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Phagocytosis and the antigen-processing abilities of macrophages derived from monocytes in spinal tuberculosis patients

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ABSTRACT

This study examined the hypothesis that there is an impairment of macrophageal function in spinal TB. We examined macrophageal functions in spinal TB patients. Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of five spinal TB patients and five healthy persons as control. The isolated monocytes were cultured with stimulation of macrophage colony-stimulating factor (M-CSF) for seven days for maturation. The phagocytic ability of the macrophages derived from monocytes was measured. Also, nitric oxide (NO), myeloperoxidase (MPO), beta-glucuronide, and acid phosphatase activity was investigated. We found that the monocytes collected from patient PBMCs were significantly fewer than those of the control group (2992.10^3 vs. 6474.10^3 (cells/mL)). There were also fewer macrophages that had adhered to sheep red blood cells (SRBC) (598.10^3 vs. 264.10^3 (cells/mL)). However, NO production (2346 vs. 325.17 (μmol/gram of protein)), and the MPO (570.7 vs. 17.4 (unit/mg), beta-glucuronide (0.149 vs. 0.123 (μmol/hour/100 mg of protein)), and acid phosphatase activities (1776.9 vs. 287.9 (μmol/hour/100 mg of protein)) of the macrophages in the spinal TB group were markedly higher than in the healthy group. Despite the low adhesion to foreign bodies, the intracellular processing of TB macrophages, including oxidative activity and lysosome function, was significantly high. These results suggested the impairment of macrophageal function in spinal TB. Possibly, there is a dominance of innate non-specific immunity in spinal TB infection.

1. Introduction

TB is still one of the public health problems in the world, even though efforts to control it by applying the directly observed treatment, short-course (DOTS) strategy have been made in many countries since 1995. According to the World Health Organization (WHO) report [1], it was estimated that there were 10 million TB cases in 2018, with 1.2 million of them (13%) being TB patients who tested positive for the human immunodeficiency virus (HIV). Among the countries with TB, Indonesia occupies the third-highest position after India and China.

At least up to 20 percent of pulmonary TB patients develop extrapulmonary TB [2,3]. Extrapulmonary TB can be found in the central nervous system, gastrointestinal tract, kidney, genitals, skin, lymphatic gland, and in osteoarticular and endometrial TB. Eleven percent of extrapulmonary TB is osteoarticular TB, and approximately half of the patients with osteoarticular TB experience a spinal TB infection [4,5].

TB is a disease transmitted by droplets, and the bacteria are caught by pulmonary macrophages [6]. Phagocytosis triggers a local

Abbreviations: DOTS, directly observed treatment, short-course; WHO, the World Health Organization; TB, tuberculosis; HIV, human immunodeficiency virus; M. tb, *Mycobacterium tuberculosis*; M-CSF, macrophage colony-stimulating factors; NO, nitric oxide; MPO, myeloperoxidase; PBMC, peripheral blood mononuclear cell; LPS, lipopolysaccharide; SRBC, sheep red blood cell; WST, water-soluble tetrazolium salt; RPMI, Rosewell Park Memorial Institute culture medium; EDTA, Ethylene diamine tetra acetic acid; PBS, Phosphate buffer saline.

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inflammatory response, characterized by the release of cytokines and chemokines, which stimulates the migration of leukocytes to the site of infection. During this process, the effectiveness of bacterial elimination determines the subsequent process before the adaptive immune response is initiated [7–9].

After entering the respiratory tract, *Mycobacterium tuberculosis* (M.tb) is finally captured by an alveolar macrophage. In most cases, the lung macrophage can, if not kill it, at least sequester and deactivate the bacteria. Even if the dormant M.tb reactivates, in most cases, the disease is limited to the lung. If the disease attacks an extrapulmonary organ, it is questionable whether the barrier, constructed by macrophages, is breached [2,3,10].

Spinal TB, as mentioned above, occupies a relatively prominent position as part of extrapulmonary TB. Moreover, spinal TB often needs surgical treatment and results in deformities and/or function limitation [11].

In this study, we aimed to figure out the basic functions of macrophages in spinal TB in defending the body against foreign bodies. We investigated phagocytes and their associated activities, including oxygen bursts, myeloperoxidase activities, and lysosomal enzyme activities, represented by acid phosphatase and beta-glucuronide, in spinal TB patients compared with the control group.

2. Material and method

This study was conducted at the Laboratory of Oxidative Stress and Molecular Biology, Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia.

Five confirmed spinal TB patients from the Department of Orthopedics and Traumatology, Cipto Mangunkusumo Hospital, Jakarta, agreed to participate by donating their blood for this study, while five healthy persons accepted to participate as control subjects. The study was approved by the local ethics committee (0824/UN2.F1/ETIK/2018).

2.1. Macrophage isolation

Ten mL of blood were taken from the cubital vein using a 20–22 gauge syringe with Ethylene diamine tetraacetic acid (EDTA). Then, the blood was diluted 1:1 in Phosphate buffer saline (PBS) in the centrifuge tube and mixed well. The blood was slowly transferred to a new centrifuge tube containing an isolation medium to form a layer. Then the tube was centrifuged for 30 min at a force of 400 g. The plasma was removed, and the buffy coat containing isolated peripheral blood mononuclear cells (PBMCs) was separated and suspended in sterile PBS [12].

The PBMC pellet was resuspended in 6 mL of sterile RPMI and distributed in 6 wells in a microplate of 6 wells, using 1 mL of PBMC suspension per well. The culture plate was incubated in 5% CO_2 for 30 min at 37 °C. Monocytes adhered to the surface of the well while lymphocytes floated in the medium. The medium was aspirated totally to eliminate the lymphocytes.

2.2. The maturation of monocytes to macrophages

Rosewell Park Memorial Institute (RPMI) 1640 medium, supplemented with 1% penicillin–streptomycin, 1% amphotericin B, and M-CSF (20 ng/mL), was added to culture plates containing the adhered monocytes. The medium was changed on the third and seventh days. On the seventh day, the monocytes differentiated into macrophages. The culture medium was complete RPMI without M-CSF, to which 1.5 mL lipopolysaccharides (LPSs) had been added, and the plate was incubated for 24 h.

The macrophages were harvested by adding TrypLE select® and counting them with a hemocytometer. Ten microliters of cell suspension were mixed with a 10 μl solution of trypan blue. The mixture was pipetted into an improved Neubauer chamber and covered by a slip glass. All the cells in the chambers, stained and not stained, were counted, and the percentage of stained cells was calculated.

2.3. The macrophage phagocytosis test [13,14]

One hundred microliters of macrophages in suspension were pipetted onto a coverslip, followed by 250 μl RPMI, then a 100 μl suspension of 2% sheep red blood cells (SRBCs), and 30 μl of LPS. The mixture was incubated for 30 min in a CO_2 incubator at 37 °C. Then, the medium was removed and the adhered cells were fixed with absolute methanol. The coverslip was dried in the air; this was followed by staining with 20% Giemsa.

Phagocytosis was determined when at least one SRBC attached to the macrophage membrane. The percentage of phagocytosis was calculated as the number of macrophages that had performed phagocytosis divided by the number of total macrophages in the field.

2.4. The water-soluble tetrazolium salt (WST) test [15]

The macrophages' ability to oxidize foreign bodies was measured using the WST-1 Cell Proliferation Assay Kit (Abnova). The principle of this measurement is based on the enzymatic cleavage of WST-1 tetrazolium salts into formazan by the cellular mitochondrial dehydrogenase that is present in living cells.

One hundred microliters of macrophages suspended in RPMI were pipetted into a 96- well microplate followed by 10 μl of the WST mixture was added in each well. After incubation for 4 h in 5% CO_2 at 37 °C, the suspension was agitated using an orbital shaker for 5 min. Finally, the optical density (OD) of the solutions was read using a spectrophotometer at 450 nm.

2.5. The nitric oxide assay [16]

The macrophages were lysed by 3 freeze-thaw cycles (−20 °C to 37 °C). NO levels were measured by the Nitric Oxide Assay Kit (Elabscience). One hundred and fifty microliters of cell lysate of 700,000 macrophages was added to a sulfate solution. After incubation for 15 min, followed with centrifugation at 4000 rpm for 10 min, a chromogenic agent was added to each supernatant. The optical density was measured at 550 nm after 20 min of incubation. The measurement results are expressed in units of μmol/gram of protein.

2.6. Myeloperoxidase activity

A myeloperoxidase assay of the macrophage lysate was performed using hydrogen peroxide (H_2O_2) as a substrate and guaiacol as a chromogen. The enzyme catalyzes the reduction of H_2O_2 with guaiacol as an electron donor:

$$2\ \text{guaiacol} + H_2O_2 \rightarrow 2\ \text{oxidized guaiacol} + 2H_2O$$

(orange) (red)

The reagent mixture consisted of PBS (0.01 M, pH 7.4) and 100 mM guaiacol. First, 0.0017% (w/w) hydrogen peroxide was prepared. One milliliter of reagent was pipetted into the well, followed by 12 μl of lysate. After 1 min, the optical density of the mixture was read at 470 nm. Data was presented as unit enzyme per milliliter.

2.7. The beta-glucuronidase activities assay [17]

The substrate for the beta-glucuronidase assay was p-nitrophenol-β-glucuronide. The cell lysate (10 μl) were added to 25 μl of the substrate and 15 μl of the sodium acetate buffer and incubated at 37 °C for 1 h. After incubation, 150 μl of glycine-NaOH was added. The OD of the solution was measured at 410 nm; the data is presented in μmol of p-nitrophenol per hour per 100 mg of protein.

2.8. The acid phosphatase activity assay [18]

The substrate for the acid phosphatase activity assay was p-nitrophenyl-phosphate. The cell lysates (10 μl) were added to 25 μl of the substrate and 15 μl of the sodium acetate buffer, and incubated at 37 °C for 1 h. After incubation, 150 μl of glycine-NaOH was added. This was mixed slowly and then OD was measured at a wavelength of 410 nm. The acid phosphatase activity is expressed in μmol of p-nitrophenol per hour per 100 mg of protein.

2.9. The total protein assay

The total protein was measured using the Warburg-Christian method [19]. The absorbance was measured at 280 nm. This protein measurement method was based on the absorbance of light by the amino acids tyrosine and tryptophan. Before the measurement of the protein, a standard curve was created using bovine serum albumin (BSA), which was divided into a range of several concentrations: 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL.

The results of the statistical analyses of each parameter were expressed as a mean ± SD and displayed in graphic form. The statistical data analysis in this study used the Statistical Product and Service Solution (SPSS) program version 20.0. The distribution of the data was examined by a Shapiro-Wilk test. The variance homogeneity was tested by Levene's test. An unpaired *t*-test was performed for normally and homogenously distributed data, while a Mann-Whitney test was performed for the non-normally distributed data.

3. Results

3.1. The characteristics of the subjects and the number of cultured macrophages

Ten people were divided into two groups in this study. The participants of the healthy group had been assessed by physicians, while the spinal TB blood samples came from individuals who had been diagnosed clinically and bacteriologically as suffering from spinal TB. The subjects' characteristics, the number of monocytes, and the number of macrophages derived from monocytes were recorded (Table 1).

Monocytes and macrophages are expressed in units of cells per milliliter. The healthy group had an average of $598 \times 10^3 \pm 218 \times 10^3$, while the spinal TB group had an average of $264 \times 10^3 \pm 113 \times 10^3$. The *t*-test showed that there was a significant difference ($p < 0.05$). However, there was no difference in the percentages of the differentiation of monocytes into macrophages in the two groups ($p > 0.05$) (Table 2). The viability of the cultured cells in this examination was above 95%. Fig. 1 shows evolution of the isolated and cultured cells of the microscopic images on day 0 of the culture, on day 3, and on day 7.

3.2. The examination of macrophage phagocytosis

3.2.1. Phagocytes per 100 macrophages

The mean number of cell phagocytes per 100 macrophages was 47.6 ± 6.3 and 23.8 ± 3 in the healthy group and spinal TB group, respectively. An unpaired *t*-test showed that there was a significant difference ($p < 0.05$) between the two groups. Fig. 2 shows microscopic images of the SRBC phagocytic macrophages.

Table 1
The characteristics of the subjects.

Group	Gender (%)	Mean age (year ± SD)*	Mean body weight (kg ± SD)**	Mean blood pressure (mmHg ± SD)
Healthy	M (80%), F (20%)	34.8 ± 10.1	77.4 ± 12.7	122/77 ± 8/13
Spinal TB	M (40%), F (60%)	37.4 ± 10.3	57 ± 11.6	114/57 ± 17/12

*t-test; the mean ages were not significantly different ($p > 0.05$).
**t-test; the mean body weights were significantly different ($p < 0.05$).

Table 2
The numbers of isolated monocytes and macrophages.

Group	Mean number of isolated monocytes (cells/mL ± SD)	Mean number of cultured macrophages (cells/mL ± SD)*	Differentiation of monocytes into macrophages (%)
Healthy	$6474.10^3 \pm 2289.6.10^3$	$598.10^3 \pm 218.10^3$	9.47
Spinal TB	$2992.10^3 \pm 2319.5.10^3$	$264.10^3 \pm 113.10^3$	11.7

*t-test; the mean numbers of cultured macrophages were significantly different ($p < 0.05$).

Table 3
A comparison of phagocytoses per 100 macrophages.

Group	Phagocytosis per 100 macrophages (mean ± SD)	Statistical test (*t*-test)
Healthy	47.6 ± 6.3	($p < 0.05$)*
Spinal TB	23.8 ± 3	

3.2.2. The phagocytosis of SRBCs by macrophages

The examination of the phagocytosis of SRBCs by macrophages was performed by observation using Giemsa staining under a light microscope. Macrophages were observed immediately after being harvested. The healthy group mean was 3.32 ± 0.33 SRBCs phagocyted per macrophage while the spinal TB group mean was 3.2 ± 0.44. The data were homogenous and normally distributed. An unpaired *t*-test indicated that the difference between the two groups was not significant ($p > 0.05$) (Table 4). The microscopic image of the macrophages' phagocytosis of SRBCs can be seen in Fig. 3. be seen in Fig. 3.

3.3. The oxidative burst

3.3.1. Macrophage activity in oxidizing antigens

The ability of macrophages to oxidize foreign bodies was assessed by a WST-1 test. The results show, as indicated in Table 5, that there is no significant difference between the two groups ($p > 0.05$).

3.3.2. Nitric oxide production

As the data were homogeneous and normally distributed, a *t*-test was performed. The analysis showed that the difference between the two groups was significant ($p < 0.05$) (Table 6). It means that high levels of NO were caused by TB.

3.3.3. Myeloperoxidase activities

The mean myeloperoxidase activity in the healthy group were 17.4 unit/mg protein ± 11, while in the spinal TB group they were 570.7 unit/mg protein ± 56.1.

The *t*-test showed that the two groups were significantly different ($p < 0.05$) on myeloperoxidase activity (Table 7).

3.4. Lysosomal enzyme activities

3.4.1. Beta-glucuronidase activities

As these data were not homogenous and not normally distributed, a non-parametric analysis was performed. The difference between the medians of the two groups was significant ($p < 0.05$), which indicated very active enzymes in the TB patients (Table 8).

3.4.2. Acid phosphatase enzyme activities

The mean for the acid phosphatase activity (unit/mg protein ± SD)

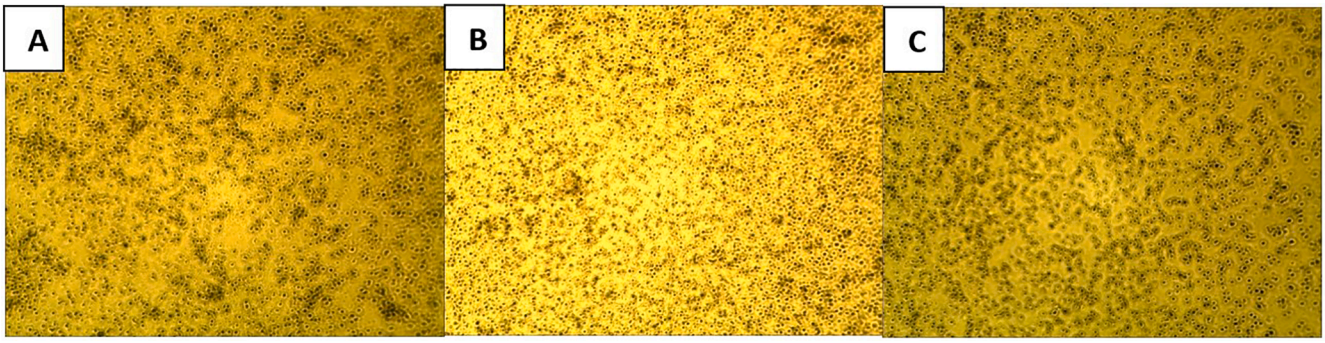

Fig. 1. Macrophages cultured from isolated monocytes using a culture medium and MCSF (400× magnification). A) Day 0 culture, B) Day 3 culture, C) Day 7 culture.

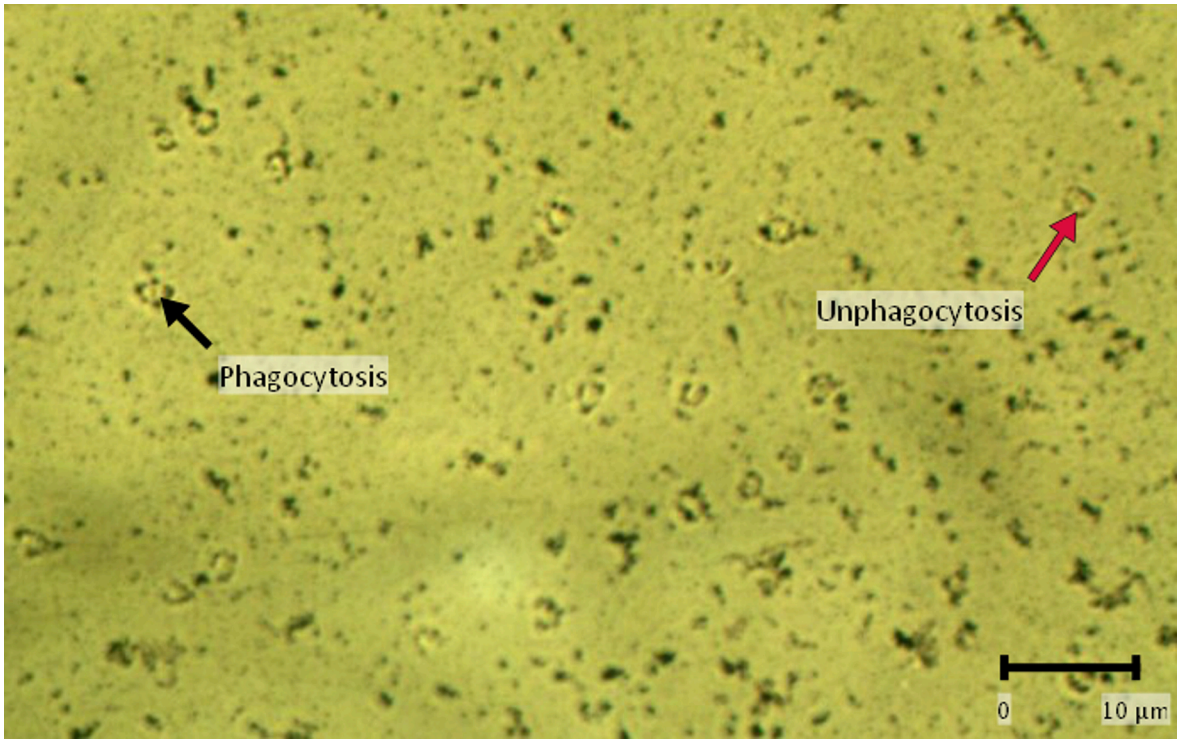

Fig. 2. Cultured macrophages' phagocytosis of SRBCs after macrophages were given SRBCs and LPSs (400× magnification).

Table 4
The number of phagocyted SRBCs per macrophage.

Group	The number of phagocyted SRBCs per macrophage (mean ± SD)	Statistical test (*t*-test)
Healthy	3.32 ± 0.33	($p > 0.05$)
Spinal TB	3.2 ± 0.44	

in the healthy group was 287.9 ± 156.5, while in the spinal TB group it was 1776.9 ± 319.2. The *t*-test showed that there was a significant difference between the level of acid phosphatase activity in the spinal TB group compared with the level of healthy group activity ($p < 0.05$) (Table 9).

4. Discussion

As in other infections, the M.tb recognized first by innate immunity, which relies mainly upon a pattern recognition mechanism, followed by phagocytosis. Granulocytes and macrophages play an essential role in this phase of immunity. These last cells are vital due to their role in innate as well as in adaptive immunities. They are considered as a hinge that mediates the alteration of innate, non-specific immunity to an adaptive or specific immunity [20].

The general conditions of the spinal TB patient are poor, indicated by low mean body weight ($p < 0.05$), a prevalent general condition that found in any chronic infection. Undernutrition can be the base of the phenomenon [21]. Most of nutritional resources are focused on combatting the infection, and the nutritional status was worsened with anorexia.

The number of circulating monocytes was much lower in spinal TB patients ($p < 0.05$). Usually, in chronic infection, there is an increase in the total number of mononuclear cells, including monocytes. The lower number of these cells suggested that there was an impairment of immunity [22]. However, the proportion of macrophages derived from monocytes in both groups was practically the same (9.47% vs. 11.7%). This data suggested that, despite the immunity impairment in the form of hypomonocytes, the monocytes' capacity to differentiate into macrophages was relatively unchanged.

Nevertheless, other functions of macrophages yielded very different

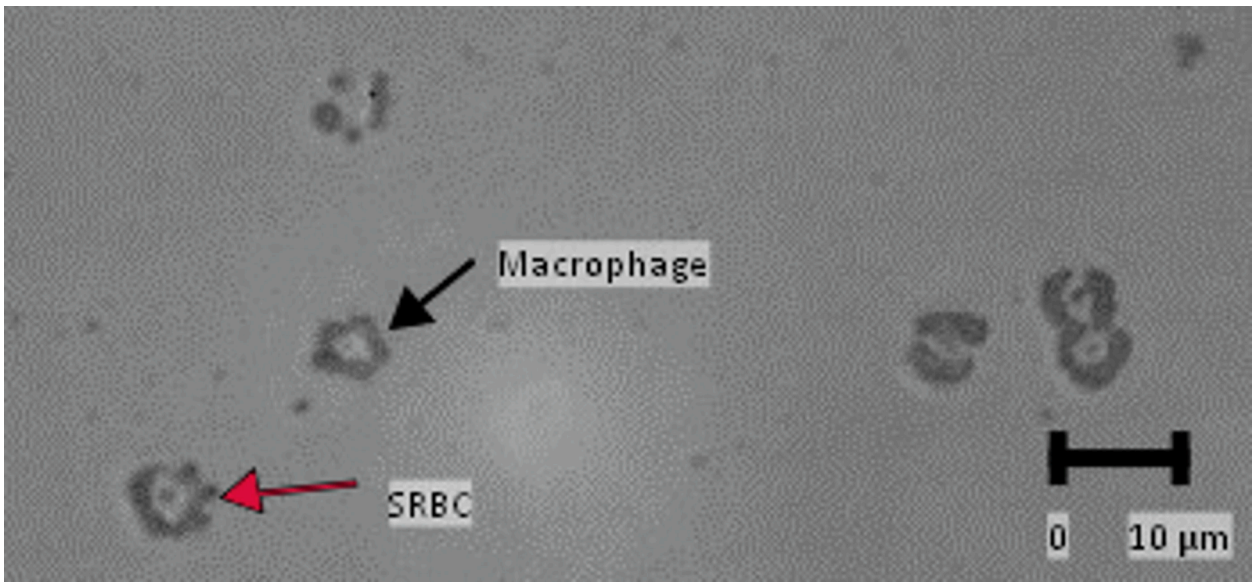

Fig. 3. Cultured macrophages were given SRBCs and LPSs with Giemsa staining (1000× magnification).

Table 5
A comparison of macrophage activity to oxidize antigens.

Group	The median activity of macrophages to oxidize antigens (absorbance, (min–max))	Statistical test (Mann-Whitney)
Healthy Spinal TB	0.491 (0.107–0.882) 0.134 (0.084–0.310)	($p > 0.05$)

Table 6
The NO levels in the healthy individuals and in the spinal TB group.

Group	Average NO levels (µmol/gr protein ± SD)	Statistical test (*t*-test)
Healthy Spinal TB	325.17 ± 182.9 2346.6 ± 468.6	($p < 0.05$)

Table 7
A comparison of myeloperoxidase activity.

Group	Myeloperoxidase activity (unit/100 mg protein ± SD)	Statistical test (*t*-test)
Healthy Spinal TB	17.4 ± 11 570.7 ± 56.1	($p < 0.05$)*

Table 8
A comparison of beta-glucuronidase activity.

Group	Beta-glucuronidase activity (µmol/hour/100 mg of protein (min–max))	Statistical test (Mann-Whitney)
Healthy Spinal TB	0.1226 (0.122–0.133) 0.1488 (0.146–0.162)	($p < 0.05$)*

Table 9
A comparison of the acid phosphatase activity.

Group	Acid phosphatase activity (µmol/hour/100 mg protein ± SD)	Statistical test (*t*-test)
Healthy Spinal TB	287.9 ± 156.5 1776.9 ± 319.2	($p < 0.05$)*

results across the two groups (Tables 5–9). The number of SRBCs that underwent phagocytosis by macrophages was lower for the spinal TB patients, and so was the average number of SRBCs that adhered to macrophages.

Adhesion is the first step of phagocytosis. The macrophage can phagocytose various particles, including foreign erythrocytes such as SRBCs, yeast, various bacteria, protozoa, viruses, and even neutral particle-like carbon or polystyrene. It is supposed that the particles adhere to macrophages by various mechanisms, mainly by Fc receptors. The receptor molecules recognize particles, including carbon, polystyrene, and SRBCs, that are coated by specific antibodies using an unclear mechanism. In this investigation, SRBC adhesion to macrophages was significantly lower in the spinal TB group than in the normal group. There were significant differences in the percentages of SRBCs that adhered to macrophages, and in the average number of SRBCs that adhered to macrophages (Tables 3 and 4). All the data suggested that spinal TB macrophages were less active in the first phase of phagocytosis.

In contrast, the intracellular phagocytosis activity was significantly higher in spinal TB macrophages. The WST test result, indicating oxidative activity, increased in both groups (Table 5). This phenomenon is called an oxygen burst and occurs in the phagosome [23]. The high levels of oxygen are used to form H_2O_2, which is the substrate of myeloperoxidase, whose activities were high in the TB patients (Table 7). According to Klebanoff, this enzyme forms ClO^- sourced from the H_2O_2 that is used to oxidize foreign bodies [24]. Nitric oxide is used by phagocytes to kill bacteria as part of innate immunity [25,26]. We also found that spinal TB macrophages produce a greater amount of this compound than control group (Table 6). Lysosomal enzyme activity was also high, as indicated by acid phosphatase and beta-glucuronidase (Tables 8 and 9). Both enzymes are known as lysosomal enzymes, organelle that are active in phagocytosis [27,28]. This organelle, containing the hydrolase class of enzymes, are essential in lysing foreign bodies, such as microorganisms that are engulfed during phagocytosis, and they form phagosomes that fuse with the lysosomes to form phagolysosomes. In a newly formed organelle, the foreign bodies are destroyed by oxidation as well as by enzymatic lysis.

All the findings and described processes are part of non-specific immunity. The entry place for most TB infection is the lung and most of the clinical symptoms are found in this organ. Lung macrophages are

essential in lung defense against various invasive microorganisms. Macrophages play a central role both in innate, non-specific immunity and in adaptive, specific immunity. The disruption of the mediator function may cause the macrophage defense function to be arrested during innate immunity. All the data in this study reflected innate, non-specific immunity; they are found in every acute phase of any infection. The strong activity and function related to phagocytosis do not definitely indicate a good defense. In granulomatosis, for instance, the activities of, and functions related to, phagocytosis are strong, but the patients are vulnerable to banal infection. If there is a defect in macrophageal function, the defense may persist only in the state of innate non-specific immunity. Apparently, in our spinal TB patients, the macrophages did not function properly, and the disease penetrated the defense and went further to other organs, including bone. Accordingly, we propose to investigate the causes of the defects in macrophageal function and to look for any methods to overcome this condition.

5. Authors' contribution

MDP contributed to the cell experiments and data analysis, AJR contributed to the conception, the design, and the data acquisition, SWA contributed to conception and the design, FCI contributed to the design, data analysis and to drafting the manuscript, and MS contributed to the conception and the design and to drafting the manuscript.

Note: MDP: Muhamad Dwi Putra; AJR: Ahmad Jabir Rahyussalim; SWA: Sri Widia A Jusman; FCI: Febriana Catur Iswanti; MS: Mohamad Sadikin.

CRediT authorship contribution statement

Muhamad Dwi Putra: Investigation, Software, Writing - original draft. **Ahmad Jabir Rahyussalim:** Resources, Supervision. **Sri Widia A. Jusman:** Conceptualization, Funding acquisition. **Febriana Catur Iswanti:** Methodology, Data curation, Writing - review & editing. **Mohamad Sadikin:** Funding acquisition, Writing - review & editing.

Conflict of interest

The authors declare no conflict of interest.

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