

28. Comparison of TLR2 1, NF- κ B p105 50, NF- κ B p65, and TNF expressions in the macrophages between multibacillary

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Comparison of TLR2/1, NF-κB p105/50, NF-κB p65, and TNF-α expressions in the macrophages between multibacillary leprosy patients with and without erythema nodosum leprosum signifying innate immune system activity



M. Yulianto Listiawan^{1*}

16 ABSTRACT

Background: Erythema nodosum leprosum (ENL) is one of the complications in multibacillary (MB) leprosy. The cause, mechanism, and treatment of ENL remain highly problematic. Various studies have identified possible involvement of the innate immune system in the pathogenesis of ENL. Increased understanding of this mechanism may be useful for the management of leprosy.

Objective: To compare the expression of Toll-like Receptor-2/1 (TLR2/1), Nuclear Factor Kappa Beta (NF-κB) p105/p50, NF-κB p65, Tumor Necrosis Factor-α (TNF-α) in the macrophages of MB patients with and without ENL as a marker of innate immune system involvement.

Methods: This study is a cross-sectional study performed on 42 MB

leprosy patients (21 with ENL and 21 without ENL) in the outpatient unit of Dr. Soetomo General Hospital from February to December 2010. Immunohistochemical staining method was conducted on all groups to determine the expression in macrophages of the dermis using the specific monoclonal antibody for TLR2/1, NF-κBp105/p50, NF-κBp65, and TNF-α. Statistical analysis was performed using Mann-Whitney U test.

Result: There was a significant difference of TLR2/1, NF-κBp105/p50, NF-κBp65, and TNF-α expression in the dermis macrophages between the MB leprosy patients with ENL and without ENL.

Conclusion: The innate immunity was activated in the ENL reaction of MB leprosy.

Keywords: Erythema nodosum leprosum, innate immunity, multibacillary leprosy

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INTRODUCTION

Leprosy is a chronic infection caused by *Mycobacterium leprae* which primarily affects the skin and peripheral nerves, and its clinical symptoms depend mainly on the patient's cellular immune system.^{1,2} Complications in leprosy including type 1 reaction and type 2 reaction or Erythema Nodosum Leprosum (ENL). Type 1 reaction is defined as acute inflammation that occurs due to delayed-type hypersensitivity. ENL is an acute inflammation allegedly caused by immune complex deposits that affect multibacillary (MB) leprosy patients.^{3,4}

Many studies on leprosy have attempted to determine the role of Tumor Necrosis Factor (TNF)-α, a type of cytokines secreted mainly by macrophages that plays a role in the innate immunity to eradicate bacteria. The primary function of TNF-α is to stimulate and attract neutrophils and monocytes to the site of infection and activate macrophages and neutrophils to treat the infection.⁵

The level of TNF-α in MB leprosy is lower than that in paucibacillary (PB) leprosy, indicating a decreased ability to localize the infection.^{6,7} Mutations in the type I membrane receptor that plays a role in innate immunity is suspected of being the cause of the decrease in TNF-α. This mutation causes a disturbance in the signal pathway that leads to the inability to produce TNF-α which serves to localize the infection.^{8,9} On the contrary, in MB leprosy with ENL there is an increase in TNF-α secretion by an unknown mechanism.^{4,10} TNF-α may also act as co-stimulator of dendritic cell that plays a role as an antigen-presenting cell (APC), so it is also involved in the acquired immunity.¹¹ The secretion of TNF-α in ENL is thought to be induced by a part of *M. leprae* cell wall which can stimulate the innate immunity of the human body.

The assessment of TNF-α expression in macrophages can be useful for leprosy patients to localize and ultimately eliminate the *M. leprae* bacteria and as a prognostic factor of cellular

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immune function.^{6,7} The acute symptoms in ENL are thought to indicate an excessive increase in innate immunity, reflected by Toll-like Receptor-2/1 (TLR2/1) expression as a part of the innate immune transduction signal pathway to produce TNF- α . Triacylated Lipoprotein (TLP) as Pathogen Associated Molecular Patterns (PAMPs) of *M. leprae* serves as a ligand for one of the Pattern Recognition Receptors (PRRs), which is TLR2/1 (a receptor in macrophages of the innate immunity in skin tissue).¹² It induces TNF- α expression, via the Nuclear Factor Kappa Beta (NF- κ B) p65 homodimer and p105/50 heterodimer pathways.¹³

More in-depth study about transduction of TLR2/1, NF- κ B p65, and p105/p50 signals in the pathogenesis of ENL is necessary in order to understand the increase of TNF- α secretion in ENL fully. Increased understanding in the pathogenesis of ENL can be used for a more appropriate treatment approach, which may reduce the morbidity of ENL and stigma in leprosy. Since this particular study on ENL has never been previously done, we conducted a study aimed to compare the expression of TLR2/1, NF- κ B p105/p50, NF- κ Bp65, and TNF- α between MB patients with ENL and without ENL as a marker of innate immune system involvement.

METHODS

This study is a cross-sectional analytical observational study with a comparison group. The observed group was MB leprosy patients with ENL reactions, while the comparison group was MB leprosy patients without ENL. The sample consisted of MB leprosy patients who met the inclusion criteria, have done history taking, and physical and bacteriological examination, from February 2010 to December 2010. The samples were collected using consecutive sampling technique.

The inclusion criteria including MB leprosy patients with or without ENL who were clinically established with a positive bacteriological

examination, aged of 12-50 years old, had no history of tuberculosis and diabetes mellitus, and were willing to sign the informed consent. We exclude patients with poor condition, patients who were pregnant or in lactation, and were treated with oral glucocorticoids for the last four days.

The diagnosis of leprosy was based on the cardinal signs of leprosy: anesthetic skin lesion, peripheral nerve enlargement along with autonomic, sensory and motor function abnormalities, and positive acid-fast bacilli (AFB) skin smear from skin lesion or ear lobe. WHO classification was used to determine the type of leprosy based on the number of skin lesions and skin smear results. If the sample showed skin lesion of >5, peripheral nerve enlargement of >1, and AFB +, then the patient was classified as MB leprosy. The diagnosis of ENL was based on history taking and clinical symptoms, including the presence of pain symptoms in the skin accompanied by the emergence of acute reddish nodules that can be accompanied by rising body temperature. ENL occurs only in patients with MB leprosy.

TLR2/1 is a surface receptor (transmembrane) that is part of PRRs that binds PAMPs of *M. leprae* (TLP) at the beginning of signal transduction that initiates reaction cascade. NF- κ B p65 and p105/p50 are heterodimers and homodimers transcription factors that play a role in the TLR2/1 signaling pathway to produce mRNA of TNF- α . TNF- α is a proinflammatory cytokine that plays a role in localized ENL clinical symptoms in skin tissues and systemic in the blood circulation.

Examination of TLR2/1, NF- κ B p65, p105/p50, and TNF- α expressions in macrophages in skin tissue was performed with immunohistochemistry. Immunohistochemical studies were performed on the macrophages of the skin tissue in both groups to determine the expression of TLR2/1, NF- κ B p65, p105/p50, and TNF- α . Examination of skin smear in the observed group was performed on two sites: skin lesions and ear lobes, and then we calculated the mean of the results. As for the comparison group, the examination was performed on the ear lobes only. The data collected were processed using the homogeneity test, normality test, and analyzed using the Mann-Whitney U test.

RESULTS

General characteristics of the subjects

In this study, there were 21 subjects of MB leprosy with ENL and 21 subjects of MB leprosy without ENL as the comparison group. The characteristics are presented in Table 1. The table shows that the age distribution of both groups was the same. Both groups had similar sex distribution, with the male being 85.7% and female 14.3%.

Table 1. Distribution of age and sex of ENL and non-ENL patients

Age Group (year)	ENL (+)		Total	ENL (-)		Total
	Male	Female		Male	Female	
15 – 25	4	0	4 (19.1%)	5	1	6 (28,7%)
26 – 35	9	2	11 (52.4%)	6	1	7 (33,3%)
36 – 45	4	0	4 (19%)	4	0	4 (19%)
46 – 55	1	1	2 (9.5%)	3	1	4 (19%)
Total	18 (85.7%)	3 (14.3%)	21	18 (85.7%)	3 (14.3%)	21

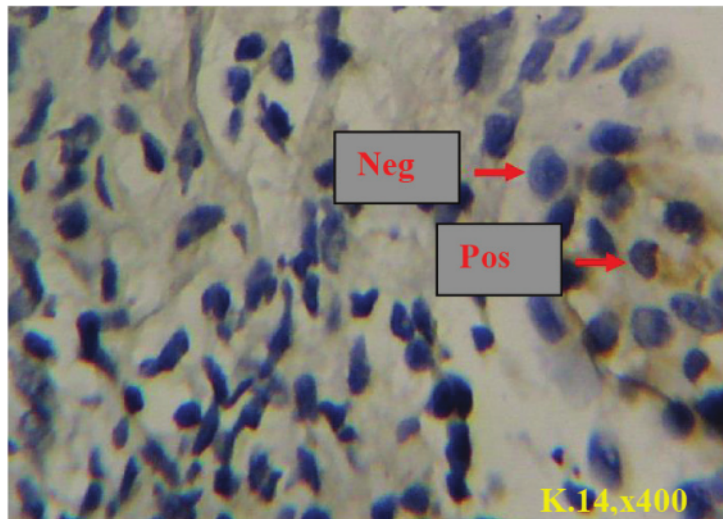


Figure 1A. The dermis samples taken from MB leprosy with ENL patient showing positive reaction to TLR2/1 in macrophage (brown color), while the negative one does not (400X magnification).

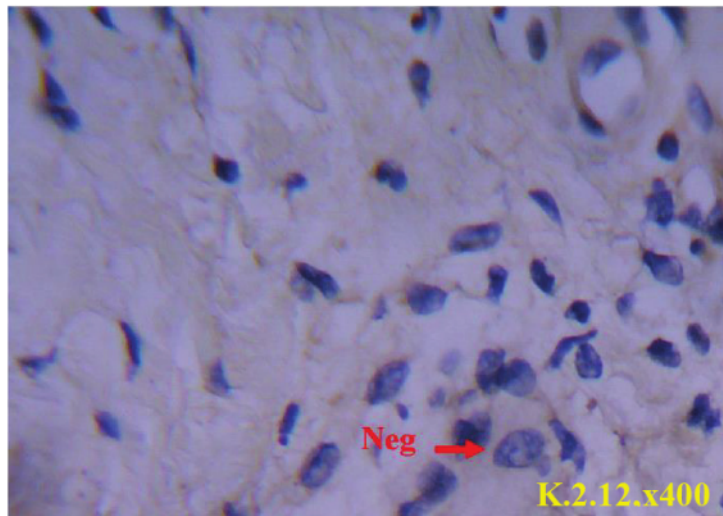


Figure 1B. The dermis samples taken from non-ENL MB leprosy patient showing no expression of TLR2/1 on the surface of the macrophage (red arrow) (400x magnification).

ENL occurrence with MDT treatment

In 21 cases of ENL, the onset of ENL symptoms was associated with multidrug therapy (MDT). Most subjects (61.9%) were still on MDT, and 8 ENL cases (38.1%) were found after the 12th treatment with MDT. There were no subjects with ENL who have not received MDT.

Calculation of bacteriological index

The bacteriological index (BI) of ENL subjects

showed that most subjects (71.4%) had 3+, while 4.8% had 4+, 14.3% had 1+, and 9.5% had 2+. In the comparison group, most subjects (81%) also had BI 3+, while 9.5% had 4+ and 9.5% had 2+.

ENL degree of severity

The results showed that 71.43% of subjects had a mild degree of severity, while the remaining 28.57% had moderate severity. The scoring of 17 clinical symptoms determined the degree of severity. The score of 1 - 17 were included as mild, 18-34 was moderate, while 35- 51 was severe.

Expression of monoclonal antibodies

MB patients with ENL expressed monoclonal antibodies against TLR/21, as seen in Figure 1A. Meanwhile, MB patients without ENL did not express monoclonal antibodies against TLR/21, as seen in Figure 1B.

In the group of MB patients with ENL, three patients did not express TLR2/1. Those who expressed TLR2/1 showed a various amount of macrophages, ranging from 1-8. In the control group, no patient expressed TLR2/1. The results of the Mann-Whitney U test showed $Z = -5.278$ with $p=0.000$ ($p<0.05$), indicating a significant difference in the macrophages expression of TLR2/1 between MB leprosy patients with ENL and without ENL.

Figure 2 shows that monoclonal antibodies against NF- κ B p105/p50 were expressed by subjects with ENL but not in the comparison group. In the ENL MB leprosy group, we found the expression of NF- κ B p105/p50 of all samples obtained, whereas in the non-ENL MB leprosy group there was no NF- κ B p105/p50 expression. Mann-Whitney U test results showed $Z = -5.942$ with $p = 0,000$ ($p <0.05$), indicating a significant difference in the macrophages expression of NF- κ B p105/p50 between MB leprosy patients with ENL and without ENL.

Figure 3 shows the expression of monoclonal antibodies against NF- κ B p65 in subjects with ENL. The overall examination results were variable. In subjects with ENL, expression of monoclonal antibodies against NF- κ B was found positive between 4-20 macrophages, while one sample showed negative expression. On the other hand, subjects without ENL did not express monoclonal antibody against NF- κ B p65. Mann-Whitney U test results showed $Z = -5.709$ with $p = 0,000$ ($p <0.05$). This result means that there is a significant difference in the expression of NF- κ B p65 between MB leprosy patients with ENL and without ENL in their macrophages.

Figure 4 and 5 shows the expression of TNF- α in ENL and non-ENL subjects, respectively. From

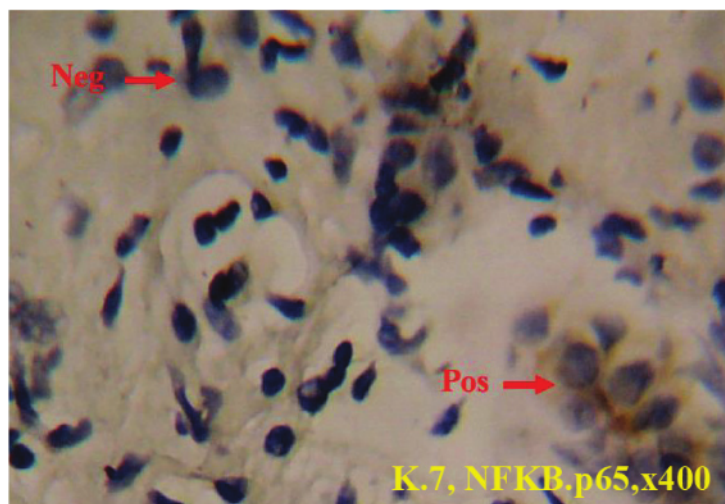


Figure 2. The dermis of ENL patients showing the expression of monoclonal antibody against NF-κB p105/p50 (400X magnification).

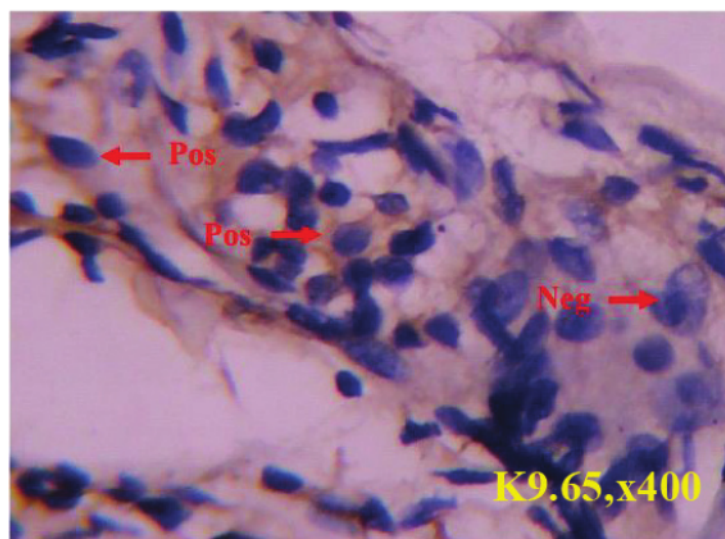


Figure 3. The samples taken from ENL patients showing the macrophages that express NF-κB p65 with positive (brown-colored) and negative reactions (red arrows) (400X magnification).

the results of all samples, there were 20 positive subjects with expression levels varying from 1-39 macrophages. Meanwhile, the non-ENL group had three patients whose expression was positive for TNF- α . Mann-Whitney U test results showed $Z = -4.77$, with $p = 0,000$ ($p < 0.05$). This result means that there is a significant difference in TNF- α expression between MB leprosy patients with ENL and those without ENL.

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DISCUSSION

In this study, we observed the expression of TLR2/1 in 21 subjects of MB leprosy with ENL and 21 subjects without ENL. It was found that all subjects without ENL did not express TLR2/1. There was a significant difference in the expression of TLR2/1 in macrophages of patients with and without ENL although there were three patients with ENL who did not express TLR2/1.

The presence of immune complexes in MB leprosy is caused by an increase in humoral immunity. Increased antibodies, along with antigen from pathogens, will lead to the formation of immune complexes and settle in the tissues. These antibodies, along with cytokines derived from new macrophages (such as IL-1) and TNF- α , are the main pathological mechanisms in ENL.¹ The presence of new macrophages and TNF- α secretion opens new horizons about the role of innate immunity in ENL.

Macrophages in MB leprosy are the site of multiplication and primary host cell for *M. leprae*.⁴ Many macrophages are deformed into leprosy cells (Virchow cells) in MB leprosy. These cells are presumably formed from incomplete bacterial degradation inside macrophages. The phospholipid layers of bacterial cells are still in the cytoplasm of the macrophages, and the cells appear as leprosy cell. Macrophages which containing *M. leprae* cannot be activated, including by the IFN- γ cytokines, which will result in the increased pathogens inside the cell.¹⁴ This small part of the *M. leprae* fragment (including TLP) will function as PAMPs. It also activates the ligand of TLR 2/1, which is a part of the PRRs and represents the function of the innate immunity.

In this study on TLR2/1 (a heterodimer as part of PRRs) is a representative of one of the mechanisms of the innate immune system that can recognize the biochemical pattern of bacteria as ligands. These ligands are part of PAMPs and are expressed by the pathogenic agent. The specificity of the recognized TLR varieties in initiating the response to ligands of various bacteria has been known. The activation function of TLR is as a direct introduction that will initiate an innate immune response, leading to regulation of phagocytic processes and initiate antibacterial activity. Also, TLR activation will lead to the release of various cytokines and differentiation of immature dendrite cells, which will increase the efficiency of the innate immune system.

Triacylated lipoprotein (TLP) is a PAMP of *M. leprae* that acts as a ligand of TLR2/1. As PAMP, TLP meets the specific descriptions: produced only by pathogenic microorganisms (*M. leprae* and *M. tuberculosis*); an essential structure in the life of mycobacteria; and a structure that is continuously

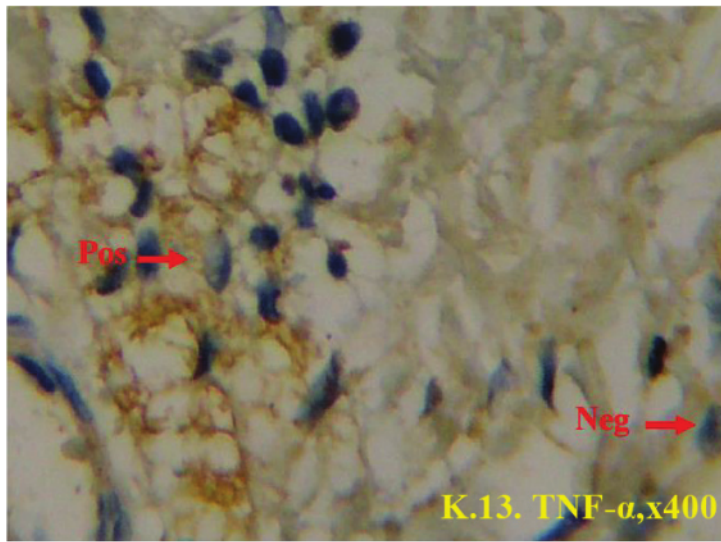


Figure 4. The expression of monoclonal antibody against TNF- α in the dermis layer of ENL patients (400X magnification).

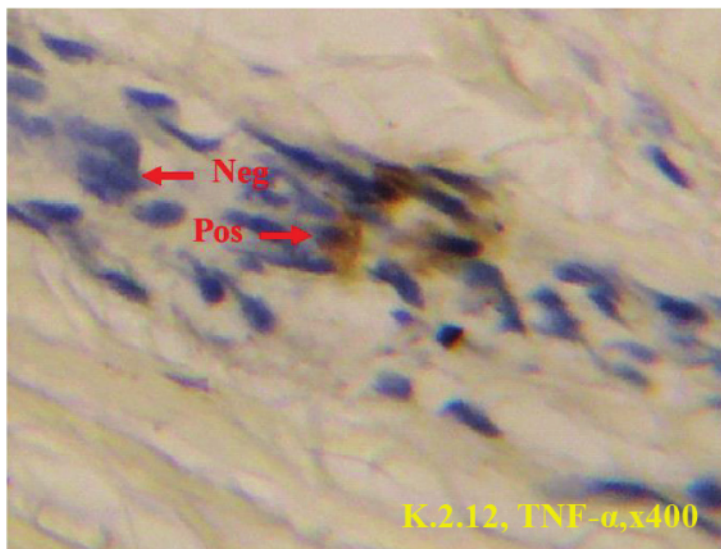


Figure 5. The expression of monoclonal antibody against TNF- α in non-ENL MB leprosy patients; the red arrows points at macrophages that expresses TNF- α with positive (brown-colored) and negative reactions (400X magnification).

shared by a group of similar bacteria.¹⁵

In this study, it was suspected that TLPs from *M. leprae*, which became fragmented by treatment or other causes, would be released from old macrophages. Then, they would be phagocytosed by new macrophages, functioning as NACP, via a functioning natural receptor line (TLR 2/1). This process did not occur in the control group that

did not express TLR 2/1. In the control group, the expression of TLR2/1 was inhibited by the dominant cytokine in the MB leprosy spectrum (IL-4). IL-4 belongs to the group of Th₂ cytokines, a group of cytokines that works in response to humoral immune.¹² It also explains that the symptoms of ENL often occur in the first six months of treatment with MDT¹⁶, which is thought to be the time of the occurrence of a TLP fragment that is part of the bacteria and acts as a ligand of TLR2/1.

In this study, there were also three samples of ENL patients who did not express TLR2/1 (samples 6, 8 and 11). These three samples were leprosy patients with clinical symptoms of ENL and history of released from treatment (RFT). This result is possibly due to activations of other TLR (such as TLR2) or other heterodimers (such as TLR2/6), which also play a role in inflammation reaction of innate immunity, with ligands in different leprosy.⁹ It is known that the factors of innate immunity do not only specifically play a role in the emergence of acute inflammatory reactions, but also the emergence of ENL. The examples of non-specific factors are other TLR roles other than TLR2/1, which can be generated through a variety of bacteria or viruses. Thus, the role of either gram-positive or gram-negative bacteria can stimulate the activation of TLR. It is something that should be watched out for and must be treated in order to avoid the excessive expression of TLR and to enable better management for the severity of ENL.

In all subjects with ENL, we found that all macrophages expressed NF- κ B p105/p50 with various levels, whereas in the control group none of the patients expressed NF- κ B p105/p50. These results indicate that the breakdown of NF- κ B p105/p50 is important to activate the transcription factors in the pathogenesis of ENL. Mann-Whitney U test performed in both groups showed significant difference of NF- κ B p105/p50 expression ($Z = -5.942$ with $p = 0.00$ [$p < 0.05$]). This result shows that the breakdown of NF- κ B p105/p50 in ENL plays a major role and does not occur in non-ENL leprosy. NF- κ B takes a key part in inflammatory and immune processes by regulating the genes coding for pro-inflammatory cytokines, chemokine adhesion molecules, growth factors and various enzymes such as cyclo-oxygenase 2 (COX2) and inducible nitric oxide synthase (iNos).¹³

In this study, we used a primary monoclonal antibody containing phosphopeptide that was derived from human NF- κ B but underwent phosphorylation of serine amino acids at site 927. Thus, macrophages that expressed monoclonal p105/p50 indicated activation of a constitutive transcription factor with varying degrees as seen in

the ENL subjects. On the other hand, in the patients without ENL, NF- κ B p105/p50 was not expressed, suggesting that this transcription factor was not involved in the infection caused by *M. leprae*.

The activation of NF- κ B1 shows the activation of innate immunity transcription factors. Since macrophages expressing NF- κ B1 may be activated with a variety of receptors (including TNF- α , IL-1, CD40L and lipopolysaccharide [LPS]), some non-receptor activations or their receptors are unknown, such as oxidative stress and ultraviolet radiation.¹⁷ The discovery of this transcription factor activation can be used to prevent excessive NF- κ B activation by avoiding various activating ligands and using various drugs that suppress NF- κ B activation.

NF- κ B p65 (also known as the RelA protein) is different from p100/p50. It has a special role because it has a section called the transcriptional activation section and can function as a homodimer or heterodimer, with other NF- κ B groups to function as a transcription factor. It is known that the heterodimer p50/RelA is the heterodimer most often found in terms of inducing NF- κ B attachment to DNA to initiate the transcription process. In this study, it was found that in 21 samples of ENL the macrophages expressed RelA, whereas in MB leprosy patients who did not have ENL at 21 their macrophages did not express RelA with significant difference ($Z = -5.709$ with $p = 0.00$ [$p < 0.05$]).

TNF- α can activate atypical pathways, via CK2 enzyme.¹⁸ TNF- α is also a marker of ENL reaction, and therefore its increase will further aggravate the occurring ENL reactions. Suppression of TNF- α production is needed to control the severity of ENL reactions. In general, NF- κ B activation is also a potential biomarker for oxidative stress.¹⁹ Associated with the advance knowledge of the field of immunology, TNF- α is one of the cytokines secreted by innate immunity during initial infection and is produced by macrophages. The production of this cytokine is lower in MB leprosy than PB type. However, in MB leprosy with ENL there is a considerable increase of TNF- α , making it as a biomarker of the onset of ENL. This study evaluated the type of membrane which has been studied rarely. The result showed a significant difference ($Z = -4.770$ with $p = 0.00$ [$p < 0.05$]) between the groups of MB leprosy with ENL and without ENL. Another study also found a similar result in which there was a significant increase of TNF- α in ENL patients compared to non-ENL patients.²⁰

According to one theory, the low level of TNF in MB leprosy is due to the polymorphism of TLR2. This study showed that TLR2/1, which is a TLR2 heterodimer, was expressed in MB leprosy with ENL patients. We also found that the inability

to express TLR2/1 in MB leprosy seems to be more caused by the immunity unresponsiveness (both innate and acquired immunity) in leprosy patients. This unresponsiveness condition is not caused by immunocompromised conditions, but rather due to the activity of *M. leprae* and the inappropriate modulation response of the host body. This condition can be seen in the ability of *M. leprae* to reproduce in macrophages chronically. Macrophages can phagocytose *M. leprae*, but due to the lack of lysosomal enzymes, only partial bacterial lysis occurs. Furthermore, due to its abundant phospholipids on its cell wall, *M. leprae* will cause metabolic disorders in the absence of an immunologic response.¹

The inability to activate immunological response is demonstrated by the inability of dermis macrophages (NAPC) to express TNF- α , an important anti-inflammatory cytokine in the onset of complicated ENL clinical symptoms.²¹ The expression of TNF- α in leprosy patients with ENL found in this study suggests an important role of TNF- α in the onset of complicated ENL symptoms. TNF was not produced in MB leprosy without reaction but produced in MB patients with reaction. It is deduced that since the fragile *M. leprae* fragments in chronic conditions of macrophages will be recognized by new macrophages (NAPCs) as ligands for natural immune responses played by TLR2/1.

The results of the study also showed ENL patients who did not express TNF- α (i.e., sample number 3). This result is possible because acute inflammation can be caused by non-TNF- α cytokines, such as IL-1 or IL-12.²² On the contrary, there are three samples of MB patients without ENL which showed TNF- α expression on their macrophages. This may be due to the three subjects have received the 10th, 6th and RFT MDTs. Each sample expressed TNF- α in a small amount of four, two, and five macrophages, respectively. The excessive expression of TNF- α in its macrophages indicates a function that leads to improvement in leprosy patients, as it can further enhance the expression of co-stimulators that increase the efficiency of acquired immunity against *M. leprae* infection.¹¹

CONCLUSION

The overall results of this research showed the activation of innate immunity in MB leprosy patients with ENL. Thus, it is necessary to revise the management approach of ENL which has been consistent with its adaptive and active immunity. In the Ministry of Health manual on the management of patients with severe ENL, the utilization consists of oral corticosteroid preparations (prednisone) in

large doses (40 mg/day), then gradually lowered for 12 weeks that it requires a reexamination to determine the period and dosage of the prednisone. A further prospective study is needed through careful observation of MB leprosy patients with and without ENL reaction using the variables similar to this study.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

ETHICAL CLEARANCE

This study protocol has been approved by the Ethics Committee of Medical Research, Dr. Soetomo General Hospital Surabaya with Ethic Certificate No. 25/Panke.KKE/15/1/2010.

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