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# Mucosal Administration of Ag85B-ESAT-6 Protects against Infection with *Mycobacterium tuberculosis* and Boosts Prior Bacillus Calmette-Guérin Immunity<sup>1</sup>

Jes Dietrich,<sup>2\*</sup> Claire Andersen,\* Rino Rappuoli,<sup>†</sup> T. Mark Doherty,\* Charlotte Green Jensen,\* and Peter Andersen\*

We have examined the intranasal administration of a vaccine against *Mycobacterium tuberculosis* (M.tb) consisting of the mucosal adjuvant LTK63 and the Ag Ag85B-ESAT-6. Vaccination with LTK63/Ag85B-ESAT-6 gave a strong and sustained Th1 response mediated by IFN- $\gamma$ -secreting CD4 cells, which led to long-lasting protection against tuberculosis, equivalent to that observed with bacillus Calmette-Guérin (BCG) or Ag85B-ESAT-6 in dimethyldioctadecylammonium bromide/monophosphoryl lipid A. Because a crucial element of novel vaccine strategies is the ability to boost BCG-derived immunity, we also tested whether LTK63/Ag85B-ESAT-6 could act as a BCG booster vaccine in BCG-vaccinated mice. We found that vaccinating with LTK63/Ag85B-ESAT-6 strongly boosted prior BCG-stimulated immunity. Compared with BCG-vaccinated nonboosted mice, we observed that infection with M.tb led to a significant increase in anti-M.tb-specific CD4 T cells in the lungs of LTK63/Ag85B-ESAT-6-boosted animals. This correlated with a significant increase in the protection against M.tb in LTK63/Ag85B-ESAT-6-boosted mice, compared with BCG-vaccinated animals. Thus, LTK63/Ag85B-ESAT-6 represents an efficient preventive vaccine against tuberculosis with a strong ability to boost prior BCG immunity. *The Journal of Immunology*, 2006, 177: 6353–6360.

The mucosa is the port of entry for many pathogens, which invade the host through respiratory, gastrointestinal, or genital surfaces. Specific lymphoid tissue associated with the mucosal surfaces of the nasal cavity and gut function both as an important site for the priming of the immune response and for the immune effector functions against pathogens that invade the host via these mucosal surfaces. One such pathogen is *Mycobacterium tuberculosis* (M.tb).<sup>3</sup> M.tb usually enters the host via the mucosal surface of the lung after inhalation of infectious droplets from an infected individual. Because the respiratory tract is the natural route of M.tb infection, mucosal immunization has received increasing attention in the field of tuberculosis (TB) vaccination, and recent studies have indeed indicated that mucosal vaccination may provide a strong protection against M.tb (1–6). One explanation for the efficiency of mucosal administration against M.tb infection could be that Ag-specific memory T cells preferentially home back to the site of vaccination (7) and that the location of T cells in the airway at the time of infection is of importance (8). In addition, another advantage with nasal delivery is that it employs needle-free vaccine administration and therefore limits the risk of transmitting infectious agents via contaminated syringes. Given the high rate of coinfection with M.tb and HIV,

especially in developing countries, this certainly suggests that this route of vaccine delivery deserves further attention.

The majority of Ags are not immunogenic when delivered mucosally and require the use of strong adjuvants or effective delivery systems. Cholera toxin and *Escherichia coli* heat-labile enterotoxin (LT) are the strongest mucosal adjuvants known so far. These toxins consist of two subunits: the A subunit, which contains the enzymatic activity, and the B subunit, which binds to the GM1 ganglioside and to other glycolipids. During the past few years, site-directed mutagenesis has permitted the generation of LT and cholera toxin mutants devoid of toxic activity, while retaining their strong mucosal adjuvant activity (9). One such adjuvant is the LT mutant LTK63 in which serine in position 63 in the A subunit has been substituted for lysine. LTK63 has totally lost its enzymatic activity and its toxic properties in vitro and in vivo (10). LTK63 has been shown to behave as a strong mucosal adjuvant in a number of animal models, when coadministered with recombinant proteins or synthetic peptides, and has been shown to prime both Ag-specific CD4 and CD8 T cells (11–13). In various studies, coadministration with candidate vaccines enhanced the cytotoxic T cell response to HIV gag-p55 (14) and respiratory syncytial virus matrix proteins (12), increased the protective efficacy of the VacA protein of *Helicobacter pylori* lysate (15), and induced Abs to tetanus toxin when delivered transcutaneously (16). Delivery via the respiratory tract improves the efficacy of the group C meningococcal conjugate vaccine (17), enhances immunity to the SAG1 Ag of *Toxoplasma gondii* (18), and induces Th1 immunity to *Bordetella pertussis* (19). Thus, several lines of evidence exist that suggest LTK63 may be a useful adjuvant for a vaccine against TB. Regarding the current vaccine against TB, bacillus Calmette-Guérin (BCG), several studies have shown that the protective efficacy of BCG wanes significantly over a period of 10–15 years, emphasizing an urgent need for a BCG booster vaccine that efficiently boosts immunity in BCG-vaccinated individuals (20). LTK63 may be particularly interesting in that respect, because it has

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<sup>3</sup> Abbreviations used in this paper: M.tb, *Mycobacterium tuberculosis*; TB, tuberculosis; LT, heat-labile enterotoxin; BCG, bacillus Calmette-Guérin; i.n., intranasal; DDA, dimethyldioctadecylammonium bromide; MPL, monophosphoryl lipid A.

been shown to induce not only a mucosal immune response but also a strong systemic response (21). Thus, an intranasally (i.n.) administered BCG-booster vaccine containing LTK63 may induce a local microbial-specific immune response that would block pathogens at the portal of entry, as well as a systemic response that would boost waning BCG-induced immunity.

Regarding the Ag part of a mucosal vaccine against TB, we recently showed that vaccination with a fusion protein consisting of Ag85B and ESAT-6 (Ag85B-ESAT-6) promoted a strong immune response, which was highly protective against TB in the mouse, guinea pig, and nonhuman primate models (22–26). However, Ag85B-ESAT-6 has never been examined as an i.n. administered vaccine.

The present study evaluated the LTK63/Ag85B-ESAT-6 vaccine administered i.n. We examined both its ability to prime a relevant response against TB and, perhaps more importantly, to boost prior BCG immunity and thereby increase the protection against infection with M.tb.

## Materials and Methods

### Animals

Studies were performed with 8- to 12-wk-old BALB/c × C57BL/6 F<sub>1</sub> female mice, purchased from Harlan. Infected animals were housed in cages contained within laminar flow safety enclosures in a BSL-3 facility. The use of mice was in accordance with both the regulations set forward by the Danish Ministry of Justice and Animal Protection Committees and in compliance with EC Directive 86/609 and the U.S. Association for the Assessment and Accreditation of Laboratory Animal Care recommendations for the care and use of laboratory animals.

### Bacteria

M.tb Erdman was grown at 37°C in suspension in Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose.

### Immunization

Mice were immunized three times at 2-wk intervals i.n. in a volume of 10 μl/nostril with experimental vaccines containing 25 μg of Ag85B-ESAT-6 and 10 μg of the adjuvant LTK63. The vaccine was mixed with LTK63 immediately before use. At the same time as the first subunit vaccination, control mice received a single dose of BCG Danish 1331 (5 × 10<sup>6</sup> CFU) injected s.c. at the base of the tail. Mice were challenged 10 wk after the first vaccination.

For boosting studies, mice received a single dose of BCG Danish 1331 (5 × 10<sup>6</sup> CFU) injected s.c. at the base of the tail and were subsequently rested for 4–8 mo. Thereafter, the mice were given two i.n. booster vaccinations with 25 μg of Ag85B-ESAT-6 in the adjuvant LTK63 at 2-wk intervals. Control groups were boosted with adjuvant alone, Ag alone, or not boosted.

### Experimental infections

The animals were challenged by the aerosol route with ~100 CFU of M.tb Erdman/mouse. The mice were sacrificed 6 wk after challenge. Numbers of bacteria in the liver, spleen, or lung were determined by serial 3-fold dilutions of individual whole-organ homogenates in duplicate on 7H11 medium. Organs from the BCG-vaccinated animals were grown on medium supplemented with 2 μg/ml 2-thiophene-carboxylic acid hydrazide to selectively inhibit the growth of the residual BCG bacteria in the test organs. Colonies were counted after 2–3 wk of incubation at 37°C.

### Lymphocyte cultures

Lymphocytes from spleens or lymph nodes (from individual mice) were obtained as described previously (22). Briefly, blood lymphocytes (PBMCs) were purified on a density gradient. Cells pooled from three to five mice in each experiment were cultured in microtiter wells (96-well plates; Nunc) containing 2 × 10<sup>5</sup> cells in a volume of 200 μl of RPMI 1640 supplemented with 5 × 10<sup>-5</sup> M 2-ME, 1% penicillin-streptomycin, 1 mM glutamine, and 5% (v/v) FCS. Based on previous dose-response investigations, the mycobacterial Ags were all used at 5 μg/ml, whereas Con A was used at a concentration of 1 μg/ml as a positive control for cell viability. All preparations were tested in cell cultures and found to be nontoxic at the concentrations used in the present study. Supernatants were harvested from cultures after 72 h of incubation for the investigation of IFN-γ.

### IFN-γ ELISA

Microtiter plates (96 wells; Maxisorb; Nunc) were coated with monoclonal hamster anti-murine IFN-γ (Genzyme) in PBS at 4°C. Free binding sites were blocked with 1% (wt/vol) BSA, 0.05% Tween 20. Culture supernatants were tested in triplicate, and IFN-γ was detected with a biotin-labeled rat anti-murine monoclonal anti-body (clone XMG1.2; BD Pharmingen). rIFN-γ (BD Pharmingen) was used as a standard.

### IFN-γ ELISPOT

The ELISPOT technique was performed as described before (22). Briefly, 96-well microtiter plates (Maxisorp; Nunc) were coated with 2.5 μg of monoclonal hamster anti-murine IFN-γ/well (Genzyme). Free binding sites were blocked with BSA followed by washing with PBS-0.05% Tween 20. Analyses were always conducted on cells pooled from three to five mice. Cells were stimulated with 5 μg of ESAT-6, Ag85B-ESAT-6, Ag85B, or the immunodominant CD4 epitope (Ag85B<sub>241–255</sub>) for 24–42 h directly in the ELISPOT plates. The cells were removed by washing, and the site of cytokine secretion was detected with a biotin-labeled rat anti-murine IFN-γ mAb (clone XMG1.2; BD Pharmingen) and phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories). The enzyme reaction was developed with 5-bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich). ELISPOT plates were counted using an AID plate reader (Autoimmun Diagnostika). The correlation between the number of cells per well and the number of spots was linear at concentrations of 2 × 10<sup>5</sup> to 2.5 × 10<sup>5</sup> cells/well. Wells with fewer than 10 spots were not used for calculations.

### FACS analysis of lymphocytes

Cells were isolated, as described above, from the blood and spleen of mice. A total of 2 × 10<sup>5</sup> cells was stimulated for 1 h with 2 μg/ml Ag and subsequently incubated for 6 h with 10 μg/ml brefeldin A (Sigma-Aldrich) and Ag. Thereafter, cells were incubated with Fc-block (BD Pharmingen), washed in FACS buffer, and stained for the indicated surface markers. Cells were then washed in PBS, permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen), and stained intracellularly with PE-labeled anti-IFN-γ mAb. After washing, cells were finally resuspended in PBS containing 0.1% sodium azide, and analyzed by FACS (BD Immunocytometry Systems).

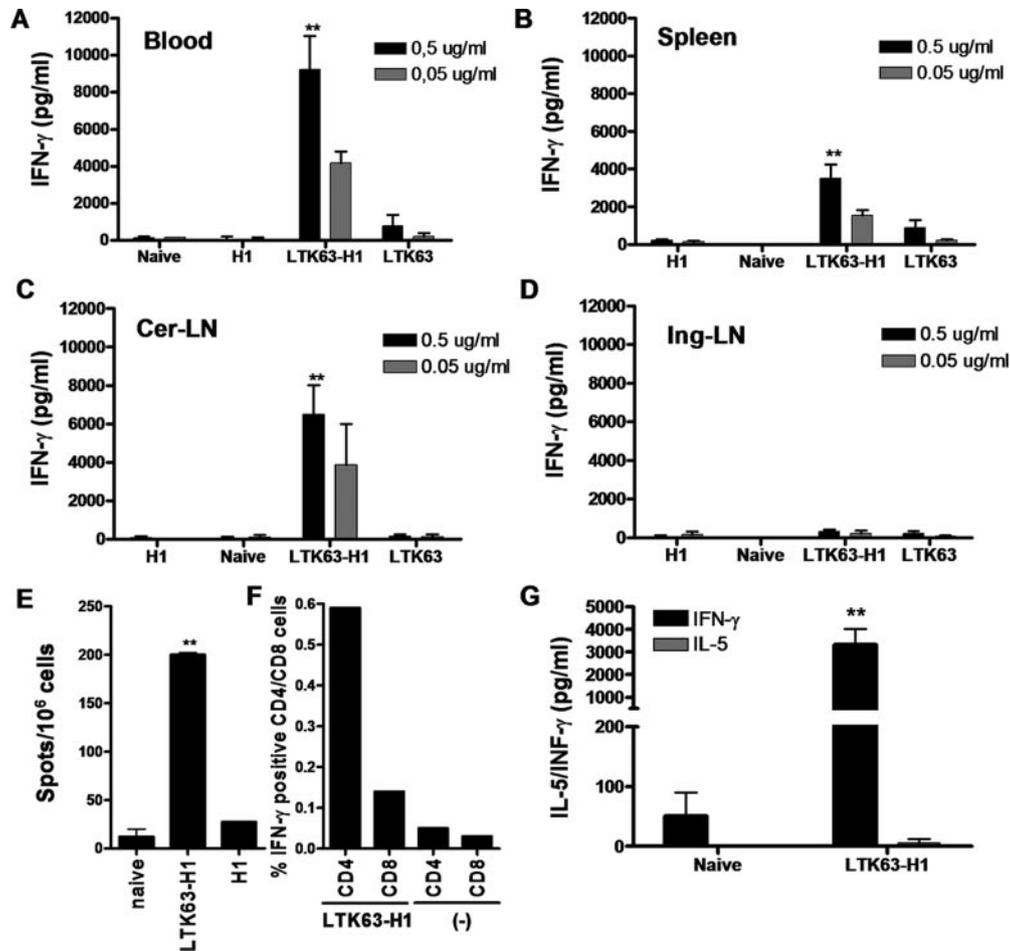
### Statistical methods

The data obtained were tested by ANOVA. Differences between means were assessed for statistical significance by Tukey's test. A value of *p* < 0.05 was considered significant.

## Results

### Immune responses following i.n. administration of Ag85B-ESAT-6

To study the recognition of Ag85B-ESAT-6 after mucosal administration of Ag85B-ESAT-6 emulsified in LTK63, the vaccine was given i.n. three times at 2-wk intervals, and the immune response was investigated by culturing splenocytes, blood cells, or cells isolated from the draining lymph nodes with different concentrations of the purified fusion protein. IFN-γ release, as assessed by ELISA, showed that Ag85B-ESAT-6 in LTK63 was highly immunogenic with strong responses in the blood and spleen (1500–4000 pg/ml IFN-γ at a concentration of 0.05 μg/ml Ag and 3500–9000 pg/ml IFN-γ at the highest Ag concentration (0.5 μg/ml)). In addition, we also observed a strong recognition (6500 pg/ml IFN-γ) by T cells isolated from the lymph nodes draining from the nasal cavities (supercervical/submandibular lymph nodes), whereas only a limited response was observed in non-draining lymph nodes (inguinal lymph nodes) (Fig. 1, A–D). The high secretion of IFN-γ, measured by ELISA, was paralleled by a substantial number of Ag-specific cells in the blood (>200/10<sup>6</sup> cells), spleen, or draining lymph nodes, as measured by in vitro ELISPOT of Ag85B-ESAT-6-stimulated cells (Fig. 1E and data not shown). To more precisely analyze the phenotype of the T cells induced by i.n. vaccination with LTK63/Ag85B-ESAT-6, splenic T cells were taken 2 wk after the final vaccination and Ag85B-ESAT-6-stimulated CD4 and CD8 T cells were analyzed for expression of IFN-γ



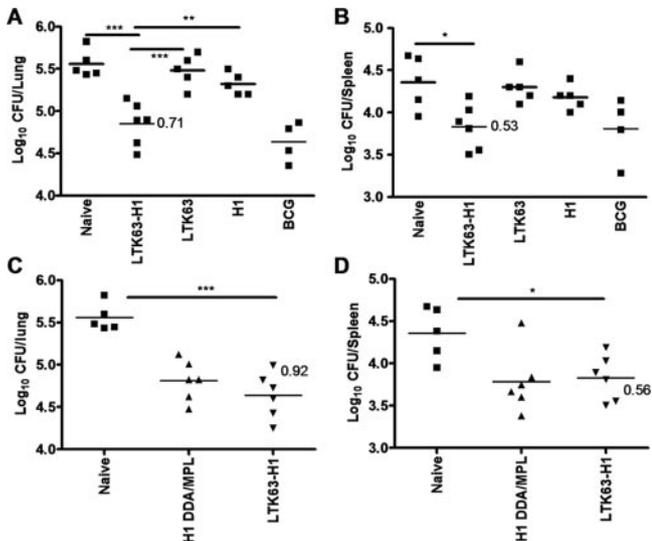
**FIGURE 1.** Recognition of Ag85B-ESAT-6 in LTK63/Ag85B-ESAT-6-vaccinated mice. *A–D*, In vitro IFN- $\gamma$  responses of cells from mice vaccinated i.n. three times with LTK63/Ag85B-ESAT-6, taken 2 wk after final vaccination from spleen, blood, cervical/submandibular lymph nodes (Cer-LN), or inguinal lymph nodes (Ing-LN) as indicated in the figure. Cells were stimulated with 0.5 or 0.05  $\mu\text{g/ml}$  Ag85B-ESAT-6 as indicated. *E*, In vitro ELISPOT analysis of Ag-stimulated splenic cells from naive mice, LTK63/Ag85B-ESAT-6 or Ag85B-ESAT-6 (H1)-vaccinated mice. *F*, FACS analysis of IFN- $\gamma$  expression by CD4/CD8 spleen cells from nonvaccinated mice or LTK63/Ag85B-ESAT-6-vaccinated mice. The figure shows percentage of IFN- $\gamma$ -positive CD4 or CD8 cells. *G*, In vitro IFN- $\gamma$  or IL-5 responses of cells from mice vaccinated i.n. three times with LTK63/Ag85B-ESAT-6. Groups significantly different from the naive groups are indicated (\*\*,  $p < 0.01$ , ANOVA and Tukey's test). Cells were pooled from five mice per group. Values represent the means and SEM of triplicate values. Results are representative of four independent experiments. H1 indicates Ag85B-ESAT-6.

by intracellular FACS analysis. Alternatively, the cells were analyzed for IL-5 secretion by ELISA. The results showed that the majority of Ag-specific IFN- $\gamma$ -producing cells were observed in the CD4 subset (0.59% of all CD4 T cells) (Fig. 1*F*). Moreover, because only a low secretion of IL-5 from Ag-specific T cells was observed, this indicated that vaccination with LTK63/Ag85B-ESAT-6 primarily induced a Th1 response, which involved IFN- $\gamma$ -secreting CD4 T cells (Fig. 1*G*).

#### Protective efficacy of LTK63/Ag85B-ESAT-6

Because Ag85B-ESAT-6 was highly immunogenic in combination with LTK63, we next analyzed the protective efficacy of this vaccine. In three independent experiments, mice were vaccinated with Ag85B-ESAT-6 in LTK63 or (as controls) with LTK63 or Ag85B-ESAT-6 alone. Because the vaccine BCG has consistently demonstrated good efficacy against TB infection in animal models (29), BCG was included in the experiment as a "gold standard" against which efficacy can be assessed (25). Six weeks after the last vaccination, the mice were subjected to an aerosol challenge with virulent M.tb. As expected, 1 wk after challenge with M.tb, a significant recall response was observed in the mice that had been vaccinated with LTK63/Ag85B-ESAT-6 compared with LTK63-

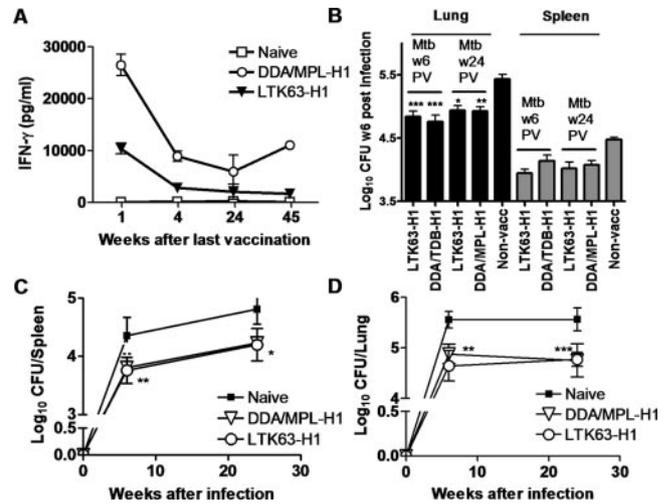
or Ag85B-ESAT-6-vaccinated mice (data not shown). Six weeks after the challenge, the mice were killed and the bacterial numbers were determined in the lungs and the spleen. The results showed that vaccination with LTK63/Ag85B-ESAT-6 lead to a bacterial (M.tb) burden of  $4.85 \pm 0.14 \log_{10}$  CFU in the lung, which was significantly lower than the bacterial burden observed in nonvaccinated mice ( $5.56 \pm 0.07 \log_{10}$  CFU;  $p < 0.001$ ). BCG-vaccinated mice showed a bacterial burden of  $4.636 \pm 0.117 \log_{10}$  CFU, which was not significantly different from the LTK63/Ag85B-ESAT-6-vaccinated mice. The same pattern was observed in the spleen (Fig. 2*B*), where only the groups having received the combination of LTK63 and Ag85B-ESAT-6 or BCG were significantly different from the naive group ( $p < 0.05$ ). We also compared the protective efficacy of an i.n. administration of LTK63/Ag85B-ESAT-6 with Ag85B-ESAT-6 administered s.c. in the strong Th1 adjuvant dimethyldioctadecylammonium bromide (DDA)/monophosphoryl lipid A (MPL), known to induce significant protection against TB (22). As seen above with BCG vaccination, LTK63/Ag85B-ESAT-6 was as efficient ( $4.64 \pm 0.11 \log_{10}$  CFU in the lung) as DDA/MPL/Ag85B-ESAT-6 ( $4.81 \pm 0.10 \log_{10}$  CFU in the lung) in inducing protection against infection with M.tb (Fig. 2, *C* and *D*).



**FIGURE 2.** Vaccination with LTK63/Ag85B-ESAT-6 induces protection against infection with *M.tb.* Bacterial burden in vaccinated mice (expressed as  $\log_{10}$  in CFU) compared with unvaccinated controls challenged by the aerosol route with virulent *M.tb.* 10 wk after the first vaccination. Six weeks postchallenge, the mice were killed and the bacterial burden (CFU) was measured in the lung (A and C) and in the spleen (B and D). The reduction in bacterial numbers in LTK63/Ag85B-ESAT-6-vaccinated mice, compared with naive mice, is indicated. (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ , ANOVA and Tukey's test). H1 indicates Ag85B-ESAT-6.

#### Vaccination with LTK63/Ag85B-ESAT-6 induces a protective memory response

Because a prerequisite of any successful vaccination is the ability to induce a memory immune response that can mediate long-lasting protection, it was important to analyze whether the immune response induced by the vaccine was a protective memory response, giving long-lasting protection. Thus, two experiments were performed. Mice were analyzed and infected 24 wk after the final vaccination (instead of 6 wk after), and bacterial numbers were determined at wk 6 postinfection. Alternatively, mice were infected 6 wk after the first vaccination, and bacterial numbers were determined in the lungs and the spleen 24 wk after infection (instead of 6 wk after). Again, mucosal administration of LTK63/Ag85B-ESAT-6 was compared with s.c. administration of DDA/MPL/Ag85B-ESAT-6, which was previously shown to induce a strong memory response. Twenty-four and 45 wk after the final vaccination, Ag85B-ESAT-6 still induced considerable secretion of IFN- $\gamma$  (~2000 pg/ml) (Fig. 3A), indicating that the response induced by LTK63/Ag85B-ESAT-6 was more than transient. Importantly, the immunity observed at wk 24, was able to mediate protection against infection with *M.tb.* that was not significantly different from that observed in vaccinated mice infected at wk 6 after the first vaccination (Fig. 3B). Again, the protection was comparable to that observed with DDA/MPL/Ag85B-ESAT-6 even though DDA/MPL induced a stronger systemic response at all the examined time points (Fig. 3A). To analyze whether the induced protection was long-lasting, bacterial numbers were examined in lung and spleen 24 wk postinfection. The results showed that, at wk 24 postinfection, the protection induced by LTK63/Ag85B-ESAT-6 (or DDA/MPL/Ag85B-ESAT-6) had not decreased (1.01  $\log_{10}$  CFU reduction in lungs from vaccinated mice compared with nonvaccinated mice) compared with the protection observed 6 wk postinfection (Fig. 3, C and D). Taken together, these data show that vaccinating with LTK63/Ag85B-ESAT-6 induced a long-lasting Th1 response, with the ability to confer significant protection



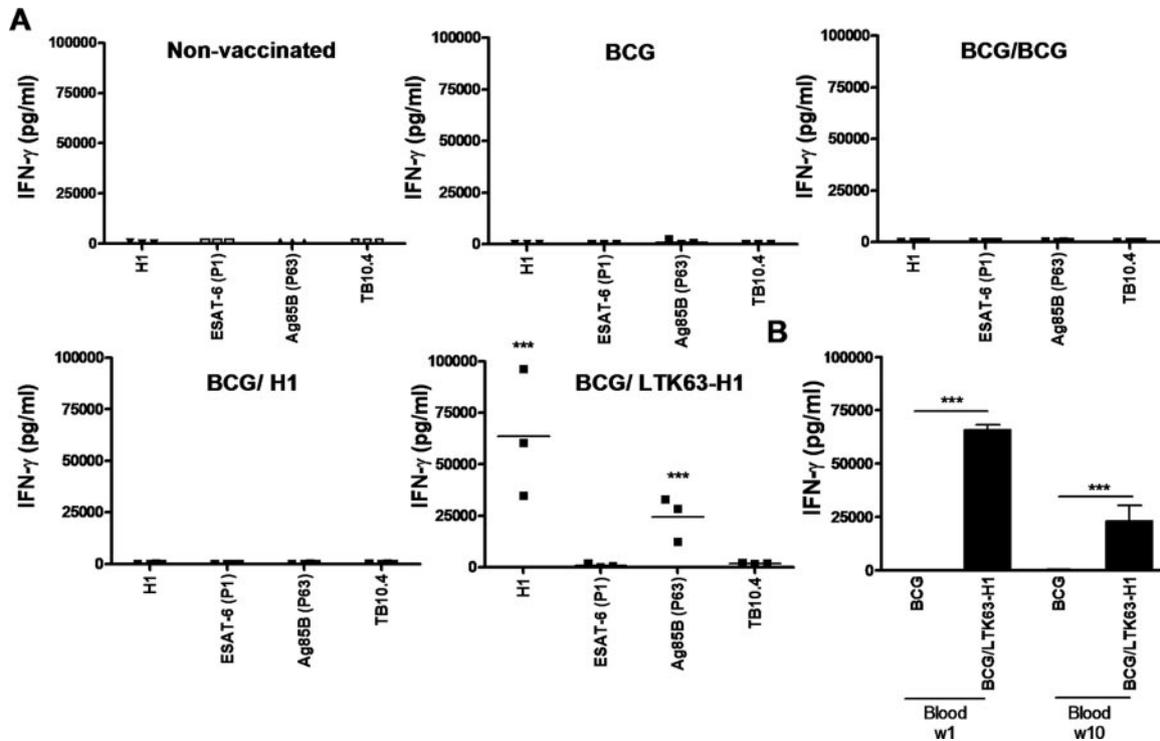
**FIGURE 3.** Vaccination with LTK63/Ag85B-ESAT-6 leads to a long-lasting protection against *M.tb.* A, In vitro IFN- $\gamma$  responses of cells from mice vaccinated i.n. three times with LTK63/Ag85B-ESAT-6, taken 1–45 wk after final vaccination from blood and stimulated with Ag85B-ESAT-6. B, Bacterial burden in vaccinated mice (expressed as  $\log_{10}$  in CFU) compared with unvaccinated controls challenged by the aerosol route with virulent *M.tb.* 6 or 24 wk (Mtb w24 PV) after the final vaccination. Six weeks postchallenge, the mice were killed and the bacterial burden (CFU) was measured in the lung. C and D, Alternatively, mice were challenged with virulent *M.tb.* 6 wk after the final vaccination and bacterial burden (CFU) was measured in the lung (C) and in the spleen (D) 6 and 24 wk postchallenge. The reduction in bacterial numbers in LTK63/Ag85B-ESAT-6-vaccinated mice, compared with naive mice, is indicated. (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ , ANOVA and Tukey's test). H1 indicates Ag85B-ESAT-6.

against infection with *M.tb.* comparable to that seen with Ag85B-ESAT-6 in the strong Th1 adjuvant DDA/MPL.

#### Boosting prior BCG vaccination with LTK63/Ag85B-ESAT-6

An important requirement for a vaccine against TB is the ability to boost prior BCG immunity. To test whether an i.n. administration of LTK63/Ag85B-ESAT-6 would be able to boost BCG immunity, mice were vaccinated with BCG, in three independent experiments, and rested for 8 mo. Thereafter, the mice were given two booster vaccinations at 2-wk intervals with LTK63/Ag85B-ESAT-6 or (as controls) the components individually or BCG. To study the booster effect of the vaccines, we analyzed the recognition of the Ag85B-ESAT-6 fusion protein, Ag85B (shared by the booster vaccine and BCG), ESAT-6 (only in the booster), and TB10.4 (only in BCG). This analysis was done 1 wk after the first booster vaccination, on cells isolated from spleen and blood. In the spleen, a low response to all the Ags tested was observed in BCG-vaccinated (nonboosted) mice. Boosting BCG with BCG or Ag85B-ESAT-6 without adjuvant, did not significantly increase the recognition of the Ags (Fig. 4A), and neither did boosting with LTK63 alone (data not shown). However, boosting with LTK63/Ag85B-ESAT-6 led to a high response against Ag85B-ESAT-6 1 wk after the vaccination ( $63,700 \pm 17,810$  pg/ml IFN- $\gamma$ ) (Fig. 4A). As expected, it was primarily a response directed against the immunodominant epitope in Ag85B (Ag85B<sub>241–255</sub>), shared between BCG and the vaccine, that was boosted ( $24,470 \pm 6,301$  pg/ml IFN- $\gamma$ ), whereas only marginal responses to ESAT-6 were seen at this early time point (Fig. 4A).

The same pattern was observed in the blood and in the lymph nodes draining the nasal cavities (Fig. 4B and data not shown). Thus, in the blood, boosting with LTK63/Ag85B-ESAT-6 led to a high response against Ag85B-ESAT-6 ( $65,989 \pm 4,164$  pg/ml



**FIGURE 4.** Vaccination with LTK63/Ag85B-ESAT-6 boosts BCG-generated immunity. *A*, Following a rest period of 8 mo, BCG-vaccinated mice received a booster vaccination as indicated in the figure. One week after the booster vaccination, cells from spleen (*A*) or blood (*B*) were stimulated in vitro with the indicated Ags and analyzed for secretion of IFN- $\gamma$  by ELISA. In addition, 10 wk after the first booster vaccination blood cells from three individual mice were stimulated in vitro with the indicated Ags and analyzed for secretion of IFN- $\gamma$  by ELISA. Groups significantly different from the BCG-vaccinated group are indicated (\*\*\*,  $p < 0.001$ ; \*,  $p < 0.05$ , ANOVA and Tukey's test). H1 indicates Ag85B-ESAT-6.

IFN- $\gamma$ ) and Ag85B (18,316 pg/ml  $\pm$  4,116 IFN- $\gamma$ ). Ten weeks after the first booster vaccination, the response was still maintained at a high level, as shown by the Ag85B-ESAT-6-specific response in the blood (23,380  $\pm$  7,022 pg/ml IFN- $\gamma$ ), indicating that the T cell response generated by booster vaccinations was more than a transient response (Fig. 4*B*). To analyze whether the increased release of IFN- $\gamma$  was due to an increase in the number of Ag-specific T cells, we examined the effect of the booster vaccination on the number of Ag-specific T cells by in vitro ELISPOT after Ag (Ag85B<sub>241-255</sub>) stimulation of isolated splenocytes or blood lymphocytes. The result showed that mice immunized with LTK63/Ag85B-ESAT-6 exhibited a highly accelerated increase in Ag85B-specific splenic (618  $\pm$  78 per 10<sup>6</sup> cells) or blood T cells (394  $\pm$  18 per 10<sup>6</sup> cells), whereas boosting BCG with BCG, Ag85B-ESAT-6, or LTK63 alone did not lead to an increase in specific T cells (Fig. 5, *A* and *B*). Vaccinating naive mice with LTK63/Ag85B-ESAT-6 did not lead to a significant increase in the number of Ag85B<sub>241-255</sub>-specific T cells 1 wk after the vaccination (Fig. 5, *A* and *B*). To analyze the nature of the boosted cells, and whether the boosted immune response was primarily a Th1 response, splenic T cells were analyzed 1 wk after the first booster vaccination with LTK63/Ag85B-ESAT-6 for IL-5 secretion (by ELISA) following stimulation with Ag85B-ESAT-6, whereas the number of splenic Ag-specific IL-4-secreting T cells were analyzed by in vitro ELISPOT after Ag stimulation. LTK63/Ag85B-ESAT-6 induced only little secretion of IL5 (703  $\pm$  109 pg/ml) from Ag-specific cells, and a low number of Ag-specific, IL-4-producing cells (25  $\pm$  10/10<sup>6</sup> cells) (data not shown). This indicated that vaccination with LTK63/Ag85B-ESAT-6 primarily boosted a Th1 response, involving IFN- $\gamma$ -secreting cells. To examine whether these IFN- $\gamma$ -secreting cells were found within the CD4 or CD8 T cell subset, cells were evaluated by intracellular

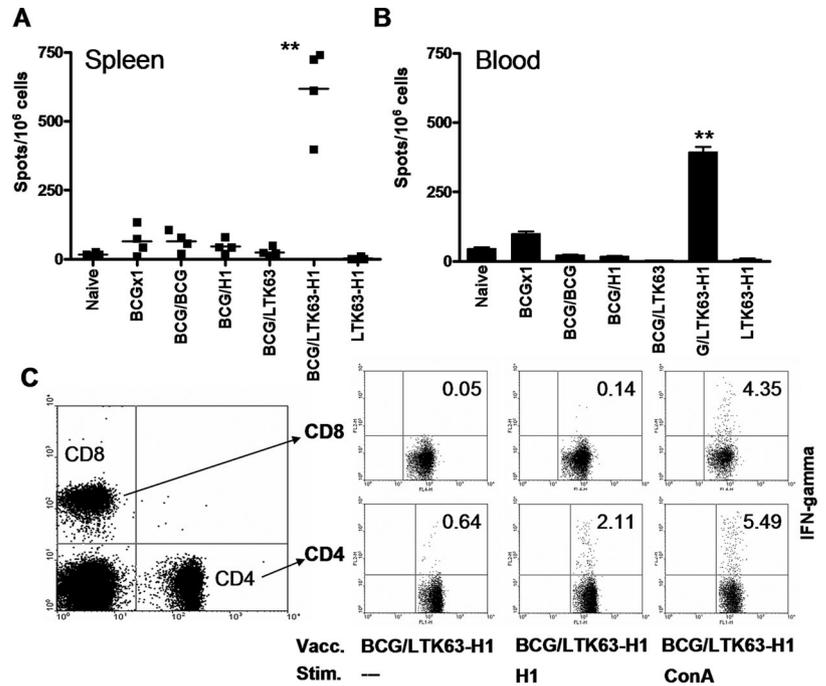
cytokine FACS analysis. The results clearly showed that the LTK63/Ag85B-ESAT-6 booster vaccine expanded Ag85B-ESAT-6-specific T cells primarily within the CD4 subset of T cells. One week after the first booster vaccination, a 1.47% (2.11–0.64%) increase in the number of Ag-specific CD4 cells was observed, whereas an increase of 0.09% was found among the CD8-positive cells (Fig. 5*C*).

Taken together, the results demonstrated that, in contrast to BCG, Ag85B-ESAT-6, or LTK63 alone, the combination of LTK63 and Ag85B-ESAT-6 efficiently boosted prior BCG-induced immunity. Furthermore, the T cell response involved was not merely a transient response and consisted primarily of IFN- $\gamma$ -secreting CD4 Th1 cells.

#### Recruitment of Ag-specific T cells to the site of infection

Because vaccination with LTK63/Ag85B-ESAT-6 was able to boost prior BCG-induced immunity, we next analyzed whether the boosted cells were indeed recruited to the site of infection after an aerosol infection with M.tb. Mice were boosted as described above, and 10 wk later, the mice were challenged by the aerosol route with virulent M.tb and the number of Ag85B-ESAT-6-specific cells in perfused lungs was analyzed by in vitro ELISPOT. One week after challenge with M.tb, an accelerated recruitment and expansion of specific T cells was found in all vaccinated groups compared with the nonvaccinated control group. In the BCG-vaccinated group, 590  $\pm$  151 spots/10<sup>6</sup> cells were isolated, which was equivalent to a  $\sim$ 10-fold increase compared with the nonvaccinated control group. The magnitude of the recall response was not potentiated further in the groups boosted with a second BCG vaccination administered i.n. (Fig. 6*A*). The group boosted with LTK63/Ag85B-ESAT-6, exhibited an impressive recall response with a very significant increase in Ag-specific lung T cells

**FIGURE 5.** The number of Ag-specific cells following a booster vaccination with LTK63/Ag85B-ESAT-6. Following a rest period of 8 mo, BCG-vaccinated mice received a booster vaccination as indicated in the figure. One week after the booster vaccination, cells from spleen (A) or blood (B) were stimulated in vitro with the indicated Ags and the number of IFN- $\gamma$ -secreting cells were analyzed by ELISPOT. C, FACS analysis of IFN- $\gamma$  expression by CD4/CD8 spleen cells from BCG-vaccinated mice boosted with LTK63/Ag85B-ESAT-6. The cells were stimulated with Ag85B-ESAT-6 (H1) as indicated. Con A was used as a positive control. The figure shows percentage of IFN- $\gamma$ -positive CD4 or CD8 cells. H1 indicates Ag85B-ESAT-6. Groups significantly different from the BCG-vaccinated group are indicated (\*\*,  $p < 0.01$ , ANOVA and Tukey's test).



( $1118 \pm 85$  spots/ $10^6$  cells) compared with the BCG group, and a 17-fold increase compared with the frequency of specific T cells in the lungs before challenge ( $67.33 \pm 15.04$ ). Comparing the number of specific T cells in the spleen and lungs preinfection and postinfection clearly showed that an enhanced recruitment and expansion was observed only in the lungs (Figs. 5A and 6B). As an important control for the nonspecific effect of boosting BCG immunity with LTK63/Ag85B-ESAT-6, we also analyzed the number of TB10.4-specific T cells. TB10.4 is expressed by BCG (and M.tb), but is not part of Ag85B-ESAT-6, and should therefore not be affected by the booster vaccinations. As expected, after infection, BCG-vaccinated mice showed an increased number of TB10.4-specific cells in the lung ( $400 \pm 104$  spots/ $10^6$  cells) compared with BCG-vaccinated mice preinfection ( $34 \pm 7$  spots/ $10^6$  cells). However, this was not increased further by vaccinating the mice with LTK63/Ag85B-ESAT-6 (Fig. 6A). As observed for Ag85B-specific T cells, boosting BCG-vaccinated mice with BCG did not lead to an increase in TB10.4-specific T cells, further demonstrating the inability of BCG to act as a booster vaccine of prior BCG-induced immunity (Fig. 6A). Finally, in agreement with our observation that vaccination with LTK63/Ag85B-ESAT-6 primarily boosts a Th1 response, no significant increase in the number of lung Ag85B-ESAT-6-specific IL-4-secreting cells was observed following infection with M.tb (data not shown). Thus, following a challenge by the aerosol route with virulent M.tb, IFN- $\gamma$ -secreting Th1 CD4 cells, boosted by vaccination with LTK63/Ag85B-ESAT-6, were recruited to, or increased at, the site of infection.

#### Protection against infection with M.tb in BCG-vaccinated animals boosted with LTK63/Ag85B-ESAT-6

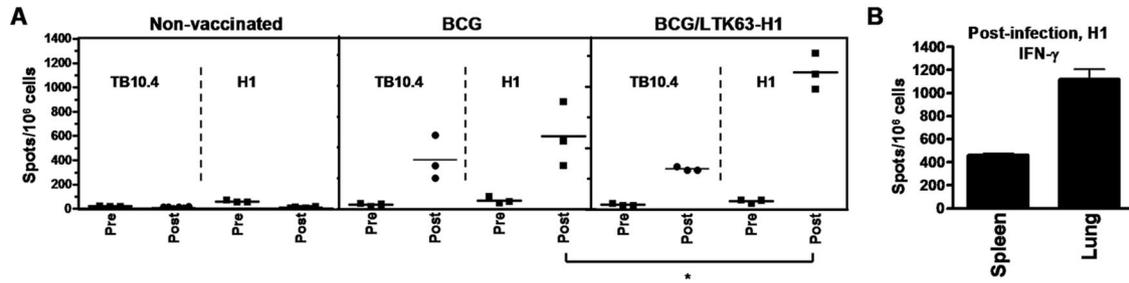
Because LTK63/Ag85B-ESAT-6 efficiently boosted prior BCG immunity, leading to an increased number of Ag85B-ESAT-6-specific cells in the lung following infection with M.tb, we finally examined whether this also led to an increased protection against TB. Mice were challenged by the aerosol route with virulent M.tb 10 wk after the first booster vaccination and killed 6 wk postchallenge. The bacterial numbers were determined in the lungs and the spleen. As observed with the immune responses, the level of protection induced by BCG was not improved further by a second

BCG vaccination. In contrast, the BCG-vaccinated mice that had received two booster vaccinations with LTK63/Ag85B-ESAT-6 showed a bacterial burden of M.tb of  $4.48 \pm 0.1156$  log<sub>10</sub> CFU, which constituted a significant decrease of 0.59 log<sub>10</sub> CFU compared with the bacterial burden observed in BCG-vaccinated mice ( $5.068 \pm 0.10$  log<sub>10</sub> CFU) ( $p < 0.01$ ). Boosting BCG with Ag85B-ESAT-6 or with LTK63 alone did not significantly improve the protective effect of BCG priming (Fig. 7A). The same pattern was observed in the spleen. There, the LTK63/Ag85B-ESAT-6-boosted mice showed a significantly increased protection ( $0.82$  log<sub>10</sub> CFU, compared with BCG-vaccinated mice;  $p < 0.01$ ) (Fig. 7B). In another independent, but similar, experiment, we observed a 0.64 log<sub>10</sub> CFU decrease in bacterial burden in the lung of LTK63/Ag85B-ESAT-6-boosted mice compared with BCG-vaccinated mice (data not shown). Taken together, these results showed that LTK63/Ag85B-ESAT-6 can boost prior BCG immunity and lead to an increased recruitment of Ag-specific T cells to the lung, which in turn provides efficient protection against infection with M.tb.

#### Discussion

Several studies have indicated that i.n. administration may be an efficient route of administration for a vaccine against TB. Thus, mucosal immunization with a novel recombinant adenoviral based vaccine expressing the M.tb immunodominant protein, Ag85A, provided efficient protection against infection with M.tb (1), and mice vaccinated via the i.n. route with culture filtrate proteins formulated in lipophilic quaternary ammonium salt, DDA, were found to be protected against experimental TB, particularly in the lung (2). Intranasal application of the BCG strain was also found to be highly protective against challenge infection with M.tb (3–6).

In the present study, we evaluated the combination of LTK63 and Ag85B-ESAT-6 administered i.n. as a vaccine against TB. Initially, we found that immunization with LTK63/Ag85B-ESAT-6 induced a strong T cell response against Ag85B-ESAT-6. This was observed in the spleen, blood, and lymph nodes draining from the nasal cavities (Fig. 1). In contrast, a s.c. immunization with Ag85B-ESAT-6 in the adjuvant DDA/MPL did not induce any significant response in the cervical lymph nodes (data not



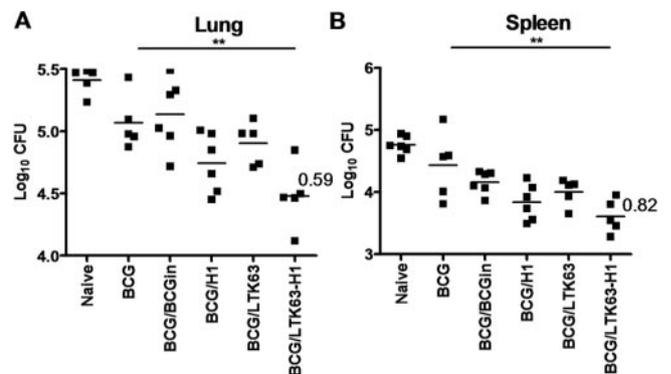
**FIGURE 6.** Recruitment of Ag-specific cells to the lung 1 wk postinfection. *A*, Mice were challenged by the aerosol route with virulent *M.tb* (post), and 1 wk after the challenge three mice were killed in each group and the number of Ag85B-ESAT-6 (H1) or TB10.4-specific cells in the lungs was analyzed by *in vitro* IFN- $\gamma$  ELISPOT and compared with the number found preinfection (pre). *B*, Comparison of the number of Ag85B-ESAT-6-specific IFN- $\gamma$ -secreting cells in lung and spleen 1 wk postinfection. (\*,  $p < 0.05$ , ANOVA and Tukey's test). H1 indicates Ag85B-ESAT-6.

shown). This indicated that *i.n.* vaccination with LTK63/Ag85B-ESAT-6 induced both a local and a systemic response. The T cell response was dominated by IFN- $\gamma$ -secreting T cells (Fig. 1*E*), the importance of which for the protection against TB has been shown in several studies (30, 31). In agreement with our results, *i.n.* administration of culture filtrate proteins formulated in DDA also gave a strong IFN- $\gamma$  response in the cervical lymph nodes (local) as well as in the mesenteric lymph nodes (systemic response). However, in the latter study, a significant Th2 response was also observed, whereas we only observed a minor Th2 response, measured by IL-5 secretion (Fig. 1*G*) or IL-4 ELISPOT (data not shown). This is probably due to the different adjuvants used in these studies, although LTK63 in some settings has been shown to induce both a Th1 and Th2 response (32). When testing the protective efficacy of LTK63/Ag85B-ESAT-6, we found that it induced a protection that was not significantly different from that of BCG, or the strong Th1 adjuvant DDA/MPL (Fig. 2). In four independent experiments, we observed protective effect of between 0.6 and 0.9  $\log_{10}$  CFU reduction in the lung. Importantly, we also found that the immune response induced by LTK63/Ag85B-ESAT-6 was sustained, and that the protection induced by the vaccine was long-lasting (Fig. 3). Interestingly, even though the protection was as good as that seen with DDA/MPL, the immune response induced by LTK63/Ag85B-ESAT-6 was considerable lower than that induced by DDA/MPL/Ag85B-ESAT-6 (Fig. 3*A*). This indicates that the quality of the T cell response induced by LTK63/Ag85B-ESAT-6, administered *i.n.* may be more relevant for the protection against *M.tb*. We are presently analyzing phenotypic differences between the T cells induced by Ag85B-ESAT-6 in LTK63 or DDA/MPL.

Because BCG is the most widely used vaccine in the world, it is perhaps more important to generate a vaccine that can boost waning BCG immunity. Vaccinating mice that had previously received BCG showed that LTK63/Ag85B-ESAT-6 effectively boosted BCG immunity. In contrast, Ag85B-ESAT-6, LTK63, or BCG did not boost prior BCG immunity (Fig. 4*A* and data not shown), the latter in agreement with several studies showing that BCG is not a suitable booster vaccine in individuals presensitized with environmental bacteria or BCG (27, 28, 33, 34). The boosting effect induced by LTK63/Ag85B-ESAT-6 was due to the Ag shared by BCG and the vaccine, Ag85B, as shown when stimulating the T cells with a peptide containing an immunodominant Ag85B epitope (Ag85B<sub>241-255</sub>) (Fig. 4). One week after the first booster vaccination, we observed a strong IFN- $\gamma$  response against Ag85B in the blood, spleen, and draining lymph nodes, as well as a strong increase in the number of Ag-specific T cells (Fig. 4). The highest response (<70,000 pg/ml IFN- $\gamma$ ) was observed in blood and spleen, indicating the ability of a mucosal administration of

LTK63/Ag85B-ESAT-6 to induce a systemic response (as well as a local response) (Fig. 4). In addition, this showed that the T cells induced by the booster immunization were of the same (or overlapping) specificity as those induced by the prior BCG vaccination. In BCG-vaccinated animals, the boosted response was clearly dominated by IFN- $\gamma$ -secreting CD4 cells (although we cannot exclude that more sensitive methods such as tetramer staining, or stimulation with specific epitopes, could identify CD8 cells not observed following stimulation with the fusion protein). Only low-level secretion of IL-4 or IL-5 was seen, indicating that LTK63/Ag85B-ESAT-6 boosted the same response as that observed when vaccinating naive mice, a response that was dominated by IFN- $\gamma$ -secreting CD4 cells (Fig. 5*C*), which amounted to 1.47% (2.11–0.64%) of all CD4 cells.

Compared with the spleen (and blood; data not shown), a strong increase in the number of Ag-specific T cells was seen 1 wk following challenge only in the lungs, indicating that the T cells boosted by the booster vaccinations were indeed expanded and/or recruited to the lungs after an aerosol infection with *M.tb* (Fig. 6). Although TB10.4-specific T cells were also recruited to the lungs, the number of recruited TB10.4-specific T cells was the same in boosted and nonboosted animals, showing that the booster vaccine only boosted the BCG-induced Ag85B-specific T cells. Because the boosted T cells were selectively recruited to the site of infection, this indicated that BCG, LTK63/Ag85B-ESAT-6, and *M.tb* induce Ag85B-specific T cells with overlapping specificities,



**FIGURE 7.** Boosting BCG with LTK63/Ag85B-ESAT-6 increases the protection against infection with *M.tb*. Bacterial burden in BCG-vaccinated mice boosted as indicated in the figure. Six weeks postchallenge, the mice were killed and the bacterial burden (expressed as  $\log_{10}$  CFU) was measured in the lung (*A*) and in the spleen (*B*). The reduction in bacterial numbers in mice boosted with LTK63/Ag85B-ESAT-6 vaccinated, compared with BCG (nonboosted) mice, is indicated (\*\*,  $p < 0.01$ , ANOVA and Tukey's test). H1 indicates Ag85B-ESAT-6.

which is of course required for the BCG boosting approach to function as intended. As observed after LTK63/Ag85B-ESAT-6 immunization of naive or BCG-vaccinated mice, the postchallenge recall response was dominated by IFN- $\gamma$ -secreting cells and only a small IL-4 response was observed. No increase in recruited cells (of Ag85B or TB10.4 specificity) to the lung was observed in the mice that had received booster vaccinations with BCG, again showing that BCG cannot be used as a BCG booster vaccine (Fig. 6). Importantly, these cytokine responses were clearly reflected in the bacterial burden in the lungs or spleen 6 wk postchallenge, which showed that boosting BCG with LTK63/Ag85B-ESAT-6 significantly decreased the bacterial burden in both lung and spleen, and thus increased the protection against TB. Compared with boosting with Ag85B-ESAT-6 or LTK63 alone (or with BCG), only the group having received booster vaccinations with the combination of Ag85B-ESAT-6 and LTK63 showed a significantly lower bacterial burden in lungs/spleen than BCG-vaccinated/nonboosted mice (Fig. 7).

In conclusion, this is the first study to show that the combination of LTK63 and the subunit fusion protein Ag85B-ESAT-6 constitutes an efficient vaccine against TB to be used as a mucosal prophylactic vaccine or a booster vaccine of prior BCG immunity. As a highly efficient mucosally administered vaccine against infection with M.tb, LTK63/Ag85B-ESAT-6 has now entered a phase I clinical study.

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## Disclosures

The authors have no financial conflict of interest.

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