Original article

Comparative studies evaluating macrophage activity of pulmonary tuberculosis patients with and without diabetes mellitus (tb-dm and non tb-dm)

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Abstract

Background**:* Macrophages, as the first defense mechanism in *Mycobacterium tuberculosis* infection, play the important role in pulmonary TB pathogenesis. The increasing prevalence of pulmonary TB is followed by the increasing prevalence of diabetes mellitus (DM). DM patients have 4,7 times higher risk to develop pulmonary TB compared to patients without DM, since DM can increase the frequency and severity of an infection, including pulmonary TB. ***Aim: To analyze macrophage activity (phagocytosis, intracellular killing, and IFN-γsynthesis) of TB-DM and TB non-DM patient. ***Method**:* This experimental study used a PBMC cultured sample from TB-DM and TB non-DM patient's which undergo observation of macrophage activity (phagocytic, intracellular killing and IFN-γsynthesis). The data were taken from microscopic observation of TB-DM and TB non-DM patients, colony growth of viable *M. tuberculosis* and the IFN-γlevel secreted by macrophages. ***Result**:* The result showed that macrophages of TB-DM patient's were less amount of phagocytosed*M. tuberculosis,* a little amount of formed vacuoles and giant cells, secrete low level of IFN-γ, and more viable *M. tuberculosis*(from subculture). ***Conclusions**:* Macrophages of TB-DM patients are reduced phagocytic activity toward *M. tuberculosis* which is this macrophages are less activated.

Keywords: macrophage; TB-DM; phagocytosis; intracellular killing; IFN-γ.

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Introduction

TB-DM is a significant health problem in Indonesia. This two diseases exacerbate clinical manifestastions and affect treatment outcomes of one another. Indonesia rank 4th worldwide for its high number of TB patients [1,2,3] World Health Organization expects that TB control will become more difficult with the increasing number of diabetes mellitus (DM) patients, due to DM is one of the risk factors for TB deterioration. Correlation between TB and DM have been reported since 1000 AD, though it is still difficult to be defined.[4,5,6]

The increasing cases of TB-DM are associated with an increase in morbidity and mortality of TB and DM. DM patients have 4,7 times higher risk to develop pulmonary TB.[4] This is due to the treatment of MDR-DM cases,which one of its aim is to restore the function of the immune system, i.e. immunostimulant.[7] Less activated alveolar macrophage of pulmonary TB patients with DM reduces the interaction between T lymphocyte and macrophage, resulting in defect of *M. tuberculosis* elimination.

The entry of *M. tuberculosis* into the macrophage and its ability to survive are the key element of the pathogenesis of tuberculosis.[8,9] On primary infection, aerosol droplet nuclei containing *M. tuberculosis* is inhaled and settle on the pulmonary alveolar epithelial cell surface expressing adhesion molecule (intracellular adhesion molecule-1/ICAM-1), thereby increasing the migration and adhesion of phagocytic cells, particularly alveolar macrophage which effectively phagocyte all particles including *M. tuberculosis*.[9,10] Immune response by macrophage in the form of phagocytosis and intracellular killing

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which contributes to the immune defense is expected to be the first in line to eliminate *M. tuberculosis* and lower the incidence of TB. However, *M. tuberculosis*are able to multiply within macrophage thereby causing tuberculosis.

This study is aimed to analyze macrophage activity (phagocytic, intracellular killing and IFN-γsynthesis) from TB-DM and TB non-DM patient's, by identifying peripheral blood mononuclear cells (PBMC) of two kinds of subjects.

Methods

DM patients were obtained from the TB patients who have a history of DM and have a blood pressure more than 140/90mmHg. TB patients were recruited from the hospitals patients that have BTA (+) and chest X-ray (+). There were 30 samples for each TB-DM and TB non-DM patients, and all of them was signed the informed consents.

Ethical clearance was provided by Gadjah Mada University (EC number 352/EC/FK/UGM/2016). Most of the laboratory works were performed in Faculty of Medicine GadjahMada University.

PBMC's Isolation (Peripheral Blood Mononuclear Cells)

As much as 20mL of peripheral blood was taken from TB-DM patients and continued with defibrination. Ten milliliters of RPMI (-) 1640 medium were added (RPMI 1640 medium without HI-PHS/Heat Inactivated Pooled Human Serum supplementation), then each 5mL were moved into a tube containing 3mL Ficoll-Histopaque, then centrifuged.[12,13] Supernatant layer was discarded, the pellet was rinsed and added with 4000 µl RPMI 1640 (+) medium (heated RPMI 1640 medium supplemented with 10% inactivated human serum with 56°C for 30 minutes/HI-PHS/ Heat Inactivated Pooled Human Serum), and then continued by mix pipetting. Monocyte viability was determined using tryphan blue exclusion (≥95%)[16] Monocyte percentage was determined using Giemsa stain on the smear of centrifuge result. Monocyte (10^5/mL) were cultured in a 24-wells tissue culture plate covered by coverslip and then it was added with RPMI 1640 medium, 7,2 pH, contains 25mM HEPES and L-glutamine without serum and antibiotic.[16]

Opsonization of *Mycobacterium tuberculosis*

An ose of platinum (10^6 CFU/mL) of *M. tuberculosis* H37Rv ATCC 27294^T (signal) strain were inserted aseptically into screw cap tube containing 4000µL of Middle brook 7H9 liquid medium and ± 6 -7 bead glass and homogenous vortex. As much as 4000µ of it was taken, then centrifuged. Supernatant was discarded, the pellet was set aside and rinsed with 5000 µl sterile PBS for 3 times. Pellet again was set aside and 4000µl RPMI 1640 (-) medium and 4000µl PHS/Pooled Human Serum were added. Next step is suction spray approximately 10 times with 26G tuberculin syringe and incubated at 37°C containing 5% $CO_2$for 20 minutes then centrifuged. The supernatant layer was discarded, the pellet was rinsed with 5000mL of sterile PBS for 3 times, then 4000mL RPMI 1640 medium (+) were added.

Co-Culture of Macrophage and *M. tuberculosis*

On the 7th day, the macrophage cells culture was added by 106 CFU/mL suspension of opsonized-*M. tuberculosis* strain H37Rv ATCC 27294T, and then it was incubated at 37°C with air containing 5% $CO_2$for 24 hours, 48 hours, 7 times 24 hours and 14 times 24 hours.[13,17]

Macrophage Activity Tests

Coverslip on the base of a 24-wells tissue culture plate was aseptically rinsed with sterile PBS for 5 times. Coverslip base was scraped to harvest the macrophage, then shaken well by mix pipetting, 200µL were taken and transferred into Eppendorf tubes. An 800mL sterile PBD were added and centrifuged. Supernatant layer was discarded, the pellet was set aside, added with 1000mL of sterile distilled water, incubated for 30 minutes at 4°C and then vortex for 5 minutes macrophage are lysis and intracellular *M. tuberculosis* free from macrophage.

As much as 30µl was taken from, dropped on a solid Middlebrook 7H10 agar medium, and incubated at 37°C with 5% CO_2 level for 7 days, 10 days, and 14 days to determine the number of bacteria surviving, not digested by macrophages; with counting colonies grown per ml (CFU / ml).[18]

CFU Measurement

The number of *M. tuberculosis* colonies which can still grow on solid Middlebrook 7H10 agar medium are counted as CFU/ml of the 7th, 10th, and 14th day.

Data Analysis

The data came from the result of the macrophage microscopic observation of the TB-DM and TB non-DM patients, viable *M. tuberculosis* colony growth, and the IFN-γlevels secreted by macrophages.

Results

The study results showed that the mononuclear cells in the buffy coat layer from the peripheral blood of the TB-DM patients consists of monocytes and lymphocytes. Monocytes of TB-DM patients which have matured into macrophages within 7 days were co-cultured with *M. tuberculosis* (*in vitro*). The results of microscopic observation showed that there

is less number of ingested *M. tuberculosis* and less formation of vacuoles and giant cell macrophages (Figure 1).

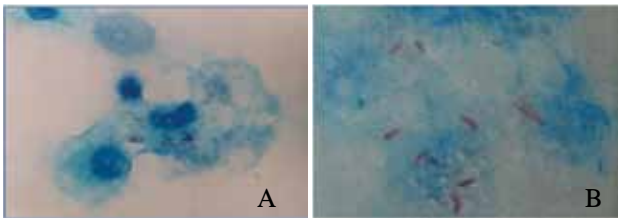

Figure 1. Macrophages ingested M. tuberculosis. a) TB-DM patients, b) TB non-DM patients (NIKON Eclipse E600 light microscope, magnification of 1700X)

Level of IFN-γ secreted by macrophages of TB-DM and TB non-DM patients can be seen in Table 1 below:

Table 1. IFN-γlevels secreted by macrophages of TB-DM and TB non-DM patients

Level of IFN-γ	TB-DM patients (pg/ml)						TB non-DM patients (pg/ml)					
	1	2	3	4	5	6	1	2	3	4	5	6
	78	97	81	103	91	81	101	132	98	111	123	132
	86	98	97	87	75	103	111	143	117	97	121	98
	93	89	101	81	97	97	96	118	81	134	117	131
	97	86	87	78	75	81	134	85	121	123	101	133
	101	89	85	76	101	81	104	98	117	97	121	111

Based on Table 2 above, the average levels of IFN-γsecreted by macrophages of TB-DM and TB non-DM patients can be seen as shown in Figure 2 below:

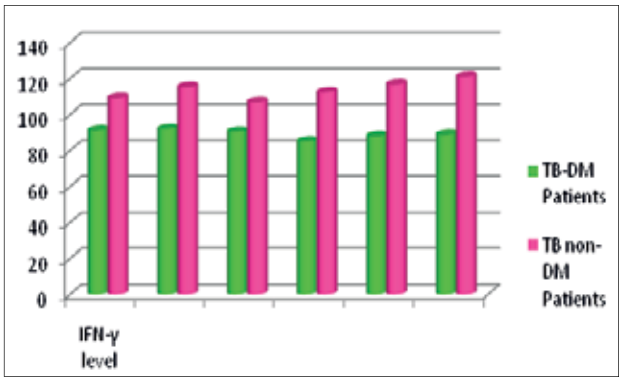

Figure 2.Average of IFN-γlevels secreted by macrophages of TB-DM and TB non-DMpatients

Average of viable M. tuberculosis colonies calculated as CFU/ml on 7th, 10th, and 14th day can be seen in Figure 3 below:

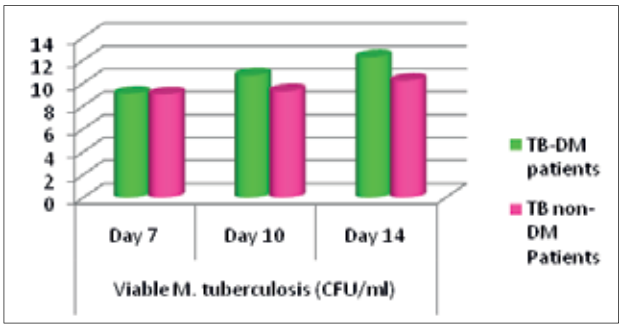

Figure 3. Average number of viable M. tuberculosis colonies (CFU / ml)

Discussions

TB-DM and TB non-DM patients' monocytes which had matured into macrophages within 7 days appears as large rounded cells with regularly spherical nucleus resembling horseshoe-shaped, have cell wall protrusions and large cytoplasm, and also macrophages showing some presence of giant cells. Additionally, this study carried out co-culture between macrophages and *M. tuberculosis (in vitro).* This was intended to provide the bacteria to invade macrophages in order to allow the phagocytosis process to take place. The interaction between opsonized *M. tuberculosis* with macrophages membranes can lead to ingestion/ engulfment and metabolic burst, which characterized by an increase in oxygen consumption (O_2), production of superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2).

The process of ingestion/ engulfment begins with the introduction of *M. tuberculosis* by macrophages receptors, membranes of macrophages will surround the bacteria in a circle (zipper mechanism), thus the bacteria are in the phagosome.[21]Phagosome then undergoes maturation and then fuse with lysosomes to form phagolysosome,[21] an organelle with antimicrobial component and acidic pH (pH ~ 6,2).

Macrophages from TB non-DM patients are more effective in performing the phagosome and lysosome fusion. In addition, macrophages are also more effective in producing oxygen radicals, NO, and various antimicrobial molecules[26-28] and may increase respiratory burst, ROI production, RNI, and IFN-γ releasing.[29,30]

Macrophages TB-DM patients' showed that there were more uningested*M. tuberculosis*. This macrophages

are less activated macrophages, because of the immune cells defects, which is this defect cannot be resolved with insulin therapy.[31]Furthermore, this macrophages have disruption of chemotaxis, phagocytosis, and antigen presenting phagocytes against *M. tuberculosis.* Patients with poorly controlled DM will upset the phagocytosis, especially if it has been in an acidosis state. This phagocytosis disruption is due to the intrinsic defect of the PMN.[32] Decreased production of IFN-γ was more significant in TB-DM patients than in TB non-DM patients. This IFN-γ production will return to normal within six months, either in patients with pulmonary TB alone or TB-DM patients, but it can be continue to decline in TB-DM patients. Also, there were changes in pulmonary vascular and alveolar oxygen tension that aggravated the patients' condition.[4,23,33]

The defects of immune cell defects, fewer macrophages activation, and decreased in phagocytic capability in TB-DM patients can support the viability of *M. tuberculosis*. In accordance with this results, it is known that there are more viable *M. tuberculosis* in TB-DM patients' macrophages than in TB non-DM patients, as shown in Figure 3.

Conclusions

Macrophages of TB-DM patients are less activated macrophages than TB non-DM patients, where there are disturbances in its phagocytosis capability (due to the intrinsic defect of the PMN) and its phagocytes antigen presenting toward *M. tuberculosis*. These are shown in the results, that the TB-DM macrophages secreted low levels of IFN-γ and there are more numbers of viable *M. tuberculosis*.

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