RESEARCH ARTICLE

An attenuated quadruple gene mutant of Mycobacterium tuberculosis imparts protection against tuberculosis in guinea pigs

Ritika Kar Bahal1,‡, Shubhita Mathur1,‡, Priyanka Chauhan1,‡ and Anil K. Tyagi1,2,§

ABSTRACT

Previously we had developed a triple gene mutant of Mycobacterium tuberculosis (MtbΔmms) harboring disruption in three genes, namely mtpA, mtpB and sapM. Though vaccination with MtbΔmms strain induced protection in the lungs of guinea pigs, the mutant strain failed to control the hematogenous spread of the challenge strain to the spleen. Additionally, inoculation with MtbΔmms resulted in some pathological damage to the spleens in the early phase of infection. In order to generate a strain that overcomes the pathology caused by MtbΔmms in spleen of guinea pigs and controls dissemination of the challenge strain, MtbΔmms was genetically modified by disrupting bioA gene to generate MtbΔmmsb strain. Further, in vivo attenuation of MtbΔmmsb was evaluated and its protective efficacy was assessed against virulent M. tuberculosis challenge in guinea pigs. MtbΔmmsb mutant strain was highly attenuated for growth and virulence in guinea pigs. Vaccination with MtbΔmmsb mutant generated significant protection in comparison to sham-immunized animals at 4 and 12 weeks post-infection in lungs and spleen of infected animals. However, the protection imparted by MtbΔmmsb was significantly less in comparison to BCG immunized animals. This study indicates the importance of attenuated multiple gene deletion mutants of M. tuberculosis for generating protection against tuberculosis.

KEY WORDS: Multi-gene mutant, BCG, Tuberculosis, Attenuation, Auxotrophic vaccines, Biotin

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of human tuberculosis (TB), continues to be a major cause of mortality. The BCG vaccine provides effective protection against severe forms of TB in children but shows variable efficacy against adult pulmonary tuberculosis (Zodpey and Shrikhande, 2007). The difference in antigenic repertoire of M. tuberculosis and BCG leads to the generation of different host immune responses which might be responsible for the limited impact of BCG on control of TB. Live attenuated vaccine strains mimic the natural course of infection and maintain a balance between attenuation and immunogenicity. Several attenuated mutants of M. tuberculosis have been tested as TB vaccine strains, only few are able to generate protection equivalent to BCG such as the panCD, cysH, proC and trpD mutants of M. tuberculosis (Sambandamurthy et al., 2002; Senaratne et al., 2007; Smith et al., 2001). Strains such as ΔlysAΔpanCD and ΔlevΔpanCD demonstrated negligible multiplication in mouse organs, yet generated protection equivalent to BCG (Sambandamurthy et al., 2005; Sampson et al., 2004). On the contrary, their prototype ΔlysA and ΔlevD strains failed to reduce the bacillary load as much as BCG (Hondalus et al., 2000; Pavelka et al., 2003). Though the attenuated M. tuberculosis strains such as MTBVC (Arbues et al., 2013; Solans et al., 2014; Spertini et al., 2015) and MtbΔsigH (Kaushal et al., 2015) have shown promising results, the success rate of TB vaccine in clinical trials is low (Tameris et al., 2013). Thus, novel strains with new combinations of gene deletions need to be evaluated for their potential as vaccine against TB.

We had previously constructed a triple gene mutant of M. tuberculosis (MtbΔmms), having deletions in genes encoding for phosphatases mtpA, mtpB and sapM that are involved in host-pathogen interaction (Bach et al., 2008; Chauhan et al., 2013; Vergne et al., 2004; Zhou et al., 2010). The mutant MtbΔmms demonstrated bacillary growth in the spleens of guinea pigs at 4 weeks post-intradermal administration along with concomitant pathological damage to spleen (Chauhan et al., 2013). Further, animals vaccinated with MtbΔmms generated a sustainable and superior protection as compared to BCG in lungs. However, MtbΔmms was unable to control hematogenous dissemination of challenge strain to spleen with no significant difference from sham-immunized animals (Chauhan et al., 2013).

In order to overcome the pathology caused by MtbΔmms during the early phase of infection, and to generate a strain that controls dissemination of challenge strain, MtbΔmms strain was modified to generate an auxotrophic mutant by disrupting bioA gene involved in biotin biosynthesis (Attwood and Wallace, 2002; Beckett, 2007; Knowles, 1989; Tang et al., 2014; Mann et al., 2009, 2013). Several studies have demonstrated essentiality of bioA for survival of mycobacteria (Keer et al., 2000; Sassetti et al., 2003; Woong Park et al., 2011). We have earlier reported that disruption of bioA renders M. tuberculosis severely attenuated for growth and virulence in guinea pig along with negligible granulomatous pathology (Kar et al., 2017). Immunization with MtbΔbioA imparted significant protection in lungs and spleen when compared to sham-immunized animals demonstrating an efficient control over the dissemination of infecting strain to the spleen (Kar et al., 2017). In this study, we generated a quadruple gene mutant (MtbΔmmsb) by disrupting bioA gene in MtbΔmms strain. Further, we evaluated in vivo attenuation and assessed the protective efficacy of MtbΔmmsb against virulent M. tuberculosis challenge in guinea pigs.

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**RESULTS**

**Disruption of bioA gene in MtbΔmms**

Prior to proceeding with the disruption of the bioA gene from MtbΔmms, unmarking of hygromycin resistance gene was carried out by employing pYUB870.Gm to obtain MtbΔmms (Hyg−) strain (Fig. S1A). Unmarking was confirmed by patching on hyg−/hyg+ agar plates (Fig. S1B) and by PCR using mptP internal primers (Fig. S1C). The M. tuberculosis strain exhibited an amplification of 391 bp (Fig. S1C, lane 1) while a 2.3 kb PCR product was observed for MtbΔmms (Fig. S1C, lane 3). With unmarked MtbΔmms (Hyg−) strain an amplification of 413 bp was observed (Fig. S1C, lane 4, 8). For the construction of MtbΔmmsb strain, recombineering method was employed (Fig. 1A). Disruption of bioA gene was confirmed by PCR where a bioA gene-up and Hyg-down primer pair resulted in an amplification of 927 bp was observed with MtbΔmmsb (Fig. 1B, lane 1), while an amplification of 927 bp was observed with bioA gene-down and Hyg-up primer pair (Fig. 1C, lane 1). The 1.1 kb and 927 bp PCR products obtained for the MtbΔmmsb were confirmed by sequencing. Further, immunoblot analysis of MtbΔmmsb cell lysate with 1:1000 dilution of polyclonal anti-BioA antiserum (kindly provided by Dr Sabine Ehrt and Dr Dirk Schnappinger, Weill Cornell Medical College, New York) (Woong Park et al., 2011) exhibited absence of ~48 kDa band, thus confirming the disruption of bioA gene from MtbΔmmsb (lane 3, Fig. 1D).

**In vitro growth kinetics**

MtbΔmmsb strain was able to grow in biotin rich MB7H9 media but it failed to grow in biotin-deficient Sauton’s media (Fig. 2A,B). The mutant resumed its growth when biotin was exogenously added in Sauton’s media, and growth of MtbΔmmsb was dependent on the amount of biotin added (Fig. 2C,D). Thus, MtbΔmmsb was auxotrophic in nature.

**Attenuation study in guinea pigs**

Attenuation was evaluated as shown in Fig. 3A. Animals infected with M. tuberculosis demonstrated a large increase in the bacillary burden from 1 to 3 weeks post-infection in the lungs and spleen (Fig. 3B and C). By 6 weeks, the bacillary load in M. tuberculosis infected animals was stabilized in lungs and spleen and remained high thereafter. As early as 1 week post-infection, MtbΔmmsb-infected animals demonstrated significantly lower bacillary load in the lungs when compared to M. tuberculosis-infected animals with

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**Fig. 1. Disruption of the bioA gene in MtbΔmmsb.** (A) The figure depicts the disruption of the bioA gene in MtbΔmms Hyg (−) strain by homologous recombination using bioA-hyg AES to generate MtbΔmmsb. Arrows show the location of the bioA gene-up, bioA gene-down, Hyg-up and Hyg-down primers employed for the confirmation of disruption of the bioA gene by PCR. (B) PCR-based confirmation of disruption of bioA gene in MtbΔmmsb by employing bioA gene-up and Hyg-down primers. A 1.1 kb PCR amplification product was obtained with the MtbΔmmsb genomic DNA as template (lane 1). Lane 2 represents M. tuberculosis genomic DNA, and lane 3 represents 100 bp ladder. (C) PCR-based confirmation of disruption of bioA gene in MtbΔmmsb by employing bioA gene-down and Hyg-up primers. A 927 bp PCR amplification product was obtained with the MtbΔmmsb genomic DNA as template (lane 1). Lane 2 represents 100 bp ladder and lane 3 represents M. tuberculosis genomic DNA. The 1.1 kb and 927 bp PCR products obtained for the MtbΔmmsb were confirmed by sequencing. (D) Immunoblot analysis for confirmation of disruption of bioA in MtbΔmmsb. 10 μg of cell lysate of M. tuberculosis (lane 1) and MtbΔmmsb (lane 3) were separated on a sodium dodecyl sulfate-polyacrylamide gel (12.5%). Anti-BioA polyclonal antiserum (1:1000 dilution) was used for immunoblot analysis. Expression of bioA (~48 kDa band) was detected in the cell lysate of M. tuberculosis. The ~48 kDa band was absent from the cell lysate of MtbΔmmsb confirming the disruption of bioA in the mutant. Protein molecular weight marker was loaded in lane 2 and 50 ng of purified bioA was loaded in lane 4.
no detectable bacilli in spleen (Fig. 3B,C). By 3 weeks post-infection the bacillary load further decreased in the lungs of animals infected with \textit{Mtb}Δ\textit{mmsb}, and only one animal displayed negligible bacillary load in spleen (Fig. 3B,C). By 6 weeks post-infection no bacilli were detected in the organs of \textit{Mtb}Δ\textit{mmsb}-infected animals (Fig. 3B,C).

\textit{Mtb}Δ\textit{mmsb}-infected animals displayed lesser organ pathology as compared to \textit{M. tuberculosis} infected animals (Fig. S2). At 1 week post-infection both \textit{M. tuberculosis}- and \textit{Mtb}Δ\textit{mmsb}-infected animals exhibited minimal involvement with scanty or no tubercles observed in lungs and spleen (minimum gross pathological score of 1) (Fig. 4A). At 3 weeks post-infection few \textit{Mtb}Δ\textit{mmsb}-infected animals exhibited moderate gross pathological damage of lung with small tubercles occasionally visible, while spleen and liver appeared essentially normal (Fig. 4B). On the other hand, numerous small tubercles were observed in the lungs and liver of animals infected with \textit{M. tuberculosis}, while spleens were moderately enlarged with several small tubercles effacing the entire organ. Importantly, at 6 and 12 weeks post-infection the organs of \textit{Mtb}Δ\textit{mmsb}-infected animals appeared essentially normal with minimal involvement of organs (Fig. 4C,D). On the contrary, \textit{M. tuberculosis}-infected animals exhibited heavy organ involvement with numerous large tubercles, necrotic areas and splenomegaly. Further, \textit{Mtb}Δ\textit{mmsb}-infected animals demonstrated lower mean weight of lung and spleen as compared to the animals infected with \textit{M. tuberculosis} at indicated timepoints, with significant difference observed at 6 and 12 weeks post-infection (Fig. 4E,F). Detailed histopathological analysis was carried out for lung and liver tissues of \textit{Mtb}Δ\textit{mmsb}- and \textit{M. tuberculosis}-infected animals at 3 and 12 weeks post-infection (Fig. 5A). At 3 weeks post-infection significantly less total granuloma fraction was observed for the lungs and liver of \textit{Mtb}Δ\textit{mmsb}-infected animals when compared to \textit{M. tuberculosis}-infected animals, which demonstrated numerous necrotic granulomas containing epitheloid cells and lymphocytes (Fig. 5C,D). At 12 weeks post-infection, \textit{M. tuberculosis}-infected animals showed increased granulomatous pathology of lungs and liver while negligible granulomatous pathology was observed for animals infected with \textit{Mtb}Δ\textit{mmsb} (Fig. 5C,D). Thus, \textit{Mtb}Δ\textit{mmsb} was attenuated for growth and dissemination in the host tissues and could be safely employed as vaccine strain.

\textbf{Protective efficacy study in guinea pigs}

Protective efficacy was evaluated as illustrated in Fig. 6A. At four weeks post-infection, the sham-immunized animals exhibited the...
The lungs of guinea pigs (were aerosolically infected with spleens of guinea pigs (t were allotted a total CFU value of 2 in lungs whereas animals with no bacilli in spleen were allotted a total CFU value of 1. *Mtb were obtained from the total homogenate of half lung of animals. Animals vaccinated with bacillary load of 5.69 log10 CFU and 4.92 log10 CFU, respectively (Fig. 6B,C). In comparison, BCG vaccinated animals exhibited a significantly reduced bacillary load in lungs (by 1.06 log10 CFU) and spleen (by 2.51 log10 CFU) as compared to the sham-immunized animals. Animals vaccinated with MtbΔmmsb also demonstrated ability to control the multiplication and spread of infecting strain with a reduction of bacillary load by 0.46 log10 CFU and 1.70 log10 CFU in lung and spleen, respectively, when compared to sham-immunized animals (Fig. 6B,C). However, MtbΔmmsb failed to provide as much protection as conferred by BCG.

At 12 weeks post-challenge, BCG-vaccinated animals demonstrated significant reduction in bacillary load in lungs by 2.37 log10 CFU and in spleen by 1.98 log10 CFU when compared to sham-immunized animals (Fig. 6D). Vaccination with MtbΔmmsb also imparted significant protection when compared to sham-immunized animals with a reduction of 1.07 log10 CFU and 0.80 log10 CFU in lungs and spleen, respectively.

Relative to 4 weeks post-challenge, BCG as well as MtbΔmmsb showed improved ability to control bacterial multiplication in lungs at 12 weeks post-challenge while both the vaccination strains showed reduced ability to control hematogenous spread to spleen at this timepoint (Fig. 6D,E). However, as seen at 4 weeks, protection generated by MtbΔmmsb was significantly less as compared to BCG.

Pathological analysis exhibited that, at 4 weeks post-infection, sham-immunized animals exhibited maximum destruction of lungs, liver and spleen with numerous small or occasional large tubercles spread throughout the organs (Fig. 7A). However, BCG-vaccinated animals displayed significant reduction in gross pathological damage of lungs, spleen and liver with scanty to moderate involvement as compared to sham-immunized animals. MtbΔmmsb-vaccinated animals displayed significantly less pathological damage when compared to sham-immunized animals with a moderate number of tubercles effacing the organs. However, lungs of MtbΔmmsb-vaccinated animals demonstrated significantly higher pathological damage as compared to BCG immunized animals (Fig. 7A). Severe pathological damage of organs was observed for sham-immunized animals at 12 weeks post-challenge with lungs and liver exhibiting extensive involvement of tissue with numerous large tubercles, scattered areas of necrosis and occasional splenomegaly (Fig. 8A). BCG-immunized guinea pigs displayed milder pathology in lungs and spleen with smaller granulomas and decreased necrosis while liver exhibited minimal involvement (Fig. 8A). However, at this time point, the animals vaccinated with MtbΔmmsb exhibited disorganized lung and spleen phenotype as compared to BCG-vaccinated individuals. The lungs and spleen of animals vaccinated with MtbΔmmsb displayed moderate to heavy involvement with numerous small tubercles and necrotic areas while liver tissue appeared similar to BCG immunized animals.

At 4 weeks post-infection, the mean lung weight of MtbΔmmsb- and BCG-vaccinated animals was significantly less when compared to sham-immunized animals (Fig. 7B). However, vaccination with MtbΔmmsb did not result in any decrease in the weight of spleen.
from that observed in sham-immunized animals (Fig. 7B). At 12 weeks post-infection the mean spleen weight of \( \text{Mtb} \Delta \text{mmsb} \)-immunized animals was significantly less in comparison to sham-immunized animals while there was no difference in mean lung weight (Fig. 8B). BCG-vaccinated animals demonstrated significantly less mean organ weight when compared to sham-immunized as well as \( \text{Mtb} \Delta \text{mmsb} \)-vaccinated animals at this timepoint (Fig. 8B).

Histopathologically, at 4 weeks post-challenge, the granuloma score and fraction was highest for lungs and liver of sham-immunized animals which exhibited a large number of necrotic granulomas. Immunization with BCG or \( \text{Mtb} \Delta \text{mmsb} \) did not result in any significant difference in the granuloma score or fraction (Fig. 7C-E). At 12 weeks post-infection while sham-immunized animals continued to demonstrate high pathology in the lungs and liver, the lungs of \( \text{Mtb} \Delta \text{mmsb} \)-immunized animals also exhibited comparable high granuloma score and fraction (Fig. 8C-E). The lungs of BCG immunized animals exhibited significantly reduced histopathological damage when compared to sham or \( \text{Mtb} \Delta \text{mmsb} \) immunized animals (Fig. 8D). However, the liver tissue of BCG- and \( \text{Mtb} \Delta \text{mmsb} \)-immunized animals appeared histopathologically comparable and exhibited significantly reduced granuloma score and fraction when compared to sham-immunized animals (Fig. 8E).

**DISCUSSION**

Only 5-10% of the individuals exposed to \( \text{M. tuberculosis} \) progress to active disease, indicating that \( \text{M. tuberculosis} \) itself is capable of triggering an effective immune response. Thus, use of live,
attenuated *M. tuberculosis* strains as vaccine candidate is a promising strategy for the development of vaccines against TB.

In this study, we have constructed a quadruple gene mutant of *M. tuberculosis* by additional disruption of *bioA* gene in *MtbΔmms* to generate *MtbΔmmsb* using recombination through allelic exchange substrate. The disruption of *bioA* gene in the mutant *MtbΔmmsb* was confirmed by PCR analysis, immunoblot assay and *in vitro* growth characterization. We observed that the growth of the mutant strain was dependent on the concentration of external biotin supplementation which could be rescued with biotin at concentrations as low as 50 nM. The results are in agreement with the previous findings by Woong Park et al. (2011), where little or no growth of *MtbΔbioA* was observed at concentrations below 25 nM in media.

Our previous findings have demonstrated that *MtbΔmms*, the prototype of *MtbΔmmsb*, showed replication in the spleen during the early phase of infection along with some pathological damage (Chauhan et al., 2013). However, in the present study we found that *MtbΔmmsb* survived for a very short period and was cleared from the lungs of infected guinea pigs by 6 weeks post-infection with almost no detectable bacilli in spleen. Moreover, while the *M. tuberculosis*-infected animals exhibited severe pathological damage in lung, spleen and liver with disease progression, *MtbΔmmsb*-infected guinea pigs exhibited negligible organ pathological damage. This implies that disruption of biotin biosynthesis improved the safety profile of multigene mutant. Moreover, ability of some *M. tuberculosis* auxotrophs to provide protection despite high degree of attenuation indicates that even with limited survival in the host, significant immune response can be triggered (Martin et al., 2006; Sakthi et al., 2016; Sambandamurthy et al., 2005; Smith et al., 2001). Nevertheless, further studies are required for evaluating the safety profile of *MtbΔmmsb* strain in immunocompromised host such as SCID mice.

Upon evaluating the protective efficacy of *MtbΔmmsb*, we observed that *MtbΔmmsb* generated significant protection in comparison to sham-immunized animals at 4 and 12 weeks post-infection in lungs and spleen of infected guinea pigs. However when compared to BCG, the protection imparted by *MtbΔmmsb* was
significantly less, which is in contrast to its prototype MtbΔmms, which demonstrated significant protection in lungs. It appears that due to the highly attenuated nature of MtbΔmmsb strain, administration of a single dose may not present certain key antigens to sufficiently trigger a sustained protective response and thus multiple immunizations with MtbΔmmsb may be required. Revaccination with live attenuated vaccines such as BCG is not supported by WHO (Leung et al., 2001). Studies in guinea pigs have also demonstrated that multiple vaccinations with BCG does not improve the efficacy and result in exacerbation of pathology.

Fig. 6. Assessment of protective efficacy of MtbΔmmsb in guinea pig model of experimental tuberculosis. (A) Experimental protocol for evaluating the protective efficacy of MtbΔmmsb against infection with virulent M. tuberculosis in guinea pigs. Guinea pigs in groups of 6 were either sham-immunized (group I) or vaccinated with 5×10⁵ CFU of BCG (group II) or with 5×10⁶ CFU of MtbΔmmsb (group III). Guinea pigs were challenged with ∼30 bacilli of virulent M. tuberculosis via the aerosol route at 12 weeks post primary immunization and euthanized at 4 weeks and 12 weeks post-challenge. (B,C) Bacillary load in the lungs and spleen of vaccinated guinea pigs at 4 weeks post-challenge. Each data point represents the log₁₀ CFU/organ for an individual animal and the bar depicts mean (±s.e.m.) for each group. Significant differences were observed for the indicated groups (unpaired t-test; two tailed; **P<0.01 and ***P<0.001). (D,E) Bacillary load in the lungs and spleen of vaccinated guinea pigs at 12 weeks post-challenge. Each data point represents the log₁₀ CFU/organ for an individual animal and the bar depicts mean (±s.e.m.) for each group. Significant differences were observed for the indicated groups (unpaired t-test; two tailed; **P<0.01 and ***P<0.001).
Baseraba et al., 2006; Moreira et al., 2002). Live attenuated M. tuberculosis-based vaccines, when administered in two doses, have demonstrated abrogation of protection generated by a single dose (Kar et al., 2017; Sampson et al., 2004). However, certain live attenuated strains have shown promise in revaccination experiments such as MtbΔlysA, MtbΔglnA1 and MTBVAC (Clark et al., 2017;...
Lee et al., 2006; Pavelka et al., 2003). It will be interesting to test the potential of \textit{Mtb}\textit{Δmmsb} in a revaccination regimen where \textit{Mtb}\textit{Δmmsb} is given at a definite time interval with BCG.

The \textit{Mtb}\textit{Δmmsb} strain needs to be modified for the removal of antibiotic resistance genes employed for its development in accordance with the recommendations on the development of live
mycobacterial vaccine candidates in the Geneva consensus (Walker et al., 2010). Additionally, it would be interesting to evaluate the safety profile of the MtbΔmmsb strain when administered via different routes such as intradermally. Also, the efficacy of MtbΔmmsb strain can be evaluated following mucosal administration, as mucosal vaccination is increasingly being recognized as a promising route for immunization against tuberculosis (Kaushal et al., 2015). Further, time to death assay and understanding of immune correlates of protection would emphasize on the future worth of the strain.

Conclusion
Although preliminary, our findings provide evidence that deletion of genes involved in signal transduction and biotin biosynthesis severely attenuates the pathogen and single immunization with the auxotroph was insufficient for reducing the bacterial burden to levels comparable to BCG. Thus, future studies are focused on testing this multi-gene mutant as a booster dose in multiple immunization protocols.

MATERIALS AND METHODS

Experimental animals
Dunken Hartley guinea pigs (Cavia porcellus, female, 250-350 g) were purchased from Disease Free Small Animal House Facility, Lala Lajpat Rai University, India. The animals were housed in individually ventilated cages under standardized conditions in Biosafety level-III facility at University of Delhi South Campus, New Delhi, India and were provided with food and water ad libitum. Animals were allowed to acclimatize and were randomized prior to initiation of experiments.

Ethics statement
Institutional Animal Ethics Committee of University of Delhi South Campus, New Delhi, India, reviewed and approved the guinea pig experiments included in this study (Ref. No. 2/IAEC/AKT/Biochem/UDSC/7.8.2013). All animals were routinely cared for, according to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India, and all efforts were made to ameliorate animal suffering. Animals were intradermally vaccinated by injecting 100 μl of suspension and were euthanized by CO2 asphyxiation whenever required during day time in Biosafety level-III facility.

Bacterial strains and culture conditions
All mycobacterial strains (M. tuberculosis Erdman, M. tuberculosis H37Rv, M. bovis BCG, MtbΔmms and MtbΔmmsb) (Table S1) were grown in Middlebrook (MB) 7H9 broth (BD Difco) supplemented with 1X-ADC (albumin-dextrose-catalase complex, Difco), 0.5% glycerol and 0.05% Tween-80 or on MB7H11 agar (BD Difco) supplemented with 1X-OADC (oleic acid-albumin-dextrose complex, Difco) and 0.5% glycerol. Antibiotics were added at a concentration of 50 μg/ml for hygromycin, 20 μg/ml for gentamicin, 30 μg/ml for chloramphenicol and 25 µg/ml for kanamycin. For vaccination and infection purposes, mycobacterial strains were grown to mid-log phase in supplemented MB7H9 medium. Subsequently, cells were washed with phosphate-buffer saline (PBS), stocks were prepared and stored at −80°C, till further use. The colony-forming unit (CFU) of the PBS stocks was determined by plating appropriate dilutions in duplicates on supplemented MB7H11 agar.

Construction of MtbΔmmsb

For the generation of quadruple gene mutant, the bioA gene was disrupted from the MtbΔmms mutant (Chauhan et al., 2013). The disruptions of mptpA, mptpB and sapM in the MtbΔmms strain were marked by kanamycin, hygromycin and chloramphenicol resistance genes, respectively. Prior to the disruption of bioA in MtbΔmms, hygromycin resistance gene was unmarked from the disrupted mptpB gene by employing modified helper plasmid pYUB870 (Table S2). The kanamycin resistance gene in PYU870 was replaced with gentamicin resistance gene to generate PYU870.Gm which was electroporated into MtbΔmms to generate MtbΔmms (Hyp−) strain. Unmarking was confirmed by patching on Hyg−/Hyp+ agar plates and PCR analysis with mptpB-specific primers (Table S2). Next, plasmid pJV53 (Table S2) was employed for the expression of recombineering proteins in MtbΔmms (Hyp−) strain to facilitate homologous recombination. The kanamycin resistance gene of vector pJV53 was replaced with gentamicin resistance gene to generate pJV53.Gm which was electroporated into MtbΔmms (Hyp−) strain to generate the recombineering strain of MtbΔmmsb expressing p60/gp61. Subsequently, a 3.4 kb linear BioA−:hyg AES (Kar et al., 2017) was electroporated into MtbΔmmsb (Hyp−) and transformants were selected on MB7H11 agar following incubation at 37°C for 3-4 weeks.

In vitro growth analysis
MtbΔmmsb and M. tuberculosis were grown as described above or in Sauton’s media (Himedia) supplemented with 0.5% glycerol and 0.05% Tween-80. Additionally, growth of MtbΔmmsb was analysed in Sauton’s media supplemented with different concentrations (10 nM to 1000 nM) of ad libitum.

In vivo attenuation studies
Groups of guinea pigs (n=6) were aerogenically infected with either M. tuberculosis or MtbΔmmsb resulting in an infection dose of ~30 bacilli in the lungs at day one post-infection and were euthanized at indicated time points post-infection for evaluating bacillary load and pathological damage. Enumeration of bacillary load, gross pathological and histopathological evaluation were carried out as described earlier (Kar et al., 2017).

Protective efficacy studies
Guinea pigs were intradermally vaccinated with either 100 μl of saline or 5×104 CFU of BCG or 5×105 CFU of MtbΔmmsb in 100 μl of saline. Twelve weeks post primary immunization guinea pigs were aerosolally challenged with low dose of M. tuberculosis H37Rv in an aerosol chamber (A4224 full body Inhalation exposure system, Glas-Col Inc., USA) resulting in 10-30 bacilli in lungs per animal at day one post-challenge. Animals were euthanized at 4 and 12 weeks post-challenge. Enumeration of bacillary load, gross pathological and histopathological evaluation were carried out as described earlier (Kar et al., 2017).

Statistical analysis
Unpaired t-test (two-tailed) was employed for comparison between groups for evaluating bacillary load, organ weight and gross pathological damage of guinea pig organs. Mann–Whitney test (two-tailed) was employed for comparison between groups for analysis of total granuloma score and fraction. Generation of graph and statistical analysis was carried out by employing Prism Software (Graph Pad software Inc., CA). Differences were considered significant when P<0.05.

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Competing interests
The authors declare no competing or financial interests.

Author contributions