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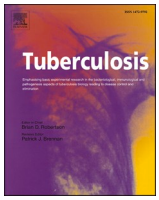

Lysosomal enzymes and the oxygen burst capability of monocyte-derived macrophages in active drug-resistant tuberculosis patients in relation to cell attachment

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ABSTRACT

Drug resistance to tuberculosis (TB) has become an obstacle in eliminating tuberculosis. The transmission of drug-resistant TB from patients increases the incidence of primary drug-resistant (DR) TB in individuals who are in close contact. Therefore, it is necessary to incorporate an immunological approach into preventive therapy. This study focuses on the activity of lysosomal enzymes, oxygen bursts, and the attachment ability of macrophages among individuals diagnosed with active drug-resistant TB compared with close contacts with latent TB or healthy cases. We measured macrophage oxygen burst ability (Water-soluble tetrazolium salt (WST) test, Nitric Oxide production, and myeloperoxidase activity) and the degradative ability of lysosomes (activity of the β-glucuronidase and acid phosphatase enzymes). Six active DR-TB patients and 18 close-contact cases (8 Latent Tuberculosis Infection (LTBI); 10 healthy) were recruited at Universitas Indonesia Hospital. The macrophage attachment of the LTBI group was higher than in the other groups. NO production, myeloperoxidase activity, β-glucuronidase, and acid phosphatase were higher in the active DR-TB group. A negative correlation was uncovered between phagocytosis and NO production, myeloperoxidase activity, and lysosomal enzymes. The difference in macrophage function is expected to be a further reference in active DR-TB treatment or preventive therapy.

1. Background

Tuberculosis (TB) is one of the most prevalent diseases caused by a single microorganism, inducing 1.4 million fatalities in 2021. The World Health Organization and the Indonesian government have established a goal to eliminate tuberculosis by 2030. However, this plan has faced significant obstacles, including the proliferation of drug-resistant tuberculosis cases. In 2021, there were 450,000 confirmed cases of multidrug- or rifampicin-resistant tuberculosis (MDR/RR-TB) worldwide, with 8,300 confirmed cases in Indonesia alone [1,2].

Mycobacterium tuberculosis causes tuberculosis, which is primarily transmitted through the inhalation of aerosols containing the bacteria. After inhalation, macrophages in the respiratory tract phagocytose the microorganisms. Subsequently, the macrophages eliminate pathogens by activating reactive oxygen species (ROS), nitric oxide synthase (NOS), and lysosomal enzymes. If macrophages effectively eliminate *Mycobacterium tuberculosis*, the majority of exposed individuals will remain healthy [3–5].

Mycobacterium tuberculosis can persist and proliferate within macrophages. At this juncture, the host immune system is believed to play a crucial role in disease progression. During bacterial phagocytosis, macrophages secrete various pro-inflammatory cytokines and

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chemokines, resulting in the formation of granulomas. The granulomas regulate the intracellular growth of *Mycobacterium tuberculosis* and restrict its spread. Ideally, if this process unfolds seamlessly, the individual will enter the latent infection stage (5–10% cases). However, if the granuloma response is insufficient, bacteria will continue to proliferate and exit the granuloma, resulting in tuberculosis in the host and the subsequent infection of the surrounding cells. Individuals who have had close contact with active TB cases are typically suspected of being infected, unwell, or afflicted with latent TB [5–7].

The transmission of drug-resistant TB from patients increases the incidence of primary drug-resistant TB in individuals who are in close contact. Therefore, an immunological approach is also a necessary part of preventive therapy. Consequently, this study focuses on the degradative ability of lysosomes (activity of the β-glucuronidase and acid phosphatase enzymes), WST assay, NO production, myeloperoxidase activity, and the phagocytosis ability of macrophages in individuals diagnosed with drug-resistant TB compared with close contacts diagnosed with latent TB or TB-free (healthy) cases.

2. Materials and methods

2.1. Subjects

Six confirmed active drug-resistant TB patients diagnosed via GeneXpert at the drug resistance clinic at Universitas Indonesia Hospital, Depok, agreed to participate by donating their blood for this study. All patients exhibited clinical symptoms and had rifampicin resistance based on sputum examinations using GeneXpert; their x-ray examinations also suggested TB infection. Eight people with latent tuberculosis infections who had histories of close contact with drug-resistant TB patients also participated (showing positive Interferon Gamma Release Assays (IGRA) examination results, while their chest x-ray, sputum, and clinical examinations were still within normal limits), as did 10 healthy people with a close-contact history with an infected person (clinical examinations, IGRA, sputum examinations, and chest x-rays prove that they were not infected). All subjects had no history of HIV, diabetes mellitus, allergies, malignant diseases, or immune disorders. The records regarding BCG vaccinations were unavailable. The ethics committee of the Faculty of Medicine, Universitas Indonesia, and Cipto Mangunkusumo Hospital approved the study (KET-987/UN2.F1/ETIK/PPM.00.02/2022).

2.2. Monocyte isolation and maturation

Blood was drawn from the cubital vein using heparin as an anticoagulant with a 20–22-gauge cannula. The blood was then diluted 1:1 with phosphate buffer saline (PBS) and thoroughly mixed in the centrifuge tube. The blood was slowly transferred to a new centrifuge tube containing Ficoll Plus (SolarBio). The tube was then centrifuged at 750×*g* for 30 min. After removing the plasma, the buffy coat containing isolated peripheral blood mononuclear cells (PBMCs) was suspended in sterile PBS. The PBMC pellet was resuspended in 6 mL of sterile Rosewell Park Memorial Institute 1640 medium (RPMI) (SolarBio) and disseminated using 1 mL per well with 10^6 cells in a six-well microplate. The plates were incubated in 5% CO_2 at 37 °C for 30 min. The entire medium was aspirated to remove all lymphocytes.

RPMI 1640 medium supplemented with 1% penicillin–streptomycin (Thermo), 1% amphotericin B (Biosera), fetal bovine serum (FBS) (Biosera) 5%, and macrophage colony-stimulating factor (MCSF) (Elabscience) (20 ng/mL) were added to culture plates containing adhered monocytes. The medium was replaced on days three and six. The culture medium was complete RPMI 1640 medium without MCSF; LPS (Santa Cruz-sc3535A) was added at 100 ng/ml; and the plate was incubated for 24 h. The macrophages were detached from the plate by adding TrypLE™ Select (THERMO) and counted with a trypan blue (Gibco)-stained hemocytometer. All the stained and non-stained cells in the chambers were enumerated, and the percentage of stained cells was determined; cell suspension with more than 95% viability was used for the culture.

2.3. The macrophage attachment test [8–10]

Attachment was determined when at least one sheep red blood cell (SRBC) was attached to the macrophage membrane. The percentage of attachment was determined by dividing the number of macrophages that had an SRBC attached by the total number of macrophages in the field. The number of sheep red blood cells attached by each macrophage cell was counted and divided by the total number of macrophage cells that had SRBC attached to their membranes. On a coverslip, 100 μl of macrophages in suspension were pipetted, followed by 250 μl of RPMI and 100 μl of a suspension of 2% SRBCs containing 100 ng/ml of LPS. The mixture was incubated in a CO_2 incubator at 37 °C for 30 min. The medium was eliminated and rinsed with PBS, and the adherent cells were fixated with methanol. The coverslip was air-dried and pigmented with 20% Giemsa.

2.4. WST-1 assay

A water-soluble tetrazolium salt-based WST-1 cell proliferation assay kit (Abcam) was used to assess the ability of macrophages to oxidize foreign bodies. The WST-1 can be reduced to a soluble formazan extracellularly by the plasma membrane NADH oxidase, involving superoxide [11]. 100 μl of macrophages (10 [5] cells) suspended in RPMI 1640 medium were pipetted into a 96-well microplate, incubated for 24 h, and 10 μl of the WST mixture was then added to each well. After 4 h of incubation in 5% CO_2 at 37 °C, the suspension was agitated using an orbital shaker for 1 min. Lastly, the solution's optical density (OD) at 450 nm was measured with a spectrophotometer.

2.5. The beta-glucuronidase activities assay [12]

The macrophages were lysed via three freezing and thawing cycles (−20 °C to 37 °C). As a substrate in this assay, p-nitrophenol-glucuronide (Sigma) was utilized. 10 μl of cell lysate was added to 25 μl (0.4 g/l) of the substrate, followed by 15 μl of sodium acetate buffer being added (0.1 M, pH 5) and incubation for 1 h at 37 °C. After incubation, 150 μl of glycine-NaOH was added, and the OD was measured at 410 nm. The data is presented in terms of enzyme units per milliliter of protein.

2.6. The acid phosphatase activity assay [13]

The substrate used was p-nitro-phenyl-phosphate (Sigma). Lysate from macrophages was added to 40 μl of substrate dissolved in citrate buffer (pH 4.8) and incubated at 45 °C for 15 min. After incubation, 0.1 M NaOH was added and the entire solution stirred gently. The OD examination was conducted at a wavelength of 410 nm. The test results are presented in terms of units of μmol p-nitro-phenyl-phosphate/hour/100 mg protein.

2.7. The nitric oxide assay

The nitric oxide assay kit (Elabscience®) determined the NO concentrations. One hundred microliters of cell lysate from 10^5 macrophages were added to a sulfate solution. After 15 min of incubation and 10 min of centrifugation at 3,100 rpm, chromogenic agents were added to each supernatant. At 550 nm, the OD was measured after 15 min of incubation. The results of the measurement are expressed in terms of mol/gram of protein.

2.8. Myeloperoxidase activity [14]

The macrophage lysate was subjected to a myeloperoxidase assay

using hydrogen peroxide (H_2O_2) (Emsure®) as a substrate and guaiacol (Sigma) as a chromogen. As an electron donor, the enzyme catalyzes the reduction of H_2O_2 using guaiacol. The final concentrations were 50 mM PBS, 100 mM guaiacol, and 0.0017% H_2O_2. One hundred 50 μL of reagent were pipetted into the well, followed by 1.75 μL of lysate. After 1 min of incubation, the OD at 470 nm was measured. The data is displayed in terms of enzyme units per milliliter.

2.9. The total protein assay

The total protein concentration was determined using the Warburg–Christian procedure. At 280 nm, absorbance was measured. Before the protein was measured, a standard curve was constructed using bovine serum albumin (Sigma).

2.10. Statistical analysis

The statistical analysis of each parameter's findings was represented graphically as a mean and standard deviation. The GraphPad Prism version 9.5.1 (733) was used to analyze the statistical data. The data distribution was analyzed using the Shapiro–Wilk test, while the homogeneity of variance was analyzed using Levene's test. An ANOVA test was conducted for normally and homogeneously distributed data, while a Mann–Whitney test was conducted for non-normally distributed data.

3. Results

3.1. The characteristics of the subjects

The 24 subjects were divided into three groups: six in the active drug-resistant tuberculosis (active DR-TB) group, eight in the latent infections (LTBI) group, and 10 in the healthy group. Active drug-resistant TB patients were newly diagnosed with GeneXpert, and the results demonstrated TB rifampicin resistance. Active drug-resistant TB patients were new active cases, exhibiting clinical symptoms and their x-ray examinations also suggesting TB infection. All patients in the LTBI group did not show any clinical symptoms; thus, we could not conduct any sputum examinations. There were more females than males in the latent TB group. The mean age in the active DR-TB group was 30.50 ± 8.67 years; for the LTBI group, it was 29.87 ± 16.97 years; and for the healthy group, it was 36.70 ± 18.98 years. The mean BMI in the active DR-TB group was 17.09 ± 3.28, which was categorized as mildly underweight. There was no difference in the smoking history between these groups (Table 1).

3.2. Isolated peripheral blood mononuclear cell (PBMCs) count and macrophage cells count

The number of isolated PBMCs in the active DR-TB group was lower ($6.63.10^6 \pm 0.17.10^6$ cells/ml) compared to the healthy group ($8.49.10^6 \pm 0.17.10^6$ cells/ml) ($p < 0.03$). The mean number of macrophages cultured in the active DR-TB group ($6.52.10^5 \pm 0.22.10^5$ cells/ml) was lower than the number cultured in the latent infection group ($10.24.10^5 \pm 0.74.10^5$ cells/ml) and the healthy group ($8.98.10^5 \pm 0.19.10^5$ cells/ml) ($p < 0.001$; $p < 0.03$). There was no difference in the percentage of differentiation among these three groups (Figs. 1 and 2).

Table 1
Characteristics of subjects.

Characteristics	DR-TB (n = 6)	LTBI (n = 8)	Healthy (n = 10)	p value
Age (years±SD)	30.5 ± 8.67	29.87 ± 16.97	36.7 ± 18.98	>0.05[a]
Sex (%)				
Male	50%	37.5%	50%	>0.05[b]
Female	50%	62.5%	50%	
BMI (±SD)	17.09 ± 3.28	20.22 ± 3.60	21.37 ± 3.17	>0.05[a]
Smoking (%)				
No	50%	62.5%	60%	>0.05[a]
Yes	50%	37.5%	40%	

DR-TB: Drug Resistant Tuberculosis, LTBI: Latent Tuberculosis Infection, BMI: Body Mass Index.
[a] One-way Anova.
[b] Kruskall-Wallis.

3.3. Macrophage attachment

The mean number of macrophages that attached SRBCs per 100 macrophage cells in the active DR-TB patients (30.79 ± 2.15 cells) was lower than in the LTBI group (48.63 ± 10.39 cells) and the healthy group (46.23 ± 1.59 cells) ($p < 0.001$). The number of SRBCs that attached to macrophages was also counted. The average number of SRBCs that attached to macrophages in the LTBI group was higher (2.03 ± 0.33 cells) than among active DR-TB patients (1.53 ± 0.25 cells) but slightly lower than in the healthy group (2.92 ± 0.42 cells) ($p < 0.05$; $p < 0.001$) (Figs. 3 and 4).

3.4. WST-1 assay

The measurement of the macrophage's NADH oxidase activity was assessed via WST assay immediately after the cells were harvested. The mean absorbance value in the active DR-TB group (0.27 ± 0.89) was lower than in the LTBI group (0.55 ± 0.20) and the healthy group (0.73 ± 0.18) ($p < 0.05$; $p < 0.001$) (Fig. 5a). The correlation test results between the attachment per 100 macrophage cells and the absorbance value of the WST test indicated a strong and significant positive relationship (Pearson correlation, $r = 0.67$, $p < 0.005$). The correlation test results between the number of SRBCs that were attached and the WST test results also demonstrated a strong positive and statistically significant relationship (Pearson correlation, $r = 0.64$, $p < 0.005$) (Fig. 5b–c).

3.5. β-Glucuronidase enzyme activity

The average activity of the β-glucuronidase enzyme in the active DR-TB group was higher (0.39 ± 0.29 U/mg protein) than in the healthy group (0.19 ± 0.16 U/mg protein) ($p < 0.05$) (Fig. 6a). The test results regarding the correlation between the attachment per 100 macrophage cells and the β-glucuronidase enzyme activity indicated a strong and significant negative relationship (Pearson correlation, $r = -0.511$, $p < 0.05$, $p = 0.011$). The test regarding the correlation between the number of SRBCs that were attached also demonstrated a strong and significant negative relationship (Pearson correlation, $r = -0.57$, $p < 0.005$) (Fig. 6b–c).

3.6. Acid phosphatase enzyme activity

The average activity of the acid phosphatase enzyme in the active DR-TB agroup was higher (7.36 ± 2.11 U/mg protein) than the activity in the healthy group (4.41 ± 1.51 U/mg protein) ($p < 0.05$) (Fig. 7a). The test results for the correlation between the attachment per 100 macrophage cells and the acid phosphatase enzyme activity indicated a weak, negative, and insignificant correlation (Pearson correlation, $r = -0.23$, $p > 0.05$). The test results regarding the correlation between the number of SRBCs that were attached and the activity of the acid phosphatase enzyme demonstrated a moderate and significant negative relationship (Pearson correlation, $r = -0.45$, $p < 0.03$) (Fig. 7b–c).

3.7. Nitric oxide levels

Upon examination, it was found that there was no significant difference between the average NO level in the active DR-TB group (117.72 ± 23.22 μmol/gr protein), the LTBI group (100.86 ± 28.89 μmol/gr protein), and the healthy group (81 .35 ± 29.12 μmol/gr protein) (one-

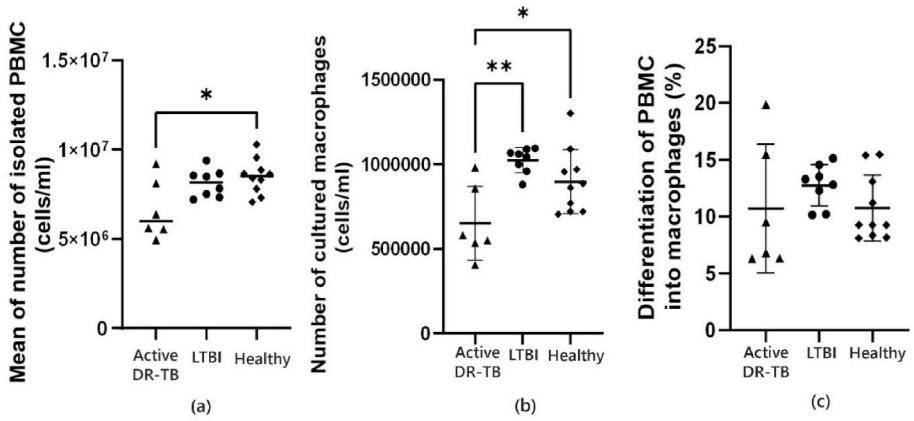

Fig. 1. (a) Mean number of cultured macrophages (cells/ml); (b) mean number of isolated PBMC (cells/ml); (c) differentiation of PBMC into macrophages (%). **p < 0.001, *p < 0.03 (*one-way* ANOVA, α 0.05).

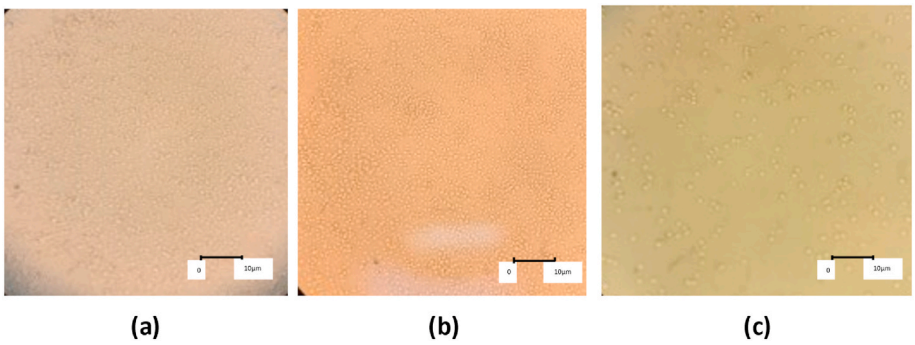

Fig. 2. Macrophages cultured from isolated monocytes using a culture medium and MCSF (100 × magnification). A) Day 0 culture; B) day 3 culture; C) day 7 culture.

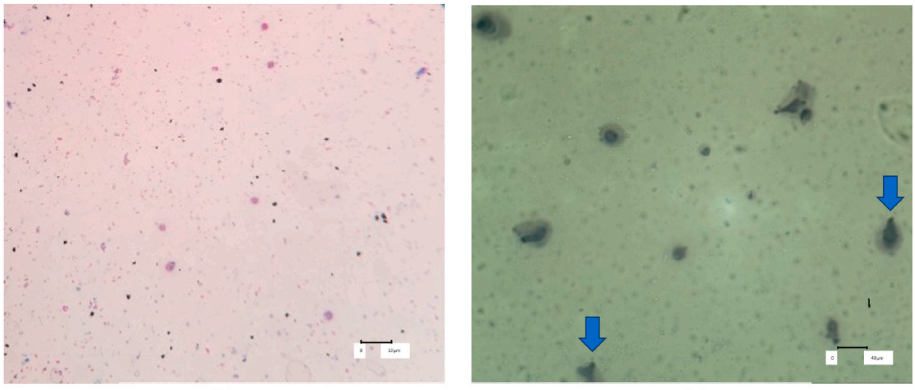

Fig. 3. Cultured macrophages attached to SRBCs after macrophages were given SRBCs and LPSs (a) 100× magnification (b) 400× magnification.

way ANOVA, p > 0.05) (Fig. 8a). The results of the test regarding the correlation between the attachment per 100 macrophage cells and the NO levels showed a moderate negative relationship and were not significant (Pearson correlation, r = −0.4, p = 0.05). The test results regarding the correlation between the number of SRBCs that were attached and the NO levels indicated a strong and significant negative relationship (Pearson correlation, r = −0.54, p < 0.005) (Fig. 8b–c).

3.8. Myeloperoxidase enzyme activity

The average activity of the myeloperoxidase enzyme in the active DR-TB group (395.14 ± 118.55 U/mg protein) was higher than in the healthy group (229.24 ± 75.33 U/mg protein) (p < 0.05) (Fig. 9a). The test results regarding the correlation between the attachment per 100 macrophage cells and myeloperoxidase enzyme activity demonstrated a moderate and significant negative relationship (Pearson correlation, r = −0.42, p < 0.05). The test regarding the correlation between the number of SRBCs that were attached and the myeloperoxidase activity also indicated a moderately negative but insignificant relationship (Pearson correlation, r = −0.37, p > 0.05) (Fig. 9b–c).

4. Discussion

M. tuberculosis is an intracellular pathogen transmitted via the inhalation of aerosol droplets containing bacteria. The immune system in the lungs, particularly macrophages, dendritic cells, monocytes, and

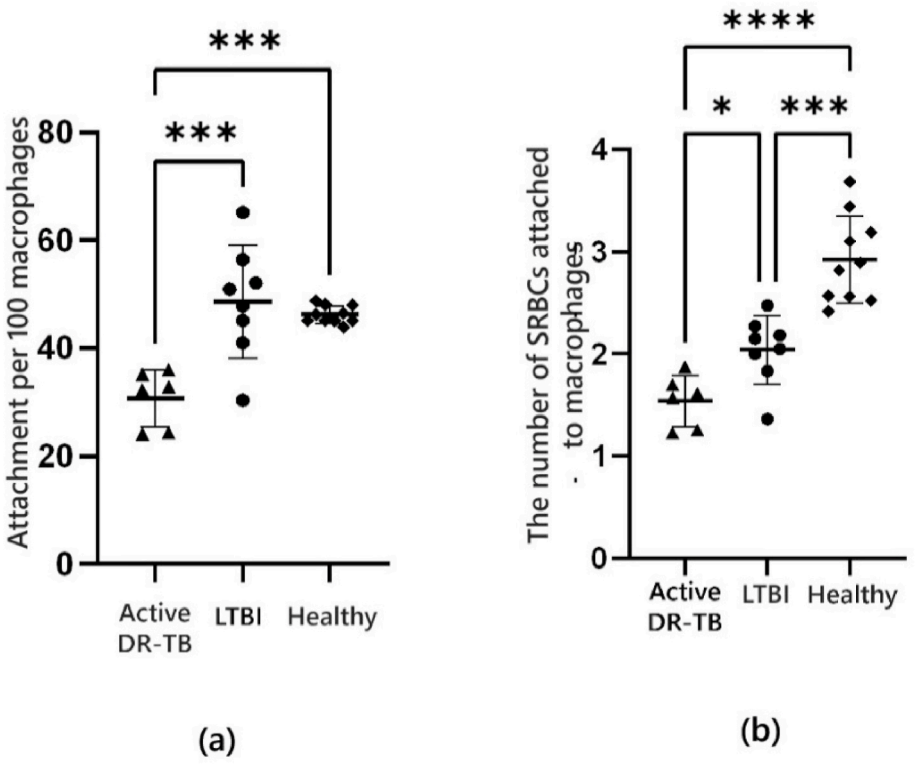

Fig. 4. (a) The number of attachments per 100 macrophages (b) the number of SRBCs attached per macrophage ****p < 0.0005, ***p < 0.001, *p < 0.05 (*one-way* ANOVA, α 0.05).

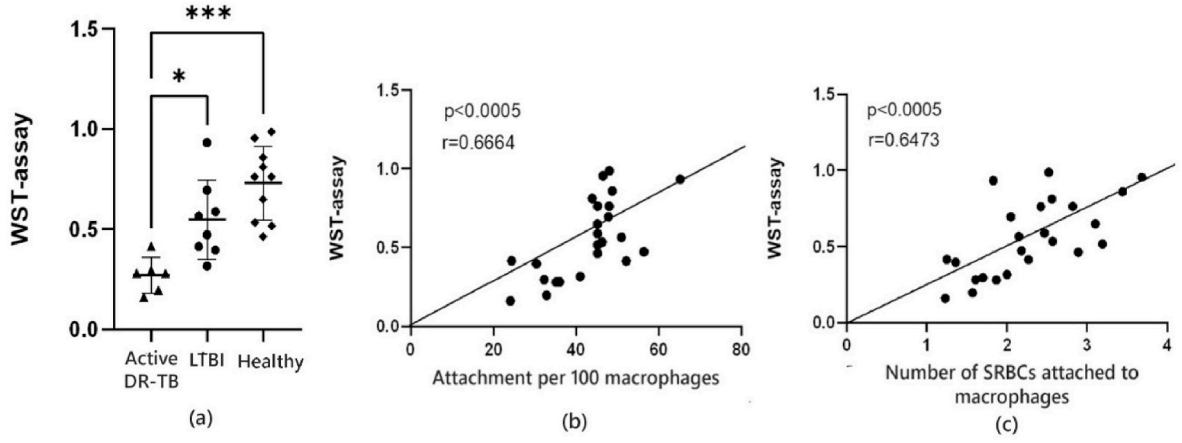

Fig. 5. (a) WST-assay (***p < 0.001, *p < 0.05 (*one-way* ANOVA, α 0.05)); (b) correlation of the number of attachments of macrophages with WST values; (c) correlation of the number of SRBCs attached to macrophages with WST values (Pearson correlation, α: 0.05).

neutrophils, is ready to eliminate these bacteria. In the initial innate immune response, resident macrophages are recruited to the site of infection (where phagocytosis will occur) to eliminate these bacteria and subsequently activate the adaptive immune system. Macrophages eliminate bacteria in various ways, such as via the activation of ROS and NOS and with the help of lysosomal enzymes. During phagocytosis, macrophages release pro-inflammatory cytokines and chemokines, which aim to recruit other immune cells to form granulomas. This process aims to limit the growth of *M. tuberculosis*; if the bacteria can be eliminated, the individual will remain healthy. However, *M. tuberculosis* can persist in the granuloma so that the individual will develop a latent infection. *M. tuberculosis* can also emerge from the granuloma, and the host will experience TB disease and can transmit it to close contacts. Mutations in chromosomes cause the resistance of M. tuberculosis to anti-tuberculosis drugs. This increase in the incidence of drug-resistant TB is associated with a more extensive hospital stay, longer treatment, higher expenses, and exacerbated mortality. Transmission through droplets from DR-TB patients will increase the incidence of new drug-resistant TB infections [15–19].

In this study, the characteristics of the active DR-TB group were characterized by a lower average BMI and were classified as mildly underweight. Inadequate nutritional status (malnutrition) has been associated with many risks of infection, including tuberculosis, and tuberculosis itself can also contribute to malnutrition. Malnutrition can cause secondary immunodeficiency, which causes individuals to be more susceptible to disease. The risk of being infected with tuberculosis increases as the protective function of immune cells such as macrophages and T cells decreases. Research concerning guinea pigs has

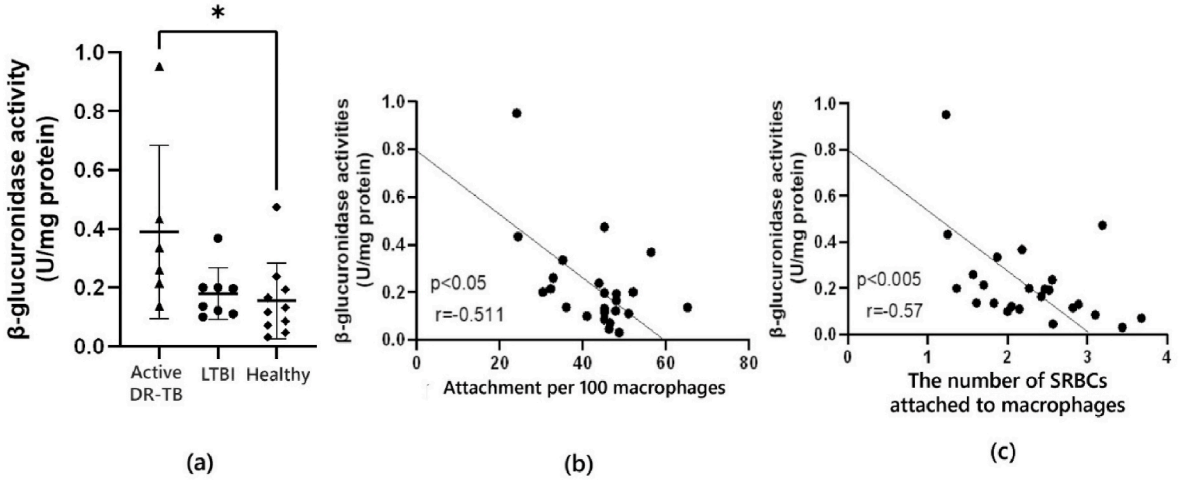

Fig. 6. (a) β-glucuronidase activity (U/mg protein) (*p < 0.05 (one-way ANOVA, α 0.05); (b) correlation of the number of attachments of macrophages with β-glucuronidase activity (U/mg protein); (c) correlation of the number of SRBCs attached to macrophages with β-glucuronidase activity (U/mg protein) (Pearson correlation, α: 0.05).

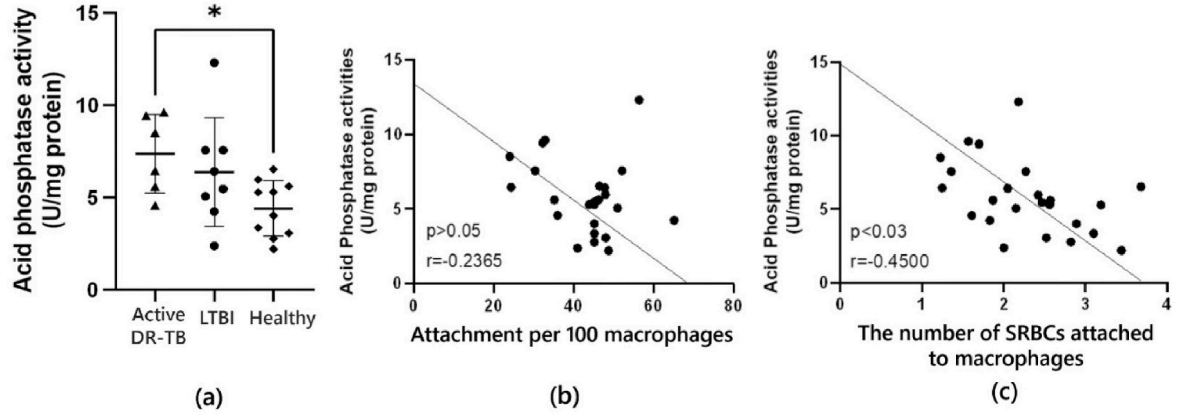

Fig. 7. (a) Acid phosphatase activity (U/mg protein) *p < 0.05 (one-way ANOVA, α 0.05); (b) correlation of the number of attachments of macrophages with acid phosphatase activity (U/mg protein); (c) correlation of the number of SRBCs attached to macrophages with acid phosphatase activity (U/mg protein) (Pearson correlation, α: 0.05).

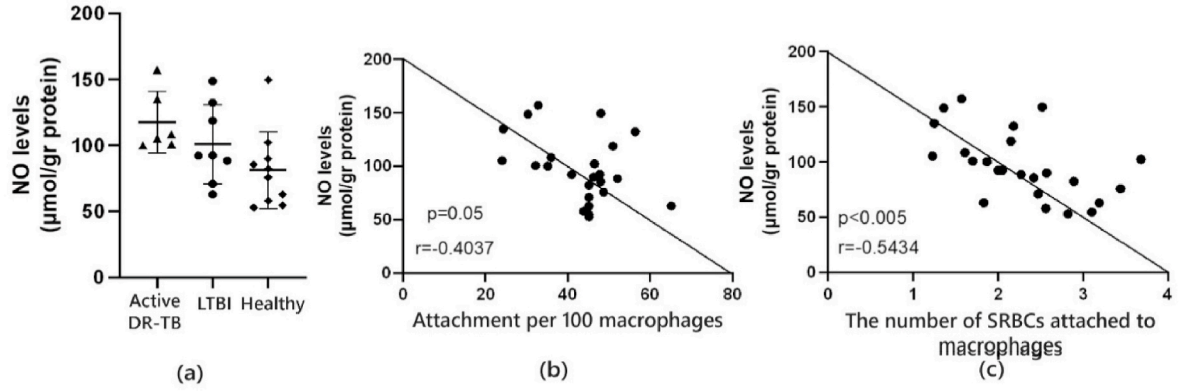

Fig. 8. (a) NO levels (μmol/gram protein) (p > 0.05, *one-way* ANOVA, α 0.05); (b) correlation of the number of attachments of macrophages with levels of NO production (μmol/gr protein); (c) correlation of the number of SRBCs attached to macrophages with the level of NO production (μmol/gr protein) (Pearson correlation, α: 0.05).

suggested that protein malnutrition reduces the protective role of macrophages and T cells against mycobacteria [20–24].

The number of PBMCs in the active DR-TB group was lower than in the healthy groups. The number of cultured macrophages also differed in each group; specifically, it was lower in the active DR-TB group than in the other two groups. The percentage of differentiation across all

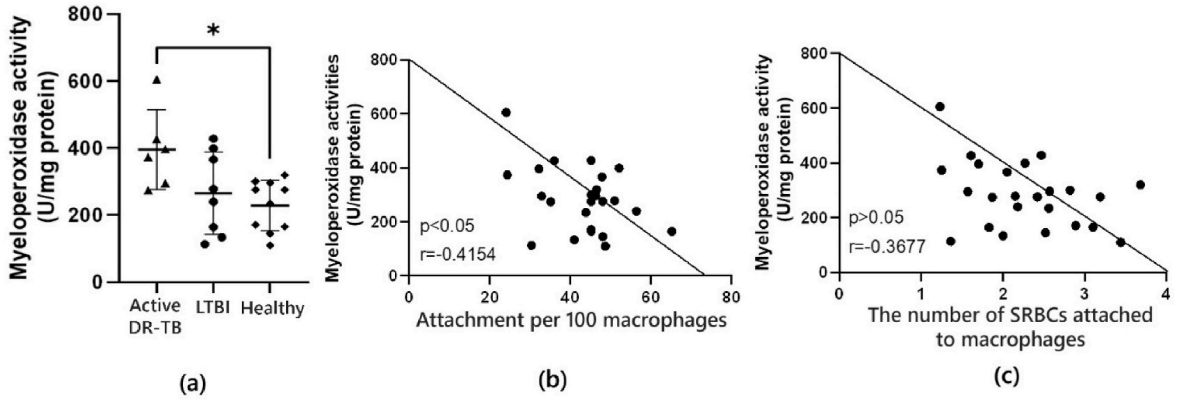

Fig. 9. (a) Myeloperoxidase activity (U/mg protein) *p < 0.05 (one-way ANOVA, α 0.05); (b) correlation of the number of attachments of macrophages with myeloperoxidase activity (U/mg protein); (c) correlation of the number of SRBCs attached to macrophages with myeloperoxidase activity (U/mg protein) (Pearson correlation, α: 0.05).

groups was not significantly different. This characteristic was also uncovered in a study by Putra et al., wherein the average monocyte isolated from bone TB patients (drug-sensitive TB) was lower than in healthy controls [25]. Several studies have demonstrated that in active and latent TB patients, there is an increased percentage of intermediate monocytes ($CD14^{+}CD16^{+}$), which can differentiate into macrophages upon their development. Nevertheless, Castano et al. found that in the TB patient group, $CD16^{+}$ has a limited number of markers related to maturation and differentiation, such as CD11b, CD11c, CD33, and CD36, thereby inhibiting the process of differentiation into macrophages [26,27].

The lower number of peripheral blood monocytes isolated in the active DR-TB group compared to other groups could be caused by chronic infections or secondary malnutrition conditions that afflicted participants. Apart from quantity, a decrease in phagocytic function also occurred in the active DR-TB group; thus, it can be concluded that there was a deterioration in the ability of the immune system both in terms of quantity and quality. This was influenced by several factors, as previously explained, such as malnutrition due to chronic infections and a shift in macrophage polarization to M2, which tends to be non-inflammatory [28,29].

Phagocytosis is composed of two steps; the first is the attachment on the surface of the macrophage, and the second step is engulfment by the macrophage. The first step is believed to be largely an immunological phenomenon through the presence of receptors on the surface of the macrophage [30]. In this study, the attachment of SRBC to macrophages was one of the determinants of the phagocytosis capability. The results of the attachment test demonstrated that the phagocytic ability of the active DR-TB group was lower than that of the latent and healthy group ($p < 0.005$). The observations also revealed that the mean number of SRBCs that were attached to macrophages in the active DR-TB group was lower than in the latent infection and healthy groups, respectively ($p < 0.05$). The number of macrophages that attached SRBCs per 100 macrophage cells in the latent infection group was not significantly different compared to the healthy group. In comparison, the number of SRBCs that were attached was lower than in the healthy group. The same phenomenon was demonstrated in a study by Putra et al.; the phagocytic activity of bone TB patients was lower than that of the healthy group [25]. During their maturation, macrophages can differentiate into several phenotypes, such as M1 and M2. M1 macrophages are also classic pro-inflammatory macrophages, whereas M2 macrophages are better known as non-inflammatory macrophages with lower antigen-presenting cell properties. A study conducted by Lastrucci et al. demonstrated that macrophages from tuberculosis patients differentiate toward M2; this could be one of the factors causing the difference in phagocytosis activity in the active DR-TB group compared with the other two groups [31]. However, this study did not analyze the population of macrophage subtypes (M1 and M2).

Phagosomes containing pathogens subsequently undergo maturation via fusion with lysosomes, which contain various enzymes, such as nuclease, protease, phosphatase, and others [32,33]. Beta-glucuronidase and acid phosphatase can represent the functions of these multiple enzymes. In this study, there was a significant difference in the activity of the β-glucuronidase enzyme between the active DR-TB and healthy groups ($p < 0.05$). The β-glucuronidase activity of the active DR-TB group was higher than that of the other two groups. The increased activity of this enzyme is believed to be related to ongoing infection. It has been reported that the beta-glucuronidase enzyme exhibits increased activity in the peritoneal fluid of patients infected with bacteria and in the cerebrospinal fluid of patient with bacterial meningitis [34,35]. The test regarding the correlation between the activity of this beta-glucuronidase enzyme and the macrophage attachment demonstrated a strong and significant negative relationship (Pearson correlation, $r = -0.511$, $p < 0.05$). The average acid phosphatase activity in the active DR-TB group was higher than in the healthy group. The findings indicating an increase in these enzymes are believed to be related to the patient's infection status. This result was also found in the previous study conducted by our research group, wherein the activity of the beta-glucuronidase and acid phosphatase enzymes was higher in the bone TB group than in the healthy group [25]. This enzyme increased in the alveolar macrophages of subjects who smoked but decreased in patients with sarcoidosis [36]. To our knowledge, no other previous studies have revealed a correlation between these parameters.

Meanwhile, the acid phosphatase enzyme activity indicated a weak negative relationship and was not related to macrophage attachment (Pearson correlation, $r = -0.23$, $p > 0.05$). This result contrasts with the correlation between acid phosphatase enzyme activity and the number of SRBCs that were attached to macrophages, suggesting a moderate and significant negative relationship (Pearson correlation, $r = -0.45$, $p < 0.03$). This finding aligns with some of the previous results, confirming the negative correlation between phagocytosis activity and macrophage activity; in this instance, it was assessed based on the activity of the lysosomal enzymes [25].

After macrophages phagocytose pathogens, ROS production will occur, mediated by NOX2 NADPH oxidase. Superoxide anion will be released into the lumen of the phagosome containing the pathogen, which will cause an increase in oxygen consumption by macrophages, hereafter referred to as an oxygen burst. Therefore, the next step will involve measuring the ability of macrophages to oxidize antigens using the WST test. This assay will align with macrophage cells' capacity to produce O^{-} in the oxygen burst process via the ability to reduce NADPH oxidase in macrophages through the WST-1 test [37–39]. The mean WST

in the active DR-TB group yielded the lowest results compared with the other two groups. These results are consistent with a study conducted by Putra et al., which also revealed that the WST values in the healthy group were higher than in the group with bone TB patients [25]. Tetrazolium was converted to formazan by cellular enzymes at a lower rate than the other two groups. Sasada found that the activity of macrophages was increased with LPS-elicitation or BCG-activation, affecting candida-killing ability (two to five times), their activity was inhibited by scavenger superoxide dismutase (SOD) and catalase enzymes. This experiment illustrates the importance of O_2^- and H_2O_2 in macrophage-killing activity [40]. The correlation test between the attachment of macrophages and the WST test revealed a strong and significant positive relationship, wherein the reduction ability of macrophages was proportional to their power to phagocytize antigens. The decrease in attachment and the ability to oxidize antigens in active DR-TB is consistent with a study conducted by Sampath et al., who found a decrease in HLA-DR expression in active DR-TB monocytes compared with latent infections and drug-sensitive TB groups. HLA-DR expression in monocytes is closely related to cells' antigen-presenting ability [41].

Aside from producing ROS, macrophages will also produce reactive nitrogen intermediates, such as nitric oxide (NO), by activating nitric oxide synthase (NOS2). The mean NO level in the active DR-TB group exhibited no significant difference from the other group ($p > 0.05$). NO production increase in patients with active tuberculosis and its decrease in patients who have been treated have also been highlighted previously by Kumar et al. [42,43] The correlation test between NO levels and the degree of attachment per 100 macrophages demonstrated a moderately negative relationship and was insignificant statistically (Pearson correlation, $r = -0.4$, $p = 0.05$). In contrast, the correlation between NO levels and the number of SRBCs that were attached to macrophages suggested a strong and significant negative relationship (Pearson correlation, $r = -0.54$, $p < 0.005$). This result indicates that the number of macrophage attachments and the number of SRBCs that were attached to macrophages are inversely proportional to the level of NO produced. This finding aligns with previous research, which demonstrated a strong and significant negative relationship between phagocytosis and nitric oxide levels. NO is known to have the capacity to induce protein ADP-ribosylation, which plays a role in actin polymerization, thereby influencing the formation of pseudopodia and phagocytosis. Jun et al. investigated the relationship between NO production by macrophages and phagocytosis through the induction of ADP-ribosylation and found that macrophages that produced large amounts of nitrite after being induced by LPS and IFN-γ exhibited a decline in phagocytosis compared with controls [44].

In addition to NO, myeloperoxidase enzymes also play a role in oxygen burst events as part of the macrophage phagocytosis process. This enzyme oxidizes substrates through a peroxidation process, requiring H_2O_2 produced by NADPH oxidase and halide compounds (such as chloride) to produce strong oxidants, such as hypochlorous acid, which has strong bactericidal capabilities. This increase in myeloperoxidase enzyme activity can indicate macrophage activity, specifically in the form of eliminating antigens or pathogens [45,46]. In this study, the mean myeloperoxidase enzyme activity in the active DR-TB group was higher than in the latent and healthy groups ($p < 0.05$). MPO exerts a microbicidal activity against *M. tuberculosis*. To fully eliminate *M. tuberculosis* growth, it required a higher MPO concentration compared to other mycobacterium (*M. leprae*) [47]. The correlation test between myeloperoxidase activity and the macrophage attachment demonstrated a moderate and significant negative relationship ($r = -0.42$, $p < 0.05$). Nevertheless, the test examining the correlation between myeloperoxidase activity and the number of SRBCs that were attached to macrophages uncovered a moderately negative relationship that was not significant ($r = -0.37$, $p > 0.05$). This result is slightly different from that yielded by the study conducted by Putra et al., which revealed a strong negative correlation between the degree of phagocytosis per 100 macrophage cells and the activity of the myeloperoxidase enzyme. Lefkowitz also reported that the increased concentration of recombinant MPO affects the phagocytosis ability of resident macrophages against candida in the murine model [48]. In the long term, myeloperoxidase deficiency will cause compensation, including increased phagocytosis [25,26].

This study has demonstrated differences in macrophage function in the active DR-TB group compared with the latent TB infection and healthy group. The phagocytic ability and oxidizing antigens (NADPH oxidase activity) in the active DR-TB group decreased compared to the latent infection group and the healthy group. However, the NO production and myeloperoxidase enzyme activity in the active DR-TB group was still higher than in the healthy groups; additionally, the activity of lysosomal enzymes exhibited no significant difference compared to the latent infection group. In the latent infection group, it was found that the number of macrophage cells that attached SRBCs was higher than in the active DR-TB group. However, the number of SRBCs that were attached to macrophages was still lower than in the healthy group but higher than in the active DR-TB group. The ability to oxidize antigens (NADPH oxidase activity) in the latent TB group was lower than in the healthy group but higher than in the active DR TB group. NO production in latent infections exhibited no significant difference compared to the healthy group and the active DR-TB agroup. The same results also emerged in relation to myeloperoxidase and lysosomal enzyme activity.

This difference in results is believed to be because in latent infections, the immune cell system has been activated in response to the presence of *M. tuberculosis*, characterized by an increase in the ability of macrophage to attach, activity of oxygen burst (NADPH oxidase, NO, and myeloperoxidase), and lysosomal enzymes. Meanwhile, in the active DR-TB group, there was a decrease in the attachment and antigen oxidation ability, although the activity of NO, myeloperoxidase, and lysosomal enzymes increased. This change in function is believed to be related to the ability of *M. tuberculosis* to influence macrophage differentiation polarization. Various studies have demonstrated that, in active TB infection and latent TB, there is an increase in the percentage of CD16 monocytes (intermediate) compared to healthy groups. The function of monocytes is associated with ROS production, phagocytosis, antigen presentation to T cells, inflammatory responses, and angiogenesis. However, research by Castano et al. found that, although there was an increase in the percentage of intermediate monocytes in active TB infection, these monocyte cells were unable to differentiate into macrophages, and there was a decrease in HLA-DR expression on the cell surface; as a result, their phagocytic ability decreased. Sampath et al. also found that there were differences in the expression on DR-TB monocytes (CD16, HLA-DR, CD64, and CD86) compared to drug-sensitive TB and latent infections, which is believed to be related to the inability to activate monocytes and to activate adaptive immune cells to fight TB infection [26,27,41,49].

This study still has drawbacks, such as not examining macrophage polarization, which, in theory, affects the activity and function of macrophages in the active DR-TB group and latent infections. The activity and function of macrophages are influenced by various factors, one of which is the presence of cytokines. In this study, no examination of the cytokines produced by these macrophages was performed. This study also did not conduct a full phagocytosis test; we simply demonstrated the attachment of macrophages to SRBCs as one of the steps of phagocytosis. This study also did not compare the drug-sensitive and drug-resistant tuberculosis macrophages. Although the study concerning bone tuberculosis (extra pulmonal-drug sensitive) also yielded similar results, we did not have data from pulmonary drug-sensitive tuberculosis.

In conclusion, this study demonstrated increasing lysosomal enzyme and oxygen burst activity in macrophages from drug-resistant tuberculosis patients and a negative correlation with macrophage attachment. Future studies should further research other factors involved in the activity of macrophages, such as the ability to produce cytokines and the characteristics and phenotypes of macrophage subtypes in drug-

resistant TB infections and latent infections.

CRediT authorship contribution statement

Febriana Catur Iswanti: Methodology, Funding acquisition, Conceptualization. **Kurnia Maidarmi Handayani:** Writing – original draft, Investigation, Data curation. **Ardiana Kusumaningrum:** Visualization, Resources, Methodology. **Tomohiko Yamazaki:** Writing – review & editing, Formal analysis. **Diah Handayani:** Validation, Project administration, Methodology, Investigation. **Mohamad Sadikin:** Validation, Supervision, Conceptualization.

Declaration of competing interest

We have no conflicts of interest to disclose.
All authors declare that they have no conflicts of interest.

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