RESEARCH ARTICLE

Well-organized Granuloma Lymphadenitis Tuberculosis Expressed Lower Macrophage Migration Inhibitory Factor (MIF) Score Compared to the Poorly-organized Granuloma

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Abstract

BACKGROUND: The case of extra-pulmonary tuberculosis (EPTB) is common and the most type of extra-pulmonary tuberculosis found is lymphadenitis TB (LnTB). Macrophage migration inhibitory factor (MIF) is correlated with TB, and low level of MIF was correlated to *Mycobacterium TB* bacteremia. Deficiency of MIF macrophage is known to be correlated to the increased of a lung pathology; however, its role on pathogenesis LnTB remains unclear. Hence, this study was conducted to analyze the correlation of MIF in several type of granuloma organization in LnTB.

METHODS: Block paraffin of the lymphoid tissue infected with *M. Tuberculosis* were analyzed with immunohistochemistry (IHC) to assess the MIF expression, by counting the immunoreactivity score (IRS) according to the intensity of stained cells and the level of staining. The histopathology type of LnTB was divided into well-organized granuloma (WOG) and poorly-organized granuloma (POG) based on the granuloma characteristics.

RESULTS: Among 100 tissues samples that fulfilled the study criteria, WOG was found in 51% cases. MIF was expressed mild positive in 21% samples, on the other hand, 79% was not expressed. There was a significant difference of MIF negative, as was found in 98% of WOG group while only 59% was found in POG group ($p<0.001$).

CONCLUSION: There is a significant correlation between MIF expression with the type of granuloma organization in LnTB. The expression of MIF in WOG group is mostly negative, as well as a half of the POG group. This results may suggests that MIF plays a role in the pathogenesis of granuloma formation in LnTB.

KEYWORDS: MIF, lymphadenitis TB, WOG, POG, immunohistochemistry

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Introduction

According to World Health Organization (WHO), lungs tuberculosis (TB) remains as a major health problem in the world.(1) Asia and Africa region, including Indonesia, are region with the highest new TB cases globally.(2) TB could also infect the extrapulmonary site, including regions in the head and neck.(3,4) The most frequent type of extrapulmonary TB (EPTB) is lymphadenitis TB (LnTB), which especially occur on the neck area.(5,6) A study in Ethiopia found that 65% of 3440 neck node was LnTB.(6) According to histopathology appearance, the type of LnTB in Indonesia was dominated by well-organized granuloma (WOG) and followed by poorly-organized granuloma (POG).(5)

The formation of granulomas in TB indicates the failure of immune system against infection, even with the presence of these granulomas, the bacteria can survive longer (latent).(7) In TB granuloma, there is a local immunoregulatory program that indicates active TB infection.(8) Thus, the better the organization of the granuloma, the worse the host defense system is. According to histopathology examination, the organization of the granuloma is important for the diagnosis of TB. In deciding the diagnosis of TB, the pathologist is more confident when found WOG criteria compare to POG. In addition to histopathological methods, molecular methods can be used to further confirm the diagnosis of patients with TB. Rapid molecular test with Gene-Xpert obtained higher sensitivity (93 *vs.* 62%) but lower specificity (68 *vs.* 83%) when compared to histopathological examination in TB diagnosis. (9) Interferon (IFN) inducible protein 10 (IP-10) has a high diagnostic accuracy with sensitivity 92% and specificity 91.9%. However, it can not be used to differentiated TB infection from TB disease.(10)

There are several factors that may affect the recovery or severity of an EPTB patient, including internal factors such as genes that affect the patient's body defense, nutritional status, and adherence to medication. HIV known as a viral infection that affect the body defense, was also documented as one of influencing factors.(11) Meanwhile, external factors that affecting the recovery of an EPTB patient are the environment as well as the resistance towards Mycobacterium TB (MTB). A prediction test results show that out of 19 subjects, 47% were multidrug resistant-TB. (12) Due to those problem, biomolecular aspect of LnTB needs to be explored in order to increase the understanding on factors that influenced diagnosis and management of this case. One of the important research focuses is the genes or cytokine that affect the patient's body defense to MTB in LnTB.(13,14) These cytokines include the macrophage migration inhibitory factor (MIF) that affects macrophages. (15,16)

MIF is produced and secreted mainly by the immune cells. MIF has the ability to activate T cells and macrophages to produce other proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1β, IL-2, IL-6, IL-8, and IFN.(17) Low level of MIF gene is more frequently identified in patients with MTB bacteremia. MIF-deficient macrophages could lead to increased lung pathology, decreased production of innate cytokines and reactive oxygen species, and impaired mycobactericidal properties. MIF also has a role as direct chemokines and promotes cell recruitment. Various clinical studies have demonstrated the utility of MIF as a biomarker for various inflammations.(16) MIF is considered to be the main mediator that reverses the inhibitory effects of glucocorticoids on the immune system. According to that role, for the treatment of inflammatory and autoimmune diseases, MIF could be used as a therapeutic target.(18,19) MIF was identified as an important factor in the innate immune response to mycobacteria and revealed that the common low-expression MIF allele confer to the increased risk of TB infection in some populations.(19) In the case of TB in human, MIF plays a cardinal role in the innate immune defense against MTB. It works through paracrine and/or autocrine pathways to detained the growth of lethal mycobacteria. The serum MIF level is an indicator associated with the spread of TB and the risk of death (19), but no study have report the assessment of MIF in LnTB by immunohistochemical methods. Moreover, the relationship between MIF expression with granuloma organization in LnTB also has not been well elucidated before. Therefore, this study was conducted to find out whether there is a correlation between MIF and the appearance of granuloma organization in lymphadenitis TB.

Methods

Study Design and Data Collection

This was a cross-sectional study with 100 samples taken at the Anatomy Pathology Laboratory of the Nusa Tenggara Barat General Hospital and Siti Hajar Hospital Mataram, Indonesia. The paraffin blocks samples were taken from patients that were diagnosed with LnTB according to histopathology criteria during January to December 2020 at both hospital and had sufficient block preparation for immunohistochemistry (IHC). Paraffin blocks with dimension less than 5 mm^3 were excluded. The examination of MIF expression was performed using IHC at the Anatomy Pathology Laboratory of Akurat Hospital Semarang. Meanwhile, general characteristics was taken from the patients' medical records. The current study has been approved by the Research Ethics Committee, Faculty of Medicine, Universitas Mataram (No. 356/UN18/F7/ETIK/2021).

WOG and POG Classification

WOG dan POG classifications were decided based on histopathology appearance with the hematoxylin eosin (HE) staining. According to the histopathology appearance, WOG was established when necrotic materials found were surrounded by epithelioid cells, lymphoid mature

cell and Langhans giant cells. On the other hand, POG was established when the necrotic materials found were histiocyte cell, lymphoid mature cell or polymorphonuclear neutrophils (PMN) cells without epithelioid cells or Langhans giant cells.(5,20)

IHC Analysis

Immunohistochemistry staining was used to assess MIF expression. The paraffin-embedded tissue sections were removed from the paraffin, added with liquid 0.01 M citrate buffer (pH 6.0) and heated in a microwave for 30 minutes. This section was immersed in 3% hydrogen peroxide for 10 minutes and then cleaned with sodium sulfate buffer. The sections were incubated for 24 hours at 40°C followed by administration of primary MIF antibody (N1C3, rabbit polyclonal IgG, 25 l, dilution 1:1000) (Cat No. GTX101162, GeneTex, Irvine, CA USA). Primary antibodies were detected using avidin biotin peroxidase solution and the signal was visualized using diaminobenzidine. The next slide was stained with Harris' hematoxylin. Observations started at 100x magnification to assess the distribution of primary antibodies and 400x magnification for cell staining evaluation using an Olympus CX21 LED light microscope (Olympus, Tokyo, Japan) and a camera mounted on the microscope in the entire field of view. There were 2 different pathologist who give expertise on each sample to control the interpretation bias.

Immunoreactivity score (IRS) to assess the expression of MIF was established according to the intensity of the stained cells and the level of staining. The percentage of stained cells were grouped as follows: score 0 = no stained cells; and score 1 = 80% stained cells. A positive staining level was indicated by the appearance of brown cells, while negative staining result was indicated by a paler color. The staining level was divided into: score 0 = uncolored; score 1 = light intensity; score 2 = moderate intensity; and score 3 = strong intensity.

The IRS score for MIF expression was assessed as the product of the percentage of stained cells (A) and staining level (B). The IRS (A X B) was divided into 4 groups: negative (score 0-1), weak (score 2-3), moderate (score 4-8), and strong (score 9-12), consecutively.(21)

Statistical Analysis

Statistical analysis using SPSS (IBM Corporation, Armonk, NY, USA) was performed in this study. Chi-square test was used to analyze the relationship between MIF expression with the type of granuloma organization. Significant level was established if $p<0.05$.

Results

There were 100 paraffin blocks that met the inclusion and exclusion criteria of this study. Female subjects were predominant than male with the ratio 3:1. The age of the subjects spreading between 3-70 years old with the mean 30.6±0.53 years old. The most frequent age was between 20-60 years old, as shown in Table 1.

Table 1. Distribution of subjects' gender and age (n=100).

Characteristics	n (%)
Gender	
Male	26 (26%)
Female	74 (74%)
Age (year old)	
< 10	5 (5%)
11-19	16 (16%)
20-60	74 (74%)
>61	5 (5%)

Based on the HE staining, 51% of the cases was WOG (Figure 1A) and 49% POG (Figure 1B). According to IHC staining for the expression of MIF, all samples were stained with the intensity of staining consecutively 36%, 43%, and 21% for negative, weak positive, and moderate positive, respectively (Figure 2). In advance, the immunoreactive score result found were 79% negative and 21% mild expression. MIF negative was found in 98% of WOG group while only found in 59% of POG group (Table 2). There was strong evidence that suggest that there were a significant

Table 2. Histopathology type finding, IHC staining, and immunoreactive score of MIF expression of subjects (n=100).

Finding	n (%)
Histopathology	
WOG	51 (51%)
POG	49 (49%)
IHC intensity staining	
Negative	36 (36%)
Weak	43 (43%)
Moderate	21 (21%)
Immunoreactive score	
Negative	79 (79%)
Mild	21 (21%)

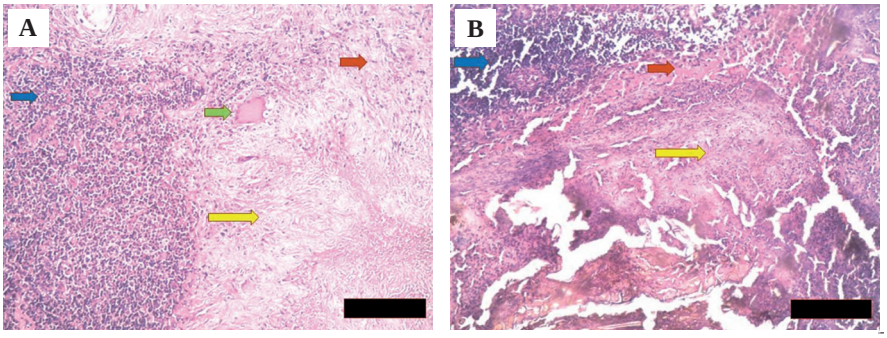

Figure 1. The histology of LnTB based on the granuloma organization. A: Histology features of WOG consist of lymphoid mature cell (blue arrow), datia Langhans giant cells (green arrow) and necrotic materials (yellow arrow) surrounded by epithelioid cells (red arrow). B: Histology features of POG consist of necrotic materials (yellow arrow), histiocyte cell (red arrow), lymphoid mature cell (blue arrow) without epithelioid cells or datia Langhans giant cells. Cells were documented under light microscope. Black bar: 10 µm.

correlation between type of granuloma organization with immunoreactive score with p=0.000 (Table 3).

Discussion

The study result showed that there were more female than male patients that were diagnosed with LnTB. Eventhough all ages could be infected with LnTB, however the group age that have the highest incidence is the productive ages (20-60 years old).

In this study, the number of subjects with WOG and POG were equal, hence these groups of sample can be used used to compare the expression of MIF. The expression of MIF in POG was significantly higher than WOG type. Based on histopathological features, WOG showed that TB infection was more severe than POG. In WOG, the granuloma was established well, its mean that the immune system was failed to protect the host. On the other hand, the granuloma on POG not completely formed. This is maybe due to the defense mechanism. Furthermore, the hypothesis is the more severe TB infection, the immune system will be more disturbed.

One of the pro-inflammatory cytokines that plays an important role in MTB is MIF. MIF, also known as glycosylation-inhibiting factor (GIF), is a regulator of innate and acquired immunity. MIF is produced by T cells and macrophages that work in the neuroendocrine system. MIF-deficient macrophages will result in decreased expression of the dectin-1 pattern recognition receptor and impaired mycobactericidal ability, thereby conferring an increased risk of TB disease in some populations.(15)

MIF is a pleiotropic cytokine that expressed by almost all cell types and tissues, and circulates in the bloodstream of a healthy adult with the volume about 5 ng/mL, on the other hand, in spinal TB is higher.(22) The characteristics of MIF differs from other proinflammatory cytokines. The MIF is more likely to be expressed and secreted

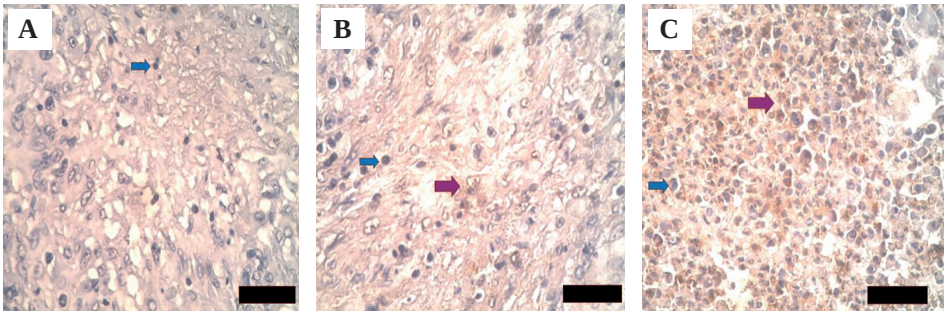

Figure 2. Immunoreactive score of MIF Expression. A: MIF negative, appearance of cytoplasm without brown staining in all field of view, and lymphoid mature cell were shown (blue arrow); B: MIF weak positive, appearance of cytoplasm with brown staining in only small field of view with intermediate intensity (purple arrow) showed staining brown in cell membrane or cytoplasm, and lymphoid mature cells were shown (blue arrows); C: MIF moderate positive, brown cytoplasm was stain in almost all field of view with the intermediate intensity (purple arrows) showed staining brown in cell membrane or cytoplasm, and lymphoid mature cells were also shown (blue arrows). Cells were analysed with intermediate intensity light microscope. Black bar: 1 µm.

Table 3. The correlation of granuloma organization and immunoreactive score of MIF expression.

Granuloma organization	Immunoreactive score of IMF			*p* -value
	Negative	Mild	Total	
WOG	50	1	51	0.000*
POG	29	20	49	
Total	**79**	**21**	**100**	

*Significant if $p<0.05$, tested with Chi-square test.

semi-constitutively (semi-constantly) into the circulation, whereas other cytokines, tend to be modulated by spikes in transcriptional activity in response to inflammatory stimuli. (23) Blood levels of MIF can be elevated in patients with arthritis, systemic lupus erythematosus (SLE), asthma, lung infections, and cancer.(23-25)

In Meliodotic disease caused by *Bulkholderia pseudomallei*, showed an increase of leukocyte MIF mRNA and plasma MIF concentrations. The increase of MIF concentration is associated with mortality. The similar result was shown in experimental in mice infected with *B. pseudomallei*, the administration of anti-MIF result in decreased of the bacterial load in the mice lungs.(26) In animal studies, macrophages MIF-deficient have shown decreased cytokine and reactive oxygen production and impaired mycobactericidal activity.(15) In certain infections caused by intracellular pathogens such as *Salmonella typhimurium* and MTB, MIF deficiency results in reduced of Th1 immune responses and may harms the host.(27) Several studies have shown that MIF undergoes post-translational modifications both covalently and structurally, as a result, it causes the changes of MIF bioactivity.(28,29)

Studies have shown that MIF can inhibit migration and increase macrophage aggregation at sites of local inflammation or infection, thus MIF is required in host defense against infection.(30) Studies of MIF-deficient animals show increased accumulation of pulmonary neutrophils while the adaptive immune response persists. MIF-deficient macrophages show decreased cytokine and reactive oxygen production and impaired mycobactericidal activity.(16,31) Two studies in the Chinese populations showed genetic variation in the MIF gene was closely associated with tuberculosis, namely that both -173 (GC+CC) SNP and -794 (7/X+8/X) increased the risk of TB.(32,33) However, in a more recent study, found no association between MIF-173 G/C and susceptibility to TB.(34) In an *in vitro* study using human THP-1 macrophages (cells used as a model for human monocytes), MIF was released rapidly from human macrophages once stimulated by mycobacteria and was fairly stable within 1 hour and then increased after four and six hours, as well as with MIF transcription also upregulated.(15) This result indicated that in acute mycobacterial infection the MIF will predominant to reduce the infection process. On the other hand, in more severe infection the MIF reversely decrease such as in the case of MTB bacteremia.(16) Due to the central role of MIF proteins in mediating broad immune responses to invading pathogens in the human system, it is presumed that polymorphisms in the MIF gene may be related to the onset and/or development of TB.(22)

In this study, the subjects with WOG had almost no MIF expression, whereas in POG the results were negative in almost half of the cases. This result was contradictory with expression of MIF in pulmonary TB and indicated that the immune system may failed to protect the spreading of MTB to the lymph nodes.(19) In line with this finding, patient with pneumonia also expressed the low MIF level. (35) However, this finding needs to be explored further. The current study still lacks of data on the concurrent pulmonary TB as well as clinical symptoms, and might be affected by the storage methods of paraffin block that may also have a potency as confounding factors. Hence, a prospective study with the analysis of concurrent pulmonary TB and clinical symptoms may need to be done in the future. The exact mechanism of MIF in the development of LnTB is also still unclear. Thus, the further research is needed to confirm it.

Conclusion

The results of this study shows that there was strong evidence that low expression of MIF is correlated with WOG LnTB. The negative expression of MIF in WOG was higher compare to POG. This may explain the role of MIF on the pathogenesis of granuloma formation in LnTB.

Authors Contribution

HK, FD, PH, and TDC were in involved in the data collection. FD and HK drafted and edited the manuscript. HK and NS gave critical review for the manuscript. All authors had agree with the final manuscript.

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