RESEARCH ARTICLE

# ESAT-6-Ag85C-polyHistag Antigen Fusion is Potential as Vaccine Candidate for Tuberculosis

Ratu Purwanti[1,*] Agnes Rengga Indrati[1], Nida Suraya[1], Maya Marinda Montain[2], Ines Atmosukarto[3], Neni Nurainy[4], Ida Parwati[1]

[1]Department of Clinical Pathology, Faculty of Medicine, Universitas Padjadjaran/Dr. Hasan Sadikin Hospital, Jl. Pasteur No.38, Bandung 40161, Indonesia
[2]Balai Besar Kesehatan Paru Masyarakat Bandung, Jl. Cibadak No.214, Bandung 40241, Indonesia
[3]Lipotek Australia, 131 Garran Rd, Acton ACT 2601, Australia
[4]PT. Biofarma Bandung, Jl. Pasteur No.28, Bandung 40161, Indonesia

*Corresponding author. Email: ratupurwanti@gmail.com

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## Abstract

**BACKGROUND:** BCG vaccine has been proven to be effective protection against tuberculosis (TB) meningitis and miliary TB. However, for protection against pulmonary TB, the results remains vary widely. Recombinant vaccine consisting of two immunodominant Mtb antigens ESAT-6-Ag85C-polyHistag (EAH) is currently being developed as a new TB vaccine candidate for booster. An immugonecity test for vaccine candidates is required in the initial phase to evaluate cellular immune response. This study was conducted to evaluate the cellular immune response by measuring interferon-gamma (IFN-γ) cytokines produced by T cells after *ex vivo* stimulation by TB EAH antigen fusion.

**METHODS:** Peripheral blood mononuclear cells supernatant samples were collected from 16 new pulmonary TB subjects, 17 pulmonary TB in treatment subjects, and 10 healthy subjects. Samples were tested for IFN-γ level with enzyme-linked immunosorbent assay (ELISA). Kruskal-Wallis test was used to test the differences between IFN-γ levels among three groups, and followed by post-hoc analysis using Mann Whitney.

**RESULTS:** The median of IFN-γ levels for new pulmonary TB, pulmonary TB in treatment, and healthy subjects were 17.09 (2.65-140.14) pg/mL, 4.36 (2.43-21.41) pg/mL, and 2.91 (2.39-3.85) pg/mL, respectively. There were significant differences of IFN-γ levels between new pulmonary TB group and pulmonary TB in treatment group ($p$=0.012), between new pulmonary TB group and healthy group ($p$=0.001), and also between pulmonary TB in treatment group and healthy group ($p$=0.035).

**CONCLUSION:** Results show that TB EAH could stimulate cell-mediated immune responses in the three groups, with the highest IFN-γ levels are found in new pulmonary TB group, suggesting a potential immunodominant antigen fusion for vaccine candidate development.

**KEYWORDS:** Ag85C, ESAT-6, immunogenicity test, IFN-γ, TB vaccine candidate, tuberculosis

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## Introduction

Even though BCG vaccine program has been implemented since 1952, Indonesia remains in the third highest tuberculosis (TB) burden country in the world.(1) The BCG vaccine has been proven to protect against TB meningitis and miliary TB, however, for protection against pulmonary TB, the results vary widely, ranging from adequate protection to absence of clinically significant benefits. The effectiveness of the BCG vaccine in adults varies greatly, and its protective effect is not significant after 10 years. (2,3) It is urgently needed to develop new TB vaccine as a booster vaccine to end TB. New TB vaccines are being

developed in numerous studies, but none of these candidates have been approved as a TB programme. Currently, recombinant *Mycobacterium tuberculosis* (Mtb) fusion protein nanoparticle-based vaccines are being developed as a new TB vaccine prototype.

Two immunodominant proteins derived from *M. tuberculosis* bacteria early secreted antigenic target 6-kDa (ESAT-6) and antigen 85C (Ag85C) were used in this new vaccine. The selection of ESAT-6 as an antigen is because this immunodominant antigen is part of the region of difference 1 (RD1), which is deleted in *Mycobacterium bovis* BCG, and has been extensively explored in vaccines.(4) Although ESAT-6 has good antigenicity, ESAT-6 immunization failed to elicit a sufficient T cell response in mice. To improve immunogenicity, it can be constructed as fusion molecules where a large immunogenic molecule might act as a carrier, as demonstrated in the ESAT-6 and Ag85B fusion molecule, the TB10.4 and Ag85B fusion protein, and the TB10.4-Ag85B-Ag85A polyprotein.(5,6) Ag85C is an immunodominant antigen belong to Ag85 complex (Ag85A, Ag85B and Ag85C). Ag85C is singularly responsible for almost 40% mycolate content of this pathogen and contributes to its virulence. In children, the antibody response to Ag85C were better than the antibody response to Ag85A and Ag85B.(7) Both ESAT-6 and Ag85C antigens will be produced as fusion proteins created by DNA tagging (a series of amino acid histidine in the C protein terminal). Fusion of ESAT-6-Ag85C-polyHis tag (EAH) antigen with a liposomal adjuvant is expected to be a potential vaccine for tuberculosis. Liposomal adjuvant in this vaccine candidate act as vaccine delivery system that can induce immune response. Liposome have depot effect that promotes stability, integrity and gradual release of vaccine. Liposome particulate also easily recognized by antigen presenting cell and activate immune response.(8)

Immunogenicity testing is required to evaluate the immune response of TB vaccine candidates. In the pre-clinical stage of the vaccine development, the immunogenicity assay vaccine was carried out *ex vivo* on peripheral blood mononuclear cells (PBMC). In Mtb infection, which is an intracellular bacterium, the immune response that plays a more important role is the cellular immune response. The immunological parameters used to assess cellular immune responses are the assessment of cytokine responses produced by T cells. One of the main cytokines produced by T cells is interferon-gamma (IFN-γ) as a defense mechanism to eliminate Mtb.(9,10) Cellular immune response in the form of T cells will increase within 1 week after being infected by Mtb, if it has passed intensive phase therapy for 2 months, the number of Mtb and activated T cells will decrease due to the healing process, whereas in healthy people T cells have not been activated because Mtb antigens have not sensitized them.(11,12) Hence in this study, IFN-γ levels were measured in new pulmonary TB, pulmonary TB in treatment, and healthy subjects to see whether there are any differences in immune responses in the three groups, to see the potential application of TB vaccine candidates as boosters.

## Methods

### Study Subject Recruitment

A cross sectional study involving new pulmonary TB, pulmonary TB in treatment, and healthy subjects were conducted. New pulmonary TB subjects were newly diagnosed active pulmonary TB patients, while pulmonary TB in treatment subjects were TB patients that have been receiving treatment for more than 2 months. Based on calculations using the Federer formula for laboratory experiments, the minimum sample required for each group was nine subjects. Subjects were recruited with consecutive sampling methods from March 2018 to January 2019 in Community Lung Health Center Bandung (*Balai Besar Kesehatan Paru Masyarakat Bandung*) and Dr. Hasan Sadikin Hospital Bandung. Healthy subjects were voluntarily obtained from PT. Biofarma Bandung and had completed physical and laboratory examinations.

TB diagnosis was made based on clinical presentation and chest X-ray, confirmed by positive acid-fast bacilli in Ziehl-Neelsen sputum smear microscopy or positive Mtb detection on rapid molecular diagnostic Expert MTB/Rif. Subjects with HIV positive, HBsAg positive, anti HCV positive, and diabetes mellitus were excluded from the study. Subjects with low PBMC count ($<1x10^6$/well) were also excluded. The protocol of this study was approved by the Ethical Committee of Dr. Hasan Sadikin Hospital, Bandung (Ref. No. LB.04.01/A05/EC/232/VII/2018).

### PBMC Stimulation and IFN-γ Measurement

Peripheral blood mononuclear cells were obtained from 8 mL of heparinized blood in BD Vacutainer® CPT (BD, Franklin Lakes, NJ, USA) and processed within 2 hours after blood collection. The PBMC collection process was carried out in the biosafety cabinet hood using sterile technique and PBMC count measured with XN-1000 hematology analyzer (Sysmex, Kobe, Japan). Fusion of ESAT-6-Ag85C-polyHis tag (EAH) antigen with liposomal adjuvant was developed

in PT. Biofarma, Bandung, Indonesia. TB EAH antigen (50 µg/mL) was added to PBMC and incubated at 37°C, supernatant were harvested after 48 hours and stored at -20°C until IFN-γ measured. IFN-γ levels examination was carried out with enzyme-linked immunosorbent assay (ELISA) method using Invitrogen™ IFN gamma Human Uncoated ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA). IFN-γ acted as antigen and would bind between capture antibody and biotin-conjugated detection antibody, streptavidin HRP and chromogen. Measurements were made at a wavelength of 450 nm.

**Statistical Analysis**

The data obtained in this study were later processed using SPSS Statistic for Windows, version 16.0 (SPSS Incorporation, Armonk, NY, USA). Kruskal-Wallis nonparametric test was used to test the differences between IFN-γ levels among three groups, and following post-hoc analysis using Mann Whitney was performed to test the IFN-γ levels between two groups. The result was considered assignificant if $p$-value≤0.05.

## Results

Initially, 57 subjects were recruited in this study, however, 14 subjects (4 subjects had hepatitis B positive, 3 subjects had diabetes mellitus, 1 subject was treated for less than 2 months, and 6 subjects had low PBMC count) were excluded from this study. (Figure 1). There were 16 total subjects in the new pulmonary TB group, 17 subjects in the pulmonary TB in treatment group and 10 subjects in the healthy group.

Baseline characteristics of the subjects were presented in Table 1. Age, leukocyte count and PBMC cell counts were presented in median (min-max) because the data were not normally distributed. There was no significant difference in median age, leukocyte value, and PBMC count between the 3 groups.

The highest median of IFN-γ after stimulation with TB EAH antigen was found in the new pulmonary TB group, and the lowest median was found in the healthy group. There were significant differences in the median of IFN-γ levels among three groups ($p$=0.001). The following post-hoc analysis test showed significant differences between all the groups as illustrated in the boxplot (Figure 2), with the highest statistical significance was between the new pulmonary TB group and healthy group ($p$=0.001). In the box plot, there were three outliers: two subjects in new pulmonary TB group (IFN-γ = 140.14 pg/mL and 55.64 pg/mL), and 1 subject in pulmonary TB in treatment group (IFN-γ = 21.41 pg/mL).

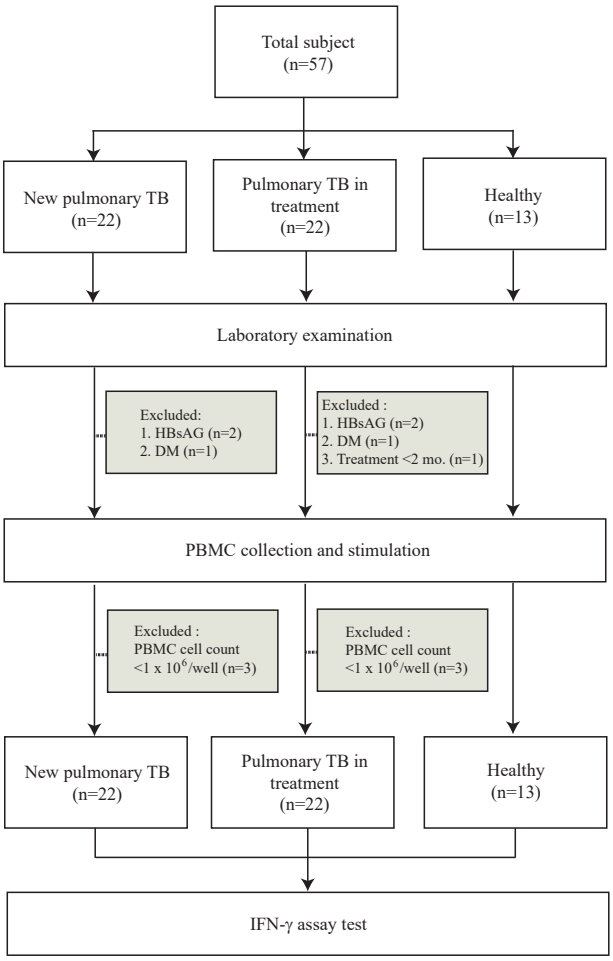


**Figure 1. Subject Recruitment Diagram.** Initially there were 22 subjects in new pulmonary TB group, 22 subjects in pulmonary TB in treatment group, and 13 subjects in healthy group. However, after the laboratory examination, PBMC collection and stimulation, some subjects are excluded from the study. Final 16 subjects in new pulmonary TB group, 17 subjects in pulmonary TB in treatment, and 10 subjects in healthy group was examined for IFN-γ assay test.

## Discussion

Baseline characteristics in this study showed pulmonary tuberculosis were more prevalent in male subject in both new-onset and TB in treatment group. Differences in social status and having more outdoor activities in males increase the likelihood of exposure to TB. Several genes that are associated with Mendelian susceptibility to mycobacteria disease (MSMD) are also X-linked, although these polymorphisms are rare, it exhibits potential immune-related genes on X chromosomes on TB immune response.

**Table 1. Subjects' characteristics and IFN-γ levels after TB EAH antigen fusion stimulation.**

| Characteristics | New Pulmonary TB (n=16) | Pulmonary TB in Treatment (n=17) | Healthy (n=10) | *p*-value |
|---|---|---|---|---|
| Gender, n (%) | | | | 0.528 |
| Male | 9 (56) | 12 (71) | 5 (50) | |
| Female | 7 (44) | 5 (29) | 5 (50) | |
| Age (years old), median (min-max) | 31 (18-65) | 26 (18-48) | 33 (24-46) | 0.428 |
| Laboratory Parameters, median (min-max) | | | | |
| Leukocyte (/mm$^3$) | 7,840 (4,310-14,920) | 7,440 (4,240-9,800) | 7,035 (4,610-11,950) | 0.135 |
| PBMC count/*well* ($10^3$) | 5,080 (1,250-17,150) | 6,100 (2,550-11,800) | 8,850 (2,760-10,540) | 0.538 |
| IFNγ level (pg/mL) | 17.09 (2.66-140.14) | 4.36 (2.43-21.41) | 2.91 (2.39-3.86) | 0.001* |

*Significant if $p<0.05$, tested with Kruskal-Wallis.

Sex hormones also modulate immune cells and contribute to Mtb eradication. Sex hormones have multiple different effects on immune cells. Estrogen is known to stimulate IFN-γ, tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-12 secretion while inhibit IL-10 production. These cytokines up-regulate antimycobacterial effector molecules. Macrophage is essential in eliminating Mtb, estradiol is known to enhance macrophage activation, while testosterone downregulates it by reducing TLR4 expression.(13) Median age of the subject in this study was 31 years old for the new pulmonary TB group and 26 years old for the pulmonary TB group on treatment. Risk factors for TB in productive age in the developing country include low education, room with no ventilation and smoking behavior.(14)

IFN-γ cytokine is thought to be associated with protective immunity in Mtb infection and is used as marker of T cell immune response.(5) Our results show that Mtb specific stimulation of IFNγ production, is the highest in new pulmonary TB followed by pulmonary TB on treatment and healthy individuals. Similar results that show lower IFN-γ levels after TB treatment were also observed by previous studies.(12,15) High IFN-γ levels in new pulmonary TB reflects the activity of the disease. It has been suggested that lower IFN-γ production after treatment of active TB indicates successful treatment that reduces bacterial load and as the consequences, the number of local and circulating IFN-γ-producing activated T cells will be reduced.(12,16) However, there is no difference in IFN-γ levels between active TB, latent TB and healthy people after ESAT-6 and CFP-10 stimulation.(17) There is no difference in the percentage of IFN-γ producing CD4 cells before and after ESAT-6 and CFP-10 stimulation. (18) Low IFN-γ levels in those studies may result from exhausted lymphocytes and nutritional status that can affect T cell function.(17,18) Prolonged exposure to a certain antigen can cause T-cell exhaustion, thus impairing effector function and preventing optimal infection control. (19,20) Malnutrition and TB are often coexist and have bidirectional association. Malnutrition can decrease the number of lymphocyte that is pivotal in immune response to TB.(21) In this study, body mass index (BMI) was used to assess nutritional status. We discovered that 56% of the subjects in new pulmonary TB group were classified in the underweight category (BMI<18.5), but they still showed adequate cellular immune response as evidenced by the elevated level of IFN-γ after stimulation.

From the boxplot, we could see two outliers in the new pulmonary TB group and one outlier in the pulmonary TB in treatment group. After investigating whether there is

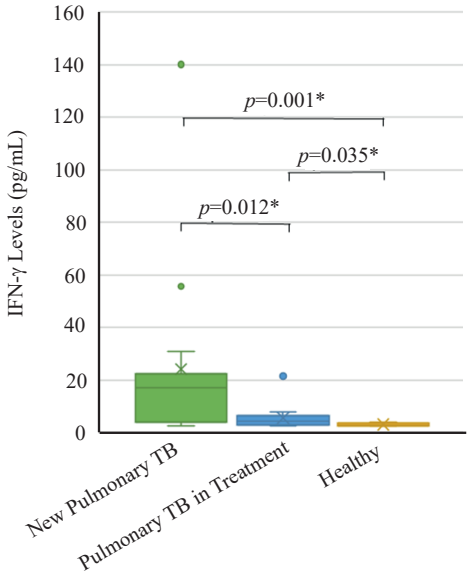


**Figure 2. Median IFN-γ levels between three groups.** The boxplot displays outliers in the new pulmonary TB group and pulmonary TB in treatment group. *Significant if p<0.05), tested with Mann-Whitney.

a relationship between IFN-γ levels and various baseline characteristics of research subjects, we found no significant differences. Therefore, the possible cause of the high IFN-γ level in this subject was the presence of other viral or bacterial infections that trigger a cellular immune response. In this study two of the subjects showed clinical symptoms such as fever, but it was difficult to prove other viral or bacterial infection because the symptoms can overlap with those of TB infection. The leukocyte counts of these three subjects were within normal limits and there are no other laboratory data that could prove the presence or absence of a viral or bacterial infection.

The results of this study indicate that stimulation of TB EAH antigen fusion with liposome adjuvants could induce *ex vivo* cellular immune response, measured by the levels of IFN-γ produced. The *ex vivo* immunogenicity test aims to assess whether the body's immune system recognizes the vaccine candidate, therefore there is no specific cut-off in IFN-γ levels. The TB EAH vaccine candidate can be continued to *in vivo* immunogenicity testing in experimental animals.(9) Future research should include functional T assay to assess the viability of T cells and assess variables that can influence T cell function. Therefore, it will reduce the bias and increase the significance of the research results.

## Conclusion

In conclusion, there were significant differences of IFN-γ levels in all three groups after TB EAH antigen fusion stimulation. This result also suggested the potential of immunodominant antigen fusion for vaccine development. However, further preclinical research is needed to evaluate the safety and efficacy of the new TB vaccine candidate.

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## Authors Contribution

IP, IA, NN and MMM were involved in planning and supervising the work. IA, NN and RP performed the measurements. IA, IP and RP processed the experimental data and performed the analysis. IP, RP, ARI and NS drafted the manuscript and designed the figures. IP and RP aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

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