D., and Barber, D.L. (2014). Cutting edge: control of Mycobacterium tuberculosis infection by a subset of lung parenchyma-homing CD4 T cells. J. Immunol. 192, 2965–2969.

26. Ottenshof, T.H., and Kaufmann, S.H. (2012). Vaccines against tuberculosis: where are we and where do we need to go? PLoS Pathog. 8, e1002607.

27. van Pintenre, L.A., Cassidy, J.P., Smedegaard, B.H., Agger, E.M., and Andersen, P. (2000). Control of latent Mycobacterium tuberculosis infection is dependent on CD8 T cells. Eur. J. Immunol. 30, 3689–3698.

28. Sousa, A.O., Mazzaccaro, R.J., Russell, R.G., Lee, F.K., Turner, O.C., Hong, S., Van Kaer, L., and Bloom, B.R. (2000). Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice. Proc. Natl. Acad. Sci. USA 97, 4204–4208.

29. Villarreal-Ramos, B., McCulay, M., Chance, V., Martin, M., Morgan, J., and Howard, C.J. (2003). Investigation of the role of CD8+ T cells in bovine tuberculosis in vivo. Infect. Immun. 71, 4297–4303.
37. Chen, C.Y., Huang, D., Wang, R.C., Shen, L., Zeng, G., Yao, S., Shen, Y., Halliday, L., Fortman, J., McAllister, M., et al. (2009). A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. PLoS Pathog. 5, e1000392.

38. Wu, Y., Woodworth, J.S., Shin, D.S., Morris, S., and Behar, S.M. (2008). Vaccine-elicted 10-kilodalton culture filtrate protein-specific CD8+ T cells are sufficient to mediate protection against Mycobacterium tuberculosis infection. Infect. Immun. 76, 2249–2255.

39. Grode, L., Seiler, P., Baumann, S., Hess, J., Brinkmann, V., Nasser Eddine, A., Mann, P., Goosmann, C., Bandermann, S., Smith, D., et al. (2005). Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Gueŕrin mutants that secrete lipoarabinomannan. J. Clin. Invest. 115, 2472–2479.

40. Wang, J., Santousson, N., Ngai, P., Zganiac, A., and Xing, Z. (2004). Activation of CD8 T cells by mycobacterial vaccination protects against pulmonary tuberculosis in the absence of CD4 T cells. J. Immunol. 173, 4590–4597.

41. Rollier, C.S., Reyes-Sandoval, A., Cottingham, M.G., Ewer, K., and Hill, A.V. (2011). Viral vectors as vaccine platforms: deployment in sight. Curr. Opin. Immunol. 23, 377–382.

42. Vordermeier, H.M., Rhodes, S.G., Dean, G., Goonetilleke, N., Huygen, K., Hill, A.V., Hewinson, R.G., and Gilbert, S.C. (2004). Cellular immune responses induced in cattle by heterologous prime-boost vaccination using recombinant viruses and bacille Calmette-Gueŕrin. Immunol. Immunopathol. 33, 461–470.

43. Williams, A., Goonetilleke, N.P., McShane, H., Clark, S.O., Hatch, G., Gilbert, S.C., and Hill, A.V. (2005). Boosting with poxviruses enhances Mycobacterium bovis BCG efficacy against tuberculosis in guinea pigs. Infect. Immun. 73, 3814–3816.

44. Verreck, F.A., Vervenne, R.A., Kondova, I., van Kralingen, K.W., Remarque, E.J., Park, C.O., and Kupper, T.S. (2015). The emerging role of resident memory T cells in tuberculosis vaccines in mice. Vaccine 33, 7217–7224.

45. Chen, Z., Gupta, T., Xu, P., Phan, S., Pickar, A., Yau, W., Karls, R.K., Quinn, F.D., Sakamoto, K., and He, B. (2015). Efficacy of parainfluenza virus 5 (PIV5)-based tuberculosis vaccine by taking advantage of characteristics of human parainfluenza type 2 virus vector showed Mycobacteria-specific immune responses by intranasal immunization. Vaccine 32, 1727–1735.

46. Meyer, J., and McShane, H. (2013). The next 10 years for tuberculosis vaccines: do we have the right plans in place? Expert Rev. Vaccines 12, 688–697.

47. Reiley, W.W., Shafiani, S., Wittmer, S.T., Tucker-Heard, G., Moon, J.J., Jenkins, M.K., Urdahl, K.B., Winslow, G.M., and Woodland, D.L. (2010). Distinct functions of antigen-specific CD4 T cells during murine Mycobacterium tuberculosis infection. Proc. Natl. Acad. Sci. USA 107, 19408–19413.

48. Watanabe, K., Matsubara, A., Kawano, M., Mizuno, S., Okamura, T., Tsujimura, Y., Inada, H., Nosaka, T., Matsuo, K., and Yasutomi, Y. (2014). Recombinant Ag85B vaccine by taking advantage of characteristics of human parainfluenza type 2 virus vector showed Mycobacteria-specific immune responses by intranasal immunization. Vaccine 32, 6710–6714.

49. Roediger, E.K., Kugathasan, K., Zhang, X., Lichty, B.D., and Xing, Z. (2008). Heterologous boosting of recombinant adenoviral prime immunization with a novel vesicular stomatitis virus-vectorised tuberculosis vaccine. Mol. Ther. 16, 1161–1169.

50. Santousson, M., Zhang, X., McCormick, S., Wang, J., Hitt, M., and Xing, Z. (2005). Mechanisms of mucosal and parenteral tuberculosis vaccinations: adenoviral-based mucosal immunization preferentially elicits sustained accumulation of immune protective CD4 and CD8 T cells within the airway lumen. J. Immunol. 74, 1798–17994.

51. Park, C.O., and Kupper, T.S. (2015). The emerging role of resident memory T cells in protective immunity and inflammatory disease. Nat. Med. 21, 688–697.

52. Orme, I.M., Robinson, R.T., and Cooper, A.M. (2015). The balance between protective and pathogenic immune responses in the TB-infected lung. Nat. Immunol. 16, 57–63.