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Single Mucosal, but Not Parenteral, Immunization with Recombinant Adenoviral-Based Vaccine Provides Potent Protection from Pulmonary Tuberculosis[1]

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Bacillus Calmette-Guérin (BCG) vaccine has failed to control the global tuberculosis (TB) epidemic, and there is a lack of safe and effective mucosal vaccines capable of potent protection against pulmonary TB. A recombinant replication-deficient adenoviral-based vaccine expressing an immunogenic *Mycobacterium tuberculosis* Ag Ag85A (AdAg85A) was engineered and evaluated for its potential to be used as a respiratory mucosal TB vaccine in a murine model of pulmonary TB. A single intranasal, but not i.m., immunization with AdAg85A provided potent protection against airway *Mycobacterium tuberculosis* challenge at an improved level over that by cutaneous BCG vaccination. Systemic priming with an Ag85A DNA vaccine and mucosal boosting with AdAg85A conferred a further enhanced immune protection which was remarkably better than BCG vaccination. Such superior protection triggered by AdAg85 mucosal immunization was correlated with much greater retention of Ag-specific T cells, particularly CD4 T cells, in the lung and was shown to be mediated by both CD4 and CD8 T cells. Thus, adenoviral TB vaccine represents a promising novel vaccine platform capable of potent mucosal immune protection against TB. Our study also lends strong evidence that respiratory mucosal vaccination is critically advantageous over systemic routes of vaccination against TB. *The Journal of Immunology,* **2004, 173: 6357–6365.**

Tuberculosis (TB)[3] is a chronic respiratory infectious disease that has afflicted humans for thousands of years. For >80 years, the bacillus Calmette-Guérin (BCG) vaccine has been the only licensed TB vaccine given to humans covering 86% of the world population in 2001 (1). However, despite the use of BCG, TB remains a global epidemic with one-third of the world population being infected and an annual rate of 8 million new cases and 2–2.5 million deaths (2). Regardless of its protection from severe forms of childhood TB, BCG fails to confer protection from adult TB (3, 4). Furthermore, BCG vaccine may cause severe complications in immunocompromised hosts (5). Thus, there is an urgent need for developing safe and effective TB vaccines that are able to confer potent protection at the respiratory mucosa.

Increasing evidence suggests that vaccination at the mucosal site is superior to vaccination at other sites in eliciting protection from mucosal infectious diseases (6). This is partially explained by the observation that memory T and B cells generated upon mucosal vaccination acquire mucosa-homing receptors and preferentially accumulate at the mucosal site of induction (7, 8). Thus, it is believed that greater immune protection may be achieved if TB vaccine is given mucosally via the respiratory tract (9, 10). However, few mucosally delivered TB vaccines, except replicating mycobacteria, could successfully trigger protective immune responses in the lung (11–14). Detrimental immunopathology that may be caused by direct application of live mycobacterial vaccines at the mucosal site, especially at the respiratory tract, is of particular safety concern (5, 9). Furthermore, although promising, recombinant plasmid DNA or protein/subunit vaccines, when given parenterally, are unable to elicit superior protection over BCG vaccine (15–17) and when given mucosally cannot lead to effective immune activation (17–20). Therefore, the development of safe and efficacious respiratory mucosal TB vaccines has remained a major challenge to TB vaccinologists.

Adenovirus with a natural tropism to the respiratory epithelium may cause mild cold symptoms of the upper airways in humans. In this regard, a live wild-type adenovirus vaccine has been given mucosally to military recruits in North America to prevent adenoviral infection (21). Thus, due to ample safety data and its low virulence, tropism to mucosal epithelial cells, and superior gene transfer efficiency, genetically engineered replication-deficient adenovirus has been widely used for the purpose of gene transfer in vivo (22). In addition to its applications in gene replacement therapy, there is an increasing interest in using adenoviral vector to deliver transgenes encoding immunogenic microbial Ags for vaccination against infectious diseases such as AIDS, herpes infection, and malaria (23–25). Adenoviral vector when used for the purpose of vaccination, as opposed to gene replacement therapy,

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[3] Abbreviations used in this paper: TB, tuberculosis; BCG, bacillus Calmette-Guérin; Ad, adenoviral; t-PA, tissue plasminogen activator signal peptide; CFP, culture filtrate protein; ICCS, intracellular cytokine staining; i.n., intranasal; *M.tb*, *Mycobacterium tuberculosis*.

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possesses type 1 immune adjuvant properties and gives rise to self-limited but prolonged high levels of Ag release, which are believed to favor long-term memory immune responses against infection (26). However, TB vaccination by using adenoviral vectors has not been explored. Given the natural tropism of adenovirus to the respiratory epithelium, recombinant replication-deficient adenoviral vector engineered to express selected immunogenic *Mycobacterium tuberculosis* (*M.tb*) Ags is an ideal candidate of respiratory mucosal TB vaccine.

In the present study, we have developed a recombinant replication-deficient adenoviral vector expressing a single immunogenic *M.tb* Ag, Ag85A (AdAg85A). We demonstrate here that a single respiratory mucosal vaccination with AdAg85A induces the activation of both Ag-specific CD4 and CD8 T cells that are different both geographically and qualitatively from those induced by parenteral vaccination, and it results in potent protection against respiratory *M.tb* challenge. Furthermore, a heterologous prime-boost regimen involving systemic priming with a plasmid DNA vaccine and respiratory mucosal boosting with AdAg85A triggers an even further enhanced protection which is remarkably superior to that by cutaneous BCG vaccination.

Materials and Methods

Construction and characterization of recombinant replication-deficient adenoviral vaccine (AdAg85A) and plasmid DNA vaccine (DNAAg85A)

Ag85A cDNA was amplified by PCR from *M.tb* genomic DNA ($H_{37}Rv$). The Ag85A segment without endogenous signal peptide was first amplified by primers (sense primer, GATGTTGTGTCTGTTCGGAG; antisense primer, GATGAGGGAAGCAAGAATGC) and was reamplified with a nested pair of primers that contained restriction sites of *Bgl*II and *Eco*RI at the 5′ end of sense and antisense primers. A 900-bp *Bgl*II/*Eco*RI fragment of Ag85A was isolated and ligated into the plasmid vector containing sequences coding for the human tissue plasminogen activator signal peptide (t-PA) in such a way that the Ag85A cDNA and t-PA signal peptide sequences were in-frame and that protein translation would start at t-PA. The t-PA/Ag85A segment was then inserted into the multicloning site of a shuttle plasmid vector, pVDB3 (27), in a position between a murine CMV promoter and a SV40 poly(A) signal in orientation. This recombinant plasmid (pJW24) was cotransfected into 293 cells along with a rescuing vector pBHG10 (28) (Fig. 1*a*) that contained the entire type 5 human adenovirus genomic DNA sequences, except the E1, E3, and packaging regions. Recombinant replication-deficient adenoviral vector AdAg85A was then rescued by homologous recombination. Similarly, the t-PA/Ag85A segment was inserted into the multicloning site of plasmid vector pCDNA3.1 to obtain plasmid DNA vaccine DNAAg85A. The correctness of AdAg85A or DNAAg85A was verified by Northern and Southern blot hybridizations. AdAg85A was amplified, purified, and titrated according to the protocols previously described (29). The secretion of Ag85A protein by AdAg85A-infected mammalian cells was verified by Western immunoblotting using a mAb (clone TD-17) and HRP-conjugated anti-mouse IgG.

Immunization with AdAg85A, DNAAg85A, or BCG and depletion of CD4 and CD8 T cells

Six- to 8-wk-old female BALB/c mice were purchased from Harlan Laboratory (Indianapolis, IN) and maintained under specific pathogen-free conditions at the McMaster University Central Animal Facility or the Animal Facility at the British Columbia Research Institute for Children's and Women's Health, University of British Columbia. For intranasal (i.n.) delivery of AdAg85A, 5×10^7 PFU of AdAg85A or Addl70-3 were diluted with PBS to a total volume of 30 μl and delivered into mouse airways in two aliquots with a fine pipette tip. For i.m. delivery, 5×10^7 PFU of AdAg85A or Addl70-3 was diluted with PBS to a total volume of 100 μl and injected into both hind legs (two separate injections of 50 μl/leg) (28). *Mycobacterium bovis* BCG (Connaught or Pasteur strain) at the dose of 5×10^5 CFU/mouse was diluted with PBS in a total volume of 100 μl and s.c. injected into mice around each hind leg (14). For DNA immunization, 50 μg of DNAAg85A was diluted in 50 μl of PBS and injected i.m into a hind leg of the mice. In some experiments, vaccinated mice were depleted of CD4 or CD8 T cells or both, 2 days before *M.tb* challenge, by four repeated weekly i.p. injections of monoclonal anti-CD4 (GK1.5) and -CD8 (2.43) T cell Abs.

Lymphocyte isolation and in vitro Ag stimulation

At various times postvaccination, spleens and popliteal lymph nodes or mediastinal lymph nodes draining the site of immunization were removed and pooled for each group. Cells were isolated and cultured as previously described (30, 31). Approximately 0.5×10^6 cells were seeded into 96-well plates in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, 100 g/ml streptomycin, and 2 mmol/L of L-glutamine at 37°C with or without Ag stimulation. The Ags used to stimulate cells included *M.tb*

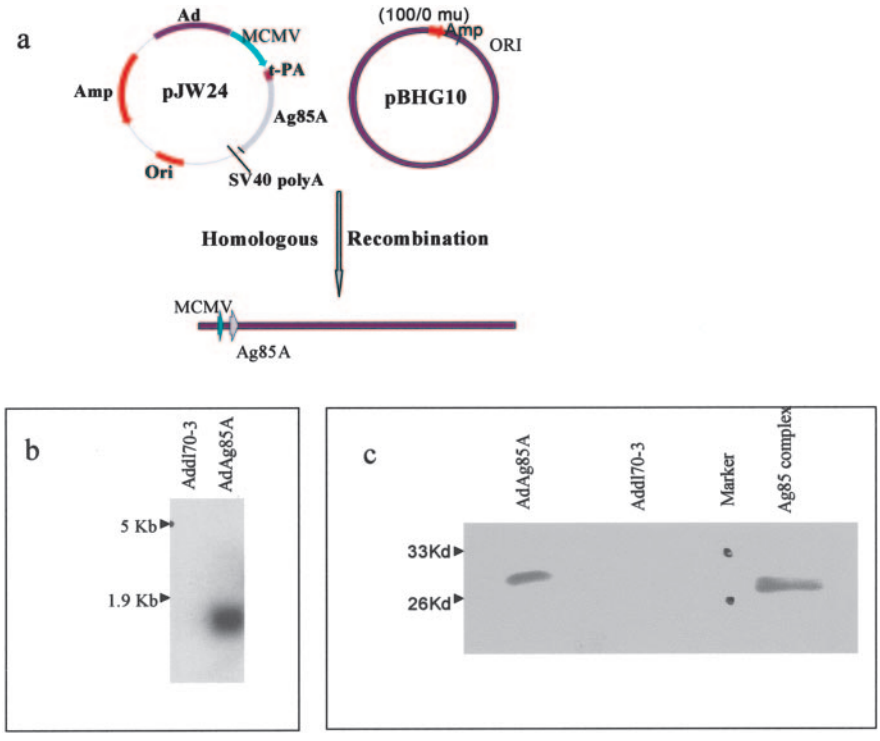

FIGURE 1. Construction of AdAg85A (*a*). A shuttle plasmid vector pJW24 was designed as shown in the diagram and constructed by multiple subcloning steps as detailed in *Materials and Methods*. The presence of adenoviral sequences in pJW24 allows homologous recombination with adenoviral rescuing vector pBHG10 to form recombinant replication-deficient adenovirus AdAg85A, which has the entire expression cassette inserted into the E1 region of the adenoviral genome. Characterization of AdAg85A by Northern blot hybridization (*b*) and Western blot (*c*). Total cellular RNA and culture supernatant were prepared from A549 cells infected with AdAg85A or control vector Addl70-3 and subjected to Northern blot hybridization with a ^{32}P-labeled Ag85A cDNA probe and Western blotting with an anti-Ag85A TD-17 mAb, respectively.

culture filtrate protein (CFP; 8 μg/ml) or purified *M.tb* Ag85 complex proteins (8 μg/ml). The culture supernatants were collected at 72 h. Stimulation with irrelevant Ags was used as control and resulted in only background levels of cytokine production (not graphed in the figures for the purpose of simplicity).

Intracellular cytokine staining (ICCS), flow cytometry analysis (FACS), and ELISPOT assay

The frequency of Ag-specific, IFN-γ-releasing T cells was examined by ICCS and FACS (31). The lungs were removed and pulmonary vasculature was perfused with 5 ml of warm buffer via the right ventricle of the heart. The lungs were cut into small pieces and subjected to 1-h collagenase (150 U/ml) digestion at 37°C and then mashed through a 70-μm Falcon cell strainer with a 5-ml syringe plunger. Approximately 1×10^7 splenocytes or lung-derived mononuclear cells were cultured in 5 ml of RPMI 1640 medium in six-well plates with or without crude BCG (10 μl/well) and Ag85 (10 μg/ml) Ag for 10 h, and were then treated with GolgiPlug (BD Pharmingen, San Diego, CA) for an additional 5 h. Cells were harvested and washed with PBS and ICCS was conducted by using Cytofix/Cytoperm kits (BD Pharmingen) according to the manufacturer's instructions. Briefly, 2×10^6 cells were first stained with surface makers of FITC-CD8α or CyChrome CD8α or allophycocyanin-CD4 or FITC-CD4 in combination with or without CyChrome-CD3, fixed, and permeabilized by Cytofix/Cytoperm solution for 20 min at 4°C, and then labeled with PE-IFN-γ Ab. A FACScan instrument was used (BD Biosciences, Sunnyvale, CA) to collect list mode data (200,000–500,000 total events) for analysis. Isolated splenocytes were stimulated with Ag85A-specific H-2^d CD4 (LTSELPG WLQANRHVKPTGS) and CD8 (MPVGGQSSF) T cell peptides for 5 h (5 μg/ml) and processed as above for ICCS and FACS analysis. In some experiments, purified splenocytes were also subjected to an ELISPOT assay (31) to compare the frequencies of Ag-specific IFN-γ- and IL-13-releasing T cells.

Pulmonary challenge with M.tb

Mice were challenged with live *M.tb* (H_{37}Rv) by i.n. inhalation at the Level III facility in McMaster University or by aerosol inhalation at the University of British Columbia. For i.n. delivery, 10,000 CFU of *M.tb* was diluted with PBS to a total volume of 25 μl and delivered into mouse lungs (14). For aerosol delivery, 100 *M.tb* bacilli per mouse were delivered via an inhalation exposure system (Glas-Col, Terre Haute, IN). The protective efficacy of vaccination with different immunization regimens was evaluated by plating serial 10-fold dilutions of lung and spleen homogenates in quadruplicates on Middlebrook 7H10 agar plates containing oleic acid-albumin-dextrose-catalase enrichment (Difco, Detroit, MI). Plates were incubated inside semisealed plastic bags at 37°C for 3 wk and colonies were counted.

Cytokine measurement and histology

The level of cytokines in the supernatants was measured by using mouse-specific ELISA kits (R&D Systems, Minneapolis, MN). The sensitivity of detection for IFN-γ, IL-4, and IL-13 was 2 pg/ml. For histology, lung tissues were fixed in 10% Formalin and processed for sectioning and H&E staining.

Data analysis

The difference comparison was conducted by using an unpaired, two-tailed Student *t* test. The difference was considered statistically significant when $p \leq 0.05$.

Results

Characterization of recombinant replication-deficient adenoviral vector expressing M.tb *Ag85A (AdAg85A)*

Since secreted *M.tb* Ag has a stronger ability to elicit immune responses compared with nonsecreted Ags (32), AdAg85A was designed to encode a secreted form of Ag85A by replacing its endogenous signal peptide with human tissue plasminogen signal peptide (t-PA) sequence (Fig. 1*a*). Expression of Ag85A is under control of a murine CMV promoter. A strong mRNA signal with the predicted size was detected in the total RNA isolated from A549 cells infected with AdAg85A but not with control vector Addl70-3 (Fig. 1*b*). Furthermore, using a mAb specific for Ag85A and Western blotting, an Ag85A protein band of ~30 kDa was detected in the culture supernatant from A549 cells infected with AdAg85A but not Addl70-3 (Fig. 1*c*).

Ag-specific type 1 immune activation by AdAg85A in vivo

We next examined whether AdAg85A was able to elicit Ag-specific immune responses in vivo and, if so, what was the nature of such Ag85A-specific immunity. BALB/c mice were injected with various doses of AdAg85A or control virus Addl70-3 via the i.m. route. We have previously demonstrated that i.m. injection of adenoviral vector represents a convenient and efficient parenteral route of gene delivery (29). As a comparison, BCG was given via the conventional cutaneous route. Total splenocytes were isolated 3 wk postimmunization and restimulated with different Ags ex vivo. Mycobacterial Ag-stimulated IFN-γ release was used as readout of type 1 immune activation. Marked elevated levels of IFN-γ were readily measured in a dose-dependent manner in cells

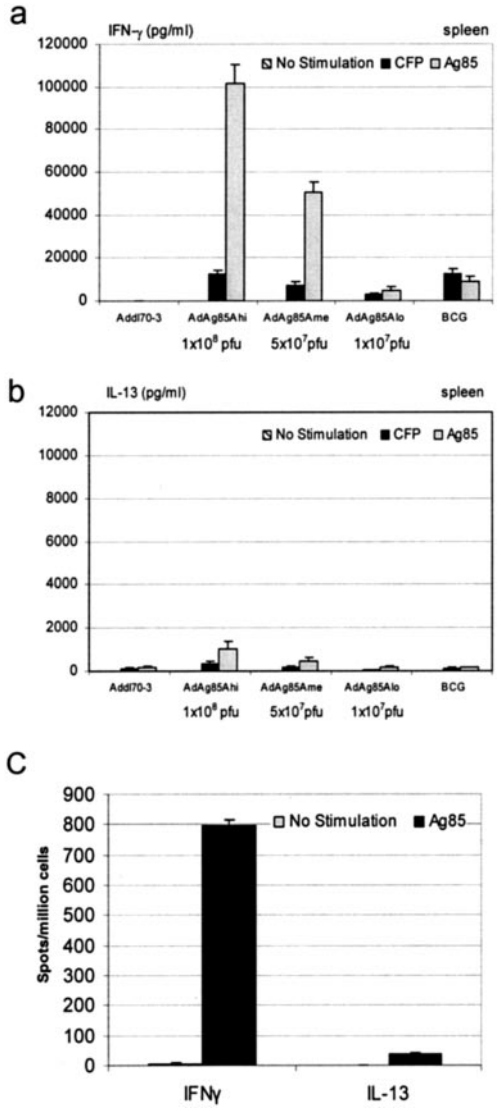

FIGURE 2. In vivo characterization of immune responses induced by AdAg85A. BALB/c mice were immunized i.m. with different doses of AdAg85A or 5×10^7 PFU of Addl70-3 or s.c. with BCG and sacrificed 3 (*a* and *b*) or 2 (*c*) wk later. Splenocytes were isolated and cultured with or without Ag85 proteins or *M.tb* CFP. The level of IFN-γ (*a*) or IL-13 (*b*) in culture supernatants was measured by ELISA. The number of IFN-γ- or IL-13-releasing cells was determined by ELISPOT assay. Data represent the mean value ± SEM of triplicate samples.

from AdAg85A-immunized, but not Addl70-3-treated, mice following stimulation with mycobacterial Ags, particularly Ag85 proteins (Fig. 2*a*). Not only were Ag85-specific IFN-γ responses elicited by AdAg85A at doses above 1×10^7 PFU much stronger than those elicited by BCG immunization (50–100 ng/ml vs 8.8 ng/ml), but the level of responses stimulated by *M.tb* CFP was also comparable to that by BCG vaccination, indicating the potent immunogenicity of AdAg85A vaccine in vivo. To verify the type 1 nature of immune activation by AdAg85A, type 2 cytokines IL-4 and IL-13 were measured. Although there were no measurable levels of IL-4 for all culture conditions (data not shown), the overall magnitude of Ag85-specific IL-13 responses was only ~1% of Ag85-specific IFN-γ responses (Fig. 2*b*), indicating that AdAg85A vaccination elicits a typical type 1 cellular immunity in vivo. To further verify the Th1 nature of immune activation caused by AdAg85A vaccination, we compared the relative frequencies of IFN-γ- and IL-13-releasing T cells in the spleen. Indeed, after AdAg85A immunization, the majority of Ag85-specific splenocytes were found to produce Th1 cytokine IFN-γ but not Th2 cytokine IL-13 (Fig. 2*c*).

Distinct distribution of cellular immune responses following parenteral and respiratory mucosal AdAg85A vaccination

We next investigated the potential difference in the distribution of immune responses elicited by i.m. parenteral and i.n. mucosal vaccinations by comparing type 1 immune responses in different organs (draining lymph nodes, spleen, and lung). Intramuscular immunization with AdAg85A elicited a potent IFN-γ response in the popliteal lymph nodes draining the site of injection after 1 wk, which then quickly declined to undetectable levels at weeks 2, 4, and 6 (Fig. 3*a*). In contrast, the responses in the spleen peaked around week 2 and then declined to levels that were maintained over weeks 4 and 6 (Fig. 3*b*). Such potent immune response in the spleen triggered by i.m. immunization was found to be accounted for primarily by Ag85A-specific CD8 T cells as assessed by ICCS immunoassay, whereby the ratio of IFN-γ-releasing CD8 vs CD4 T cells was ~8:1 (Fig. 3*c*). In comparison, i.n. mucosal immunization led to much smaller immune responses both in the mediastinal lymph nodes draining the respiratory tract and in the spleen (Fig. 3, *a–c*). We next examined the level of immune responses in

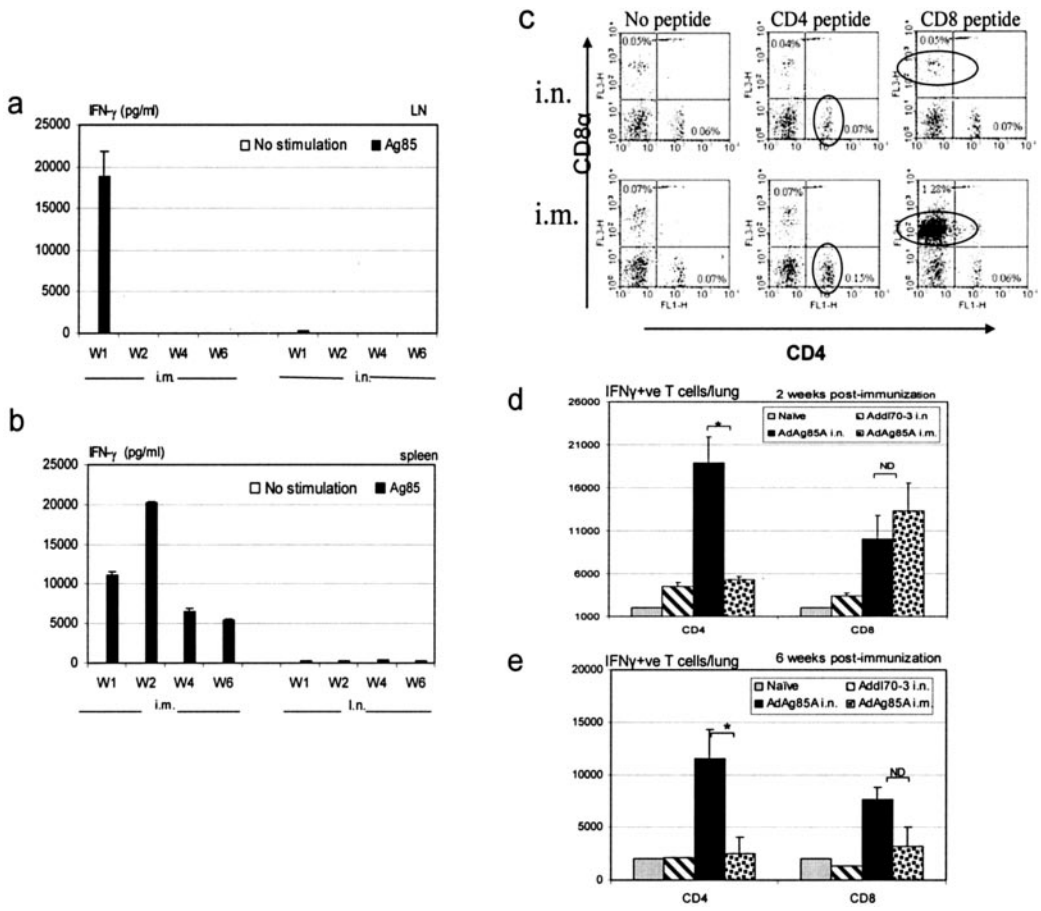

FIGURE 3. Immune activation in local draining lymph nodes (LN), spleen, and lung induced by i.m. parenteral or i.n. mucosal immunization with AdAg85A. Splenocytes (*a*) and lymph node-derived cells (*b*) were isolated from mice immunized i.m. or i.n. with AdAg85A at weeks 1, 2, 4, and 6 and cultured with or without stimulation by Ag85 complex proteins, and the level of IFN-γ was measured by ELISA. Data represent the mean value ± SEM of triplicate samples. In separate experiments, splenocytes were isolated 2 wk after i.n. or i.m. immunization with AdAg85A and stimulated with CD4 T cell- or CD8 T cell-specific peptides and ICCS was conducted following surface immunostaining (*c*). The percentage in the *lower right* and *upper left quadrants* represents the frequency of Ag85A-specific IFN-γ-releasing CD4 and CD8 T cells out of the total cells, respectively. Total lung mononuclear cells were isolated at weeks 2 (*d*) or 6 (*e*) postimmunization with PBS (naive), control vector Addl70-3 i.n., or AdAg85A i.n. or AdAg85A i.m. and stimulated with Ag85 proteins and *M.tb* CFP. The number of Ag85A-specific IFN-γ-releasing CD4 and CD8 T cells was examined by intracellular IFN-γ staining and the results are expressed as the absolute number of IFN-γ-positive (IFN-γ+ve) cells per lung per group per time. Results are expressed as the mean ± SEM from four to six mice per vaccinated group per time and average value from two mice per control group per time. ND, Not statistically significant different; ∗, $p < 0.05$.

the lung. Since in contrast to the lymph nodes and spleen, the lung contains many cell types other than lymphocytes, ICCS was used to analyze mycobacterial Ag-specific CD4 and CD8 T cell responses. At 2 wk postimmunization, mucosal immunization with AdAg85A induced both CD4 and CD8 T cell responses as illustrated by marked increases in the number of Ag-specific IFN-γ-releasing T cells as compared with that in mice receiving mucosal delivery of PBS or Addl70-3 (Fig. 3*d*). In comparison, parenteral immunization with AdAg85A failed to induce a CD4 T cell response while it resulted in a CD8 T cells response comparable to the level of CD8 T cell responses induced by i.n. immunization (Fig. 3*d*). At 6 wk postimmunization, both Ag-specific CD4 and CD8 T cell responses induced by mucosal vaccination remained at high levels in the lung (Fig. 3*e*). In comparison, although there was still little CD4 T cell response in the lung induced by i.m AdAg85A immunization, CD8 T cell responses declined by ~75% at 6 wk (Fig. 3*e*). These results suggest that parenteral and respiratory mucosal AdAg85A vaccinations give rise to differential immune activation profile and distribution, with the former primarily inducing a robust systemic activation of CD8 T cells and the latter inducing the activation/accumulation of both CD4 and CD8 T cells in the lung.

Potent protection from pulmonary TB conferred by respiratory mucosal AdAg85A vaccination and contribution by CD4 and CD8 T cells

Having characterized the immune responses, we compared immune protection from pulmonary *M.tb* challenge conferred by parenteral and mucosal AdAg85A vaccinations. At 4 wk after i.m. AdAg85A vaccination, while *M.tb* infection in the lung was reduced by ~0.5 log, there was little reduction in the spleen (Fig. 4, *a* and *b*). By 12 wk, i.m. AdAg85A vaccination-mediated protection completely vanished. In stark contrast, i.n. AdAg85A vaccination reduced *M.tb* infection in the lung by 1.3 and 1.5 log at weeks 4 and 12 postvaccination, respectively (Fig. 4, *a* and *b*). The CFU in the spleen was also significantly reduced at these times by mucosal vaccination. In keeping with potent immune protection, there was much reduced lung histopathology upon airway *M.tb* challenge in mice immunized i.n. with AdAg85A (Fig. 4*c*). In comparison, a much greater extent of histopathology was observed in the lung of i.m. AdAg85A-immunized hosts (Fig. 4*c*). Potent immune protection mediated by mucosal AdAg85A vaccination is associated with higher levels of retention of Ag85A-specific CD4 and CD8 T cells in the lung (Fig. 3, *d* and *e*). To investigate whether both CD4 and CD8 T cells contributed to immune protection, i.n. AdAg85A-vaccinated mice were depleted of either CD4 or CD8 T cells or both, before *M.tb* challenge, with anti-CD4 and CD8 Abs. Although i.n. AdAg85A reduced *M.tb* infection in the lung by >1.6 log, depletion of either CD4 or CD8 T cells markedly attenuated such protection and depletion of both subsets completely abolished such protection in the lung (Fig. 4*d*) and spleen (data not shown). These results suggest that both CD4 and CD8 T cells participate in mucosal AdAg85A vaccination-mediated protection.

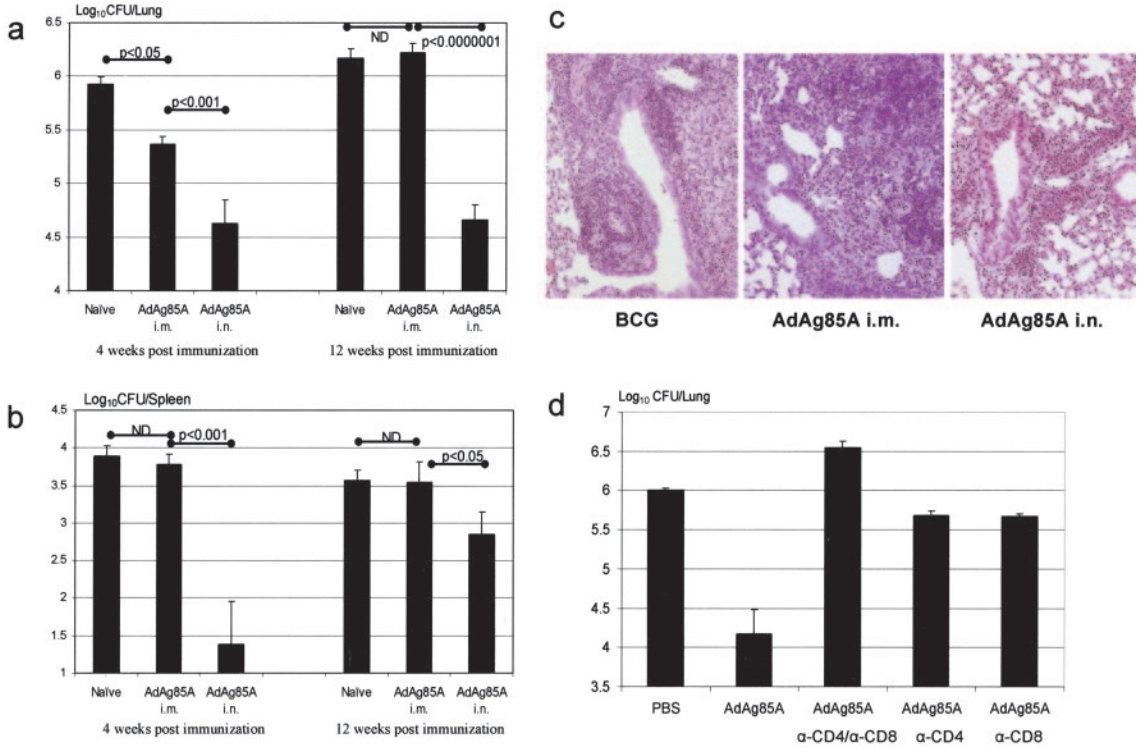

FIGURE 4. Sustained immune protection from pulmonary *M.tb* challenge by respiratory mucosal, but not parenteral, vaccination with AdAg85A. Immunized or nonimmunized (naive) mice were challenged via the airway at either 4 or 12 wk postimmunization and the level of *M.tb* infection was determined in the lung (*a*) and spleen (*b*) 4 wk postchallenge. Results are expressed as the mean ± SEM from 7 to 10 mice/group. H&E staining of lung sections from mice at 12-wk postimmunization with s.c. BCG or i.m. or i.n. AdAg85A (*c*). In separate experiments, groups of mice were immunized i.n. with AdAg85A for 4 wk and challenged via the airway with *M.tb*. Two days before challenge, some groups of mice were injected weekly i.p. with anti-CD4, anti-CD8 T cell Abs, or both for a total of 4 wk. These and control mice were sacrificed at 4 wk after *M.tb* challenge and the level of *M.tb* infection was determined in the lung (*d*). Results are expressed as the mean ± SEM from seven mice per group.

Single respiratory mucosal AdAg85A vaccination confers improved protection from M.tb *challenge over that by cutaneous BCG vaccination*

Given potent protection by single i.n. AdAg85A vaccination, we then compared i.n. AdAg85A vaccination with conventional cutaneous BCG vaccination. To decide on the strain of BCG to be used, we first compared the level of protection mediated by using a 0.5×10^6 CFU dose of Connaught and Pasteur BCG strains. Although s.c. vaccination with both Connaught and Pasteur BCG strains similarly reduced *M.tb* infection in the lung (1–1.2 log) and spleen (1.1–1.3 log), the Connaught strain was slightly more potent (data not shown). Thus, the Connaught BCG was used for all of the following *M.tb* challenge experiments. Based on our previous (14) and current observations, s.c. BCG vaccination at 4 and 12 wk consistently leads to 1–1.2 log reduction of infection in the lung. In comparison, i.n. AdAg85A vaccination leads to 1.3–1.7 log reduction (Fig. 4, *a* and *d*), thus already suggesting that i.n. AdAg85A vaccination leads to an improved immune protection over that by s.c. BCG. To further verify, mice were vaccinated or treated in parallel for 6 wk with a control adenoviral vector (Addl70-3), i.m. AdAg85A, i.n. AdAg85A, or s.c. BCG. After airway *M.tb* challenge, they were sacrificed at 4, 6, and 8 wk for colony assay. Intranasal delivery of Addl70-3 marginally enhanced protection only at 4 wk compared with naive mice (Table I). Intramuscular AdAg85A provided limited protection. Again, s.c. BCG led to a 0.9–1.3 log reduction of infection in the lung and a 0.5–0.8 log reduction in the spleen at weeks 4, 6, and 8 postchallenge (Table I). In comparison, single i.n. AdAg85A immunization conferred the greatest extent of protection. At weeks 4, 6, and 8 postchallenge, the level of infection was reduced by 2.3, 2.1, and 1.5 log in the lung and 2.4, 2.2, and 1.5 log in the spleen, respectively (Table I). These results suggest that airway mucosal AdAg85A immunization confers improved protection over s.c. BCG vaccination. We have recently reported that compared with s.c. BCG vaccination, respiratory mucosal BCG vaccination could also improve anti-TB protection, reducing *M.tb* infection by ~1.5 log and 1.4 log in the lung and spleen, respectively (14). Thus, respiratory mucosal AdAg85A vaccination could accomplish a level of immune protection both in the lung and spleen that is comparable to or even better than respiratory mucosal BCG vaccination.

Intramuscular priming by DNAAg85A and respiratory mucosal boosting by AdAg85A confer the most potent immune protection

We next investigated whether systemic (i.m.) DNAAg85A vaccine priming and respiratory mucosal (i.n.) AdAg85A boosting could further improve protection. Groups of mice were immunized in parallel as depicted in Fig. 5*a*. Repeated i.m. DNAAg85A immunizations reduced *M.tb* infection by ~0.9 log in the lung at 4 wk postchallenge but such protection subsided both in the lung and spleen by 8 wk postchallenge (Fig. 5, *b–e*). The same was true with the mice receiving i.m. DNAAg85A priming and systemic (i.m.) AdAg85A boosting (Fig. 5, *b–e*). In contrast, systemic priming with two repeated DNAAg85A injections and respiratory mucosal boosting once with AdAg85A provided the most potent protection of all of the tested vaccines/regimens, including s.c. BCG vaccination both at 4 and 8 wk postchallenge (Fig. 5, *b–e*), and this level of protection was also better than single i.n. AdAg85A vaccination at 8 wk postchallenge (4.42 ± 0.18 CFU/lung; 2.74 ± 0.16 CFU/spleen). Again, such further enhanced immune protection was found to be associated with more increased numbers of Ag-specific CD4 and CD8 T cells in the lung (data not shown). These results suggest that systemic priming with DNAAg85A vaccine and airway mucosal boosting with AdAg85A vaccine represent an effective way to achieve a robust immune protection against pulmonary TB.

Discussion

Although different recombinant TB vaccines have been described in the past decade (9, 10, 15, 16, 32–40), the exploration of adenoviral vector for TB vaccination has not been reported. We have now demonstrated that adenoviral-based recombinant TB vaccine, AdAg85A, elicits potent type 1 CD4 and CD8 T cell immune activation and single airway mucosal, but not i.m., immunization with AdAg85A provides potent protection against pulmonary *M.tb* challenge. Of importance, such protection is improved over that by conventional cutaneous BCG vaccination at least for the periods of time of observation. Airway mucosal AdAg85A immunization confers an even further dramatically enhanced protection in the mice that were systemically primed by DNAAg85A vaccine. To our knowledge, this is the first demonstration of superior immune

Table I. *Comparison of immune protection by a single immunization with different vaccines/regimens at various times after airway M.tb Challenge (log_{10} CFU/organ)*[a]

	4 wk		6 wk		8 wk	
	Lung	Spleen	Lung	Spleen	Lung	Spleen
Naive	5.476 ± 0.058	3.187 ± 0.1065 (n = 25)	5.717 ± 0.125	3.756 ± 0.186 (n = 17)	5.702 ± 0.110	4.192 ± 0.128 (n = 28)
Add170–3 (i.n.)	4.886 ± 0.185¶	2.536 ± 0.351¶ (n = 7)	5.649 ± 0.079	3.796 ± 0.165 (n = 7)	5.629 ± 0.129	4.066 ± 0.135 (n = 11)
AdAg85A (i.m.)	4.991 ± 0.299¶	2.327 ± 0.128§ (n = 14)	5.516 ± 0.117	3.791 ± 0.062 (n = 11)	5.071 ± 0.168¶	3.248 ± 0.159¶ (n = 10)
AdAg85A (i.n.)	3.102 ± 0.343†***	0.706 ± 0.255†**** (n = 15)	3.687 ± 0.308†**	1.547 ± 0.308†*** (n = 16)	4.249 ± 0.121†	2.752 ± 0.179†** (n = 16)
BCG (s.c.)	4.610 ± 0.078¶	2.653 ± 0.179§ (n = 19)	4.538 ± 0.086†	2.946 ± 0.142§ (n = 13)	4.371 ± 0.114†	3.400 ± 0.195§ (n = 14)

[a] Mice were immunized with AdAg85A i.m. or i.n. or with BCG s.c. for 6 wk and were then challenged via the airway with *M.tb.* Naive mice or mice treated i.n. with a control adenoviral vector (Add170–3) were set up as control. A dose of 5×10^7 PFU of adenoviral vector and a dose of 0.5×10^6 CFU of Connaught BCG were administered. Mice were sacrificed at 4, 6, and 8 wk postchallenge. The number of tuberculous bacilli in the lung and spleen was determined by colony enumeration assay. Data are expressed as mean ± SEM from pooled two to three separate experiments per time point per treatment. *n,* Number in parentheses represent the total number of mice per group per time used for analysis. ¶, $p < 0.05$; §, $p < 0.00001$; †, $p < 0.00000001$ as compared to the naive group; **, $p < 0.005$; ***, $p < 0.001$; ****, $p < 0.0001$ as compared to BCG group (the difference is considered statistically significantly different when $p \leq 0.05$).

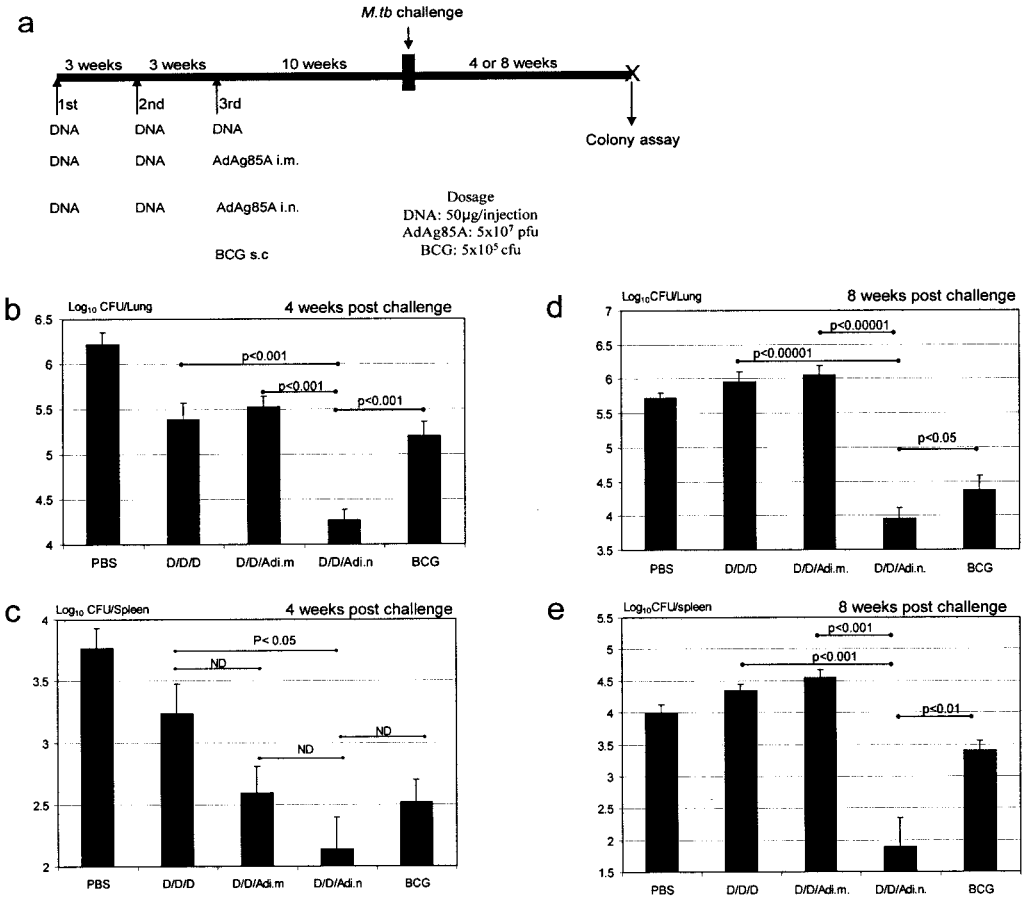

FIGURE 5. Immune protection by heterologous prime-boost vaccination regimens. Schematic diagram of experimental design (*a*). Three repeated injections of DNAAg85A vaccine were conducted at a 3-wk interval. In some groups, AdAg85A was given i.n. or i.m. as the final booster. As control, some mice received only a single i.n. or i.m. injection of AdAg85A or s.c. BCG. Mice were challenged via the airway with *M.tb* at 10 wk after the last immunization and the level of infection in the lung and spleen was determined by colony enumeration assay at 4 wk (*b* and *c*) or 8 wk (*d* and *e*) postchallenge. Results are expressed as the mean ± SEM from 8 to 12 mice/group per time.

protection conferred by the use of recombinant TB vaccine(s) expressing a single *M.tb* Ag. Thus, adenoviral TB vaccine represents a promising novel TB vaccine capable of potent mucosal immune protection. Our study also illustrates the critical advantage of respiratory mucosal vaccination over parenteral vaccination for protection against pulmonary TB.

Respiratory mucosal immunization with AdAg85A results in a preferential accumulation/retention of Ag-specific CD4 and CD8 T cells in the lung but not in the distant lymphoid organs. This is in contrast to parenteral AdAg85A vaccination that leads to the accumulation of Ag-specific CD8 T cells predominantly in the spleen. Of interest, regardless of a high number of such CD8 T cells in the spleen of i.m. immunized mice, there was a relative lack of protection in the spleen. This could result from a greater systemic dissemination due to high levels of lung infection and perhaps also from a poorer protective property of these cells. Recently, strong evidence has emerged that memory T cells can be localized not only to the secondary lymphoid organs but also to the peripheral tissues (41), and it is the memory T cells present in the lung that protect the host from secondary infection (42). Although parenteral AdAg85A immunization also leads memory CD8 T cells to be recruited into the lung, only mucosal vaccination results in sustained accumulation/retention of both CD4 and CD8 T cells in the lung. In this regard, Gallichan and Rosenthal (43) have shown that long-term mucosal CTL memory was observed only with mucosal, but not systemic, immunization with an adenoviral herpes vaccine. At the present time, it remains to be determined whether the quality of CD8 memory T cells in the lung generated by the two routes of AdAg85A immunization differs and what are the mechanisms by which mucosal AdAg85A immunization favors memory T cell retention in the lung. Evidence suggests that the interactions of $\alpha_4\beta_1$ integrin/VCAM-1 and LFA-1/ICAM-1 adhesion molecules play a role in the airway tissue homing and retention of memory T cells (44). Thus, it is very likely that the T cells preferentially retained in the lung of airway mucosal-immunized hosts potently protect from pulmonary TB. This notion is further supported by our observation that depletion of both CD4 and CD8 T cells abolishes immune protection and that further enhanced protection by heterologous systemic prime/airway mucosal boost vaccination is closely associated with more increased numbers of Ag-specific T cells retained in the lung.

In the last decade or so, a number of new TB vaccines have been under development and these include recombinant plasmid DNA- and protein/peptide-based and viral-based vaccines (9, 10, 15, 16, 32, 38). Although nonorganism-based recombinant vaccines such as DNA and protein/peptide vaccines are believed to be safe, even with repeated injections and/or adjuvant, none of those vaccine formulations confers the level of immune protection that exceeds that by BCG vaccine in experimental models (in most studies including our current one, 0.3–1.3 and 0.3–1.0 log reduction of *M.tb* infection in the lung and spleen, respectively, with DNA or subunit TB vaccination; compared with 0.5–1.4 log and 0.6–1.4 log in the

lung and spleen with BCG, respectively) (15, 16, 18). Moreover, the limited accessibility to the pulmonary mucosal surface or low efficiency of gene transfer has restricted the airway mucosal use of these TB vaccines. D'Souza et al. (18) have explored the use of lipid-encapsulated DNA TB vaccine for i.n. immunization but the resultant immune protection is still unsatisfactory (0.7–1.0 and 0.1–0.3 log reduction in the lung and spleen, respectively). Recombinant vaccinia viral vector has been used to express selected *M.tb* Ags but this type of viral TB vaccine by itself cannot elicit strong immune protection. In comparison to all of these nonmycobacterial recombinant forms of TB vaccines, single airway mucosal vaccination with our AdAg85A vaccine elicits an improved protection over cutaneous BCG vaccination (1.3–2.4 and 1.0–2.5 log reduction of *M.tb* infection in the lung and spleen, respectively, with i.n. AdAg85A compared with 0.9–1.3 and 0.5–1.3 log reduction with s.c. BCG, respectively). Furthermore, this level of immune protection is also comparable to or even better than that by airway mucosal BCG vaccination (1.5–1.7 and 1.0–1.5 log reduction in the lung and spleen with i.n. BCG, respectively) (14).

More recently, recombinant BCG- and auxotrophic *M.tb*-based vaccines have also been evaluated. Compared with wild-type BCG, recombinant BCG vaccine expressing ESAT-6 was found to confer a similar protection in mouse models while it conferred better protection in the spleen, but not in the lung, of guinea pig models (34). Recombinant BCG overexpressing Ag85B appears to hold great promise because it provides better protection than BCG in guinea pig models (33). Sambandamurthy et al. (37) have reported that an auxotrophic *M.tb* vaccine elicits immune protection similar to wild-type BCG (~1.1 and 1.0 log reduction of *M.tb* infection in the lung and spleen, respectively). The heterologous prime-boost vaccination regimens have been used to enhance the immunogenicity of TB vaccine. In this regard, plasmid DNA TB vaccines were used for priming and protein or vaccinia viral TB vaccine was used for boosting. Such regimens enhanced protection to the levels similar to those of BCG control (45, 46). In comparison, our systemic DNAAg85A prime and mucosal AdAg85A boost regimen triggers a remarkably improved immune protection (1.8–2.0 and1.7–2.1 log reduction of *M.tb* infection the lung and spleen, respectively) not only over single BCG but also over single i.n. AdAg85A vaccination set up in the same experiments. Up to now, there was only one TB vaccination regimen (airway mucosal BCG priming and modified vaccinia Ankara boosting) that has demonstrated a similar or better level of protection in mouse models of TB (2.5 and 1.5 log reduction in the lung and spleen, respectively) (47). However, in our view, airway mucosal vaccination with replication-deficient adenoviral TB vaccine will be safer than mucosal vaccination with live replication-competent BCG in both immune-competent and -deficient hosts since the former only causes transient and mild inflammatory responses in the lung, whereas the latter will inevitably induce chronic granulomatous pathology and may also lead to uncontrollable systemic infection in the case of immune-compromised hosts (5). It needs to be noted that we have only evaluated the tissue level of infection for immune protection in our current study and that long-term studies remain to be conducted to determine whether mucosal adenovirus vaccination could also improve the survival of various strains of *M.tb*-infected mice.

In summary, we have provided the evidence that recombinant replication-deficient adenoviral-based TB vaccine represents a promising novel platform of TB vaccine. One of the major advantages associated with this form of TB vaccine is its effectiveness in respiratory mucosal immune activation. When delivered mucosally into the respiratory tract, it confers robust protection against pulmonary TB on its own and when mucosally used in DNA vaccine-primed hosts, it accomplishes a remarkable level of protection. The level of protection triggered by adenoviral-mediated mucosal vaccination could be even better than the current BCG vaccine given parenterally or mucosally. Of note, BCG vaccination seems to hold off lung infection more constantly than AdAg85A vaccination. It is likely that activation of multiple T cell clones of different Ag specificity by BCG vaccination (AdAg85A produces only Ag85A-specific T cells) may confer an advantage of long-term lung protection. We are currently investigating whether multivalent or mixture-based adenoviral TB vaccines expressing Ag85 and the *M.tb* Ags such as ESAT-6, missing from BCG (48), could further improve such immune protection. Our current findings also warrant further evaluation in TB models involving the use of guinea pigs and other large animals.

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