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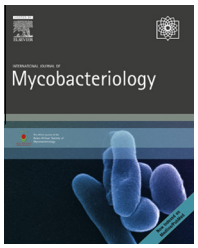

Cellular and humoral immunogenicity of recombinant *Mycobacterium smegmatis* expressing Ag85B epitopes in mice

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ABSTRACT

Objective/background: The search for new vaccines more efficacious than bacille Calmette–Guérin for tuberculosis prevention is of paramount importance for the control of the disease. The expression of *Mycobacterium tuberculosis* antigens in *Mycobacterium smegmatis* is one of the current strategies for the development of new-generation vaccines against tuberculosis. The objective of this study was to evaluate the immunogenicity in mice of *M. smegmatis* expressing epitopes from Ag85B antigen.

Methods: *M. smegmatis* expressing three T cell epitopes from *M. tuberculosis* Ag85B (P21, P26, and P53) was constructed (rMs064). rMs064 was used to immunize BALB/C mice for immunogenicity evaluation. The present study investigates the capacity of rMs064 to induce specific cellular and humoral immune responses against the expressed epitopes. Cytokine production upon stimulation with Ag85B peptides and specific total immunoglobulin G and immunoglobulin G subclasses were determined.

Results: The results showed a significant production of interleukin-12 and interleukin-23 when splenocytes were stimulated with P21, P26, and P53 peptides, and interferon-γ after stimulation with P21 in animals immunized with rMs064 compared with controls. The total immunoglobulin G and its subclasses showed significant increases against the Ag85B epitopes in the sera of rMs064-immunized mice compared with the control groups.

Conclusion: The results of this study support the future evaluation of rMs064 as a vaccine candidate against tuberculosis in challenge experiments.

Introduction

Tuberculosis (TB) remains one of the primary infectious-disease burdens in most part of the world [1,2]. One-third of the world's population is already infected with *Mycobacterium tuberculosis*, in which 10% of infected individuals carry a lifetime risk of developing the disease [1]. The use of the bacille Calmette–Guérin (BCG) vaccine shows variable efficacy

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against pulmonary TB, and confers protection only against the severe forms of the disease in children [3,4]. The development of an effective vaccine has been highlighted as one of the most effective means toward the control of TB [2].

Mycobacterium smegmatis is a nonpathogenic, rapidly growing, and commensal strain of *Mycobacterium* species. The most important advantage of *M. smegmatis* as a vaccine vector is due to the genetic and structural homology of this strain with *M. tuberculosis* [5,6]. *M. smegmatis* also has similarities in cell-wall lipid moiety, and shares the same mechanisms of cell-wall synthesis with those of *M. tuberculosis* [6]. In addition, *M. smegmatis* is superior in activating and inducing the maturation of dendritic cells (DCs) compared to BCG [7]. In terms of activating adaptive immunity, *M. smegmatis* is a strong inducer of effector and memory T cells [8,9]. Autophagy has been described as an important mechanism in the defense against TB; in this regard, a recent study has showed that the lipid components of *M. smegmatis* have the capacity to initiate and modulate autophagy in murine macrophages independent of mammalian-target-of-rapamycin signaling pathway [10].

Experimental vaccines based on the cell-wall components of *M. smegmatis* elicited cross-reactive responses against *M. tuberculosis* antigens in mice [11–13]. Recombinant *M. smegmatis* expressing the 6-kDa early secreted antigen (ESAT-6) and culture-filtrate protein (CFP)-10 has been shown to reduce the bacterial load in the lungs of immunized mice challenged with a virulent *M. tuberculosis* strain [14]. These antecedents support the interest in the evaluation of *M. smegmatis* as a live vaccine vector for the expression of recombinant proteins, in particular those from *M. tuberculosis* as a potential strategy for the development of new-generation vaccines against TB.

Ag85 complex consists of highly homologous 30–32 kDa CFPs (85A, 85B, and 85C) of *M. tuberculosis* [15]. These antigens are associated with mycolyl-transferase activity in vitro, and catalyze the synthesis of the glycolipid of mycobacterial cell wall [16]. The Ag85 components, also known as fibronectin-binding proteins, interact with fibronectin at a specific fibronectin-binding motif of the host cell [17]. Fibronectin plays an important role in bacteria–host interactions by binding with microbial surface components, leading to the initiation of infection [18]. Ag85B has been shown to promote excellent immunogenicity in experimental animal models, and it is essential in the induction of cellular and humoral immunity [19,20].

The reports related with the potential importance of the humoral immune response in protection against TB have opened a new avenue in the development of new-generation vaccines against TB [21,22]. Ag85B is considered among the *M. tuberculosis* antigens, which can potentially induce protective antibodies based on reports of a better prognosis in TB patients with circulating immunoglobulin G (IgG) antibodies against *M. tuberculosis* Ag85 complex [23].

Taking into consideration these antecedents, the possibility to express Ag85B epitopes in *M. smegmatis* to induce protective immune responses against TB is an interesting possibility; in this study, epitopes from Ag85B antigen (P21, P26, and P53) were cloned into *M. smegmatis* (rMs064), and the cellular and humoral immunogenicity was evaluated in mice.

Materials and methods

Construction of rMs064

Strain

M. smegmatis mc2155 strain was used. Cultures were grown in Middlebrook 7H9 supplemented with 0.2% (volume/volume [v/v]) glycerol, 0.5% (v/v) Tween 20, and 10% (v/v) oleic–albumin–dextrose–catalase for 48 h with agitation (200 rpm) at 37 °C. The purity of the culture was evaluated by Ziehl–Neelsen staining [24].

Selection of Ag85B epitopes

Epitopes $P21_{101–115}$ (LTSELPQWLSANRAV), $P26_{126–140}$ (SMAGSSAMILAAYHP), and $P53_{261–275}$ (THSWEYWGAQLNAMK) were selected from *M. tuberculosis* Ag85B protein based on previous reports [25], and the presence of B epitopes in these sequences was demonstrated using the ABCpred Bioinformatics prediction tool (http://www.imtech.res.in/raghava/bcepred/) [26].

Genetic transformation

A multi-epitope construct including P21, P26, and P53 epitopes from Ag85B with codon usage optimized for mycobacteria was synthesized by Geneart (Bavaria, Germany). The DNA fragment was fused to the MTB8.4 protein gene, into the pNMN012 mycobacterial shuttle plasmid under the control of the *M. tuberculosis* Hsp65 antigen promoter, followed by the *M. tuberculosis* MPT63 signal sequence. The genetic transformation of *M. smegmatis* was carried out by electroporation [27]. A negative control strain was obtained by the transformation of *M. smegmatis* with pNMN012 (rMs012). The expression of the epitopes was determined by Western blotting using anti-6× His antibodies (Abcam, Cambridge, UK).

Peptides

Peptides corresponding with the Ag85B epitopes P21, P26, and P53 were commercially synthesized (1st BASE Laboratories, Singapore Science Park II, Singapore).

Evaluation of cellular and humoral Immunogenicity

Immunization schedule

Male BALB/c mice (6–8 weeks), supplied by the Animal Research and Service Centre, Universiti Sains Malaysia, were used in the experiments. All procedures were carried out according to the standard international regulations and guidelines of laboratory animal experimentation [28], and approved by the Ethical Committee for Experimentation in Animals of the Universiti Sains Malaysia. Three groups of animals ($n = 5$ per group) were inoculated subcutaneously with 2×10^6 CFU of rMs064, rMs012 (both strains were suspended in 100 μL of phosphate-buffered saline [PBS]), or PBS alone. Two doses were administered in a 2-week interval. Blood samples were taken at 35 days after the first immunization. The blood was centrifuged and the serum stored at −20 °C until use. Subsequently, the mice were sacrificed, and the spleens were aseptically removed to assess the cellular immune response.