International Journal of Mycobacteriology 5 (2016) 7–13

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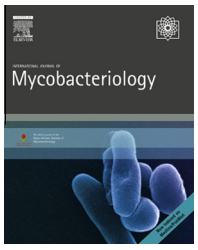

Cellular and humoral immunogenicity of recombinant *Mycobacterium smegmatis* expressing Ag85B epitopes in mice

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ARTICLE INFO

Article history:
Received 26 July 2015
Received in revised form
22 September 2015
Accepted 27 September 2015
Available online 17 October 2015

Keywords:
Ag85B
Cytokine
Epitopes
Mycobacterium smegmatis
Mycobacterium tuberculosis
Tuberculosis

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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Peer review under responsibility of Asian African Society for Mycobacteriology.
http://dx.doi.org/10.1016/j.ijmyco.2015.09.006

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.

Determination of extracellular cytokine production

The splenocytes from immunized mice were cultured (2×10^5 cells/mL) in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, USA) supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C. The cultures were stimulated with 10 μg/mL of P21, P26, and P53 peptides. Following the 72-h incubation, cell-free supernatant was harvested and assayed for the presence of interferon (IFN)-γ, interleukin (IL)-12 (IL-12p70), IL-23, and IL-4 by enzyme-linked immunosorbent assay (eBioscience, San Diego, USA) according to the manufacturer's instructions.

Determination of specific total IgG and IgG subclass antibodies

The specific total IgG and IgG subclass levels were determined by enzyme-linked immunosorbent assay against P21, P26, and P53 peptides. Briefly, 96-well MaxiSorp plates (Nunc, Rochester, USA) were coated with each peptide (1 μg/mL) in coating buffer, incubated overnight at 4 °C, washed five times with PBS containing 0.1% Tween 20, and blocked with blocking buffer (Roche, Mannheim, Germany) for 1 h at 37 °C. The plates were incubated with the mice sera (in triplicates) at a dilution of 1:50, and incubated for 2 h at 37 °C. The plates were washed five times, and horseradish-peroxidase-conjugated goat antimouse IgG or anti subclass IgG1, IgG2a, and IgG2b (Dako, Carpinteria, CA, USA) were added at 1:1000 dilution for 1 h at 37 °C. The enzyme reaction was developed with 2,2′-azino-di(3-ethyl-benzothiazolesulfonate) (ABTS; Roche). The reaction was stopped after 30 min with 2 N H_2SO_4 and the optical density determined with a microplate reader (Tecan, San Jose, USA) at 405 nm.

Statistical analysis

The results were analyzed using one-way analysis-of-variance test and the differences between groups were determined by Tukey's post-test. A p value was considered significant.

Results

The replicative plasmid pNMN064 was electroporated into *M. smegmatis* mc^2 155 to produce rMs064, and the expression of the recombinant protein was detected as a 22-kDa band by Western blotting (Fig. 1).

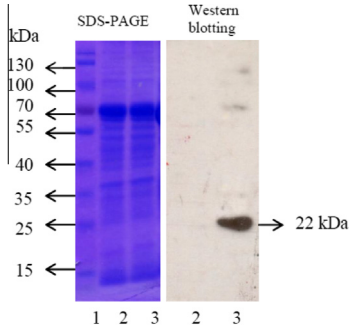

Fig. 1 – Expression of 85B epitopes in *Mycobacterium smegmatis* by Western blotting. (1) Prestained protein marker; (2) rMs012 cell lysate; (3) rMs064 cell lysate. SDS–PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

To assess the induction of specific cellular responses, the splenocytes obtained from mice immunized with rMs012 and rMs064 were stimulated with P21, P26, and P53 peptides. The production of IFN-γ, IL-12, IL-23, and IL-4 cytokines was then evaluated. A significant IL-12 production was observed in P21-, P26-, and P53-stimulated splenocytes in rMs064-immunized mice compared with the other groups (Fig. 2A).

A significant increase in the production of IL-23 upon stimulation with all three epitopes was observed in rMs064-immunized mice (Fig. 2B).

Splenocytes from rMs064-immunized mice showed significant highest production of IFN-γ when stimulated with P21 peptide compared to the other groups (Fig. 2C).

Regarding IL-4, there was no increase of this cytokine upon stimulation with any of the epitopes (data not shown).

A significant increase was observed in the level of total IgG against all Ag85B epitopes after the immunization of mice with rMs064 compared to the other groups (Fig. 3A). Enhanced levels of specific IgG1 subclass were detected against P21, P26, and P53 epitopes of Ag85B (Fig. 3B). However, specific IgG2a antibodies showed a significant increase only against P26 epitope (Fig. 3C), while specific IgG2b production was significantly increased against P53 (Fig. 3D). The subclass responses against the individual Ag85B epitopes indicate that rMs064 stimulated both the T helper cell type 1 (Th1)- and T helper cell type 2-associated IgG subclasses (IgG2a, IgG2b, and IgG1, respectively).

Discussion

Ag85B epitopes designated as P21, P26, and P53 were successfully cloned into pNMN012 vector and expressed in *M. smegmatis*. These three Ag85B epitopes overlapped with regions of the Ag85B protein recognized by guinea pigs and purified-protein-derivative-positive individuals [25]. It has been demonstrated that these epitopes were immunogenic in mice when expressed in BCG [29], and contain predicted B cell epitopes as determined with the ABCpred Bioinformatics prediction tool (data not shown), which support their evaluation as vaccine candidates.

M. smegmatis has several advantages, which make it an attractive carrier for the expression of *M. tuberculosis* antigens/epitopes. *M. smegmatis* is a fast grower, nonpathogenic, has genetic and antigenic homology with *M. tuberculosis*, and, as such, has the possibility to express *M. tuberculosis* antigens in their native conformation. Furthermore, induction of DC maturation with increased expression of major histocompatibility complex class I and class II molecules, induction of autophagy, and strong generation of effector and memory T cells have been previously proposed [5–10].

M. smegmatis overexpressing whole Ag85B protein protects mice in a challenge model with *M. tuberculosis* [30]. The same effect was obtained in guinea pigs with recombinant BCG overexpressing Ag85B, and this vaccine candidate showed cross-protective responses against *Mycobacterium leprae* [31,32]. The expression of a fusion protein of Ag85B and ESAT-6 in *M. smegmatis* produced an immunotherapeutic

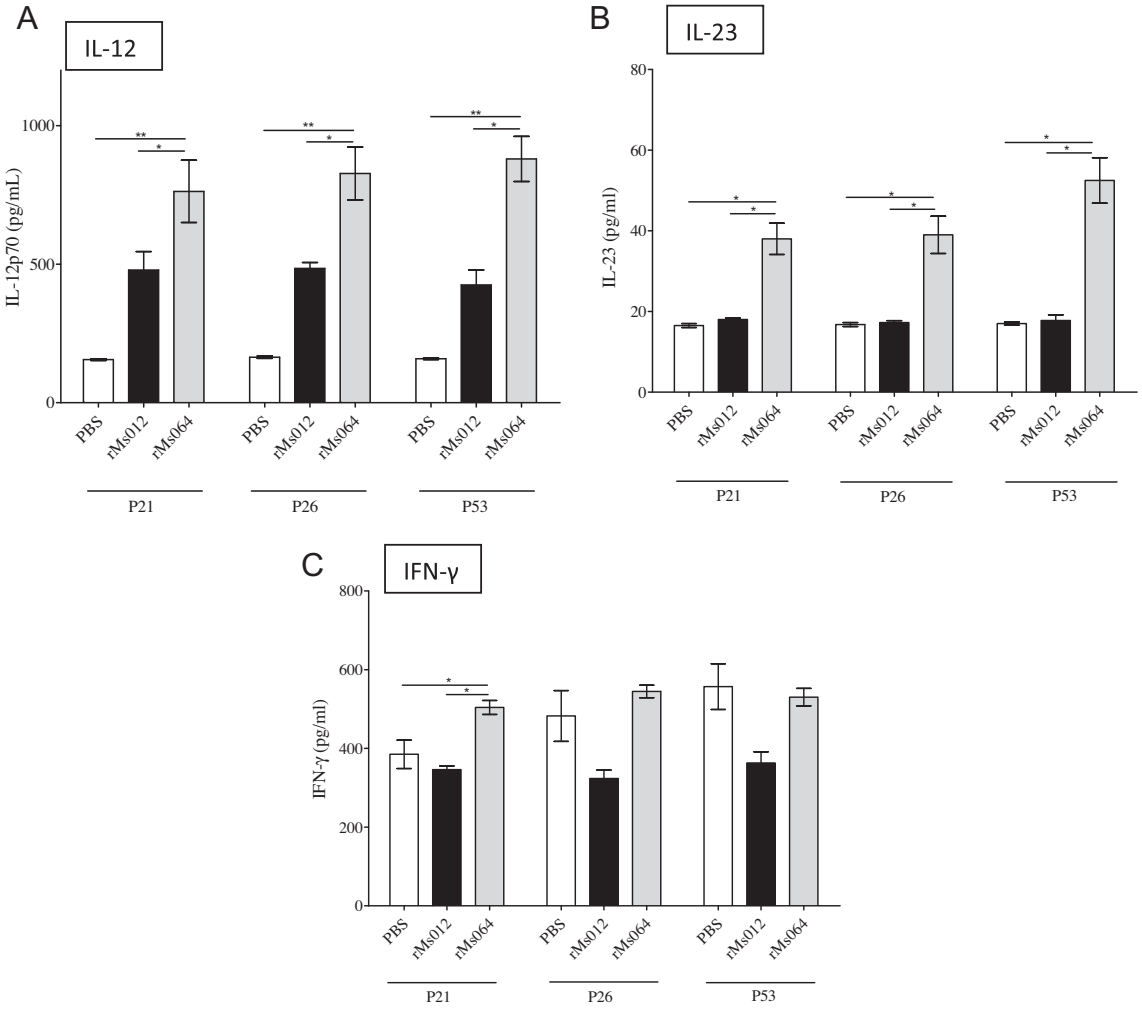

Fig. 2 – Production of (A) interleukin-12 (IL-12p70), (B) interleukin-23, and (C) interferon-γ against P21, P26, and P53. BALB/c mice (n = 5 per group) were immunized with either phosphate-buffered saline, rMs012, or rMs064. Results are presented as mean ± standard error of the mean. IFN = interferon; IL = interleukin; PBS = phosphate-buffered saline. $^{*}p < .05$. $^{**}p < .01$.

effect against TB in mice [33]. *M. smegmatis* vaccine candidates expressing Ag85C, MPT51, and HspX (designated as mc2 CMX) also induced both specific IgG2a and IgG1, and protect mice against *M. tuberculosis* [34].

The capacity of *M. smegmatis* expressing epitopes of Ag85B to stimulate specific cellular immune response was demonstrated by the production of IL2, IL23, and IFN-γ after the stimulation of splenocytes from rMs064-immunized mice.

IL-12 is produced by phagocytic cells and plays a key role in host defense against *M. tuberculosis* infection [35]. The production of IL-12p70, cytokine made up of p35 and p40 heterodimer chains, is mainly produced upon the phagocytosis of *M. tuberculosis* [36]. IL-12 mediates the activation and development of Th1 CD4 and CD8 T lymphocytes [37]. IL-12 exerts its protective roles against mycobacterial infection mainly through the induction of IFN-γ; thus, it acts as a link between the innate and adaptive host response [38].

IL-23 is produced by activated monocytes, macrophages, and DCs in response to mycobacterial infection, and is considered an important cytokine for the protection against *M. tuberculosis* after natural infection or vaccination [39,40]. The synergistic action of IL-12 and IL-23 promotes the recruitment of antigen-specific $CD4^{+}$ T cells in the draining lymph nodes of *M. tuberculosis*-infected lungs [41]. Mice deficient in both IL-12- and IL-23-secreted cytokines are susceptible to infection with *M. tuberculosis* [36]. Other groups expressing a fusion protein of heparin-binding hemagglutinin and human IL-12 have reported prophylactic and/or therapeutic effect against TB in mice [42]. The expression of IL-12 and granulysin in *M. smegmatis* produced a therapeutic effect against TB in a model of infection in mice [43].

IFN-γ is a dominant cytokine involved in antigen-specific T cell immunity in response to *M. tuberculosis* infection [44]. The importance of IFN-γ and tumor necrosis factor-α as major cytokines in mycobacterial infection is proven by the activation, under the influence of these cytokines, of the inducible form of nitric oxide synthase and the production of reactive nitrogen intermediates in murine macrophages, which is a potent antimycobacterial effector mechanism [45]. Wang et al. [33] reported an increase in the production of IFN-γ in

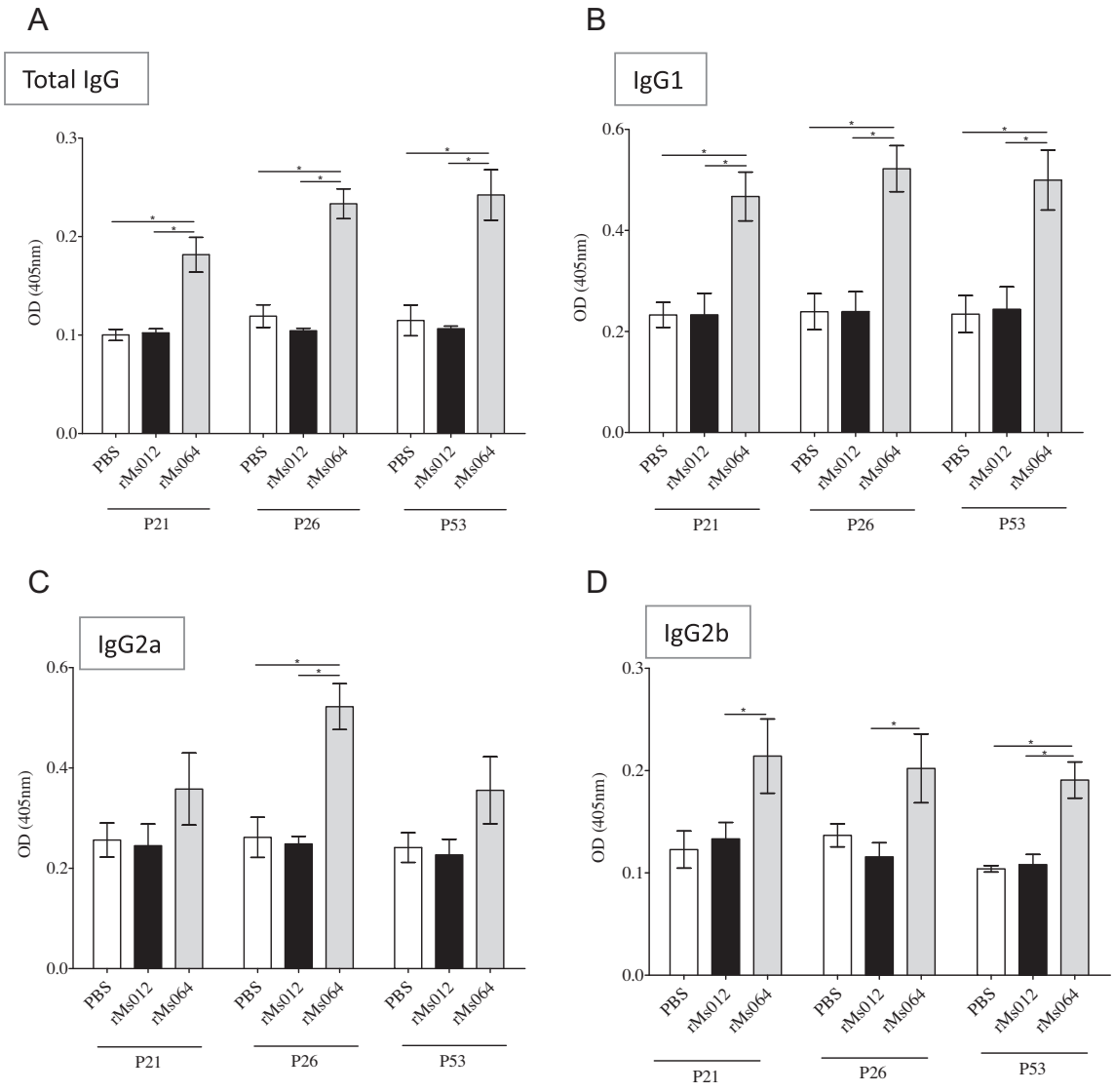

Fig. 3 – Enzyme-linked immunosorbent assay. (A) Total immunoglobulin G, (B) IgG1, (C) IgG2a, and (D) IgG2b response against P21, P26, and P53. BALB/c mice ($n = 5$ per group) immunized with either phosphate-buffered saline, rMs012, or rMs064. Results are presented as mean ± standard error of the mean. IgG = immunoglobulin G; PBS = phosphate-buffered saline. * $p < .05$.

C57BL/6 mice immunized with rMs expressing Ag85B-ESAT-6 fusion protein.

The humoral immunogenicity of the Ag85B epitopes expressed in rMs was evident in the study of total IgG and subclasses.

The induction of specific antibodies has been reported following immunization with rMs expressing a fusion protein of the ESAT-6 and CFP-10 proteins from *M. tuberculosis* [14]. It has been reported that antigen Ag85B is highly expressed during mycobacterial multiplication, as indicated by messenger RNA copy number in mouse lung tissue infected with virulent *M. tuberculosis* [46]. In this regard, the induction of specific humoral responses recognizing Ag85B epitopes after the immunization with rMs064 could be an important consideration according to the potential role of antibodies in the protection against TB [47–49].

It has been reported that specific antibodies against *M. tuberculosis* antigens could contribute to the protection by different mechanisms, among them interference with mycobacterial adhesion, toxin neutralization, growth inhibition, opsonization, agglutination of the pathogen, enhancement of cytokine release, complement activation, promotion of phagosome–lysosome fusion, activation of cell-mediated immunity, antibody-dependent cellular cytotoxicity, and enhancement of antigen presentation [50,51].

Proteins of the Ag85 complex, including Ag85B, have enzymatic mycolyl-transferase activity related with the biogenesis of cord factor, and bind to fibronectin and elastin, which are associated with the survival and virulence of *M. tuberculosis* [52]. In this regard, the elicitation of antibodies against epitopes of Ag85B could interfere with important functions of *M. tuberculosis*. It is important to note that epitope P53

contains an essential amino acid for the mycolyl-transferase enzymatic activity [52].

The elicitation of humoral cross-reactive immune responses against *M. tuberculosis* has been reported with proteoliposomes and liposomes obtained from *M. smegmatis* [11–13]. Liposomes containing lipids of *M. smegmatis* induced protective immunity against *M. tuberculosis* in mice [53]. Considering these antecedents, the specific humoral immune response induced by rMs064 against the Ag85B epitopes could be reinforced by additional responses elicited by other cross-reactive components of the carrier strain.

Conclusion

The demonstration of the cellular and humoral immunogenicity of rMs064 expressing selected epitopes from Ag85B stimulating Th1 immune responses supports the further evaluation of this vaccine candidate in challenge experiments with *M. tuberculosis* in mice.

Conflicts of interest

There is no conflict of interest to declare.

Acknowledgments

The authors acknowledge the financial contribution under the Long-Term Research Grant Scheme (203/PSK/6722001), Department of Higher Education, Ministry of Education, Malaysia.

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