A live attenuated vaccine confers better protection than BCG against *Mycobacterium tuberculosis* Beijing in mice

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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

68 variability of efficacy of BCG, there is a strong rationale for development of alternative live
69 attenuated vaccines that would perform better than BCG.

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

73 A few live vaccine candidates based on recombinant BCG and attenuated *M. tuberculosis* are
74 currently in preclinical or clinical development [8, 16, 17]. These include VPM1002, a
75 recombinant BCG strain expressing listeriolysin and lacking the urease component UreC [18-
76 21]; and MTBVAC, a *M. tuberculosis* mutant inactivated in FadD26, involved in the synthesis
77 of the virulence lipids PDIM, and the master transcriptional regulator PhoP [22-25].
78 Noteworthy, such live vaccine candidates are also considered to be used as boosters, on the
79 top of BCG, and MTBVAC already showed promise in this direction, significantly reducing TB
80 disease [26].

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

87 We recently isolated *M. tuberculosis* mutants generated in the GC1237 strain, which belongs
88 to lineage 2, with an impaired capacity to prevent phagosome acidification in macrophages
89 and to survive in these cells [36]. One of these mutants, inactivated in the Rv1503c gene,
90 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::Tn∆*phoPR* 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'-GGGATCGCCAATCTCTACG-5') and H2 (5'-GCCTTCACCTTCCTGCAC-3'). One clone exhibiting the expected PCR amplification profile for allelic replacement was retained for further studies and named GC1237 Rv1503c::Tn∆*phoPR*.

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::TnΔ*phoPR*, 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::Tn*ΔphoPR*. Mice were vaccinated subcutaneously with $5x10^6$ 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 10^6 CFU of the vaccine strains in PBS (Saline, BCG Danish or GC1237Rv1503::Tn*ΔphoPR*). 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::Tn∆*phoPR*. 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::Tn∆*phoPR* 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.

197 **2.5 Protection assay in guinea pigs**

198 Animals were individually identified using subcutaneously implanted microchips (Plexx, the
199 Netherlands) to enable blinding of the analyses wherever possible. Group sizes were
200 determined by statistical power calculations (Minitab, version 16) performed using previous
201 data (SD, approximately 0.5) to reliably detect a difference of 1.0 $\log_{10}$ in the mean number
202 of colony-forming units (CFU) per millilitre. Groups of eight female Dunkin-Hartley guinea-
203 pigs (250 g) were s.c. vaccinated with saline, BCG Danish 1331 ($5x10^4$ CFU in 250 µl), or the
204 GC1237 Rv1503c::Tn∆*phoPR* vaccine candidate ($5x10^6$ CFU in 100 µl). Six weeks after,
205 animals were aerosol-challenged with a low dose (10–50 CFU/animal) of *M. tuberculosis*
206 H37Rv, generated from a suspension at $3x10^6$ CFU/mL using a modified Henderson
207 apparatus and AeroMP control unit. Four weeks post-challenge, animals were euthanized by
208 intraperitoneal injection of sodium pentobarbital (Dolethal, Vetoquinol UK Ltd) and lungs
209 and spleen were removed aseptically. The spleen minus a small apical section and the
210 combined left apical, cardiac, right cardiac and right diaphragmatic lung lobes were
211 homogenized in 5 and 10 mL sterile water, respectively. Serial dilutions were plated (0.1 mL
212 per plate, in duplicate) on Middlebrook 7H11 selective agar (bioMerieux UK Ltd). After 3–4
213 weeks incubation at 37 °C, colonies were counted to measure CFU/mL of homogenate. Total
214 CFU was calculated by multiplying CFU/mL by the homogenate volume. Where no colonies
215 were observed, a minimum detection limit was set by assigning a count of 0.5 colonies,
216 equating to 5 CFU/mL. Samples for histopathology were processed and analysed as
217 described [40]. Pair-wise analysis of the log transformed CFU values was performed using
218 the Mann-Whitney non-parametric test to compare between the groups. The histopathology
219 scores for the lung were the product of a subjective scoring system [40]. Therefore,

220 statistical analysis was not performed on these data, but a two-sample *t*-test was used to
221 compare the number of lesions in the spleen.
222 **2.6 Immunogenicity assays in mice**
223 In order to evaluate immunogenicity of GC1237 Rv1503c::Tn∆*phoPR* mutant, C57BL/6 mice
224 were vaccinated with BCG or GC1237 Rv1503c::Tn∆*phoPR* mutant subcutaneously (1 x 10^6
225 CFU/mouse). Nine weeks after vaccination, non-vaccinated, BCG- or GC1237
226 Rv1503c::Tn∆*phoPR* mutant-vaccinated groups were sacrificed for analysis. The lungs and
227 spleens were removed and used for the preparation of single-cell suspensions. Lung cell and
228 splenocyte were treated with 2 μg/ml PPD for 12 h, and the level of IFN-γ secreted from the
229 lung and spleen cells were measured with ELISA. For the analysis of functional $CD4^+$ and
230 $CD8^+$ T cells secreting IFN-γ, TNF-α, and IL-2 , individual lung and spleen cells were prepared
231 and cultured with stimulation of with 2 μg/ml PPD for 12 h in the presence of GolgiPlug and
232 GolgiStop (BD, Bioscience). First, the cells were washed with PBS, and the Fc receptor was
233 blocked with anti-CD16/32 blocking antibody at 4°C for 15 min. Surface molecules were
234 stained with fluorochrome-conjugated antibodies against Thy1.2, CD4, CD8 and CD44 and
235 using the LIVE/DEAD™ Fixable Dead Cell Kit for 30 min at 4°C. The cells were then washed
236 with PBS, fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 30 min at
237 4°C. The permeabilized cells were washed twice with Perm/Wash (BD Biosciences) and
238 stained anti-IFN-γ, anti-TNF-α and anti-IL-2 Abs for 30 min at 4°C. Cells were washed
239 twice with Perm/Wash and fixed with IC fixation buffer (eBioscience) for flow cytometry
240 analysis.
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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

268 the live *M. tuberculosis*-derived vaccine MTBVAC [43]. In these mice, protection was
269 observed with both BCG and the double Rv1503c/*phoPR* mutant (with *P* values of 0.09 and
270 0.07, respectively), however the double mutant protected equally to BCG against H37Rv (Fig
271 2C). Similar results were obtained in guinea pigs, a widely used animal model to evaluate
272 protective efficacy of live and subunit TB vaccine candidates. In this model, the
273 Rv1503c/*phoPR* double mutant protected equally to BCG against *M. tuberculosis* H37Rv (Fig.
274 3). Altogether, these results indicate that the Rv1503c/*phoPR* double mutant is as protective
275 as BCG against *M. tuberculosis* H37Rv in small rodent animal models.
276 The H37Rv strain belongs to the Euro-American lineage (lineage 4) of *M. tuberculosis*.
277 Because our vaccine candidate was generated in a Beijing background, we next sought to
278 assess whether it could confer better protection than BCG against *M. tuberculosis* strains of
279 this lineage. Indeed, BCG is was reported to poorly protect against *M. tuberculosis* Beijing in
280 several animal models [32, 33], and it has been suggested that the same might happen in
281 humans, which may explain, at least in part, the global spread of this lineage worldwide [30,
282 31].
283 C57BL/6J mice were immunized with either saline, BCG or the Rv1503c/*phoPR* double
284 mutant. Pre-infection immunogenicity analysis revealed that the double mutant induced an
285 increased production of IFNγ, compared to BCG, in the lungs and spleen (Fig. 4A). The
286 mutant also induced more TNFα-producing effector $CD4^+$ T cells in the lungs, and more
287 multi-functional effector $CD4^+$ T cells in the spleen, compared to BCG (Fig. 4B). More TNFα-
288 producing CD8+ T cells were also observed in the spleen of mice vaccinated with the double
289 mutant, compared to BCG (Fig. 4B).
290 Nine weeks after vaccination, vaccinated mice were challenged with *M. tuberculosis* M2, a
291 strain of the Euro-American lineage 4, or HN878, a Beijing strain of the East-Asian linage 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 IFNγ 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 IFNγ 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.

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Authors' contribution

Conceptualization & Methodology, F.L., K.H., K.W.K, S.C., F.C., W.M., C.G., G.J.B., A.W., S.J.S., Y.P., and O.N.; Resources, P.B. and B.G.; Writing, F.L., Y.P. and O.N.; Proofreading and editing, S.C., A.W., and S.J.S.; Supervision, Y.P. and O.N.

Conflict of interests

The authors declare no conflicting interests.

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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 10^6CFUs 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::Tn∆*phoPR*. 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::Tn∆*phoPR*. **, *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::Tn∆*phoPR* 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)

493 were vaccinated subcutaneously with 10^6 CFU BCG Danish (1331) in PBS (100uL) or 10^6 CFU
494 the GC1237 Rv1503c::Tn∆*phoPR* strain. Control mice received saline. Eight weeks after
495 vaccination, mice were subjected to intranasal challenge with *M. tuberculosis* H37Rv aiming
496 for an infective dose level of 200 CFU/mouse. Lungs from infected mice were harvested 4
497 weeks after challenge and homogenates were prepared prior to plating onto 7H11 medium
498 for CFU scoring. Data show mean and S.E.M.
499
500 **Figure 3. Efficacy studies in guinea pigs against *M. tuberculosis* H37Rv infection. (A, B)**
501 Dunkin Hartley guinea pigs (n=8 per group) were vaccinated subcutaneously with $5x10^4$ CFU
502 BCG Danish (1331) or $5x10^6$ CFU GC1237 Rv1503c::Tn∆*phoPR* strain. Control guinea pigs
503 received saline. 16 weeks after vaccination, guinea pigs were subjected to a nose-only
504 aerosol challenge with *M. tuberculosis* H37Rv aiming for an infective dose level of 10-20
505 CFU/animal. Lungs (A and B left) and spleens (A and B right) from infected guinea pigs
506 infected were harvested 4 weeks after challenge. Homogenates were prepared from lung
507 and spleen prior to plating onto Middlebrook 7H11 agar medium for CFU scoring (A, left and
508 right, respectively). Lung and spleen sections from the same animals were also taken for
509 histological examination (B, left and right respectively)). Data show mean and S.E.M. ns, not
510 significant; *, P<0.05; **, P<0.01.
511
512 **Figure 4. Immunogenicity studies in mice.** C57BL/6 mice (n=6 per group) were vaccinated
513 subcutaneously with 10^6 CFU BCG Pasteur (1173P2) or 10^6 CFU of the GC1237
514 Rv1503c::Tn∆*phoPR* mutant. Control mice received saline. Nine weeks after vaccination,
515 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 (mm^2) of inflamed area was calculated and shown in dot graphs **(D)**. Data show mean and S.E.M. **, $P<0.01$; ***, $P<0.001$.