Comparison of Immunological Surface Marker of CD4⁺ Lymphocytes in Patients with Pulmonary Tuberculosis Pre- and Post-Intensive Phase of Treatment

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ABSTRACT

Immunological surface marker of CD4⁺ lymphocytes such as interleukin-2 (IL-2) is a potential marker for tuberculosis (TB) diagnosis and treatment evaluation. This study aimed to compare the expression of CD4+ lymphocyte IL-2 in patients with pulmonary TB, pre- and post-intensive phase of treatment. TB patients were diagnosed with GenXpert and confirmed with Mycobacterium tuberculosis (Mtb) culture. The expression of CD4⁺ lymphocytes IL-2, measured by flow cytometry, was compared between newly diagnosed TB patients and TB patients who completed intensive phase of TB treatment. A student's t-test was used to compare the expression of CD4⁺ lymphocyte IL-2 between pre- and posttreatment groups. Thirty-six patients were recruited and divided evenly into two groups. The mean age of participants was 38±12.43 years for pre-treatment group and 39.44±11.50 years for post-treatment group, while the mean body mass index was 21.18±1.93 and 21.03±1.65 in pre- and post-treatment group, respectively. The expression of IL-2 on CD4⁺ lymphocytes was significantly lower in pretreatment compare to post-treatment group (68.43±18.74 vs. 80.59±10.76, p=0.040). Further analysis showed that the expression of CD4⁺ lymphocytes IL-2⁺ GM-CSF⁺ was significantly lower (p<0.001) in pre-treatment compared to the posttreatment group (4.47±2.88 vs. 10.19±4.76, respectively). The area under the ROC curve (AUC) of CD4⁺ lymphocyte IL-2 was 0.70 (95%CI: 0.52, 0.87), suggesting its potential as a predictor of sputum conversion. In conclusion, the expression of CD4⁺ lymphocyte IL-2 was significantly higher in post-intensive TB treatment group compared to the newly diagnosed TB patients, suggesting its potential as a biomarker for rapid TB diagnosis and treatment evaluation.

INTRODUCTION

Mycobacterium tuberculosis (Mtb) is a causative agent of TB, one of the most prevalent respiratory infection.¹ Most people infected with Mtb can actually be cured with prompt diagnosis and a 6-months treatment, consists of 2 months intensive phase with isoniazid, rifampicin, pyrazinamide, and ethambutol (HRZE), followed by a 4-month continuation phase with isoniazid and rifampicin (HR) or other combinations.² However, TB remains a major public health problem, and is responsible for an estimated 1.2 million deaths and 10 million new cases annually.¹ The situation is getting worse due to the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of Mtb.³

Mtb infection stimulates host immune response, one of which is by inducing CD4 T cells lymphocytes activation. Increased Mtb specific CD4+ lymphocytes immunological surface markers such as human leukocyte antigen-DR (HLA-DR), granulocyte-macrophage colony-stimulating factor (GM-CSF), CD38, interleukin 2 (IL-2), and interferon gamma (IFNγ) were observed after Mtb infection.⁴⁺⁶ IL-2 is essential for cellular immunity and granuloma formation as it induces T cells lymphocytes proliferation after Mtb infection.⁷ Moreover, IL-2 induces the production of cytotoxic lymphocytes, natural killer cells, and lymphocyte-induced killer cells.⁸ Successful TB treatment, Keywords: Tuberculosis, CD4⁺ lymphocytes IL-2, immunological marker

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marked with smear and culture sputum conversion, was associated with changes in expression of Mtb specific CD4+ T cells and their biomarkers. 9

Diagnosis of pulmonary TB remains complex and requires clinical, radiological, bacteriological, and molecular examination.⁹ Sputum culture is currently the golden standard for pulmonary TB diagnosis and treatment evaluation, but it is not really effective and efficient since it takes a long time (3-6 weeks).¹⁰ A previous study suggested that IFN- γ release assay (IGRA) is a promising alternative for TB diagnosis;¹¹ however, IGRA cannot be used to evaluate the treatment or to distinguish active TB from latent TB.¹² Cellular immunity, particularly of CD4+ T cells are responsible for protection against Mtb infection.¹³ Therefore, the interest now shifts to study the expression of immunological surface of Mtb specific CD4+ T cells such as IL-2 for a rapid diagnostic and treatment evaluation.^{4, 5, 9}

Previous studies showed that the expression of CD4⁺ T cells IL-2 could be used as a marker for TB diagnosis and treatment evaluation. The expression of CD4⁺ T cells IL-2 was significantly higher in people infected with Mtb compared to healthy population.¹⁴⁻¹⁶ The expression of CD4⁺ T cells IL-2 could also be used to distinguish active TB from latent TB as it was significantly higher in the latter group.^{17, 18} Studies also demonstrated that latent TB

infection and smear negative TB patients had higher frequencies of Mtb specific CD4+ T cells co-producing IFN- γ /IL-2/TNF- α compared to smear positive active TB patients.^{19, 20} A significantly higher frequencies of Purified Protein Derivative (PPD)-specific CD4+ T cells IFN-γ+IL-2+TNF- α^+ was observed following TB treatment.²¹ However, a study in 2008 suggested that the expression of IL-2 was a less useful marker for TB diagnosis due to its low amounts of release.¹⁴ Another study also agreed that IL-2 alone cannot be used to monitor TB treatment, although its expression declined after treatment.²² Further study is required due to these contradictory findings. The aim of this study was to compare the expression of CD4+ T cells IL-2 in patients with pulmonary TB, pre- and postintensive phase of treatment, and to investigate its potential as a biomarker for rapid TB diagnosis and treatment evaluation.

MATERIALS AND METHODS

Study design and patients

A cross sectional study was conducted to compare the expression of IL-2 and its combination with other CD4+ lymphocytes immunological markers on newly diagnosed pulmonary TB with positive sputum and TB patients with negative sputum, after two months of intensive therapy. Patients diagnosed with pulmonary TB (confirmed with GenXpert and Mtb culture examination), aged 18-55 years old and had BMI \geq 18.5 kg/m² were recruited at primary health centers and clinics in Banda Aceh, Aceh province, Indonesia. Pulmonary TB patients who had finished the intensive two-months treatment of HRZE and had converted (negative) sputum were recruited and matched with the newly diagnosed pulmonary TB patients. Patients with HIV, diabetes, malignances, were taking steroids or undergoing chemotherapy were not included in the study. The expression of IL-2, defined as percentage of IL-2 on CD4+ T cells, were measured using venous blood flow cytometry with Intra-Cellular Cytokine Staining (ICS) approach, and was conducted at Flow Cytometry Laboratory, Life Sciences Institute, National University of Singapore. Sputum examination was conducted using GenXpert at Dr. Zainoel Abidin Hospital, Aceh, and Mtb culture at Universitas Airlangga - Dr. Soetomo Hospital, Surabaya, Indonesia.

An approval from Institutional Review Board of the School of Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia, and written informed consent from all study participants were obtained prior to the study.

Measurements of IL-2

PPD-specific Mtb was used to stimulate venous blood collected from the patients, while no antigen was given to

the control. Co-stimulatory antibody of CD28 and CD49d were added at the temperature of 37° C. After 2 h of incubation, Brefeldin A was added to inhibits cytokines release from Mtb-specific CD4⁺ T cells. The Streck Cell Preservative (Streck, La Vista, NE, USA) was added to white blood cell integrity and the antigenic sites, at a 1:1 ratio.

Apart from IL-2 marker on CD4+ T cells, other markers such as CD38. HLA-DR. GM-CSF. and IFNv⁺ were also measured. In brief, red blood cells were lysed with NH₄Cl solution and the cells were mixed with surface label antibody mix before antibody labeling. The mix of intracellular antibody (CD154, IFNy, GM-CSF, CD4) was added to the cells after being mixed with permeabilization buffer. The UltraComp eBeads[™] Compensation Beads (Life Technologies Corporation, Carlsbad, CA, USA) was used as single-color controls. Those positive for CD154 and/or cytokine positive in the PPD stimulated samples were identified as Mtb-specific CD4+ T cells antigen. Positive Mtb-specific CD4+ T cells responses were defined by a frequency of CD4+ specific marker of 0.05% or more, and non-stimulated sample was used to quantify the background of non-specific antibody staining. The preand post- treatment phenotype of the Mtb-specific CD4+ T cells was later compared. The procedure conducted using Cytek Aurora spectral analyzer flow cytometer (Cytek Biosciences, Inc., Fremont, CA, USA) while FlowJo software (Tree Star Inc.) was used to analyze the flow cytometry data.

Statistics

A Student's t-test, conducted using SPSS version 20, was used to compare participants' characteristics and the expression of CD4⁺ T cells IL-2 between pre- and posttreatment groups, with significant difference defined when p<0.05. Discrimination power of studied biomarker was evaluated using receiver operating characteristic (ROC) curve analysis, where areas under the curve (AUC) and the corresponding 95% confident interval were calculated.

RESULTS

Study participants' characteristics

At the end of the study, a total of 36 TB patients were enrolled, 18 patients in pre-treatment group and 18 patients in post-treatment group. The mean age was 38±12.43 years for pre-treatment group and 39.44±11.50 years for post-treatment group. Similar mean of BMI was found in both groups, 21.18±1.93 vs. 21.03±1.65 (**Table 1**).

Variables	Pre-intensive phase treatment (Mtb positive)	Post-intensive phase treatment (Mtb negative)	t	p-value
Gender (n)				
Male	11	13		
Female	7	5		
Age; mean (±SD)	38 ± 12.43	39.44 ± 11.50	-0.36	0.359
BMI (kg/m^2); mean (±SD)	21.18 ± 1.93	21.03 ± 1.65	0.21	0.415
Expression of IL-2; mean (±SD)	68.43 ± 18.74	80.59 ± 10.76	97.00	0.040a

Table 1. Characteristics of study participants and expression of IL-2 on CD4+ lymphocytes.

*Significant at 0.05; ^a Analyzed with Mann-Whitney test

Expression of CD4+ T cells IL-2

Individual expression of CD4⁺ T cells IL-2 is shown on **Figure 1**. The expression of CD4⁺ T cells IL-2 was higher in

pulmonary TB patients' post-intensive phase of treatment compared to newly diagnosed group with positive acidfast staining sputum (pre-treatment group).



Figure 1. Individual expression of CD4+ lymphocytes specific IL-2 between pre-intensive phase treatment (Mtb positive) and post-intensive phase treatment (Mtb negative).

Table 1 showed flow cytometry result of both groups, whereas the expression of IL-2 was significantly higher in post-treatment group with converted sputum compare to pre-treatment group with positive sputum (80.59 ± 10.76 vs. 68.43 ± 18.74 ; p=0.040) (**Table 1**).

Expression of CD4+ lymphocytes specific IL-2 and its combination with other markers

This study also compared the expression of CD4⁺ T cells IL-2 and its combination on pulmonary TB patients, pre- and post-intensive phase of treatment, as shown in **Figure 2** and **Table 2**. The expression of CD4⁺ T cells IL-2⁺GM-CSF⁺ in pre- treatment group was significantly lower (p<0.001) compare to the post- treatment patients, 4.47 ± 2.88 and 10.19 ± 4.76 , respectively. No significant differences were observed in expression of other combination of CD4⁺ lymphocytes specific IL-2.



Figure 2. Expression of CD4+ lymphocytes specific IL-2+CD38+ (A), IL-2+ HLA-DR+ (B), IL-2+ GM-CSF+ (C), and IL-2+ IFNγ+ (D) on pulmonary TB patients, pre- and post- intensive phase treatment.

Table 2. Expression of specific CD4+ lymphocyte IL-2 and its combination with other biomarkers in pulmonary TB patients, pre- and post- intensive phase treatment.

CD4+ lymphocyte IL-2 and its	Pre-intensive phase	Post-intensive phase	t	p-value
combination	treatment (Mtb positive)	treatment (Mtb negative)		
IL-2+ CD38+	2.16 ± 2.12	3.04 ± 2.36	1.190	0.242ª
IL-2+ GM-CSF+	4.47 ± 2.88	10.19 ± 4.76	4.366	<0.001 a
IL-2+ HLA-DR+	1.32 ± 0.76	0.94 ± 0.68	-1.543	0.132ª
IL-2+IFN-γ+	5.09 ± 3.38	5.22 ± 3.48	0.107	0.916 ^a

^a Analyzed with independent t-test

Receiver operating characteristic (ROC) curve

The area under the ROC curve (AUC) of CD4+ T cells IL-2 expression was 0.70 (95%CI: 0.52, 0.87, p=0.040). AUC \geq

0.7 suggesting that the biomarker was potential as a predictor of sputum conversion (**Figure 3**).



Figure 3. ROC curve of CD4+ lymphocytes specific IL-2 expression

DISCUSSION

In regards to study characteristics, the proportion of men in this study was higher than women, corresponding to the national data that showed the prevalence of pulmonary TB in Indonesian male was 1.5 times higher compare to female.²³ This also support the data from World Health Organization (WHO), which suggested higher prevalence of TB among men than women in general population.¹ The mean age of studied participants in both groups were less than 50 years, in accordance with Indonesian data that showed about 75% of TB patients aged from 15-50 years old.²³ These findings might be associated with higher risk of Mtb exposure on men in their productive age. It might also reflect public, biological, and cultural factors that influence different chances of Mtb transmission between men and women.

This study found that the expression of CD4+ T cells IL-2 was significantly higher in pulmonary TB patients postintensive phase treatment compare to the pre-treatment group. ROC analysis in this study also suggested that the expression of CD4+ T cells IL-2 could be used to predict sputum conversion in pulmonary TB patients' postintensive phase of treatment. As mentioned in previous studies, an increase of specific CD4+ T cells IL-2 was observed following TB treatment.¹⁹⁻²¹ Decreased proportions of IL-2 producing Mtb-specific CD4+ T cells were found in individuals with positive smear pulmonary TB, compared with negative smear TB and latent TB.²⁰ Increased expression of IL-2 after TB treatment may be the result of the central memory T cells expansion, caused by decreased bacterial load.²² Reductions of mycobacterial load due to treatment alter the function of specific CD4+ T cells and increase production of IL-2. Higher expression of IL-2 in post-treatment TB patients was also in line with hypothesis of intracellular immune response, highlighted the effective role of CD4+ T cells-produced IFNy, TNF α , and IL-2 in Mtb infection control.24 Different result was reported in another study in 2004 that showed a decreased of IL-2 concentration in TB patients 2 months after treatment.8

This study also compared the expression of CD4⁺ T cells IL-2 combined with CD38, HLA-DR, GM-CSF, and IFN γ , and found a significantly higher expression of CD4⁺ T cells IL-2⁺ GM-CSF⁺ in post-treatment group. This finding was different from previous study that showed a higher expression of CD4 T cells IL-2⁺GM-CSF⁺ in active

pulmonary TB patients with positive sputum compared to latent or converted sputum TB patients.¹⁵ Nevertheless, the increased of IL-2+GM-CSF+ expression in posttreatment group was in line with the hypothesis that Mtb infection induces intracellular immune response, thus increases secretion of CD8+ T and CD4+ T cells IFNγ, TNFα, and IL-2.24 The intracellular immune response might explain why the expression of CD4+ T cells IL-2+GM-CSF+ was higher in post-treatment group in this study. Furthermore, the production of GM-CSF⁺ increase after Mtb infection and is accumulated throughout the time as an adaptive immune response of the lungs against Mtb infection, and last up to 24 weeks after infection.25 All these explain why we found that the combined expression of CD4+ T cells IL-2+GM-CSF+ was significantly higher in pulmonary TB patients with converted acid fast staining sputum after 2 months (8 weeks) of intensive phase of treatment.

A relatively small number of participants is one of the limitations of this study, however this number is enough to provide preliminary information about the expression of CD4⁺ T cells IL-2 between pulmonary TB patients, preand post-intensive phase of treatment. Moreover, the nature of cross-sectional design used in this study make it impossible to determine case-effect relationship, thus, further prospective study is needed.

LIMITATIONS

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CONCLUSION

A rapid yet accurate method for diagnosis and treatment evaluation is warranted to eliminate TB. This study showed that the expression of CD4⁺ lymphocyte IL-2 was significantly higher in pulmonary TB patients who had completed the 2-months intensive phase of treatment compared to the newly diagnosed patients, suggesting its potential as a biomarker to diagnose TB as well as to evaluate the success of treatment. Further studies are required to evaluate the possibility of using CD4⁺ lymphocyte IL-2 expression with and without other immunological surface markers in TB diagnosis and treatment evaluation.

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