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Original Article

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

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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 infection. 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.

INTRODUCTION

Tuberculosis (TB) is among the most deadly health threats to humankind. 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.[15–19] Furthermore, being a respiratory transmissible virus, SeV provides a basis for vaccines that elicit potent antigen-specific mucosal immune responses.[19–21] 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.[21,23,24] 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

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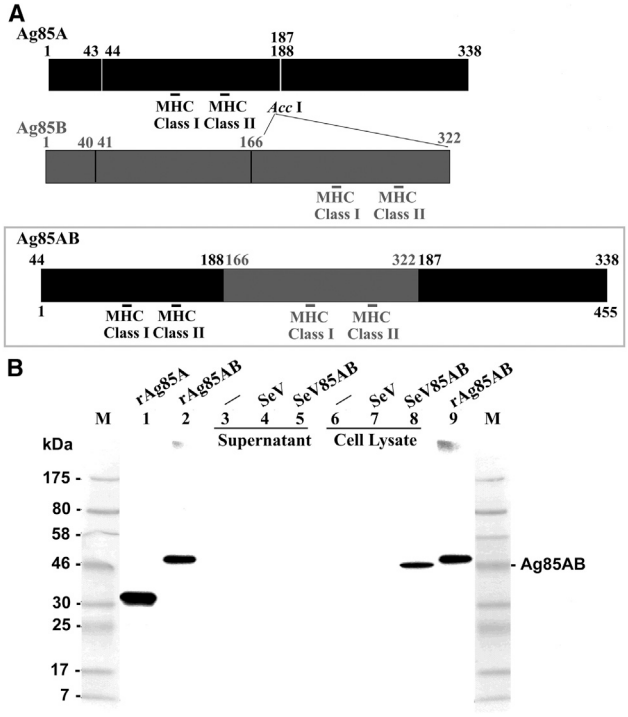

Figure 1. Construction and Characterization of SeV85AB

(A) *fbpA/B* gene was amplified from a chimeric *fbpA/B* plasmid DNA containing the full-length mature structural gene of Ag85A and fragments of Ag85B. (B) Western blotting of culture supernatant and cell lysate from infected cells using antiserum to Ag85A. Lane 1, rAg85A protein; lanes 2 & 9, rAg85AB protein; lane 3, supernatant and lane 6, extract of control LLC-MK_2 cells; lane 4, supernatant and lane 7, extract of cells infected with wild-type SeV; and lane 5, supernatant and lane 8 extract of cells infected with SeV85AB.

T cell immunity.[26] Third, as a RNA virus, SeV expresses antigens without using host transcriptional machinery. This is in contrast to MVA85A and AdAg85A, both of which use DNA-based vectors encoding vaccine antigens under the CMV promoter, which may be prone to transcriptional silencing in human cells.[27] Fourthly, being a RNA virus, it does not undergo reverse transcription, so SeV always remains in the RNA phase during its entire life cycle. This feature avoids possible risk of integration into the human genome and highlights its safety as a vaccine vector for use in humans.

Herein, we for the first time report construction of a replication-deficient recombinant SeV85AB vaccine encoding *Mtb* immuno-dominant antigen Ag85A plus fragments of Ag85B[28] and vaccination of BALB/c mice. A single mucosal dose of SeV85AB induced robust T cell responses and substantial protection against *Mtb* challenge, which was largely mediated by $CD8^+$ T cells. Interestingly, high levels of lung-resident memory $CD8^+$ T cells were induced by SeV85AB vaccination, the first anti-TB vaccine found to do this. These lung-resident memory T cells were probably responsible for enhanced $CD8^+$ T cell recall responses that were seen upon subsequent *Mtb* challenge infection. Additionally, the SeV85AB vaccine was able to compensate 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 (T_{RM}) 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[28] 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-MK_2 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 ($SeV85AB^{hi}$, 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 $SeV85AB^{hi}$-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., $TB10.3/4_{20–28}$ (GYAGTLQSL), 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 $SeV85AB^{hi}$ 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 Ag85A/B 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

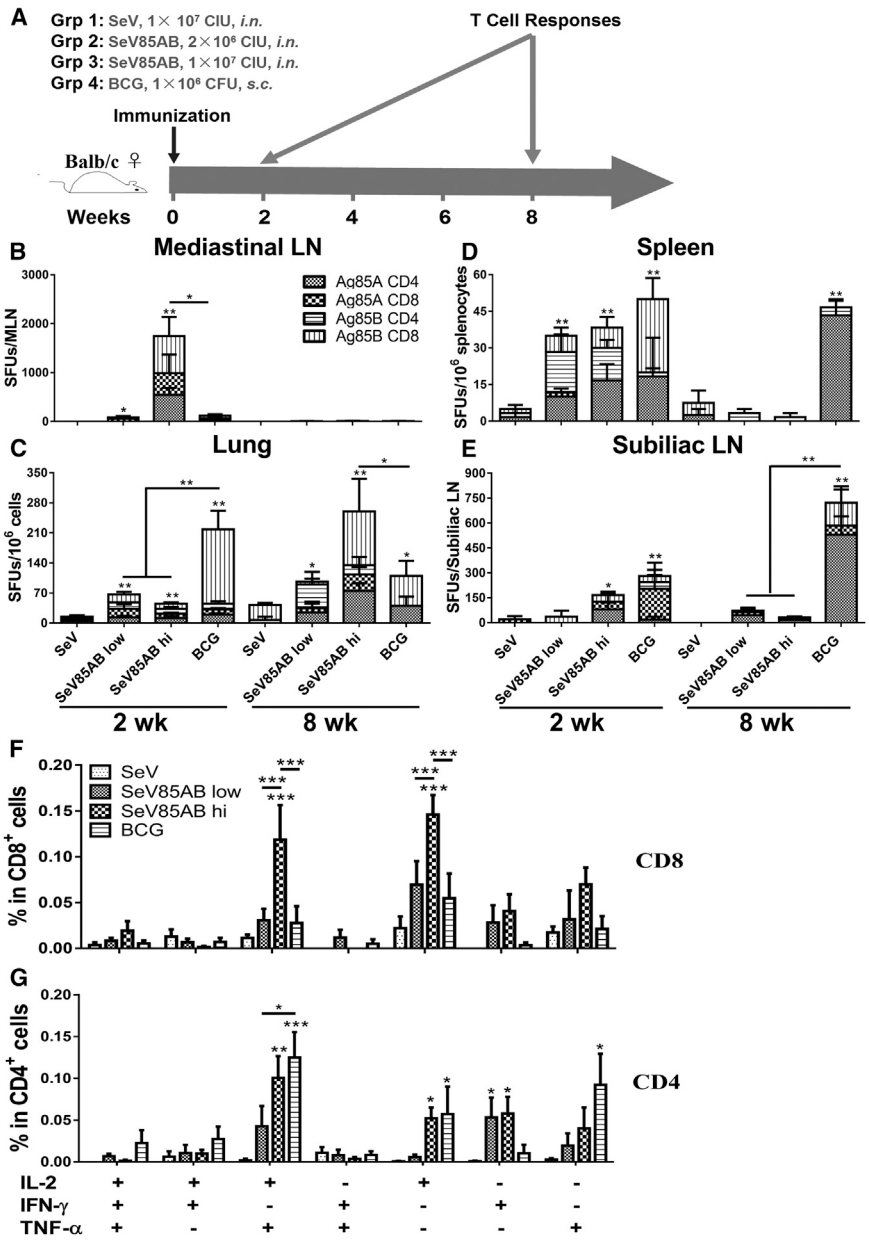

Figure 2. Primary Antigen-Specific T Cell Responses Induced by SeV85AB Vaccination

(A) Immunization schedule. BALB/c mice were vaccinated i.n. as indicated, then were sacrificed at 2 weeks and 8 weeks after vaccination for assays of cellular immune responses. (B–E) The IFN-γ responses by ELISPOT assay at 2 weeks and 8 weeks, respectively. The cells from mediastinal LN (B), lung (C), spleen (D), and subiliac LN (E) were stimulated for 20 hr with Ag85A or Ag85B dominant peptides specific for $CD4^+$ T or $CD8^+$ T cells (5 μg/mL), respectively. (F and G) Multi-functional Ag85AB-specific $CD8^+$ (F) and $CD4^+$ (G) T cell responses in the lung at 8 weeks. LN = lymph node. The T cell responses were compared with SeV control or as indicated. The data are representative of two independent experiments with at least five mice per group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

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

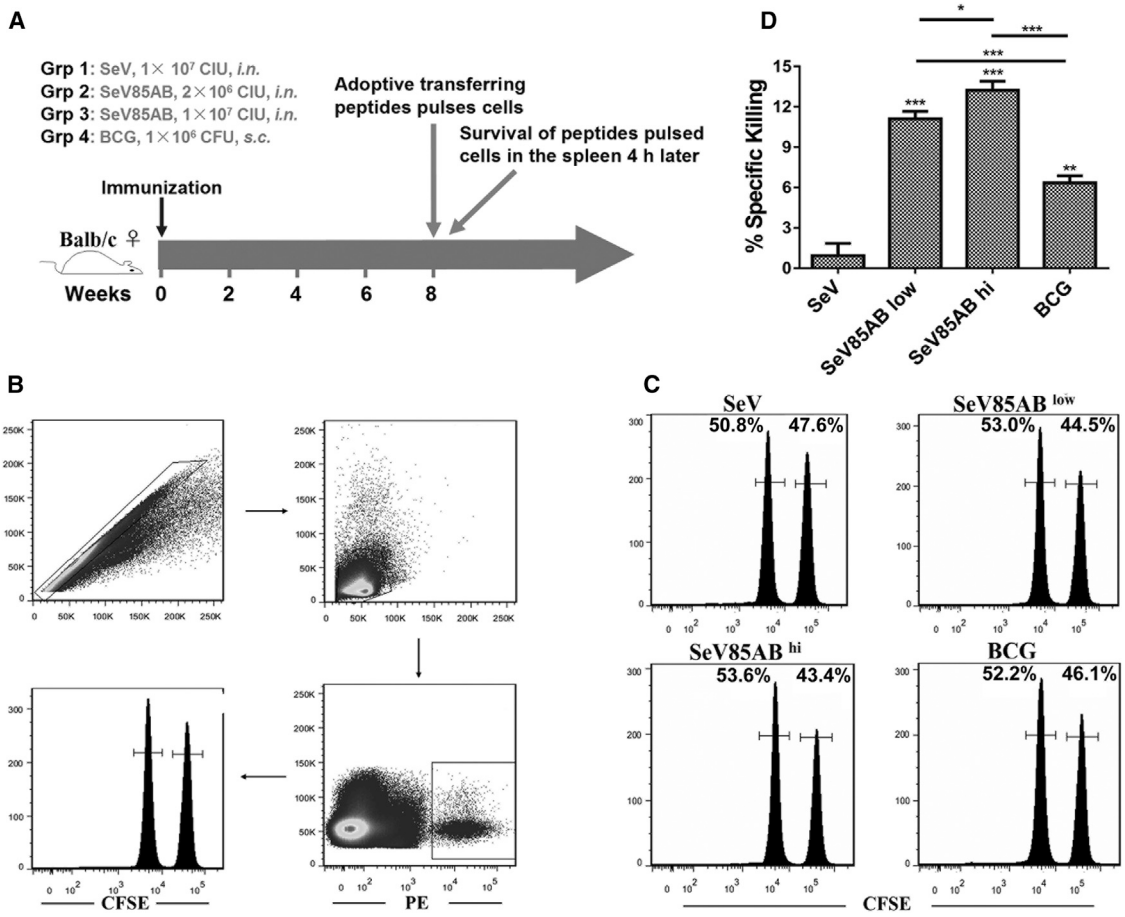

Figure 3. Enhanced In Vivo Cytolytic Activity of Antigen-Specific CD8⁺ T Cells Induced by SeV85AB Vaccination

(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 × [(% $CFSE^{hi}$ / % $CFSE^{lo}$) / (% $CFSE^{hi}$ in naive mice / % $CFSE^{lo}$ in naive mice)]). (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.

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

T_{RM} 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

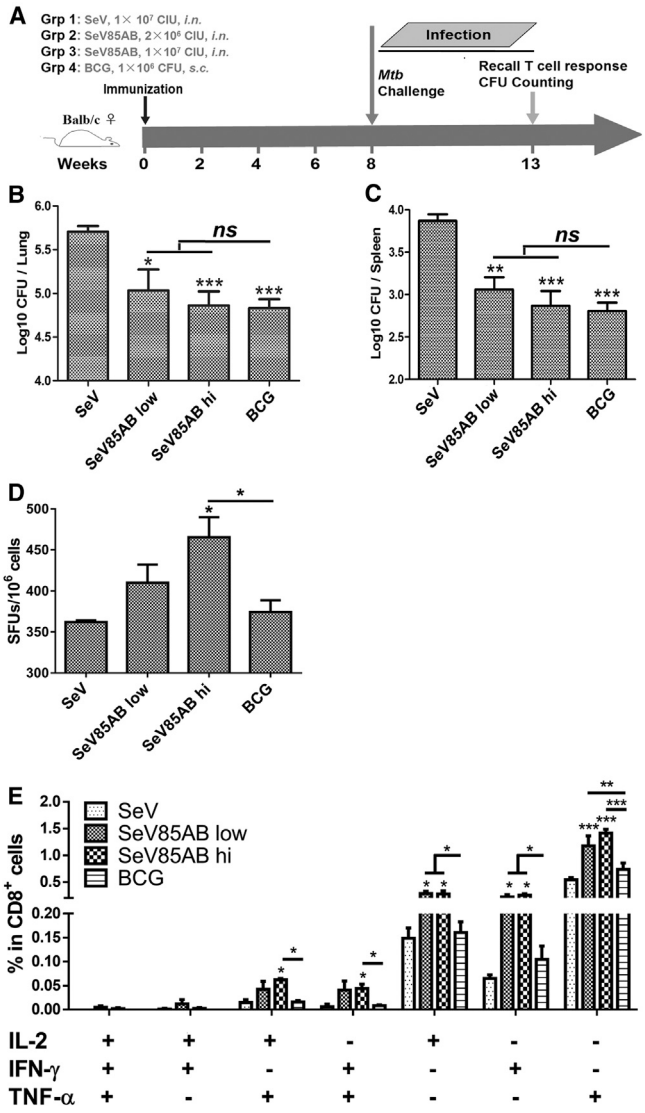

Figure 4. Vaccine Protective Efficacy and Ag85AB-Specific Recall T Cell Responses against *Mtb* Challenge

(A) Immunization and infection schedule. Groups of mice were immunized then aerosol challenged 8 weeks later with virulent *Mtb* H37Rv. At 5 weeks after challenge, protective efficacy and recall $CD8^+$ T cell responses in the lung were characterized. (B and C) Protective efficacy against *Mtb* infection. Numbers of live bacteria in homogenates of lung, excluding right superior lobe (B) and spleen (C), were counted as CFU after 3-week incubation on 7H11 agar and transformed as $\log_{10}$. (D and E) Recall T cell responses against Ag85AB stimulation in *Mtb* challenge infection determined by IFN-γ ELISPOT assay (D) or intracellular cytokine staining (ICS) assay (E). The percentages of $CD8^+$ T cells in the pooled right superior lung lobes that produced one, two, or three of the indicated cytokines are shown (E). The data are the mean values of two independent experiments with at least five mice per group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, when compared with the SeV control or as indicated.

further supported by the staining results using CD103[31] as a marker of lung resident $CD8^+$ T cells (Figures 6E–6G). 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 T_{RM} 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,[4,42–45] 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

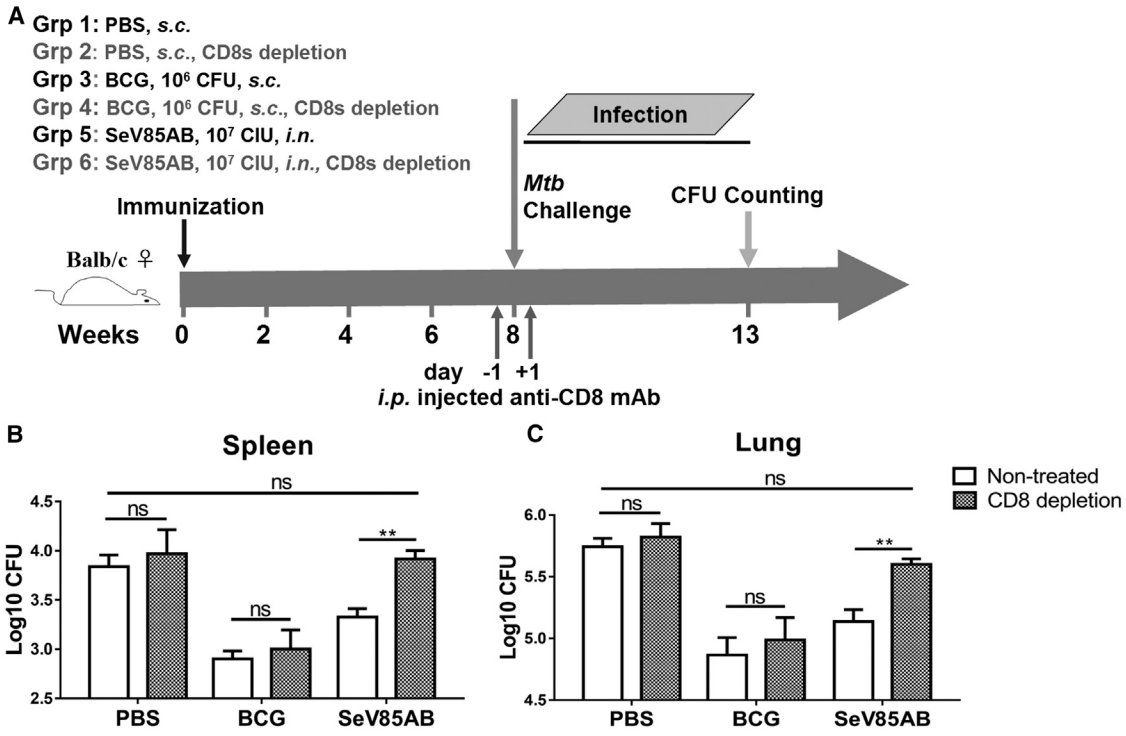

Figure 5. Crucial Role of CD8⁺ T Cells in SeV85AB-Induced Protection against *Mtb*

(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.

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.[47] 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.[48]

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.[4–8] Indeed, mucosal delivery has been shown to be more immunogenic than parenteral immunization with several virus-based anti-TB vaccines,[49] including MVA85A,[4] AdAg85A,[50] 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.[51] 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.[52] 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.[32,53] Because the activation of antigen-specific

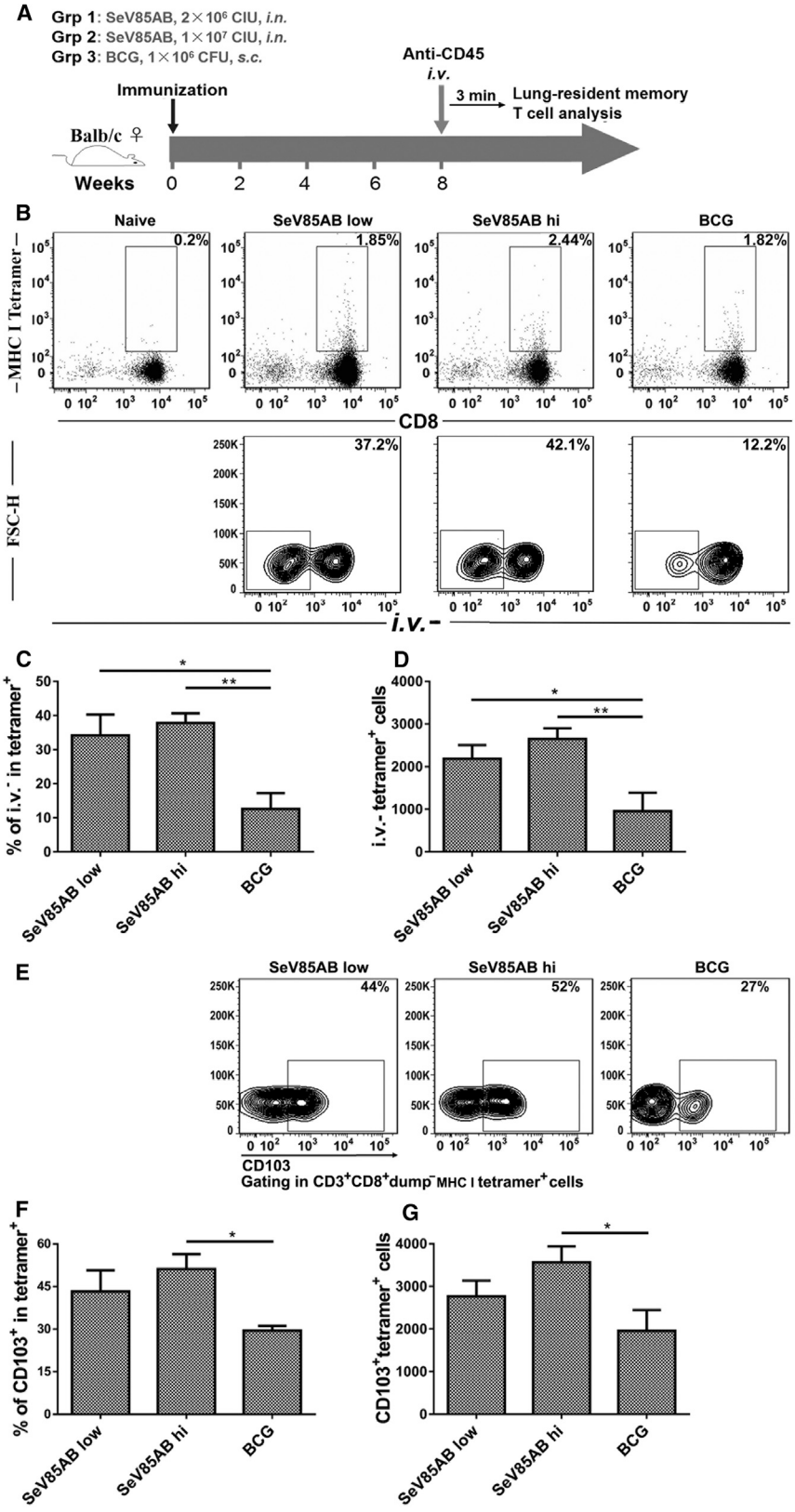

Figure 6. Establishment of Lung-Resident Memory of CD8⁺ T Cells by SeV85AB Vaccination

(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.

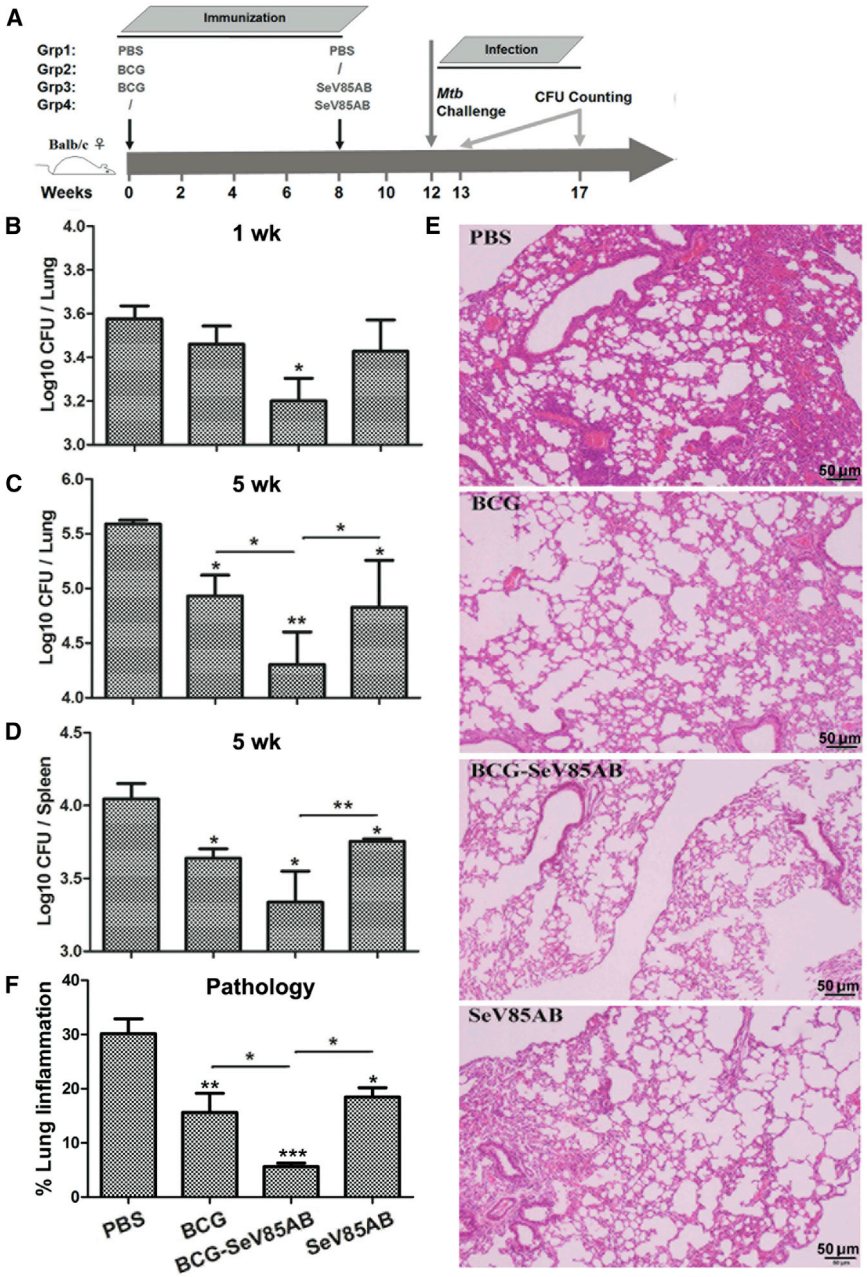

Figure 7. Protection Conferred by BCG Immunization Can Be Boosted by a Single Dose of Intranasal SeV85AB Vaccination

(A) Immunization and infection schedule. BCG-primed mice were i.n. boosted with 1×10^7 CIU 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 $\log_{10}$. (E) Representative histological appearance of tissue sections from the right superior lobes of infected lung (H&E staining). (F) The percentage of granlomatous 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.

$CD8^+$ 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

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.[54] A hPIV5-vectored anti-TB vaccine expressing Ag85A has also shown substantial efficacy, although its protective mechanism still remains unknown[55] 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 I clinical trial.[22] 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.[56] It also gave significant clinical improvements in patients with peripheral arterial disease.[56] 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.[28] *fbpA/B* was amplified and ligated into pSeV(+)18/dF cDNA containing the F-gene-defective SeV genome.[57] 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 $LLCMK_2$ cells that were infected with vaccinia virus vTF7-3 containing the phage T7 RNA polymerase gene.[58] Cytoplasmic extracts were prepared from the transfected $LLCMK_2$ 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 2×10^6 or 1×10^7 cell infectious units (CIU) of SeV85AB in 20 μL PBS or vaccinated subcutaneously with 1×10^6 colony forming units (CFU) of BCG Danish strain in 100 μL PBS around the hind legs. Empty SeV vector (1×10^7 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 1×10^7 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,[59,60] the peptides of Ag85A TFLTSELPGWLQANRHVKPT (amino acids [aa] 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 GLSMAASSALTL (aa 124–135). The peptides of Ag85B HSWEYWGAQLNAMKGDLQ (aa 262–279), and IYAGSLSAL (aa 144–152) and ALLDPSQGMGPSLIG (aa 151–165) were used as dominant epitopes for inducing $CD4^+$ and $CD8^+$ T cell responses, respectively, along with the Ag85B peptide GPSSDPAWERNDPTQQIP (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 BioLegend; CD4-Alexa Fluor 647 (clone RM4-5), CD8-FITC (clone 53-6.7), 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 53-6.7), 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), CXCR3-PE (clone CXCR3-173), 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% CO_2 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 permeablization 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 (LPGWLQANRHVKPT) that was bound to the BALB/c major histocompatibility complex (MHC) class II allele I-E^d tetramer and conjugated with APC was provided by the NIH tetramer core facility (Order No. 12018), and the peptide (MPVGGQSST)[61] that was bound to the BALB/c MHC I allele H-$2L^d$ tetramer and 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 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.[62] 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 (4×10^6 $CFSE^{hi}$ and $CFSE^{lo}$, 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.[17] 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 $CD8\alpha^+$ myeloid dendritic cells (DCs),[63] 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).[64] 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.[65]

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.W., H.-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., D.B.L., and K.-W.W. 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

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