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Efficacy of Tuberculosis Vaccine Candidate pcDNA3.1-rpfB in Inhibiting the Growth of *Mycobacterium tuberculosis In Vitro* with Mycobacterial Growth Inhibition Assay

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Abstract

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (Mtb). Bacille Calmette-Guérin (BCG) is the only licensed vaccine against TB, and it is effective in children but not in adults. The Vaccine Research Team, Department of Microbiology FKUI has developed a DNA-based TB vaccine candidate pcDNA3.1-*rpf*B. This candidate induces immune responses in mice, but its potency is unknown. The gold standard for potency testing of TB vaccine is the challenge method. The BSL3 animal laboratory for the challenge method is currently unavailable at FKUI. Therefore, mycobacterial growth inhibition assay (MGIA) was used as a preliminary test before the *in vivo* challenge test was conducted. The principle of MGIA is to reculture Mtb in a Mycobacteria Growth Indicator Tube (MGIT^{TM}) from co-cultured Mtb with mammalian cells that have been previously treated with pcDNA3.1-*rpf*B, pcDNA3.1 (negative control), and BCG (positive control). MGIT^{TM} shows the time to positivity, which is the time that has lapsed until a positive growth of Mtb is detected. In addition, measurements of interferon (IFN)γ levels by enzyme-linked immunosorbent assay were carried out. This study concluded that pcDNA3.1-*rpf*B can inhibit the growth of Mtb *in vitro* and showed no statistical difference from BCG. The IFNγ levels from co-culturing did not correlate with the level of inhibition of the growth of Mtb *in vitro*.

Keywords: mycobacterial growth inhibition assay (MGIA), Mycobacterium tuberculosis, resuscitation-promoting factor B (rpfB), vaccine

Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb). TB is one of the ten diseases that caused the most death in the world in 2020. Globally, an estimated 10 million people suffer from TB, and 1.3 million of them die in 2020 [1]. According to the data from the Ministry of Health of the Republic of Indonesia, Indonesia has an estimated 845,000 cases TB, and 13,947 of them died in 2020 [2].

Bacille Calmette-Guérin (BCG) is the only licensed vaccine that is currently most available and used against TB in the world. BCG comes from an attenuated virulent strain of *Mycobacterium bovis*. This vaccine can prevent TB in children but not in adults [3]. With the World Health Organization's (WHO) goal to eradicate TB by 2035, development of a new vaccine that is better than BCG is crucial and is a component of the strategy to fight the TB epidemic [3, 4, 5].

The development of pcDNA3.1-*rpf*B as a TB vaccine candidate in the form of DNA vaccine was carried out by the Vaccine Research Team of the Department of Microbiology, FKUI [6]. This pcDNA3.1-*rpf*B was constructed from vector plasmid pcDNA3.1 of *Escherichia coli* strain DHα and resuscitation-promoting factor B (*rpf*B) genes obtained from the Beijing strain of Mtb genome. This recombinant pcDNA3.1-*rpf*B plasmid successfully induced humoral immune response, which is characterized by the formation of anti-RpfB antibodies confirmed by Western blot, it can also express RpfB protein in mammalian cells (CHO-K1 cells) as evidenced by immunostaining, and stimulate cellular immune responses in the form of T cell proliferation and interferon (IFN)γ production [6, 7]. However, the efficacy of the pcDNA3.1-*rpf*B TB vaccine candidate is unknown because no measurement of its efficacy in inhibiting Mtb growth has been measured either *in vivo* or *in vitro*.

The widely used method for efficacy testing of TB vaccine candidates is the challenge test through the inhalation of Mtb by mice [8]. The challenge process requires a high level of laboratory biosafety with special equipment for the inhalation of Mtb by mice. This facility is currently unavailable at the FKUI Microbiology

Laboratory. Therefore, in this study, the mycobacterial growth inhibition assay (MGIA) was developed as a preliminary test to measure the potency of TB vaccine candidate pcDNA3.1-*rpf*B in inhibiting Mtb growth *in vitro*.

In general, the MGIA involved the co-culture of mammalian cells with Mtb within a certain time. The mammalian cells used in this study were mouse splenocytes and peripheral blood mononuclear cells (PBMCs) that had previously been immunized with the pcDNA3.1-*rpf*B vaccine seed and then compared with the positive (BCG vaccine) and negative (pcDNA3.1 only) controls. After co-culturing, Mtb was recultured in the Mycobacteria Growth Indicator Tube (MGIT™) system. This system provides a time to positivity (TTP) value, which is the time from the beginning of culture to the occurrence of positive fluorescence in the MGIT™ [9]. The pcDNA3.1-rpfB and BCG groups were expected to have a higher TTP value than the negative control.

Materials and Methods

Research location and ethics: The research was conducted at the Microbiology Laboratory, Faculty of Medicine, Universitas Indonesia and approved by the Health Research Ethics Committee of the Faculty of Medicine through the Certificate of Passing the Ethics Review No. KET-1020/ UN2.F1/ETIK/PPM.00.02/2020.

Primers and plasmid: The primers and pcDNA3.1-*rpf*B used in this study were designed and constructed accordance with our previous research [6]. Given the patent purpose, we did not include the sequence of primers in this report. Meanwhile, pCDNA3.1 was obtained from the stock of the Department of Microbiology, Medical Faculty, Universitas Indonesia.

Isolation of pcDNA3.1-*rpf*B and pcDNA3.1: Recombinant plasmid pcDNA3.1-rpfB and plasmid pcDNA3.1 were isolated from a suspension of *E. coli* DH5α. Stock colonies of *E. coli* DH5α carrying the recombinant plasmid pcDNA3.1-*rpf*B and *E. coli* DH5α carrying pcDNA3.1 were obtained, and each was cultured in 500 mL liquid LB containing 0.1 g/mL ampicillin and then incubated in a shaker incubator at 200 rpm and 37 °C for 20 h. The bacterial suspension was then centrifuged at 5,000 rpm for 10 min. Plasmids were isolated from the pellets formed, in accordance with the procedure listed in the Hispeed Plasmid Midi Kit (QIAGEN).

Confirmation of *rpf*B gene by polymerase chain reaction (PCR): To ensure that the *rpf*B gene was built in the recombinant plasmid pcDNA3.1-*rpf*B, we confirmed the plasmid isolation by PCR. For this purpose, a forward *rpf*B (F_*rpf*B) primer and a reverse *rpf*B (R_*rpf*B) primer were used. In addition, PCR was carried out to verify the correct direction of gene insertion using a forward CMV (F_CMV) and a R_*rpf*B primers. PCR was conducted under an initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s, final elongation at 72 °C for 7 min, and final temperature at 25 °C with a total of 40 cycles.

Mouse immunizations: The animals used for this study were BALB/c mice aged 6-8 weeks. The mice were randomly divided into three groups: (1) one group immunized with 100 µg/100 µL TB vaccine candidate pcDNA3.1-*rpf*B intramuscularly, (2) one group injected intramuscularly with 100 µg/100 µL pcDNA3.1 as the negative control, and (3) one group immunized intraperitoneally with 100 µL BCG vaccine (Serum Institute of India) as the positive control. After the first injection, a booster was performed on days 14 and 28. One week after the last booster, the mice were terminated, blood was obtained from the heart, and the spleen was isolated.

Isolation of mouse splenocytes: Mouse spleen was isolated aseptically, and the splenocytes were extracted using Roswell Park Memorial Institute (RPMI) medium containing L-glutamine and 10% fetal bovine serum (RPMI-Sp). This process was carried out until the spleen appeared transparent. Afterward, the splenocytes were counted with a Neubauer counting chamber, and a splenocyte suspension was prepared with a final concentration of 3 x 10^6 cells in a total volume of 600 L RPMI-Sp.

Mouse PBMCs and serum isolation: To obtain PBMCs, we obtained ±1 mL of mouse blood from the heart aseptically using a 1 mL disposable tuberculin syringe and collected it in an ethylenediaminetetraacetic acid tube. Then, ±0.5 mL blood was obtained again aseptically for serum isolation. The PBMCs were separated by histopaque-1077. Afterward, the PBMCs were counted using a Neubauer counting chamber, and a PBMC suspension with a final concentration of 3 x 10^6 cells was prepared in a total volume of 600 L RPMI medium containing L-glutamine and 25 mM Hepes (RPMI-Pb). For serum separation, ±0.5 mL blood in a centrifuge tube was centrifuged at 10,000 rpm for 10 min. The supernatant, which was the serum, was then separated.

MGIA: For the Mtb–splenocyte co-culture, 300 µL splenocyte suspension was placed in the wells of a sterile 48-well plate and added with 300 µL 0.0005 McFarland (McF) Mtb suspension. For the control of mammalian cells, splenocyte suspension was added to the well with a final concentration of 3 x 10^6 cells in 600 µL RPMI-Sp without the addition of Mtb suspension. For bacterial control, 300 µL Mtb 0.0005 McF and 300 µL RPMI-Sp suspension were added to the wells without the addition of splenocyte suspension. For the Mtb–PBMC co-culture, 270 µL PBMC suspension and 30 µL autologous serum were placed in the wells of a sterile 48-well plate and

added with 300 µL 0.0005 McF Mtb suspension. For the control of mammalian cells, PBMC suspension and autologous serum were added to the wells at a final concentration of 3 x 10^6 cells in 600 µL RPMI-Pb without the addition of Mtb suspension. For the bacterial control, 300 µL suspension of Mtb 0.0005 McF and 300 µL RPMI-Pb were added without the addition of PBMCs and autologous serum. The co-culture was incubated for 96 h at 37 °C and 5% CO_2. After 96 h, the co-culture samples were transferred to a screw-cap centrifuge tube and centrifuged at 12,000 rpm for 10 min to separate the Mtb. From the supernatant formed, 300 µL was obtained and stored for cytokine assay, whereas the pellet was resuspended with the rest of the supernatant. A total of 300 µL resuspension was cultured into the MGIT™ unit and incubated in the BACTEC™ system until positive results were detected.

IFNγ level measurement using enzyme-linked immunosorbent assay (ELISA): The MGIA procedure above involved a storage step of 300 µL co-culture supernatant for cytokine testing. The cytokine testing carried out on these samples was the measurement of IFNγ levels by ELISA. The ELISA steps were performed in accordance with the procedures listed in the ELISA Kit. Absorbance was measured by an ELISA reader at 450 nm.

Results

Confirmation of *rpf*B gene by PCR: Based on the results of electrophoresis (Figure 1), the amplicon bands of the *rpf*B gene, amplified with primers F_*rpf*B and R_*rpf*B and with primers F_CMV and R_*rpf*B, were between the 1,000 and 1,500 bp marker bands, respectively.

This finding indicates the isolated recombinant plasmid contained the *rpf*B gene. This result can be ascertained because with primers F_rpfB and R_*rpf*B, the *rpf*B gene was 1,089 bp, which corresponds to the size of the *rpf*B gene itself [6, 7]. In addition, with primers F_CMV and R_*rpf*B, the *rpf*B gene was about 1,100 bp because of the additional length of the *Eco*R1 restriction site (6 bp), *Hind*III restriction site (6 bp), and Kozak sequence (9 bp) [6].

Mtb reculture in MGIT™ from Mtb-splenocytes co-culture: In the Mtb-splenocyte co-culture, the MGIT™ results showed that the mean TTP values of the pcDNA3.1-*rpf*B group was 140.028 h, that of the pcDNA3.1 group (negative control) was 138.787 h, and that of the BCG group (positive control) was 151.770 h. To determine the level of significance between treatments, we analyzed the data statistically using analysis of variance (ANOVA), followed-up by post-hoc least significant difference (LSD) test (Figure 2). Statistical analysis showed that the mean TTP of the pcDNA3.1-*rpf*B group was not significantly different from that of the BCG group ($p = 0.057$), and it was higher than that of the pcDNA3.1 only group, although not significantly different ($p = 0.828$). The mean TTP of the BCG group was higher and significantly different from that of the pcDNA3.1 only group ($p = 0.039$). The MGIT™ showed that the control splenocytes in each treatment group gave negative results.

Mtb reculture in MGIT™ from Mtb–PBMC co-culture: In the Mtb–PBMC co-culture, the MGIT™ results showed that the mean TTP of the pcDNA3.1-*rpf*B group was 236.425 h, that of the pcDNA3.1 group (negative control) was 213.783 h, and that of the BCG group (positive control) was 239.456 h. To determine the levelof significance between treatments, we statistically analyzed the data using ANOVA followed by post-hoc LSD test (Figure 3). Statistical analysis showed that the mean value of TTP for the pcDNA3.1-*rpf*B group was

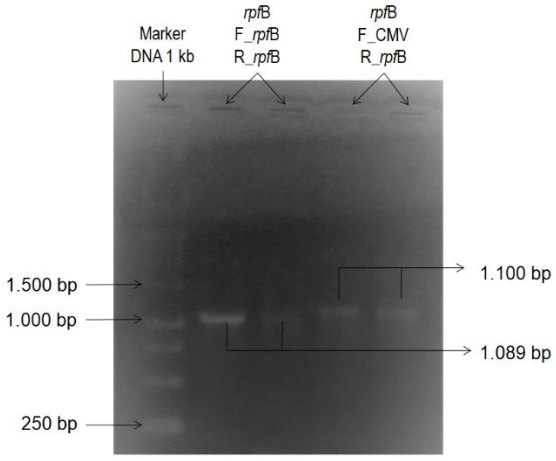
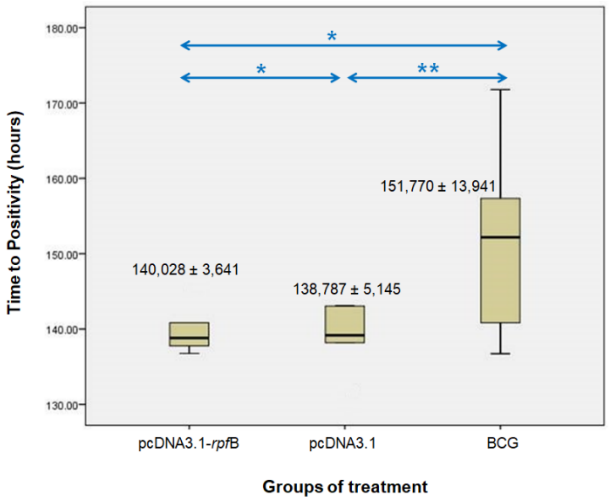


Figure 1. Electrophoresis Results of PCR Product of *rpf*B Gene



(Note: * = not significantly different, ** = significantly different)

Figure 2. Statistical Test Results of TTP Values from Mtb–splenocytes Co-culture

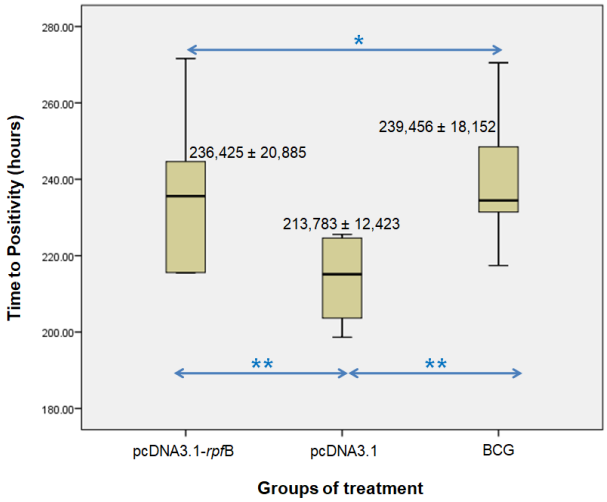
not significantly different from that of the BCG group ($p = 0.769$) but significantly higher than that of the

pcDNA3.1 group (p = 0.041). The MGIT™ showed that the PBMC control in each treatment group gave negative results.

IFNγ levels from Mtb–splenocytes co-culture: The ELISA showed that the mean value of IFNγ levels in the pcDNA3.1-*rpf*B group was 1.424 pg/mL, that of the pcDNA3.1 group was 1.377 pg/mL, and that of the BCG group was 1.613 pg/mL. ANOVA was carried out to determine the level of significance between treatments (Figure 4). No significant difference was observed between the mean IFNγ levels of each treatment group (p = 0.788). T-test was performed to determine the level of significance between each treatment with its splenocyte control. Each treatment had a mean IFNγ level that was significantly different from that of the splenocyte control. The mean level of IFNγ in the pcDNA3.1-*rpf*B group was significantly different from that of the control splenocytes (p = 0.007). For the pcDNA3.1 and BCG groups, the mean level of IFNγ were significantly different from that of the control splenocytes (p = 0.000).

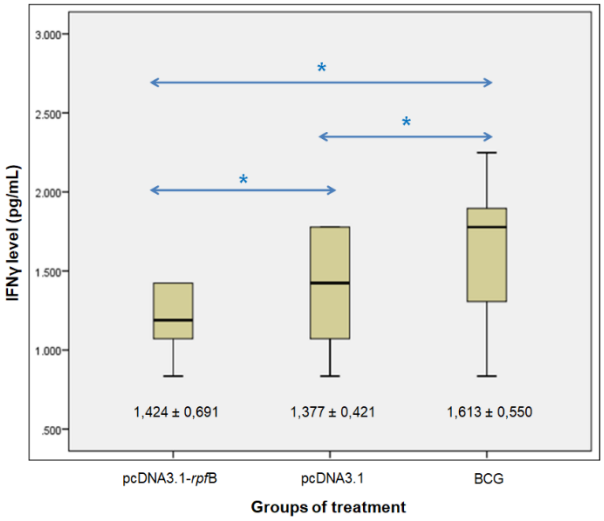
IFNγ levels from Mtb–PBMC co-culture: The ELISA showed that the mean IFNγ levels in the pcDNA3.1-*rpf*B, pcDNA3.1, and BCG groups were 3.859 pg/mL, 4.035 pg/mL, and 4.742 pg/mL, respectively. Kruskal-Wallis test was carried out to determine the level of significance between treatments (Figure 5). No significant difference was observed between the mean IFNγ level of each treatment group (p = 0.440). T-test was performed to determine the level of significance between each treatment and its PBMC control. No significant difference was noticed between the mean IFNγ levels in the pcDNA3.1-*rpf*B group and the control PBMCs (p = 0.429). However, a significant difference was identified between pcDNA3.1 and BCG groups and their control PBMCs, with both showing a significant difference at p = 0.000.

Correlation between TTP values and IFNγ levels: The results of the MGIT™ were in the form of TTP values and those of ELISA in the form of IFNγ levels. Each value was obtained from the results of Mtb-mammalian cell co-culture and analyzed for statistical correlation. Pearson correlation test showed no correlation between the TTP values and IFNγ levels in the Mtb–splenocytes (p = 0.911) and Mtb–PBMC (p = 0.648) co-cultures. This result indicates that the level of IFNγ is not directly correlated with the inhibition of Mtb growth *in vitro* based on the TTP value.



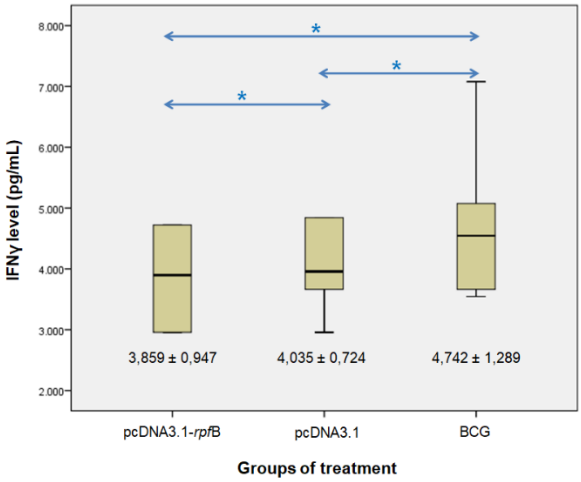
(Note: * = not significantly different, ** = significantly different)

Figure 3. Statistical Test Results of TTP Values from Mtb–PBMC Co-culture



(Note: * = not significantly different)

Figure 4. Statistical Test Results of IFNγ Levels from Mtb–splenocytes Co-culture



(Note: * = not significantly different)

Figure 5. Statistical Test Results of IFNγ Levels from Mt – PBMC Co-culture

Discussion

This pcDNA3.1-*rpf*B TB vaccine candidate was developed in the form of a DNA vaccine because this type has numerous advantages. DNA vaccines can trigger an immune response involving B and T cells. In addition, this vaccine contains no live components. Thus, the risk of disease caused by the vaccine is zero. The development of DNA vaccine is relatively easy and inexpensive. DNA vaccines do not require special facilities for storage, unlike RNA vaccines which require very cold storage conditions [10].

Various studies mentioned that BCG is effective in preventing TB in children but not in adults. BCG can provide protection against Mtb because it induces the response of T helper 1 (Th1) CD4 cells and CD8 cytotoxic T cells [10]. A research proved that the induction of Th1 and Th17 cells is needed to form an ideal protection against TB [11]. BCG can induce Th1 response but fails to induce Th17 response in the lungs, which results in the low efficiency of the BCG vaccine.

This pcDNA3.1-*rpf*B vaccine candidate is expected to have better efficacy than BCG or as a companion to BCG vaccine in building an immune response against Mtb infection because the RpfB protein encoded by the *rpf*B Mtb gene has the highest biological and immunological characteristics among other proteins in the Rpf family [6, 7]. The RpfB protein is highly expressed during Mtb resuscitation. Various studies have also shown that the rpfB protein has an important role in Mtb replication *in vivo* [12].

The mammalian cells used in this study were splenocytes and PBMCs. Splenocytes were selected because they consist of various populations of immune cells, such as T lymphocytes, B lymphocytes, dendritic cells, and macrophages that have different immune functions. PBMC was selected because they consist of T lymphocytes, B lymphocytes, natural killer cells, and monocytes, which also play an important role in the immune response of the body [13].

The BACTEC™ MGIT™ system was selected in this MGIA because it has numerous advantages over conventional Mtb culture systems. Mtb is categorized as slow growth bacteria, with a doubling time of 10–20 h [14, 15]. Thus, conventionally cultured Mtb can only be detected positive in 5-8 weeks. This system can reduce the time for obtaining test results to a matter of days. This is achievable because each MGIT™ unit contains Middlebrook 7H9 liquid medium, PANTA antibiotic blend, OADC growth supplement, and is equipped with a fluorescence indicator. All of these conditions can facilitate the Mtb culture to reach a concentration equivalent to 10^5–10^6 colony forming units and allow the detection of positive results. In addition, the BACTEC™ MGIT™ system is sensitive to detecting mycobacteria and has a large incubation capacity [16].

The mean TTP value from the co-culture of Mtb–splenocytes showed that splenocytes in the BCG group had a higher potency for inhibiting Mtb growth *in vitro* than the negative control. Splenocytes treated with the TB vaccine candidate pcDNA3.1-*rpf*B had the potency for inhibiting Mtb growth *in vitro*, which was not different from the BCG group. On the other hand, the mean TTP value from the Mtb–PBMC co-culture showed that PBMCs in the pcDNA3.1-*rpf*B and BCG groups had a higher potency for inhibiting Mtb growth *in vitro* than the negative control. In addition, PBMCs in the pcDNA3.1-*rpf*B TB vaccine candidate group had Mtb growth inhibition potency in *vitro*, which is similar to that of the BCG group.

From this MGIA study based on the TTP value, the Mtb–splenocyte and Mtb–PBMC co-cultures gave similar results. The results showed that the TB vaccine candidate pcDNA-3.1-*rpf*B had Mtb growth inhibition potency *in vitro* which is similar to that of the BCG vaccine. This finding can be a good support for the further development of the pcDNA3.1-*rpf*B TB vaccine candidate.

Marsay *et al.* [17] used MGIA to examine the inhibition of Mtb growth by splenocytes of mice immunized with BCG compared to naive controls. Parra *et al.* [18, 19] also used MGIA to examine the inhibition of Mtb growth by splenocytes of mice immunized with BCG compared to naive controls and five novel vaccines. MGIA is a WHO-supported method for testing TB vaccine candidates. The WHO's goal in supporting the MGIA is to develop functional tests that can be applied to all TB vaccine candidate clinical trials; thus, they can help in identifying the correlation between potential TB vaccine candidates and built-up immunity [20].

The levels of IFNγ in the supernatant from the co-culture of Mtb and mammalian cells in this study were measured by ELISA. This step was performed because IFNγ was the dominant type of cytokine produced *in vivo* by BaLB/c mice treated with pcDNA3.1-*rpf*B in the previous study. The results of statistical analysis on the average IFNγ level were not in line with the results of statistical analysis of the average TTP value. However, if further observed, the level of values was linear, with the BCG group having the highest value and the pcDNA3.1 group having the lowest value. Each treatment group had higher levels of IFNγ that were significantly different from the control mammalian cells (splenocytes and PBMCs). This results indicates that the addition of Mtb suspension to splenocytes and PBMC co-cultures can increase IFNγ production.

Statistical analysis was carried out to determine the correlation between the TTP values and IFNγ levels in

the co-cultured Mtb and mammalian cells. Data from the MGIT™ test results in the form of TTP values and ELISA data in the form of IFNγ levels were analyzed by the Pearson correlation test. This test showed no correlation between the TTP values and IFNγ levels in the Mtb-splenocyte and Mtb-PBMC co-cultures. This finding indicates that IFNγ levels are not directly correlated with TTP values, that is, they are not directly related to Mtb growth inhibition *in vitro*.

No statistical correlation existed between the TTP values and IFNγ levels due to a complex immune response system. After infection, Mtb stimulates CD4, CD8 T cells, and other immune cells to secrete IFNγ, which dominates the strong type-1 immune response. IFNγ then plays a role in activating the bactericidal action in the host cell. However, in addition of IFNγ, other cytokines, such as interleukin 10 (IL-10), are present in the system. In contrast to IFNγ, IL-10 is considered an inhibitory cytokine. The presence of IL-10 is important for the adequate balance between inflammatory and immunopathological responses. Elevated levels of IL-10 favor mycobacterial survival in host cells [15]. Therefore, given that other cytokines are counterproductive, this condition may be the cause of the absence of a statistical correlation between the TTP values and IFNγ levels.

Conclusion

This study revealed that the TB vaccine candidate pcDNA3.1-*rpf*B has efficacy for inhibiting Mtb growth *in vitro*, but it is not significantly different from that of the BCG, according to the MGIA. IFNγ levels in co-cultured Mtb and mammalian cells did not correlate with the level of inhibition of Mtb growth *in vitro*.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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