A live attenuated vaccine confers better protection than BCG against

*Mycobacterium tuberculosis* Beijing in mice

Florence Levillain\(^a,\#\), Hongmin Kim\(^b,\#\), Kee Woong Kwon\(^b\), Simon Clark\(^c\), Felipe Cia\(^d\), Wladimir Malaga\(^b\), Priscille Brodin\(^a\), Brigitte Gicquel\(^f,\#\), Christophe Guilhot\(^b\), Gregory J. Bancroft\(^d\), Ann Williams\(^c\), Sung Jae Shin\(^b\), Yannick Poquet\(^a,\#\#\), Olivier Neyrolles\(^a,\#\#\,*

\(^a\) Institut de Pharmacologie et Biologie Structurale, IPBS, Université de Toulouse, CNRS, UPS, Toulouse, France

\(^b\) Department of Microbiology, Institute for Immunology and Immunological Disease, Yonsei University College of Medicine, Seoul, South Korea

\(^c\) Public Health England, Salisbury, United Kingdom

\(^d\) London School of Hygiene and Tropical Medicine, London, United Kingdom

\(^e\) University of Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, Center for Infection and Immunity of Lille, Lille, France

\(^f\) Unité de Génétique Mycobactérienne, Institut Pasteur, Paris, France

\(^g\) Department of Tuberculosis Control and Prevention, Shenzhen Nanshan Center for Chronic Disease Control, Shenzhen, China

\(^\#\) These authors contributed equally to the work

\(^\#\#\) These authors co-supervised the work

\(*\) Corresponding author. E-mail address: olivier.neyrolles@ipbs.fr

Keywords: Tuberculosis, *Mycobacterium tuberculosis*, BCG, Vaccine
ABSTRACT

Tuberculosis still claims more lives than any other pathogen, and a vaccine better than BCG is urgently needed. One of the challenges for novel TB vaccines is to protect against all Mycobacterium tuberculosis lineages, including the most virulent ones, such as the Beijing lineage. Here we developed a live attenuated M. tuberculosis mutant derived from GC1237, a Beijing strain responsible for tuberculosis outbreaks in the Canary Islands. The mutant strain is inactivated both in the Rv1503c gene, responsible for surface glycolipid synthesis, and in the two-component global regulator PhoPR. This double mutant is as safe as BCG in immunodeficient SCID mice. In immune-competent mice and guinea pigs, the mutant is as protective as BCG against M. tuberculosis strains of common lineage 4 (Euro-American). By contrast, in mice the vaccine is protective against a M. tuberculosis strain of lineage 2 (East-Asian, Beijing), while BCG is not. These results highlight differences in protection efficacy of live attenuated M. tuberculosis-derived vaccine candidates depending on their genetic background, and provide insights for the development of novel live vaccines against TB, especially in East-Asian countries where M. tuberculosis strains of the Beijing family are highly dominant.
1. Introduction

With an estimated 1.6 million deaths in 2017 according to the World Health Organization, tuberculosis (TB) is among the top 10 causes of death worldwide, and the leading cause of death from a single pathogen [1]. The only TB vaccine currently in use, Bacillus Calmette Guérin (BCG), is known to protect efficiently against disseminated forms of TB in infants [2], and even protects against other childhood infectious diseases, possibly through trained immunity-related mechanisms [3]. Due to their broad antigen content, long-lasting and natural adjuvant properties, live attenuated vaccines, such as BCG, are generally considered most promising for conferring durable immunity, compared to subunit vaccines [4]. Protection conferred by BCG was reported to last up to 10-15 years, and even longer in some instances [5-7]. Yet, the efficacy of BCG to prevent *Mycobacterium tuberculosis* infection or TB reactivation in adults is too variable, ranging from nil to 80%, and novel vaccines or vaccination strategies against TB are highly needed [2, 8, 9]. BCG revaccination at adolescence was long considered a possible strategy to boost protection conferred by BCG administered at birth. Several large-scale studies showed that BCG revaccination confers only modest, if any, improved protection [10, 11], most likely due to immune sensitization following pre-exposure to environmental mycobacteria [8, 12, 13]. These results were challenged by a recent study conducted in South Africa that reported BCG revaccination protected against sustained *M. tuberculosis* infection, as reflected by sustained QuantiFERON-TB Gold In-tube assay conversion, with an efficacy of 45% [14]. The apparent discrepancy between these results and those from previous studies are thought to be due, at least in part, to the low level of exposure to environmental mycobacteria in the Cape Town area. This will nevertheless need to be evaluated, and these results will need to be confirmed in other settings and on longer time periods. In the meantime, and given the
variability of efficacy of BCG, there is a strong rationale for development of alternative live attenuated vaccines that would perform better than BCG.

To be considered for advancing in the preclinical pipeline, live vaccine candidates other than BCG need to be at least as safe as BCG and more effective than BCG, considered as a benchmark, in small animal models of TB, such as mice and guinea pigs [15].

A few live vaccine candidates based on recombinant BCG and attenuated *M. tuberculosis* are currently in preclinical or clinical development [8, 16, 17]. These include VPM1002, a recombinant BCG strain expressing listeriolysin and lacking the urease component UreC [18-21]; and MTBVAC, a *M. tuberculosis* mutant inactivated in FadD26, involved in the synthesis of the virulence lipids PDIM, and the master transcriptional regulator PhoP [22-25].

Noteworthy, such live vaccine candidates are also considered to be used as boosters, on the top of BCG, and MTBVAC already showed promise in this direction, significantly reducing TB disease [26].

One of the major challenges in TB vaccine development is to develop a vaccine that confers protection against all *M. tuberculosis* strains, including the most virulent ones, such as those of lineage 2 (East-Asian or Beijing) [27-29]. Indeed BCG is thought to poorly protect against strains of the Beijing lineage, which might explain, at least in part, the global spread of this lineage [30, 31]. This hypothesis is supported by experiments in TB animal models in some reports [32, 33], but not in others [34, 35], which calls for more studies in this context.

We recently isolated *M. tuberculosis* mutants generated in the GC1237 strain, which belongs to lineage 2, with an impaired capacity to prevent phagosome acidification in macrophages and to survive in these cells [36]. One of these mutants, inactivated in the Rv1503c gene, was affected in glycolipid synthesis and was found to be attenuated both in macrophages
and in vivo in mice [36]. Here we exploited this mutant to generate a double mutant in both Rv1503c and the phoPR operon, involved in mycobacterial virulence [37]. The resulting double mutant was found to be as safe as BCG in immune-deficient SCID mice and as protective as BCG against common strains of lineage 4 (e.g. the laboratory strain H37Rv) in mice and guinea pigs. Strikingly, this mutant was protective against a Beijing isolate, while BCG was not. These results highlight the importance of taking the genetic background of *M. tuberculosis* into consideration when generating novel live attenuated *M. tuberculosis*-based vaccines, and provide clues for the development of TB vaccines with broader efficacy against multiple strains and lineages of the TB bacillus.
2. Materials and methods

2.1 Media and bacterial strains

All *M. tuberculosis* strains and BCG (Danish strain 1331) were grown in Middlebrook 7H9 culture medium (Difco, Sparks MD) supplemented with 10% albumin-dextrose-catalase (ADC, Difco), glycerol, 0.05% Tween 80, kanamycin (25 µg/mL) and/or hygromycin (50 µg/mL) in the case of the mutants. *M. tuberculosis* GC1237 and the Rv1503c transposon insertion mutant (Rv1503c::Tn) were previously described [36].

2.2 Construction of the *phoPR* deletion mutant

Construction of the GC1237 Rv1503c::TnΔphoPR double mutant was performed using the thermosensitive bacteriophage phWM27 constructed previously [38]. This construct harbours a DNA fragment overlapping the *phoPR* genes from H37Rv and carrying a deletion/insertion replacing the 3’ end of *phoP* and the 5’end of *phoR* by a hygromycin-resistance marker. This fragment was cloned into the recombinant bacteriophage phAE87 [39]. The phWM27 bacteriophage was transferred into the recipient strain GC1237 Rv1503::Tn as described previously [39] and hygromycin resistance colonies were selected on 7H11 OADC Hyg (50µg/ml) agar plates incubated at 37°C. Six individual clones were analysed by PCR amplification using primers phoE (5’-CTTGTCGATCAGTCCGCCT-3’) and phoF (5’-GACACGAAAGCAGCAACCC-3’), located upstream and downstream respectively on the *M. tuberculosis* genome from the DNA fragment carried by the phWM27 bacteriophage, and the *hyg* gene specific primers H1 (5’-GGGATCGCAATCTCTAGCCTAGC-5’) and H2 (5’-GCCTTCACCTTCTGCAC-3’). One clone exhibiting the expected PCR amplification profile for allelic replacement was retained for further studies and named GC1237 Rv1503c::TnΔphoPR.

2.2 Ethics and animal experiments
Intranasal safety experiments in SCID mice and protection assays in C3H/HeNRj mice were conducted in strict accordance with French laws and regulations in compliance with the European community council directive 68/609/EEC guidelines and its implementation in France. All protocols were reviewed and approved by the Comité d’Ethique Midi-Pyrénées (reference MP/03/07/04/09) and the Comité d’Ethique FRBT (APAFIS #11404).

Intravenous safety experiments in CB17 SCID mice and protection assays in CB6F1 mice and in guinea pigs were approved by the UK Home Office (HO) regulations for animal experimentation which requires a HO-approved licence and approval from local ethical committees of Public Health England, Porton Down (Licence number PPL30/3236) and London School of Hygiene and Tropical Medicine (LSHTM) Animal Welfare and Ethical Review Board (Authorization # 70/6934).

Immunogenicity and efficacy experiments in C57BL/6J mice infected with Beijing and non-Beijing TB strains, were carried out accordingly following the guidelines of the Korean Food and Drug Administration (KFDA). The experimental protocols used in this study were reviewed and approved by the Ethics Committee and Institutional Animal Care and Use Committee (Permit Number: 2015-0041) of the Laboratory Animal Research Center at Yonsei University College of Medicine (Seoul, Korea).

2.3 Safety assays

For intranasal testing of residual virulence, 3 groups of severe combined immunodeficient (SCID) mice were infected via the intranasal route with *M. tuberculosis* wild-type (GC1237, Beijing strain), or the Rv1503c::Tn (500 CFU/animal) or 1,000 CFUs BCG Pasteur. For intravenous assays, two groups of 8, female CB17 SCID mice were subjected to challenge with a single dose of bacteria (nominally $10^6$ CFU/mouse) of either BCG Danish 1331 or the candidate vaccines GC1237 Rv1503c::Tn or GC1237 Rv1503c::TnΔphoPR, in a total volume of
200 μL diluted in pyrogen-free sterile saline. Actual number of bacteria administered to each group was assessed by CFU counting of the diluted inocula on the day of challenge (see Figure legend). Percentage of body weight change was calculated over time. Data are presented as survival curves compared to the gold standard BCG Danish 1331 using the Kaplan-Meier method. Statistical differences were assessed using GraphPad Prism 7.01 software using the Log-Rank (Mantel-Cox) test with Bonferroni correction with statistical significance considered to be a P value equal or smaller than 0.05.

### 2.4 Protection assay in mice

For protection assays in CB6F1/Crl mice, groups of 6, female mice aged 6-8 weeks were used. The following three experimental groups were evaluated: saline, BCG Danish 1331, and GC1237 Rv1503c::TnΔphoPR. Mice were vaccinated subcutaneously with 5x10⁶ CFU/mouse in 100 μL pyrogen-free sterile saline. Six weeks after vaccination mice were subjected to an aerosol challenge with *Mycobacterium tuberculosis* H37Rv aiming for an infective dose level of 100 CFU/mouse. Lungs and spleens from mice infected with H37Rv were harvested 6 weeks after challenge. For logistic reasons lungs were homogenized and plated on the same day as harvest on complete 7H11 agar plates, while spleens were kept refrigerated overnight and homogenates were prepared prior to plating corresponding dilutions for CFU counting. The CFU data obtained were analysed using One-way ANOVA statistical test followed by Tukey’s test for multiple comparison tests to compare mean values of the various experimental groups. GraphPad Prism 7.01 software was used for the statistical analysis. A P value equal or less than 0.05 was considered significant.

For protection assays in C3H/HeNRj mice, 3 groups of 6 8–10 weeks old females were vaccinated subcutaneously (100 μL) with 10⁶ CFU of the vaccine strains in PBS (Saline, BCG Danish or GC1237Rv1503c::TnΔphoPR). Eight weeks post vaccination, mice were intranasally
challenged with 500 CFU of H37Rv in 25 μL of PBS. Bacterial burden was assessed 4 weeks post challenge by plating homogenized lungs on solid medium.

For comparative protection assays against *M. tuberculosis* M2 and HN878, 6-7-week old female C57BL/6 mice were used. The following three experimental groups were evaluated against *M. tuberculosis* M2 and HN878: saline, BCG Pasteur 1173P2, and GC1237 Rv1503c::TnΔphoPR. Mice were vaccinated subcutaneously with 1x10^6 CFU/mouse in 300 μl pyrogen-free sterile saline. Nine weeks after vaccination mice were subjected to an aerosol challenge with *M. tuberculosis* strain M2 or HN878 aiming for an infective dose level of 200-250 CFU/mouse. The protective efficacy of BCG Pasteur 1173P2, and GC1237 Rv1503c::TnΔphoPR against *M. tuberculosis* strain M2 and HN878 was determined at 6 weeks from challenge through analysis of the histopathology and bacterial growth in the lung and spleen. For the lung histopathology analysis, the right-superior lobes were preserved overnight in 10% formalin and embedded in paraffin. The lung was sectioned at 4–5 μm and stained with H&E. For the bacterial growth analysis, the lung and spleen were homogenized, and serially diluted samples were plated onto Middlebrook 7H11 agar plates (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 10% OADC (Difco Laboratories), 2 μg/ml 2-thiophenecarboxylic acid hydrazide (Sigma-Aldrich, St. Louis, MO, USA) and amphotericin B (Sigma-Aldrich). After incubation at 37°C for 3-4 weeks, the bacterial colonies were counted. The comparison of lung inflamed area and CFU data obtained were analysed using One-way ANOVA statistical test followed by Tukey’s test for multiple comparison tests to compare mean values of the various experimental groups. GraphPad Prism 7.01 software was used for the statistical analysis. A *P* value equal or less than 0.05 was considered significant.
2.5 Protection assay in guinea pigs

Animals were individually identified using subcutaneously implanted microchips (Plexx, the Netherlands) to enable blinding of the analyses wherever possible. Group sizes were determined by statistical power calculations (Minitab, version 16) performed using previous data (SD, approximately 0.5) to reliably detect a difference of $1.0 \log_{10}$ in the mean number of colony-forming units (CFU) per millilitre. Groups of eight female Dunkin-Hartley guinea-pigs (250 g) were s.c. vaccinated with saline, BCG Danish 1331 ($5 \times 10^4$ CFU in 250 µl), or the GC1237 Rv1503c::Tn$\Delta$phoPR vaccine candidate ($5 \times 10^6$ CFU in 100 µl). Six weeks after, animals were aerosol-challenged with a low dose (10–50 CFU/animal) of *M. tuberculosis* H37Rv, generated from a suspension at $3 \times 10^6$ CFU/mL using a modified Henderson apparatus and AeroMP control unit. Four weeks post-challenge, animals were euthanized by intraperitoneal injection of sodium pentobarbital (Dolethal, Vetoquinol UK Ltd) and lungs and spleen were removed aseptically. The spleen minus a small apical section and the combined left apical, cardiac, right cardiac and right diaphragmatic lung lobes were homogenized in 5 and 10 mL sterile water, respectively. Serial dilutions were plated (0.1 mL per plate, in duplicate) on Middlebrook 7H11 selective agar (bioMerieux UK Ltd). After 3–4 weeks incubation at 37 °C, colonies were counted to measure CFU/mL of homogenate. Total CFU was calculated by multiplying CFU/mL by the homogenate volume. Where no colonies were observed, a minimum detection limit was set by assigning a count of 0.5 colonies, equating to 5 CFU/mL. Samples for histopathology were processed and analysed as described [40]. Pair-wise analysis of the log transformed CFU values was performed using the Mann-Whitney non-parametric test to compare between the groups. The histopathology scores for the lung were the product of a subjective scoring system [40]. Therefore,
statistical analysis was not performed on these data, but a two-sample t-test was used to compare the number of lesions in the spleen.

2.6 Immunogenicity assays in mice

In order to evaluate immunogenicity of GC1237 Rv1503c::TnΔphoPR mutant, C57BL/6 mice were vaccinated with BCG or GC1237 Rv1503c::TnΔphoPR mutant subcutaneously (1 x 10^6 CFU/mouse). Nine weeks after vaccination, non-vaccinated, BCG- or GC1237 Rv1503c::TnΔphoPR mutant-vaccinated groups were sacrificed for analysis. The lungs and spleens were removed and used for the preparation of single-cell suspensions. Lung cell and splenocyte were treated with 2 µg/ml PPD for 12 h, and the level of IFN-γ secreted from the lung and spleen cells were measured with ELISA. For the analysis of functional CD4^+ and CD8^+ T cells secreting IFN-γ, TNF-α, and IL-2, individual lung and spleen cells were prepared and cultured with stimulation of with 2 µg/ml PPD for 12 h in the presence of GolgiPlug and GolgiStop (BD, Bioscience). First, the cells were washed with PBS, and the Fc receptor was blocked with anti-CD16/32 blocking antibody at 4°C for 15 min. Surface molecules were stained with fluorochrome-conjugated antibodies against Thy1.2, CD4, CD8 and CD44 and using the LIVE/DEAD™ Fixable Dead Cell Kit for 30 min at 4°C. The cells were then washed with PBS, fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 30 min at 4°C. The permeabilized cells were washed twice with Perm/Wash (BD Biosciences) and stained anti-IFN-γ, anti-TNF-α and anti-IL-2 Abs for 30 min at 4°C. Cells were washed twice with Perm/Wash and fixed with IC fixation buffer (eBioscience) for flow cytometry analysis.
3. Results and Discussion

We previously isolated several *M. tuberculosis* mutants defective for phagosomal maturation arrest and intracellular survival in macrophages [36]. These mutants were generated in a *M. tuberculosis* strain, GC1237, that belongs to the East-Asian/Beijing family (lineage 4), and which was responsible for TB outbreaks in the Canary Islands [41]. Two of these mutants, in Rv1503c and Rv1506c, were further characterized as defective in the biosynthesis of various surface glycolipids and were found attenuated in mice [36]. Based on these results, we sought to explore whether such mutants may represent promising live attenuated vaccine candidates for TB.

We further explored the residual virulence of the Rv1503c-inactivated mutant in immune-deficient SCID mice in two models: a mildly severe model of intranasal infection, and a more stringent model of intravenous infection. When given intranasally, the mutant was as safe as BCG over a 140-day period (Fig. 1A). However, the more stringent intravenous model revealed that this mutant retained significant virulence, with the infected animals starting to show signs of disease, as reflected by weight loss, and to die 40 days post-infection (Fig. 1B,C). For this reason, we sought to further attenuate the Rv1503c-inactivated mutant through genetic deletion of the two-component system PhoPR, which regulates a large number of virulence genes in *M. tuberculosis* [37, 42]. The double mutant was found as safe as BCG in the stringent intravenous model (Fig. 1D,E).

Next, the protective efficacy of the Rv1503c/phoPR double mutant against *M. tuberculosis* laboratory strain H37Rv (lineage 2) was assessed in two mouse lines and in guinea pigs. In CB6F1 mice, a cross between BALB/c and C57BL/6 mice, the mutant was as protective as BCG (Fig 2A,B). We also used C3H/HeNRj mice, which were recently showed to exhibit better protection against *M. tuberculosis* than BALB/c or C57BL/6 animals when vaccinated with
the live *M. tuberculosis*-derived vaccine MTBVAC [43]. In these mice, protection was observed with both BCG and the double Rv1503c/phoPR mutant (with *P* values of 0.09 and 0.07, respectively), however the double mutant protected equally to BCG against H37Rv (Fig 2C). Similar results were obtained in guinea pigs, a widely used animal model to evaluate protective efficacy of live and subunit TB vaccine candidates. In this model, the Rv1503c/phoPR double mutant protected equally to BCG against *M. tuberculosis* H37Rv (Fig. 3). Altogether, these results indicate that the Rv1503c/phoPR double mutant is as protective as BCG against *M. tuberculosis* H37Rv in small rodent animal models.

The H37Rv strain belongs to the Euro-American lineage (lineage 4) of *M. tuberculosis*. Because our vaccine candidate was generated in a Beijing background, we next sought to assess whether it could confer better protection than BCG against *M. tuberculosis* strains of this lineage. Indeed, BCG is reported to poorly protect against *M. tuberculosis* Beijing in several animal models [32, 33], and it has been suggested that the same might happen in humans, which may explain, at least in part, the global spread of this lineage worldwide [30, 31].

C57BL/6J mice were immunized with either saline, BCG or the Rv1503c/phoPR double mutant. Pre-infection immunogenicity analysis revealed that the double mutant induced an increased production of IFNγ, compared to BCG, in the lungs and spleen (Fig. 4A). The mutant also induced more TNFα-producing effector CD4+ T cells in the lungs, and more multi-functional effector CD4+ T cells in the spleen, compared to BCG (Fig. 4B). More TNFα-producing CD8+ T cells were also observed in the spleen of mice vaccinated with the double mutant, compared to BCG (Fig. 4B).

Nine weeks after vaccination, vaccinated mice were challenged with *M. tuberculosis* M2, a strain of the Euro-American lineage 4, or HN878, a Beijing strain of the East-Asian lineage 4.
Six weeks later, lungs and spleen were recovered for CFU and histo-pathological analyses. As previously reported [44], BCG did not confer significant protection against the Beijing strain of *M. tuberculosis* (Fig. 5A). Remarkably, the GC1237 Rv1503c/phoPR double mutant conferred significant long-term (15 weeks post-vaccination) protection to both the M2 and the HN878 strains (Fig. 5A). As previously reported [45], lung cells from mice infected with the HN878 Beijing strain produced less IFN\(\gamma\) than lung cells from mice infected with the non-Beijing strain, when restimulated *ex vivo* with PPD (Fig. 5B). Nevertheless, increased protection against HN878 conferred by the Rv1503c/phoPR double mutant was accompanied by an increased production of IFN\(\gamma\) by lung cells from the mutant-vaccinated mice, compared to unvaccinated mice (\(P=0.0015\)) or to BCG-vaccinated mice (\(P=0.09\); Fig. 5B). In line with bacterial growth control results, Rv1503c/phoPR double mutant vaccination resulted in slightly better restoration of inflamed lesion than BCG vaccination in HN878 infected mice, but BCG vaccination displayed more mitigated lung lesion than Rv1503c/phoPR double mutant vaccination in M2 infected mice compared to non-vaccinated animals (Fig. 5C,D).

4. Conclusion

To our knowledge our report is the first to date of a live attenuated *M. tuberculosis*-derived vaccine candidate generated in a Beijing/lineage 2 background. Although this vaccine confers equal protection to BCG against *M. tuberculosis* H37Rv and M2 strains, which both belong to lineage 4, in mice and guinea pigs, it confers protection against HN878, a Beijing strain, while BCG does not, at least in mice. This may be explained, in part, by differential antigen expression in *M. tuberculosis* Beijing and non-Beijing strains, such as that reported in the DosR regulon [46, 47], which will require further investigation. These
results suggest that combining live attenuated TB vaccines generated in multiple genetic backgrounds might be a promising approach to develop a multivalent vaccine with broader efficacy against all *M. tuberculosis* strains, including the Beijing family that is particularly dominant in Eastern Europe and South-East Asia.

Acknowledgments

This work was supported by the European Commission (contracts NEWTBVAC n°241745 and TBVAC2020 n°643381), Centre National de la Recherche Scientifique, Université Paul Sabatier, Agence Nationale de la Recherche (ANR-11-EQUIPEX-0003), Fondation pour la Recherche Médicale (contract DEQ2016 0334902), Fondation Bettencourt Schueller. The funding bodies did not have a role on the study design, collection, analysis or interpretation of the data or the writing of the manuscript or in the decision to publish the manuscript.

This work was supported by the Department of Health, UK. The views expressed in this publication are those of the authors and not necessarily those of the Department of Health.

The authors acknowledge P. Constant, F. Moreau, and C. Berrone (IPBS, BSL3 laboratories) and the Genotoul ANEXPLO and the Biological Investigations Group (PHE) facilities.

Authors' contribution


Conflict of interests

The authors declare no conflicting interests.
References


Legends to figures

**Figure 1. Safety of the vaccine candidates.** (A) SCID mice (n=5 per group) were infected intranasally with 1,000 CFUs of BCG (Pasteur), 500 CFUs of the single mutant Rv1503c::Tn [36], or 500 CFUs of the GC1237 parental strain. Mice were killed when reaching the humane endpoint, defined as the loss of >20% of bodyweight in accordance with ethics committee guidelines. The median survival time was of 62 days for mice infected with GC1237. (B,C) SCID mice (n=8 per group) were infected intravenously with saline, 10^6 CFUs of BCG Danish (1331) or 10^6 CFUs of the single mutant Rv1503c::Tn. Mice were killed (B) when reaching the humane endpoint, defined as the loss of >20% of bodyweight (C), in accordance with ethics committee guidelines. The median survival time was of 42 days for mice infected with GC1237 Rv1503c::Tn. (D,E) SCID mice (n=8 per group) were infected intravenously with 3x10^5 CFUs of BCG Danish (1331) or 7x10^5 CFU of the double mutant Rv1503c::TnΔphoPR. Mice were killed (D) when reaching the humane endpoint, defined as the loss of >20% of bodyweight (E), in accordance with ethics committee guidelines. The median survival time was of 79 days for mice infected with BCG, and of 79.5 days for mice infected with GC1237 Rv1503c::TnΔphoPR. **, P<0.01; ***, P<0.001; ****, P<0.0001.

**Figure 2. Efficacy studies in mice against M. tuberculosis H37Rv infection.** (A,B) CB6F1 mice (n=6 per group) were vaccinated subcutaneously with 1.3x10^6 CFU BCG Danish (1331) or 7x10^6 CFU the GC1237 Rv1503c::TnΔphoPR strain. Control mice received saline. Six weeks after vaccination, mice were subjected to an aerosol challenge with M. tuberculosis H37Rv aiming for an infective dose level of 100 CFU/mouse. Lungs (A) and spleens (B) from infected mice infected were harvested 6 weeks after challenge and homogenates were prepared prior to plating onto 7H11 medium for CFU scoring. (C) C3H/HeNRj mice (n=6 per group)
were vaccinated subcutaneously with $10^6$ CFU BCG Danish (1331) in PBS (100uL) or $10^6$ CFU the GC1237 Rv1503c::TnΔphoPR strain. Control mice received saline. Eight weeks after vaccination, mice were subjected to intranasal challenge with *M. tuberculosis* H37Rv aiming for an infective dose level of 200 CFU/mouse. Lungs from infected mice were harvested 4 weeks after challenge and homogenates were prepared prior to plating onto 7H11 medium for CFU scoring. Data show mean and S.E.M.

**Figure 3. Efficacy studies in guinea pigs against *M. tuberculosis* H37Rv infection. (A, B)** Dunkin Hartley guinea pigs (n=8 per group) were vaccinated subcutaneously with $5 \times 10^4$ CFU BCG Danish (1331) or $5 \times 10^6$ CFU GC1237 Rv1503c::TnΔphoPR strain. Control guinea pigs received saline. 16 weeks after vaccination, guinea pigs were subjected to a nose-only aerosol challenge with *M. tuberculosis* H37Rv aiming for an infective dose level of 10-20 CFU/animal. Lungs (A and B left) and spleens (A and B right) from infected guinea pigs infected were harvested 4 weeks after challenge. Homogenates were prepared from lung and spleen prior to plating onto Middlebrook 7H11 agar medium for CFU scoring (A, left and right, respectively). Lung and spleen sections from the same animals were also taken for histological examination (B, left and right respectively)). Data show mean and S.E.M. ns, not significant; *, P<0.05; **, P<0.01.

**Figure 4. Immunogenicity studies in mice.** C57BL/6 mice (n=6 per group) were vaccinated subcutaneously with $10^6$ CFU BCG Pasteur (1173P2) or $10^6$ CFU of the GC1237 Rv1503c::TnΔphoPR mutant. Control mice received saline. Nine weeks after vaccination, lungs and spleen were harvested for immunogenicity study. (A) Lung and spleen cells were
stimulated with PPD (2 μg/mL) at 37°C for 12 h. IFNγ production was quantified by ELISA in the cell culture supernatant. (B) Lung (upper panels) and spleen (lower panels) cells were stimulated with PPD (2 μg/mL) at 37°C for 12 hours in the presence of GolgiStop and GolgiPlug and stained for FACS analysis. The frequency of IFNγ-, TNFα- and IL-2-producing CD4+CD44+ (left panels) or CD8+CD44+ (right panels) T cells was determined by intracellular cytokine staining. Data show mean and S.E.M. *, P<0.05; **, P<0.01; ***, P<0.001.

Figure 5. Efficacy and immunogenicity studies in mice infected with M. tuberculosis M2 and HN878. Mice were vaccinated as in Fig. 4. Nine weeks after vaccination, mice were subjected to an aerosol challenge with M. tuberculosis M2 or HN878 aiming for an infective dose level of 200 CFU/mouse. Six weeks after challenge, mice were sacrificed and lungs were collected for analysis. (A) Lungs were homogenized and plated onto 7H11 medium for CFU scoring. (B) IFNγ production by isolated lung cells was performed as in Fig. 4A. (C) The lung lesions were visualized by H&E staining of the superior lobe of the right lung. The percentage and area (mm²) of inflamed area was calculated and shown in dot graphs (D). Data show mean and S.E.M. **, P<0.01; ***, P<0.001.