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## Preclinical assessment of a new live attenuated *Mycobacterium tuberculosis* Beijing-based vaccine for tuberculosis

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1 **A live attenuated vaccine confers better protection than BCG against**

2 ***Mycobacterium tuberculosis* Beijing in mice**

3

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26 **ABSTRACT**

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

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43

## 44 1. Introduction

45 With an estimated 1.6 million deaths in 2017 according to the World Health Organization,  
46 tuberculosis (TB) is among the top 10 causes of death worldwide, and the leading cause of  
47 death from a single pathogen [1]. The only TB vaccine currently in use, Bacillus Calmette  
48 Guérin (BCG), is known to protect efficiently against disseminated forms of TB in infants [2],  
49 and even protects against other childhood infectious diseases, possibly through trained  
50 immunity-related mechanisms [3]. Due to their broad antigen content, long-lasting and  
51 natural adjuvant properties, live attenuated vaccines, such as BCG, are generally considered  
52 most promising for conferring durable immunity, compared to subunit vaccines [4].  
53 Protection conferred by BCG was reported to last up to 10-15 years, and even longer in  
54 some instances [5-7]. Yet, the efficacy of BCG to prevent *Mycobacterium tuberculosis*  
55 infection or TB reactivation in adults is too variable, ranging from nil to 80%, and novel  
56 vaccines or vaccination strategies against TB are highly needed [2, 8, 9]. BCG revaccination at  
57 adolescence was long considered a possible strategy to boost protection conferred by BCG  
58 administered at birth. Several large-scale studies showed that BCG revaccination confers  
59 only modest, if any, improved protection [10, 11], most likely due to immune sensitization  
60 following pre-exposure to environmental mycobacteria [8, 12, 13]. These results were  
61 challenged by a recent study conducted in South Africa that reported BCG revaccination  
62 protected against sustained *M. tuberculosis* infection, as reflected by sustained  
63 QuantiFERON-TB Gold In-tube assay conversion, with an efficacy of 45% [14]. The apparent  
64 discrepancy between these results and those from previous studies are thought to be due,  
65 at least in part, to the low level of exposure to environmental mycobacteria in the Cape  
66 Town area. This will nevertheless need to be evaluated, and these results will need to be  
67 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

91 and *in vivo* in mice [36]. Here we exploited this mutant to generate a double mutant in both  
92 Rv1503c and the *phoPR* operon, involved in mycobacterial virulence [37]. The resulting  
93 double mutant was found to be as safe as BCG in immune-deficient SCID mice and as  
94 protective as BCG against common strains of lineage 4 (e.g. the laboratory strain H37Rv) in  
95 mice and guinea pigs. Strikingly, this mutant was protective against a Beijing isolate, while  
96 BCG was not. These results highlight the importance of taking the genetic background of *M.*  
97 *tuberculosis* into consideration when generating novel live attenuated *M. tuberculosis*-based  
98 vaccines, and provide clues for the development of TB vaccines with broader efficacy against  
99 multiple strains and lineages of the TB bacillus.

100

101

## 102 **2. Materials and methods**

### 103 **2.1 Media and bacterial strains**

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

### 109 **2.2 Construction of the *phoPR* deletion mutant**

110 Construction of the GC1237 Rv1503c::TnΔ*phoPR* double mutant was performed using the  
111 thermosensitive bacteriophage phWM27 constructed previously [38]. This construct  
112 harbours a DNA fragment overlapping the *phoPR* genes from H37Rv and carrying a  
113 deletion/insertion replacing the 3' end of *phoP* and the 5' end of *phoR* by a hygromycin-  
114 resistance marker. This fragment was cloned into the recombinant bacteriophage phAE87  
115 [39]. The phWM27 bacteriophage was transferred into the recipient strain GC1237 Rv1503  
116 ::Tn as described previously [39] and hygromycin resistance colonies were selected on 7H11  
117 OADC Hyg (50µg/ml) agar plates incubated at 37°C. Six individual clones were analysed by  
118 PCR amplification using primers *phoE* (5'-CTTGTCGATCAGTCCGCCT-3') and *phoF* (5'-  
119 GACACGAAAGCAGCAACCC-3'), located upstream and downstream respectively on the *M.*  
120 *tuberculosis* genome from the DNA fragment carried by the phWM27 bacteriophage, and  
121 the *hyg* gene specific primers H1 (5'-GGGATCGCCAATCTCTACG-5') and H2 (5'-  
122 GCCTTCACCTTCCTGCAC-3'). One clone exhibiting the expected PCR amplification profile for  
123 allelic replacement was retained for further studies and named GC1237 Rv1503c::TnΔ*phoPR*.

### 124 **2.2 Ethics and animal experiments**

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

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

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

### 142 **2.3 Safety assays**

143 For intranasal testing of residual virulence, 3 groups of severe combined immunodeficient  
144 (SCID) mice were infected via the intranasal route with *M. tuberculosis* wild-type (GC1237,  
145 Beijing strain), or the Rv1503c::Tn (500 CFU/animal) or 1,000 CFUs BCG Pasteur. For  
146 intravenous assays, two groups of 8, female CB17 SCID mice were subjected to challenge  
147 with a single dose of bacteria (nominally  $10^6$  CFU/mouse) of either BCG Danish 1331 or the  
148 candidate vaccines GC1237 Rv1503c::Tn or GC1237 Rv1503c::Tn $\Delta$ *phoPR*, in a total volume of



149 200  $\mu$ L diluted in pyrogen-free sterile saline. Actual number of bacteria administered to each  
150 group was assessed by CFU counting of the diluted inocula on the day of challenge (see  
151 Figure legend). Percentage of body weight change was calculated over time. Data are  
152 presented as survival curves compared to the gold standard BCG Danish 1331 using the  
153 Kaplan-Meier method. Statistical differences were assessed using GraphPad Prism 7.01  
154 software using the Log-Rank (Mantel-Cox) test with Bonferroni correction with statistical  
155 significance considered to be a *P* value equal or smaller than 0.05.

#### 156 **2.4 Protection assay in mice**

157 For protection assays in CB6F1/Crl mice, groups of 6, female mice aged 6-8 weeks were  
158 used. The following three experimental groups were evaluated: saline, BCG Danish 1331,  
159 and GC1237 Rv1503c::Tn $\Delta$ *phoPR*. Mice were vaccinated subcutaneously with  $5 \times 10^6$   
160 CFU/mouse in 100  $\mu$ L pyrogen-free sterile saline. Six weeks after vaccination mice were  
161 subjected to an aerosol challenge with *Mycobacterium tuberculosis* H37Rv aiming for an  
162 infective dose level of 100 CFU/mouse. Lungs and spleens from mice infected with H37Rv  
163 were harvested 6 weeks after challenge. For logistic reasons lungs were homogenized and  
164 plated on the same day as harvest on complete 7H11 agar plates, while spleens were kept  
165 refrigerated overnight and homogenates were prepared prior to plating corresponding  
166 dilutions for CFU counting. The CFU data obtained were analysed using One-way ANOVA  
167 statistical test followed by Tukey's test for multiple comparison tests to compare mean  
168 values of the various experimental groups. GraphPad Prism 7.01 software was used for the  
169 statistical analysis. A *P* value equal or less than 0.05 was considered significant.

170 For protection assays in C3H/HeNRj mice, 3 groups of 6–10 weeks old females were  
171 vaccinated subcutaneously (100  $\mu$ L) with  $10^6$  CFU of the vaccine strains in PBS (Saline, BCG  
172 Danish or GC1237Rv1503::Tn $\Delta$ *phoPR*). Eight weeks post vaccination, mice were intranasally

173 challenged with 500 CFU of H37Rv in 25  $\mu$ L of PBS. Bacterial burden was assessed 4 weeks  
174 post challenge by plating homogenized lungs on solid medium.

175 For comparative protection assays against *M. tuberculosis* M2 and HN878, 6-7-week old  
176 female C57BL/6 mice were used. The following three experimental groups were evaluated  
177 against *M. tuberculosis* M2 and HN878: saline, BCG Pasteur 1173P2, and GC1237  
178 Rv1503c::Tn $\Delta$ phoPR. Mice were vaccinated subcutaneously with  $1 \times 10^6$  CFU/mouse in 300  $\mu$ l  
179 pyrogen-free sterile saline. Nine weeks after vaccination mice were subjected to an aerosol  
180 challenge with *M. tuberculosis* strain M2 or HN878 aiming for an infective dose level of 200-  
181 250 CFU/mouse. The protective efficacy of BCG Pasteur 1173P2, and GC1237  
182 Rv1503c::Tn $\Delta$ phoPR against *M. tuberculosis* strain M2 and HN878 was determined at 6  
183 weeks from challenge through analysis of the histopathology and bacterial growth in the  
184 lung and spleen. For the lung histopathology analysis, the right-superior lobes were  
185 preserved overnight in 10% formalin and embedded in paraffin. The lung was sectioned at  
186 4–5  $\mu$ m and stained with H&E. For the bacterial growth analysis, the lung and spleen were  
187 homogenized, and serially diluted samples were plated onto Middlebrook 7H11 agar plates  
188 (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 10% OADC (Difco  
189 Laboratories), 2  $\mu$ g/ml 2-thiophenecarboxylic acid hydrazide (Sigma-Aldrich, St. Louis, MO,  
190 USA) and amphotericin B (Sigma-Aldrich). After incubation at 37°C for 3-4 weeks, the  
191 bacterial colonies were counted. The comparison of lung inflamed area and CFU data  
192 obtained were analysed using One-way ANOVA statistical test followed by Tukey's test for  
193 multiple comparison tests to compare mean values of the various experimental groups.  
194 GraphPad Prism 7.01 software was used for the statistical analysis. A *P* value equal or less  
195 than 0.05 was considered significant.

196

## 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<sub>10</sub> 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<sup>4</sup> CFU in 250 µl), or the  
204 GC1237 Rv1503c::TnΔ*phoPR* vaccine candidate (5x10<sup>6</sup> 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<sup>6</sup> 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 $\Delta$ *phoPR* mutant, C57BL/6 mice  
224 were vaccinated with BCG or GC1237 Rv1503c::Tn $\Delta$ *phoPR* mutant subcutaneously ( $1 \times 10^6$   
225 CFU/mouse). Nine weeks after vaccination, non-vaccinated, BCG- or GC1237  
226 Rv1503c::Tn $\Delta$ *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  $\mu$ g/ml PPD for 12 h, and the level of IFN- $\gamma$  secreted from the  
229 lung and spleen cells were measured with ELISA. For the analysis of functional CD4<sup>+</sup> and  
230 CD8<sup>+</sup> T cells secreting IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 , individual lung and spleen cells were prepared  
231 and cultured with stimulation of with 2  $\mu$ g/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- $\gamma$ , anti-TNF- $\alpha$  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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242

243

### 244 3. Results and Discussion

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

253 We further explored the residual virulence of the Rv1503c-inactivated mutant in immune-  
254 deficient SCID mice in two models: a mildly severe model of intranasal infection, and a more  
255 stringent model of intravenous infection. When given intranasally, the mutant was as safe as  
256 BCG over a 140-day period (Fig. 1A). However, the more stringent intravenous model  
257 revealed that this mutant retained significant virulence, with the infected animals starting to  
258 show signs of disease, as reflected by weight loss, and to die 40 days post-infection (Fig.  
259 1B,C). For this reason, we sought to further attenuate the Rv1503c-inactivated mutant  
260 through genetic deletion of the two-component system PhoPR, which regulates a large  
261 number of virulence genes in *M. tuberculosis* [37, 42]. The double mutant was found as safe  
262 as BCG in the stringent intravenous model (Fig. 1D,E).

263 Next, the protective efficacy of the Rv1503c/*phoPR* double mutant against *M. tuberculosis*  
264 laboratory strain H37Rv (lineage 2) was assessed in two mouse lines and in guinea pigs. In  
265 CB6F1 mice, a cross between BALB/c and C57BL/6 mice, the mutant was as protective as  
266 BCG (Fig 2A,B). We also used C3H/HeNRj mice, which were recently showed to exhibit better  
267 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 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 IFN $\gamma$ , compared to BCG, in the lungs and spleen (Fig. 4A). The  
286 mutant also induced more TNF $\alpha$ -producing effector CD4<sup>+</sup> T cells in the lungs, and more  
287 multi-functional effector CD4<sup>+</sup> T cells in the spleen, compared to BCG (Fig. 4B). More TNF $\alpha$ -  
288 producing CD8<sup>+</sup> 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 lineage 4.

292 Six weeks later, lungs and spleen were recovered for CFU and histo-pathological analyses. As  
293 previously reported [44], BCG did not confer significant protection against the Beijing strain  
294 of *M. tuberculosis* (Fig. 5A). Remarkably, the GC1237 Rv1503c/*phoPR* double mutant  
295 conferred significant long-term (15 weeks post-vaccination) protection to both the M2 and  
296 the HN878 strains (Fig. 5A). As previously reported [45], lung cells from mice infected with  
297 the HN878 Beijing strain produced less IFN $\gamma$  than lung cells from mice infected with the non-  
298 Beijing strain, when restimulated *ex vivo* with PPD (Fig. 5B). Nevertheless, increased  
299 protection against HN878 conferred by the Rv1503c/*phoPR* double mutant was  
300 accompanied by an increased production of IFN $\gamma$  by lung cells from the mutant-vaccinated  
301 mice, compared to unvaccinated mice ( $P=0.0015$ ) or to BCG-vaccinated mice ( $P=0.09$ ; Fig.  
302 5B). In line with bacterial growth control results, Rv1503c/*phoPR* double mutant vaccination  
303 resulted in slightly better restoration of inflamed lesion than BCG vaccination in HN878  
304 infected mice, but BCG vaccination displayed more mitigated lung lesion than  
305 Rv1503c/*phoPR* double mutant vaccination in M2 infected mice compared to non-  
306 vaccinated animals (Fig. 5C,D).

307

#### 308 **4. Conclusion**

309 To our knowledge our report is the first to date of a live attenuated *M. tuberculosis*-derived  
310 vaccine candidate generated in a Beijing/lineage 2 background.

311 Although this vaccine confers equal protection to BCG against *M. tuberculosis* H37Rv and M2  
312 strains, which both belong to lineage 4, in mice and guinea pigs, it confers protection against  
313 HN878, a Beijing strain, while BCG does not, at least in mice. This may be explained, in part,  
314 by differential antigen expression in *M. tuberculosis* Beijing and non-Beijing strains, such as  
315 that reported in the DosR regulon [46, 47], which will require further investigation. These

316 results suggest that combining live attenuated TB vaccines generated in multiple genetic  
317 backgrounds might be a promising approach to develop a multivalent vaccine with broader  
318 efficacy against all *M. tuberculosis* strains, including the Beijing family that is particularly  
319 dominant in Eastern Europe and South-East Asia.

320

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332

### 333 **Authors' contribution**

334 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.,  
335 Y.P., and O.N.; Resources, P.B. and B.G.; Writing, F.L., Y.P. and O.N.; Proofreading and  
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337

### 338 **Conflict of interests**

339 The authors declare no conflicting interests.



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469 **Legends to figures**

470 **Figure 1. Safety of the vaccine candidates. (A)** SCID mice (n=5 per group) were infected  
471 intranasally with 1,000 CFUs of BCG (Pasteur), 500 CFUs of the single mutant Rv1503c::Tn  
472 [36], or 500 CFUs of the GC1237 parental strain. Mice were killed when reaching the  
473 humane endpoint, defined as the loss of >20% of bodyweight in accordance with ethics  
474 committee guidelines. The median survival time was of 62 days for mice infected with  
475 GC1237. **(B,C)** SCID mice (n=8 per group) were infected intravenously with saline, 10<sup>6</sup> CFUs  
476 of BCG Danish (1331) or 10<sup>6</sup>CFUs of the single mutant Rv1503c::Tn. Mice were killed (B)  
477 when reaching the humane endpoint, defined as the loss of >20% of bodyweight (C), in  
478 accordance with ethics committee guidelines. The median survival time was of 42 days for  
479 mice infected with GC1237 Rv1503c::Tn. **(D,E)** SCID mice (n=8 per group) were infected  
480 intravenously with 3x10<sup>5</sup> CFUs of BCG Danish (1331) or 7x10<sup>5</sup> CFU of the double mutant  
481 Rv1503c::Tn $\Delta$ phoPR. Mice were killed (D) when reaching the humane endpoint, defined as  
482 the loss of >20% of bodyweight (E), in accordance with ethics committee guidelines. The  
483 median survival time was of 79 days for mice infected with BCG, and of 79.5 days for mice  
484 infected with GC1237 Rv1503c::Tn $\Delta$ phoPR. \*\*, P<0.01; \*\*\*, P<0.001; \*\*\*\*, P<0.0001.

485

486 **Figure 2. Efficacy studies in mice against *M. tuberculosis* H37Rv infection. (A,B)** CB6F1 mice  
487 (n=6 per group) were vaccinated subcutaneously with 1.3x10<sup>6</sup> CFU BCG Danish (1331) or  
488 7x10<sup>6</sup> CFU the GC1237 Rv1503c::Tn $\Delta$ phoPR strain. Control mice received saline. Six weeks  
489 after vaccination, mice were subjected to an aerosol challenge with *M. tuberculosis* H37Rv  
490 aiming for an infective dose level of 100 CFU/mouse. Lungs (A) and spleens (B) from infected  
491 mice infected were harvested 6 weeks after challenge and homogenates were prepared  
492 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 $\Delta$ 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  $5 \times 10^4$  CFU  
502 BCG Danish (1331) or  $5 \times 10^6$  CFU GC1237 Rv1503c::Tn $\Delta$ 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 $\Delta$ 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

516 stimulated with PPD (2 µg/mL) at 37°C for 12 h. IFN $\gamma$  production was quantified by ELISA in  
517 the cell culture supernatant. **(B)** Lung (upper panels) and spleen (lower panels) cells were  
518 stimulated with PPD (2 µg/mL) at 37°C for 12 hours in the presence of GolgiStop and  
519 GolgiPlug and stained for FACS analysis. The frequency of IFN $\gamma$ -, TNF $\alpha$ - and IL-2-producing  
520 CD4<sup>+</sup>CD44<sup>+</sup> (left panels) or CD8<sup>+</sup>CD44<sup>+</sup> (right panels) T cells was determined by intracellular  
521 cytokine staining. Data show mean and S.E.M. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

522

523 **Figure 5. Efficacy and immunogenicity studies in mice infected with *M. tuberculosis* M2**

524 **and HN878.** Mice were vaccinated as in Fig. 4. Nine weeks after vaccination, mice were  
525 subjected to an aerosol challenge with *M. tuberculosis* M2 or HN878 aiming for an infective  
526 dose level of 200 CFU/mouse. Six weeks after challenge, mice were sacrificed and lungs were  
527 collected for analysis. **(A)** Lungs were homogenized and plated onto 7H11 medium for CFU  
528 scoring. **(B)** IFN $\gamma$  production by isolated lung cells was performed as in Fig. 4A. **(C)** The lung  
529 lesions were visualized by H&E staining of the superior lobe of the right lung. The percentage  
530 and area (mm<sup>2</sup>) of inflamed area was calculated and shown in dot graphs **(D)**. Data show  
531 mean and S.E.M. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

532