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INTERFERON GAMMA EXPRESSION CD8⁺-T LYMPHOCYTE WITH ESAT-6-CFP-10 FUSION ANTIGEN STIMULATION

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ABSTRACT

Tuberculosis (TB) is an infectious disease in the world causing a global problem. Vaccination with Purified Protein Derivative (PPD) still cannot prevent tuberculosis in Indonesia. Interferon-gamma (IFN- γ) produced by CD8⁺-T lymphocyte has an important role in eliminating *Mycobacterium tuberculosis*. The vaccine candidate antigenic test was done to observe the inducible ability of IFN- γ as a main protection cytokine. This study aim was to research the difference of IFN- γ expression CD8⁺-T lymphocyte percentage with ESAT-6-CFP-10 fusion antigen stimulations as a TB vaccine candidate. This research is a quasi-experimental study design in the laboratory by ESAT-6-CFP-10 fusion antigen-stimulated Peripheral Blood Mononuclear Cells (PBMC) culture in vitro in TB patients, latent TB and healthy subjects' groups. IFN- γ expression CD8⁺-T lymphocyte percentage were examined by flow cytometry BDFACSCalibur with results: without antigen fusion stimulation IFN- γ expression CD8⁺-T lymphocyte percentage mean (TB patients 2,560, latent TB 2,173 and healthy people 2,153) and with antigen fusion (TB patients 3,039, latent TB 2,471 and healthy people 2,405). There was no significant difference in fusion antigen stimulation PBMC between TB patients, latent TB and healthy subjects' group and also within groups.

Key words: ESAT-6-CFP-10 fusion antigen, CD8⁺-T lymphocyte, tuberculosis

INTRODUCTION

Tuberculosis caused by *Mycobacterium tuberculosis* is a community disease that is still a global issue. World Health Organization (WHO) estimated 9.6 million new cases of TB (5.4 million cases were males, 3.2 million cases were females and 1 million cases were children) with 1.5 million deaths in 2014. Worldwide tuberculosis cases in 2014 were estimated around 281 cases in 100,000 population. South East Asia and West Pacific Region contributed 58% of cases with the proportion of 28% in Africa, 23% in India, 10% in Indonesia, also 10% in China population. Currently, Indonesia occupies the second position after India. Tuberculosis is one of the infectious diseases that cause death accompanied with Multi-Drug Resistant (MDR), Extensively Drug Resistant (XDR) and Total Drug Resistant (TDR). Vaccine administration with Purified Protein Derivative (PPD) cannot prevent the high prevalence of TB cases in the world as well as in Indonesia, so far.^{1,2}

M.tuberculosis infection starts with bacteria entry by inhalation. Alveolar macrophages and dendritic cells then phagocytose bacteria. *M.tuberculosis* in the macrophage will give signals so that class II MHC will be produced in the endoplasmic reticulum. This molecule will cause the *M.tuberculosis* fragment to be processed by the macrophage and presented on its surface until the CD4⁺-T lymphocyte receptor recognized it. Activated CD4⁺-T lymphocyte will produce cytokines, which are necessary to destroy and control the *Mycobacterium tuberculosis* growth. The main cytokines produced are IFN- γ , Tumor Necrosis Factor- α (TNF- α) and Interleukin-2 (IL-2).

Interferon-gamma (IFN- γ) plays an vital role in eliminating *Mycobacterium tuberculosis*. Interferon-gamma strengthens the phagocyte potential of the infected *Mycobacterium tuberculosis* macrophage by stimulating phagolysosome production.^{3,4} Interferon-gamma stimulates free radical production that can destroy *M.tuberculosis* components. *Mycobacterium tuberculosis* can be destroyed by IFN- γ produced by CD8⁺-T cell. CD4⁺ Tcell alone cannot control the *M.tuberculosis* growth but is required by CD8⁺-T cell to increase IFN- γ production.

Interferon-gamma will stimulate macrophage-containing TB bacteria to increase reactive Nitrogen Intermediate (RNI) which is required to destroy the *M.tuberculosis*. CD8⁺-T lymphocyte acts in macrophages activation through IFN- γ production. CD8⁺-T lymphocyte has a cytolytic function by stimulating macrophage to recognize mycobacterial antigen presented by Major Histocompatibility Complex (MHC) class I molecule in the surface of infected macrophage. CD8⁺-T lymphocyte is required to release the intracellular *M.tuberculosis* inside the infected macrophage.²⁻⁶ The similar response with the innate immune response to infection is a paradigm in the invention of new tuberculosis vaccine, which emphasizes mainly on an immune response that makes interferon- γ (IFN- γ) as the main protective cytokine. The essential roles of IFN- γ compared to various cytokines have been studied in many kind of research. Factors that regulate IFN- γ and IL-17 production are important to be known in order to develop vaccine that overcomes *M.tuberculosis* infection effectively.⁷

Early secreted antigenic target-6kDa (ESAT-6) is an immunodominant protein with low molecular weight. First identified from short-term culture filtrate *M.tuberculosis* using cells that secrete IFN- γ in *M.tuberculosis* infected mice. Early secretory antigenic target-6 kDa (ESAT-6 or Rv3875 or ESXA) proteins and culture filtrate protein 10 (CFP10 or Rv3874 or ESXB) are protein antigens with a low molecular weight produced by live and metabolized *M.tuberculosis* actively. ESAT-6 and CFP-10 are potent immunogens coded by Region of Difference (RD)-1 region of *M.tuberculosis* and cannot be found in the BCG strain, *Mycobacterium bovis*. The cellular immune response towards this specific antigen showed a good association with *M.tuberculosis* infection.⁸ This study done on mice showed that ESAT-6 and CFP-10 were potent antigens in inducing T lymphocyte response with abundant IFN- γ production.^{9,10}

Previous research done by Colangeli in 2000 stated that CFP-10 recombinant was a strong antigen toward T lymphocyte and produced IFN- γ in 70% PBMC of latent TB patients.¹¹ Early secretory antigenic target-6 kDa (ESAT-6) is an antigen that induces IFN- γ production by T lymphocyte in early TB infection.¹²⁻¹⁵

A combination of ESAT-6 and CFP-10 has been a real breakthrough in the field of latent TB infection detection research. Strong antigenicity comes from ESAT-6 and CFP-10 combination has been used to develop T lymphocyte examination such as skin test, ELISPOT and other blood tests that are already available to diagnose both latent and active TB infections.¹⁶

Based on this background, the researchers were interested in knowing the percentage difference of CD4⁺-T lymphocyte and of CD8⁺-T lymphocyte that express IFN- γ with ESAT-6-CFP-10 antigen fusion stimulation as the TB vaccine candidates.

METHODS

This study used a quasi-experimental study design in the laboratory. This study used an in-vitro method in Peripheral Blood Mononuclear Cells (PBMC) culture previously stimulated with ESAT-6 and CFP-10 antigen fusion of *Mycobacterium tuberculosis* in three sample groups from December 2016 until March 2017.

The percentage of CD8⁺ T lymphocyte that expressed IFN- γ was measured by FACSCalibur flowcytometry method. All PBMC donors were adult subjects (\geq 18 year old) that agree to fill and sign the informed consent to join the study.

Lung TB patients were new cases of active lung TB patients diagnosed by a Karang Tembok Hospital pulmonologist. The diagnosis was established based on history, physical examination and laboratory examination in which the BTA was positive and also thorax photo. The TB patients had not received any antituberculosis drugs. Latent tuberculosis (TB) person was a person that had no symptoms of TB. The latent TB subject was on duty in outpatient and inpatient unit in Karang Tembok Hospital Surabaya for more than six months. The latent TB subject had direct contact with

pulmonary TB patients but the sputum examination showed that the BTA was negative. Thorax photo examination was within normal limits and the tuberculin test was negative with diameter of the test 10 mm or more. Healthy subject criteria were negative tuberculin test (diameter \leq 10 mm) with no lung abnormality seen in the radiology examination.

Exclusion criteria for all PBMC donor were subjects with a history of abnormality in the liver, kidney, diabetes mellitus, hepatitis B, HIV infection and also a history of immunosuppressant and corticosteroid therapy. Active lung TB PBMC donor was excluded if known to have extra-pulmonary TB and new pulmonary TB case with any other lungs abnormalities, while latent TB was excluded if the subject had been diagnosed as pulmonary TB or extra-pulmonary TB and if the subject was suffering from a respiratory infection. A healthy person had never been diagnosed with pulmonary TB or extra-pulmonary TB and was not suffering from respiratory infection.

Control of application settings, positive control and negative control used unstained and single stained-PBMC of a normal person for each fluorochrome-label antibody used in this study.

Optimization was done before the examination was started to determine antigen level that would be used. Six PBMC of healthy person sample tubes was prepared. Each tube contain 1 millions cell, then ESAT-6-CFP-10 fusion antigen with concentrations of 1.06 μ g/mL, 2.13 μ g/mL, 5.3 μ g/mL and 10.6 μ g/mL were added. One tube was the negative control, without antigen addition and the other tube was the positive control with phytohemagglutinin (PHA) addition. All tubes were cultured for 5 days in CO₂ 5% incubator then the lymphocyte proliferation index was measured by (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) test. The level of ESAT-6-CFP-10 fusion antigen chosen in this study was determined based on the highest proliferation index. The highest proliferation index was found in the antigen level of 5.3 μ g/mL so that it was decided to use 5.3 μ g/mL ESAT-6-CFP-10 fusion antigen level in this study.

Peripheral blood mononuclear cells isolated from heparinized blood was done toward 16 samples of active lung TB, 16 samples of latent TB and 16 samples of healthy subjects. Each sample then were divided into 2 tubes i.e. tube with ESAT-6-CFP-10 fusion antigen stimulation and without the antigen. The antigen used in this study was a protein produced by the Indonesian Ministry of Health from living and active *M.tuberculosis* coded by Rv3875 (ESAT6) and Rv3874 (CFP10) gene located in a region of difference (RD)-1 region with the concentration of 5.3 μ g/mL.

The tubes were incubated for 3 days in 37^oC with a 5% CO₂ concentration and then the Golgi stop was added in the third day of incubation continued with 4-hour incubation. PBMC culture was then washed twice with Phosphate Buffer Saline (PBS) (2 mL/wash) and centrifuged at 1,800 rpm for 5 minutes until the pellet was obtained. The pellet was then added with 300 μ L of staining buffer and the CD8 staining was done. A hundred microliter PBMC sample was put in

Falcon tube and added with 5 µL anti-human CD8 FITC in the bottom of the tube. The tube then was vortexed and incubated for 20 minutes. The sample was added with 1 mL of staining buffer then centrifuged at 1,800 rpm for 5 minutes and the supernatant was discharged.

The sample was added with 250 µL of citofix/citoperm buffer then vortexed and incubated for 20 minutes at 40C. Fixation and cell permeability procedure was done with the addition of 1 mL of permeabilization wash then centrifuged at 1,800 rpm for 5 minutes; then the supernatant was discharged. Intracellular cytokine staining was done by addition of 5 µL of PE Mouse anti-human IFN-γ, vortexed and incubated in a dark room for 30 minutes at 40C. The sample was added with 1 mL of permeabilization wash and centrifuged at 1,800 rpm for 5 minutes and the supernatant was discharged (this washing process was done twice); then flow cytometry analysis was done with FACSCalibur.¹⁷

The differences of CD8+ T lymphocyte that express IFN-γ after ESAT-6-CFP-10 fusion antigen stimulation in PBMC culture between active pulmonary TB, latent TB and healthy people were statistically analyzed. The statistical analysis was done using Varian Analysis (One Way Anova) by computer program SPSS v.23, with p=0.05.

RESULT AND DISCUSSION

The research subjects were grouped based on the age and sex. Based on sex, the group of active TB was found dominated by males with a proportion of 81%, while the latent TB group was conversely dominated by females (69%). In the healthy group the number of the subjects was equal between males and females (Table 1). Male proportion of the active lung TB subjects was dominated by male subjects compared to female. The same result was obtained in the WHO Global Tuberculosis Report in 2015, where the comparison between males, and females was 1.4 from the whole new cases.¹The latent TB proportion in this study found dominated by females (69%). Pena found similar results in which the female proportion was 60% and Pallock *et al.*, 63.6%.^{18,19} This result was suspected to be correlated with the economic status wherein a developing country the females in their productive age had a higher mobility.²

According to the WHO Global Tuberculosis Report in 2015, the most of new TB cases in Indonesia were found in patients with aged range 55-64 years old. The same result was found in this study, where most of the subjects were more than 50 years old (30% of all active lung TB subjects).¹

Statistic analysis showed that data distribution of the percentage of CD8+-T lymphocyte that expressed IFN γ in active lung TB, latent TB, and healthy subjects with ESAT-6-CFP-10 fusion antigen stimulation were normally distributed (One-Sample Kolmogorov-Smirnov Test, p>0.05). Mean percentage of the CD8+-T cell that expressed IFN γ with ESAT-6-CFP-10 fusion antigen stimulation in active TB group was the highest among the three groups, while the lowest mean was shown by the healthy subjects group.

The three groups showed increased CD8+-T cell that express IFNγ with ESAT-6-CFP-10 fusion antigen stimulation percentage. Analysis done with ANOVA showed no statistically difference of the CD8+-T cell that express IFNγ with ESAT-6-CFP-10 fusion antigen stimulation percentage in active TB, latent TB, and healthy subjects (p>0.05).

This research showed no statistical difference of the percentage of CD8+-T cell that expressed IFN γ with ESAT -6-CFP-10 fusion antigen stimulation among those three groups. The same results were found by Wang *et al.* in which no significant difference was found between active TB and healthy persons and also by Rozot *et al.* which also found no difference between active and latent TB.^{20,21}

Wang *et al.* stimulated PBMC of active TB using ESAT-6-CFP-10 fusion antigen with a concentration of 10 µg/mL and incubated for 24 hours.²⁰ This research stated that IFNγ played a role in eliminating TB by macrophage activation rather than produced by NK cell. IFN γ production by CD8+ -T lymphocyte was increased in fusion antigen stimulation as well as found in this research, but IFNγ production by NK cell was found lower. IFN γ production by NK cell was not studied in this research.

Latent TB patients have a various immune response that may be influenced by the bacterial count. The bacterial count can be so low until it is almost eradicated so that it can be said that IFN γ produced by CD8++ T lymphocyte was correlated with TB clinical manifestation.²²

Table 1. Characteristics of the subjects

Characteristics	Active lung TB	Latent TB	Healthy controls
Sex			
Male	13 (81%)	5 (31%)	8 (50%)
Female	3 (19%)	11 (69%)	8 (50%)
Mean age ± SD	40 ± 16.11 years	40.44 ± 9.16 years	37±10.89 years
≤ 20	3 (19%)	0 (0%)	1 (8%)
21 – 30	2 (13%)	3 (19%)	3 (19%)
31 – 40	4 (25%)	6 (38%)	5 (30%)
41 – 50	2 (13%)	5 (30%)	5 (30%)
> 50	5 (30%)	2 (13%)	2 (13%)
Total	16 (100%)	16 (100%)	16 (100%)

Table 2. Percentage of CD8+T lymphocyte that express IFN γ with ESAT-6-CFP-10 fusion antigen stimulation in active lung TB, latent TB and healthy subjects

Group	N	Mean (%) \pm SD	Lowest value (%)	Highest value (%)	P
Active TB	16	3.039 \pm 1.468	1.0	5.1	0.459
Latent TB	16	2.471 \pm 1.168	0.8	4.6	
Healthy subjects	16	2.405 \pm 1.963	0.9	7.1	

Sillah *et al.* found different results, i.e. CD8+T lymphocyte that expressed IFN γ was lower in healthy controls compared to active TB at the beginning of the diagnosis.²³ Day *et al.* found no difference in frequency of IFN γ produced by CD8+T lymphocyte towards ESAT-6-CFP-10 fusion antigen stimulation between active and latent TB patients. Further analysis towards these differences showed suboptimum immunity ability of the CD8+T lymphocyte and a decrease of apoptosis in active TB. Further research is required to know the apoptotic function in this active TB.²²

The increase of IFN γ production from CD8+T lymphocyte could be found in all groups of this study although there were no significant differences that described the CD8+T lymphocyte role in producing IFN γ . A previous research by Caruso *et al.* supported this result that in mice where its CD4+T lymphocyte had been taken away still produced enough IFN γ to overcome the TB infection.²⁴

This research has some limitations, ie in the field of the optimization test used to determine the concentration of ESAT-6-CFP-10 fusion antigen. ESAT-6-CFP-10 fusion antigen concentration used in this study was only done in 1.06 μ g/mL, 2.13 μ g/mL, 5.3 μ g/mL, and 10.6 μ g/mL. The optimization test was not done in a concentration range between 2.13 μ g/mL-5.3 μ g/mL, also ranging between 5.3 μ g/mL -10.6 μ g/mL. This research also did not study other IFN γ -producing cell such as NK cells.²⁵

latent TB, and healthy people), there was no difference in the percentage of CD8+T lymphocyte that expressed IFN γ with and without ESAT-6-CFP-10 fusion antigen stimulation. A further study about IFN- γ immune respond from CD8+T lymphocyte towards ESAT-6-CFP-10 fusion antigen stimulation with optimum fusion antigen concentration that can produce the expected immune response are required.

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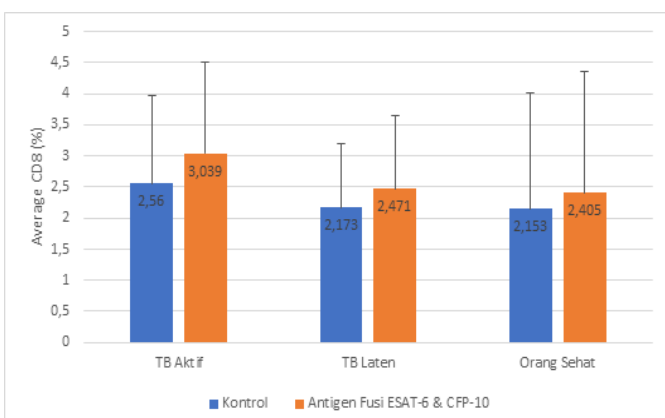


Figure 1. Percentage CD8+T lymphocyte that express IFN- γ with and without ESAT-6-CFP-10 fusion antigen stimulation in active TB, latent TB and healthy people

CONCLUSION AND SUGGESTION

There was no difference in PBMC with ESAT-6-CFP-10 fusion antigen stimulation between active TB, latent TB, and healthy subject groups. In each group (active TB,

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