The multistage vaccine H56 boosts the effects of BCG to protect cynomolgus macaques against active tuberculosis and reactivation of latent *Mycobacterium tuberculosis* infection

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It is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis*. Infection typically remains latent, but it can reactivate to cause clinical disease. The only vaccine, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), is largely ineffective, and ways to enhance its efficacy are being developed. Of note, the candidate booster vaccines currently under clinical development have been designed to improve BCG efficacy but not prevent reactivation of latent infection. Here, we demonstrate that administering a multistage vaccine that we term H56 in the adjuvant IC31 as a boost to vaccination with BCG delays and reduces clinical disease in cynomolgus macaques challenged with *M. tuberculosis* and prevents reactivation of latent infection. H56 contains Ag85B and ESAT-6, which are two of the *M. tuberculosis* antigens secreted in the acute phase of infection, and the nutrient stress–induced antigen Rv2660c. Boosting with H56/IC31 resulted in efficient containment of *M. tuberculosis* infection and reduced rates of clinical disease, as measured by clinical parameters, inflammatory markers, and improved survival of the animals compared with BCG alone. Boosted animals showed reduced pulmonary pathology and extrapulmonary dissemination, and protection correlated with a strong recall response against ESAT-6 and Rv2660c. Importantly, BCG/H56-vaccinated monkeys did not reactivate latent infection after treatment with anti-TNF antibody. Our results indicate that H56/IC31 boosting is able to control late-stage infection with *M. tuberculosis* and contain latent tuberculosis, providing a rationale for the clinical development of H56.

Introduction

Novel tuberculosis (TB) vaccines in clinical trial are all designed as prophylactic vaccines and are, in most cases, based upon antigens recognized by the immune system during the early stage of infection (1). In animal models these vaccines do not prevent *Mycobacterium tuberculosis* infection but result in control of bacterial growth at a lower level than in unvaccinated animals, although eventually some or all the animals develop clinical disease (2). In humans, vaccination with bacillus Calmette-Guérin (BCG) can limit disease spread in children but not the establishment of *M. tuberculosis* infection, as demonstrated by the immense numbers of latently infected individuals and those with active TB, despite widespread BCG use. Latent infection can reactivate to cause clinical disease, which is exacerbated by HIV infection or other forms of immune suppression (3, 4). Among the candidates in the first generation of new TB vaccines is a fusion of the antigens Ag85B (Rv1886) and ESAT-6 (Rv3875) (H1), both secreted in the acute phase of infection, with an extensive track record as a vaccine (5–10). H1 was designed to improve the efficacy of BCG but was not designed to prevent reactivation from latent infection.

The ability to establish a lifelong, persistent infection is a characteristic feature of *M. tuberculosis*. As the bacillus adapts to conditions in the host, a significant part of the bacterial population is believed to transform from metabolically active to a state of nonreplicating persistence with low metabolic activity and a fundamentally changed gene expression profile (11, 12). Thus, in latent TB, it is likely that some of the bacteria exist in a different state compared with active disease, and preventing reactivation may require targeting several different bacterial antigens expressed at various metabolic states. To enhance long-term efficacy, our strategy is to develop a multistage vaccine that combines classical preventive vaccine target antigens and key latency-associated antigens highly expressed as the bacteria adapt to survive in the host (13).

We recently performed a comprehensive evaluation of latency-associated antigens in a large, multi-partner consortium. Among more than 100 different molecules, we selected the nutrient stress–induced antigen Rv2660c as a partner for Ag85B and ESAT-6 in the multistage subunit vaccine H56 (14). Rv2660c expression is

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Conflict of interest: Peter Andersen is named coinventor on a patent application covering the use of H56 as a vaccine. All rights have been assigned to Statens Serum Institut, a not-for-profit Danish governmental institute.

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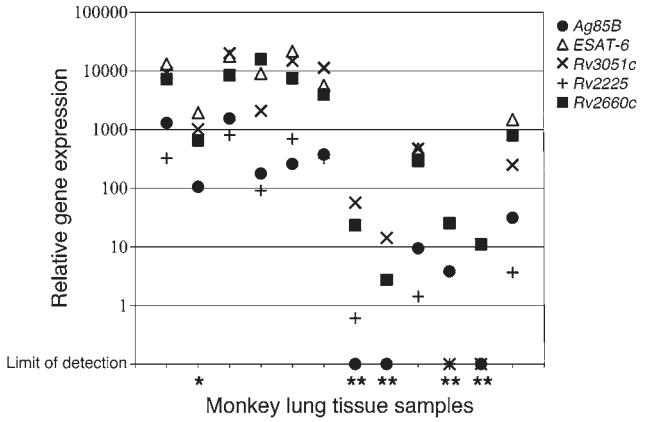

Figure 1
Expression of *M. tuberculosis* genes in infected monkeys. Relative *M. tuberculosis* gene expression was measured by qRT-PCR in lung samples from infected monkeys. Data are presented as relative gene expression. Those data points not shown were eliminated due to high background from presumed DNA contamination for the genes indicated, **Rv2225* and ***ESAT-6*.

increased 100- to 300-fold in non-replicating, nutrient-starved cultures, which makes this gene the most strongly upregulated of all nutrient starvation–induced genes identified (15). Hypoxia is a key feature of caseous granulomas (16), which are present in active and latent *M. tuberculosis* infection, and the set of genes induced under extended low-oxygen conditions, termed the enduring hypoxia response, also includes Rv2660c (17, 18). Moreover, immunologic responses to Rv2660c during latent infection was identified in two different African populations (19). In mice, H56 subunit vaccine was demonstrated to effectively contain late-stage infection and reduce bacterial burden more efficiently than BCG at week 24 after infection. It furthermore prevented recrudescence of TB in mice when given after exposure and thus shows great potential as both a preventive and post-exposure vaccine (14). Here, we tested this vaccine in non-human primate models, which are important in evaluating immunogenicity, safety, and efficacy of new vaccine candidates due to their close phylogenetic relationship with humans. Cynomolgus macaques are susceptible to *M. tuberculosis*, and the outcome of infection clinically and pathologically resembles that of human infection (20–23). We tested H56 in both high- and low-dose challenge models, as a boost to BCG. The adjuvant IC31 (Intercell) was chosen, as it been shown to promote a strong Th1 response and was recently demonstrated to have an excellent safety profile in the first TB clinical trial (24). Our data support that this regimen results in efficient containment of subclinical *M. tuberculosis* infection, preventing clinical disease as well as reactivation after treatment with anti-TNF antibody.

Results

Quantitative analysis of Ag85B, ESAT-6, and Rv2660c gene expression in M. tuberculosis–infected monkeys

Expression of the antigens contained in the H56 vaccine was measured by quantitative RT-PCR (qRT-PCR) in 12 lung (granuloma) samples from 3 monkeys following BCG vaccination and subsequent low-dose challenge with *M. tuberculosis*. The BCG-vaccinated monkeys were those that developed active disease (see below) and were infected for 4.5–8 months prior to necropsy. These monkeys were used because bacterial numbers in the lesions were sufficient for analysis, ranging from 10^3 to 10^7 CFU/g granuloma tissue. For comparison, we measured expression of *M. tuberculosis* genes *Rv3051c* and *Rv2225*, previously found to be expressed at high and low levels, respectively, in chronically infected rabbits (K.M. Guinn and D.R. Sherman, unpublished observations). Consistent with previous studies in mice and rabbits (14, 25), *Rv2660c* showed high relative expression in each sample (Figure 1). *ESAT-6* was also expressed, at levels similar to *Rv2660c*. In contrast, *Ag85B* transcript was detected at relatively low levels at these time points (10–100 times below the level of *Rv2660c*), and in 3 samples was below the limit of detection (Figure 1). These data indicate that *Rv2660c* and *ESAT-6* were expressed at high levels in *M. tuberculosis* bacilli from primate granulomas up to 8 months after infection.

Experimental design and vaccine safety

Two experiments were performed with the aim of examining whether the H56/IC31 vaccine would enhance the immunogenicity and protection induced by BCG (Figure 2). In experiment 1, we examined whether H56/IC31 as a BCG booster vaccine could provide protection against high-dose *M. tuberculosis* challenge (500 CFU). This dose was chosen to establish clinical disease in all control animals, and the primary end point was delayed and reduced clinical disease in vaccinated animals. In experiment 2, vaccination against a low-dose challenge (25 CFU) was evaluated to determine whether the vaccine would result in higher rates of latent infection. Without vaccination, low-dose infection results in approximately 50% of animals developing latent infection, while the remaining monkeys develop active disease (22, 26). Groups of animals were left unvaccinated or were vaccinated with BCG or with BCG followed by two H56/IC31 boosts 4 weeks apart. There was no evidence of systemic toxicity in any of the animals receiving H56/IC31. Injection sites were observed for local erythema, induration, and/or tenderness for up to 48 hours after injection. There were no apparent local reactions at injection sites in animals primed with BCG followed by H56/IC31 8 or 12 weeks later. Animals were challenged 6–8 weeks after the last boost. In both experiments, monkeys were followed with serial clinical, microbiological, immunological, and radiographic assessments for 40–64 weeks (Figure 2). We monitored responses to vaccine antigens ESAT-6, Ag85B, and Rv2660c, as well as to CFP10, which is an immunogenic protein present in *M. tuberculosis* but not in BCG (27).

Experiment 1: high-dose challenge

Vaccine-promoted antigen-specific immune responses. Antigen-specific T cell proliferative responses to the vaccine antigens were evaluated in BCG-vaccinated animals (n = 6) and BCG-vaccinated animals boosted twice with H56/IC31 (n = 6). PBMCs were obtained at baseline, week 14 (immediately before the H56/IC31 booster), and at week 17 (3 weeks after the second booster) (Figure 3). Negligible proliferative responses to the antigens were observed in BCG-immunized animals, but following booster vaccinations with H56/IC31, we observed increased proliferative response to all vaccine antigens and to PPD. There was no background proliferation from unstimulated PBMCs (data not shown). The highest responses were observed 3 weeks after the second H56/IC31 boost. Stimulation with Ag85B induced the strongest responses (stimulation index [SI], 7.95 ± 10.3), followed by ESAT-6 and Rv2660c.

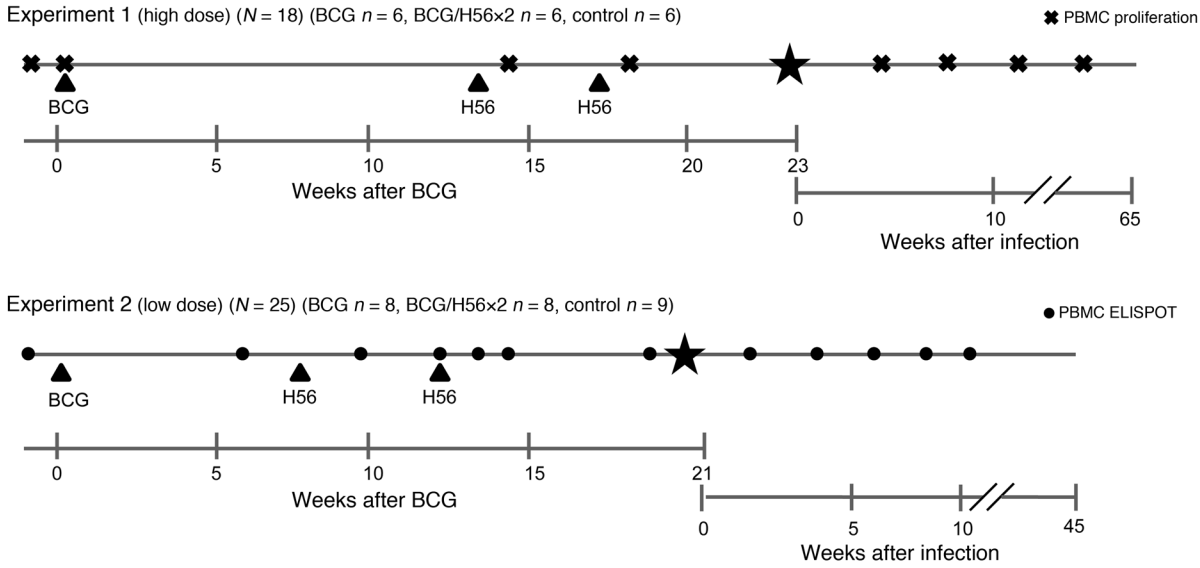

Figure 2
Overview of experimental design. Experiment 1 included 3 arms (n = 6/group): (a) control; (b) BCG only; (c) BCG followed by H56 at 14 and 17 weeks. All groups were infected with high-dose *M. tuberculosis* (500 CFU) at 23 weeks after BCG (star). PBMC lymphocyte proliferation assay was performed at the indicated intervals (depicted by bold ×). Animals were followed for up to 65 weeks after infection. Experiment 2 included 3 arms: (a) control group (n = 9); (b) BCG vaccination only (n = 8); (c) BCG followed by H56 at week 8 and a second dose of H56 at week 12 (n = 8). All groups were challenged with low-dose *M. tuberculosis* (25 CFU) (star) infection at 20 weeks after BCG vaccine. PBMC ELISPOT was performed at the indicated intervals (filled circles). Animals were followed for up to 45 weeks after infection.

Clinical parameters after M. tuberculosis challenge. The three groups of monkeys were infected with *M. tuberculosis* (500 CFU), and blood samples taken every 4 weeks up to week 64 after infection (Figure 2). We measured erythrocyte sedimentation rate (ESR) as a marker for inflammation. Increased ESR can signify progression to active TB in *M. tuberculosis*–infected macaques (22, 28). Following infection, non-vaccinated animals rapidly showed elevated ESR, and by 16 weeks after infection 3 of the 6 animals showed abnormal ESR levels greater than 2 mm (Figure 4A and Supplemental Table 1). With the exception of one animal (2861I; Supplemental Table 1), ESR levels in the unvaccinated group became elevated as clinical disease advanced, and at week 40 all the animals in this group had been euthanized. BCG-vaccinated animals had a delayed increase in ESR, and only 4 animals developed increased ESR. Boosting BCG with H56/IC31 had a clear effect, resulting in only one ESR-positive animal in more than 1 year of observation after challenge. When mean time to ESR conversion (i.e., the mean time for animals in a group to develop an ESR greater than 2) was compared

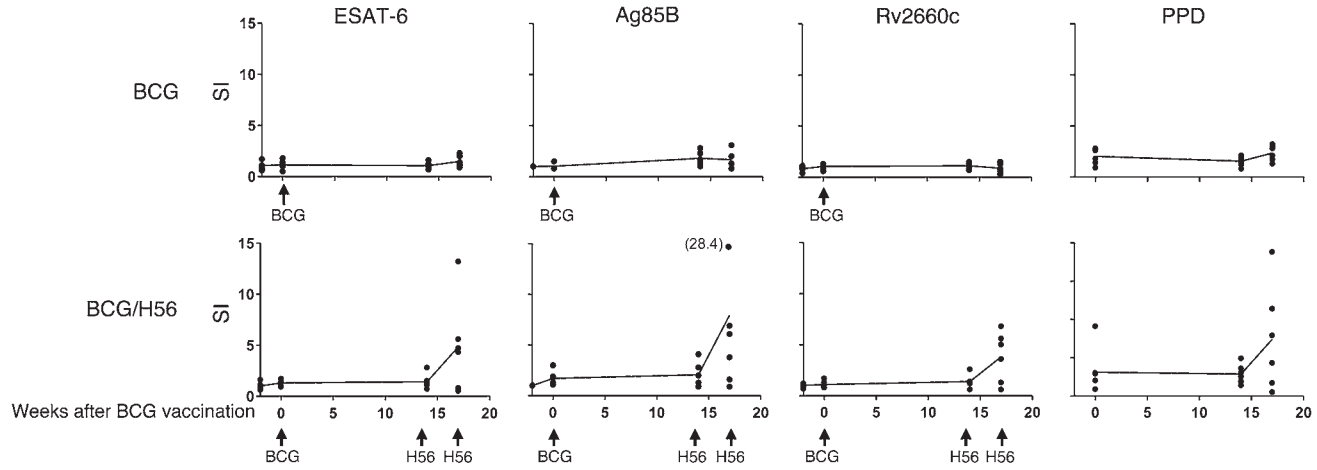

Figure 3
Vaccine-induced antigen-specific lymphoproliferative responses. Lymphoproliferation responses to vaccine antigens in vaccinated monkeys (BCG and BCG followed by H56 boost), represented as SI of PBMCs in response to mycobacterial antigen ESAT-6, Ag85B, Rv2660c, or PPD (positive control) compared with media alone. Results represent individual monkeys. The time points for BCG and H56 booster vaccinations are indicated.

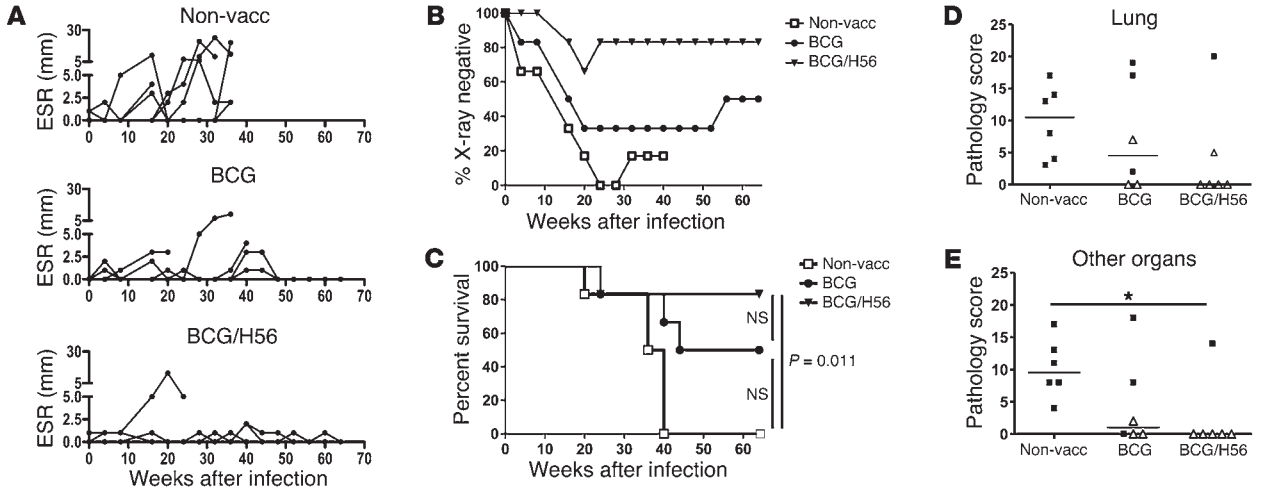

Figure 4

Clinical outcome after high-dose *M. tuberculosis* infection. Monkeys were infected with *M. tuberculosis* (500 CFU). (**A**) ESR was determined on whole blood from individual animals up to 64 weeks after infection. Non-vacc, non-vaccinated. (**B**) The percentage of X-ray-negative animals after challenge. Individual X-ray findings are shown in Supplemental Table 1. If an animal was positive and then had to be euthanized, it remained positive in our summation for the curves depicted. (**C**) Animals were examined clinically and euthanized according to preestablished humane end points to generate the survival curve. Analysis of Kaplan-Meier survival curves was performed with log-rank (Mantel-Cox) test taking into account that there were 3 groups. (**D** and **E**). Pathology scores of lungs (**D**) and other organs (**E**). Individual organ scores are given in Supplemental Table 2. The figure shows the scores for both euthanized (filled squares) and surviving (open triangles) animals. Each data point represents an animal and the median is represented as a horizontal bar. *$P < 0.05$, 1-way ANOVA, Kruskal-Wallis/Dunn's multiple comparison test.

among the groups, BCG had a modest effect on its own (mean ESR conversion of 28.0 weeks), which was not significantly different from that of non-vaccinated animals (17.3 weeks). Boosting BCG with H56/IC31 resulted in only one ESR-positive animal, and the group had a mean ESR conversion of 52.0 weeks, which was significantly longer than that of non-vaccinated animals ($P < 0.05$, 1-way ANOVA, Kruskal-Wallis test, Dunn's multiple comparison test). In summary, vaccination significantly delayed and reduced TB-associated inflammation as measured by ESR.

The first radiological changes were observed 4 weeks after *M. tuberculosis* challenge in BCG-primed and non-vaccinated animals, and the two groups had a similar progressive development of X-ray changes affecting 66%–100% of monkeys from week 20 (Figure 4B). The main radiological diagnosis was bilateral progressive bronchopneumonia with multiple nodules (6 of 6 in non-vaccinated animals and 4 of 6 in BCG-vaccinated animals), and two animals even showed consolidation/atelectasis of lung lobes. In the BCG/H56 monkeys, only two animals had detectable X-ray abnormalities. One animal had a minor X-ray change that quickly resolved. In the other animal, changes were seen from week 16, after which this animal (7531C) developed progressive disease and was euthanized 12 weeks later (Figure 4B and Supplemental Table 1). The difference in mean time to X-ray conversion (i.e., the mean time for animals in a group to become X-ray positive) between the BCG (30.7 weeks) and BCG/H56 (48.7 weeks) groups was not significantly different. However, only in the BCG/H56-vaccinated animals did we observe a significantly delayed mean time to X-ray conversion compared with non-vaccinated animals (48.7 versus 12 weeks) ($P < 0.05$, 1-way ANOVA, Kruskal-Wallis test, Dunn's multiple comparison test).

Animals in the non-vaccinated group had progressive development of disease, and all had to be euthanized before week 40 (mean survival time, 37.3 weeks) (Figure 4C). In the BCG group, animals displayed a less aggressive course of infection, with 50% surviving the 64-week observation period (mean survival time, 49.3 weeks) (Kaplan-Meier analysis: not significant, $P = 0.11$, log-rank [Mantel-Cox] test). In the BCG/H56 group, 5 of 6 (83%) monkeys survived until termination of the experiment (mean survival time, 57.3 weeks), and this was significantly different from the non-vaccinated animals ($P = 0.02$) (Figure 4C). Finally, the animals in this group also showed increased weight gain and decreased temperature fluctuations compared with BCG animals and/or non-vaccinated animals (Supplemental Figure 1).

Pathology scores at necropsy. Separate lung lobes were scored for pathology (see Methods) and results summed to obtain the total lung score per animal (Figure 4D). The mean pathology score in "other organs," i.e., heart, spleen, liver, and kidney, was analyzed as a measure of extrapulmonary dissemination (Figure 4E and Supplemental Table 1). At necropsy, all non-vaccinated animals showed extensive lung pathology, characterized by the presence of multiple granulomas in lungs, and the pathology scores for the other organs were high, demonstrating substantial dissemination of the infection. The median pathology score in the non-vaccinated group was 10.5 in both the lung and in other organs, but the variation was significant, with some animals having substantial pathological changes (Figure 4, D and E, and Supplemental Table 1). The BCG-only group did not have statistically significant differences in pathology compared with the control group according to this scoring system. However, there was variable protection induced by BCG in that 2 of 3 surviving animals in the BCG group had pathology scores of 0 in lungs and other organs (Supplemental Table 1). In contrast to the control and BCG-only groups, only 1 of the 5 surviving animals in the BCG/H56 group showed any detectable pathology in the lungs (this animal had a modest

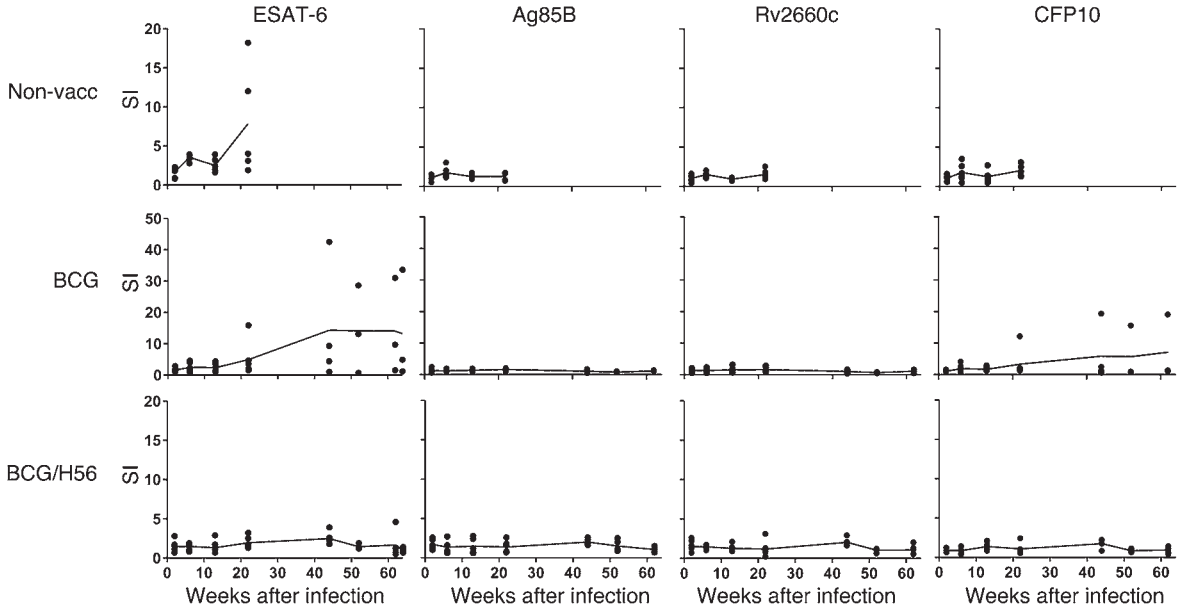

Figure 5
Antigen-specific T cell responses after high-dose *M. tuberculosis* infection. Lymphoproliferative responses in non-vaccinated and BCG- and BCG/H56-vaccinated monkeys are depicted as SI of PBMCs in response to the vaccine antigens ESAT-6, Ag85B, and Rv2660c or to CFP10 used as a marker of infection. Results for individual monkeys are shown.

score of 5). None of the 5 H56-vaccinated monkeys that survived the 64 weeks of infection had any pathology outside the lung ($P < 0.05$, non-vaccinated versus H56-boosted). Animal 7531C, which was euthanized at week 28, was the only animal in the BCG/H56 group with extensive pathology (Figure 4 and Supplemental Table 1). These differences were significant compared with the non-vaccinated group when including all organs in the comparison ($P < 0.05$, 1 way ANOVA multiple test). Samples were analyzed for histopathological characteristics, and in the non-vaccinated group, severe, necrotizing tuberculous pneumonia was common, with central areas of caseous necrosis and degenerating neutrophils. In the vaccinated groups, more moderate histopathological findings were reported, but animals with high pathology scores (BCG animals 7947B and 197BJ, BCG/H56 animal 7531C; Supplemental Table 2) all had areas with extensive caseous necrosis.

Taken together, these data support that H56/IC31 boost after BCG vaccination delayed and reduced clinical disease and pathology and resulted in increased survival following high-dose challenge.

Antigen-specific T cell responses after M. tuberculosis challenge. We monitored antigen-specific lymphoproliferative responses for up to 64 weeks after challenge. After challenge, we included CFP10 antigen which, like ESAT-6, has been demonstrated to be a sensitive marker of progression of *M. tuberculosis* infection (27) but is not included in H56 or BCG. As described above, the non-vaccinated animals showed an accelerated course of infection, and the only antigen they recognized was ESAT-6 (SI at week 22, 7.84 ± 3.14) (Figure 5). Surprisingly, we did not find the expected responses to CFP10 in this group. The BCG-vaccinated group responded vigorously to both infection markers, ESAT-6 and CFP10. Increasing responses were seen after week 24, with a plateau from week 44 onward. In the BCG/H56 group, antigen-specific responses remained low throughout 64 weeks of infection (Figure 5), suggesting very efficient control of bacterial replication.

Experiment 2: low-dose challenge

Vaccine-induced antigen-specific immune responses. In the low-dose experiment, vaccine-induced antigen-specific T cell responses were measured by PBMC production of IFN-γ via ELISPOT. In the BCG group, only modest responses to Ag85B were found, whereas neither ESAT-6 nor Rv2660c was recognized, since these antigens are not present in BCG (Figure 6). The frequency of specific T cells decreased from week 6 to week 19. In the BCG/H56 group, responses were observed to all three vaccine antigens and culture filtrate proteins (CFPs) (responses to this crude mix of *M. tuberculosis* proteins reached levels of ~270 spots/200,000 cells). The response to Ag85B waned after BCG vaccination but returned to more than 60 SFU/200,000 cells after the second H56 injection (Figure 6). No responses were found to the two other antigens before the boost, but detectable responses to ESAT-6 and RV2660c were measured after both H56 injections. IL-2 ELISPOTs were performed on the cryopreserved samples with the same stimulators. IL-2 responses to all components from the H56 molecule were observed in most monkeys in the boosted group as compared with the BCG group. Both ESAT-6 and Rv2660c were less frequently recognized than Ag85B (and CFP), but none of the BCG-only animals recognized these antigens, underlining the effect of the H56 vaccination on all its antigen constituents (Supplemental Figure 2). We performed Luminex multiplex assay for 23 cytokines and chemokines (see Methods) on cryopreserved PBMCs obtained before and after challenge and stimulated with vaccine antigens; overall responses were low using this method in the BCG and BCG/H56 groups, and there was no correlation of any of these cytokines or chemokines with outcome of challenge (data not shown).

Clinical parameters after M. tuberculosis challenge. In the low-dose model, approximately 50% of monkeys develop latent or subclinical (termed "percolator") infection, and 50% develop primary tuberculosis (22). Non-vaccinated animals showed an accelerated

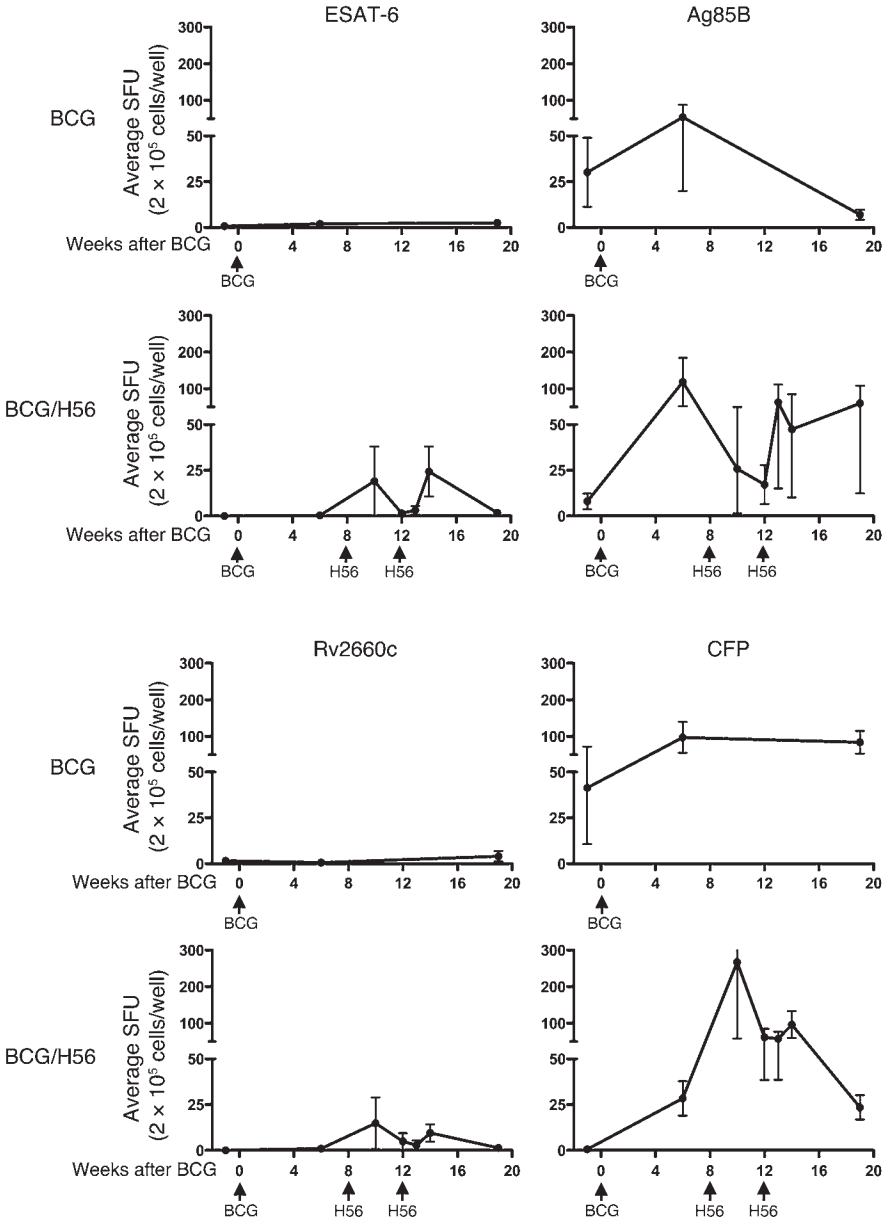

Figure 6
Vaccine-induced antigen-specific IFN-γ responses. IFN-γ ELISPOT responses to vaccine antigens in vaccinated monkeys (BCG and BCG/H56). Antigen- or CFP-specific IFN-γ spot-forming units (SFU/200,000 cells) were measured at serial time points after vaccination by PBMC ELISPOT. Results are depicted as mean ± SEM ($n = 8$). Media background responses were subtracted from each antigen-specific result for each respective monkey at each time point.

development of ESR responses after challenge compared with the vaccinated groups (Figure 7). By 4 weeks after challenge, the non-vaccinated animals had ESR values up to 40 mm, and 6 of the 9 control monkeys developed positive ESR within the first 32 weeks of infection. In the BCG-only group, one monkey had an increased ESR at 4 weeks and rapidly progressed to disease. By 16 weeks, 3 of the 8 animals had high ESR values (≥20 mm). In the BCG/H56 group, there was a delay in positive ESR values compared with non-vaccinated and BCG-only monkeys, and only two monkeys had sustained increases in ESR over the course of 32 weeks. These data suggest that vaccine-induced immune responses allow early containment of infection after challenge.

Disease outcome (i.e., latent/subclinical or active disease) was determined at 8 months after infection using our published criteria (22). While the sample sizes were too small for adequate statistical power, a greater proportion of BCG/H56-vaccinated monkeys (75%) developed latent/subclinical infection compared with the non-vaccinated (44%) and BCG-only (37.5%) groups, supporting that H56 boost results in better control of infection and less active disease. One of the 8 non-vaccinated monkeys developed active disease severe enough to warrant early euthanasia at 12 weeks after infection. Two of the BCG-vaccinated monkeys required early euthanasia at 17 and 18 weeks after infection. None of the BCG/H56-vaccinated monkeys required early euthanasia, and these remained clinically stable to 30 weeks after infection (Figure 7B). In the BCG/H56-vaccinated group only two animals progressed to active disease throughout the course of the experiment, and these animals were euthanized at weeks 36 and 44.

No differences were observed in the overall rate of abnormal chest X-ray development, presence of *M. tuberculosis* growth by either gastric aspirate or BAL, or weight loss between control and vaccine groups (data not shown). These changes tended to be more

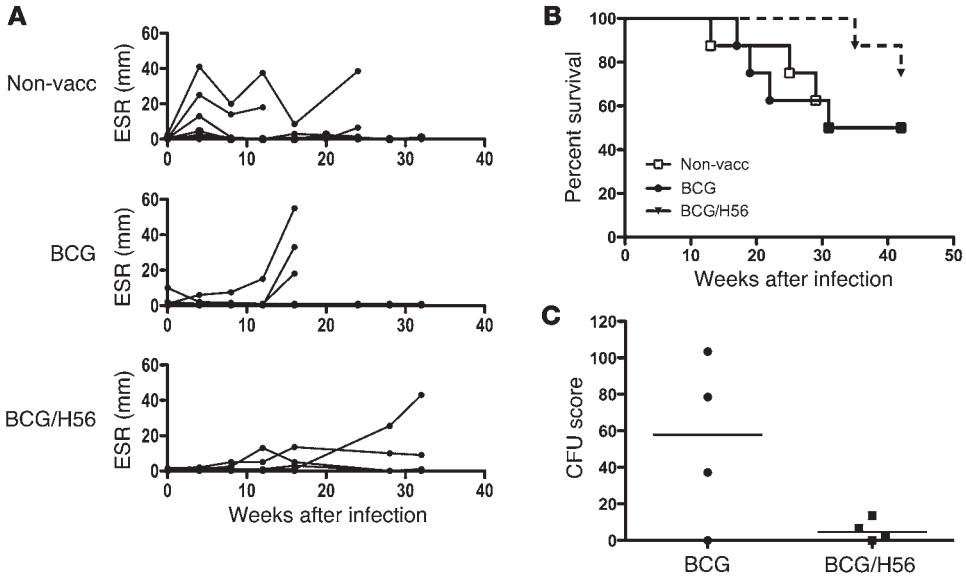

Figure 7

Clinical parameters after low-dose *M. tuberculosis* challenge. Monkeys were infected with 25 CFU *M. tuberculosis*. (**A**) Each line represents serial ESR results of an individual monkey in the non-vaccinated control, BCG, or BCG/H56 group up to 32 weeks after infection. (**B**) Animals were examined clinically and euthanized according to preestablished humane end points. Survival curves for the different groups are given in **B**, representing up to 45 weeks after infection. (**C**) BCG (n = 4) and BCG/56 (n = 4) monkeys with latent infection were treated with anti-TNF antibody for 5 weeks, then subjected to necropsy. At necropsy, 25–40 samples per animal were plated for bacterial numbers, and a CFU score calculated that reflects overall bacterial burden in the animal, as previously described (22). Each data point represents an animal, and the median is represented as a horizontal bar.

subtle and slowly progressing in the low-dose challenge model compared with the high-dose challenge model.

Reactivation of latent M. tuberculosis infection. To test the hypothesis that H56 would improve the control of latent infection, we compared reactivation of latent infection in BCG- and BCG/H56-vaccinated animals. We previously reported that anti-TNF antibody causes rapid reactivation in approximately 70% of latently infected macaques (29) and neutralization of TNF in latently infected humans sharply increases the risk of reactivation. Thus, we reasoned that TNF neutralization was a stringent test of the ability of a vaccine to prevent reactivation. Four latently infected monkeys from both the BCG and BCG/H56 groups were treated for 5 weeks with anti-TNF antibody, then subjected to necropsy. Although the numbers of animals per group were too low for robust statistical analysis, 3 of the 4 BCG-only monkeys reactivated the latent infection, as demonstrated by increased bacterial numbers (Figure 7C) and extrapulmonary dissemination, similar to our previous reports of reactivation of non-vaccinated animals (29). In stark contrast, none of the BCG/H56 animals demonstrated reactivation of latent infection, and all had low bacterial burdens. These data suggest that the inclusion of H56 boost in a preexposure vaccine can prevent reactivation of latent infection.

Antigen-specific T cell responses after M. tuberculosis challenge. PBMC ELISPOTs for mycobacterial antigen-specific production of IFN-γ were performed after *M. tuberculosis* challenge. In our experience, increased IFN-γ production is not generally seen until 4–6 weeks after infection without vaccination (22). Thus, IFN-γ production prior to 6 weeks after challenge most likely reflects vaccine-induced recall responses. CFP10 antigen was included as a marker of *M. tuberculosis* infection (distinct from vaccine recall) in the post-challenge analysis. Animals given BCG/H56 had recall responses to Ag85B, ESAT-6, and Rv2660c within the first 6 weeks of infection, before both non-vaccinated controls and BCG-only animals (Figure 8). Responses to CFP10 were initially very low but dramatically increased at 6 weeks in the non-vaccinated group. As previously reported (22), IFN-γ responses to CFP10 in the initial phase of infection likely reflect increased bacterial burden. Peak responses to CFP10 among BCG monkeys occurred later than in the control group.

Antigen-specific IFN-γ responses were analyzed to determine whether immunologic responses could predict protection. Monkeys vaccinated with BCG/H56 that developed latent/subclinical infection had pre-challenge IFN-γ responses to ESAT-6, Rv2660c, and Ag85B after H56 boost. These animals were also characterized by recall responses to the vaccine antigens and in particular a very pronounced early recall response (1–3 weeks after infection) against ESAT-6 and Rv2660c. In contrast, BCG/H56-vaccinated monkeys that developed active disease had only minimal vaccine-promoted responses and importantly did not show any recall response to Rv2660c or to ESAT-6 (Figure 9 and Supplemental Figure 4). Monkeys vaccinated with BCG only did not demonstrate early recall responses to vaccine antigens, regardless of infection outcome (Figure 8 and data not shown). Thus, an early recall response against ESAT-6, Rv2660c, and Ag85B was associated with latent infection.

Gross disease at necropsy. All non-vaccinated monkeys with active disease (n = 4) had extrapulmonary disease; however, none of the BCG monkeys with clinically classified active disease (n = 4) had extrapulmonary disease. This confirms other findings in which human BCG vaccination was associated with prevention of disseminated disease (30, 31). In the BCG/H56 group, only two animals developed active disease, but both of these animals had extrapulmonary disease. Although there were clearly too few animals

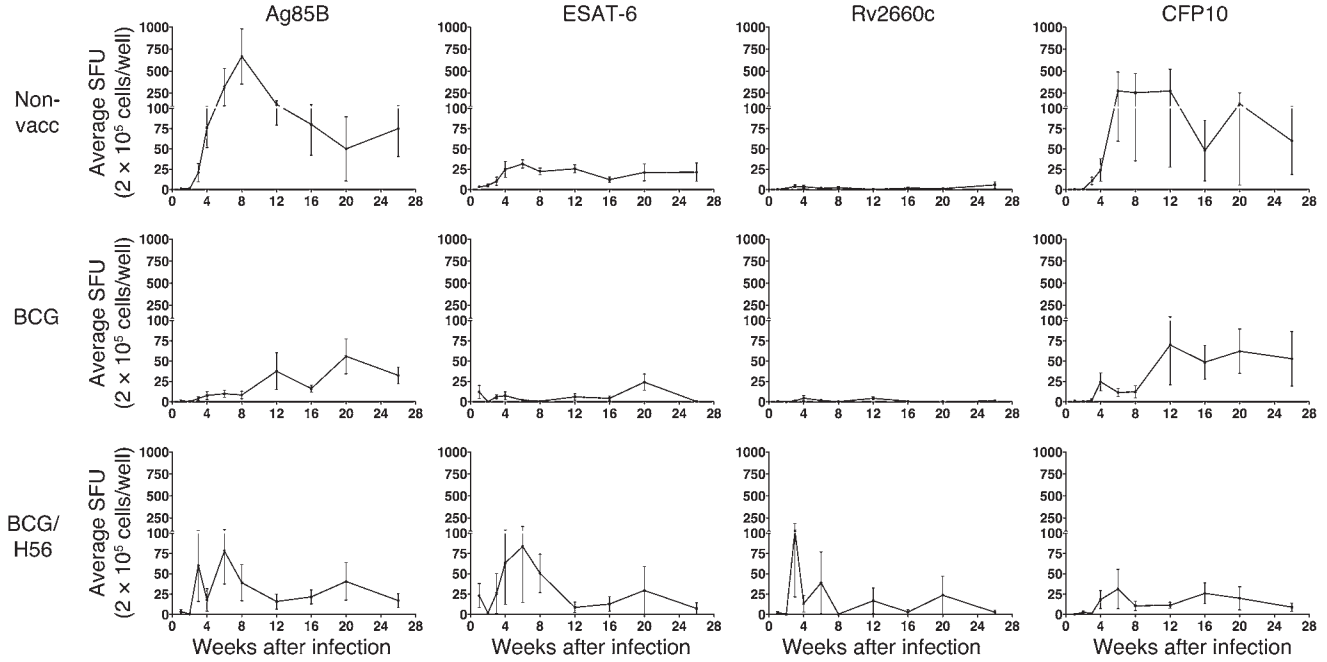

Figure 8
Antigen-specific IFN-γ ELISPOT responses after low-dose *M. tuberculosis* infection. Antigen-specific IFN-γ responses to the vaccine antigens ESAT-6, Ag85B, and Rv2660c and to CFP10 in non-vaccinated and BCG- and BCG/H56-vaccinated monkeys were determined by ELISPOT (depicted as SFU/2 × 10^5 PBMCs). Results are depicted as mean ± SEM (n = 8–9 per group). Media background responses were subtracted from each antigen-specific result for each respective monkey at each time point.

for any statistical assessment, this tendency is different from the high-dose study, where none of the BCG/H56-vaccinated animals developed extrapulmonary disease. This difference may result from the accelerated vaccination schedule in the low-dose experiment where the interval between BCG and H56 boosting (8 weeks) was 5 weeks smaller than in the high-dose trial (13 weeks). Microscopic histopathology of lesions from monkeys with active disease was not different in vaccinated and unvaccinated monkeys (22, 26).

Discussion

Given the high prevalence of BCG vaccination in developing countries, a new TB vaccine will most likely not replace BCG, but instead be used in addition to BCG (32). Animal studies have shown that a strategy based upon boosting or supplementing BCG with a subunit vaccine can indeed increase protection against infection with *M. tuberculosis* (33, 34). A characteristic feature of BCG is that vaccination protects against severe childhood forms of TB but is unreliable against the highly infectious pulmonary form of TB characteristically found in adults. The booster vaccines currently in clinical trials may improve or boost BCG efficacy in the shorter term, but they have not been designed to contain latent TB and prevent reactivation of disease (13). In the present study, we demonstrate that vaccination of non-human primates with the triple fusion Ag85B–ESAT-6–Rv2660c (H56) in the Th1 adjuvant IC31 (24, 35) promotes an immune response targeted to all three protein components, including the latency-associated antigen Rv2660c. This was seen with proliferation and IFN-γ and IL-2 ELISPOT analyses. We also demonstrate high expression of the genes for *Rv2660c* and *ESAT-6* and 10- to 100-fold lower expression of *Ag85B* in granulomas from monkeys infected with *M. tuberculosis* for up to 8 months. The low expression of *Ag85B* and high expression of *ESAT-6/Rv2660c* in the stage of infection where *M. tuberculosis* encounters a fully developed adaptive immune response are in agreement with previous observations from the mouse model (14, 36). Our data in aggregate indicate that in the macaque TB model, the BCG/H56 regimen is more protective than BCG alone, as demonstrated by containment of infection, efficient prevention of clinical disease, no reactivation in response to TNF neutralization, improved overall survival of the animals, and reduced pathological changes at necropsy. These findings are in agreement with a recent evaluation of this vaccine in the mouse model, where the most pronounced feature was its ability to control the infection even at very late time points of infection (14). The efficient containment of subclinical infection in the H56-boosted animals resulted in limited disease spread, and several animals had minimal disease and pathology at necropsy (Figure 4). This result is in contrast to findings from studies testing other vaccines in non-human primates, where substantial pathology was observed in boosted groups (37), in some cases at a higher level than in animals receiving only BCG (28). The finding that a vaccine (i.e., H56) can prevent reactivation of true latent infection is, to our knowledge, novel in the study of vaccines against TB and supports the hypothesis of a multi-stage vaccine generating long-term protection against disease.

In agreement with data obtained in the mouse model (14), Rv2660c was not recognized in non-vaccinated animals during infection, whereas early recall responses to Rv2660c during infection were found in the H56-boosted animals. This confirms that Rv2660c is indeed expressed during infection and available for immune recognition, but that this small molecule is of a relatively low immunogenicity and therefore subdominant to antigens such as ESAT-6 and

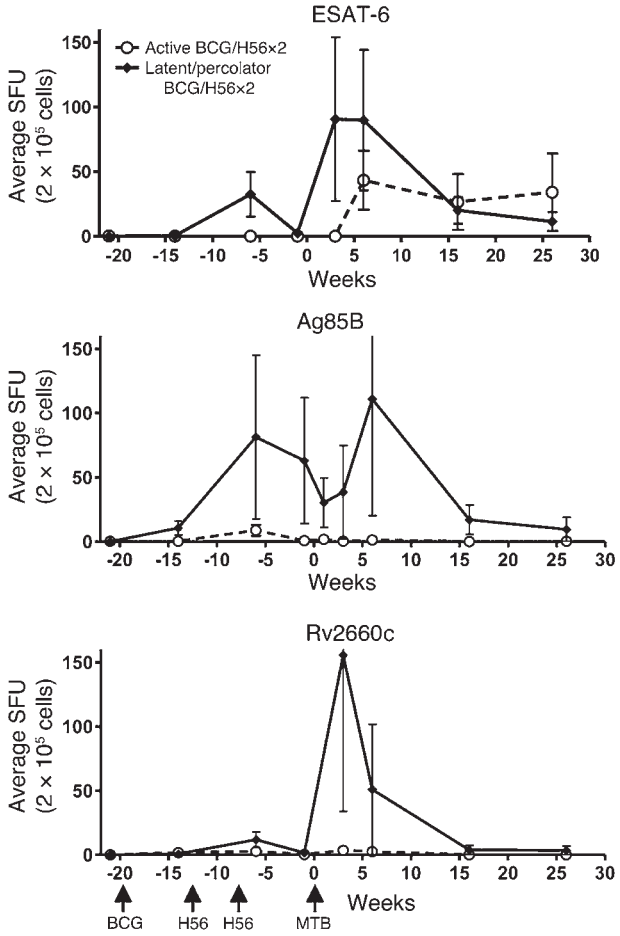

Figure 9
Antigen-specific IFN-γ ELISPOT responses correlated with outcome of low-dose infection. Monkeys were divided into "active" and "latent/percolator" groups based on previously established clinical, microbiologic, and radiologic criteria. ELISPOT results for these categorized monkeys were compared before and after *M. tuberculosis* infection. Antigen-specific IFN-γ ELISPOT results are depicted as spots per 2 × 10^5 PBMCs. Results are shown as mean ± SEM. Individual animals are shown in Supplemental Figure 4.

Ag85B during the complex immune response promoted by the natural infection. Importantly, *M. tuberculosis*–infected individuals recognize Rv2660c, and both this molecule and its coexpressed neighbor on the genome, Rv2659c, were recently found to be recognized at a low but clearly detectable level in latently infected individuals (19).

In our experience, ESAT-6 and CFP10 induced IFN-γ production at 6 weeks after infection correlated with development of active disease due to higher bacterial burdens (and thus increased antigenic stimulation) in non-human primates, as has been reported in other animal models (27, 38). As ESAT-6 is a component of the H56 vaccine, we introduced the CFP10 antigen for the post-challenge monitoring of T cell responses. Our data in general suggest that increased ESR and ESAT-6/CFP10 T cell responses predict later clinical disease. We have previously suggested that the IFN-γ release assay (IGRA) may have prognostic potential and that it may be possible to establish a cut-off value for the detection of latently infected individuals that will later develop disease (39). The lack of CFP10 responses and modest ESAT-6 responses in the non-vaccinated animals (as opposed to the BCG-vaccinated group) that received the high-dose challenge (Figure 5) suggest that IGRA values should be interpreted with great caution in individuals with progressive disease, as they may develop anergy.

We also analyzed our data for possible predictors of protective immunity. As the BCG/H56-vaccinated monkeys included animals that were protected and animals that developed disease, we had the opportunity to compare T cell responses that differed in these two groups. H56/IC31-boosted monkeys with the infection in the latent/subclinical stage had vaccine-promoted and recall responses to all three vaccine antigens and a very pronounced early recall response (1–3 weeks after infection) against ESAT-6 and Rv2660c. Animals that later developed active disease showed negligible responses to all antigens and were characterized by the complete absence of a recall response to the vaccine antigens. To our knowledge, this is the first demonstration in vaccinated monkeys that specific responses to vaccine antigens, both before and after infection, correlated with infection outcome in individual monkeys; this was only observed in the BCG/H56 vaccination group and not the BCG-only group. We did not find differences in IFN-γ responses beyond 4 weeks after infection that correlated with good disease outcome, as others have (40). Pre-challenge IL-2 responses did not correlate with outcome of infection.

While a number of vaccine efficacy studies have been published using the high-dose cynomolgus or rhesus macaque models of infection (8, 37, 41, 42), to our knowledge this is the first vaccine trial in which both low- and high-dose *M. tuberculosis* challenges were performed. The H56 booster vaccine gave similar outcomes in the two models, and in particular very low levels of the inflammation marker ESR and low infection-driven responses to CFP10 were striking observations in both experiments and indicated efficient control of bacterial replication. Last, the H56 booster in our study resulted in very efficient containment of latent infection and prevented anti-TNF–induced reactivation of TB, demonstrating its ability to provide long-term protection against clinical disease. Improved bacterial burden was observed in H56-vaccinated mice during chronic murine infection (14), and the addition of Rv2660c to another vaccine backbone had a similar effect (J. Dietrich, unpublished observations), suggesting that the H56 vaccine–induced response results in a more stable and protected state of latent infection that is less prone to reactivation. If H56 in human clinical trials also promotes a response that controls the late stages of *M. tuberculosis* infection and contains latent TB (i.e., prevents reactivation), this vaccine could have a huge impact on TB transmission and the global TB epidemic. We have therefore recently initiated clinical development of H56.

Methods

RNA isolation from infected tissue

Granulomas were dissected from the lungs of three BCG-vaccinated *M. tuberculosis*–infected macaques (low dose) that developed active TB. Tissue was snap-frozen in liquid nitrogen and stored at –80°C. Frozen tissue was placed in TT1XT bags (Covaris), held in liquid nitrogen 1 minute, and pulverized in a CryoPrep (Covaris) on setting 5. Samples were immediately transferred to the attached glass tube, with 1 ml TRIzol (Invitrogen) added, and maintained on ice. Samples were processed for tissue disruption and RNA extraction with a Covaris S2 machine, on degas 60 setting. Samples were transferred to a 15-ml tube and centrifuged at 3,500 *g* for 10 minutes,

and the pellet was resuspended in 1 ml TRIzol, added to Lysing Matrix B (Qbiogene), and beat in a bead beater (FastPrep 120) 3 times at 6.5 for 30 seconds. Supernatant was added to Heavy Phase Lock Gel (Eppendorf), with 300 μl chloroform added, and the sample was mixed for 2 minutes and centrifuged at maximum speed (17,000 *g*) for 10 minutes at 4°C. The aqueous layer was removed, 540 μl of 70% EtOH added, and the sample mixed and placed on ice. The sample was then processed on an RNeasy Column (QIAGEN), per the manufacturer's instructions. RNA was DNAse (RQ1 DNAse, Promega) treated at 37°C for 45 minutes and processed on an RNeasy column, with final elution in 50 μl RNase-free water.

qRT-PCR

cDNA was generated as previously described (17), except that here we used 8.5 μl RNA in a 20-μl final volume reaction. Primary amplification of cDNA was done with Advantage 2 Polymerase (Clontech) with 5 μl cDNA and 0.2 μM each flanking primer (sequences below), with the following reaction conditions: 95°C 3 minutes, followed by 25 cycles of 95°C 20 seconds, 58°C 1 minute, 72°C 1 minute, and a final 72°C 7 minutes. Real-time PCR was done with 0.05 μl of the primary reaction, 4.5 μM of each primer, and 1.25 μM TaqMan probe (Applied Biosystems, 6-FAM dye) in 5 μl total. Reactions were run and analyzed on a Bio-Rad CFX-384 real-time PCR machine. Primer and probe sequences are described in Supplemental Figure 3.

Experimental vaccine

The vaccine antigen construct H56 (Ag85B–ESAT-6–Rv2660) was formulated with the adjuvant IC31. Doses used were 50 μg antigen protein and 400 μl IC31. The adjuvant IC31 consists of a mixture of the peptide KLK (NH_2-KLKL_5KLK-COOH) (1,250 mmol/ml) and the oligodeoxynucleotide ODN1a [oligo-$(dIdC)_{13}$] (50 nmol/ml) provided by Intercell.

Experimental animals

Cynomolgus macaques (*Macaca fascicularis*) ranging in age from 1.6 to 3.7 years and weighing 2.2–3.2 kg were used for high-dose trial studies. Low-dose studies were performed on adult cynomolgus macaques older than 4 years and between 5 and 8 kg. Prior to the studies, the macaques underwent a rigorous battery of diagnostic and clinical procedures (e.g., physical examination, complete blood count [CBC] with differential, ESR, serum chemistry profile, thoracic radiography, and standard tuberculin skin testing [TST]).

Animal infection and clinical assessments after infection

Under sedation, cynomolgus macaques were inoculated intratracheally with either 500 CFU per monkey in 1-ml volume (high-dose trial) or 25 CFU per monkey in a 2-ml volume (low dose trial) of *M. tuberculosis* virulent Erdman strain. In the high-dose trial, the infection was allowed to proceed until the macaques reached disease states that ranged from no apparent disease to advanced disease. Weight, temperature, CBC, serum chemistry profile, direct fecal examination, rectal culture, PPD skin test, and Sediplast Westergren ESR were recorded monthly. In the low-dose trial, monthly microbiologic (*M. tuberculosis* growth by monthly gastric aspirate and BAL), clinical (exam, weight, symptoms), and immunologic (PBMC ELISPOT, ESR [Wintrobe method]) assays were performed and recorded for analysis. In both trials, chest radiographs were taken at baseline, monthly, and when clinically warranted. Radiographs were evaluated by a board-certified thoracic radiologist with extensive experience in pulmonary TB.

Immunization

Experiment 1. As shown in Figure 1, monkeys (n = 6) were primed with 0.1 ml BCG Danish strain or injected with saline delivered intradermally. Thereafter, the monkeys were vaccinated intramuscularly in the gluteal muscle 2 times with 50 μg H56 formulated with IC31 at weeks 14 and 17 after BCG treatment. At week 23 the animals were challenged with *M. tuberculosis* and followed for 64 weeks.

Experiment 2. BCG/H56 monkeys (n = 8) were given BCG (0.1 ml intradermal injection), then H56/IC31 at 8 and 12 weeks after BCG inoculation. The BCG group (n = 8) was given BCG (0.1 ml intradermal injection) only. There were 9 unvaccinated animals followed for this study. Vaccinated groups were challenged with *M. tuberculosis* (25 CFU) 20 weeks after BCG administration and followed for 8 months until clinical classification (active disease or latent infection) could be determined.

Cellular immune response assays

Heparinized blood was diluted with complete RPMI at 1:5 dilution for lymphoproliferation (LPA) assays and stimulated with PPD (10 μg/ml) or the indicated antigens at 10 μg/ml, or not stimulated. Stimulated cells were incubated at 37°C for 5 days, and [^{3}H]thymidine (1 μCi per well) was added for the final 6 hours of incubation. Results are reported as SI, the fold increase in cpm over the unstimulated control. PBMC ELISPOT for mycobacterium-specific IFN-γ was performed on the low-dose-challenge monkeys as previously described (43). In monkeys given BCG/H56 (n = 8), the frequency of antigen-specific (ESAT-6 peptide, Ag85B protein, Rv2660c peptide) IFN-γ–producing T cells was measured at baseline and 6, 10, 12, 13, 14, and 19 weeks after BCG administration. Each ELISPOT also included positive control (phorbol dibutyrate [PDBu] and ionomycin), negative control (no antigen stimulation), and stimulation with CFP conditions as described previously (43). Only samples in which an adequate number of spot-forming units were observed in the positive control condition were used for analysis. Monkeys given BCG (n = 8) had PBMC ELISPOTs performed at baseline and 6 and 19 weeks after BCG vaccination. After challenge, CFP10 peptides were added as another stimulator. Media background was subtracted from the antigen-specific response at each time point.

A more thorough investigation of vaccine-induced cytokine responses was undertaken using cryopreserved PBMCs obtained from both vaccine groups at 6 weeks and 1 week prior to *M. tuberculosis* challenge and 3 weeks after challenge. After thawing, PBMCs (200,000 PBMCs/ well) were stimulated with vaccine antigens (ESAT-6, Ag85B, Rv2660c), media, or positive control antigens (anti-CD3 and PDBu/ionomycin) with co-stimulatory molecules anti-CD28 and anti-CD49d (10 μg/ml) for 72 hours. Multiplex cytokine analysis (G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12/23[p40], IL-13, IL-15, IL-17, MCP-1, MIP-1α, MIP-1β, sCD40L, TGF-α, TNF, VEGF, and IL-18) was performed using a non-human primate pre-mixed Milliplex kit (Millipore) according to the manufacturer's instructions. IL-2 ELISPOT was also performed on cryopreserved PBMCs obtained from both vaccine groups at 1 and 6 weeks prior to *M. tuberculosis* challenge. Non-human primate-specific antibodies for IL-2 detection were used (3440M-2AH-Plus kit, Mabtech Inc.) with a previously described protocol (43). Cells (200,000 PBMCs/well) were stimulated with ESAT-6, Ag85B, and Rv2660c, media (negative control) and CFPs (positive control) for 48 hours and data generated in spot-forming units (43).

Reactivation of latently infected macaques

Low-dose-infected animals that were declared to have latent infection (i.e., clinically stable with normal ESR, normal chest X-ray, absence of clinical signs, and negative for growth of *M. tuberculosis* from gastric aspirate and BAL beyond 3 months after infection) were treated with anti-TNF antibody (Adalimumab, Abbott) (4 mg/kg/dose subcutaneously every 10 days) to induce reactivation (29) at least 8 months after *M. tuberculosis* challenge. During the course of anti-TNF treatment, serial clinical, microbiologic, and radiographic assessments were performed for evidence of reactivation. After 5 weeks of anti-TNF antibody treatment, monkeys were subjected to necropsy.

Necropsy procedures and pathology scores

Animals were anesthetized with either a cocktail (5:1:1) of Zoletil 50 (Virbac Laboratories) plus Atrosite and ILIUM Zylasil-100 (Troy Laboratories) (high-dose experiment) or ketamine (Butler Schein Animal Health) given intramuscularly at 0.1 ml/kg body weight. Animals were euthanized with an i.v. overdose of sodium pentobarbital (Beuthanasia, Intervet/Schering-Plough Animal Health, or Delvet Veterinary Laboratories). For the high-dose experiment, the gross extent of pathology from mycobacterial infection was recorded. Each lung was examined for superficial lesions, and the gross dissemination of mycobacterial infection (e.g., number of visible granulomas) and other pathological findings were recorded for right and left lung separately, spleen, liver, kidney, thoracic lymph node, and other sites (pericardium, stomach, bone). Scoring of lungs and other organs was recorded separately, taking into account both the number (i.e., score 0, no visible granulomas; score 1, 1–3 granulomas; score 2, 4–10 granulomas; score 3, more than 10 granulomas; score 4, miliary pattern with numerous lesions) and size (i.e., score 1,1–2 mm; score 2, 3–4 mm: score 3, >4 mm. Each organ (with pathological changes) could obtain a minimal score of 2, e.g., 1–3 small lesions (1–2 mm), and a maximum score of 10, e.g. miliary TB with several coalescent large granulomas, of lesions. All individual pathology scores are recorded in Supplemental Table 2.

Statistics

Statistical analyses were performed as indicated in Results: For ESR, X-ray, and pathology, the 3 animal groups were compared with 1-way ANOVA, Kruskal-Wallis/Dunn's multiple comparison test. Analysis of Kaplan-Meier curves was performed with log-rank (Mantel-Cox) test. As the group number exceeded 2, the Bonferroni-corrected threshold used for each individual comparison equaled the significance level (i.e., 0.05) divided by the number of groups ($n = 3$). If a P value was less than this Bonferroni-corrected threshold, then the comparison was considered statistically significant. A P value less than or equal to 0.5 was considered significant. All tests were done using Prism Software (GraphPad Software).

Study approval

All experimental manipulations and protocols for the low-dose infection experiments were approved by the University of Pittsburgh School of Medicine IACUC. The University of Pittsburgh animals were housed and maintained in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited (AAALAC-accredited) facility in accordance with standards established in the Animal Welfare Act and the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23. Revised 1985). For the high-dose experiments, all experimental manipulations and protocols were approved by the LWM IACUC. All animals used in the study were housed in facilities accredited by the Philippine Association for Laboratory Animal Science (PALAS) in accordance with standards established in the Animal Welfare Act and the *Guide for the Care and Use of Laboratory Animals*.

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