Hyper-attenuated MTBVAC erp mutant protects against tuberculosis in mice

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\textbf{A B S T R A C T}

Safety of individuals at risk of immune suppression is an important concern for live vaccines. The new-generation tuberculosis vaccine candidate MTBVAC, a genetically engineered doubly attenuated \textit{Mycobacterium tuberculosis} mutant with deletions in \textit{phoP} and \textit{fadD26} virulence genes has demonstrated comparable safety in different relevant animal models and superior protection in mice as compared to the only currently licensed tuberculosis vaccine \textit{Mycobacterium bovis} BCG. Here we describe the construction of a highly attenuated MTBVAC-based live vaccine by an additional gene inactivation generated in erp of MTBVAC. The gene product of \textit{erp} is an exported repeated protein (Erp), a virulence factor described to be involved in intracellular replication of \textit{M. tuberculosis}. The resultant strain, MTBVAC erp\textsuperscript{−}, was tested in severe combined immunodeficiency (SCID) mouse model showing to be severely attenuated when compared to BCG and MTBVAC. Experiments conducted in immunocompetent mice revealed that the hyper-attenuated profile observed with MTBVAC erp\textsuperscript{−} strain did not compromise its protective efficacy profile in comparison with BCG. These results postulate MTBVAC erp\textsuperscript{−} as a potential tuberculosis vaccine candidate for use in high-risk populations of immune suppression (e.g., due to HIV infection), where the use of BCG is not recommended.

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1. Introduction

Tuberculosis (TB) remains a major global health problem and \textit{Mycobacterium tuberculosis}, the causative agent of human TB, is responsible for infecting a third of the world’s population. In 2011, there were 8.7 million new cases of TB worldwide and 1.4 million people who died from the disease [1]. With TB causing a quarter of the deaths in people living with HIV [1] and the emergence of increasingly drug resistant strains of \textit{M. tuberculosis}, an effective vaccine is needed now, more than ever, to reduce the burden of this disease [1].

The only existing licensed vaccine against TB is a modified attenuated strain of \textit{Mycobacterium bovis}, bacille Calmette–Guerin (BCG), which has been used worldwide since 1921 when it was first administrated to a newborn with a household contact of TB. BCG confers protection against severe forms of TB (meningitis and military TB) in children but lacks consistency in preventing pulmonary disease, the most common form responsible for transmission by respiratory route [2]. Even though BCG is considered very safe, serious immunodeficiency states have been associated with increased risk of systemic BCG dissemination post-vaccination [3]. The risk groups for which vaccination with BCG is not recommended include patients with primary and secondary immunodeficiency and HIV-infected individuals, including HIV-infected newborn infants [2]. There has been particular concern for the role of HIV infection in the safety of BCG vaccination [4]. A recent retrospective study documented a high frequency of BCG infection with complications in HIV-infected infants [5]. Current WHO vaccination policy does not recommend BCG vaccination of newborn infants with a known HIV-infection status, with or without infection symptoms [2].

Very substantial efforts have been made over the past decade to develop vaccines against tuberculosis, including vaccines targeting
individuals at risk of immune suppression [6]. Various preventive strategies in the current Global TB Vaccine Portfolio include recombinant BCG strains, boosting subunit vaccines for use in BCG-immunized individuals, or live attenuated M. tuberculosis strains at different stages of development [6].

MTBVAC, a live attenuated M. tuberculosis vaccine based on two independent stable deletion mutations without antibiotic resistance markers in the virulence genes phoP and fadD26 [7], is currently the first and only vaccine to fulfil the Geneva consensus criteria for progressing new live mycobacterial vaccines to clinical evaluation [8,9]. MTBVAC confers superior protection in mouse model and presents equivalent safety profile as compared to BCG [7]. As a result, MTBVAC is the first vaccine of this kind to enter clinical trials (NCT02013245).

Considering individuals at risk of immune suppression as a possible target population for MTBVAC, here we describe the profile of the hyperattenuated vaccine based on MTBVAC with an additional inactivation in the erp gene. This gene codifies for an exported protein (Erp), a virulence factor of M. tuberculosis implicated in bacterial intracellular replication both in vitro and in vivo [10]. Disruption of the erp locus in M. tuberculosis and M. bovis has been shown to lead to attenuation of virulence [10,11]. Erp was initially thought to be present in M. tuberculosis complex members only, but today it is known to be part of an extracellular protein family specific for mycobacteria [12] implicated in early-time infection evolution [13] and recognized as a virulence determinant required for cell wall integrity and intracellular survival in Mycobacterium marinum [14].

The present work focuses specifically on the construction and extensive preclinical characterisation of MTBVAC erp−, which, in addition to phoP and fadD26 deletions, contains a marked deletion in the erp locus of MTBVAC. This new generation live hyperattenuated vaccine based on M. tuberculosis is conceived with the potential to be administrated to risk groups of acquiring immune suppression (e.g., as a result of HIV-infection). MTBVAC erp− is significantly more attenuated than MTBVAC and BCG in preclinical studies in SCID mice and confers similar protection to BCG in C57/BL6 mice, which is lower than the efficacy conferred by MTBVAC.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Escherichia coli HB101 and the mycobacterial strains M. tuberculosis H37Rv [15], M. tuberculosis Mt103 (clinical isolate) [7], M. bovis BCG Pasteur and MTBVAC [7] were used in this work. Mycobacterial strains were grown at 37 °C in 7H9 medium (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80 and 10% albumin–dextrose catalase (ADC, Middlebrook), on 7H10 plates supplemented with 0.5% glycerol and 10% albumin–dextrose catalase (ADC, Middlebrook) or 7H11 plates supplemented with 0.5% glycerol, 10% albumin–dextrose catalase (ADC, Middlebrook), polymixin B 50 μg/ml, trimetoprim 0.02 mg/ml and amphotericin B 0.01 mg/ml. Resulting mutants were grown in the same media. E. coli HB101 used for cloning procedures was grown at 37 °C in LB broth or on LB agar plates. When required, kanamycin (Km) was used at 20 μg/ml, gentamicin was used at 10 μg/ml for 7H10 medium or 20 μg/ml for LB medium and sucrose was used at 4% (w/v).

2.2. Plasmid construction and allelic replacement

A 2.2 Kb erp-containing fragment was amplified from Mt103 genomic DNA using erpF (AGCGGCCCTCGATGCCGTTGTCAGC) and erpRv (AAGGGGCCCATACTGGTGTCAGC) primers. It was cloned in pGEM-Teasy® vector. Res-site flanked Km resistant marker from pCG122 [7] was cloned into BamHI and EcoRI sites into erp gene resulting in a deletion of 431 bp in the ORF (pLZ2). Plasmid pLZ3 was constructed by inserting an 8955 bp Apal fragment containing erp::km fragment from pLZ2 into the Apal site of pLQ200-ynIE [7]. Suicidal vector in mycobacteria, pLZ3, can be used to replace genomic material by double recombination. PCR primers were designed to characterize the new construction and confirm double recombination in desired position, erpMUT1 (ACGTCGA-GATGTTGATCAGCATAC) placed upstream of the recombined region and erpMUT2 (CAGAGTTCCACGACCGT) placed downstream, res 1 and res 2 [7] were also used for that purpose.

2.3. Infection of mouse alveolar macrophage cell line MH-S

MH-S cells (2 × 10^6 cells/well) were seeded in 24-well plates (TPP) in DMEM medium enriched with 10% foetal calf serum and glutamine 2 mM and were infected with bacterial suspension of Mt103, MTBVAC or MTBVAC erp− at a multiplicity of infection of 1:1. Cultures were incubated at 37 °C in 5% CO2 atmosphere. After 4h of infection, cell monolayers were washed three times with PBS to remove any non-internalized bacteria. At various times post-infection (4h, 24 h, 72 h or 168 h), infected cells were treated with 0.1% triton X-100 to lyse macrophages and appropriate dilutions were plated on 7H10 plates to count colony forming units (CFU) of intracellular bacteria.

2.4. Mt103 and Mt103 erp− competition in SCID mice

A group of five eight-week old CB17 SCID mice was simultaneously infected with 10^6 CFU of viable Mt103 and Mt103 erp− by the intraperitoneal route. Three weeks post-infection, mice were euthanized and lungs and spleen were harvested to measure viable bacteria by plating on 7H11 and 7H11+ Km 20 μg/ml to discriminate Mt103 from Mt103 erp−. Statistical analysis was performed using GraphPad Prism software.

2.5. Determination of survival in SCID mice

Groups of five eight-week old CB17 SCID mice were intraperitoneally inoculated with 10^6 CFU of Mt103, Mt103 erp−, M. bovis BCG Pasteur, MTBVAC or MTBVAC erp−. Experimental endpoint was determined by survival and mouse weight lower than 17 g was established as euthanasia end point. Statistical analysis was performed using GraphPad Prism software.

2.6. Vaccination and protection efficacy

Groups of six C57BL/6 mice were intradermally vaccinated with 10^3 CFU of viable M. bovis BCG Pasteur, MTBVAC or MTBVAC erp− saline was used as control. Four weeks post-infection, mice were challenged with approximately 10^3 CFU of M. tuberculosis H37Rv strain via intratracheal inoculation. Eight weeks post-challenge, mice were sacrificed and viable mycobacteria present in lungs and spleen were measured by plating the appropriate dilutions on 7H11 plates. Statistical analysis was performed using GraphPad Prism software.

2.7. Immunogenicity induced in C57BL/6 by vaccine candidates

Groups of five C57BL/6 mice were immunized subcutaneously with 10^3 CFU of viable M. bovis BCG Pasteur, MTBVAC or MTBVAC erp−. Four weeks post-immunization mice were euthanized and spleen and lungs were extracted. Spleens were ground and cells suspensions were treated with red blood cell lysing buffer
Mutants

using

were obtained. DNA was amplified using pair of primers, erpMUT1 and res1, and erpMUT2 and res2. Mutants 1 and 2 were obtained from Mt103 and mutants 3 and 4 were obtained from MTBVAC. Mutants 2 and 4 were chosen (grey arrows).

(SIGMA) to eliminate erythrocytes. Lungs were homogenized in 10 mM HEPES–NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2 buffer and crushed in gentleMACS dissociator (MACS) and incubated with red blood cell lysing buffer (SIGMA). Splenocytes or lung cells (10⁶ cells/well) were seeded in 96-well plate (TPP) and incubated in RPMI media supplemented with 10% foetal calf serum, glutamine 2 mM, penicillin 100 U/ml, streptomycin 100 μg/ml and β-mercaptoethanol 50 μM, in presence of PPD (SSI) 1 μg/ml, at 37°C in 5% CO₂ atmosphere. After 18 h of incubation, cells were marked with α-IFN-APC (BD) and α-CD4-FTTC (BD) using BD Cytofix/Cytoperm Fixation/Permeabilization kit according to manufacturer recommendations. Cells were analyzed by flow cytometry using FACSArria (BD). Statistical analysis was performed using GraphPad Prism software.

3. Results

3.1. Erp inactivation attenuates M. tuberculosis in vivo

Different erp mutants in MTBVAC and in wild-type Mt103 (parental strain of MTBVAC) (Fig. 1a) were obtained by using double recombination system [16]. A suicide vector carrying a sacB gene (lethal in mycobacteria), xylE gene (gives colouration in the presence of catechol), Gm' (confers resistance to gentamicin) and an erp⁻ deleted locus including a kanamycin resistance marker was electroporated into Mt103 and MTBVAC. Single recombination mutants were selected onto plates containing kanamycin and gentamicin, presenting a yellow colouration in presence of catechol and unable to grow in presence of sucrose. Double recombination mutants were selected onto plates containing kanamycin, which were unable to grow on plates containing gentamicin, did not present colouration in presence of catechol and were able to grow in presence of sucrose. Correct recombination was analyzed by PCR using primers flanking the recombinated region erpMUT1 and erpMUT2 and primers placed in res sites of the construction [7]. We selected colonies which amplified both PCRs (Fig. 1b).

The attenuation profile of a single erp mutant in Mt103 strain was assessed in a competition experiment inoculating CB17 SCID mice intraperitoneally with an equivalent amount of Mt103 and Mt103 erp⁻ CFU per mouse and measuring bacterial burden in lungs and spleen at three weeks post-infection. Results showed that Mt103 displaced Mt103 erp⁻ by 1 log₁₀ order (Fig. 2), indicating that erp inactivation affected capacity of bacteria to replicate in vivo.

3.2. MTBVAC erp⁻ hyperattenuation in vivo is due to a synergic effect of three mutations

First, we aimed at studying whether erp inactivation conferred additional attenuation of MTBVAC in a cellular model of infection. Thus, murine cell line MH-S was used as model of alveolar macrophages for replication experiments comparing Mt103, MTBVAC and MTBVAC erp⁻. At 3 days post-infection Mt103 replicated within MH-S cells 2 log₁₀ orders, reaching 3 log₁₀ orders after seven days post-infection. MTBVAC and MTBVAC erp⁻ exhibited very similar impaired replication capacity with less than 1 log₁₀ CFU increase in seven days (Fig. 3a).

Next, we studied MTBVAC erp⁻ attenuation in CB17 SCID mice, in comparison with Mt103, Mt103 erp⁻, BCG Pasteur and MTBVAC following intraperitoneal administration. Comparison of survival of animals that received Mt103 or Mt103 erp⁻ confirmed data obtained with the competition experiment in SCID mice, as mice inoculated with Mt103 erp⁻ showed significant survival by 2 additional weeks as compared to wild-type. SCID mice inoculated with MTBVAC erp⁻ survived between 42 and 51 weeks (Fig. 3b), with viable bacteria in the range of 6.2 × 10⁴ to 7.7 × 10⁵ CFU recovered in lungs after that period. When we analyzed survival times of MTBVAC and MTBVAC erp⁻ groups, data revealed a dramatic attenuation profile of MTBVAC erp⁻ strain, as these animals survived for approximately 1 year as compared to MTBVAC group, which succumbed by day 120. Data shown in Fig. 3 indicate that the highly attenuated profile of MTBVAC erp⁻ is due to a synergic effect of the three inactivated genes in MTBVAC, namely phoP, fadD26, and erp (Fig. 3). Finally, by a respiratory inoculation route in SCID mice, both vaccine candidates MTBVAC and MTBVAC erp⁻ were significantly safer than BCG Pasteur, in agreement with previous data in this animal model of infection generated with the MTBVAC prototype, SO2 phoP mutant, published by Martin et al. [17].
Fig. 3. MTBVAC erp− hyper attenuation is a result of a non-additive synergic effect of phoP− and fadD26 deficiencies. (a) Intracellular replication in MH-S cell line of Mtb103, MTBVAC and MTBVAC erp−. Statistical analysis was done applying a 2-way ANOVA test, using post hoc Bonferroni test with confidence intervals of 95% (**equivalent to p < 0.0001). (b) Survival experiment in SCID mice. Mice were infected by intraperitoneal route and experiment was followed until decease of animals. Statistical analysis of survival was done applying a Gehan–Breslow–Wilcoxon test with a confidence interval of 95% (**equivalent to p < 0.001), all group differences were significant p < 0.001.

3.3. MTBVAC erp− confers similar protection to BCG in mice

Since a high level of attenuation could compromise protective efficacy, we tested efficacy of MTBVAC erp− as compared to BCG and MTBVAC following challenge with H37Rv in C57BL/6 mice. When compared to saline control group, BCG and MTBVAC erp− showed similar but significant ability to confer control of lung bacterial burden, whereas MTBVAC showed a significantly improved protection (Fig. 4a). A similar result was obtained in spleen (Fig. 4b).

3.4. Immunogenicity of the vaccine candidate MTBVAC erp−

In order to analyze immunogenicity of MTBVAC erp−, CD4+/IFNγ+ and CD8+/IFNγ+ cell activation were measured following four weeks of vaccination with M. bovis BCG, MTBVAC or MTBVAC erp−. CD4+/IFNγ+ and CD8+/IFNγ+ cells were observed in all vaccinated groups (Fig. 5a). A lower percentage of CD4+/IFNγ+ was observed in the lungs and spleen of mice vaccinated with the MTBVAC erp− strain, which was not significant when compared to MTBVAC or BCG (Fig. 5a and c). No differences in CD8+/IFNγ+ were found between the three groups neither in lungs nor in spleen (Fig. 5b and d).

4. Discussion

Although BCG administration prevents against the most severe forms of TB disease common mainly in infants and children in the developing world, the failure of BCG to protect against pulmonary TB, the transmissible form of the disease, makes this vaccine unfit to stop the global TB pandemic, mainly striking the poorest parts of the world [2]. As a result, there is an urgent need for effective vaccines that could arrest the social and medical problem caused by TB. One of the advantages of using live attenuated M. tuberculosis vaccines is the presence of major antigens lost during BCG attenuation [7,18], thus providing a rational solution for replacing BCG. Moreover, BCG vaccination is contraindicated in patients with primary and secondary immunodeficiencies including HIV-infected people [2]. As a result, development of effective TB vaccines that are safer than BCG for use in this population is also urgently needed.

The main challenge in the development of live vaccines is achieving a satisfactory level of safety attenuation without severely compromising vaccine immunogenicity and protectiveness. There are examples in the existing literature of live-attenuated M. tuberculosis mutants as vaccine candidates that even though highly attenuated, protection levels against infection are compromised, as observed with M. tuberculosis single and double auxotroph mutants, including H37RvΔleu [19]
H37RvΔlys [20] and H37RvΔpanCD [21] or H37RvΔleuΔpanCD [22] or H37RvΔlysΔpanCD [23], evidenced the great challenge in developing new safe and effective vaccines.

Here we present the highly attenuated MTBVAC erp− mutant as a vaccine candidate with protective capacity against TB for use in patients in risk of immune suppression. The hyper-attenuation phenotype of MTBVAC erp− observed in SCID mice is superior when compared to wild-type Mt103 and the single Mt103 erp− mutant. The erp mutation introduced in MTBVAC (phoP−, fadD26) presents a synergic effect in attenuation that does not affect the protection capacity of the vaccine against TB. When compared to BCG, protection conferred in this model is similar, even though MTBVAC erp− is much more attenuated.

MTBVAC was constructed by generation of two unmarked stable deletion mutations conferring PhoP− and DIM− deficient phenotypes [7], whereas MTBVAC erp− is Kanamicin resistant. For the future development of MTBVAC erp− as a prophylactic strategy for use in immunocompromised individuals, a stable deletion mutation without antibiotic resistance cassette must be generated and the final construct characterized for safety and stability in order to fulfill the Geneva consensus requirements for progressing new live mycobacterial vaccines to clinical trials [8,9]. Regulatory authorities have approved for first time in the history of human TB vaccines, the entry of the live-attenuated M. tuberculosis MTBVAC vaccine in clinical trials. The construction of a hyper-attenuated MTBVAC derivate suitable for use in high-risk groups of immune suppression can help stop the dramatic social and medical problem as result of HIV and TB co-infection.

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Contributors: Conceived and designed the experiments: CM, BG, NA, JB, MM. Performed the experiments: LS, NA, ABG. Analyzed the data: CM, BG, LS, NA, JB, MM. Wrote the paper: CM, LS, NA.

Conflict of interest: BG and CM are co-inventors on a composition of patent matter “tuberculosis vaccine” filed by the University of Zaragoza. LS, SU, NA, BG and CM are co-inventors on a composition of matter patent “Triple mutante del complejo Mycobacterium tuberculosis erp−, phoP− y DIM−” filed by the University of Zaragoza. There are no other conflicts of interest.

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