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Evaluation of Tuberculosis Vaccine Candidate, pcDNA3.1-*rpfD* using Mycobacterial Growth Inhibition Assay (MGIA)

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ABSTRACT

Resuscitation-promoting factor D (RpfD) is a protein involved in the resuscitation of dormant bacteria. A new tuberculosis vaccine carrying the *rpfD* gene has been successfully constructed, pcDNA3.1-*rpfD*. It was demonstrated that this vaccine exhibits cellular and humoral immune responses. Therefore, within this study, the efficacy of this new vaccine candidate was evaluated using mycobacterial growth inhibition assay (MGIA). MGIA is a functional assay that measures the complex host immune response, peripheral blood mononuclear cell (PBMC) and splenocyte from BALB/c mice against mycobacteria. With BACTEC™ MGIT™ 960 automated system, the effect of vaccination on bacterial growth was reported as a time to positivity (TTP) in hours. The mean of TTP from the vaccinated group (both pcDNA3.1-*rpfD* and BCG) was higher than the negative control group. These results suggest that pcDNA3.1-*rpfD* may be effective in controlling tuberculosis growth and may provide a clue for the development of the tuberculosis vaccine. In addition, despite previous evidence that IFNγ was essential for tuberculosis immunity, IFNγ (interferon gamma) production was found not to be correlated with mycobacterial inhibition. Therefore, these findings offer an alternative method to evaluate vaccine candidates than the assessment using IFNγ only.

1. Introduction

Tuberculosis (TB) is a burden for the world, reaching 10 million new cases and 1.2 million deaths per year, driving the deadliest contagious disease (WHO 2020). The only clinically approved vaccine against TB is Bacille Calmette-Guérin (BCG). It gives reliable protection against severe TB in children, but its efficacy is extremely variable in adults (Fine 1995). This limitation urges the need for a new TB vaccine to control the spread of the infection. Although there are many TB vaccines in the pipeline, their trial phase is still overshadowed by unpromising results. Therefore, continuous efforts are carried out for developing TB vaccines including methods to validate correlates of protection in early screening.

In 2017, a new tuberculosis DNA vaccine, pcDNA3.1-*rpfD* has been successfully constructed (Rakhmawati 2017) . It is composed of the pcDNA3.1 vector and the resuscitation-promoting factor D (*rpfD*) gene. The pcDNA3.1 vector was designed to allow replication in bacterial cells and expression of the cloned gene in mammalian cells (Invitrogen 2010). The cloned gene, *rpfD* encodes a protein that plays a role in the resuscitation of *Mycobacterium tuberculosis* (Mukamolova *et al.* 2002). Rakhmawati 2017 demonstrated that pcDNA3.1-*rpfD* induced cellular immune responses characterized by the production of IL-12 and IFNγ, important cytokines in controlling tuberculosis infection. Humoral immune response was also successfully induced which was defined by the production of IgG anti-RpfD antibodies in BALB/c mice, suggesting that it may serve as a potent vaccine candidate. Moreover, Pratama 2019 evinced that the strongest induced IgG subclass was IgG2a which bears an important role in facilitating *M. tuberculosis* eradication by complement (Macedo *et al.* 2011) or antibody-dependent cell-mediated cytotoxicity (ADCC) (Kips *et al.* 1985).

Following the successful completion of *in vitro* study, TB vaccine candidates must enter the extensive *in vivo* study to establish safety, efficacy, and potency. Mouse models can be used to aid in the identification

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of correlates protection through challenge studies (Ordway and Orme 2001). Nevertheless, not all laboratories support this risky-challenge assay, and more so as TB disease progresses, animals may experience TB symptoms, pain and will eventually die of pulmonary insufficiency. In the effort of overcoming this problem, mycobacterial growth inhibition assay (MGIA) has been used as an assay for down-selecting TB vaccine or 'gating' before in vivo assay (Tanner 2015). Therefore, only the strong candidate is permitted for *in vivo* challenge and thus fulfilling the "protection" in the animal model principle. Besides, MGIA has been established to produce output that was relevant to the results of the challenge assay. Yang *et al.* 2016 proved that there was a significant correlation between the inhibitory effect based on the MGIA and the challenge assay, supporting the MGIA as an alternative method for the challenge assay. In this study, BACTEC MGIT-based MGIA was adapted for assessment of vaccine candidate, pcDNA3.1-*rpfD* efficacy by measuring mycobacterial survival after incubation with PBMCs or splenocytes from BALB/c mice.

2. Materials and Methods

2.1. Isolation and Confirmation of Recombinant pcDNA3.1-*rpfD* Plasmid

Recombinant pcDNA3.1-*rpfD* and pcDNA3.1 plasmid control were isolated from *Escherichia coli* DH5α using Hispeed Plasmid Midi Kit (QIAGEN) following manufacturer instruction. The presence of the *rpfD* gene in the isolated plasmid was confirmed using conventional PCR with primer F_*rpfD* and R_*rpfD*, or using primer set F_cmV/R_*rpfD* for correct direction of inserted gene (Rakhmawati 2017).

2.2. Mice Immunization

Eighteen BALB/c mice within aged of 6—8 weeks were divided into 3 groups of experiment. Mice were immunized intramuscularly with 100 µg/100 ul pcDNA3.1-*rpfD* as treatment group, pcDNA3.1 as negative control group or with 100 ul (equal to 2-8 x 10^5 CFU) of BCG SII vaccine intraperitoneally (Rakhmawati 2017). All immunization was carried out three times within two weeks interval. One week after last immunization, mice were terminated followed by blood collection and sera separation, PBMC and splenocytes isolation. Ethical approval for this study was obtained from Komite Etik Penelitian Kesehatan FKUI—RSCM, number 616/UN2.F1/ETIK/PPM.00.02/2020.

2.3. Sera, PBMC and Splenocytes Isolation

Blood from terminated mice were collected via cardiac puncture, followed by centrifugation for sera separation and PBMC isolation using density-gradient centrifugation with Histopaque® (Sigma) (Riedhammer *et al.* 2014). Mice splenocytes were isolated from fresh spleen aseptically. Both PBMC and splenocytes were adjusted to 3 x 10^6 cells in total 300 ul of RPMI 1640 (Gibco), 2 mM L-glutamine with additional 25 mM HEPES for PBMC or 10% of FBS (Sigma) for splenocytes.

2.4. *In Vitro* Mycobacterial Growth Inhibition Assay

The following method was adopted from Zelmer *et al.* 2016 and Tanner *et al.* 2019 with slight modification. Prepared splenocytes and PBMC were co-cultured with 0.0005 McFarland of *Mycobacterium tuberculosis* H37Rv strain, followed by incubation 37°C with 5% CO_2 for 96 hours. For PBMC group, 10% of autologous sera was added to the co-culture. After incubation, co-culture were centrifuged followed by 300 µl isolation of supernatant for cytokine analysis and obtained pellet were inoculated in to MGIT tube (BD Biosciences) and incubated until they were positively detected.

2.5. IFNγ Measurement

IFNγ concentration in co-cultured supernatants were measured using mouse IFN gamma ELISA kit (Invitrogen) according to the manufacturer's instructions. Briefly, the assay was performed with incubating 100 µl undiluted supernatant into pre-coated wells followed by incubation at room temperature (RT) for 1 h. After incubation and washing step, biotin conjugate and streptavidin-HRP were added subsequently followed also by 1h incubation at RT for each reagent and vigorous washing steps. Finally, stabilized chromogen substrate was added followed by 30 minutes incubation at RT. Reaction was stopped by adding stop solution followed by absorbance measurement using ELISA reader Elx800 BioTek at 450 nm and cytokine concentration was calculated in comparison to standard IFNγ concentration curve.

2.6. Statistical Analysis

Statistical Package for the Social Sciences (SPSS) was used to perform statistical analysis with p-value >0.05.

3. Results

3.1. Isolation and Confirmation of Recombinant pcDNA3.1-*rpfD* Plasmid

Electrophoresis result showed that the isolated plasmids were at the length of around 5,428 bp and 5,893 bp, corresponds to the size of pcDNA3.1 and pcDNA3.1-*rpfD*, respectively (Figure 1a). Furthermore, the PCR product showed a band around 465 bp, indicating the *rpfD* gene was successfully amplified. Besides, the PCR product showed that the direction of the *rpfD* gene insertion was correct, as evidenced by the presence of a band around 673 bp (Figure 1b).

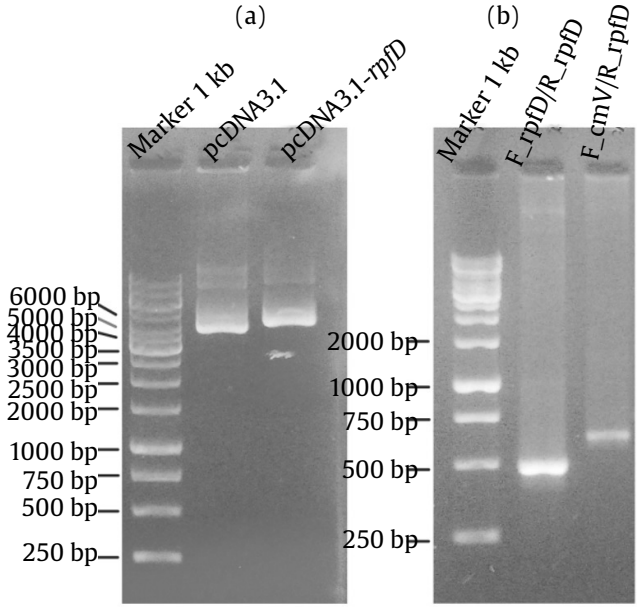

Figure 1. Electrophoresis result of (a) pcDNA3.1 and pcDNA3.1-*rpfD* plasmid (b) PCR product using primer sets of F_*rpfD*/R_*rpfD* and F_cmV/R_*rpfD*

3.2. Evaluation of Seed Vaccine Protection Based on Time to Positivity

Immune cells (PBMCs or splenocytes) were co-cultured with *M. tuberculosis* for 96 hours to give immune cells time for delivering their responses. The effect was expected since the incubation condition was set to 37°C and 5% CO_2, which favors the growth of mammalian cells. Using the BACTEC™ MGIT™ system, mycobacterial growth in each tube was determined by TTP in hours. The higher TTP was observed in the test and positive control groups compared to the negative control group, as depicted in Figure 2.

Regarding splenocyte co-culture, the mean of TTP from the test group (pcDNA3.1-*rpfD*), negative control group (pcDNA3.1), and positive control group (BCG) was 323 (±31), 200 (±77), and 283 (±8) hours, respectively. The mean of TTP from the test group was higher than the negative control group ($p = 0.047$). Likewise, the TTP of the positive control group compared to the negative control group ($p = 0.196$). As for the test group, it has a higher mean than the positive control group ($p = 0.081$). There was no significant difference in TTP from the pcDNA3.1 group and *M. tuberculosis* control. As a control, *M. tuberculosis* was incubated in RPMI-S media without splenocytes, indicating that the inhibitory effect was derived from splenocytes.

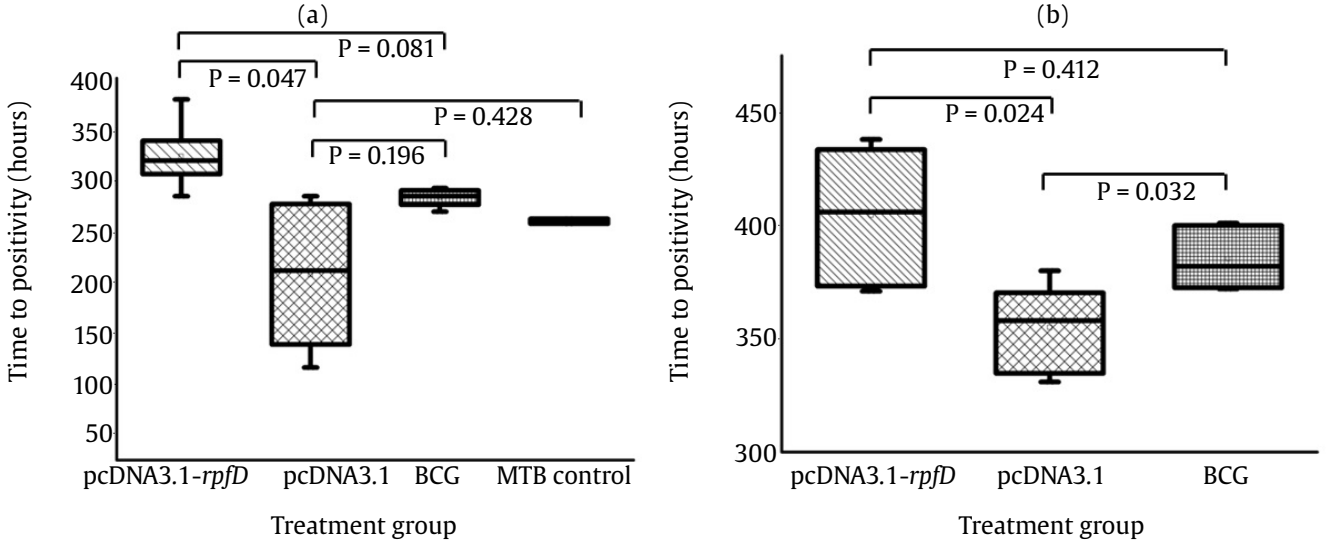

Figure 2. Comparison of MGIA responses expressed in TTP between study groups. (a) TTP of *M. tuberculosis* co-cultured with splenocyte, (b) TTP of *M. tuberculosis* co-cultured with PBMC

A similar trend was observed in the TTP of PBMC co-culture. The mean of TTP from the test group (pcDNA3.1-*rpfD*), negative control group (pcDNA3.1), and the positive control group (BCG) was 404 (±31), 355 (±19), and 385 (±14) hours, respectively. The mean of TTP from the test group was higher than the negative control group ($p = 0.024$). Likewise, the mean TTP of the positive control group compared to the negative control group ($p = 0.032$). Meanwhile, the test group has a higher mean than the positive control group ($p = 0.412$).

3.3. Analysis of IFNγ Levels Based on ELISA

To understand the immune response after vaccination, we measured the cytokine associated with TB infection, IFNγ. The mean of IFNγ concentration from splenocyte-*M. tuberculosis* co-culture (after subtraction of the absorbance from the nil control) was not significant between the test group, negative control, and positive control group ($p = 0.387$). Likewise, there was no significant difference between these treatment groups and the splenocyte control group (Figure 3). The splenocyte control group was splenocytes from each treatment group incubated without *M. tuberculosis*.

IFNγ was also detected in the supernatant of PBMC+*M. tuberculosis* co-culture. However, no significant differences were observed in pcDNA3.1-*rpfD*, pcDNA3.1, and BCG groups. PBMC control group, when compared with these treatment groups, also has no significant difference in IFNγ levels (Figure 4).

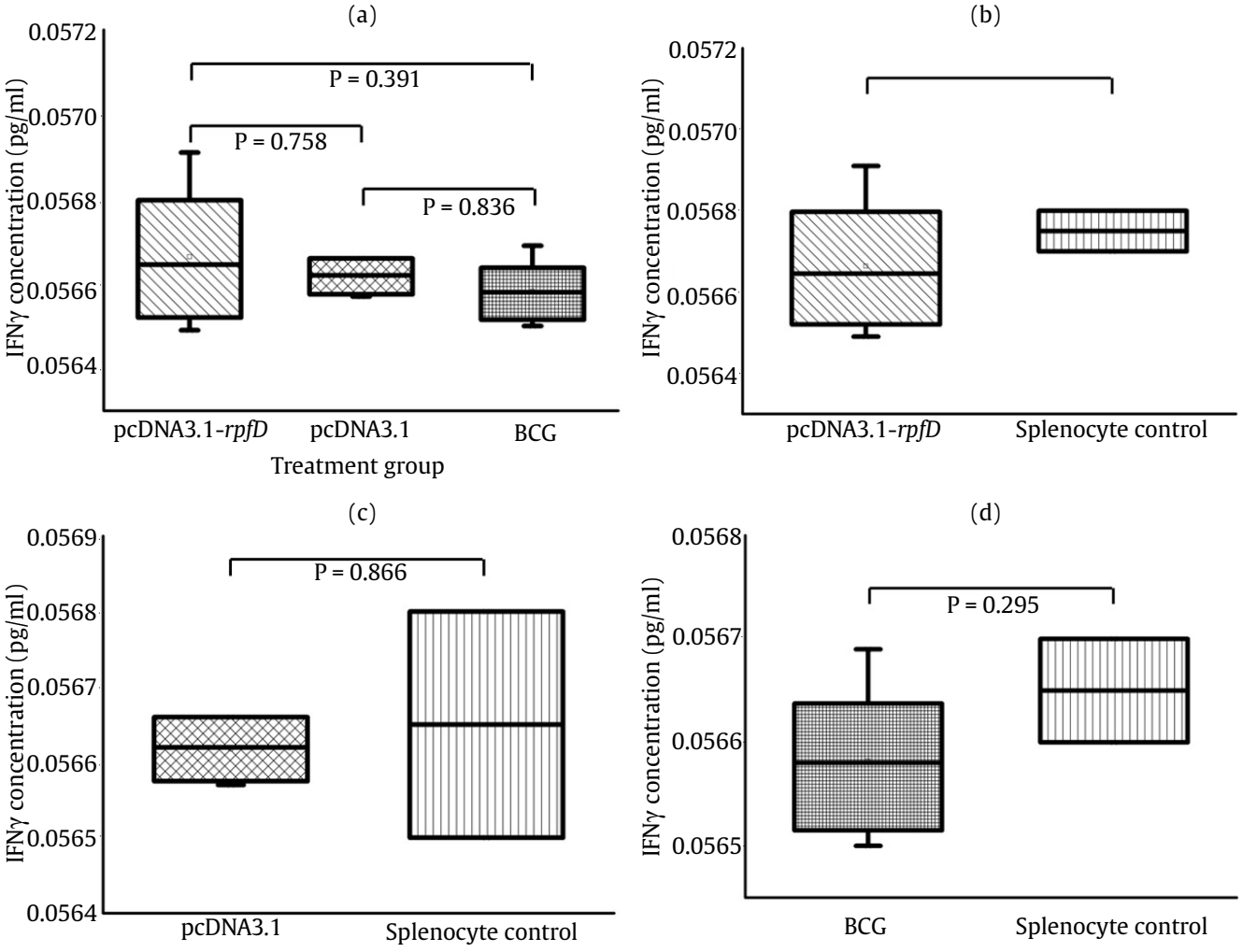

Figure 3. Comparation of IFNγ levels from splenocytes co-culture supernatant after 96 hours incubation. (a) pcDNA3.1—*rpfD*, pcDNA3.1, and BCG group have IFNγ levels of 0.05666, 0.05669, and 0.05658 pg/ml respectively, (b) splenocytes from mice injected with pcDNA3.1—*rpfD*, pcDNA3.1, and BCG that cultured without *M. tuberculosis* has IFNγ levels of 0.05677, (c) 0.05668, (d) 0.05665 pg/ml

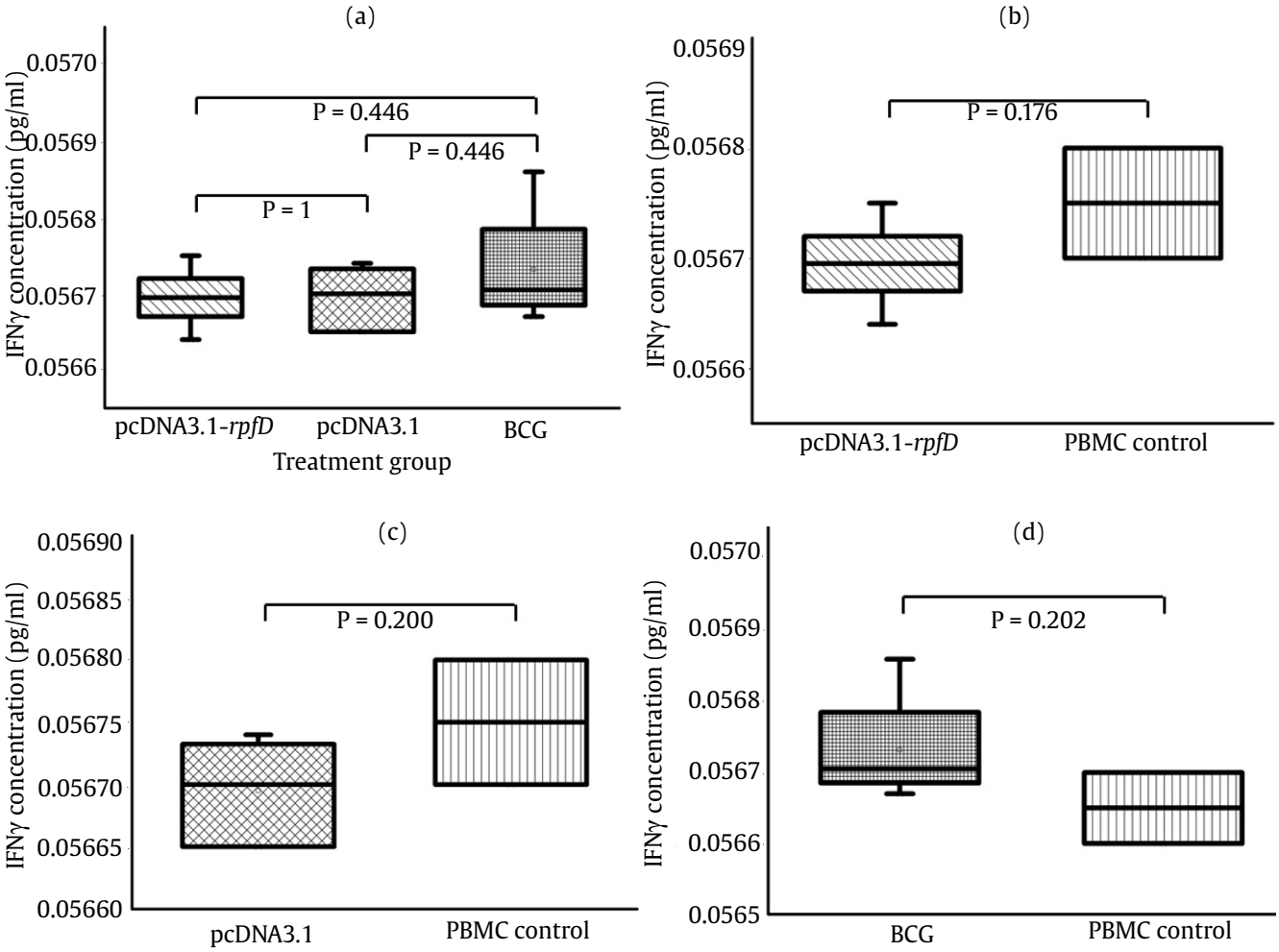

Figure 4. Comparation of IFNγ levels from PBMC-*M. tuberculosis* co-culture supernatant after 96 h incubation. (a) pcDNA3.1-*rpfD*, pcDNA3.1, and BCG group have IFNγ levels of 0.05669, 0.05670, and 0.05673 pg/ml respectively, (b) PBMC from mice injected with pcDNA3.1—*rpfD*, pcDNA3.1, and BCG that cultured without *M. tuberculosis* has IFNγ levels of 0.0567, (c) 0.0568, (d) 0.0567 pg/ml

3.4. Correlation of IFNγ Levels with Time to Positivity

The TTP value represented the ability of mammalian cells to inhibit the growth of *M. tuberculosis* in this study. The higher the TTP value, the greater the ability of mammalian cells to inhibit bacterial growth. The TTP value was then correlated with IFNγ levels considering that this cytokine was known to play a role in the immune response against tuberculosis. Statistical analysis showed that the Pearson correlation value between IFNγ and TTP of PBMC co-culture was -0.008. Meanwhile, the Pearson correlation value between IFNγ and TTP from splenocyte co-culture was 0.073.

4. Discussion

In this study, MGIA was conducted using vaccinated mice's PBMCs or splenocytes co-cultured with *M. tuberculosis*. This procedure was carried out on a 48-well plate, which was more favored than culturing in a rotating tube as it increased mammalian cell viability and cytokine production (Jensen *et al.* 2017; Tanner *et al.* 2019). The number of cells used in this study was 0.0005 McF and 3 x 10^6 for mycobacteria and mammalian cells, respectively. Regarding the number of mycobacteria, it was determined that the inhibition effect of mammalian

cells toward mycobacteria was still observable along with the research time-wise. Besides, the number of mycobacteria of 0.0005 McF was known to have ten days of TTP (data not shown); Zelmer *et al.* 2016 described that the effect of the immune response against mycobacteria was expected to be seen. As for mammalian cells, despite the more mammalian cells, the greater the effect of the immune response was, increasing mammalian cells was undesirable. It was reported that mammalian cells exceeding 5 x 10^6 could cause an event called yellowing culture media in 96 hours, leading to unhealthy mammalian cells (Painter *et al.* 2020).

The addition of autologous serum to the co-culture of the PBMCs ensures the effect of vaccination against *M. tuberculosis*. The addition of serum has been shown to enhance the effect of the immune response on bacterial growth in human PBMC cultures (Tanner *et al.* 2021). Meanwhile, a study by Pratama 2019 showed that pcDNA3.1-*rpfD* was proven to induce IgG2a anti-RpfD antibodies in the serum of BALB/c mice. The IgG2a subclass is known to play a role in the immune response through ADCC (Kips *et al.* 1985) and the complement system (Macedo *et al.* 2011). After the co-culture period was completed, all cells were centrifuged, followed by a re-culture process without lysis of mammalian cells because the lysis process, with water, PBS-Tween, saponins, SDS, or without lysis did not affect the re-culture results (Tanner *et al.* 2019).

The output of MGIA is the ability to inhibit the growth of *M. tuberculosis*, which can be expressed in TTP. The use of the BACTEC™ MGIT™ system has the advantage of detecting bacterial growth faster and providing more accurate growth measurement results (Pfyffer *et al.* 1997; Brennan *et al.* 2017). Although co-culture observations with a microscope at 400x magnification did not show any morphological differences (data not shown), statistical analysis of TTP values in splenocyte and PBMC cultures showed significant differences between the treatment groups (Figure 2). These results indicate that PBMC cells from mice immunized with pcDNA3.1-*rpfD* or with BCG could inhibit the growth of tuberculosis. Control of mammalian cells constituting PBMCs and splenocytes without *M. tuberculosis* cells was also carried out. The TTP measurement of control mammalian cells was negative for the use of oxygen in the MGIT tube within 42 days (data not shown). These results indicated that the positive growth results in the MGIT tube, by decreasing oxygen levels in the tube, are indeed due to the growth of *M. tuberculosis* and not due to the presence of mammalian cells. These results also confirm the research by Tanner *et al.* 2019 that BACTEC MGIT-based MGIA can be performed without mammalian cell lysis.

Indeed, the PBMC and spleen are composed of immune cells that have a role in inhibiting the growth of *M. tuberculosis*. Dendritic cells (DCs) that respond to the RpfD antigen from the injection site may migrate to the spleen or lymph nodes. The expression of RpfD protein has been demonstrated by Rakhmawati 2017 that anti-RpfD antibodies were detected in the serum of mice injected with pcDNA3.1-*rpfD*. These DCs are thought to present antigen to CD8+ T cells followed by differentiation into effector cells and finally leaving the spleen (Bronte and Pittet 2013). Effector T cells can be taken up and participate in inhibiting bacterial growth during the co-culture period. In addition, T cells can differentiate into memory cells (Abbas *et al.* 2018). When induced with *M. tuberculosis* during co-culture, these T cells can respond to the presence of bacteria.

Growth inhibition of *M. tuberculosis* also occurs with monocyte response. Antigens released by transfected cells may be captured by APCs, processed via the endocytosis system, and then presented to CD4+ T (Gurunathan *et al.* 2000; Klutzer and Weiner 2008; Li and Petrovsky 2016). These CD4+ T cells can differentiate into Th1 in the presence of the IL-12 cytokine (Abbas *et al.* 2018). Regarding IL-12, it was determined that immunization with pcDNA3.1-*rpfD* triggered higher levels of IL-12 compared to the control group (Rakhmawati 2017). Furthermore, Th1 cells produce IFNγ, which plays a role in macrophage activation (Abbas *et al.* 2018). The growth of *M. tuberculosis* in co-culture was prevented in the test group, pcDNA3.1-*rpfD*, assuming that activated macrophages were taken up and participated in inactivity during the co-culture period.

Considering the importance of IFNγ in eradicating *M. tuberculosis*, in this study, IFNγ levels were measured using ELISA. The results showed that IFNγ was detected in each treatment group. However, IFNγ levels were not significantly different between the immunized group and the negative control group. These results may arise due to injection treatment in the negative control group with pcDNA3.1. This plasmid does not express the RpfD protein, but

possible that it can express other proteins such as the neomycin resistance gene, which is part of the pcDNA3.1 plasmid (Invitrogen 2010). Neomycin resistance gene expression might triggered the production of IFNγ. Thus, in the negative control, IFNγ was also detected by ELISA even though the production of IFNγ was triggered by a different cause. Furthermore, statistical differences could not be detected when comparing IFNγ levels between the pcDNA3.1-*rpfD* and the negative control groups. This limitation can be avoided by adding a naive BALB/c mouse not given injection treatment in subsequent studies. This naive control group can be used as a reference to normalize the data (Yang *et al.* 2016). On the other hand, the measurement time may have contributed to the absence of differences in levels of IFNγ. In this study, the measurement of IFNγ levels was carried out on the 4th day. It is possible that at that time, the levels of IFNγ in culture are no longer sensitive to make a difference at that point. The MGIA study that found differences in IFNγ levels was carried out by Marsay *et al.* 2013 by measuring the level of IFNγ gene expression at 12 hours.

In this study, it was found that there was no relationship between IFNγ levels and the ability to inhibit the growth of *M. tuberculosis*, as indicated by Pearson correlation analysis. In line with previous reports by Hoft *et al.* 2002; Kampmann *et al.* 2004; Fletcher *et al.* 2013; Joosten *et al.* 2018; Tanner *et al.* 2019. This phenomenon is due to the complexity of the immune response to *M. tuberculosis*, which involves various cytokines likes IL-12, IL-18, IL-23, and TNFα, which were not measured in this study. In addition, the killing of *M. tuberculosis* itself is carried out by effector cells such as macrophages, CD4+ T cells, and CD8+ T cells, not by cytokines (Crevel *et al.* 2002; Schaaf and Zumla 2009). Therefore, in addition to IFNγ, it is preferred to measure the number or ratio of effector cells in co-culture (Silver *et al.* 1998). These findings suggest that complex immune mechanisms exerted by other cytokines and their interactions with immune cells may be responsible for the inhibition. Therefore, the MGIA results can be used as a complete method to assess vaccine potency to accompany cellular response analysis.

In conclusion, this study provides evidence that pcDNA3.1-*rpfD* conferred protection against *M. tuberculosis* as reflected by longer TTP compared to the negative control. As this vaccine candidate yielding greater potential, it may be feasible to proceed to the *in vivo* challenge assay with animal models. Moreover, the result was supported by the positive control group, BCG which has already known as an agent in preventing tuberculosis infection. Even though IFNγ levels did not show any correlation with TTP, this finding was in line with previous evidence. Suggesting that MGIA may serve as an additional method for evaluating vaccine candidates.

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