Gluthathione S-transferase-resuscitation-promoting factor B recombinant protein of *Mycobacterium tuberculosis* induces the production of interferon-γ and interleukin-12 in mice splenocytes

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pISSN: 0853-1773 • eISSN: 2252-8083
https://doi.org/10.13181/mji.v28i3.2444
Med J Indones. 2019;28:234–40

Received: January 07, 2018
Accepted: May 16, 2019

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ABSTRACT

BACKGROUND As the only TB vaccine available, Bacillus Calmette-Guérin shows variable efficacy in adults and does not provide protection against the resuscitation of latent TB infections. Resuscitation-promoting factor B (RpfB) is a protein produced by *Mycobacterium tuberculosis* during the resuscitation phase and is promising as a novel TB vaccine. This study was aimed to analyze the immunogenicity of the gluthathione S-transferase (GST)-RpfB recombinant protein on mice splenocytes *in vitro*.

METHODS After induction with isopropyl β-D-1-thiogalactopyranoside, the protein was extracted by sonication followed by solubilization in 8 M urea buffer. Protein was then re-natured and purified with a GST chromatography column. The isolated protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot using anti-GST antibodies, and its concentration was determined using the Bradford method. Each group of splenocytes was treated with 25 μg/ml of the recombinant protein (GST-RpfB), GST, and phytohemagglutinin. Antigen induction was repeated twice at 24 and 72 hours. The supernatant was collected at 96 hours and interferon gamma (IFNγ), interleukin (IL-12, IL-4, and IL-10) levels were measured with enzyme-linked immunosorbent assays.

RESULTS GST-RpfB recombinant proteins were expressed in the form of inclusion bodies with a molecular weight of approximately 66 kDa. Based on the independent t-test, GST-RpfB stimulated IFNγ and IL-12 production but not IL-4 and IL-10.

CONCLUSIONS The GST-RpfB protein has been immunogenically proven and is a potential candidate as a novel subunit TB vaccine.

KEYWORDS immunogens, *Mycobacterium tuberculosis*, recombinant fusion proteins, RpfB, spleen

Tuberculosis (TB) infection remains one of the leading causes of death worldwide, particularly in Indonesia. Within 2013, 670,000 people have died due to TB, and 210,000 of these were HIV-positive individuals.[1] Latent TB infection is the most common form; in 5% to 10% of individuals, latent infection develop into active TB.[2,3] Currently, Bacillus Calmette-Guérin (BCG) is the only vaccine used to prevent TB infection. The BCG vaccine is relatively safe and can protect children against TB, but in adults it is less effective in prevention and long-term protection from infections. The infection cases mainly occur in the endemic areas.[4] As the BCG vaccine has a relatively low success rate shown in a number of clinical trials; major efforts are being made to develop novel vaccine candidates that are more effective against TB. Several new TB candidate vaccines (e.g., H4, DAR-901, and VPM1002) are now in clinical and late-stage development.[5] However, additional studies to develop alternative vaccines are still warranted.

The control of *Mycobacterium tuberculosis* infection is mainly mediated by cellular immunity, especially by CD4 T cells.[6] Polyclonal T cell activation through simultaneous vaccination is necessary for the production of multiple cytokines. This is the foundational basis for the development of TB vaccines. Various cytokines are known to be associated with protection against TB, such as interferon gamma (IFNγ), tumor necrosis factor-alpha, and interleukins (IL-2, IL-6, and IL-12).[7] Although the humoral immune response against TB infection remains elusive, the responses of several cytokines, such as IL-4 and IL-10, cannot be divided to regulate the balance of both the cellular and humoral responses.[8] *In vivo* studies on mice showed that the resuscitation-promoting factors B and D (RpfB, RpfD) proteins are immunogenic.[9] In humans, the immunogenic properties of all Rpf proteins can be detected in latent TB patients using immunoglobulin release assays. Moreover, RpfA, RpfB, RpfD, and RpfE induce IFNγ production in patients with active TB.[10]

In this study, the potency of glutathione S-transferase (GST)-RpfB recombinant protein construction in inducing the protective cellular immune system to TB infection were analyzed in experimental mice. The results of this study will determine whether this candidate protein could potentially be used as a TB vaccine. This study will form the basis for further studies on this protein.

METHODS

Bacterial strains and mice splenocytes

Escherichia coli BL21 containing a pGEX6P-1 vector with the insertion of the *rpfB* gene from *M. tuberculosis* Beijing (MRB4 [*E. coli* BL21pGEX6P-1 rpfB]) and pGEX6P-1 (MRB6 [*E. coli* BL21pGEX6P-1]) was used in this study. Splenocytes from 4–6-week-old "Deutschland, Denken, and Yonken" (DDY) mice were collected and used in this study. The ethical approval was from the Ethical Committee, Faculty of Medicine, Universitas Indonesia, No. 906/UN2.F1/ETHICS/2014.

Protein expression and pellet collection

MRB4 and MRB6 strains were grown in 5 ml Luria-Bertani (LB) medium amended with 100 µg/ml of ampicillin. The cultures were incubated at 37°C while agitated at 200*g* overnight. Bacteria were subsequently sub-cultured in 500 ml of liquid LB medium, and incubated at 28°C with 200*g* agitation. At an optical density at 600 nm of 0.4, isopropyl β-D-1-thiogalactopyranoside was added to the culture for a final concentration of 0.1 mM, followed by incubation under the same conditions for 4 hours. Subsequently, the cultures were incubated on ice for 30 min, and then centrifuged at 5,000*g* for 10 min at 4°C. After the centrifugation, the pellets were collected and stored at -30°C until processing.

Extraction, solubilization, and protein refolding

Extraction of the recombinant protein (GST-RpfB) was performed through sonication and centrifugation of bacterial pellets using a lysis buffer (50 mM tris, 10% glycine, 0.1% triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 100 µg/ml lysozyme), TNMFX-2M urea buffer (50 mM tris-base, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], and 2M urea), and TNMFX-0.1% triton X-100 buffer (50 mM tris, 150 mM NaCl, 1 mM EDTA, and 0.1% triton X-100). After centrifugation, the pellet was solubilized using 8 M urea buffer, and then centrifuged again to obtain the supernatant containing the GST-RpfB recombinant protein.

GST-RpfB was dissolved into 8 M urea buffer and further diluted into 20 ml of refolding buffer consisting of 50 mM tris-HCl, 100 mM NaCl, 1 mM reduced glutathione, 1 mM dithiothreitol, and 1 mM PMSF. The solution was diluted with a stirrer and incubated overnight at 4°C.

Isolation of recombinant proteins

The re-naturated GST-RpfB recombinant protein was further isolated using an affinity chromatography column (Fermentas, catalog number 16107) against GST, based on the manufacturer's instructions. Columns containing the slurry were equilibrated for 1 hour in a shaker with 10 ml of the refolding buffer described above. The equilibrium buffer was then discharged, and protein samples were added to the column. Incubation was continued in the shaker for 1 hour. Non-binding proteins were drained from the column and were stored as flowthrough. The columns were washed twice with the refolding buffer. GST-RpfB bound in the resin was subsequently eluted 3–4 times with 3 ml of buffer consisting of 50 mM tris,

150 mM NaCl, and 10 mM reduced glutathione. The GST-RpfB isolation result was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using an anti-GST antibody. The protein concentration was determined using Bradford assay.

Splenocyte preparation

The splenocytes used in this study were isolated from DDY mice 4–6 weeks of age. The mice were bred at the Animal Laboratory, Department of Microbiology, Faculty of Medicine, Universitas Indonesia. Mice were euthanized by cervical dislocation. The spleens were isolated under aseptic conditions, homogenized in Roswell Park Memorial Institute (RPMI)-1640 medium, and then centrifuged at 2,000*g* for 5 min at 4°C. The obtained pellets were washed twice with the medium, and then centrifuged again under the same conditions. The density of splenocyte was adjusted to 2×10^5 cells/ml.

Cellular immunity test on mouse splenocytes

Using a 96-well culture plate, splenocytes were cultured in RPMI-1640 medium containing L-glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonate, 10% fetal bovine serum, 100 µg/ml penicillin-streptomycin, and 100 µg/ml antibiotic-antimycotic. In this immunogenicity test, three culture groups were stimulated separately: with 25 µg/ml GST-RpfB or GST-RpfD or 25 µg/ml GST protein. About 1–2% of phytohemagglutinin (PHA) mitogen (Invitrogen) was used as the positive control. A negative control group that was not stimulated was included. Antigen induction was repeated twice, 24 and 72 hours after initial induction. The supernatants of each group were collected after 96 hours of incubation, followed by centrifugation at 1,000*g* for 15 min. The production of IL-12 and IFNγ were measured using commercial enzyme-linked immunosorbent assay kits (Cusabio Biotech, USA).

Statistical analysis

Data were statistically analyzed with an independent t-test. A difference with $p < 0.05$ was considered statistically significant.

RESULTS

Expression, isolation, and purification of GST-RpfB recombinant protein

In order to isolate the recombinant protein, the GST-RpfB protein that formed in the inclusion bodies

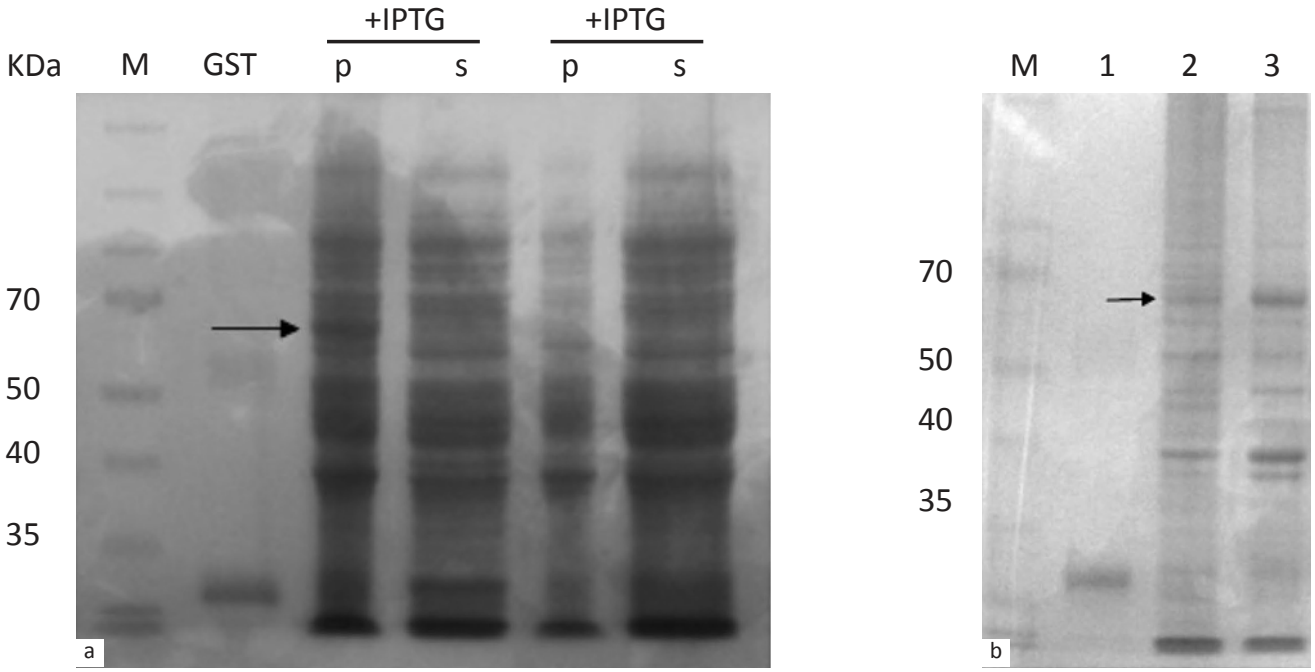

Figure 1. Detection and confirmation GST-RpfB expression using SDS-PAGE. (a) SDS-PAGE from crude cells; (b) SDS-PAGE after solubilizing and refolding; Lane 1, GST protein; Lane 2 and 3, supernatants (8 M urea buffer). The black arrow indicates the protein band that is identical to the molecular mass of GST-RpfB. KDa=kilodalton; M=marker; IPTG=isopropyl β-D-1-thiogalactopyranoside; p=pellet; s=supernatant; GST-RpfB=glutathione S-transferase-resuscitation-promoting factor B; SDS-PAGE=sodium dodecyl sulfate -polyacrylamide gel electrophoresis

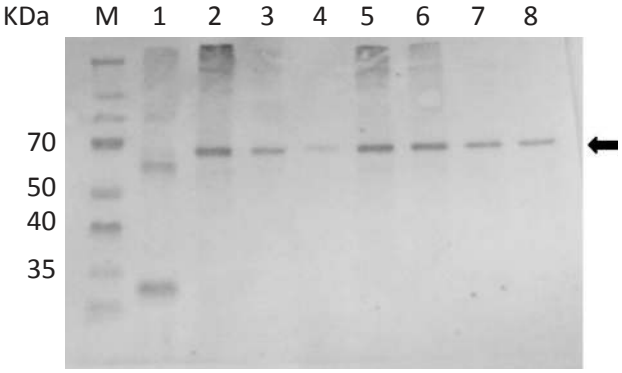

Figure 2. Western blot of GST-RpfB after isolation using the affinity of the chromatography column to GST. Lane 1, GST; Lane 2, flowthrough; Lane 3 and 4, wash; Lane 5–8, eluate. The black arrow indicates the protein band identical to the molecular mass of GST-RpfB. KDa=kilodalton; M=Marker; GST-RpfB=glutathione S-transferase-resuscitation-promoting factor B

were extracted by centrifugation using TNMFX-2M urea buffer and TNMFX-0.1% triton X-100 buffer, followed by solubilization with 8 M urea buffer (Figure 1). The results showed that the GST-RpfB recombinant can be solubilized in the buffer and can be found in the supernatant fraction. Western blot analysis with anti-GST antibodies against protein re-naturation, which was isolated in the affinity chromatography column, confirmed that the protein present was the GST-RpfB recombinant protein (Figure 2).

Cellular immunity response

The responses of cytokines IL-12 and IFNγ indicated that the splenocyte cultures stimulated with GST-RpfB had significant differences ($p < 0.05$) compared to the negative control. The stimulated splenocyte culture also showed a higher levels of IL-12 and IFNγ compared to the response of the splenocytes stimulated with GST protein ($p < 0.05$; Figure 3). Analysis of IL-4 and IL-10 in mice splenocytes showed that recombinant protein (RpfB) was able to generate a significantly greater response (IL-4 production) than the negative control ($p < 0.05$). Meanwhile, there was no difference in the response (IL-4) between the splenocyte culture stimulated with GST-RpfB compared to that stimulated with only the GST protein. Splenocytes that were stimulated with PHA (positive control) had a significantly higher concentration of IL-4 than in splenocytes stimulated with GST-RpfB or the GST protein ($p < 0.05$), validating the results of the assay (Figure 3).

DISCUSSION

TB has become a major problem in the world since the disease has caused a large number of deaths, including in Indonesia.[1] The spread of this disease can be reduced by several methods, one of those is vaccination. However, the BCG vaccine that is currently available is relatively ineffective. Finding new candidate vaccines (as a substitute or a companion to the BCG vaccine) is still a challenge, even though several candidate vaccines were already undergoing clinical trials.[1] This encourages a study of TB proteins that can potentially be used as a vaccine. In this study, RpfB recombinant proteins used in conjunction with the GST protein was assessed for its capabilities in inducing cellular immunity to TB. The use of GST in this study was expected to increase solubility and facilitate the isolation and purification processes of the recombinant proteins.[11] Proteins were purified with the GST gene fusion system that has been used successfully in a variety of immunological studies and in vaccine production.[12] Nevertheless, the GST-RpfB recombinant protein from this study was more commonly found in the pellet fraction, which was suspected to be inclusion bodies. Although various attempts have been made to create the GST-RpfB recombinant protein in supernatant fractions, such as by lowering the temperature and reducing the concentration of IPTG (data not shown), pellet fractions remain the dominant method to create recombinant proteins.

The purified GST-RpfB recombinant protein was tested for its immunogenicity with three times antigen exposure in mice splenocytes *in vitro*. Among the four tested cytokines, the GST-RpfB recombinant protein gave strong IFNγ and IL-12 responses. These two cytokines were proven to play a significant role in protection against TB where they can control both active and latent TB.[13] IFNγ is an immunostimulator and an immunomodulator cytokine that plays an important role in major histocompatibility complex (MHC) class II activation, which leads to the type 1 helper (TH1) response as a major response in TB protection. During TB infection, IFNγ is produced in both CD4+ and CD8+ T cells, but can also be secreted by infected macrophages dependent on IL-12. Although IFNγ production alone is insufficient to control *M. tuberculosis* infection, it is required for protection against the pathogen. IL-12 is an pro-inflammatory cytokine that plays an important

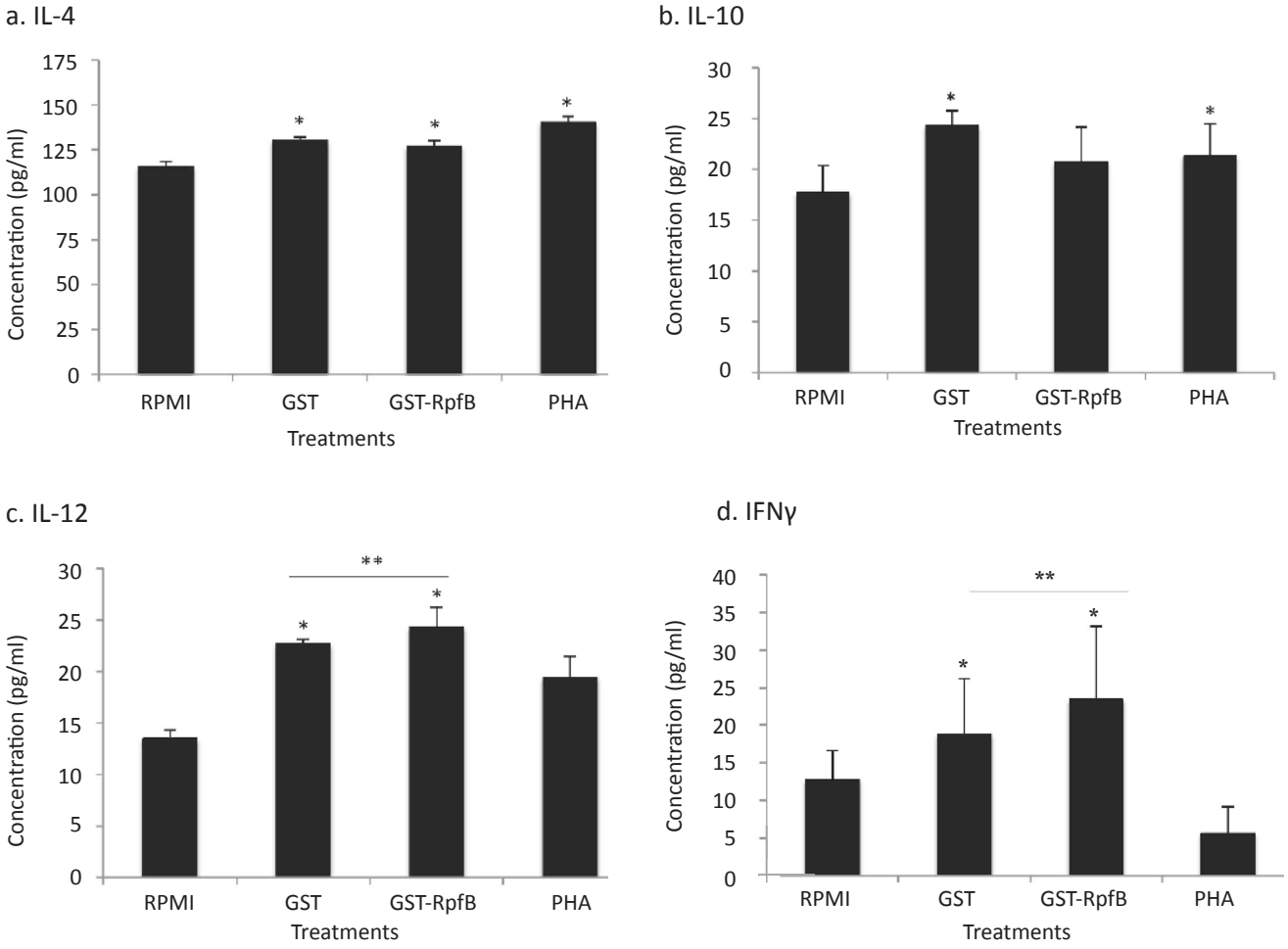

Figure 3. IL-4, IL-10, IL-12, and IFNγ responses of splenocytes treated by RPMI (negative control), GST, GST-RpfB, and PHA (positive control). Asterisk symbols (*) indicate significant differences ($p < 0.05$) to RPMI; double asterisk (**) show significant differences ($p < 0.05$) between GST and GST-RpfB. IL=interleukin; IFNγ=interferon gamma; RPMI=Roswell Park Memorial Institute; GST-RpfB=glutathione S-transferase-resuscitation-promoting factor B; PHA=phytohemagglutinin

role in naive Th cell differentiation into Th1, which also stimulates the production of IFNγ by reducing the suppressive effect of IL-4 on IFNγ. In addition, macrophages also secrete IL-12 to induce IFNγ production by T cells, which increases phagocytosis and oxidative burst. In patients with multidrug-resistant tuberculosis (MDR-TB), IFNγ improves the function of the macrophages function against *M. tuberculosis*. Monocyte-derived macrophages of MDR-TB patients demonstrate a defective immune response to *M. tuberculosis*, but pre-treatment with recombinant human IFN-γ improves their immune response. This indicates that IFNγ can be used as an adjuvant for the treatment of patients with poor responsiveness to TB therapy.[14] This result shows that IFNγ and IL-12 are major cytokines induced by the Rpf protein. This is consistent with previous Rpf immunogenicity studies derived from *M. luteus*. These were highly homologous with RpfB, which showed strong immunogenicity.[9] An *in vivo* study on C57BL/6 and BALB/c mice infected with *M. tuberculosis* vaccinated with BCG found IFNγ- and IL-12- specific responses against the GST-RpfB recombinant protein in mice splenocytes 4 weeks after infection.[15] These results were similar for other mice strains, such as C3H/Hen, in which immunization with a multiphasic vaccine (including Rpf proteins of *M. tuberculosis*) was shown to offer protection against infection by the disease.[9] The Rpf protein stimulates IgG1 and IgG2a responses, T cell proliferation, and the upregulation of IFNγ and IL-12 in C57BL/6 mice. Vaccination of mice with the Rpf protein can also inhibit the growth and proliferation of *M. tuberculosis* H37Rv.[16] Another study demonstrated that dendritic cells induced by RpfB had increased production of cell surface molecules (i.e., CD80, CD86, MHC class I, and MHC class II) and pro-inflammatory cytokines, among which was IL-12.[17]

Conversely, this GST-RpfB recombinant protein did not induce the production of IL-4 and IL-10 in mice splenocytes. IL-4 is a cytokine that differentiates naive Th cells into Th2, elevating the CD4 response in the mature plasma cells to produce antibodies. This results show that the GST-RpfB recombinant protein did not stimulate IL-4 production, indicating that this antigen mainly induced a cellular response rather than a humoral response. Previous studies of Rpf vaccinated mice also showed there was no stimulation of IL-4 cytokine production.[17] On the other hand, IL-4 is known to downregulate Th1 cells and have negative effects on IFNγ and IL-12 production.[7,18] Meanwhile, IL-10 is known to have anti-inflammatory activity that could inhibit the action of macrophages where they can help eliminate *M. tuberculosis*.[7] In contrast to IFNγ, IL-10 is considered to be an anti-inflammatory cytokine produced by T cells during infection by *M. tuberculosis*. It leads to the downregulation of IL-12 production by the macrophages, which subsequently decreases IFNγ production by T cells. IL-10 directly limits Th1 immunity and exacerbates diseases.[7] However, the PHA mitogen used in this study revealed an opposing function; it was assumed that PHA stimulated Th2 cells and inhibited Th1 cells, which stimulated the production of IL-4 and IL-10 but inhibited the production of IL-12 and IFNγ.

Based on the stimulated cytokine profile of this study, the GST-RpfB recombinant protein has the potential to be developed for clinical use as a TB vaccine. This results revealed that cellular immunity arises as a response to the GST-RpfB protein, where the protein is able to induce the cytokines that are known to be the major response of cellular immunity to *M. tuberculosis* infection in humans. Although this current research is carried out on test animals (as a requirement of the vaccine research), this seed vaccine can perhaps be used in humans in the future, especially to prevent latent TB.[2,3] Moreover, the binding of the RpfB protein to GST may lead to increase cellular immunity responses in the splenocytes. Since GST is a protein naturally found in worms (*Schistosoma japonicum*), recombination of RpfB with GST may lead to double protective immunity in areas with endemic worms and TB infections. Worm infections are known to play a role in the low efficacy of the TB vaccine due to the opposing effects of the cellular immune response.[19,20] However, the protective efficacy of GST-RpfB against single-infection *M. tuberculosis* or co-infection with helminths requires further research.

This study was conducted on Balb/C mice, which is a common animal model that has been used widely in vaccine research. This mice strain is easy to raise and maintain, and generally gives uniform immune responses to all tested antigens. Assessing the GST-RpfB recombinant fusion protein on animal models is a necessary early step in evaluating its immunogenicity and antigenicity. As immune responses consist of both cellular and humoral responses, the cellular immune response found in this study need to be complemented by a humoral immunity profiling in future study. It is necessary to better understand candidate seed vaccines that have the ability to protect against bacterial infections in tested animals (e.g. a challenge test before continuing on with clinical trials).

In conclusion, the GST-RpfB recombinant protein used in this study demonstrated a capability in inducing IFNγ and IL-12 in mice models. These two cytokines are known to play a role in cellular immune response against *M. tuberculosis* infection in humans.

Conflict of Interest

The authors affirm no conflict of interest in this study.

Acknowledgment

The authors would like to thank the Ministry of Research, Technology & Higher Education.

Funding Sources

This research was funded by Ministry of Research, Technology & Higher Education.

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