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Lipoprotein LpqS deficient *M. tuberculosis* mutant is attenuated for virulence *in vivo* and shows protective efficacy better than BCG in guinea pigs

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ABSTRACT

Bacterial lipoproteins are a functionally diverse class of membrane anchored proteins. Lipoproteins constitute nearly 2.5% of the *Mycobacterium tuberculosis* proteome. Inactivation of genes coding for individual lipoproteins results in attenuated phenotype of the mutants. LpqS is a lipoprotein highly conserved among slow growing pathogenic mycobacteria. Our previous study has shown that the *lpqS* gene deletion mutant of *M. tuberculosis* (MtbΔlpqS) poorly replicates in THP1-(human acute monocytic leukemia cell line) derived macrophagic cell line. In addition, guinea pigs, when infected with the mutant strain exhibited significantly reduced bacterial burden and pathological damage in the infected tissues in comparison with the parental strain infected group. Subsequently, we evaluated the protective efficacy of the mutant by immunization of guinea pigs through aerosol and subcutaneous routes. We observed that immunization of guinea pigs with MtbΔlpqS offered superior protection in lungs as compared to BCG. In addition, MtbΔlpqS also prevented the haematogenous spread of the disease which was evident from the significantly reduced splenic bacillary load compared to saline vaccinated animals. The gross pathological observations and the histopathological observations well corroborated the bacterial findings. We also observed that aerogenic route of immunization imparts superior protection compared to subcutaneous route of immunization. These findings well establishes the efficacy of *M. tuberculosis* mutant in imparting protection against pulmonary TB.

1. Introduction

Q4 Tuberculosis (TB) has been a global threat for several years and is the second leading cause of death from a single infectious disease worldwide, after HIV. In the year 2013, nine million new TB cases were registered. The number of people succumbed to TB was estimated to be 1.5 million [1]. Emergence of MDR and XDR strains has become serious threats to global control of tuberculosis. The directly observed treatment short course (DOTS) adopted by the WHO is highly effective as a therapeutic strategy at reducing prevalence of the disease in targeted regions, but it does not address directly ongoing transmission of infection to susceptible individuals. Variable efficacy of BCG vaccine [2], TB-HIV coinfection, treatment involving lengthy antibiotic course remain major obstacles in TB eradication. Thus current needs include novel vaccines, rapid diagnostic methods and development of newer antimycobacterial drugs.

Host–pathogen interactions are very elaborate and decisive for mycobacterial survival. Secreted and cell surface associated proteins are ideally positioned to participate in host pathogen interactions and they play crucial role in the pathogenesis of mycobacteria. Considerable fraction of such exported proteins in *Mycobacterium tuberculosis* was identified as lipoproteins [3,4]. However, very few lipoproteins remain characterized till date based on their potential implication in pathogenesis and immunogenicity. Earlier studies have established that deletion of these lipoproteins from the genome of *M. tuberculosis* resulted in attenuated virulence exhibited by the mutants in animal models of infection [5–10]. An *M. tuberculosis* lipoprotein mutant Δ*lpqH* displayed high attenuation *in vivo* in C57BL/6 mice [7]. LprG deletion mutants of both *M. tuberculosis* and *Mycobacterium bovis* strains were found to be highly attenuated for growth and survival in mice model of infection [8–10]. Disruption of *lppX* results in attenuated virulence in BALB/c mice [6]. Thus the crucial role played

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by lipoproteins in the pathogenesis of *M. tuberculosis* remains well established. We have previously described the construction and complementation of the *M. tuberculosis* lipoprotein mutant MtbΔlpqS and have shown that the lipoprotein mutant exhibited poor replication *in vitro* in Macrophagic cell line [11]. Here, we have further established the attenuated virulence phenotype of the *M. tuberculosis* mutant MtbΔlpqS *in vivo*. Several studies have evaluated the vaccine potency of such attenuated *M. tuberculosis* mutants in animal models of tuberculosis [12–16]. *M. tuberculosis* mutants like *ΔRD1ΔpanCD* strain and *ΔPhoPΔfad Mtb* strain [14,15,17] have shown promising results and is under clinical evaluation. Here, we have also evaluated protective efficacy of the single knockout mutant MtbΔlpqS in the guinea pig model of tuberculosis infection.

2. Materials & methods

2.1. Bacterial strains and culture conditions

M. bovis BCG—Pasteur and *M. tuberculosis* H_{37}Rv were grown in Middlebrook 7H9 broth containing 10% OADC (Becton Dickinson), 0.2% glycerol and 0.05% Tween 80 or on Middlebrook 7H11 agar containing OADC (Becton Dickinson), 0.5% glycerol and the antifungal agent cycloheximide (100 mg/ml) (Sigma-Aldrich). For the growth of MtbΔlpqS, all of the above media were supplemented with hygromycin.

2.2. Growth kinetics of MtbΔlpqS mutant at acidic pH

The wild-type *M. tuberculosis* H_{37}Rv, MtbΔlpqS and CΔlpqS were grown to log phase, washed twice and diluted to an OD_{600} of 0.02 in 7H9-OADC media having a pH of 5.5 and supplemented with 0.05% Tween 80. The cultures were grown in the shaker incubator with gentle shaking at 80 rpm at 37 °C. All experiments were performed in duplicates. Aliquots of these cultures were withdrawn for $OD_{600\,nm}$ readings at specified time points as indicated.

2.3. Growth kinetics of MtbΔlpqS disrupted mutant under hypoxic conditions

To assess the growth of the MtbΔlpqS mutant under reduced oxygen tension, *M. tuberculosis* H_{37}Rv, MtbΔlpqS and CΔlpqS cultures were grown under conditions that mimic Wayne dormancy model. Oxygen starved cultures were grown by diluting aerobic exponential preculture to 0.02 OD in glass bottles containing 7H9 + OADC in a shaker incubator with gentle shaking at 80 rpm. Each culture was sealed using a rubber sealer septum to prevent air exchange. Aliquots of cultures were taken from the sealed bottles using a sterile needle through the rubber lining for $OD_{600\,nm}$ readings at specified time points to measure the growth of *M. tuberculosis*. Viable counts at specified time points were determined by plating appropriate dilutions of the cultures on Middle brook 7H10 agar. Mean values with SEM from three independent experiments are shown.

2.4. Experimental animals

Pathogen free 200–300 g female outbred Dunkin Hartley guinea pigs were housed in stainless steel cages and were provided with *ad libitum* food and water in a BSLIII facility (National JALMA Institute of Leprosy and Other Mycobacterial Diseases, Agra, India). All the guinea pig experimental protocols included in this study were reviewed and approved by the animal ethics committee of the institute.

2.5. Infection of guinea pigs to evaluate the virulent phenotype of the mutant

Guinea pigs ($n = 6$) were infected with 50–100 bacilli of each of virulent *M. tuberculosis* H_{37}Rv, MtbΔlpqS mutant and the complemented strain CΔlpqS *via* the respiratory route in an aerosol chamber (Inhalation Exposure System, Glasscol Inc., IN, USA). Animals from each group were then euthanized by i.p. injection of Thiopentone sodium (100 mg/kg body weight) (Neon Laboratories Ltd., India) and dissected aspectically at two different time points (5 weeks and 10 weeks post infection).

2.6. Bacterial enumeration

A portion of left caudal lung lobe and caudal portion of spleen were aseptically removed, weighed and homogenized separately in 5 ml saline in a Teflon glass homogenizer foe measurement of bacillary load. Appropriate dilutions of the homogenates were inoculated on to MB7H11 agar plates in duplicates and incubated at 37 °C in a CO_2 incubator for three to four weeks. The number of colonies were counted and expressed as $\log_{10}$ CFU/g of tissue.

2.7. Necropsy procedure, gross pathological and histopathological evaluation

Guinea pigs euthanized by i.p. injection of Thiopentone sodium were aseptically dissected. Lung and spleen were examined for gross pathological changes and scored using the modified Mitchison scoring system by veterinary pathologists. 5 μm thick sections of formalin fixed and paraffin embedded lung and spleen tissues were cut onto glass slides and stained with hematoxylin and eosin for histopathological examination. The granuloma formation, type and extent of necrosis in the lung and spleen were assessed and total granuloma score assigned for lung sections [18]. A portion of lung from the animals that were euthanized at 10 weeks post infection were stored in RNA later at −80 °C for the isolation of RNA to be used for RT-PCR studies.

2.8. RNA extraction and real time PCR

RNA samples were extracted from the lung tissues of three animals from each group ($n = 6$). Lung tissues stored in RNA later were homogenized and further disrupted with 0.1 mm zirconia beads in a mini-bead beater after adding Trizol reagent (Invitrogen). Total RNA was purified using an RNeasy purification kit (Qiagen). Contaminating DNA in the RNA sample was digested with RNase-free DNase I. The purity of the RNA was determined by measuring the absorbance at 260 and 280 nm. The first-strand cDNA was synthesized from 800 μg total RNA using Quantitect Reverse Transcriptase Kit (Qiagen). Real-time quantitative RT-PCR9 (qRT-PCR) for the cytokines IFN-γ and IL-10 was performed in an ABI 7500 system (Applied Biosystems) using TaqMan assays. Each reaction was repeated three times with independent RNA samples. Negative controls consisting of no reverse transcriptase and no template mixtures were run with all reactions.

2.9. Evaluation of the protective efficacy of the MtbΔlpqS mutant

For evaluation of protective efficacy of MtbΔlpqS mutant, guinea pigs ($n = 6$) were immunized with 5×10^5 CFU of BCG (Pasteur strain) or MtbΔlpqS in 100 μl of saline by subcutaneous (S) route or aerosol (A) route. In the control group, guinea pigs were immunized with 100 μl of saline. The animals were then challenged 5 weeks post immunization with 50–100 bacilli of virulent *M. tuberculosis* H_{37}Rv *via* the respiratory route in an aerosol chamber. Five weeks after infection, animals were euthanized and dissected

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