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A multistage tuberculosis vaccine that confers efficient protection before and after exposure

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All tuberculosis vaccines currently in clinical trials are designed as prophylactic vaccines based on early expressed antigens. We have developed a multistage vaccination strategy in which the early antigens Ag85B and 6-kDa early secretory antigenic target (ESAT-6) are combined with the latency-associated protein Rv2660c (H56 vaccine). In CB6F1 mice we show that *Rv2660c* is stably expressed in late stages of infection despite an overall reduced transcription. The H56 vaccine promotes a T cell response against all protein components that is characterized by a high proportion of polyfunctional CD4$^+$ T cells. In three different preexposure mouse models, H56 confers protective immunity characterized by a more efficient containment of late-stage infection than the Ag85B-ESAT6 vaccine (H1) and BCG. In two mouse models of latent tuberculosis, we show that H56 vaccination after exposure is able to control reactivation and significantly lower the bacterial load compared to adjuvant control mice.

Currently, effective vaccines are lacking for many diseases, in particular chronic and intracellular infections where protection is thought to depend on T cell–mediated immunity. Tuberculosis is one of the best examples of this category, and central to the success of *Mycobacterium tuberculosis* (*Mtb*) as a pathogen is its ability to subvert host immune responses and establish latent infection[1]. The BCG vaccine is currently the only tuberculosis vaccine approved for human use. However, in the last five years there has been substantial progress in the tuberculosis vaccine field, with several vaccines either in clinical trials or on their way through preclinical development[2]. These vaccines are all designed as prophylactic vaccines for preinfection administration and are in most cases based on antigens recognized by the immune system during the early stage of infection[3]. Evidence from mouse models suggests that although these new vaccines represent improvements over BCG, none of them results in sterilizing immunity[4,5]. Similarly, BCG does not prevent the establishment of latent persistent tuberculosis infection, as clearly demonstrated by the immense numbers of latently infected individuals worldwide and the reactivation of clinical disease as a consequence of HIV infection and immune suppression[6,7]. Among the candidates in the first generation of new tuberculosis vaccines developed to replace or supplement BCG is a fusion of the two early secreted antigens, Ag85B and ESAT-6, that have an extensive track record both as individual vaccine antigens[4,8–11] and as the H1 fusion protein[4,11–14]. Although H1 as a booster may improve upon the efficacy of BCG, recent epidemiological modeling has highlighted the importance of reducing transmission by protecting infected people from progressing to active tuberculosis by vaccination either before or after exposure[15].

Mtb differs from many other pathogens in its ability to survive in an intracellular habitat for years. It achieves this long-term intracellular persistence by controlling phagosomal maturation, preventing phagosomal fusion with the lysosome and reducing acidification of the phagosome[16]. To restict *Mtb* growth, infected monocytes attract additional monocytes, macrophages and T cells, and an organized aggregate (granuloma) is formed. Mimicking conditions thought to reflect the environment inside the granuloma *in vitro* and evaluating the transcriptional response has been the subject of intensive research in recent years, and strategies have included hypoxia[17,18] and nutrient starvation[19]. Our hypothesis is that it is possible to selectively target the bacteria in the persistent stage of infection by combining antigens with preventive vaccine activity, such as Ag85B and ESAT-6, with targets preferentially expressed as the bacteria adapt to long-term persistence in the immune host. Here we show that as *Mtb* adapts to persistence, it downregulates many genes characteristic of the early stage of infection. Among the genes that are expressed at unchanged levels in the early and late stages of infection is *Rv2660c*. Expression of this gene has previously been reported to be 80- to 300-fold increased in nutrient-starved cultures, making it the most strongly upregulated of all the nutrient starvation–induced genes identified[19], and *Rv2660c* is also part of the transcriptional response to hypoxia[18,20] and activated macrophages[21]. In this study we report on the design, construction and evaluation before and after *Mtb* exposure of a new multistage vaccine, H56, that combines Ag85B, ESAT-6 and Rv2660c.

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RESULTS

Gene expression in various stages of *Mtb* infection

We compared the gene expression profile of 28 selected *Mtb* genes in the early (weeks 2 and 3 after infection) and late (weeks 20 and 30 after infection) stages of infection in the lungs of CB6F1 mice infected by the aerosol route. We reverse-transcribed and amplified total RNA isolated from individuals mice before we quantified the PCR products in individual real-time PCR reactions[22,23]. Use of multiple internal controls allowed accurate quantification of transcripts in each sample across multiple specimens. The 28 genes represented markedly different classes of genes with roles in metabolism, stress responses or host-pathogen interactions.

In two independent studies, we found a reduction in the general expression profile in the later stages of infection, where 19 of the 28 genes, were expressed at a level at least 50% lower than in the early stages. The expression of the classical heat shock protein HspX (*hspX*, Rv2031c) was lower but still highly expressed at the late stage of infection (**Table 1**). Six genes (*fadE5*, *Rv0244c*; *sigE*, *Rv1221*; *Rv2030c*; *Rv2660c*; *sigB*, *Rv2710* and *ppsD*, *Rv2934*) were expressed at the same level in the early and late stages of infection (**Table 1**). Apart from *ppsD (Rv2934)*, these genes are all involved in stress responses.

Initial evaluation of H56 and its protein components

To construct the H56 vaccine, we purified the recombinant H56 fusion protein (Ag85B-ESAT6-Rv2660c) from *Escherichia coli* (**Fig. 1a**) and formulated it with a cationic adjuvant (CAF01)[14]. Groups of CB6F1 mice were vaccinated three times with doses from 0.01 to 10 μg. Six weeks later, we challenged the mice with *Mtb* (Erdman) by the aerosol route and enumerated bacteria in the lungs 6 weeks later (**Fig. 1b**). Vaccinated mice were protected at all H56 doses, but the best protection occurred with a 5-μg dose, resulting in a 1.25 ± 0.08 $\log_{10}$ reduction of lung colony-forming units (CFU) compared to the adjuvant control group ($P < 0.001$), but not significantly different from BCG (1.01 ± 0.12 $\log_{10}$).

After determining the optimal dose, we compared immune responses and protection by H56 and its individual components. In all groups, we found vaccine-specific responses, but the immunogenicity of the single proteins varied considerably, with Ag85B being the most immunogenic and Rv2660c being the least (**Fig. 1c**). H56 vaccination promoted a stronger response against all three components than vaccines based on the individual antigens. Six weeks after vaccination, we challenged the mice and determined the bacterial loads in their lungs 6 weeks later (**Fig. 1d**). Both Ag85B and ESAT-6 vaccination induced moderate protection, but only Ag85B was statistically different from the control

Table 1 Expression pattern of 28 selected *Mtb* strain H37Rv genes *in vivo*

			Experiment 1				Experiment 2	
Rv number[a]	Group	Function	3 weeks p.i. RGCN[b]	20 weeks p.i. RGCN[b]	Fold reduction[c]	*t* test *P* value	Fold reduction[c]	*t* test *P* value
Rv0005	IP	*gyrB*, DNA gyrase	708	187	3.8	0.0016	3.1	0.074
Rv0011c	CW	Conserved transmembrane protein	288	115	2.5	0.0315	2.0	0.1600
Rv0244c	LM	*fadE5*, lipid degradation	397	309	1.3	0.0148	1.2	0.0002
Rv0569	DosR	Conserved protein	879	287	3.1	0.0570	2.2	0.4982
Rv0640	IP	50S ribosomal protein L11	537	98	5.5	0.0000	3.0	0.0001
Rv0700	IP	30S ribosomal protein S10	377	58	6.5	0.0060	2.9	0.0168
Rv1221	Adapt	*sigE*, heat shock response	1.303	982	1.3	0.1867	0.6	0.3147
Rv1284	Starv	Starvation, pH stabilizer	656	155	4.2	0.0216	2.9	0.1108
Rv1793	Esx	*esxN*, ESAT-6 family protein	433	117	3.7	0.0016	2.1	0.103
Rv1886c[d]	Ag85	Antigen 85A–85C cell wall mycoloylation	339	83	4.1	0.0917	2.8	0.0169
Rv1926c	CW	MPT63, immunogenic protein	108	11	10.2	0.0051	5.2	0.0355
Rv1980c	CW	MPT64, immunogenic protein	514	61	8.4	0.0085	4.3	0.0488
Rv2005c	DosR	Conserved protein	439	142	3.1	0.0287	2.6	0.1196
Rv2030c	DosR	Conserved protein	534	520	1.0	0.9388	1.0	0.0077
Rv2031c	Adapt	*hspX*, chaperone	9874	3962	2.5	0.0154	1.4	0.0001
Rv2347	Esx	*esxP*, ESAT-6–like protein	350	82	4.3	0.0002	2.7	0.1271
Rv2497c	IM	*pdhA*, pyruvate dehydrogenase	301	46	6.5	0.0329	5.8	0.024
Rv2623	DosR	TB31.7, hypothetical protein	336	195	1.7	0.1517	2.0	0.080
Rv2660c	Starv	Hypothetical protein	956	860	1.1	0.7836	0.9	0.3755
Rv2710	Adapt	*sigB*, RNA polymerase σ factor	3782	2016	1.8	0.0342	1.1	0.0109
Rv2878	CW	MPT53, soluble secreted antigen	125	22	5.8	0.0000	3.4	0.1073
Rv2933	LM	*ppsC*, biosynthesis of polyketides	215	77	2.8	0.0224	2.6	0.6044
Rv2934	LM	*ppsD*, biosynthesis of polyketides	246	166	1.5	0.2942	1.1	0.0938
Rv3130c	DosR	*tgs1*, synthesis of triacylglycerol	4087	1945	2.1	0.0185	1.4	0.0002
Rv3155	IM	NADH dehydrogenase I, respiration	5484	172	31.9	0.0856	5.2	0.1242
Rv3291c	Starv	Transcriptional regulator	2947	818	3.6	0.0308	2.6	0.0363
Rv3804c	Ag85	Antigen 85A, cell wall mycoloylation	193	61	3.2	0.0018	2.3	0.0002
Rv3875	Esx	*esxA*, ESAT-6 family protein, ESAT-6	3969	894	4.4	0.0005	2.8	0.0001

Genes that are part of the H56 vaccine are shown on a gray background. CW, cell wall associated; Starv, gene induced by nutrient starvation[18]; LM, lipid metabolism; Esx, part of one of the five Esx secretion systems.; IP, information pathway; IM, intermediary metabolism; DosR, part of the DosR regulon[20]; Adapt, involved in adaptation; Ag85, antigen 85; Vir, virulence.

[a]Genes are annotated as described on Tuberculist (http://tuberculist.epfl.ch/). [b]Relative gene copy numbers. Quantitative RT-PCR was used to measure the expression profile in lungs of individual mice after infection. [d]Because of high sequence homology, the results are given for the combination of Ag85A, Ag85B and Ag85C.

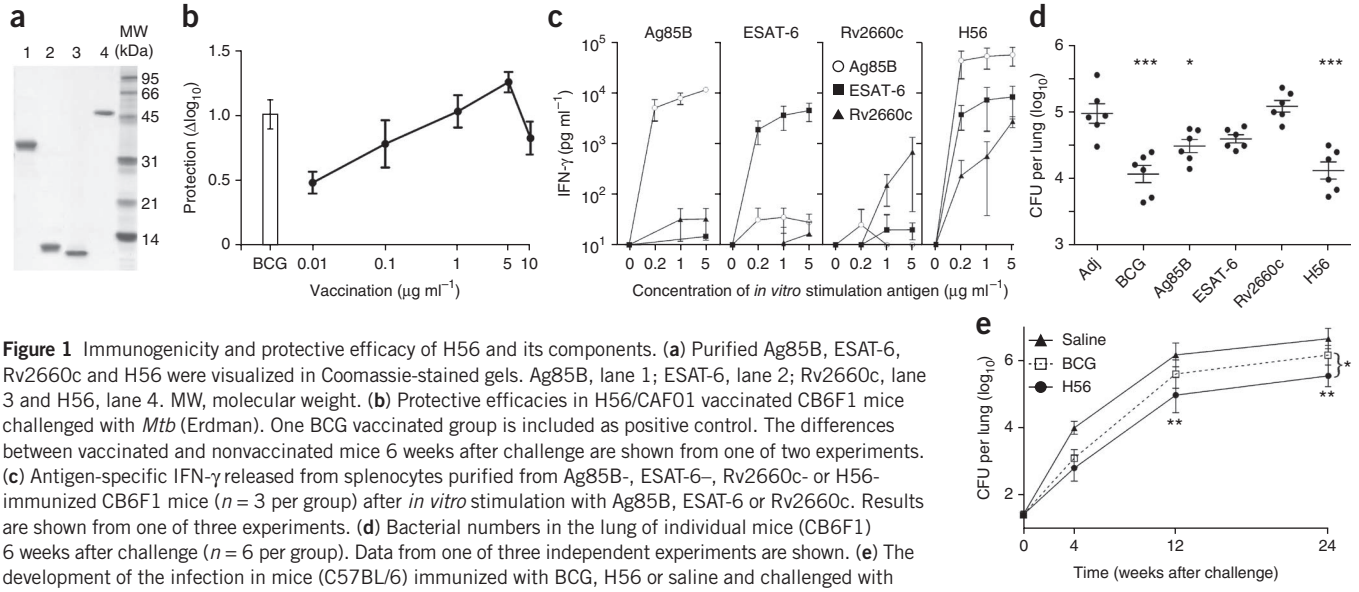

Figure 1 Immunogenicity and protective efficacy of H56 and its components. (**a**) Purified Ag85B, ESAT-6, Rv2660c and H56 were visualized in Coomassie-stained gels. Ag85B, lane 1; ESAT-6, lane 2; Rv2660c, lane 3 and H56, lane 4. MW, molecular weight. (**b**) Protective efficacies in H56/CAF01 vaccinated CB6F1 mice challenged with *Mtb* (Erdman). One BCG vaccinated group is included as positive control. The differences between vaccinated and nonvaccinated mice 6 weeks after challenge are shown from one of two experiments. (**c**) Antigen-specific IFN-γ released from splenocytes purified from Ag85B-, ESAT-6–, Rv2660c- or H56-immunized CB6F1 mice ($n = 3$ per group) after *in vitro* stimulation with Ag85B, ESAT-6 or Rv2660c. Results are shown from one of three experiments. (**d**) Bacterial numbers in the lung of individual mice (CB6F1) 6 weeks after challenge ($n = 6$ per group). Data from one of three independent experiments are shown. (**e**) The development of the infection in mice (C57BL/6) immunized with BCG, H56 or saline and challenged with *Mtb* (H37Rv) was followed by enumerating the bacilli in the lung of individual mice ($n = 6$) over a period of 24 weeks. In **d** and **e**, one-way analysis of variance (ANOVA) was used for group comparisons. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to the adjuvant control group or BCG ({}). Data are means ± s.e.m.

group ($P < 0.05$). Rv2660c was of low immunogenicity (**Fig. 1c**) and did not protect the mice (**Fig. 1d**). In contrast, H56 vaccination efficiently controlled bacterial growth ($P < 0.001$) to the same degree as the BCG control vaccine (**Fig. 1d**). We then conducted a 24-week evaluation of H56 and BCG in mice challenged with *Mtb* (H37Rv). At all time points investigated, the H56-immunized group had significantly lower bacterial counts in the lungs than the control group ($P < 0.01$), but at the two late time points (12 and 24 weeks after challenge) H56 also performed significantly better than the BCG vaccine ($P < 0.05$) (**Fig. 1e**).

Comparative evaluation of H56 and H1

We decided to compare H56 vaccination to vaccination with the H1 fusion on which it is based. H56-vaccinated mice responded with higher levels of interferon-γ (IFN-γ) to both Ag85B and ESAT-6 than H1-vaccinated mice and also had a specific (IFN-γ) response against Rv2660c that was not present in the H1-vaccinated mice (**Fig. 2a**). All mice, including a BCG-vaccinated control group, were challenged with *Mtb*, and the course of the infection was followed for 24 weeks. At the time of challenge, we found strong responses to the vaccine components Ag85B and ESAT-6 in both H1- and H56-vaccinated mice, whereas we found no significant immune responses against these antigens in BCG-vaccinated mice (**Fig. 2b**). As the infection developed, the strength of the immune responses specific for all three vaccine antigens increased. In the initial phase of infection, the immune response to Rv2660c was at the same level as in nonvaccinated mice but from week 6 and onward we could detect an increasing response in H56-vaccinated mice (**Fig. 2b**). By flow cytometry we observed a large population of cytokine-positive, antigen-specific $CD4^+$ T cells that could be isolated from perfused lungs 6, 12 and 24 weeks after infection. The $CD4^+$ T cells were directed to all three components of H56 but had the

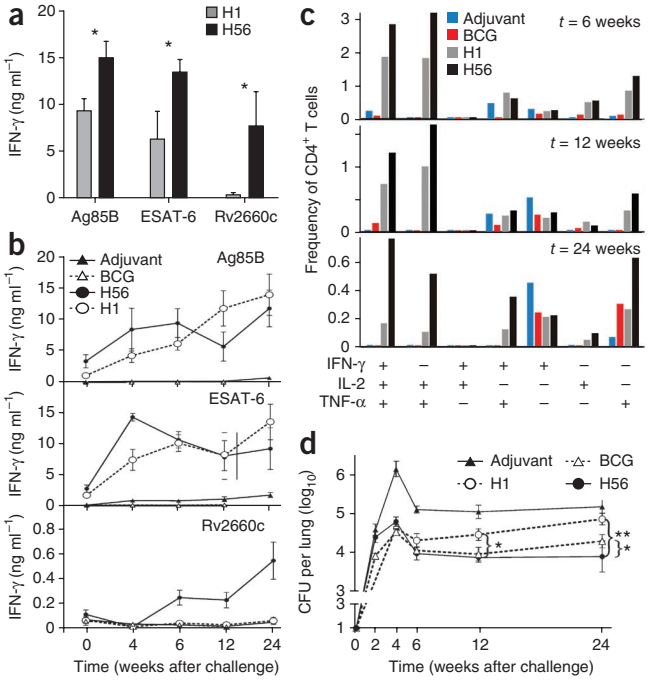

Figure 2 Immune responses and vaccine efficacy of H56 compared to H1. (**a**) Antigen-specific IFN-γ release from *in vitro*–stimulated splenocytes isolated from mice (CB6F1) vaccinated with H1 or H56. Representative results from one of three experiments are shown. $^*P < 0.05$, one-way ANOVA. (**b**) IFN-γ released from splenocytes isolated after challenge with *Mtb* (Erdman) from groups of CB6F1 mice vaccinated with CAF01 adjuvant, BCG, H1 or H56 and stimulated *in vitro* with the indicated antigens. Results from one of two independent experiments are shown. (**c**) The frequency of Ag85B-specific $CD4^+$ T cells ($CD44^{high}$) producing IFN-γ, TNF-α or IL-2 measured in cells isolated from perfused lungs from mice immunized with CAF01, BCG, H1 or H56. The cytokine profile in individual cells was measured by multicolor flow cytometry by gating for lymphocytes and $CD4^+$ T cells. All possible combinations of cytokine expression were tabulated, and, after subtracting the background (non stimulated samples), the results for the seven combinations expressing at least one of the cytokines are shown. Each of the panels **a**–**c** show results from the same experiment. Two independent experiments were performed. (**d**) The bacterial load (CFUs) in the lungs of individual mice. The results are pooled values from two experiments, and each time point represents results from 10–12 mice per group. $^*P < 0.05$; $^{**}P < 0.01$ using one-way ANOVA. Data are means ± s.e.m.

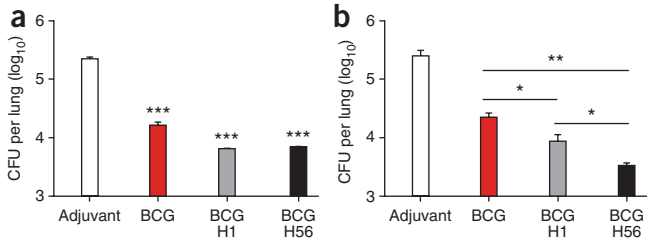

Figure 3 Evaluation of H56 as a BCG booster. (**a**,**b**) Bacterial numbers (CFUs) 6 (**a**) and 24 (**b**) weeks after challenge with *Mtb* (Erdman) in the lung ($n = 6$ per group) of mice (CB6F1) vaccinated with BCG and boosted twice with either H1 or H56. Representative data from one of two experiments are shown as $\log_{10}$ CFU. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$, one-way ANOVA with Tukey's post test. Data are means ± s.e.m.

strongest response to Ag85B and ESAT-6. We found no detectable vaccine-specific $CD8^+$ T cells in the lungs (**Supplementary Fig. 1**). H56-vaccinated mice preferentially accumulated Ag85B-specific, triple-positive (interleukin-12 (IL-2)$^+$ tumor necrosis factor-α (TNF-α)$^+$IFN-γ$^+$), double-positive (IL-2$^+$TNF-α$^+$ and IFN-γ$^+$TNF-α$^+$) and TNF-α single-positive $CD4^+$ T cells in the lung at weeks 6, 12 and 24 after infection (**Fig. 2c**). We also found polyfunctional $CD4^+$ T cell subsets in H1-vaccinated mice but the frequencies were lower at all time points (**Fig. 2c**). In the adjuvant and BCG vaccinated group, the majority of the infection-induced Ag85B- and ESAT-6–specific $CD4^+$ T cells were found in the IFN-γ$^+$ single-positive population. These differences were particularly evident at the late time point 24 weeks after infection (**Fig. 2c**).

The bacterial load in infected mice showed the same overall profile as we had seen before when comparing the H56 vaccine with BCG (**Fig. 1e**). In the early stage of infection (<week 4) the three vaccines gave a similar level of protection, and the bacterial numbers were reduced by approximately 1.5 $\log_{10}$ in all vaccination groups at week 4 after infection relative to the saline control group (**Fig. 2d**). However, from week 12 onward, H56 was superior to H1 ($P < 0.05$) and at week 24 also to BCG ($P < 0.05$). The sustained H56 vaccine activity at a time point where both H1 and BCG had waning activity indicate an additional advantage of H56 and the Rv2660c antigen in late stages of infection.

Boosting BCG with H56 and H1

Three months after mice were vaccinated with BCG, we investigated the ability of H56 to boost the BCG vaccine. Peripheral blood mononuclear cells (PBMCs) showed minimal responses 1 week after the final vaccination in the BCG group, whereas both H1- and H56-boosted groups had Ag85B- and ESAT-6–specific responses, and H56-boosted mice also had a Rv2660c-specific response (**Supplementary Fig. 2**).

Six weeks after vaccination, the mice were challenged, and the bacterial counts in the lungs were enumerated 6 and 24 weeks after challenge. Six weeks after challenge, all mice in the BCG-vaccinated groups had significantly fewer bacilli in the lungs than the control mice ($P < 0.001$) (**Fig. 3a**). Both H1- and H56-boosted mice had slightly lower bacterial counts than the BCG-vaccinated mice (**Fig. 3a**). However, 24 weeks after infection, H56 boosting was better and the bacterial loads in these mice (1.87 ± 0.04 $\log_{10}$ CFU reduction) were significantly lower than both the BCG-vaccinated (1.05 $\log_{10}$ CFU reduction, $P < 0.01$) and the H1-boosted mice (1.46 ± 0.11 $\log_{10}$ CFU reduction, $P < 0.05$, **Fig. 3b**). In agreement with the observations above (**Fig. 2c**), the $CD4^+$ T cells recruited to the lungs in H56-boosted mice were predominantly vaccine-specific polyfunctional T cells. This confirms the ability of the H56 vaccine to promote high-quality (polyfunctional) T cell responses even in BCG-vaccinated mice. (data not shown).

Vaccination with H56 after exposure

To establish a model for vaccination after exposure, we infected mice by aerosol exposure followed by antibiotic treatment (**Fig. 4a**). After treatment, the bacterial load was low but we obtained robust

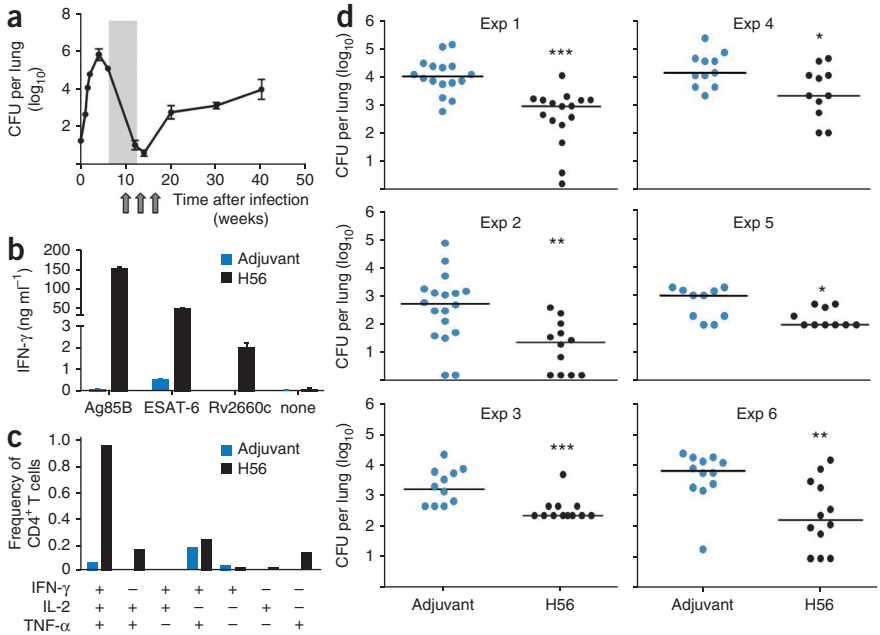

Figure 4 Vaccination with H56 after exposure. (**a**) The model used for evaluation of the H56 vaccine after exposure. Mice infected with *Mtb* (Erdman) were treated with antibiotic for 6 weeks (shaded area). After treatment, mice were killed and bacteria were enumerated in the lungs at the indicated time points ($n = 6$). The arrows indicate the vaccination time points used in **b**, **c** and **d**. $\log_{10}$ CFUs are given as mean values ± s.e.m. (**b**) IFN-γ released from PBMCs isolated 35 weeks after infection with *Mtb* (Erdman) from mice vaccinated at weeks 10, 13 and 16 and from nonvaccinated mice ($n = 16$ per group, pooled PBMCs). PBMCs were stimulated *in vitro* with Ag85B, ESAT-6 or Rv2660c (**c**) At the same time point, cytokine profiles of antigen-specific $CD4^+$ T cells were measured in Ag85B-stimulated splenocytes by flow cytometry as described in **Figure 2c**. (**d**) The protective efficacy of H56 was measured in two different laboratories. Experiments 1–3 were done in CB6F1 mice at Statens Serum Institut and experiments 4–6 were done in C57BL/6 mice at Unitat de Tuberculosi Experimental. In experiments 1–3, bacteria were enumerated in the lungs of individual mice 35 weeks (exp. 1 and 3) and 43 (exp. 2) weeks after challenge. In experiments 1 and 2, mice received two vaccinations, and in experiment 3, three. In experiments 4–6, mice received two vaccinations, and the bacterial load was measured 23 weeks after infection. The CFU values are shown as scattered plots with the median indicated ($n = 12$–16 per group in each experiment). We used the Mann-Whitney *U* test for comparison among groups. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Data are means ± s.e.m.

reactivation from week 22 after infection onward. In six independent experiments, we used H56 formulated in CAF01 adjuvant to vaccinate mice after exposure. We measured vaccine-induced immune responses in PBMCs 35 weeks after infection (**Fig. 4b**). In agreement with the hierarchy of responses found after preventive vaccination with H56 (**Fig. 1c**), vaccination after exposure with H56 gave a T cell response that was focused toward Ag85B and ESAT-6, with modest recognition of Rv2660c. Adjuvant control mice were characterized by a low but detectable response against ESAT-6 (**Fig 4b**). Thirty-five weeks after infection, we characterized vaccine-specific $CD4^+$ T cells (there was no specific recognition in the $CD8^+$ subset) by flow cytometry. H56 vaccination resulted in a four- to six-fold increase in antigen-specific $CD4^+$ T cells compared to control mice (from 0.2–0.3% to 1–1.5% of accumulated cytokine-positive cells) (**Fig. 4c**). The H56 vaccine–specific T cells were polyfunctional $CD4^+$ T cells expressing IFN-γ, IL-2 and TNF-α (data are shown for Ag85B, but ESAT-6 gave a similar pattern) (**Fig. 4c** and data not shown). This distribution was in clear contrast to the control mice, in whom the infection-induced T cell response towards Ag85B and ESAT-6 was at a much lower frequency and almost exclusively found among IFN-γ^+TNF-α^+ double-positive $CD4^+$ T cells (**Fig. 4c**).

We evaluated the protective efficacy of H56 vaccination after exposure in mice killed 23–43 weeks after infection by assessing the bacterial load in lung homogenates from individual mice. In six independent experiments performed in two different laboratories, we found that H56 consistently provided a significant degree of protection against reactivation in mice (**Fig. 4d**). This was demonstrated in both the CB6F1 mouse model and in a similar model established in C57BL/6 mice (**Fig. 4d**). We found some variation in the bacterial burden, but there was statistically significant ($P < 0.05$) protection in all experiments (0.8–1.6 $\log_{10}$ reduction of CFU). Histological examination of lung samples showed normal lung tissue with a few scattered, small, lymphocyte-rich granulomas in both vaccinated and adjuvant control groups (data not shown). In some experiments the tendency was fewer and smaller granulomas in the vaccinated group, but these differences were not significant.

DISCUSSION

We have presented a vaccine strategy designed to improve the long-term containment of *Mtb* and prevent reactivation by vaccination either before or after exposure. We hypothesize that it is possible to target the bacteria in the late stage of infection and prevent reactivation by combining early protective antigens (here Ag85B and ESAT-6) with proteins that are preferentially expressed as the bacteria adapt to long-term persistence in a host with a fully developed adaptive immune system. We selected Rv2660c because it is associated with the major transcriptional response as the bacteria adapt to nutrient starvation[19] and because we found here that it is stably expressed in late stages of infection characterized by reduced overall transcription level of most of the genes analyzed. We have shown that vaccination both before and after exposure with the triple fusion H56 results in an immune response that encompasses all three protein components and protects mice against tuberculosis. Protection was evident in the various phases of the infection up to at least 24 weeks after infection. In the late persistent stage of infection, the effect of adding Rv2660c was particularly pronounced, and we found the H56 vaccine to be almost ten times more efficient at reducing the bacterial number than the H1 vaccine. In agreement with the activity of H56 against the late stages of infection, we observed a detectable recognition of Rv2660c from week 6 of infection. Notably, we detected this response only in mice primed with the Rv2660c-containing vaccine. Rv2660c is of low immunogenicity in CB6F1 mice, and our data suggest that whereas responses to this antigen remain below the detection level during primary *Mtb* infection, prior vaccination with H56 primes a Rv2660c-specific response that is recalled during infection. BCG expression of Rv2660c has been shown *in vitro*[24], but, owing to its attenuation, BCG probably does not persist long enough to promote a response to latency antigens[25]. Nevertheless, late-stage vaccine activity was also evident in BCG boost experiments; H1 and H56 gave similar levels of protection at week 6, but H56 boosted a response that was significantly more efficient in the late stages of infection. We also found that H56 had a very substantial activity when administered after exposure in modified Cornell models[26], and it promoted immune responses that contained the infection with CFU counts ten to 50 times below that found in adjuvant control mice.

We have shown that the late persistent stage of infection is characterized by reduced transcription of prominent antigens that are expressed during the early stage of infection and that genes involved in protein translation or metabolism are downregulated, indicating a lower metabolic activity and replication. Six genes (*fadE5*, *sigE*, *Rv2030c*, *Rv2660c*, *sigB* and *ppsD*) are expressed at the same level in the early and late stages of infection, and, with the exception of *ppsD*, these genes are all involved in stress responses that characterize the long-term adaptation to a host with a fully developed adaptive immune response. The σ factors are key bacterial transcription regulators implicated in the adaptation of *Mtb* to environmental changes, intracellular stress and bacterial virulence[27,28]. Rv2030c is part of the DosR regulon induced by hypoxic conditions[29]. FadE5 is involved in the lipid degradation that is the primary source of energy and building blocks during latency. The function of Rv2660c is presently unknown, but the protein is highly upregulated under nutrient starvation[19] and in the enduring response to hypoxia[18], both conditions thought to reflect the intracellular conditions in the persistent stage of infection. *In vivo* expression profiles from *Mtb* residing in human granulomas suggest that *Rv2660c* is expressed during the latent persistent stage of human disease (http://www.tbdb.org/cgi-bin/data/rtpcr.pl?sd=GC11h&ex=Dan2&s=submit), and recent data have shown that latently infected individuals have a higher and more frequent Rv2660c-specific T cell response than individuals with active tuberculosis disease[30].

Rv2660c does not confer a protective immune response on its own in our studies, something we observed both at early (**Fig. 1d**) and late (data not shown) time points. Although we do not fully understand this discrepancy, we believe it relates to immunogenicity and T cell help. Rv2660c has a low immunogenicity in CB6F1 mice, but the immune responses are amplified five-to tenfold by incorporating it into H56, and that may bring responses up to protective levels. This is in agreement with previous observations where improved immunogenicity was obtained by incorporating ESAT-6 or TB10.4 (a small ESAT-6 family protein) into fusions constructs containing the highly immunogenic Ag85B molecule[4,31,32]. It is possible that Rv2660c only reaches its true potential in concert with immunogenic antigens, because the immune response toward these early antigens provides sufficient IL-2 to expand clones of late-reactive T cells.

Recent reports from a number of disease models have shown that T cells with multiple effector functions, such as the concomitant production of IFN-γ, TNF-α and IL-2, are functionally superior to their single-positive counterparts[33–38]. We used multiparameter flow here to analyze the quality of the T cell response at the site of infection in the mice immunized with the various vaccines. These analyses showed that the vaccine promoted $CD4^+$ T cells that were almost exclusively polyfunctional, and after post-exposure vaccination these

cells replaced the IFN-γ^+TNF-α^+ double-positive effector cells characteristic of the infection-driven response. The background for the induction and maintenance of polyfunctional T cells possibly relates to the use of the CAF01 adjuvant system. CAF01 is known to efficiently target antigens to activated dendritic cells over prolonged periods of time[14], resulting in strong and long-lived polyfunctional $CD4^+$ memory T cells[39]. On the basis of the unique activity found after vaccination with H56 before and after exposure, it is our hope that reactivation and, consequently, outbreak of active tuberculosis could be delayed and transmission reduced, and we have therefore recently initiated clinical development of H56.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

C.A. conceived of the study, produced H56, conducted preexposure vaccine studies and prepared the manuscript. T.H. developed the CB6F1 latency model and conducted latency studies in this model. J.D. conducted the BCG boost studies. P.-J.C. developed the C57BL/6 latency model and conducted latency studies in this model. A.I. conducted preexposure vaccine studies. G.D. designed and performed gene expression analyses. G.K.S. designed and performed gene expression analyses. J.P.C. performed histological evaluation of lung specimens. R.B. contributed to the latency vaccine studies. P.A. conceived of the study and prepared the manuscript. All authors discussed the results and commented on the manuscript at all stages.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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ONLINE METHODS

Mice. We obtained CB6F1 mice from Harlan Scandinavia and C57BL/6 mice from Charles River Laboratories or Harlan Iberica. All experiments performed in Denmark were done according to Danish Ministry of Justice and Animal Protection Committees, Danish Ministry of Justice and in compliance with European Community Directive 86/609. Experiments carried out in Spain were approved and supervised by the Animal Care Committee of Germans Trias i Pujol University Hospital in agreement with EU laws. At Colorado State University, all procedures were approved by the Colorado State University Institutional Animal Care and Use Committee.

Cloning and purification. We amplified *Rv2660c* from *M. tuberculosis* H37Rv chromosomal DNA with the 5′-**GGATGTTCGCA**GTGATAGCGGGCGTC GAC-3′ and 5′-TTAGTGAAACTGGTTCAATCCCAGTATC-3′ primers and fused it to *Ag85B-ESAT6*[4]. Stop codon is underlined, and the sequence overlap to the ESAT-6 sequence is in bold. *E. coli* BL21-SI cells (Invitrogen) harboring either *Ag85B, ESAT-6, Rv2660c, Ag85B-ESAT6* or *Ag85B-ESAT6-Rv2660c* produced the recombinant proteins as aggregated inclusion bodies. These cells were washed three times in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% deoxycholic acid and dissolved in 8 M urea, 100 mM Na_2PO_4, 10 mM Tris-HCl pH 8.0 (buffer A) before being applied to metal affinity columns (Clontech). Bound proteins were washed five times by alternating between 10 mM Tris-HCl pH 8.0, 60% isopropanol and 50 mM NaH_2PO_4 pH 8.0 before being eluted in buffer A supplemented with 200 mM imidazole. Pooled fractions were dialyzed against 3 M urea, 10 mM Tris-HCl pH 8.5 and applied to anion-exchange columns (Pharmacia) before being eluted and dialyzed against 25 mM NaH_2PO_4 pH 8.0, 10% glycerol, 150 mM NaCl, 0.05% Tween20. Finally, we determined protein concentrations and analyzed the purity by scanning stained gels.

Vaccine evaluations. PBMCs and splenocytes were isolated and cultured, and immune responses were measured as previously described[4,39]. Antigens were emulsified in cationic liposomes[14] and used for vaccination in doses from 0.01 to 10 μg. Mice were vaccinated with 200 μl subcutaneously at the base of the tail three times, 2 weeks apart. Negative control mice received three equivalent doses of CAF01, and positive control mice received a single dose of 5×10^6 CFU BCG Danish 1331 (Statens Serum Institut). When H56 was used as a BCG booster vaccine, mice were vaccinated with 5×10^6 CFU BCG, rested for 3 months and then vaccinated twice with 5 μg H56 in CAF01 2 weeks apart. Ten weeks after the first vaccination, virulent *M. tuberculosis* Erdman or H37Rv were delivered via the respiratory route at approximately 100 CFU per mouse. Six mice per group were killed at various time points and lung homogenate from single mice plated at threefold serial dilutions. For vaccine evaluation after exposure, mice infected for 6 weeks were treated with isoniazid (100 mg ml^{-1}) and rifabutin (100 mg ml^{-1}) (Becton Dickinson) in the drinking water for 6 weeks. Immunizations were commenced at week 10 after infection and given two or three times with 3 weeks' interval. Control groups received CAF01 without antigen. Mice were killed 23, 35 or 43 weeks after infection, and CFUs from 12–16 mice per group were determined. We established a similar model in C57BL/6 mice at Unitat de Tuberculosi Experimental Badalona. Mice were infected with *M. tuberculosis* H37Rv, and the infection was controlled by a weekly oral administration of rifapentine (10 mg per kg body weight) and isoniazid (25 mg per kg body weight) at weeks 6–16 after infection.

Transcriptional analysis. Lungs were homogenized, and unbroken *M. tuberculosis* bacilli were resuspended in Trizol and lysed by bead beating (MP Biomedicals). Total RNA was isolated (Qiagen), precipitated, cleaned by two DNase digestions (Promega) and resuspended in 50 μl RNase-free water (Applied Biosystems). Reverse transcription was performed in 20-μl reactions with 8.5 μl total RNA, 12.5 μM random hexamers, 0.5 mM dNTPs, 20 U RNase inhibitor (Fermentas) and 0.5 μl PowerScript with provided buffers (Clontech). Multiplex amplifications were performed on each sample with 6 μl cDNA, 1 mM dNTPs and 78 nM of each gene-specific primer sets (ftp://smd-ftp.stanford.edu/tbdb/rtpcr/taqman_oligos.fa). PCR conditions were 95 °C for 5 min followed by 15 cycles of 95 °C for 30 s, 60 °C for 20 s and 68 °C for 1 min. Second amplifications were performed with 6 μl from each first-amplification reaction for ten cycles with the same PCR conditions as in the first amplification. Finally, quantitative real-time PCR was performed with 0.05 μl of the second multiplex reaction and a gene-specific primer and probe set in 10 μl 1X Probes Master (Roche Applied Science). The final concentration was 0.3 mM for each of the primers and 0.1 mM for the probe.

Statistical analyses. Immune responses and protective efficacies of the vaccines were compared by one-way ANOVA followed by Tukey's multiple comparison test of the means. For vaccination after exposure, the protective efficacies were compared by a non-parametric Mann–Whitney *U* test. The expression data from the early and late time points of infection were compared by a *t* test for the individual genes. $P < 0.05$ was considered statistically significant.