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Research Report

CELLULAR IMMUNITY ACTIVATION METHOD BY STIMULATING RD1 COMPLEX PROTEINS AS VIRULENCE MARKER ON *Mycobacterium tuberculum* TO ESTABLISH DIAGNOSIS ON TUBERCULOSIS AND LATENT TUBERCULOSIS INFECTION

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ABSTRACT

This study was intended to invent a simpler and more affordable method to establish diagnosis on Tuberculosis (TB) and Latent Tuberculosis infection (LTBI). Similar to "Quantiferon TB Gold In Tube" (QFT-GIT) and T.SPOT.TB methods, the researchers also utilized "early secreted antigenic target 6kDa" (ESAT-6) and "cultur filtrate protein 10kDa" (CFP-10) proteins to be induced on the specimen. ESAT-6 and CFP-10 are commercial products used to induce interferon gamma (INF-γ) which were to be read using sophisticated and expensive equipment. This study was intended to conduct an analysis on effective cocktail protein modification, i.e. ESAT-6, CFP-10 and Ag85A/B/C, with high validity to detect cellular immunity activity through in vitro examination on peripheral blood monocyte cells of Tuberculosis-suspected patients or patients with latent tuberculosis infection. Peripheral Blood Monocyte Cells (PBMCs) activity on children tuberculosis patient or Latent Tuberculosis Infection (LTBI), adult tuberculosis patient or LTBI, which induced by cocktail protein modification and not induced, were analyzed microscopically. The activity of PBMCs on children and adult tuberculosis patient or LTBI induced by RD1 secretory proteins: ESAT-6, CFP-10, Ag85A/B/C was higher compared to PBMCs which had not been induced by the secretory proteins. Cellular debris and monocyte cells with abnormal shapes were found on PBMCs which had been induced by RD1 secretory proteins at 8th day after culture.

Key words : *cellular immunity activation, Region of Difference 1 (RD1) complex proteins stimulation, virulence markers, Mycobacterium tuberculosis, diagnosis on Latent Tuberculosis Infection (LTBI).*

ABSTRAK

Penelitian ini bertujuan menemukan inovasi metode yang lebih sederhana dan terjangkau dalam hal biaya. Hampir serupa dengan QFT-GIT dan T-SPOT.TB assay, penelitian ini juga akan menggunakan protein "early secreted antigenic target 6kDa" (ESAT-6) dan "cultur filtrate protein 10kDa" (CFP-10) untuk induksi pada spesimen. Kedua produk komersial tersebut, ESAT-6 dan CFP-10, telah digunakan secara komersial untuk menginduksi interferon gamma (INF-γ) yang kemudian akan dibaca dengan alat yang canggih dan berbiaya tinggi. Studi ini bertujuan melakukan analisis modifikasi cocktail protein yang efektif, yaitu ESAT-6, CFP-10 and Ag85A/B/C, dengan validitas tinggi untuk deteksi aktivitas imunitas seluler pada uji in vitro kultur sel monosit darah tepi dari pasien dengan suspek Tuberkulosis atau infeksi Tuberkulosis laten. Aktivitas sel monosit Peripheral Blood Monocyte Cells (PBMCs) pada pasien tuberculosis aktif/tuberculosis laten pada anak/tuberculosis dewasa/LTBI yang diberi induksi dengan modifikasi cocktail protein dan yang tidak diberi induksi, dianalisis secara mikroskopis . Aktivitas sel-sel monosit PBMCs dari pasien tuberculosis aktif/tuberculosis laten pada anak/tuberculosis dewasa/LTBI yang diberi induksi campuran protein sekretorik RD-1 : ESAT-6, CFP-10, Ag85A/B/C lebih tinggi dibandingkan dengan sel-sel monosit PBMCs dari pasien tuberculosis aktif/tuberculosis laten pada anak/tuberculosis dewasa/LTBI yang tidak diberi induksi campuran protein sekretorik RD-1 : ESAT-6, CFP-10, Ag85A/B/C. Terdapat beberapa debris sel dan bentukan

abnormal dari sel monosit pada kultur hari kedelapan dari sel-sel monosit PBMC pasien tuberculosis aktif/tuberculosis laten pada anak/tuberculosis dewasa/LTBI yang diberi induksi campuran protein sekretorik RD-1 : ESAT-6, CFP-10, Ag85A/B/C.

Kata kunci: *aktivasi imunitas seluler, stimulasi protein kompleks RD-1, marka virulensi, Mycobacterium tuberculosis, diagnosis infeksi tuberculosis laten.*

INTRODUCTION

Tuberculosis (TB), an infectious contagious disease caused by *Mycobacterium tuberculosis* still becomes one of global health issues especially in developing countries. World Health Organization estimated that almost a third of world population had ever infected by TB with 8.7 million new cases found and annual mortality rate as much as 1.4 million.[1,2] According to Indonesian Ministry of Health, Indonesia positioned the fourth rank among countries with highest TB cases and pulmonal TB had infected many individuals at productive age and become the second highest cause of death in Indonesia.[3]

The burden of TB in Indonesia is increasing as the increase of new resistant TB cases found in Indonesia. The new TB cases are resistant to standard medication regiment, such as *Multi Drug Resistant TB/MDR-TB,* followed by *Emerging Extended Drug Resistant TB/XDR-TB,* and *Extreme Extended Drug Resistant TB/XXDR-TB*. The load of TB in Indonesia becomes greater as the emerge of co-morbid TB cases with Human Immunodeficiency Virus (*TB-HIV*).[1,3] Immunocompromised condition caused by HIV might improve the risk of TB infection, reactivation of dormant *Mycobacterium tuberculosis* on Latent Tuberculosis Infection (LTBI) patients, and mortality rate caused by TB.[1,4,5] WHO estimated about 10% of LTBI could develop to TB.[2,6] Therefore, tuberculosis was the main opportunistic infection in TB endemic areas, including Indonesia.[2,4]

Lower new TB cases found compared to expectancy rate of TB cases indicated that there were many TB cases which is happening on communities remained unidentified and has not been covered by TB governance.[7] One of the causes of this problem was inaccurate and improper implementation of TB diagnosis establishment in Indonesia. In order to establish diagnosis on TB, the patient should undergo laboratory examination in health care institutions. However, many people living in remote areas in Indonesia found difficulties in accessing health facilities due to distance and length of time to be taken to reach health care centers. This problem became harder because of bad roads, limited means of transportation, limited electricity availability and coverage, limited health care facility, and low quality and quantity of human resources owned by health care facilities in Indonesia.[3]

The first important step to be taken in conducting an effective and efficient TB prevention effort is improving case finding by applying proper method and establishing accurate diagnosis on TB. A quick and accurate TB diagnosis is the foundation in determining adequate medication.[2] Quick and accurate detection and identification on TB infection enables quick and adequate medication given to the patient in order to prevent pulmonal tissue damage and transmission of the disease. However, confirming diagnosis on tuberculosis was not easy, especially on primary tuberculosis cases, extra-pulmonal tuberculosis cases (i.e. *pleural tuberculosis, cerebrospinal fluid (CSF), pericardial tuberculosis,* and *ascetic tuberculosis*), TB on children, and TB-HIV co-infection cases. Confirming TB diagnosis becomes more difficult as the improvement of *Non-tuberculous Mycobacteria (NTMs)* infection prevalence.[8]

Common methods to diagnose TB such as through microscopic examinations on dyed *Acid Fast Bacilli (AFB)* and specimen culture require sophisticated health facility and skilled health facility operator. These methods also possess some disadvantages. Microscopic examination on dyed AFB has limited sensitivity and specificity while specimen culture takes too long to produce its results. The results of specimen culture can only be achieved 2-8 weeks later.[8] Tuberculin Skin Tests (TSTs) have been used worldeide for more than a century as an aid in diagnosing both LTBI and active tuberculosis but a valid TSTs requires proper administration by the Mantoux method with intradermal injection of 0,1ml of tuberculin-purified protein derivative (PPD) into the volar surface of the forearm. In addition, patients must return to a health-care provider for test reading, and inaccuracies or bias can exist in reading the test.[9] The most recent method, *Interferon Gamma Release Assays (IGRA)* is quite promising.[7] This method offers accurate sensitivity and specificity in shorter time period.[10,11] However, this method is still quite expensive and requiring sophisticated instruments making this method quite hard to be applied in health care centers in Indonesia. A quick, accurate, and affordable method to diagnose active tuberculosis infection and Latent Tuberculosis Infection (LTBI) becomes an urgent need.[12]

This study was conducted to invent a simpler and more affordable method in diagnosing tuberculosis infection and Latent Tuberculosis Infection (LTBI). Similar to QFT-GIT and T-SPOT.TB assay, in this study, the researchers use ESAT-6 and CFP-10 proteins to be induced into patient *Peripheral Blood Monocyte Cells (PBMCs)* specimen.[13,14] ESAT-6 and CFP-10 are commercial proteins which are used to induce interferon gamma (INF-γ) and the result will be examined using sophisticated and expensive equipment.[15,16] Different from previous studies, in this study the researchers do not only use ESAT-6 and CFP-10 proteins but also use

other proteins from *Region of Difference 1 (RD1)* protein family, such as Ag85A/B/C to be induced into suspected TB patient and LTBI patient's PBMCs suspension through in vitro manner.[17,18] Specific proteins from RD1 family can be found on all virulent strains and all clinical isolation of *Mycobacterium tuberculosis* and *Mycobacterium bovis*.[19,20] RD1 protein family perform enzymatic function, namely to metabolize lipid on *Mycobacterium tuberculosis* cell wall.[21] These proteins were associated to virulence and immunogenicity of *Mycobacterium tuberculosis*.[22,23] After being induced, the monocyte cells undergo microscopic examination under light microscope to examine the activation of monocyte cells.

MATERIAL AND METHOD

This study was categorized as laboratory experimental research. This study was conducted by comparing treatment groups consisting of Peripheral Blood Monocyte Cell (PBMCs) culture sample collected from TB-suspected patients and LTBI patients which had been induced by RD1 secretory protein compounds and control group consisting of monocyte cell which had not received treatment (i.e. RD1 secretory protein induction). PBMCs suspension samples were collected from children TB patients/LTBI,[24,25,26] and adult TB patients/LTBI based on random consecutive method.[27,28] The samples were collected by collecting 5-10 mL median cubital vein blood based on vena puncture method using syringe. The collected blood was put into flasks containing heparin anti-coagulant. The flasks were shaken slowly to mix the blood with the anti-coagulation agent and preventing the blood from coagulation. PBMCs preparation was conducted based on Ficoll-Histopaque 1077 technique. PBMCs culture was incubated for 7 to 10 days enabling the monocyte cells to differentiate into macrophage. This process produced 10^5 macrophages in each well. At the 4th day, treatment on PBMCs samples was conducted namely by inducing ESAT-6, CFP-10, Ag85A/B/C protein compounds into several wells while the viability of PBMCs was observed using inverted microscope and Giemsa colorization. RD1 secretory protein compounds (ESAT-6, CFP-10, and Ag85A/B/C) were prepared by culturing *Mycobacterium tuberculosis H37Rv* on Lowenstein Jensen medium. The culture was incubated for 3-4 weeks enabling the culture to reach its logarithmic phase. 1 ose of *Mycobacterium* colony was collected and put into 10 mL Middle Brook 7H9 medium which had been prepared before. The suspension was incubated in CO_2 incubator at 37 °C for 2-3 weeks with loosened cap. After 2-3 weeks incubation period, the flasks containing *Mycobacterium tuberculosis* cultured in Middle Brook 7H9 medium were centrifuged for 30 seconds-1 minute until the mixture became homogenous. The mixture was rested for an hour until sedimentation formed. 200 μL supernatant was collected and used as "treatment" (TX) in this study.

RESULTS AND DISCUSSION

This study was conducted from November 2014 to February 2015. The samples of this study consisted of Peripheral Blood Monocyte Cells (PBMCs) samples collected from healthy patients with negative Tuberculin Skin Test (TST) result (K2), and PBMCs samples collected from child tuberculosis patients/LTBI, and adult tuberculosis patients/LTBI (K1). Treatment (TX) was given to several K1 samples at the fifth day after incubation. The results were compared with K1 samples which had not received treatment. PBMC culture was cultivated daily by feeding (i.e. changing the medium daily). Microscopic examination was conducted at the 8th day.

Table 1. Results of Peripheral Blood Monocyte Cells Activity Examination

Result of Examination	K2	K1 (Without TX)	K1 (Receiving TX)
Amount of Monocyte/100lp	11.56	49.22	80.33

Note:
K1: PBMCs samples collected from children tuberculosis patients/LTBI, and adult tuberculosis patients/LTBI.
K2: PBMCs samples collected from healthy individual with negative Tuberculosis infection.
TX: Treatment (ESAT-6, CFP-10, Ag85A/B/C secretory protein compounds induction).

Table 2. Results of Peripheral Blood Monocyte Cells Qualitative Analysis

Microscopic Examination	K2	K1 (Without TX)	K1 (Receiving TX)
Cellular Debris, Abnormality in Monocyte Cells Morphological Appearance	Negative	Negative	Positive

Note:
K1: PBMCs samples collected from children tuberculosis patients/LTBI, and adult tuberculosis patients/LTBI.
K2: PBMCs samples collected from healthy individual with negative Tuberculosis infection.
TX: Treatment (ESAT-6, CFP-10, Ag85A/B/C secretory protein compounds induction).

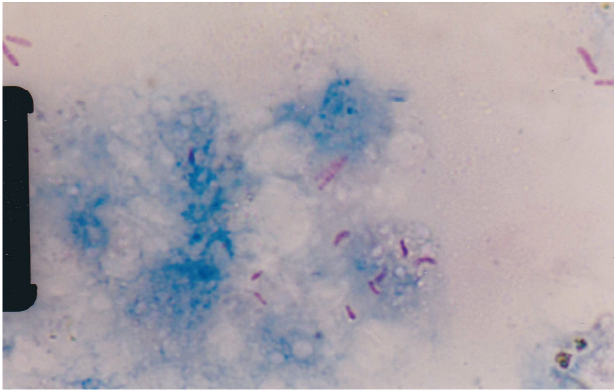

Figure 1. Result of microscopic analysis on *Mycobacterium tuberculosis H37Rv* suspension preparation cultured in Middle-Brook 7H9 medium (ZN, 100x). Acid Fast Bacilli are identified as red thin bacillus.

Mycobacterium tuberculosis H37Rv suspension cultured in Middle-Brook 7H9 medium, was stained by Ziehl Nielsen staining for detection. It is seen that the morphology of Mycobacterium tuberculosis is red thin rod/bacillus.[29]

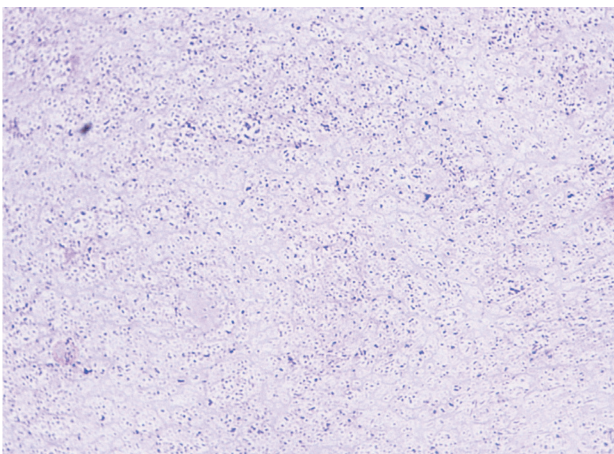

Figure 2. Patient PBMCs culture preparation at the 5th day (before receiving treatment) (Giemsa coloration, 10x): Monocyte cell started growing (indicated by dark blue nucleus with round structure).

PBMCs was cultured and incubated for 7 to 10 days enabling the monocyte cells to differentiate into macrophage. But we do the feeding of the PBMCs culture day by day to keep them alive.

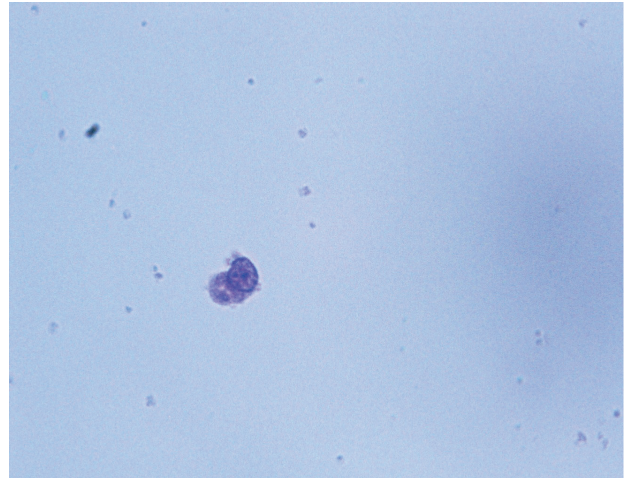

Figure 3. Patient PBMCs culture at the 5th day (Giemsa coloration, 100x) before treatment: Dark blue kidney-shaped monocyte nucleus started developing into macrophage.

Monocyte cell start to become a macrophage by developing the pseudopodia from its cellular membrane.

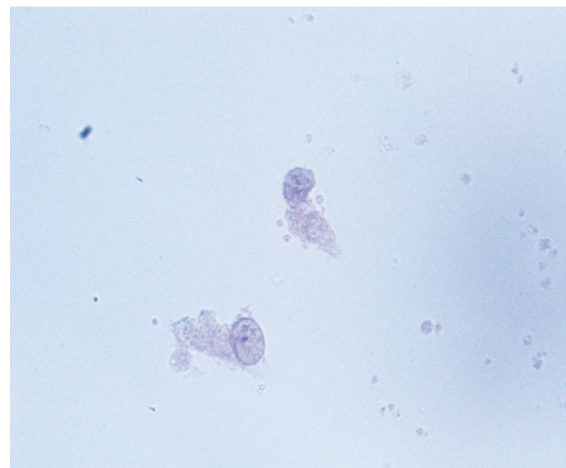

Figure 4. Patient PBMCs culture at 8th day (after treatment) (Giemsa, 100x): Monocytes are interacting with macrophage.

After treatment, there were a lot of active macrophages formed from monocytes. It is seen that the two macrophages is active and did the engulfment.

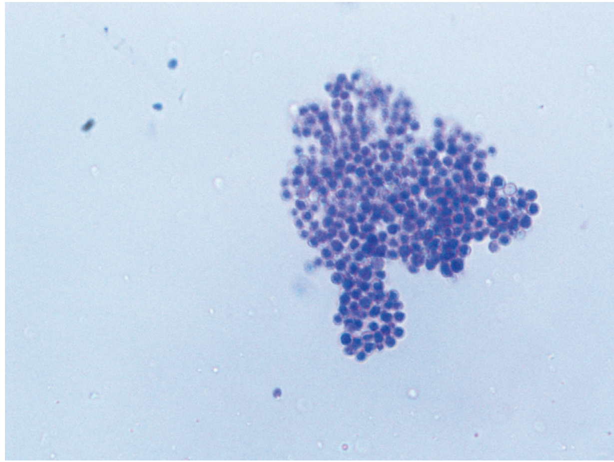

Figure 5. Patient PBMCs culture preparation at 8th day (after treatment) (Giemsa, 100x): Monocyte-lymphocyte cells are interacting with *M. tuberculosis* infected macrophage.

After treatment, there were many monocyte and lymphocyte cells came to interact with macrophages which had been infected by *Mycobacterium tuberculosis*.

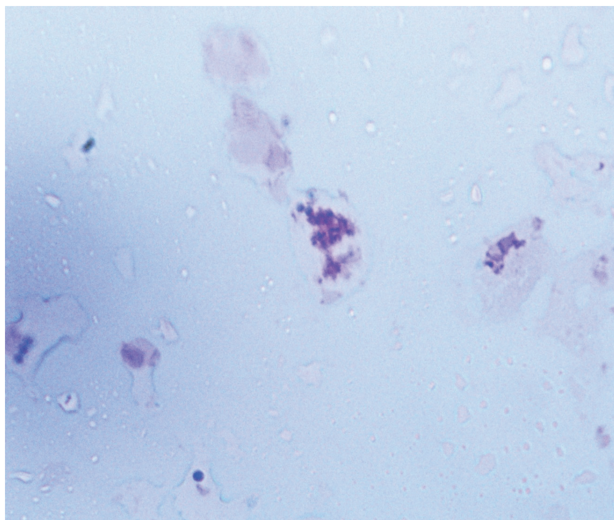

Figure 6. Patient macrophage preparation at 8th day (after treatment) (Giemsa, 100x): macrophage nuclei undergoing pyknosis (probably caused by autophagy).

After treatment, some of macrophages became pyknosis. Pyknosis is the irreversible condensation of chromatin in the nucleus of a cell undergoing necrosis or apoptosis.

Interaction between macrophage and *Mycobacterium tuberculosis* (i.e. the role of macrophage as response made by host) was very important in Tuberculosis infection. Complement receptors (CR1, CR2, CR3, and CR4), mannose receptors (MR), and other molecular receptors on the surface of the cells played significant role in binding the microorganism to the phagocytes. Interaction between phagocyte molecular receptors and mycobacteria might be mediated by *Lipoarabinomannan (LAM)*, glycoprotein found on the surface of mycobacteria. *Prostaglandin E2 (PGE2)* and *Interleukin 4 (IL4)*, cytokines produced by *T-helper 2* (Th2) cells, expression regulation, the function of MR and CR receptors, and interferon γ (INF-γ) could reduce receptors expression resulting in reducing the ability of mycobacteria to attach to macrophage cells. Surfactant proteins, CD14 receptors, and scavenger receptors also functioned in mediating mycobacteria attachment.[30]

Microorganisms underwent phagocytosis were to be degraded through hydrolysis at acid condition after phagolysosome fusion. This process indicated a significant antimicrobial mechanism performed by phagocyte cells. Meena LS and Rajni (2010) proposed a hypothesis stating that phagolysosome fusion inhibition referred to a mechanism in which *Mycobacterium tuberculosis* survived in macrophage cell.[31] Previous studies reported that *mycobacterial sulphatides*–a derivative of *multiacylatedtrehalose 2-sulphate*- had an ability to inhibit phagolysosome fusion.[32,33] Previous in vitro studies showed that *Mycobacterium tuberculosis* produced a huge amount of ammonia which could be the factor affecting this inhibition process.[34,35] There were several functions of macrophage antimicrobial effectors including improving Reactive Oxygen Intermediates (ROI), Reactive Nitrogen Intermediates (RNI), and other mechanisms mediated by cytokines.[30] Hydrogen peroxide (H_2O_2), one of ROI produced by macrophage through oxidative reaction was identified as the first molecular effectors affecting mycobactericidal effect of mononuclear phagocyte cells. Previous laboratory researches showed that *Mycobacterium tuberculosis* infection might induce accumulation of macrophage on pulmonal tissue and H_2O_2 production. However, H_2O_2 production improvement by alveolar macrophage cells was not specific on TB infection. Moreover, alveolar macrophage cells produced less H_2O_2 compared to blood monocyte cells.[4]

Through interferon gamma (INF-γ) and Tumor Necrosis Factor alpha (TNF-α), phagocyte cells produced nitric oxide (NO) and other RNI through Inducible Nitric Oxide Synthase ($INOS_2$) using L-arginine as substrate. The significance of these toxic nitric substances as host immune response against *Mycobacterium tuberculosis* had been proven in in vitro examination, especially by using murine.[30]

Another mechanism as the result of interaction between macrophage cells and *Mycobacterium tuberculosis* was antimicrobial effect mediation by IFN-γ and TNF-α. Previous reports indicated human IFN-γ macrophage effect on *Mycobacterium tuberculosis* replication was varied from inhibition to enhancement. 1,25-$(OH)_2D_3$ itself (or combined with IFN-γ and TNF-α) might activate macrophage to inhibit and kill *Mycobacterium tuberculosis* inside human body.[36]

Other potential mechanism associated with macrophage defense response to *mycobacterium tuberculosis* was

apoptosis or "Programmed Cell Death. Lee et al (2009) showed that apoptosis by macrophage might reduce the viability of *Mycobacterium tuberculosis*.[37]

Until recently, biomolecular processes taking place in macrophage after *Mycobacterium tuberculosis* infecting the host andhow the bacteria survive on these processes are still being studied.[38] This study is attempted to compare the monocyte cells of healthy individuals and TB patient monocyte cells and TB patient monocyte cells which have been induced by ESAT-6, CFP-10, and Ag85A/B/C secretory protein compounds through microscopic examination. The results indicated improvement on monocyte cell activity of TB patient peripheral blood sample after secretory protein induction compared to the activity of TB patients which had not received secretory protein compounds. The result also showed that monocyte cell activity of TB patient was higher than healthy individual (control group). The result also indicated abnormal monocyte cells morphological appearance of TB patient monocyte cells after receiving ESAT-6, CFP-10, and Ag85A/B/C induction. The abnormal appearance was probably caused by macrophage/monocyte cells undergoing autophagy.

CONCLUSION

Monocyte cells activity of child tuberculosis patient, children with LTBI, and adult tuberculosis patient was higher than healthy individual monocyte cells activity.

Monocyte cells activity of child tuberculosis patient, children with LTBI, and adult tuberculosis patient receiving treatment (i.e. RD1 secretory proteins induction) was higher compared to monocyte cells which did not receive treatment.

Cellular debris and abnormal monocyte cell appearance were found at 8th day examination on child tuberculosis patient, children with LTBI, and adult tuberculosis patient monocyte cells sample after receiving RD1 secretory proteins (ESAT-6, CFP-10, and Ag85A/B/C) induction.

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