Deletion of zmp1 improves Mycobacterium bovis BCG-mediated protection in a guinea pig model of tuberculosis

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A B S T R A C T
Having demonstrated previously that deletion of zinc metalloprotease zmp1 in Mycobacterium bovis BCG increased immunogenicity of BCG vaccines, we here investigated the protective efficacy of BCG zmp1 deletion mutants in a guinea pig model of tuberculosis infection. zmp1 deletion mutants of BCG provided enhanced protection by reducing the bacterial load of tubercle bacilli in the lungs of infected guinea pigs. The increased efficacy of BCG due to zmp1 deletion was demonstrated in both BCG Pasteur and BCG Denmark indicating that the improved protection by zmp1 deletion is independent from the BCG sub-strain. In addition, unmarked BCG Δzmp1 mutant strains showed a better safety profile in a CB-17 SCID mouse survival model than the parental BCG strains. Together, these results support the further development of BCG Δzmp1 for use in clinical trials.

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1. Introduction

Tuberculosis (TB) caused by the bacterial pathogen Mycobacterium tuberculosis, is a major cause of human morbidity and mortality. It is estimated that 1.5 million people, among them 80,000 children, died of the disease in 2013. About 9 million new cases of TB, including 550,000 in children, occur each year and one third of the world’s population, 2 billion people, are latently infected according to WHO (Global Tuberculosis Report 2014, WHO; http://www.who.int/tb/publications/global_report/en/). Approximately 5% of these latently infected individuals will develop active TB at a later time point. WHO has declared elimination of TB by the year 2050 a global target [1]. The low decline in the incidence of TB observed in the past years is insufficient to reach this goal. Even the most stringent implementation of diagnostic techniques and medication currently available will not suffice.

Continuous in vitro passaging of Mycobacterium bovis, the causative agent of bovine tuberculosis, gave rise to the currently available attenuated TB live vaccine. Deletion of the Region of Difference 1 (RD1), encoding the specialized protein secretion system ESX-1, is considered the primary genetic alteration responsible for attenuation [2]. Named after its inventors, the live vaccine M. bovis bacille Calmette-Guérin (BCG) was first applied to humans in 1921. Still today, BCG is the only licensed TB vaccine and more than 4 billion doses have been applied [3]. BCG confers consistent protection against TB meningitis and disseminated TB in children. However, protective efficacy of BCG against the most prevalent form of TB, adult lung TB, is highly variable. Protective efficacy varied between 0% and 85% in different trials [4,5]. According to WHO, improvement of TB vaccines has high priority and is a corner stone in TB elimination.

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Vaccine development from bench to field is a long lasting process and the last decade witnessed considerable research efforts to develop more protective TB vaccines. Candidates include recombinant BCG [6], attenuated strains of M. tuberculosis [7], recombinant Mycobacterium smegmatis [8], subunit vaccines [9] and recombinant viruses [10]. Many more candidate vaccines have been explored, however, the attrition rate proved to be high [11,12]. A few advanced candidates are currently tested for safety in clinical trials. MVA85A, a recombinant strain of modified vaccinia virus Ankara expressing antigen 85A from M. tuberculosis was tested for its protective efficacy in a large scale clinical trial. MVA85A, given as a booster after primary vaccination with BCG, was well tolerated but failed to show a beneficial effect over BCG vaccination alone [13]. Clinical trials with recombinant BCG strains expressing Clostridium perfringens perfringolysin [14] were stopped because of adverse side effects [11]. Therefore, there is a continuing need for new TB vaccines to be developed which are both effective and safe.

M. tuberculosis open reading frame Rv0198c encodes the zinc metalloproteinase Zmp1. Rv0198c has considerable sequence and structural homology with human peptidases neprilysin and endothelin converting enzyme (ECE) [15]. Zmp1 preferentially cleaves small peptides and has an optimal activity at a slightly acidic pH [16]. Deletion of zmp1 attenuates M. tuberculosis, induces innate, inflammasome- and IL-1β-dependent immune mechanisms and increases the ratio of mycobacteria localizing in late endosomal compartments of infected macrophages instead of in early endosomal compartments [17]. In vitro and in vivo immunization experiments in mice indicated that a BCG zmp1 deletion mutant is more immunogenic than its parental strain [18]. In cattle, BCG zmp1 deletion mutant strains induced an enhanced antigen specific immune response [19] considered to be a correlate of protection [20]. Here, we report on the protective efficacy of BCG zmp1 deletion mutants in the guinea pig model of TB infection and the safety of the BCG zmp1 deletion mutants in a CB-17 SCID mouse survival model.

2. Materials and methods

2.1. Recombinant BCG zmp1 mutant strains

BCG Pasteur SmR zmp1::aph is a zmp1 deletion mutant of a streptomycin resistant BCG Pasteur strain. Two consecutive Nhel fragments within zmp1 were substituted by an aph cassette conferring kanamycin resistance. The strain has been described [17]. BCG Denmark Δzmp1 is a derivative of BCG Denmark resulting from transformation with zmp1 replacement vector pzm1SacBaphHyg. Besides the Nhel deletions, the zmp1 allele in pzm1SacBaphHyg carries a triple translation stop. In the vector, the Δzmp1 allele is flanked by a hygromycin and a kanamycin resistance cassette for (transient) positive selection and levan sucrose gene sacB [21] for negative selection. Integration and subsequent removal of vector backbone from transformants was checked by Southern blot analyses and antibiotic susceptibility testing [19]. Vector pzm1SacBaphHyg was also used to construct an unmarked Δzmp1 mutant in BCG Pasteur SmS strain named BCG Pasteur SmS Δzmp1. BCG Pasteur SmS was made electrocompetent, transformed with plasmid pzm1SacBaphHyg and transformants were selected on 7H10 OADC plates containing hygromycin (25 mg L⁻¹). Following counter selection of single cross over transformants on sucrose, deletion of the zmp1 locus and removal of the vector backbone was confirmed by Southern blot analysis and phenotypic (hygromycin, kanamycin) susceptibility testing.

2.2. Growth of mycobacteria

BCG strains were grown in Middlebrook 7H9 supplemented with oleic acid albumin dextrose catalase (OADC) (10% v/v) until OD₆₅₀=0.4 to 0.6. Bacterial aliquots were frozen and stored at −80 °C. Serial dilutions of thawed aliquots were plated on 7H10 OADC agar plates and the number of colony forming units (CFU) was determined after 3–4 weeks of incubation at 37 °C. The M. tuberculosis H37Rv (National Collection of Type Cultures (NCTC) 7416) challenge stock was generated from a chemostat culture grown to steady state in a defined medium which has been previously described [22].

2.3. Vaccination

Groups of 8 pathogen-free Dunkin–Hartley guinea pigs, weighing between 250 and 300 g, purchased from a commercial supplier (Harlan, UK), were used to evaluate the relative efficacy of vaccination with BCG zmp1 mutants and standard BCG Danish 1331 (Statens Serum Institute, Copenhagen, Denmark). BCG was administered subcutaneously in a single dose (5 × 10⁶ CFU). One vaccination group received phosphate buffered saline (PBS) as a negative control. Individual animals were identified using subcutaneously implanted microchips (PLEXX BV, The Netherlands). Guinea pig experimental work was conducted according to UK Home Office legislation for animal experimentation and was approved by the local ethics committee. All animals were weighed weekly and observed daily in order to monitor any adverse effects.

2.4. Aerosol challenge

Guinea pigs were infected with a low aerosol dose (10–50 CFU retained dose in the lung) of M. tuberculosis H37Rv [23] 12 weeks after vaccination. Aerosol challenge was performed using a fully contained Henderson apparatus as previously described [24,25] in conjunction with the AeroMP (Biacea) control unit [26]. Fine particle aerosols of M. tuberculosis H37Rv, with a mean diameter of 2 μm (diameter range 0.5–7 μm) [24] were generated using a Collison nebulizer and delivered directly to the animal snout. The aerosol was generated from a water suspension containing 5 × 10⁶ CFU/ml M. tuberculosis H37Rv. The Henderson apparatus allows controlled delivery of aerosols to the animals and the reproducibility of the system and relationship between inhaled CFU and the concentration of organisms in the nebulizer has been described previously [25].

2.5. Post mortem procedures

Four weeks after challenge, guinea pigs were euthanised by an intraperitoneal injection of pentobarbital sodium (Euthatal). A post-mortem examination was performed immediately. The thoracic “pluck” (comprising lung, trachea, heart and mediastinum) was dissected and removed. The left cranial, right cranial and right caudal lung lobes were placed in sterile containers for bacteriological analyses. The remaining tissue, including left middle, left caudal, right middle and right accessory lobes were fixed in 10% (v/v) neutral-buffered formalin (NBF) for histological examination. Sections of spleen were sampled separately either for bacteriological analysis or placed in 10% NBF for histological examination.

2.6. Histopathological examination

A representative sample from each lung lobes, sampled consistently between animals, and spleen, were processed routinely and embedded in paraffin wax. Sections (approximately 5 μm)
were stained with haematoxylin and eosin. Selected sections were stained with Alizarin red for the presence of calcium. The pathologist was blinded to the treatment groups.

2.7. Morphometric analyses of lung and spleen histology sections

For each animal, the area of lung granulomas relative to the total lung area was analysed with an image analysis software (Matlab Version 7.9.0.529, The MathWorks TM).

2.8. Bacterial analysis

Tissues for determination of CFU were homogenized in 5 ml of sterile deionized water using a rotating blade macerator system (Ystral, UK). Viable counts were performed on the macerate by preparing serial dilutions in sterile deionized water and plating 100 µl aliquots onto Middlebrook 7H11 OADC agar (BioMerieux, UK). Plates were incubated at 37 °C for 3 weeks before counting the number of M. tuberculosis colonies (CFU). Protection was primarily assessed by measuring bacterial load in lungs and spleen comparing the groups of animals vaccinated with BCG zmp1 mutants with the control groups (BCG Danish 1331 and buffered saline). A statistical significant reduction in bacterial load in the vaccinated animals when compared with the control groups was considered as a protective effect of the vaccine.

2.9. CB-17 SCID mouse infection

Groups of 7–8 female CB-17 SCID mice were intravenously challenged with a single dose of the candidate vaccines Pasteur SmSm Δzmp1, BCG Denmark Δzmp1 or the parental strains BCG Pasteur SmSm or BCG Denmark, in a total volume of 200 µl diluted in pyrogen-free saline. Titrated frozen stocks of mycobacteria were thawed, centrifuged and resuspended in saline and injected. Parallel aliquots from the prepared inoculum was plated for colony counts. Pellets of centrifugations were compact and looked comparable. The supernatants were clear. Bacterial suspensions were tested for lack of clumping prior to use in animals.

All animals included in the experiments were weighed at least once a week and observed daily in order to comply with ethical requirements and to monitor any potential adverse effects related to BCG administration. In line with the UK Home Office guidelines for welfare of experimental animals, animals were euthanized when they reached the defined endpoint of 20% weight loss.

2.10. Statistics

The CFU data from the protection experiments were analysed by non-parametric Mann–Whitney U-test to compare median values of the various experimental groups using Minitab software version 15. A p value of <0.05 was considered significant. Lung histom- etry data were represented graphically, compared and evaluated by Mann–Whitney U-test for statistically significant differences (p < 0.05) with the GraphPad Prism software. Survival curves and median survival times (MST) of each group of CB-17 SCID mice was compared using the Kaplan–Meier method. Statistical differences were assessed using GraphPad Prism using the Log Rank (Mantel–Cox) test.

3. Results

3.1. Protective efficacy of BCG Pasteur SmR zmp1::aph in guinea pigs

We have previously characterized a zmp1 deletion mutant of a streptomycin resistant derivative of BCG Pasteur [17], referred here to as BCG Pasteur SmR zmp1::aph. This mutant proved to be more immunogenic than its parental strain in various in vitro and in vivo immunological murine assays as indicated by increased presenta- tion of MHC class II-restricted antigens, increased and accelerated delayed-type hypersensitivity (DTH) response, antigen-specific lymphocyte proliferation and the frequency of antigen-specific interferon-γ producing lymphocytes [18].

In the absence of cor- relates of protection [27], vaccine candidates such as the zmp1 mutants described here, have to be tested in animal models to demonstrate their ability to protect against virulent challenge prior to further testing in clinical trials. The guinea pig has been used extensively for this purpose and has aided in identifying promising vaccine candidates [28]. To assess the protective effi- cacy of the BCG Pasteur SmR zmp1::aph strain in the guinea pig model a proof of concept study was conducted. In a head to head comparison, guinea pigs were vaccinated subcutaneously with BCG Pasteur SmR zmp1::aph, BCG Danish (standard positive control) or saline (negative control). The vaccine candidate was well tolerated, as was the comparator. The weight of the vaccinated animals increased similar as the weight of the animals in the non-vaccinated control group. Twelve weeks later, the guinea pigs were challenged with a low aerosol dose (10–50 CFU) of M. tuberculosis H37Rv. Four weeks post challenge, guinea pigs were euthanized and the bacterial load of M. tuberculosis in the lungs and the spleens was determined. The median bacte- rial load in the lungs of unvaccinated guinea pigs was 6.33log10, compared to 4.53log10 in BCG Danish vaccinated animals and 4.11log10 in BCG Pasteur SmR zmp1::aph vaccinated animals (Fig. 1A). In the lungs, vaccination with both BCG Danish and the BCG Pasteur SmR zmp1::aph mutant resulted in a statistically significant reduction in bacterial load compared to non-vaccinated control (1.80log10, p = 7.8 × 10−2 and 2.22log10, p = 7.8 × 10−5, respectively) and the mutant conferred a statistically significant reduction of 0.42log10 as compared to the BCG Danish control (p = 0.019).

Microscopic-examination of lung parenchyma from animals in the saline group revealed numerous, often coalescing tuberculous granulomas, comprising abundant epithelioid macrophages, scattered lymphocytes, often around the periphery of the lesion, neutrophils and central necrosis. Large areas of lung parenchyma were affected (median = 16.39%) (Fig. 1B and D). By contrast, the lungs of BCG Danish and BCG Pasteur SmR zmp1::aph vaccinated animals had smaller, primarily non-necrotic granu- lomas, with fewer neutrophils than those lesions in the lungs of the control animals (Fig. 1D). Whilst the lung of animals vaccinated with BCG Danish contained fewer, large confluent lesions and scattered, smaller lesions with central, necrotic foci, necrosis was not observed in animals vaccinated with BCG Pasteur SmR zmp1::aph. The affected area in the lungs of BCG Danish vaccinated animals was slightly but not significantly (p = 0.16) larger than the affected area in the lungs of BCG Pasteur SmR zmp1::aph vaccinated animals (median 4.48% vs. 2.95%) (Fig. 1B).

BCG vaccine strains reduced the bacterial load in the spleen similarly (3.95log10 for BCG Danish and 3.90log10 for BCG Past- eur SmR zmp1::aph, p = 0.48); the median CFU in the spleens of non-vaccinated guinea pigs was 5.61log10 compared to 1.66log10 (p = 0.00045) and 1.71log10 (p = 0.00042) in BCG Danish and BCG Pasteur SmR zmp1::aph vaccinated guinea pigs, respectively (Fig. 1C). Tuberculous granuloma were observed in the spleen of all animals in the saline control group. By contrast, only one animal in the BCG Danish-vaccinated group, and two animals in the BCG Pasteur SmR zmp1::aph-vaccinated group contained lesions in the spleen. A decrease in severity of lesions (or any at all) was present in the spleen of animals vaccinated with either BCG Danish or BCG Pasteur SmR zmp1::aph.
3.2. Protective efficacy of BCG Denmark Δzmp1

In the proof of concept study described above, the streptomycin resistant BCG Pasteur SmR zmp1::aph mutant showed improved protective efficacy in guinea pigs compared to BCG Danish. However, the use of this strain in humans is compromised by its antibiotic resistance phenotype. The replacement of zmp1+ by the zmp1::aph allele introduced a kanamycin resistance cassette, and the presence of an rpsl mutation already present in the parent BCG Pasteur SmR strain [29] confers streptomycin resistance. According to the “Geneva consensus recommendation for novel live TB vaccines”, live vaccines must be free of antibiotic resistance markers [30]. In addition, differences in protective efficacy in pre-clinical tests and clinical vaccine trials have been attributed to genetic, biochemical and immunological differences in the BCG sub-strains used [4,5,31,32]. To exclude the possibility that the difference in protective efficacy of BCG Pasteur SmR zmp1::aph and the comparator BCG Danish reflects the use of distinct BCG sub-strains, we generated an unmarked zmp1 deletion mutant in BCG Danish 1331 background (BCG Denmark Δzmp1) [19] and tested the protective efficacy of this mutant as described above. BCG Denmark Δzmp1 vaccine candidate was well tolerated by the guinea pigs. They showed similar weight gain profiles as the control animals. Four weeks after the aerosol M. tuberculosis challenge, the median lung CFU counts were 6.27 log10 in non-vaccinated, 4.57 log10 in BCG Danish vaccinated and 3.66 log10 in BCG Denmark Δzmp1 vaccinated guinea pigs (Fig. 2A). Importantly, the Δzmp1 mutant conferred a statistically significant improved protection over BCG Danish against growth of tubercle bacilli in the lung (0.91 log10; p = 0.019). Vaccination significantly reduced granuloma and necrosis formation in the lung and the Δzmp1 vaccinated animals were slightly but not significantly better protected than BCG Danish vaccinated animals against pathological changes in the lung as assessed by histology (Fig. 2B and D). The median of the affected area of the lung was 33.63% for non-vaccinated, 5.62% for BCG Danish and 4.75% for BCG Denmark Δzmp1 vaccinated animals.

Median spleen CFU counts were 5.05 log10 for non-vaccinated, 1.08 log10 BCG Danish vaccinated (p = 0.00045) and 1.08 log10 for BCG Denmark Δzmp1 (p = 0.00045) vaccinated animals (Fig. 2C). The growth of M. tuberculosis in the spleen was not significantly different in animals vaccinated with BCG Danish and BCG Δzmp1 (p = 0.65).

In summary, vaccination with either of the zmp1 deletion mutants consistently confers better protection in the lung than the BCG Danish control strain against M. tuberculosis challenge in the guinea pig model and this protection is independent of the genetic background of the BCG sub-strain.

3.3. Virulence of unmarked Δzmp1 mutants in CB-17 SCID mice

Having demonstrated that the improved protection of the candidate vaccine is a consistent property of zmp1 deletion and independent of the BCG sub-strain, we constructed a Δzmp1 deletion mutant without antibiotic resistance marker also in the BCG Danish background for further evaluation in the CB-17 SCID mouse model.
Fig. 2. Protective efficacy of BCG Denmark Δzmp1 against tuberculosis. Vaccinated (BCG Denmark Δzmp1; BCG Danish) or non-vaccinated guinea pigs were challenged with virulent M. tuberculosis via the aerosogenic route. M. tuberculosis CFU bacterial burden within lungs (A) and spleens (C) was determined four weeks after challenge. M. tuberculosis CFU counts in individual guinea pigs are given along with the median and quartiles. (B) Quantitation of lung area (%) of vaccinated and non-vaccinated guinea pigs four weeks after infection with M. tuberculosis. (D) Photomicrographs of lung sections from infected guinea pigs (unvaccinated [left]; BCG Danish vaccinated [middle]; BCG Denmark Δzmp1 vaccinated [right]). Haematoxylin and eosin.

Pasteur SmS background, BCG Pasteur SmS Δzmp1. We addressed possible unexpected adverse effects of unmarked zmp1 deletion by infecting immune-compromised CB-17 SCID mice and monitoring their survival. CB-17 SCID mice were infected with BCG Denmark, BCG Denmark Δzmp1, BCG Pasteur SmS and Pasteur SmS Δzmp1. The input doses (CFU/mouse) are given in Table 1. The median survival time of CB-17 SCID mice infected with the BCG Pasteur SmS Δzmp1 deletion mutant was 186 days and significantly longer than the median survival time of CB-17 SCID mice infected with the same dose of the corresponding BCG Pasteur parental strain (Fig. 3; Table 1; p < 0.0001). Likewise, all CB-17 SCID mice infected with BCG Denmark reached their humane endpoint before the first mouse infected with BCG Denmark Δzmp1 did, the median survival times being 127 and 278 days (Fig. 3; Table 1; p < 0.0001), hence demonstrating enhanced safety of the Δzmp1 mutants over their respective parental BCG strains.

4. Discussion

BCG is still the only licensed TB vaccine despite its limited protective efficacy against adult lung TB [3]. Even a modest improvement in potency over BCG would translate into hundreds of thousands of saved lives in the next years. The past decade has seen numerous efforts to improve vaccine-mediated protection against tuberculosis and several vaccine candidates have entered in clinical trials. It has been hypothesized that BCG, derived from virulent M. bovis is over-attenuated and therefore only confers limited protection [33]. However, an episode of tuberculosis

<table>
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<th>Strain</th>
<th>Actual input (CFU/mouse)</th>
<th>Median survival time (days)</th>
<th>Significance (p value)</th>
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<tbody>
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<td>BCG Pasteur SmS</td>
<td>1.0 × 10^6</td>
<td>135.5</td>
<td>&lt;0.0001</td>
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<tr>
<td>BCG Pasteur SmS Δzmp1</td>
<td>0.98 × 10^6</td>
<td>186.5</td>
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<tr>
<td>BCG Denmark</td>
<td>1.2 × 10^6</td>
<td>127.0</td>
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<tr>
<td>BCG Denmark Δzmp1</td>
<td>1.8 × 10^6</td>
<td>278.0</td>
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Fig. 3. Virulence of BCG Δzmp1 in CB-17 SCID mice. Kaplan–Meier survival curves of CB-17 SCID mice infected with a defined dose of BCG Pasteur SmS, BCG Pasteur SmS Δzmp1, BCG Denmark and BCG Denmark Δzmp1 (for details of statistical analysis see: Table 1).
likewise only confers limited protection against subsequent reinfection [34]. The limited post-infective immunity may indicate that M. tuberculosis escapes immune surveillance and that the underlying mechanisms are preserved in BCG [18]. Mutants of M. tuberculosis and M. bovis BCG deficient in zmp1 zinc metalloproteinase are unable to arrest phagosome maturation [17] and a BCG zmp1 mutant has been demonstrated to enhance immunogenicity of mycobacterial antigens [18,19]. Our previous investigations mainly focused on virulence, cellular and immunological aspects of zmp1 mutant strains and on the characterization of the biochemical and structural properties of the enzyme [15,16,35]. We now add an important aspect towards exploitation of BCG zmp1 deletion mutants as TB vaccine by characterizing the protective and safety properties of corresponding BCG mutants. Protective efficacy of BCG zmp1 deletion mutants was tested in the guinea pig model which is considered the most relevant small animal model of pulmonary tuberculosis [36]. Guinea pigs are highly susceptible towards a small number of virulent M. tuberculosis bacteria and eventually succumb to infection due to weight loss and decreased pulmonary function. BCG confers significant protection (approx. 100-fold reduction in CFU counts) in this model and improved protection over BCG is a most stringent criterion, which has only been met by few vaccine candidates [28,37–40]. Two completely independent experiments with zmp1 mutant strains in two different BCG genetic backgrounds were conducted in a head-to-head comparison. Each experiment included a positive control (vaccination with BCG Danish) and a negative control (saline). Compared to BCG Danish, which conferred approximately 1.8 log10 protection in terms of CFU counts in the lung, both zmp1 mutants of BCG Pasteur and BCG Denmark further improved the level of protection by 0.4–0.9 log10.

Attenuated live vaccines (e.g. BCG or attenuated M. tuberculosis) alone or in combination with subunit booster vaccines are often considered as the most promising approach for developing a better TB vaccine [30]. However, safety issues are a concern for any live vaccine since a high incidence of HIV is found in areas where TB is endemic. A novel live vaccine should therefore at least be as safe as BCG. Safety of BCG Denmark Δzmp1 and BCG Pasteur SmS Δzmp1 mutants was tested in immunocompromised CB-17 SCID mice in parallel with their parental strains. Median survival time was prolonged for 50–150 days for each of the mutant strains, indicating that zmp1 deletion mutants of BCG Denmark and BCG Pasteur are attenuated as compared to their parental strains. Attenuation of BCG Denmark Δzmp1 and BCG Pasteur SmS Δzmp1 mutants in this experiment is in agreement with attenuation of M. tuberculosis zmp1 deletion mutant in immune competent mice [17], while wild-type like persistence of BCG Pasteur SmR zmp1::aph was seen in immune competent mice [18].

Based on superior TB protective efficacy in the highly stringent guinea pig model and the good safety profile, BCG Δzmp1 mutants are promising candidates for further vaccine development. The initial proof of concept protection study was conducted with a streptomycin resistant derivative of BCG Pasteur. For subsequent studies, unmarked BCG Δzmp1 deletion mutants were constructed in order to meet the “Geneva consensus recommendation for novel live TB vaccines” [30]. These strains may now be exploited for “Good manufacturing production” for the final stages of pre-clinical development and subsequent clinical trials.

Conflict of interest statement

PS and ECB are inventors of BCG zmp1.

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