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## Macrophage Activity Test Of Pulmonary Tuberculosis Patients With Diabetes Mellitus (TB-DM)

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### ABSTRACT

**Background:** Control of pulmonary TB is getting more and more complicated as the number of patients with diabetes mellitus (DM) is increasing. The increasing prevalence of DM is followed by the increasing prevalence of pulmonary TB. Diabetes Mellitus 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 TNF- $\alpha$  synthesis) of TB-DM patients.

**Method:** The research was an experimental study used a PBMC cultured sample from TB-DM patient's which undergo observation of macrophage activity (phagocytic, intracellular killing and TNF- $\alpha$  synthesis). The data were taken from microscopic observation of TB-DM patients, colony growth of viable *Mycobacterium tuberculosis* and the TNF- $\alpha$  level secreted by macrophages.

**Result:** Microscopic observation showed that there were less amount of phagocytosed *M. tuberculosis* (in macrophage/intracellular level) and there was a little amount of formed vacuoles and giant cells. Furthermore, macrophages in TB-DM patients secrete low level of TNF- $\alpha$ , and there were more viable *M. tuberculosis* from this macrophage.

**Conclusions:** Macrophages of TB-DM patients are less activated, with reduced phagocytic activity toward *M. tuberculosis*..

**Keywords:** macrophage, TB-DM, phagocytosis, intracellular killing, TNF-  $\alpha$ .

### INTRODUCTION

Tuberculosis (TB) is a major cause of death among bacterial infection diseases. TB affects 9,4 million people and kills 1,7 million of world population annually. The etiology is *Mycobacterium tuberculosis* bacteria which has a rod shape, aerobe characteristic, and acid-fast feature. Indonesia rank 4th worldwide for its high number of TB patients<sup>1,2,3</sup> 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.<sup>4,5,6</sup>The increasing cases of TB-DM are associated with an increase in morbidity and mortality of TB and DM. DM patients have<sup>4,7</sup> times higher risk to develop pulmonary TB.<sup>4</sup> 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.<sup>7</sup> 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.<sup>8,9</sup> 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*.<sup>9,10</sup> Immune response by macrophage in the form of phagocytosis and intracellular killing 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* is able to multiply within macrophage thereby causing tuberculosis.

The increase in cases of TB-DM that associated with the increased of morbidity and mortality due to TB-DM, which is DM patients have an immune respond disorder, thereby facilitating *M. tuberculosis* infection. Thus, it is important to know more about the treatment of cases of TB-DM, one of which emphasizes the restoration of immune abnormalities. Therefore, to be able to find out, it is necessary to do the initial research with emphasis on the activity of macrophages from TB-DM patients.

This study is aimed to analyze macrophage activity (phagocytic, intracellular killing and TNF- $\alpha$  synthesis) from TB-DM patients, by identifying peripheral blood mononuclear cells (PBMC) of TB-DM patients.

## 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 (+). Healthy individu were the individu who have BTA (-) and chest X-ray (-). There were 18 samples of DM patients, TB patients, and healthy individu were recruited for this study, and all of the samples was signed the informed consents.

Ethical clearance was provided by Gadjah Mada University (EC numb. 36/EC/2.08.16). 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 RPMI1640 medium (Sigma-Aldrich Inc., R0883) 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 (Sigma-Aldrich Inc., 10771), then centrifuged.<sup>12,13,14</sup> Supernatant layer was discarded, the pellet was rinsed and added with 4000  $\mu$ l 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 ( $\geq 95\%$ ).<sup>16</sup> 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 RMPI 1640 medium, 7,2 pH, contains 25mM HEPES and L-glutamine without serum and antibiotic.<sup>16</sup>

## Oponization of *Mycobacterium tuberculosis*

An ose of platinum ( $10^6$  CFU/mL) of *M. tuberculosis* H37Rv ATCC 27294<sup>T</sup> (signal) strain (derived from cultures of tuberculosis laboratory, Tropical Disease Centre Institute, Airlangga University) were inserted aseptically into screw cap tube containing 4000 $\mu$ L of Middlebrook 7H9 liquid medium and  $\pm 6-7$  bead glass and homogenous vortex, then 4000  $\mu$ L suspension was added and centrifuged. Supernatant was discarded, the pellet was set aside and rinsed with 5000  $\mu$ l sterile PBS for 3 times. Pellet again was set aside and 4000 $\mu$ l RPMI 1640 (-) medium and 4000 $\mu$ l 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<sub>2</sub> 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 7<sup>th</sup> day, the macrophage cells culture was added by  $10^6$  CFU/mL suspension of oponized-*M. tuberculosis* strain H37Rv ATCC 27294<sup>T</sup>, and then it was incubated at 37°C with air containing 5% CO<sub>2</sub> for 24 hours, 48 hours, 7 times 24 hours and 14 times 24 hours.<sup>13,15,17</sup>

### Macrophage Activity Tests and TNF- $\alpha$ Levels Measurement

Coverslip on the base of a 24-wells tissue culture plate was aseptically rinsed with sterile PBS for 5 times.

Sixty  $\mu$ l supernatant was taken from 24-well tissue culture plate aseptically, then test by used an ELISA kit (88-7346-76, Ready-SET-Go!, eBioscience, DTA00C). TNF- $\alpha$  levels was measured by ELISA reader.

Coverslip base of a 24-well tissue culture plate was scraped to harvest the macrophage, then shaken well by mix pipetting, 200 $\mu$ L were taken and transferred into eppendorf tubes. An 800mL sterile PBS 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 $^{\circ}$ C and then vortex for 5 minutes macrophage are lysis and intracellular *M. tuberculosis* free from macrophage.

Thirty  $\mu$ l was taken from, dropped on a solid Middlebrook 7H10 agar medium, and incubated at 37 $^{\circ}$ C with 5% CO<sub>2</sub> 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).<sup>18</sup>

### CFU Measurement

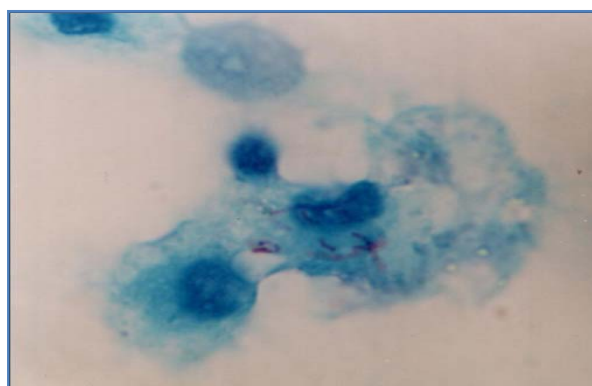
The number of *M. tuberculosis* colonies which can still grow on solid Middlebrook 7H10 agar medium are counted as CFU/ml of the 7th, 10th, and 14th day.

### Data Analysis

The data came from the result of the macrophage microscopic observation of the TB-DM patients, viable *M. tuberculosis* colony growth, and the TNF- $\alpha$  levels secreted by macrophages by used statistic test.

### 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 from macrophages of TB-DM patients showed that there is less number of ingested *M. tuberculosis* (red) and less formation of vacuoles and giant cell macrophages (blue) (Figure 1).



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

TNF- $\alpha$  levels secreted by macrophages of TB-DM patients, TB patients non-DM, and healthy individuals can be seen in Table 2.

Based on Table 2, the average levels of TNF- $\alpha$  secreted by macrophages of TB-DM patients, TB patients without DM, and healthy individual can be seen as shown in Figure 2.

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

### DISCUSSIONS

Approximately 10-20% buffy coat is consisted of monocytes, the rest were lymphocytes.<sup>19</sup> Buffy coat is located between the upper layer (plasma) and the bottom layer (erythrocytes and granulocytes sediment) from PBMCs of TB-DM patients, consisting monocytes and lymphocytes. TB-DM patients' monocytes which had matured into macrophages within 7 days appears as large rounded cells with regularly spherical nucleus resembling kidney/horseshoe (horseshoe-shaped), have cell wall protrusions and large cytoplasm, and also macrophages showing some presence of giant cells. While lymphocytes appeared as having large nuclei, round shape, heterochromatin features with a large nucleus-cytoplasm ratio.<sup>11,12,19,20</sup> This study eliminated the presence of the lymphocytes using the PBS rinsing method in order to avoid the bias results from the process of intracellular killing because the lymphocytes have a role in producing IFN- $\gamma$  as a mediator of macrophage activation.

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. *M. tuberculosis*

invasion process in macrophages can take place after an opsonization<sup>21,22</sup> followed by binding and uptake of the complement receptors (CR1, CR3, and CR4),<sup>21,23</sup> mannose receptor, surfactant, and Toll-Like Receptors (TLR). 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.<sup>21</sup> Phagosome then undergoes maturation and then fuse with lysosomes to form phagolysosome,<sup>21</sup> an organelle with antimicrobial component and acidic pH (pH ~ 6,2).

Macrophages from the activated TB 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<sup>26-28</sup> and may increase respiratory burst, ROI production, RNI, and TNF- $\alpha$  releasing.<sup>29,30</sup>

Macrophages of TB-DM patients are activated macrophages, but because the TB-DM patients have defects in their immune cells, the macrophages become less activated. Furthermore, macrophages in DM patients have disruption of chemotaxis, phagocytosis, and antigen presenting phagocytes against *M. tuberculosis*. This defect cannot be resolved with insulin therapy.<sup>14</sup> 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.<sup>12</sup>

This results showed that there were decreased production of TNF- $\alpha$  in pulmonary tuberculosis patients with DM rather than non-diabetic TB patients. It is according to the literature mentioned that Th-1 response levels, IFN- $\gamma$ , as well as the production of IL-1  $\beta$  and IL-6 are more profound in pulmonary tuberculosis patients with DM rather than non-diabetic TB patients. Decreased production of IFN- $\gamma$  was more significant in patients with pulmonary tuberculosis with uncontrolled diabetes than in patients with controlled DM TB. This IFN- $\gamma$  production will return to normal within six months, either in

patients with pulmonary TB alone or pulmonary TB patients with uncontrolled DM, but will continue to decline in pulmonary TB patients with uncontrolled DM. Also, there were changes in pulmonary vascular and alveolar oxygen tension that aggravated the patients' condition.<sup>4,23,33</sup>

TNF- $\alpha$  proved to be effective in eliminating *M. tuberculosis*, this cytokine will increase the phagocytosis capability and induces apoptosis of infected macrophages. Both TNF- $\alpha$  and IFN- $\gamma$  will increase the intracellular killing macrophages process by producing reactive NO. TNF- $\alpha$  also plays a role in maintaining granuloma integrity, which formed when macrophages infected by *M. tuberculosis*. Macrophages that are unable or only slightly expresses TNF- $\alpha$  would be very easily infected and difficult to eliminate *M. tuberculosis*.

The presence of viable *M. tuberculosis* showed that this bacteria is able to escape and survive the macrophage phagocytosis and intracellular killing macrophage. Immune cell defects, fewer macrophages activation, and decreased in phagocytic capability in patients with TB-DM can support the viability of *M. tuberculosis*. In accordance with these results, it is known that there are more viable *M. tuberculosis* in TB-DM patients' macrophages as shown in Figure 3.

*M. tuberculosis* can also produce lipoarabinomannan (LAM), which can inhibit the activation of macrophages and is an important virulence determinant of these bacteria. LAM can inhibit the functions induced IFN- $\gamma$  thus disrupts the microbicidal function of macrophages, inhibiting the activity of protein kinase C, and TNF- $\alpha$  evocation.<sup>34</sup>

## CONCLUSIONS

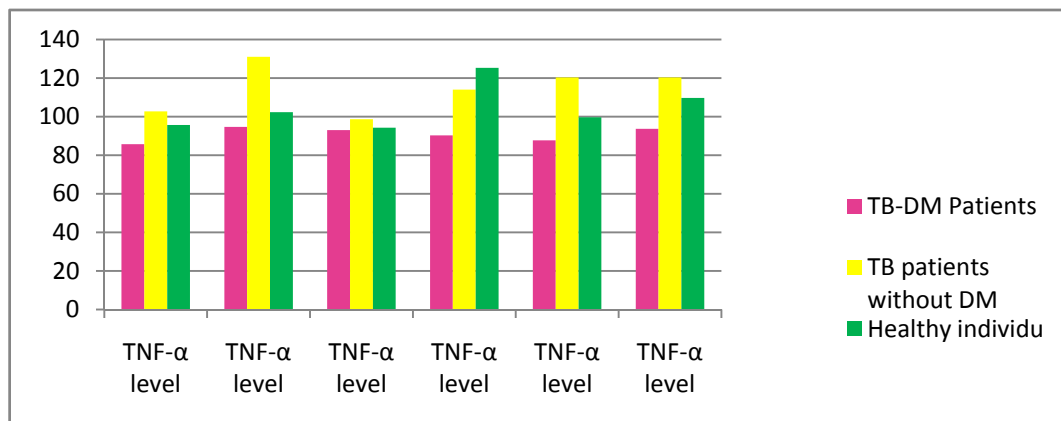
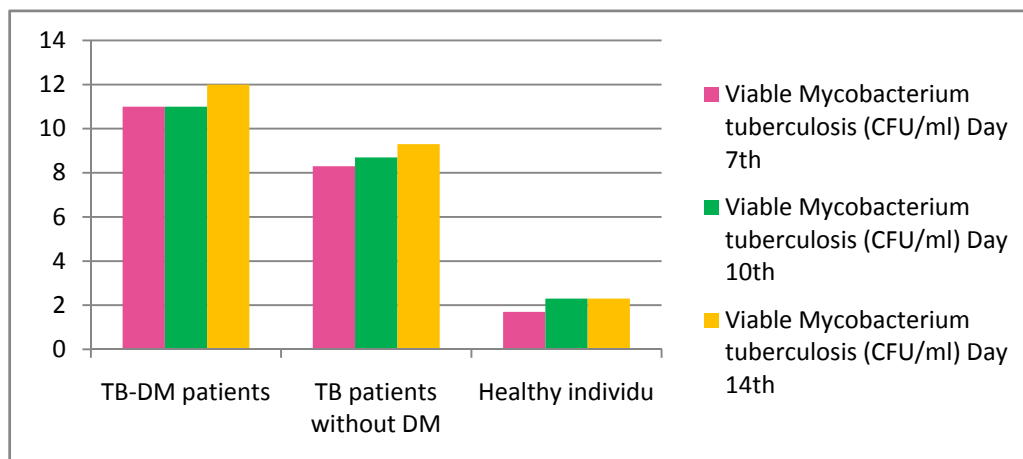
Macrophages of TB-DM patients are less activated macrophages, where there are disturbances in its phagocytosis capability (due to the intrinsic defect of the PMN) and its phagocytes antigen presenting toward *M. tuberculosis*. These are shown in the results, that the TB-DM macrophages secreted low levels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

**Table 2.** TNF- $\alpha$  levels secreted by macrophages of TB-DM patients, TB patients without DM, and healthy individuals

|               | TB-DM patients (pg/ml) |    |     |     |    |     | TB patients without DM (pg/ml) |     |     |     |     |     | Healthy Individuals (pg/ml) |     |    |     |     |     |
|---------------|------------------------|----|-----|-----|----|-----|--------------------------------|-----|-----|-----|-----|-----|-----------------------------|-----|----|-----|-----|-----|
|               | 1                      | 2  | 3   | 4   | 5  | 6   | 1                              | 2   | 3   | 4   | 5   | 6   | 1                           | 2   | 3  | 4   | 5   | 6   |
| TNF- $\alpha$ | 78                     | 97 | 81  | 103 | 91 | 81  | 101                            | 132 | 98  | 111 | 123 | 132 | 89                          | 103 | 86 | 127 | 99  | 101 |
|               | 86                     | 98 | 97  | 87  | 75 | 103 | 111                            | 143 | 117 | 97  | 121 | 98  | 98                          | 107 | 98 | 113 | 97  | 97  |
|               | 93                     | 89 | 101 | 81  | 97 | 97  | 96                             | 118 | 81  | 134 | 117 | 131 | 100                         | 97  | 99 | 136 | 103 | 131 |

**Table 3.** Mean and standard deviation of TNF- $\alpha$  levels secreted by macrophages of TB-DM patients, TB patients without DM, and healthy individuals

|            | TB-DM patients (pg/ml) |                    |                    |                    |                    |                    | TB patients without DM (pg/ml) |                    |                    |                    |                    |                    | Healthy Individuals (pg/ml) |                    |                    |                    |                    |                    |
|------------|------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|-----------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
|            | 1                      | 2                  | 3                  | 4                  | 5                  | 6                  | 1                              | 2                  | 3                  | 4                  | 5                  | 6                  | 1                           | 2                  | 3                  | 4                  | 5                  | 6                  |
| Mean<br>SD | <u>41.2</u><br>2.2     | <u>48.1</u><br>2.3 | <u>43.6</u><br>2.5 | <u>44.1</u><br>2.7 | <u>41.5</u><br>2.1 | <u>48.1</u><br>2.3 | <u>45.1</u><br>2.0             | <u>46.7</u><br>2.3 | <u>47.1</u><br>2.3 | <u>44.8</u><br>2.1 | <u>46.5</u><br>2.1 | <u>47.2</u><br>2.5 | <u>44.8</u><br>2.1          | <u>42.6</u><br>2.4 | <u>49.1</u><br>2.6 | <u>47.6</u><br>2.4 | <u>46.5</u><br>2.1 | <u>44.0</u><br>2.5 |
|            | <u>46.2</u><br>2.7     | <u>41.3</u><br>2.3 | <u>42.1</u><br>2.4 | <u>44.3</u><br>2.6 | <u>47.3</u><br>2.1 | <u>41.3</u><br>2.4 | <u>48.3</u><br>2.7             | <u>48.1</u><br>2.1 | <u>49.1</u><br>2.6 | <u>44.5</u><br>2.0 | <u>42.8</u><br>2.6 | <u>42.7</u><br>2.6 | <u>42.8</u><br>2.6          | <u>41.3</u><br>2.4 | <u>44.8</u><br>2.3 | <u>41.5</u><br>2.1 | <u>44.1</u><br>2.7 | <u>42.6</u><br>2.4 |
|            | <u>44.0</u><br>2.5     | <u>43.1</u><br>2.3 | <u>41.2</u><br>2.2 | <u>43.6</u><br>2.5 | <u>42.3</u><br>2.6 | <u>42.6</u><br>2.4 | <u>47.6</u><br>2.6             | <u>47.6</u><br>2.4 | <u>44.8</u><br>2.3 | <u>45.7</u><br>2.4 | <u>42.8</u><br>2.6 | <u>47.3</u><br>2.3 | <u>44.1</u><br>2.7          | <u>43.6</u><br>2.5 | <u>41.2</u><br>2.2 | <u>45.1</u><br>2.0 | <u>43.1</u><br>2.3 | <u>41.5</u><br>2.1 |

**Figure 2.** Average of TNF- $\alpha$  levels secreted by macrophages of TB-DM patients, TB patients non-DM, and healthy individuals.**Figure 3.** Average number of viable *M. tuberculosis* colonies (CFU / ml).

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