Major article: 3,500

Abstract 200

Revaccination in guinea pigs with the live-attenuated *Mycobacterium tuberculosis* MTBVAC improves BCG protection against tuberculosis

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Abstract

Background. The need for an effective vaccine against human tuberculosis (TB) has driven the development of different candidates and vaccination strategies. Novel live attenuated vaccines are being developed which promise greater safety and efficacy, than BCG, against TB. We combined BCG with MTBVAC to evaluate whether efficacy of either vaccine would be affected upon re-vaccination.

Methods. In a well-established guinea pig model of aerosol TB infection, BCG and MTBVAC vaccination schedules were compared in prime-boost combination or alone. Efficacy was determined by a reduction in bacterial load at four weeks post-challenge.

Results. Efficacy data suggests MTBVAC immunity is longer lasting than BCG when given as a single dose. Long and short intervals between BCG prime and MTBVAC boost resulted in improved efficacy in lungs compared to BCG alone. A shorter interval between MTBVAC prime and BCG boost resulted in improved efficacy in lungs compared to BCG alone. A longer interval resulted in protection equivalent to BCG.

Conclusions. These data indicate that rather than boosting waning BCG, it is a combination of the two vaccines, which gave a stronger immunity to *M. tuberculosis* infection. This work supports development of MTBVAC as a revaccination strategy to improve upon BCG in vaccinated people living in TB-endemic countries.

Key words: Tuberculosis, vaccine, MTBVAC, guinea pig, aerosol

Introduction

Tuberculosis (TB) remains one of the deadliest diseases and is present in all regions of the world. In 2015, an estimated 10.4 million people developed TB and 1.8 million died from the disease [1]. TB is slowly declining each year but, given that most deaths from TB are preventable, the death toll from the disease is still unacceptably high. The World Health Organisation (WHO) has developed a post-2015 global TB strategy, the overall goal of which is to end the global TB epidemic, with corresponding 2035 targets of a 95% reduction in TB deaths and a 90% reduction in TB incidence (both compared with 2015) [2]. The only licenced vaccine, BCG (*Bacillus Calmette-Guerin*) is widely used and provides protection against TB meningitis and disseminated TB in children. However, the efficacy of the vaccine in preventing pulmonary TB is unclear, with studies showing 0-80% protection [3]. Mass vaccination of adults in high TB burden countries with a new, effective tuberculosis (TB) vaccine will be key to global elimination of TB [4, 5] and development of such a vaccine is an international research priority [2].

A common strategy for TB vaccine development is aimed towards enhancing the protection afforded by neonatal BCG vaccination by applying a second vaccine which boosts the immune responses to targeted antigens of *Mycobacterium tuberculosis*. This is regarded as heterologous boosting since the boost vaccines are sub-unit approaches consisting of single or multiple antigen targets delivered as a protein in adjuvant (H1,H4,M72,ID93,HBHA) [6, 7] or viral vectors (MVA85A, Ad85A, Aeras402) [6]. Revaccination with BCG (homologous boosting) has been considered and evaluated both clinically and in animal models. Multiple vaccinations of BCG in animal models have been shown to have variable effects on protective efficacy ranging from improved efficacy [8] to exacerbated disease [9] but differences in experiment design are behind some of this variation. A second BCG vaccination given 4 weeks after the first was equivalent in protection to a single BCG in guinea pigs [10], but when the interval between the vaccinations was much longer (11 months), revaccination was significantly better than a single BCG. In humans BCG revaccination does not confer additional protection against development of TB disease [11-13], and this strategy is not endorsed by the World Health Organization [14]. However, Hatherill et al. report that BCG revaccination of adults infected with *M. tuberculosis* is safe, has a similar reactogenicity profile compared to single BCG vaccination at birth and that clinical trials of live recombinant BCG or attenuated mycobacterial vaccines may be considered for targeted populations including latently infected adults [15]. This opens the possibility of using revaccination with novel live-attenuated vaccines rather than sub-unit vaccines to stimulate immunity in adults where the protection afforded by neonatal BCG has waned. Over the last decade several recombinant live-attenuated mycobacterial vaccines [6, 7, 16-20] have been developed and some have been evaluated in first-in-human Phase 1 clinical trials on the basis that they are safer than BCG and or afford greater protection in pre-clinical animal models. Today only two live-attenuated vaccines are in the clinical development pipeline towards efficacy testing in high-burden countries. One is the recombinant BCGΔ*ureC*::*hly* (VPM1002) which has successfully reached Phase IIa safety and immunogenicity evaluation in healthy newborns (NCT02391415). The other candidate vaccine is MTBVAC, a live-attenuated *M. tuberculosis* strain with two deleted genes

phoP and *fadD26*, which are essential for *M. tuberculosis* virulence, constructed in the genetic background of the clinical isolate Mt103 [16]. In rigorous preclinical (and Good Manufacturing Practices) characterization studies, MTBVAC has shown promising safety and efficacy in different, relevant TB animal models [16]. As a result MTBVAC successfully entered first-in-human Phase 1 clinical evaluation in healthy adults in Lausanne, Switzerland in 2013 [21]. This first-ever Phase 1 trial with a vaccine of this kind is considered a milestone in TB vaccinology. Currently MTBVAC is being tested for safety and immunogenicity in healthy newborns in South Africa (NCT02729571), as its main target product profile is as a preventive newborn tuberculosis vaccine that could eventually replace BCG [22]. MTBVAC is also being developed as a preventive vaccine for use in adolescents and adults (BCG vaccinated at birth) living in high-burden countries. It is estimated that vaccines targeted at adolescents and adults could have a much greater impact on the TB burden over a short time horizon (2024–2050) and could also be cost-effective [23, 24].

The present work sought to investigate, preclinically, the effects of revaccination strategies with two live mycobacterial vaccines, using BCG or MTBVAC, and combinations of the two. The principal aim was to determine if the efficacy of a single BCG immunisation could be affected upon revaccination. Using a guinea pig aerosol challenge model of *M. tuberculosis* infection, these revaccination regimens were compared for their ability to limit bacterial replication in the lungs and spleens. Since the interval between the two consecutive vaccine administrations had an impact upon efficacy in previous BCG-BCG revaccination studies, the effect of short and long prime-boost intervals was evaluated. The effect of the length of time prior to challenge on the efficacy of BCG or MTBVAC given as single vaccines was also determined.

Methods

Vaccinations

The studies were conducted according to UK Home Office Legislation for animal experimentation and approved by a local ethical committee at Public Health England, Porton Down, UK. Dunkin Hartley guinea pigs weighing between 250-350 grams, and free from pathogen-specific infection were randomly assigned to vaccine groups and identified using subcutaneously implanted microchips (PLEXX BV, The Netherlands) to enable blinding of the analyses wherever possible. Group sizes were determined by statistical power calculations (Minitab version 16) performed on previous data giving an average standard deviation of approximately 0.5 with the aim to reliably detect a difference between the median colony forming units (CFU) per ml of 1.0 $\log_{10}$.

MTBVAC was produced and characterised by Biofabri (Porriño, Spain), in compliance with Good Manufacturing Practices, as a freeze-dried preparation following the European Pharmacopoeia monograph and the WHO Recommendations to Assure the Quality, Safety and Efficacy of BCG Vaccines. The BCG was a commercial formulation of the Danish strain from Statens Serum Institute (Copenhagen, Denmark).

Waning BCG efficacy study: Two groups of animals were immunized subcutaneously on the nape with $5x10^4$ CFU of BCG (SSI) in a volume of 250 µl, at either 11 (n=6) or 3 (n=8) months pre-infection. An unvaccinated group (n=8) was used as a negative control. All animals were rested and then challenged on the same day by the aerosol route.

Heterologous revaccination study: The schedule for the different immunisation groups is shown in Figure 1. At week 0, for groups 5 to 8 and week 14 for groups 1 to 4 animals were immunized subcutaneously on the nape with $5x10^4$ CFU of BCG (SSI) in a volume of 250 µl or with $5x10^5$ CFU MTBVAC in a volume of 100 µl. Animals in groups 1, 2, 5 and 6 were revaccinated either at 6 weeks (groups 1 and 2) or 20 weeks (groups 5 and 6) following the prime vaccination. The revaccination was performed with either subcutaneous BCG (SSI) ($5x10^4$ CFU in 250 µl) or MTBVAC ($5x10^5$ CFU in 100 µl), as for the prime vaccine. Following immunisation, all animals were rested until challenge at week 30.

Challenge

Challenge for each of the studies was by the aerosol route with *M. tuberculosis* strain H37Rv grown in batch culture under defined conditions [25]. The animals were challenged using a contained Henderson apparatus in conjunction with an AeroMP control unit [10, 26, 27]. Fine particle aerosols of *M. tuberculosis*, with a mean diameter of 2µm, were generated in a Collison nebulizer and delivered directly to the snout of each animal [26]. The suspension in the Collison nebulizer was adjusted to deliver an estimated retained, inhaled, low dose of approximately 10-20 CFU to lungs of each animal [26]. The suspension of *M. tuberculosis* in the nebulizer was plated onto Middlebrook 7H11 OADC selective agar to measure the concentration in order to confirm retrospectively that the expected dose had been delivered.

Assessment of protection

Protection was determined by measuring bacterial burden at 4 (heterologous revaccination study), or 10 (waning BCG study) weeks post-challenge, guinea pigs were killed by an overdose with sodium pentobarbital given by the intraperitoneal route. At necropsy, lungs and spleen were removed as described previously [28].

For bacterial load analysis, each tissue was homogenized in 5 (waning BCG study) or 2 ml (heterologous revaccination study) sterile phosphate buffered saline (PBS). Each tissue homogenate was serially diluted in sterile PBS and 100 µl of each dilution plated, in duplicate onto Middlebrook 7H11 OADC selective agar. Plates were incubated at 37°C for up to 4 weeks. Following incubation, colonies were enumerated (CFU) and the concentration of bacilli per ml of each sample was calculated. Bacterial load data were expressed as Log_{10} CFU/ml.

Histological analysis was performed only on the heterologous revaccination study. Tissue representative from each lung, sampled consistently between animals, was processed routinely (formaldehyde fixation) and embedded in paraffin wax. Sections (approximately 5 µm) were stained

with haematoxylin and eosin. The nature and severity of the lesions were assessed blind using a subjective scoring system. Each lung lobe was assigned a score as previously described [29]. Scores from each lobe were combined. A mean score from lung lobes was calculated for each group. Group mean histopathology scores were compared between groups and compared with bacterial loads.

Statistical analysis

Efficacy was determined by pairwise comparisons between each vaccine group versus the control group and were considered statistically significant if $P<0.05$. The bacterial load in each vaccine group for all experiments was compared using Mann-Whitney test. No adjustment was made for multiple comparisons between different vaccine groups versus the control. The histology score in each vaccine group was compared using 2-group T-test (Minitab version 16).

Results

Waning BCG efficacy study.

A single immunisation of BCG given 3 months before infection provided significant protection from disease (as measured by reduced bacterial load) in both lung (P= 0.0014) and spleen (P= 0.01) compared to the unvaccinated control group (Figure 2). However, this protective effect was lost in both the lung (P=0.12) and spleen (P=0.06) when the interval between BCG vaccination to infection was increased to 11 months. No significant differences were observed in either the lung (Figure 2A) or the spleen (Figure 2B) when directly comparing the two BCG groups (P=0.12 and P=0.14, respectively).

Heterologous re-vaccination study.

In both lung and spleen all vaccine groups had significantly improved protection compared with the unvaccinated control group (Figure 3).

In the lungs, with a short revaccination interval, BCG prime-MTBVAC revaccination (*P*=0.02) and MTBVAC prime-BCG revaccination (*P*=0.04) groups had significantly lower CFU compared to BCG alone. The BCG prime-MTBVAC revaccination group had significantly lower CFU (*P*=0.02) following a long revaccination interval compared to the BCG alone control group, whereas the MTBVAC-BCG group had an equivalent bacterial load compared to the BCG group (*P*=0.27) (Figure 3).

The MTBVAC alone vaccine group had significantly lower CFU (*P*=0.03) compared to BCG alone when given 30 weeks prior to challenge whereas the two vaccines gave equivalent efficacy (*P*=0.23) when vaccination was 16 weeks prior to challenge. No significant difference was observed between the BCG only groups comparing short and long vaccine to challenge interval (*P*=0.09) (Figure 3).

In the spleen, no detectable *M. tuberculosis* was observed in animals vaccinated with either BCG prime-MTBVAC revaccination (short interval) regimen or BCG alone (<5 CFU/ml). CFU was detected in 1 of 8 animals in each of the other vaccine groups except for BCG given alone following a long interval, where CFU was observed in 2 of 8 guinea pigs.

Histopathology

All vaccine groups had a statistically significantly lower group mean lung and spleen histopathology score compared to the unvaccinated control group, except for the long interval regimen for MTBVAC-BCG (*P*=0.07),. In the lung, the severity of microscopic lesions were similar in animals in each of the vaccine groups (lesion score between 3-4 and little necrosis) (Figure 4). Representative images of the lungs from each group of animals (Figure 5) show that the pathology was similar in all of the vaccinated groups and was notably reduced compared to the unvaccinated controls.

Lesions were not observed in the spleen of the vaccine groups except for the BCG-MTBVAC (long interval) and MTBVAC-BCG (long interval) vaccine regimens. However, lesions were observed in the spleens of one animal from each of those groups.

Discussion

Novel live attenuated vaccines and regimens are being developed with an aim to replace the current vaccine BCG with promise of greater safety and efficacy. MTBVAC is based on the genetic deletions of two major virulence factors, the transcription factor regulator PhoP and the virulence associated cell-wall lipid PDIM, from a clinical isolate of the Euro-American *M. tuberculosis* lineage, which is the most widespread lineage commonly transmitted between humans by the aerosol route [16]. MTBVAC is the only live attenuated *M. tuberculosis* vaccine candidate in clinical development, presenting a full spectrum of specific mycobacterial antigens to the host immune system, which is highly desirable for a tuberculosis vaccine, since protective antigens are yet to be definitively identified [30]. In the first-in-human Phase 1a trial in healthy, BCG-naïve, HIV-uninfected adults in Switzerland, MTBVAC was safe, with similar reactogenicity to licensed BCG Vaccine SSI, and demonstrated robust immunogenicity in a dose-dependent manner [21].

Building on convincing results from Phase 1a, clinical development of MTBVAC is advancing into two specific target populations, healthy neonates and *M. tuberculosis* uninfected and infected adults. In Sept 2015, MTBVAC entered a Phase 1b safety and immunogenicity study in newborns with a safety arm in healthy, BCG-vaccinated at birth, *M. tuberculosis* uninfected adults (NCT02729571). Most adults in tuberculosis endemic countries have received BCG vaccine in infancy and have been exposed to *M. tuberculosis*. Demonstration of safety, immunogenicity, and optimal dose selection in an adult study population in tuberculosis-endemic settings is key to advancement of MTBVAC into adult efficacy trials. In the present study we used a re-vaccination approach combining *M. bovis* BCG with the live attenuated *M. tuberculosis* vaccine MTBVAC to evaluate whether efficacy (and safety) of BCG would be affected upon revaccination with MTBVAC, expecting no interference between both vaccines. Previous efficacy data using the standard short-term guinea-pig protection experiment, evaluating three independent dose levels of MTBVAC ($5x10^3$ CFU, $5x10^4$ CFU and $5x10^5$ CFU) by the subcutaneous route, showed similar protection which was dose independent four weeks after aerosol challenge (16 weeks post vaccination) [16]]. Surprisingly, in the present study efficacy of BCG was improved following MTBVAC revaccination and the improved protection afforded by

prime MTBVAC was unaffected following BCG-revaccination. Although, these findings should be confirmed in a repeated experiment, the results of the present study provide strong support for using MTBVAC as a safe and effective revaccination strategy in adolescents and adults living in high-burden TB-endemic countries, who are BCG vaccinated at birth and or pre-sensitized to *M. tuberculosis*.

Impact of vaccine-challenge intervals

The interval between BCG vaccination and challenge was investigated as an indication of the duration of protective immunity induced by vaccines. This is usually determined by measuring markers of immunological memory [31, 32] but is rarely tested in terms of a delayed interval between vaccination and challenge [33, 34]. In the heterologous re-vaccination study reported here (Figure 3), a thirty-week interval between BCG and challenge resulted in a reduced efficacy, which was significantly different compared to a shorter interval of 16 weeks. In a separate study (Figure 2), we demonstrated that the efficacy of BCG was lost if the vaccination to challenge period was 11 months. These data support reported studies also demonstrating this effect [35-37], and this evidence for waning efficacy of BCG is important to inform future studies because pre-clinical testing of subunit strategies to boost BCG is often confounded by the potent efficacy of BCG alone. If the boost is given distally from the BCG prime vaccination there is more chance that an improved effect can be observed. Studies involving such long prime-boost intervals can be costly and time consuming and it would be preferable to be able to demonstrate immunologically that an effective boost response had been induced, in order to accelerate development.

In contrast to published data and those reported here on waning of BCG, the MTBVAC vaccine had improved efficacy relative to BCG when the vaccination to challenge interval was longer. This suggests that MTBVAC immunity is longer lasting than BCG because the protective efficacy of MTBVAC is maintained with a longer interval between vaccination and challenge, whereas the

protection afforded by BCG is reduced when compared to the short vaccine-to-challenge interval.

Impact of prime-boost interval

Short and long revaccination interval comparisons were made to test the hypothesis that the potency of BCG or other live attenuated priming vaccines is diminished by a heterologous vaccine if given too soon after the prime. The data showed that, if anything the reverse was true. The combination of BCG and MTBVAC in a revaccination regimen improved protection in the lungs of guinea pigs compared to the efficacy observed by either vaccine alone. Interestingly, the strongest protection was observed with the short re-vaccination interval since only BCG-MTBVAC revaccination performed better than BCG alone with the longer re-vaccination interval. This implies that the observed effects could be due to a combination of the two vaccines giving a stronger immunity to *M. tuberculosis* infection, rather than due to a boosting of waning BCG immunity. The strong protection of these vaccine regimens was such that very few bacteria could be detected in the spleen and most were at least below the limit of detection of the CFU assay (Figure 4). Whilst this demonstrates high levels of potentially sterilising immunity at the spleen level, it was not possible to determine whether any of the regimens had a significantly stronger protective effect than BCG in the spleen. The histopathology analysis demonstrated that all of the vaccine regimens reduced pathology broadly in agreement with the primary bacteriology read out. However, the pathology scoring system did not have the sensitivity to differentiate between the BCG-MTBVAC revaccination regimens and BCG. Additionally, the MTBVAC-BCG (long interval) group was significantly protected compared to the unvaccinated group by CFU but the histopathology analysis indicated that the difference was not statistically significant. This is because the scoring system is semi-quantitative based upon the extent and nature of the pathology rather than absolute values. Therefore, the histopathology data should be considered only as supportive of the primary readout of vaccine efficacy (CFU) and not interpreted as a stand-alone marker of efficacy.

A BCG-BCG control was not included in the protection study because published data (pre-clinical and clinical) suggest that this strategy does not improve protection [9, 11-13, 28]. However, there are differences in study design between the published studies and those reported here, notably in the time intervals between BCG vaccination, re-vaccination, challenge and necropsy which make it difficult to draw precise conclusions. Therefore, BCG-BCG is important as an internal control and must be considered for future studies.

No BCG revaccination strategy is currently supported as described in the WHO position paper [12-15] and our own preclinical studies in guinea pigs support the view that revaccination with BCG does not provide improved efficacy in the guinea pig compared to a single dose of BCG [28]. Basabara et al. have reported that multiple vaccinations with BCG can have adverse effects when guinea pigs are subsequently challenged with *M. tuberculosis*, with exacerbation of pathology and excessive inflammation [9]. No such worsening of pathology was observed in our studies and the pathological changes were either similar or fewer in animals given BCG and MTBVAC, than in animals where the single vaccines were given. There are several differences between the Basaraba et al. study and ours, particularly the number of vaccinations (3 vs 2, respectively) and the intervals between each inoculation (3 vs 6 or 20, respectively) and any of these differences may explain the lack of adverse events seen in our study. Moreover, revaccination with a novel live-attenuated vaccine such as MTBVAC may be deemed an improvement to a BCG–BCG regimen as MTBVAC contains all the genes present in BCG, plus the *M. tuberculosis* genes deleted in *M. bovis* and BCG, including the human T-cell epitopes lost in BCG [38]. It is our hypothesis that a vaccine such as MTBVAC, based on the human TB pathogen, should be more efficient at inducing specific protective immunity against human TB disease caused by *M. tuberculosis*. Most if not all of the novel live vaccines in clinical trials are safer than BCG and offer a better prospect for use in latently infected people [15]. For example new vaccines including MTBVAC have demonstrated reduced reactivity [16]. In addition, clinical trials of the novel live vaccines which are currently on-going include cohorts that receive both BCG and the novel vaccine NCT02729571. Preclinical studies such as the strategy described in this paper

could provide important safety data in preparation for and in support of MTBVAC clinical trials in populations who have previously been vaccinated with BCG (mainly living in TB-endemic countries).

Preclinical investigation of re-vaccination is important to provide data for clinical trials where novel vaccines may be given in human populations who have or will receive BCG [39]. Here we report evaluation of the protective efficacy of MTVBAC and BCG in revaccination regimens. These results suggest that MTBVAC could be used as an effective vaccine administered at birth and as a revaccination strategy to improve upon BCG in adolescents and adults vaccinated at birth and living in high-burden TB-endemic countries.

Footnote page

Acknowledgements

The work was funded by TBVI-Norad Grant and supported by European H2020 grant TBVAC2020 643381. This work was supported by the Department of Health, UK. The views expressed in this publication are those of the authors and not necessarily those of the Department of Health. We thank the staff of the Biological Investigations Group at PHE Porton for assistance in conducting studies. The authors also gratefully acknowledge TBVI Preclinical and Clinical Development Teams for their dedication, expertise and continuing support in the preclinical, GMP and clinical development of MTBVAC. We thank Unizar Partner Biofabri and in particular, the team dedicated to the industrial development and production of MTBVAC, including Eugenia Puentes, Conchita Fernandez, Alberto Parra, Juana Doce, Esteban Rodriguez and Oswaldo Alvarez. We also thank Dr. Nacho Aguilo for his scientific contribution on preclinical TB immunity and for his incessant dedication to studying the protective mechanisms of MTBVAC in different models *in vivo*.

Conflicts of interests: Carlos Martin is co-inventor on a composition of matter patent “tuberculosis vaccine” and Biofabri is the exclusive licensee for MTBVAC. There are no other conflicts of interests.

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Figure legends

Figure 1. Study Schedule showing vaccination and re-vaccination intervals. (x =vaccination).

Figure 2. – Bacterial load determined in lungs (A) and spleen (B) of guinea pigs given a single subcutaneous injection of BCG Danish 1331 at either 3 or 11 months prior to aerosol-infection with *M. tuberculosis* and both compared to an unvaccinated, challenged control group. Values ($\log_{10}$ CFU/ml) for each individual animal are shown, group medians are presented for each vaccine group (horizontal bar). *P* values presented for each pairwise comparison between groups.

Figure 3. Bacterial load determined in both lungs (A) and spleen (B). Values ($\log_{10}$ CFU/ml) for each individual animal are shown, group medians are presented for each vaccine group (horizontal bar). * *P*= 0.05.

Figure 4. Group mean (and standard error of the mean for both consolidation and caseation/necrosis) lung pathology scores represented for each vaccine group. Black bar: consolidation, white bar: caseation and necrosis. * *P*= </=0.05. # No observed lesions.

Figure 5. Representative light photomicrographs of lungs from guinea pigs from each vaccine group. Main panel (50×) and inset photomicrograph (200x). Preceding challenge with the H37Rv strain of *M.* tuberculosis, groups of guinea pigs were given the following vaccine regimens; MTBVAC-BCG vaccine regimen as short (A) and long (D) prime-boost interval, BCG-MTBVAC vaccine regimen as short (B) and long (E) prime-boost interval, MTBVAC given in a short (C) and long (F) vaccination to challenge interval, BCG vaccination short (G) and long (H) vaccination to challenge interval, and (I) unvaccinated control group. No adverse pathology was observed as a result of re-vaccination. Haematoxylin and eosin.

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Figures [Clark et al. Revaccination in guinea pigs with the live-attenuated *Mycobacterium tuberculosis* MTBVAC improves BCG protection against tuberculosis]

Figure 1.

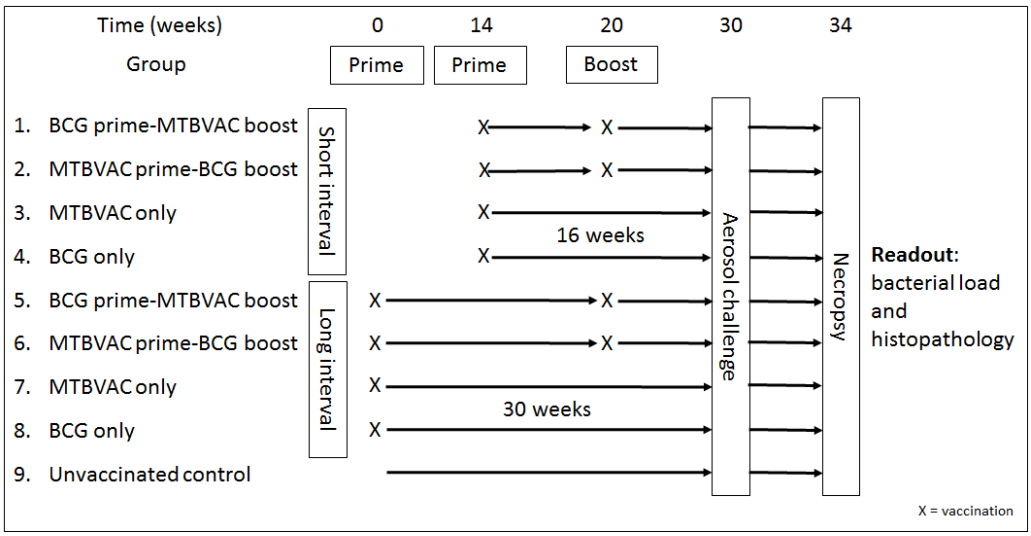


Figure 2.

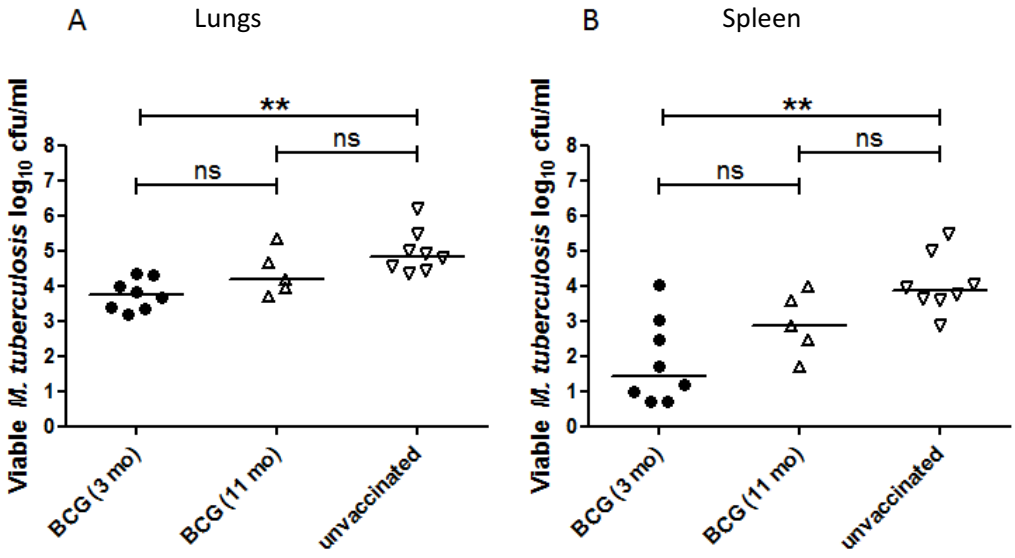

A
Lungs
B
Spleen
Viable M. tuberculosis log10 cfu/ml
**
ns
ns
BCG (3 mo)
BCG (11 mo)
unvaccinated

Figure 3.

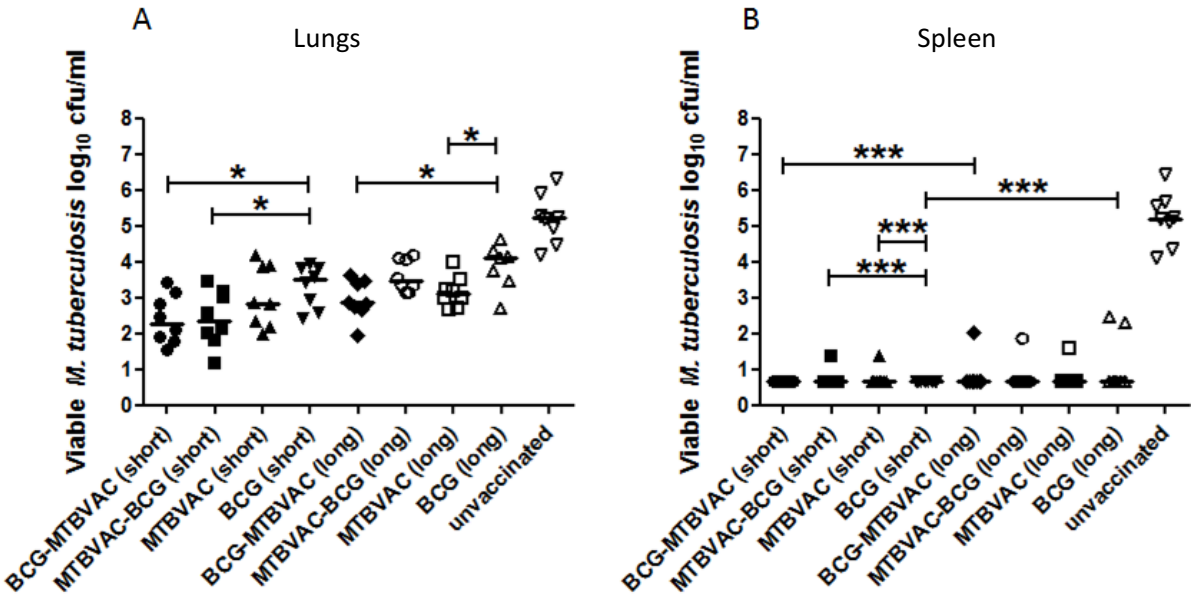
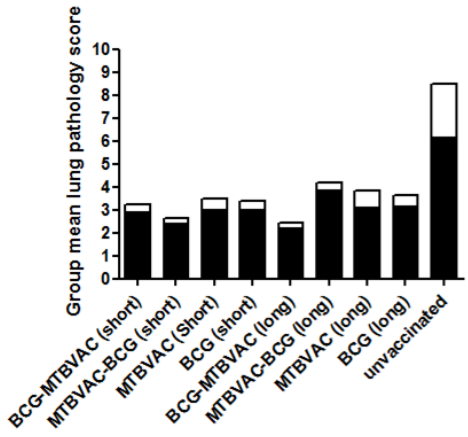
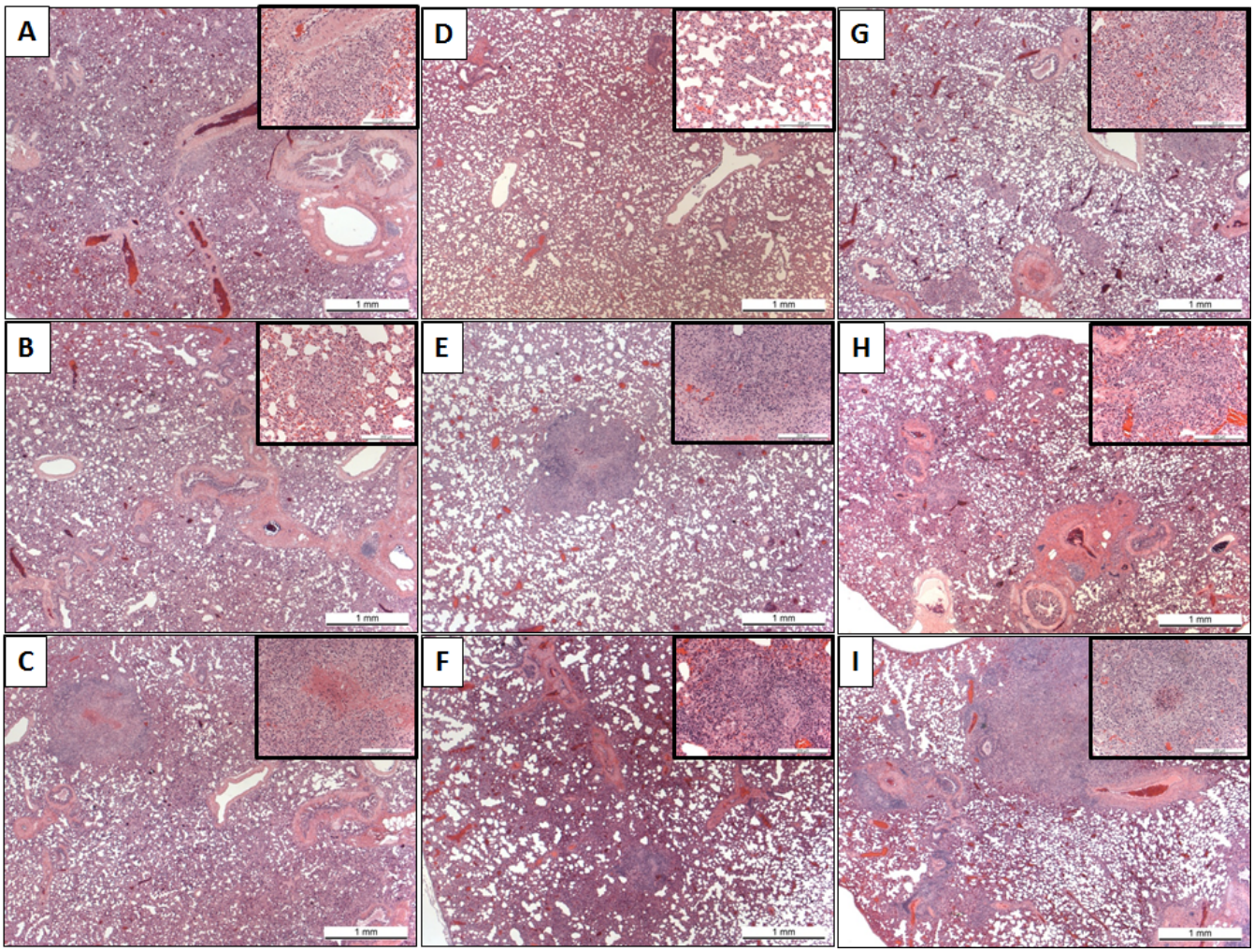


Figure 4.


Group mean lung pathology score
10
9
8
7
6
5
4
3
2
1
0
BCG-MTBVAC (short)
MTBVAC-BCG (short)
MTBVAC (Short)
BCG (short)
BCG-MTBVAC (long)
MTBVAC-BCG (long)
MTBVAC (long)
BCG (long)
unvaccinated

1 Figure 5.



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