Global Medical and Health Communication

Online submission: https://ejournal.unisba.ac.id/index.php/gmhc
DOI: https://doi.org/10.29313/gmhc.v10i2.9797

GMHC. 2022;10(2):97–103
pISSN 2301-9123 │ eISSN 2460-5441

RESEARCH ARTICLE

Effect of ESAT-6 on Phagocytosis Activity, ROS, NO, IFN-γ, and IL-10 in Peripheral Blood Mononuclear Cells of Pulmonary Tuberculosis Patients

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Abstract

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTB) that lives intracellularly. MTB can inhibit lysosomal enzymes and phagolysosomal fusion, which is challenging to eliminate. These are due to ESAT-6/CFP-10 originating from the RD1 region genome that expresses the Esx-1 type VII secretion system. Esx-1 encodes Esx-A (ESAT-6) and Esx-B (CFP-10), potential vaccine candidates still under research and development. ESAT-6/CFP-10 is predicted to affect macrophage phagocytic activity, IFN-γ, ROS/NO, and IL-10 levels. Several studies have begun to focus on the ESAT-6 antigen due to the high levels of ESAT-6 antibody found in pleural effusion and granuloma fluid. They can last up to 1 year compared to CFP-10 in experimental animals. This study aimed to analyze the effect of ESAT-6 on the phagocytic activity of macrophages, ROS/NO, IFN-γ, and IL-10 in peripheral blood mononuclear cells (PBMCs) cultures of pulmonary TB patients. It is experimental laboratory research with a post-test-only control group design with PBMCs from October 2019 to December 2020 in the Aretha Laboratory Bandung. There were two groups: the negative group (without ESAT-6) and the positive group (with ESAT-6). Six subjects were sampled at the Pindad Hospital in Bandung, and the research was carried out at the Aretha Laboratory in Bandung. Statistical analysis using paired t test. There was a significant difference between the negative and positive groups ($p<0.05$). ESAT-6 can decrease macrophage phagocytic activity, intracellular ROS/NO, and IFN-γ but increase IL-10 levels.

Keywords: ESAT-6, IFN-γ, IL-10, macrophages, *Mycobacterium tuberculosis*, NO, ROS

Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTB), which usually affects the lungs.[1–4] *Mycobacterium tuberculosis* is an intracellular bacteria that lives and reproduces in macrophages cells and can withstand lysosomal enzymes and inhibit phagolysosomal fusion—making it difficult to eliminate due to the presence of early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10). In a dormant state, they can hide in cells for long periods without being tracked by the immune system, making the global eradication of MTB very difficult.[5] Prevention efforts in the form of BCG immunization are the most effective intervention in controlling TB disease. However, it has not been able to provide an optimal protective effect. The weakness of BCG is the loss of the genome region RD1 which expresses the Esx-1 type VII secretion system, causing the protective effect is not maximal. Esx-1 encodes Esx-A (ESAT-6) and Esx-B (CFP-10), which are potential vaccine candidates that are still being researched and developed, and are used as markers of diagnostic of TB disease. ESAT-6 and CFP-10 are also virulence and pathogenicity factors for MTB which are predicted to cause cytolysis of alveolar epithelial cells and macrophages. The virulence mechanism and pathogenicity between ESAT-6 and CFP-10 are still being investigated but are predicted to drive the cytolysis of alveolar epithelial cells and macrophages.[6] Several studies have begun to focus on the ESAT-6 antigen due to the high levels of ESAT-6 antibodies found in pleural effusions[7] and granulomas.[8] ESAT antibody levels can last up to 1 year compared to CFP-10 in experimental animals infected with MTB.[9] Several studies using the ESAT-6 antigen in vitro on peripheral

Received: 24 May 2022; Revised: 17 August 2022; Accepted: 17 August 2022; Published: 20 August 2022

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blood mononuclear cells (PBMCs) patients with TB showed an increase in IL-10 levels, while IFN-g levels were still disputable.[10,11] ESAT-6 is predicted to cause phagosome rupture so that MTB avoids elimination and translocates to the cytosol of macrophages.[12] ESAT-6 is predicted to be an MTB virulence factor that can inhibit macrophages in phagocytosis activity and inhibits the production of ROS/NO in eliminating MTB.[13] ESAT-6 is an antigen that is predicted to induce IFN-γ but can inhibit the production of IFN-γ.[11] ESAT-6 can increase the production of IL-10.[11]

This study aims to determine whether ESAT-6 can affect phagocytosis activity of macrophages, intracellular ROS/NO levels, IFN-γ levels, and IL-10 levels. The object used is peripheral mononuclear blood cells (PMBCs) cultures of adult active pulmonary TB patients in vitro.

Methods

This research is an experimental laboratory study with a post-test-only control group design with peripheral blood mononuclear cells (PBMCs) cultures in adult active pulmonary TB patients in vitro from October 2019 to December 2020 in the Aretha Laboratory Bandung. Subjects used in this study were adult active pulmonary TB patients from a national hospital in Bandung, Indonesia, who met the inclusion criteria. The inclusion criteria for this study were patients with active pulmonary TB aged >18 years, diagnosed for the first time, had not been able to receive anti-TB oral therapy, and had never been used as study subjects. Exclusion criteria were TB patients with severe malnutrition, liver disease, kidney disorders, heart disease, diabetes, malignancy, and HIV. The diagnosis of TB is made by a specialist in internal medicine based on clinical symptoms, chest x-ray, sputum, and rapid molecular tests. After establishing the diagnosis, we asked the patient to participate in the study. The subject data was recorded in the checklist, and a blood sample of ±10 mL from the vein was taken. A total of ±60 mL of blood from 6 patients from Pindad Hospital Bandung were brought to the Aretha Laboratory Bandung for research. The samples were divided into two groups: a negative control (NC) group (without induction) and positive control (PC) group (induced by ESAT-6). The PC group was induced by ESAT-6 first, then macrophages phagocytosis activity, intracellular ROS/NO, IFN-γ, and IL-10 levels were measured. The blood samples suspended into PBMCs were cultured in RPMI 1640 media for five days, followed by phorbol 12-myristate 13-acetate (PMA) brand Biovision 1544-5 so that monocytes proliferate into macrophages. Into the 5-day-old PBMCs culture, added 5 μg/mL ESAT-6 brand Abbexa/abx 169018 (except NC), incubated for 24 hours, and laboratory tests were carried out. ESAT-6 dose of 5 μg/mL was determined based on reference and preliminary studies, which provide the most optimal proliferation of PBMCs cultures. In the macrophages phagocytosis activity test, latex beads suspension was added. They were incubated for 1–2 hours, then counted the number of macrophages cells that phagocytized latex particles. Intracellular ROS levels were measured using a DCFDA-cellular ROS detection assay kit (ab113851) reagent analyzed by flow cytometer. While the intracellular NO levels were analyzed using the Greiss method. IFN-γ levels were analyzed using the human IFN-γ ELISA kit reagent (Elabscience, E-EL-H0108). IL-10 levels were analyzed using the human IL-10 ELISA kit (430601). The univariate analysis consisted of a frequency distribution (mean, standard deviation) and the Shapiro-Wilk test (normally distributed sample data). The bivariate analysis conducted was paired t test to determine whether there were differences between groups. All statistical calculations were carried out using IBM SPSS Statistic software version 25. This study was approved by the Health Research Ethics Committee of the University of Padjadjaran with letter 1499/UN6.KEP/EC/2019.

Results

The research subjects came from six TB patients aged >18 years at Pindad Hospital Bandung, consisting of 3 male patients and 3 female patients based on inclusion criteria. The results of the comparison of NC and PC are shown in Table. There was a significant difference between NC and PC with $p<0.05$. There was a decrease in the phagocytosis activity of macrophages, ROS, NO, and IFN-γ in PC compared to NC, as well as an increase in IL-10 levels in PC compared to NC.

Discussion

This study requires a large number of mononuclear cell samples from TB patients, considering that each well requires 5×10^5 cells, and analysis carried out as many as five tests. PBMC culture must produce mononuclear cells

Table Differences in the Mean Phagocytosis Activity of Macrophages, Intracellular ROS/ NO, IFN-γ, and IL-10 in the NC and the PC Groups

PBMC Culture Sample	Phagocytosis Activity of Macrophage (Mean[a]±SD)	Intracelluler ROS (Mean[a]±SD)	Intracelluler NO (Mean[b]±SD)	IFN-γ (Mean[c]±SD)	IL-10 (Mean[d]±SD)	T Test
NC	20.63±0.26	3.34±0.29	17.13±0.96	16.65±0.13	9.06±1.50	$p<0.05^*$
PC	10.30±1.24	2.16±0.79	12,16±0.59	11.28±0.11	45.28±1.17	

Note: [a]mean percentage; [b]mean concentration of NO (µmol/mL); [c]mean concentration of IFN-γ (pg/mL); [d]mean concentration of IL-10 (pg/mL); NC: negative control group without ESAT-6; PC: positive control group with ESAT-6; *paired t test, it means if $p<0.05$

according to research needs, then approximately six patients' blood samples are needed. TB, according to the inclusion criteria, is an in-vitro model. ESAT-6 was used as a model for the virulence and pathogenesis of MTB bacterial infection, based on previous studies. The dose of ESAT-6 was determined based on references and analysis results in the laboratory, where the optimal amount gave the highest proliferation index of PBMC cells.

Monocytes derived from PBMC cultured cells of TB patients were induced with PMA, so they differentiated into macrophages. The phagocytosis ability of macrophages was assessed using a fluorescence latex bead as a microorganism model to be phagocytosed by macrophages. The analysis of the phagocytosis activity of macrophages showed that the highest average percentage was in the NC group compared to the PC group. The most increased phagocytosis activity of macrophages in the NC group was due to the optimal phagocytosis ability of macrophages (innate immunity) because it came from peripheral blood, which was not directly infected by MTB. The phagocytosis activity of macrophages includes nonspecific cellular immunity. The phagocytosis activity of macrophages is stimulated by various stimuli such as microbes and their products, antigen-antibody complexes, inflammation, sensitized T lymphocytes, cytokines, and trauma. The most potent activating cytokine is IFN-γ. The activated macrophages have an increased number of lysosomes microscopically and produce and release chemical mediators (IL-1, IL-12) that are useful in activating lymphocytes and releasing other cytokines. The fluorescence latex bead binds to receptors on the surface of macrophages cells, stimulates, engulfs, and destroys them through respiratory burst killing. Antigen-presenting cells (APC), together with MHC class I/II, stimulate macrophages to produce cytokines and chemokines to attract other cells to the site of infection.[14]

In the PC group in Table, there was a decrease in the average percentage of macrophages phagocytosis activity after ESAT-6 was induced. ESAT-6 is vital in phagosome rupture and MTB cytosolic translocation, which causes impaired phagocytosis activity. ESAT-6 secrets by MTB through Esx-1/Esx-A (type VII secretory system), which can inhibit phagosome maturation. They damage the phagosome membrane, so these bacteria escape the elimination process by phagolysosomes and can cause macrophages' death.[12] In a study by Houben et al.[15] using cryo-electron microscopy, MTB, and *Mycobacterium leprae* could translocate from phagolysosomes into the myeloid cell's cytosol, as well as ESX-1-mediated entry of mycobacterial cytosol. It depends on the secretion of ESAT-6 and CFP-10 as mycobacterial virulence. Simeone et al.[16] showed that *Mycobacterium marinum* and wild-type MTB strains could induce phagosome rupture and translocate to the cytosol. Whereas *Mycobacterium marinum* and MTB secreted low levels of ESAT-6 and BCG could not induce phagosome rupture and translocated to the cytosol. ESAT-6 is predicted to inhibit the acidification and maturation of phagolysosomes by lowering the pH and inhibiting the accumulation of ATP and GTP vacuolar enzymes in macrophages.[13] Phagocytosis activity of macrophages (chemotaxis, adhesion, ingestion, degranulation), formation of lysosomal membranes, phagocytosis vacuoles, phagolysosomal fusion, phagolysosomal maturation, phagolysosomal acidification, and a mechanism of suppression burst killing (producing ROS and NO) requires the activation

of cytokines, especially IFN-γ which is produced mainly by CD4+ (Th1) T cells. Decreased phagocytosis activity of macrophages could also be caused by reduced levels of IFNγ and increased levels of IL-10 after ESAT-6 induction.[11]

Levels of PMA induction on PBMC cells, besides stimulating monocyte differentiation into macrophages, also stimulated macrophages to produce ROS and RNI in the NC group. Due to innate immunity, intracellular ROS levels in NC were higher than in PC. NC macrophages cells are derived from the peripheral blood of TB patients and do not experience direct infection compared to pulmonary macrophages cells. NADPH-oxidase produces ROS in macrophages activated by cells undergoing inflammation, injury, and infection by bacteria or viruses, as well as IFN-γ and TLRs (in this study, induced by PMA as a control). ROS will reduce oxygen molecules to H_2O_2 (respiratory burst killing).[17]

In the PC group in Table, which ESAT-6 had induced, the intracellular ROS levels decreased. Therefore, ESAT-6 is predicted to inhibit intracellular ROS levels. Mahesh et al.[18] found that cofilin is an actin-depolymerizing protein or actin filament, which plays an essential role in phagocytosis, phagosome acidification, and phagolysosomal fusion in macrophages cells, decreased levels due to ESAT-6 produced by MTB. Decreased cofilin levels due to ESAT-6 can reduce intracellular ROS levels in macrophages. The study of Seghatoleslam et al.[19] in Th1 cells and human macrophages induced by ESAT-6 and the combination of ESAT-6/CFP-10 showed a decrease in intracellular NO and ROS levels. ROS produced by NADPH phagocyte oxidase also induces the iNOS pathway (producing NO).[17] The reduction in ROS levels will affect a decrease in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription, which plays a role in the expression of iNOS levels, causing a decrease in intracellular NO levels. ESAT-6 is predicted to induce macrophages cell apoptosis which is seen in decreased phagocytosis activity and decreased levels of intracellular ROS in the PC group.[19] The interruption in the acidification process of phagolysosomes and lysis of lysosomal membranes, decreased levels of IFN-γ, and increased levels of IL-10 after ESAT-6 induction, caused a decrease in ROS levels.

Levels the results of the analysis of NO levels showed in Table, in which the highest mean concentration was found in the NC group. The mean NO concentration in NC was higher than in PC and the intervention group, due to innate immunity, according to the macrophage's phagocytosis activity test and previous intracellular ROS levels. NC macrophages cells are derived from the peripheral blood of TB patients and do not experience direct infection compared to pulmonary macrophages cells. iNOS produce NO in macrophages that are activated by increased levels of ROS. ROS activates NF-κB. This protein complex regulates DNA gene transcription and plays an essential role in the immune response against bacteria by inducing proinflammatory cytokines such as TNF-α, IL-12, IFN-γ, and iNOS.[19] The iNOS enzyme will convert NO into RNI with the help of the NADPH-oxidase enzyme in the form of peroxynitrite ($ONOO^-$), which can damage lipids, proteins, and bacterial DNA.[14]

In the PC group in Table, which ESAT-6 had induced, the mean of NO concentration decreased, so ESAT-6 was predicted to inhibit NO levels. The study by Xie et al.[20] on rat alveolar macrophages cells induced by ESAT-6/CFP-10 fusion showed a decrease in intracellular NO levels, which was predicted to be due to inhibition of macrophages cells proliferation, inhibition of NO production, and inhibition of apoptosis. The study conducted by Seghatoleslam et al.[19] in Th1 cells and human macrophages induced by ESAT-6 or the combination of ESAT-6/CFP-10 showed a decrease in intracellular NO and ROS levels. ROS produced by NADPH-oxidase can also be caused by the iNOS pathway (producing NO). Decreased levels of ROS will affect the decrease in transcription of NF-κB, which plays a role in the expression of iNOS levels, causing a reduction of intracellular NO levels. NO production requires the iNOS pathway, which ROS activates. In comparison, ROS is produced by NADPH oxidase, which involves the excretion of IFN-γ as an activator produced by Th1 cells. Therefore, the decrease in intracellular NO levels was caused by decreased levels of ROS, decreased levels of IFN-γ, and increased levels of IL-10 after ESAT-6 induction.

The results of the analysis of IFN-γ levels showed in Table that the highest mean concentration was in the NC group compared to the PC group. IFN-γ levels in NC are higher than in PC due to the natural immune response (innate immunity). NC macrophages cells are derived from the peripheral blood of TB patients and do not experience direct infection compared

to pulmonary macrophages cells. Macrophage cells in the NC group were derived from PBMC cells from TB patients, which differentiated into macrophages cells with the help of PMA as a control. PMA is often used as a promoter in research to activate protein kinase and NF-κB enzymes for cell proliferation. Macrophages and T cells were stimulated to produce IFN-γ as a control.

In the PC group in Table, ESAT-6 induced a decrease in IFN-γ levels. ESAT-6 is predicted to inhibit IFN-γ levels. ESAT-6 can induce IFN-γ but can hinder IFN-γ production. Wang et al.[21] found that ESAT-6 can decrease transcription of IFN-γ and reduce the activating transcription factor-2 (ATF-2) and c-Jun expression, which usually binds to the proximal IFN-γ promoter that stimulates mRNA expression in human PBMC cells. ESAT-6 can inhibit the production of IL-17 and TNF-α and increase the production of IL-2. ESAT-6 can directly inhibit T cell response to Ag MTB by interfering with the T cell receptor (TCR) signaling pathway in ZAP-70. Higher concentrations of ESAT-6 can directly inhibit the production of IFN-γ as a T cell response to MTB infection or can inhibit TCR activation by reducing T cell activation without affecting TCR signaling.[11] Kumar et al.[22] demonstrated that induction of ESAT-6 in human mononuclear cells could reduce IFN-γ levels by downregulating MHC class II and binding to TLR2. While Abebe et al.[11] reported decreased levels of IFN-γ, TNF-α, and IL-10 during TB infection or close contact. They also said increased IFN-γ, TNF-α, and IL-10 in clinical TB during therapy 6–9 months after PBMC cells induced by ESAT-6/CFP-10 fusion. Wibowo et al.,[23] Bastian et al.,[24] and Marpaung et al.[25] reported no difference in IFN-γ levels after stimulation with ESAT-6/CFP-10 fusion in patients with active pulmonary TB and latent TB. It differs from Setiawan and Nugraha,[26] Safutri et al.,[27] and Prihantika et al.,[10] where the levels of IFN-γ in PBMC cells with active TB were higher than in latent TB PBMC cells after being stimulated by ESAT6/CFP-10 fusion. The differentiation in results may be due to the different types and doses of antigens, the status of TB patients who are primarily malnourished, and the possibility of recurrent pulmonary TB disease. It was predicted because the T-cell immune response to ESAT-6 was more persistent in the long term than acute CFP-10. ESAT-6 is thought to have virulence and pathogenic effects that last longer than CFP-10.[9] Decreased levels of IFN- can be caused by an increase in IL-10, which also acts as an anti-inflammatory.[11] The reduction levels of IFN-γ can lead to decreased macrophages phagocytosis activity, inhibition of phagolysosomal acidification, inhibition of phagolysosomal maturation, reduced ability of suppression burst killing (reduced production of ROS and NO), inhibition of apoptosis and autophagy, inhibition of leukocyte aggregation to the site of infection, causing MTB to translocate to the cytosol, multiply in macrophages cells (dormant), and can spread if macrophages cells die so that MTB infection becomes difficult to eradicate.

The results of the analysis of IL-10 levels showed in Table, that the highest mean concentration was in the PC control group compared to the NC. In the PC group that ESAT-6 had induced, there was an increase in the average concentration of IL-10 levels. Various studies have been carried out which prove that IL-10 levels have increased in patients with active pulmonary TB. Research by Abebe et al.,[11] Setiawan and Nugraha,[26] Fatima et al.,[28] and Prihantika et al.,[10] found an increase in IL-10 levels in PBMC cells of patients with active pulmonary TB after ESAT-6/CFP-10 induction. The growth in IL-10 after being induced by ESAT-6 is predicted to be due to a shift from Th1 to Th2 due to decreased CD4+ T cell proliferation,[10,26] and regulatory T cells (Tregs) which act as suppressor T cells are involved in increasing IL-10 production.[10,11] IL-10 is produced mainly by Th2 cells, regulatory T cells (suppressor T cells/Tregs), macrophages, Th17 cells, B cells, neutrophils, and some dendritic subsets. The shift in the balance of Th1 cells to Th2 cells is predicted to be caused by increased levels of IL-10, mainly produced by Th2 cells. Macrophage cells also produce IL-10 through programmed cell death protein 1 (PD1) to reduce apoptosis and stimulate Tregs to secrete IL-10 due to decreased production of CD4+ cells.[29] Safutri et al.[27] showed an increase in IL-4 In PBMC cells of active TB patients after being induced by ESAT-6/CFP-10. IL-4 is a cytokine mainly produced by Th2 cells, which is also predicted to shift from Th1 cells to Th2 cells. Regulatory T cells (suppressor T cells/Tregs) are a type of T cell that can suppress the immune system, significantly inhibiting the proliferation of CD4+ and CD8+ (Th1) by producing anti-inflammatory cytokines such as IL-10. Stringari et al.[30] study in PBMC cells with active TB patients showed an increase in the population of Tregs cells compared to PBMC cells in healthy people.

The increase in IL-10 levels was thought to be due to a decrease in IFN-γ levels after ESAT-6 induction. IL-10 is an anti-inflammatory cytokine or human cytokine synthesis inhibitory factor (CSIF), mainly produced by Th2 cells, Tregs, macrophages, B cells, neutrophils, and some subsets of dendritic cells. IL-10 can inhibit MHC class II, inhibit the proliferation of CD4+ T cells that differentiate into Th1 cells, and inhibit IL-12 excretion so that IFN-γ production decreases.[14] IL-10 can inhibit macrophages' phagocytosis activity and phagolysosomal maturation and reduce ROS/NO, facilitating MTB's survival, virulence, and pathogenicity.

The limitation of this study is conducting in-vitro research, which uses PBMC cells of patients with active pulmonary TB outside the patient's body, which may have a different effect from the actual situation. In addition, in this study, we did not use MTB bacteria directly but only used ESAT-6 as a model for the virulence and pathogenicity of MTB in PBMC cells of active pulmonary TB patients, so it might give different results if the study was carried out directly either in-vivo or directly on patients with active pulmonary TB.

Conclusions

Virulence and pathogenicity of the ESAT-6 antigen can cause a decrease in macrophages phagocytosis activity, a decrease in intracellular macrophages ROS/NO, a reduction in IFN-γ, and an increase in IL-10 levels in PBMC cells with active pulmonary TB in vitro. There were significant differences in macrophages phagocytosis activity, intracellular RO/NO levels, ROS levels, and IL-10 levels after ESAT-6 induction in PBMC cultures of adult active pulmonary TB patients. Therefore, ESAT-6 is the primary vaccine candidate that should be added to the BCG vaccine. However, further research is needed on the effects of ESAT-6 in vivo.

Conflict of Interest

There was not a conflict of interest in this article.

Acknowledgment

Research funding is from the doctoral grant from the Ministry of Research, Technology and Higher Education of the Republic of Indonesia, contract number: PRJ-1039/LPDP.4/2019.

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