The live Mycobacterium tuberculosis phoP mutant strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs

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Abstract

The Mycobacterium tuberculosis phoP mutant strain SO2 has previously been shown to have reduced multiplication in mouse macrophages and in vivo using the mouse intravenous-infection model. In this study we demonstrate that the M. tuberculosis SO2 is highly attenuated when compared with the parental M. tuberculosis MT103 strain and also more attenuated than BCG in severe combined immunodeficiency disease (SCID) mice. Complementation of the M. tuberculosis SO2 with the wild-type phoP gene restored the virulence of the strain in the SCID mice, confirming that the attenuated phenotype is due to the phoP mutation. In Balb/c mice subcutaneously vaccinated with either M. tuberculosis SO2 or BCG, the proportions of CD4+ and CD8+ populations measured in the spleen were significantly higher in the M. tuberculosis SO2 vaccinated group. In addition, the proportion of antigen-stimulated CD4+CD8− cells expressing IFN-γ was significantly higher in the M. tuberculosis SO2 vaccinated group when compared with the BCG group. Balb/c mice subcutaneously vaccinated with the M. tuberculosis SO2 strain were also protected against intravenous challenge with M. tuberculosis H37Rv at levels comparable to mice vaccinated with BCG, as measured by reduced bacterial counts in lung and spleens. Guinea pigs subcutaneously vaccinated with the M. tuberculosis SO2 strain were protected against aerosol challenge with M. tuberculosis H37Rv delivered at different doses. A high dose aerosol challenge of M. tuberculosis SO2 vaccinated guinea pigs resulted in superior levels of protection when compared with BCG vaccination, as measured by guinea pig survival and reduction in disease severity in the lung.

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1. Introduction

Tuberculosis remains one of the leading causes of infectious disease mortality throughout the world [1]. The HIV/AIDS pandemic, the deterioration of public health infrastructures in developing countries, and the emergence of multi-drug resistance forms of tuberculosis have further contributed to the spread of the disease [2]. Given the variable protective efficacy generated by Mycobacterium bovis BCG (Bacillus Calmette–Guérin) vaccination against tuberculosis, there is a concerted effort worldwide to develop better vaccines that could be used to reduce the burden of tuberculosis. Rational attenuated mutants of Mycobacterium tuberculosis are vaccine candidates that offer some potential in this area. As an eradication strategy, the concept of mass vaccination is a relatively cost-effective alternative to expensive chemotherapy as a means to reduce the global burden of tuberculosis. The aims of the ‘classical’ live vaccine are to generate host responses that mimic natural infection, but without causing disease [3]. In this context, M. bovis BCG is the only vaccine available for the prevention of tuberculosis in humans. The live, attenuated BCG vaccine, originally derived by serial passage of a virulent strain of M. bovis, has been used to prevent tuberculosis since 1921. The BCG is effective against severe forms of childhood tuberculosis but appears to be of limited efficacy against adult pulmonary disease in endemic areas [4]. It is now clear that a number of BCG vaccine strains have evolved that differ from the original BCG after many years of growth passages in different laboratories. Recent genomic comparisons have made it possible to determine the precise order of genetic events and revealed the existence of M. tuberculosis-specific regions that have been deleted from all BCG strains as the deletion of RD1 [5]. The absence of RD1 in BCG removes immunodominant antigens such as ESAT-6, which have recently been shown to be important for protection against M. tuberculosis challenge in the guinea pig model [6].

In establishing a set of criteria to measure and demonstrate that a vaccine is superior to BCG the minimum requirements would be that it is at least as attenuated as BCG in stringent animal model systems, e.g. SCID mice, it would reduce tissue damage in the lungs equivalent to BCG following challenge, lower the bacterial burden and enhance survival after virulent challenge. In a concerted effort to improve on the protective efficacy afforded by BCG, several broad approaches to tuberculosis vaccine development are being pursued. One approach is to use non-viable subunit vaccines to deliver immunodominant mycobacterial antigens. Both protein and DNA vaccines induce partial protection against experimental tuberculosis infection in mice, however, their efficacy has generally been equivalent to or less than that afforded by BCG [7–9]. The second approach utilises live vaccines modified by genetic manipulation of BCG to express new antigens and/or cytokines [10]. Alternatively, attenuated strains of M. tuberculosis are produced by random mutagenesis or targeted deletion of known genes. Rational attenuated mutants of M. tuberculosis are also potential vaccine candidates against tuberculosis. A particular advantage of this approach is that many potential immunologically important genes are conserved, unlike in BCG substrains where these are deleted [5]. The relatively recent development of sophisticated biological tools has facilitated the ability to genetically manipulate M. tuberculosis [11–13]. These advances, in combination with the completion of the M. tuberculosis genome sequence [14], have increased our understanding of the contribution of individual genes to M. tuberculosis virulence [15,16]. Several studies have described the development of M. tuberculosis auxotrophic mutant strains with different levels of attenuation and potential as vaccine candidates in animal models [17,18]. Two-component regulatory signal transduction systems (TCS) are important elements in the adaptive response of prokaryotes to a variety of environmental stimuli [19] and are also implicated in virulence regulation [20]. The phoP transcription factor, a component of the M. tuberculosis PhoPR TCS is strongly upregulated in a clinical isolate demonstrated to be a cause of multidrug resistant tuberculosis [21]. Recently, it has been shown that phop is involved in the regulation of complex mycobacterial lipids implicated in the virulence of M. tuberculosis [22]. In previous work, the M. tuberculosis phoP SO2 strain was constructed by a single gene disruption [23] and it was established that the M. tuberculosis SO2 exhibited impaired multiplication in vitro within mouse cultured macrophages, and in vivo in a mouse infection model. Here, we have extended these studies and demonstrate that M. tuberculosis SO2 strain is highly attenuated in the SCID mouse model and is effective as a vaccine against tuberculosis in mice and guinea pigs. Moreover, we show that vaccination of guinea pigs with M. tuberculosis SO2 is superior to BCG in conferring protection to guinea pigs against virulent M. tuberculosis challenge as measured by guinea pig survival and severity of lung disease.

2. Methods

2.1. Protein extraction and immunoblotting

Polyclonal antibodies against PhoP protein were obtained from rabbits that received four doses of PhoP (0.5 mg), at weeks 0, 4, 8, 12 and 16, respectively. The anti-PhoP antibodies were detected by ELISA (ZEU-Immunotec Zaragoza, Spain). Monoclonal antibodies against ESAT-6 were kindly provided by Stewart Cole [24]. Cell-free protein extracts of mycobacteria were prepared from early log-phase cultures grown on Middlebrook 7H9-ADC using standard procedures [25]. Protein extracts of M. tuberculosis were filtered through a 0.22 µm pore size Millex-GP filter (Millipore, Bedford, MA). Culture filtrate was harvested from M. tuberculosis H37Rv grown for 5–6 weeks and filtrate proteins precipitated with 45% (w/v) ammonium sulphate. Western Blot analysis was carried out using standard procedures. Horseradish peroxidase-labelled goat anti-rabbit antibodies
OADC (Difco Laboratories). After 1 month of culture, the brook 7H9 medium (Difco Laboratories) supplemented withing doses equivalent to 2 viable specific pathogen free (SPF) mice were obtained from Charles River (Burgonux Cellls, France). For aerosol infection, mice were placed in the exposure chamber of an airborne infection apparatus (Glascol Inc., Terre Haute, IN, USA). For aerosol infection, the nebulizer compartment was filled with 7 ml of a M. tuberculosis suspension to provide an approximate uptake of 20 viable bacilli within the lungs. Ten mice were used per experimental group. For intravenous infection, groups of seven mice were infected with 200 μl PBS containing doses equivalent to 2 × 10^7, 2 × 10^6 and 2 × 10^5 viable M. bovis BCG Pasteur and 5.4 × 10^6, 5.4 × 10^5 and 5.4 × 10^4 viable M. tuberculosis phoP strain via a lateral tail vein. The significance in differences of survival times among treated mice was determined using the Mantel–Haenszel test. Viable counts were performed on serial dilutions of the homogenate, plated onto Middlebrook 7H11 + OADC agar and examined after 3 weeks for growth. For histological analysis, tissues were fixed in formalin-buffered saline and were embedded in paraffin. Five-micrometer thickness sections were cut and sections were stained with Ziehl-Neelsen.

2.3. Determination of cellular immunity activation in Balb/c mice following M. tuberculosis SO2 and BCG subcutaneous vaccination

Groups of four Balb/C mice were sacrificed at days 7, 14, 21, 28, 45 and 60 post-subcutaneous vaccination with 8 × 10^7 cfu of M. bovis BCG (Phipps) or 2.5 × 10^6 cfu SO2. The spleens were collected and placed in 2 ml of RPMI medium and 10% fetal calf serum (GIBCO, Invitrogen Corporation) containing 0.5 mg/ml collagenase type 2 (Worthington, NJ, USA), and 2 U/ml of DNase (GIBCO), and incubated for 1 h at 37°C at 5% CO2. They were then passed through a 70 μm cell sieve (Falcon, Becton Dickinson 70 μm Nylon 35-2350), crushed with a syringe plunger, and rinsed with medium. Cells were centrifuged, the supernatant was removed and red cells were eliminated with lysis buffer [26]. After centrifugation and washing with RPMI medium, cells were resuspended in FACS buffer (PBS 1x, pH 7.2, 1% BSA), and counted. Cell surface labeling was performed by incubating 10^6 cells with 100 μl of monoclonal antibodies against CD4-FITC or CD8-FITC diluted 1:20 in PBS containing 1% BSA and 0.1% sodium azide during 20 min at 4°C, and analyzed with a FACScan cytometer.

The M. tuberculosis H37Rv strain was grown in Middlebrook 7H9 medium (Difco Laboratories) supplemented with OADC (Difco Laboratories). After 1 month of culture, the bacterial mass was separated and the culture filtrate was harvested. Culture filtrate antigens were precipitated with 45% (w/v) ammonium sulfate, washed and re-dissolved in PBS. For cell stimulation, 1 × 10^6 spleen cells were resuspended in 100 μl RPMI medium per culture well and incubated with 10 μg of M. tuberculosis culture filtrate antigens suspended in 100 μl PBS for 72 h at 37°C with 5% CO2. Cells and culture medium were centrifuged, the supernatant was discarded and after counting and checking viability, 2.5 × 10^3 cells per tube were CD4⁺ or CD8⁺ cell surface labelled as described above. After washing, cells were resuspended and incubated for 20 min at 4°C in 0.1% saponin dissolved in PBS. Intracellular IFN-γ was detected by incubating the cells for 20 min at 4°C in the dark with 100 μl of a 1:20 dilution of phycoerythrin (PE)-labelled monoclonal anti-IFN-γ. Cells were fixed with 100 μl of 4% paraformaldehyde diluted in PBS. Samples were analyzed after 20 min with a FACScan cytometer. Isotype controls were Ab-FITC (1:20 dilution) + Ab-PE (1:20 dilution).

2.4. Protective efficacy of M. tuberculosis SO2 in Balb/c mice

All of the animals were kept under controlled conditions in the P3 High Security Laboratory of the Animal Facility at the Pasteur Institute in Paris and in agreement with the European Union directives for protection of experimental animals. Groups of Balb/C mice (seven per group) were vaccinated subcutaneously in the base of the tail with 10^7 cfu of either M. tuberculosis phoP strain or M. bovis BCG (Pasteur). At 8 weeks post-vaccination, all mice were challenged by the intra-venous route with 2.5 × 10^6 cfu of M. tuberculosis H37Rv. Mice were then sacrificed 4 weeks post-challenge. Viable counts were performed on serial dilutions of the homogenate, plated onto Middlebrook 7H11 + OADC agar and examined after 3 weeks for growth of M. tuberculosis. The protective efficacy of M. tuberculosis SO2 was distinguished from the M. tuberculosis phoP on the basis of the kanamycin resistance phenotype of the latter strain.

2.5. Protective efficacy of M. tuberculosis SO2 in guinea pigs

Guinea pig experimental work was conducted according to UK Laws for animal experimentation and was approved by a local ethical committee at Health Protection Agency, Porton Down, UK. Female Dunkin-Hartley guinea pigs were obtained from (UK Home Office) accredited commercial suppliers (David Hall, Burton-on-Trent, UK or Harlan Ltd. UK, Bicester, UK) and were full barrier reared.

2.6. Low dose challenge

Groups of six were vaccinated subcutaneously in the nape of the neck with 250 μl of either: 5 × 10^5 cfu BCG Pasteur; 5 × 10^4 M. tuberculosis SO2; or saline. Animals were rested
for a period of 12 weeks prior to aerosol challenge using a contained Henderson apparatus as previously described [27]. Fine particle aerosols of M. tuberculosis H37Rv, with a mean diameter of 2 μm, (diameter range, 0.5–7 μm) were generated using a Collision nebulizer and delivered directly to the animal snout. The aerosol was generated from a water suspension containing 2 × 10⁶ cfu/ml in order to obtain an estimated retained, inhaled dose of approximately 10–50 cfu/lung.

Protection was assessed at 4 weeks post-challenge. Animals were killed by peritoneal overdose of sodium pentobarbital. Spleen and lung tissue (the left cranial and middle lobes, right middle lobe and right caudal lobes) were aseptically removed and placed into sterile containers. Material retained, inhaled dose of approximately 10–50 cfu/lung.

PhoP gene The construction of the M. tuberculosis SO2 strain from a clinical isolate of M. tuberculosis has been described previously [23]. Evidence for the involvement of the phoP gene in global regulation of mycobacteria gene circuits was provided by the observation of changes in bacillus size and for a clinical isolate of M. tuberculosis in the presence of sodium pentobarbital. Spleen and lung tissue (the left cranial and middle lobes, right middle lobe and right caudal lobes) were aseptically removed and placed into sterile containers. Material was stored at −20 °C then processed to enumerate the number of bacteria. Tissues were homogenised in 10 ml (lung) or 5 ml (spleen) of sterile deionised water using a rotating blade macerator system (Ystral). Viable counts were performed on serial dilutions of the homogenate, plated onto Middlebrook 7H11 + OADC agar and examined after 3 weeks for growth of M. tuberculosis. The data was log₁₀ transformed for analysis and the numbers of viable M. tuberculosis in each vaccine group were compared with the saline control group by Student's t-test.

2.7. High dose challenge

Groups of six guinea pigs were vaccinated subcutaneously with 5 × 10⁴ cfu of M. tuberculosis SO2 or BCG (Danish 1331) 10 weeks prior to aerosol challenge with M. tuberculosis. Aerosol challenge was performed as described above, using a suspension of 5 × 10⁵ cfu/ml in order to deliver approximately 500 cfu to the lungs. Following challenge, the animals were housed at ACDP containment level 3, were monitored regularly for weight changes and were killed humanely at 180 days post-challenge or at the humane endpoint (20% loss of maximal body weight). Post-mortem collection and processing of samples was as described above with the exception that lung consolidation was measured using image analysis on sections of formalin fixed lung tissue stained with Haematoxylin & Eosin (H&E). Animal survival was compared using Kaplan Meier survival estimates and Log Rank distribution analysis was used to identify statistically significant differences. The cfu and lesion consolidation data were analyzed by ANOVA, using Fisher's pairwise comparisons to compare mean values of the groups.

3. Results

3.1. Characterization of M. tuberculosis phoP

The construction of the M. tuberculosis SO2 strain from a clinical isolate of M. tuberculosis has been described previously [23]. Evidence for the involvement of the phoP gene in global regulation of mycobacteria gene circuits was provided by the observation of changes in bacillus size and for a clinical isolate of M. tuberculosis in the presence of sodium pentobarbital. Spleen and lung tissue (the left cranial and middle lobes, right middle lobe and right caudal lobes) were aseptically removed and placed into sterile containers. Material was stored at −20 °C then processed to enumerate the number of bacteria. Tissues were homogenised in 10 ml (lung) or 5 ml (spleen) of sterile deionised water using a rotating blade macerator system (Ystral). Viable counts were performed on serial dilutions of the homogenate, plated onto Middlebrook 7H11 + OADC agar and examined after 3 weeks for growth of M. tuberculosis. The data was log₁₀ transformed for analysis and the numbers of viable M. tuberculosis in each vaccine group were compared with the saline control group by Student's t-test.

The attenuation of the M. tuberculosis SO2 strain was also compared with BCG in SCID mice following intravenous delivery. Groups of SCID mice were inoculated with a range of doses (2 × 10⁴, 2 × 10⁵ and 2 × 10⁶ cfu) of BCG Pasteur or with M. tuberculosis SO2 strain (5.4 × 10⁵, 5.4 × 10⁶ and 5.4 × 10⁷ cfu) via a lateral tail vein. Histological staining of infected alveolar macrophages from a sub-group of mice sacrificed at three weeks post-infection revealed fewer acid fast bacilli in lungs of mice infected the M. tuberculosis SO2 strain when compared with BCG (data not shown). All mice inoculated with the highest dose of BCG (2 × 10⁷ cfu) died after 92 days post-infection (median survival time of 89 ± 3.5
Fig. 2. Attenuation of *M. tuberculosis* SO2 in SCID mice. (a) Survival curve of SCID mice infected (*n* = 10) by the aerosol route with 20 cfu of *M. tuberculosis* SO2, *M. tuberculosis* SO2 complemented with pSO5 (SO2 + pSO5) and *M. tuberculosis* MT103. Means of the survival days were, >245 days (SO2), 62.1 ± 5.88 (SO2 + pSO5) and 36.7 ± 0.67 (MT103). (b) Survival curves of SCID mice (*n* = 7) infected by intravenous injection with 5.4 × 10^6 cfu of *M. tuberculosis* SO2 and 2 × 10^5 *M. bovis* BCG Pasteur. In contrast, all mice infected with the highest dose of SO2 (5.4 × 10^6 cfu) survived after 120 days (Fig. 2b). At the time of death, the bacterial loads in the lungs of BCG infected mice with 2 × 10^5 cfu were at least 100-fold higher when compared with the *M. tuberculosis* SO2 infected mice with 5.4 × 10^6 cfu.

3.3. Quantitative CD4+ and CD8+ responses of Balb/c vaccinated mice

In order to compare cellular immunity activation induced by vaccination with *M. tuberculosis* SO2 and BCG, spleen cell suspensions from groups of at least four Balb/c mice subcutaneously vaccinated with *M. tuberculosis* SO2 and BCG Phipps were collected on days 7, 14, 30, 45 and 60 post-vaccination, and the relative proportions of CD4+ and CD8+ cells determined by cytofluorometry (Fig. 3). Vaccination with *M. tuberculosis* SO2 induced significantly more CD4+ cells after 14 days of vaccination, when compared with BCG vaccination and significantly more CD8+ cells after 45 days. These splenocytes were stimulated with *M. tuberculosis* culture filtrate-derived whole antigens. After 3 days, the lymphocyte populations were analyzed by flow cytometry, combining antibodies specific for CD4+/CD8+ detection and intracellular synthesis of IFN-γ. Vaccination with *M. tuberculosis* SO2 induced a significantly higher proportion of CD4+/IFN-γ+ producing cells after 45 days of vaccination when compared with BCG (Fig. 3). Apart from one time-point, the proportion of CD8+/IFN-γ+ producing cells was consistently higher in the *M. tuberculosis* SO2 group (significantly different at day 14). Data obtained from mice immunised with the parental *M. tuberculosis* MT103 strain revealed cell populations and IFN-γ expression levels similar to that measured following *M. tuberculosis* SO2 immunisation (data not shown).

3.4. Protective immunity generated by *M. tuberculosis* SO2 in Balb/c mice

Having established that *M. tuberculosis* SO2 strain was attenuated in SCID mice, we were interested to determine whether the observed decrease in virulence conferred any protective properties to the mutant strain. We subcutaneously vaccinated Balb/c mice with either *M. tuberculosis* SO2 strain or BCG (Pasteur). At 8 weeks post-vaccination, all mice were challenged by the intra-venous route with 2.5 × 10^5 cfu of *M. tuberculosis* H37Rv. The mice were then sacrificed at 4 weeks post-challenge. The levels of protection were determined by evaluating the numbers of viable *M. tuberculosis* H37Rv recovered from the lungs and the spleen in both groups of mice (Fig. 4). Both vaccines conferred similar but significant levels of protection compared to the saline-treated controls (*p* < 0.05). Growth inhibition of *M. tuberculosis* H37Rv was recorded in both lungs and spleen, with reductions of approximately 1.5 log10 and 1.3 log10 cfu, respectively.

3.5. Protective immunity of *M. tuberculosis* SO2 in guinea pigs

The results obtained from the mouse vaccination experiments indicated that the attenuation of *M. tuberculosis* SO2 strain afforded it vaccine characteristics that were comparable with BCG Pasteur. However, it is widely recognized that guinea pigs constitute a more relevant model of human tuberculosis, with many similarities in the progression and pathology of the disease. Thus, this animal model is a more challenging system with which to evaluate vaccine efficacy. In order to investigate the protective efficacy of the *M. tuberculosis* SO2 strain, we performed experiments involving low-dose (10–50 cfu) and high-dose (500 cfu) aerosol-challenge of vaccinated animals. Groups of six guinea pigs were vac-
Fig. 3. Cellular immune responses in M. tuberculosis SO2 and BCG vaccinated mice. Balb/C mice were vaccinated by the subcutaneous route with $8 \times 10^3$ cfu of M. bovis BCG (Phipps) or $2.5 \times 10^3$ cfu M. tuberculosis SO2. The results are presented as the percentage of total CD4+/CD8+ populations in lymph nodes at time intervals after vaccination and the percentage of cells expressing IFN-γ from the total CD4+/CD8+ population after M. tuberculosis whole antigen stimulation. *Denotes significant statistical differences between the groups at the time points indicated ($p < 0.05$).

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Denoted subcutaneously with either M. tuberculosis SO2 or BCG. Ten weeks post-vaccination all guinea pigs were challenged with inhaled doses of M. tuberculosis H37Rv.

Animals receiving the low-dose challenge were sacrificed at 4 weeks and bacterial loads enumerated in lungs and spleens. Protective efficacy was determined by comparing the numbers of viable M. tuberculosis H37Rv recovered from the organs of guinea pigs in each treatment group. In this experiment, the reduction of cfus in lungs and spleens was significantly different between non-vaccinated control animals and those vaccinated with BCG or M. tuberculosis SO2 ($p = 0.005$). However, no significant difference was found between the vaccinated groups (Fig. 5).

Guinea pigs challenged with the high-dose were killed at day 180 post-challenge or at the humane end point (20% loss of maximal body weight). For the purposes of analyzing survival, animals killed at the humane end-point were considered to have died due to infection. The levels of protection were determined by comparing the survival times of guinea pigs in each treatment group. The progression of lesion development was also studied in the vaccinated/challenged guinea pigs and compared with that observed in non-vaccinated/challenged animals. During the post-challenge phase of the experiment all of the non-vaccinated and four of the BCG vaccinated guinea pigs were euthanased at the humane end-point, prior to the last time-point (180 days) because of severe and progressive disease (Fig. 6a). In contrast, all of the guinea pigs vaccinated with the M. tuberculosis SO2 strain survived for the duration of the study. Guinea pigs vaccinated with the M. tuberculosis SO2 strain survived significantly longer than guinea pigs vaccinated with BCG ($p = 0.018$) which in turn survived significantly longer than control guinea pigs treated with saline ($p = 0.0049$). In addition, the guinea pigs vaccinated with the M. tuberculosis SO2 strain gained weight and did not present with any visible or clinical signs of disease. The extent of pulmonary disease, as measured by total lung consolidation, also differed between the treatment groups. The highest level of disease progression was observed, not unexpectedly, in the non-vaccinated guinea pigs and a mean percentage consolidation of 76% was measured in this group.
of animals (Fig. 6b and c). The coalescence of granulomas was also pronounced in the BCG vaccinated guinea pigs with a mean 70% consolidation measured in the lungs. In contrast, there was less consolidation (approximately 50%) in the M. tuberculosis SO2 vaccinated guinea pigs and this was significantly ($p<0.05$) reduced compared with both the non-vaccinated and BCG vaccinated animals (Fig. 6c). This reduction in disease severity was also reflected in the bacterial counts from lung and spleen homogenates. In the vaccinated groups, there were a differences in the levels of growth inhibition of M. tuberculosis H37Rv in both organs. The numbers of cfu recovered from the guinea pigs vaccinated with M. tuberculosis SO2 were reduced by more than $1 \times 10^{10}$ compared to that from guinea pigs vaccinated with BCG and this reduction was statistically significant ($p<0.05$) in the spleen (Fig. 6d). These data demonstrated that the M. tuberculosis SO2 strain was superior to BCG in conferring enhanced survival to infected guinea pigs, reduction in the severity of the disease in the lung, and dissemination of infection to the spleen.

4. Discussion

The use of vaccines to control tuberculosis in human populations has proved a formidable challenge for almost a century. The development and widespread administration of the BCG vaccine since the early 1920s was originally hailed as a major breakthrough with the promise to eradicate the world of the scourge of tuberculosis. However, the early promise was not realised and from the results of a large number of efficacy trials, it became clear that the BCG vaccine, in its existing form, was of limited use in controlling disease, particularly in adults in disease endemic areas of the developing world [4]. With a greater understanding of the virulence of M. tuberculosis and the patterns of immune responses that lead to the generation of protective immunity, there is renewed optimism that vaccines superior to M. bovis BCG can be developed. The observation that the highest levels of protection are obtained when the host is vaccinated with
live _M. bovis_ BCG indicates that viability and persistence are key attributes required for a successful tuberculosis vaccine. In this study, we used the _M. tuberculosis_ SO2 strain as a prototype single dose live vaccine and showed that, in addition to being more attenuated than BCG in SCID mice, it elicited high levels of protection in mice and superior protection in guinea pigs. The _phoP_ gene is part of a two-component system (along with _phoR_) that shows a high degree of similarity to other two-component systems, including _phoP/phoQ_, a well described system that controls the transcription of key virulence genes in intracellular pathogens such as _Salmonella_ sp. [20]. It also controls expression of many other genes not

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Fig. 6. Protective efficacy of _M. tuberculosis_ SO2 and BCG vaccinated guinea pigs against high dose challenge with _M. tuberculosis_ H37Rv. (a) Survival curve of vaccinated and non-vaccinated guinea pigs after aerosol infection with _M. tuberculosis_ H37Rv. (b) The extent of pulmonary disease and disseminated infection as measured by total lung consolidation. Values for individual animals euthanased at humane end-point are indicated by an "x". Dashed line indicates the mean % value for the group (n in SO2 corresponds to two animals). (c) Low power magnification (30x) images of representative lung lobe sections taken from guinea pigs in each of the treatment groups. Bar represents 1 mm. (d) Mean cfu counts in the spleen and lungs of vaccinated and non-vaccinated guinea pigs.
directly involved in virulence [19]. The removal of virulence genes per se does not appear to be crucial for attenuation of *M. tuberculosis*. A pantothenic acid (vitamin B5), was shown to persist in SCID mice without being able to cause disease [17]. Single leucine auxotrophs are also highly attenuated and incapable of in vivo replication in SCID mice [29]. Thus, there is now a well-established proof of principle that vaccine strains based on *M. tuberculosis* can be successfully attenuated while retaining the genes that are deleted in *M. bovis* BCG.

The role of secreted antigens as determinants of protective immunity has been well described and vaccination with the major extracellular antigens does produce significant levels of protection against challenge [29]. However, subunit vaccines containing single or combinations of antigens, while capable of eliciting protective immunity, are generally not as efficient as BCG [30]. To overcome this limitation, recombinant BCG strains have been created with the aim to either overexpress immunodominant antigens (e.g. Ag85B) that are already present in BCG [31] or by re-introducing antigen coding genes (e.g. *esat-6*) [6] from *M. tuberculosis* that have been lost in BCG. In both these examples, the levels of protection surpassed that conferred by BCG when the recombinant strains were used as vaccines in guinea pigs [31,6]. These results highlighted the important role played by secreted antigens in eliciting protective immunity. Because of the pleiotropy associated with the *phoP* mutation, we were interested to determine whether it had any effect on synthesis of immunodominant secreted antigens. Using Western blotting, we found no significant differences on the in vitro production of ESAT6 in the *M. tuberculosis* SO2 strain compared to the parental *M. tuberculosis* MT03. However, without deleting these antigen-coding genes from the *M. tuberculosis* SO2 strain we cannot rule out that their de novo synthesis contributes to the protective efficacy of the *M. tuberculosis* SO2 vaccine in mice and guinea pigs.

The search for vaccines better than BCG was often, in the past, predicated on the notion that the loss of virulence in BCG was in itself a factor contributing to its lack of complete protective efficacy [32]. Thus, it was reasoned that new attenuated mutants of *M. tuberculosis* with reduced virulence might prove to be more effective as vaccines. However, recent work has demonstrated that natural infection with *M. tuberculosis* and vaccination with BCG do not differ in their capacity to produce protective immunity against tuberculosis [33]. This raises the questions as to whether it is possible to improve on the BCG by rational attenuation of *M. tuberculosis*. In this context, the observation that the *M. tuberculosis* SO2 strain proved to be more attenuated than BCG in the SCID mouse model, even when delivered at a 10-fold higher dose, is particularly relevant. That the attenuation was caused by the mutated *phoP* gene was proven beyond doubt by the restoration of virulence in the wt *phoP* complemented strain.

In the vaccination experiments carried out in the Balb/c mice, the levels of protection conferred by *M. tuberculosis* SO2 and BCG were similar both in the lungs and spleen up to four weeks post challenge. When we compared the relative proportions of CD4+ and CD8+ cells from the spleens of vaccinated mice, a higher percentage of both CD4+ and CD8+ cells was found in *M. tuberculosis* SO2 vaccinated mice when compared with the BCG vaccinated mice. Moreover, when these cells were stimulated with culture filtrate derived antigen, a significant higher percentage of CD4+ and CD8+ cells was measured in *M. tuberculosis* SO2 vaccinated mice after 45 and 60 days post-vaccination. Although not significant at every time point, a similar trend was measured for CD8+/IFN-γ+ in the *M. tuberculosis* SO2 vaccinated mice. The data suggests that vaccination with *M. tuberculosis* SO2 results in enhanced T cell activation compared with BCG vaccination, as measured by IFN-γ synthesis. As protective immunity against *M. tuberculosis* generally depends on the generation of a Th1 type cellular immune response, characterized by secretion of IFN-γ from antigen-specific T cells, we can speculate that the relatively high levels of T cell activation induced by *M. tuberculosis* SO2 contributes to its ability to mount a potent protective response.

By using several test model systems and conditions we were able to demonstrate the relative capacity of the animal models to reveal differences in vaccine efficacy between the *M. tuberculosis* SO2 strain and the BCG. Having demonstrated equivalence of the vaccines in the mouse model, we...
compared with the unvaccinated controls and demonstrated in comparison with BCG. In terms of survival, the animals culosis did this to generate a stringent level of challenge that might be relative high dose aerosol challenge with \( H_37RV \) and used a period of study up to 180 days. We have demonstrated vaccine potential \([38]\). We, therefore, used a relatively high dose aerosol challenge study with a double leucine and pantothenate auxotrophic mutant of \( M. tuberculosis \), protection levels equivalent to \( M. bovis \) BCG were generated in the lungs and spleen of vaccinated guinea pigs, with reduced dissemination of infection to the spleen induced by both vaccines, five weeks after aerosol challenge with \( M. tuberculosis \) \([34]\). In another study using recombinant BCG expressing ESAF-6, protection levels superior to \( M. bovis \) BCG were only observed in the spleen \([6]\) suggesting that the improved protection was restricted to its ability to prevent dissemination of infection from the lung. In guinea pigs challenged with low dose of \( M. tuberculosis H_37RV \) the levels of protection conferred by vaccination with \( M. tuberculosis \) SO2 and BCG were similar both in the lungs and spleen up to 4 weeks post-challenge. Both vaccines gave highly efficient protection, reducing the cfu in lungs and spleens by approximately 2 logs compared with the saline control groups. However, there was no statistically significant difference between the two vaccine groups. With such a short period post-challenge, we can surmise that it would be difficult to demonstrate the superior efficacy of a novel vaccine over BCG. This is because, at this time point the cfu in the organs of the BCG-vaccinated animals are so low that the assay does not have the discriminatory power to demonstrate a significant further reduction in cfu. In other guinea pig survival studies, it has been shown that whilst BCG vaccination provides statistically significant protection compared to unvaccinated controls (or ineffective vaccines), this protection is only partial even against low-dose challenge with \( M. tuberculosis \). In low-dose challenge studies, conducted over 60–80 weeks post-challenge, some BCG controls failed to protect any of the guinea pigs \([35]\) whilst others protected a small (20–30) percentage of the animals \([36,37]\). In contrast, a high challenge dose can result in disease more severe than that conventionally used to evaluate the protective efficacy of TB vaccines. However, this may be required where it is necessary to discriminate between candidates that have demonstrated vaccine potential \([38]\). We, therefore, used a relatively high dose aerosol challenge with \( M. tuberculosis \) \( H_37RV \) and used a period of study up to 180 days. We did this to generate a stringent level of challenge that might demonstrate the potential protective efficacy of the \( M. tuberculosis \) SO2 strain and also provide a level of discrimination in comparison with BCG. In terms of survival, the animals in the BCG vaccinated group were significantly protected compared with the unvaccinated controls and demonstrated an overall level of protection similar to that seen in other studies, despite the relatively higher challenge dose used in our study. However, we also found a statistically significant increase in the protective efficacy of the \( M. tuberculosis \) SO2 strain compared with BCG, as measured by several indicators including prolonged survival and the degree of lesion consolidation in the lung. This less severe form of disease may have directly lead to the prolonged survival of the \( M. tuberculosis \) SO2 vaccinated animals.

The inability of the BCG vaccination to provide 100% protection has been exploited to demonstrate the superior efficacy of other candidate vaccines \([35,36]\). Also, in a recent comparative testing of 24 tuberculosis vaccine candidates in the guinea pig model (a 3-year study funded by the EU Fifth Framework Programme) \([38]\) two vaccine candidates, the recombinant modified vaccinia virus Ankara (MVA85A) and fowlpox vectors expressing antigen 85A used to boost guinea pigs previously vaccinated with BCG, and a single dose of \( M. tuberculosis \) SO2 vaccine, were found to protect better than BCG Danish 1331 as measured by animal survival. The former candidate has been tested in Phase 1 of human trials and the MVA85A vaccine shown to significantly boost cell-mediated immune responses in volunteers previously vaccinated with BCG \([39]\).

The results described in our study demonstrate that \( M. tuberculosis \) SO2, when delivered as a single dose, is a superior vaccine to BCG by a number of criteria. It is more attenuated than BCG in SCID mice. It confers protective immunity to mice at least as good as BCG and generates more potent CMI responses. It protects guinea pigs against low dose challenge equivalent to BCG. However, against a more stringent challenge, it confers 100% survival to guinea pigs under circumstances where BCG confers 33% survival. This protection is associated with reduced severity of disease and bacterial burden. Some of the variability in levels of protection observed in the different animal model systems may be due the choice of BCG strain used in the participating laboratories. If the \( M. tuberculosis \) phoP mutant strain is to progress to clinical trials it will need to be formulated appropriately and compared with the BCG strains currently registered for use in humans. Nevertheless, the results described here may provide a rational starting point for the development of a new generation of live vaccines against tuberculosis based on inactivation of virulence pathway regulated by PhoP. It has many attributes desirable of a tuberculosis vaccine. It has a defined mutation which reduces virulence but allows persistence in the host. In addition, it has a complete repertoire of genes coding for immunodominant antigens. Unlike other vaccine candidates tested, it may potentially provide high levels of protection when delivered as a single dose. It should be possible to add additional mutations to address safety concerns associated with clinical development of a new vaccine \([40]\). If this can be achieved then vaccines based on \( M. tuberculosis \) SO2 may be likely candidates to progress to comparative vaccine efficacy trials in humans and to eventually replace BCG.
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References


