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RESEARCH

PERCENTAGE OF CD3$^+$ T LYMPHOCYTES EXPRESSING IFN-γ AFTER CFP-10 STIMULATION

(Persentase Limfosit T-CD3$^+$ yang Mengekspresikan Interferon Gamma Setelah Stimulasi Antigen CFP-10)

Yulia Nadar Indrasari[1], Betty Agustina Tambunan[1], Jusak Nugraha[1], Fransiska Sri Oetami[2]

ABSTRAK

Tuberkulosis (TB) merupakan penyakit infeksi menular, disebabkan oleh Mycobacterium tuberculosis. Respons imun adaptif yang diperantarai oleh limfosit T berperan sangat penting dalam menyingkirkan bakteri intraseluler. Hasilan sitokin IFN-γ merupakan mekanisme efektor utama dari limfosit T. Pengembangan vaksin yang efektif dalam melawan infeksi TB mempertimbangkan faktor yang mengatur hasilan IFN-γ. CFP-10 merupakan antigen yang disekresikan oleh Mycobacterium tuberculosis. Antigen ini dikenal sebagai komponen vaksin potensial untuk TB. Tujuan penelitian ini adalah membandingkan respons imun seluler yaitu persentase limfosit T-CD3$^+$ yang mengekspresikan IFN-γ setelah dirangsang antigen CFP-10 di pasien TB paru kasus baru, TB laten dan orang sehat. Penelitian ini menggunakan desain eksperimen murni di laboratorium secara in vitro pada kultur PBMC pasien TB paru kasus baru, TB laten dan orang sehat. Subjek penelitian adalah 8 pasien TB paru kasus baru, 7 TB laten dan 7 orang sehat di RS Khusus Paru Surabaya. Pemeriksaan persentase limfosit T-CD3$^+$ yang mengekspresikan IFN-γ dengan metode Flow cytometry (BD FACSCalibur). Hasil dianalisis dengan Kruskal-Wallis atau ANOVA satu arah. Rerata persentase limfosit T-CD3$^+$ yang mengekspresikan IFN-γ di TB paru kasus baru setelah stimulasi antigen CFP-10 (4,36%) lebih tinggi daripada sebelum stimulasi (3,50%) (nilai P=0,015). Rerata persentase limfosit T-CD3$^+$ yang mengekspresikan IFN-γ di TB laten setelah stimulasi antigen CFP-10 (3,96%) lebih tinggi dibandingkan sebelum stimulasi (2,50%) tetapi tidak bermakna (nilai P=0,367). Rerata persentase limfosit T- CD3$^+$ yang mengekspresikan IFN-γ di orang sehat setelah stimulasi (1,66%) lebih rendah daripada sebelum stimulasi (2,89%) tetapi tidak bermakna (nilai P=0,199). Perubahan persentase limfosit T-CD3$^+$ yang mengekspresikan IFN-γ setelah stimulasi antigen CFP-10 antarkelompok tidak berbeda bermakna (nilai P=0,143). Berdasarkan hasil telitian ini dapat disimpulkan bahwa terdapat peningkatan persentase limfosit T-CD3$^+$ yang mengekspresikan IFN-γ di TB paru kasus baru setelah stimulasi antigen CFP-10. Hal ini menunjukkan limfosit T-CD3$^+$ yang mengekspresikan IFN-γ berperan dalam perlindungan terhadap infeksi TB paru.

Kata kunci: *CD3+, IFN-γ, TB paru kasus baru, flow cytometry, CFP-10*

ABSTRACT

Tuberculosis (TB) is an infectious disease, caused by Mycobacterium tuberculosis. Cytokine IFN-γ production is the main effector mechanism of T lymphocytes. The development of an effective vaccine against TB infections has to consider the factors regulating the production of IFN-γ. CFP-10 is an antigen secreted by Mycobacterium tuberculosis, known as a component of a potential vaccine for TB. The purpose of this study was to compare the cellular immune response that is the percentage of CD3$^+$ T-lymphocytes that expresses IFN-γ after stimulation of CFP-10 antigen in patients with new cases of pulmonary TB, latent TB, and healthy people. This study used a true experimental design in the laboratory in vitro in cultured PBMC of patients with 8 new cases of pulmonary TB, 7 latent TB and 7 healthy people in the Pulmonary Hospital Surabaya. Examination of the percentage of CD3$^+$ T-lymphocytes expressing IFN-γ was done by Flow cytometry (BD FACSCalibur). Results were analyzed by Kruskal-Wallis and one way ANOVA. The mean percentage of CD3$^+$ T-lymphocytes expressing IFN-γ in new cases of pulmonary TB after stimulation of CFP-10 antigen (4.36%) was higher than before stimulation (3.50%) (P value=0.015). The mean percentage of CD3$^+$ T-lymphocytes expressing IFN-γ in latent TB after CFP-10 antigen stimulation (3.96%) was higher than before stimulation (2.50%) but was not significant (P value=0.367). The mean percentage of CD3$^+$ T-lymphocytes expressing IFN-γ in healthy people after stimulation (1.66%) was

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lower than before stimulation (2.89%) but not significant (P value=0.199). Changes in the percentage of $CD3^+$ T-lymphocytes expressing IFN-γ after stimulation of CFP-10 antigen were not significantly different between groups (P value=0.143). Based on this study, it could be concluded that there were an increase in the percentage of $CD3^+$ T-lymphocytes expressing IFN-γ in new cases of pulmonary TB after stimulation of CFP-10 antigen. This showed that $CD3^+$ T-lymphocytes expressing IFN-γ played a role in protection against pulmonary tuberculosis infection.

Key words: CD3+, IFN-γ, new cases of pulmonary TB, flow cytometry, CFP-10

INTRODUCTION

Tuberculosis (TB) is a contagious infectious disease caused by *Mycobacterium tuberculosis* bacteria. Tuberculosis is also considered as one of the most deadly infectious diseases in the world (ranking second after HIV), thus becoming a primary global health problem.[1] The incidence of tuberculosis (TB) cases in 2013 was estimated at 9 million, or about 126 cases per 100,000 population with the proportion of Asia (56%), Africa (29%), Middle East (8%), Europe (4%) and America (3%). Six (6) countries with the largest TB incidence rates in 2013 were India (2 million-2.3 million), China (900,000–1.1 million), Nigeria (340,000–880,000), Pakistan (370,000–650,000), Indonesia (410,000–520,000) and South Africa (410,000–520,000). Indonesia is currently ranked fourth in the world after India, China and South Africa.[1]

Previous researches on immune response to *Mycobacterium tuberculosis* bacteria in TB patients and individuals with a positive tuberculin reaction focused on the role of T lymphocytes as a major component of adaptive immune response. The adaptive immune response is mediated by T and B lymphocytes. The adaptive immune response mediated by T lymphocytes plays a very important role in the elimination of *Mycobacterium tuberculosis*. The development of effective vaccines against TB infection considers factors that regulate *IFN-γ* production.[2]

Early secreted antigenic target of 6-kDa (ESAT-6) and *culture filtrate protein* (CFP-10) have recently been proven as antigens secreted by *Mycobacterium tuberculosis* bacteria, but not derived from *bacillus Calmette-Guerin* (BCG) of *Mycobacterium bovis*, a cellular immune response to those specific antigens has a good association with TB infection.[3] The secreted antigens are produced in the early phase of infection and induce protective immunity, as well as stabilize *bacterial load* in lung rapidly. These antigens (Ag85, ESAT-6, and *culture filtrate protein* [CFP-10]) have been known as components of potential vaccine for TB.[4]

Many people infected with *Mycobacterium tuberculosis* are healthy people with tuberculin reaction and immune protection (latent TB). Patients with active TB have an ineffective immunity and quite severe clinical manifestation. Therefore, an understanding of how TB latent can control TB infection is needed so it does not develop further. This understanding is also expected to provide an important description about the mechanisms of protective immunity against TB infection and it useful for effective TB vaccine development.[2] This research aimed to examine the cellular immune response of *$CD3^+$ T lymphocytes* after *CFP-10* stimulation in new cases of pulmonary TB, latent TB, and healthy people.

METHODS

This research was a pure in vitro experimental laboratory study on cultured *peripheral blood mononuclear cells* (*PBMC*). The research was conducted from May to December 2015. The samples of the research were divided into three sample groups stimulated by *CFP-10* antigens of *Mycobacterium tuberculosis*, namely Group 1 consisted of eight new pulmonary TB cases, Group 2 consisted of seven latent TB cases and Group 3 consisted of seven healthy people.

Blood samples in heparin tubes, were processed, then isolated and PBMC cultured using a CO_2 incubator. Cultured *PBMC* samples were then divided into two different treatment groups namely one group with *CFP-10* stimulation and without *CFP-10* stimulation. Following that, the percentage of *IFN-γ* expressed on *$CD3^+$ T-lymphocytes* in those three sample groups was examined using *flow cytometry FACSCalibur* in the Clinical Pathology Laboratory, Dr. Soetomo Hospital, Surabaya.

Next, the normality test of data distribution was performed using Shapiro Wilk test. Kruskal-Wallis one-way ANOVA test was then carried out to analyze the difference in the percentage of $CD3^+$ *T lymphocytes* expressing *IFN-γ* before and after *CFP-10* stimulation between the cultured PBMC group of new pulmonary TB cases, latent TB cases, and healthy people. The level of significance was then determined using the value of P, less than 0.05.

RESULTS AND DISCUSSION

New cases of pulmonary tuberculosis were mostly found in the age group of 26–34 years (37.5%). Meanwhile, latent TB cases were mostly found in the age group of 26-34 years (57.1%). TB cases in healthy people (100%) were found in the age group of 20-25 years.

The results of Kruskal-Wallis test, showed that there was no significant difference in the percentage of *CD3+ T-lymphocytes* expressing *IFN-γ* before *CFP-10* stimulation between the groups of new pulmonary TB cases, latent TB cases and healthy people (P=0.620). Similarly, the results of ANOVA test showed that there was no significant difference in the percentage of *CD3+ T-lymphocytes* expressing *IFN-γ* after *CFP-10* stimulation between the groups of new pulmonary TB cases, latent TB cases and healthy people (P=0.198).

In addition, the results of paired t test showed that there was a significant increase in the percentage of *CD3+ T-lymphocytes* expressing *IFN-γ* after *CFP-10* stimulation in the group of new pulmonary tuberculosis cases (P=0.015) as seen in Table 2. Similarly, a research conducted by Winkler *et al*[5] also showed that there was an increased frequency of *CD3+ T-lymphocytes* expressing *IFN-γ, IL-2* and *TNF-γ* in patients with active pulmonary tuberculosis compared to controls after *PPD* (*purified protein derivative*) stimulation. Increased cytokine responses generated from *Th1 lymphocytes* to *PPD* stimulation showed a shift of naïve cellular subpopulations towards specific *T lymphocyte* activation for *Mycobacterium* in order to produce *IFN-γ*.[5]

A research conducted by Abramo *et al*[6], showed that patients with active pulmonary TB generated higher levels of *MIG (monokine induced by IFN-γ)* and *IFN-γ* as well as a higher frequency of cells producing *MIG (monokine induced by IFN-γ)* levels, compared to the control group (post-BCG vaccine) after stimulation by fusion protein, ESAT-6/CFP-10, specific to *Mycobacterium tuberculosis*. Similar results were also obtained on *PPD (purified protein derivative)* stimulation.[6]

On the other hand, the analysis results of Wilcoxon test showed that there was no significant increase in the percentage of *CD3+ T-lymphocytes* expressing *IFN-γ* after *CFP-10* stimulation in the group of latent tuberculosis (P=0.367) as seen in Table 2. This condition was thought due to the number of memory *T lymphocyte* clones which was less in recognizing *CFP-10* antigens. So, the proliferation of *T lymphocytes* and the increase in the number of *IFN-γ* were not too high.

Table 1. Characteristics of research subjects

Characteristics of research subjects	New pulmonary TB cases (n=8)	Latent TB (n=7)	Healthy people (n=7)
Sex			
Males	5 (62.5%)	2 (28.6%)	2 (28.6%)
Females	3 (37.5%)	5 (71.4%)	5 (71.4%)
Group of Age			
20-25 years old	1 (12.5%)	2 (28.6%)	7 (100%)
26-34 years old	3 (37.5%)	4 (57.1%)	-
35-44 years old	2 (25%)	1 (14.3%)	-
45-54 years old	2 (25%)	-	-

Table 2. Changes in the percentage of *CD3+ T lymphocytes* expressing *IFN-γ* before and after CFP-10 stimulation in each group

Groups	N	The percentage change of *CD3+ T lymphocytes* expressing *IFN-γ*		P
		Before CFP-10 stimulation	After CFP-10 stimulation	
New pulmonary TB cases	8	3.50±2.33	4.36±2.37	0.015*
Latent TB	7	2.50±3.25	3.96±4.13	0.367
Healthy People	7	2.89±2.79	1.66±1.22	0.199
P		0.620	0.198	

Note: * significant, $\alpha = 0.05$

Table 3. Changes in the percentage of *CD3+ T lymphocytes* expressing *IFN-γ* after CFP-10 stimulation among the groups

Groups	N	The percentage change of CD3+ T lymphocytes expressing IFN-γ after CFP-10 stimulation			P
		Median	Mean	Standard deviation	
New pulmonary TB cases	8	0.52	0.86	0.89	
Latent TB	7	0.26	1.45	5.28	0.143
Healthy people	7	-0.40	-1.23	3.00	

The analysis results of Wilcoxon test on the group of healthy people showed that there was no significant increase in the percentage of *CD3+ T-lymphocytes expressing IFN-γ* after *CFP-10 stimulation* (P=0.199) as shown in Table 2. This was because the *T lymphocyte* memory had not yet been formed in healthy people who had never been exposed to *Mycobacterium tuberculosis*, so *T lymphocyte* proliferation response to *CFP-10* antigen exposure tended to be low. The results of this research were similar to the study conducted by Tsao *et al*[7] stating that there were no increased levels of serum *IFN-γ* in healthy subjects. The research conducted by Tsao *et al*[7] focused on the correlation between *IFN-γ* level and *IL-2 receptor-γ* in *bronchoalveolar lavage* liquid (BAL) and serum with a clinical grade and management of pulmonary tuberculosis. The results of the research stated that patients with a higher-grade of pulmonary TB (with advanced lung lesions, fever, or weight loss) showed an increase of *IFN-γ* and also *soluble interleukin-2 receptor-γ* (SIL-2R-γ) derived from *epithelial lining fluid* level (ELF) samples compared to those of lower-grade pulmonary TB patients. A similar correlation was also found in *soluble interleukin-2 receptor-α* (SIL-2R-α) serum levels in this research, but not in IFN-γ.[7]

In addition, the analysis of Kruskal-Wallis test showed that there was no significant difference in changes in the percentage of *CD3+ T-lymphocytes* expressing *IFN-γ* before and after *CFP-10 stimulation* between groups (P=0.143) as shown in Table 3. The results of this research were different from the results of a study conducted by Borgström *et al*[8] stating that there was a significant increased production of *IFN-γ* in patients with active TB compared to healthy people after stimulation of specific antigens of *Mycobacterium tuberculosis*, *ESAT-6* and *CFP-10* (P<0.05). The significant increased production of *IFN-γ* in patients with active TB after the stimulation was also correlated with the proliferative response of *T lymphocytes*. This was apparently due to the long exposure to *Mycobacterium tuberculosis* in those active pulmonary tuberculosis patients, but who did not yet showed clinical symptoms because the immune response of those patients to *Mycobacterium tuberculosis* was able to neutralize, thus, *T lymphocyte* memory had been formed despite newly clinically diagnosed as new pulmonary TB cases.[8]

The individual variations of the percentage of *CD3+ T-lymphocytes* expressing *IFN-γ* between groups in this research were wide enough. As a result, it may cause changes in the percentage of *CD3+ T-lymphocytes* expressing *IFN-γ* between the groups were not significantly different. An increase in the percentage of *CD3+ T-lymphocytes* expressing *IFN-γ* was only found in the group of new pulmonary TB cases. It indicated that clones of *CMT lymphocytes* (central memory T cells) transforming into *T lymphocyte* memory after *CFP-10* stimulation did not survive longer than *EMT lymphocytes* (effector memory T cells). In the group of latent tuberculosis, intracellular *Mycobacterium tuberculosis* bacteria were not exposed to the immune system (dormant) so that there was no immunological activation.[9]

Early secreted antigenic target of 6-kDa (*ESAT-6*) and *culture filtrate protein 10* (*CFP-10*) of *Mycobacterium tuberculosis* are considered as potent antigens of human *T lymphocytes* and also as potential tuberculosis vaccine candidates. *ESAT-6* and *CFP-10* secretion were required for virulence and pathogenicity of *Mycobacterium tuberculosis* and bacterial growth within the macrophages. A research conducted by Wang *et al*[10] evaluated both *ESAT-6* and *CFP-10* effects on the capacity of human *T lymphocytes* in producing *IFN-γ* and *T lymphocyte* proliferation response to the activation of *T-cell receptor* (TCR) showed that recombinant *ESAT-6* could inhibit the production of *IFN-γ* by *T lymphocytes*, but not to *CFP-10*.[10]

CONCLUSION AND SUGGESTION

Based on the results of this research, it could be concluded that the percentage of *CD3+ T-lymphocytes* expressing *IFN-γ* increased after *CFP-10* stimulation in cultured PBMC group of new pulmonary TB cases. There was no difference in changes in the percentage

of *CD3+ T lymphocytes* expressing *IFN-γ* before and after *CFP-10* stimulation between the cultured PBMC group of new pulmonary TB cases, latent TB cases and healthy people. Thus, it could be said that *CFP-10* effects on the capacity of *T lymphocytes* in producing *IFN-γ* were less. Therefore, further research on the effector *T lymphocyte* response and *cytokine* responses generated after *ESAT-6* stimulation or *ESAT-6/CFP-10* protein fusion is needed to determine the activities of fusion immunogenic antigens, *ESAT-6/CFP-10* as TB vaccine candidates.

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