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Research article

Comparison of interleukin 4 levels in leprosy and non-leprosy patients at Dr. Muhammad Hoesen Palembang General Hospital

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Key words: Leprosy, Interleukin-4, T-helper, Hospital.

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Abstract

Leprosy is a chronic granulomatous infectious disease caused by the mycobacterium leprae which injure the skin and peripheral nervous system. The spectrum and clinical type of leprosy are based on cellular immunity responses namely T-helper 1 (Th1) and T-helper 2 (Th2). This study aims to compare serum IL-4 levels between lepers and non-lepers in Dr. Mohammad Hoesin Palembang General Hospital. This research is an observational analytic study with a case-control design conducted at Dr. Mohammad Hoesin Palembang General Hospital. IL-4 examination is carried out using the ELISA method at the Molecular Biology Laboratory, Faculty of Medicine, Sriwijaya University in January to February 2019. The case and control groups were 40 lepers and 40 medical personnel and paramedics working in Dr. Mohammad Hoesin Palembang General Hospital and family patients. The study included IL-4 levels, respondent status, clinical type of leprosy, and characteristics of respondents. In the leprosy group, the average age is 35 years, and most of them are male (27). In the non-leprosy group, the average age is 36.5 years, and most of them are female (24). There are more MB patients (36) than PB type (4) while type BL (19), type LL (10) type BB (7) and type BT and BB (2). The median value of IL-4 levels in leprosy patients is 209.63 pg/ml range from 103-243 pg/ml while the median value of IL-4 levels in non-leprosy patients is 63.38 pg/ml range from 32-125 pg/ml. Mann-Whitney analysis results show a p-value of 0.000. There is a significant difference between Interleukin 4 levels in lepers and non-lepers where IL-4 levels are higher in lepers than non-lepers.

Introduction

Leprosy is still a problem for countries in the world, especially in developing countries [1]. This chronic disease is caused by the mycobacterium leprae which injure the skin, oral mucosa, respiratory tract, and peripheral nervous system that can cause permanent disability [2]. In the world, the permanent disability rate due to leprosy is still high, 6.6 per 1,000,000 residents [3]. Southeast Asia region has the highest Leprosy cases where Indonesia ranked in the third place with the highest prevalence is in West Papua. (Indonesian Ministry of Health, 2018). In South Sumatra, it is noted that new leprosy cases are the highest in 2015 with a stagnant level-II disability rates over the past 10 years (>5%) [4].

Leprosy has three distinctive clinical features; numb lesions, peripheral nerve damage, and the presence of acid-resistant bacilli bacteria [5]. According to WHO, leprosy is divided into multibacillary (PB) and multibacillary (MB) types (WHO, 2017), while Ridley Jopling divides leprosy into tuberculoid type, (TT) borderline tuberculoid (BT), mid borderline (BB), borderline lepromatous (BL), and lepromatous (LL) [6]. In those clinical spectrum, TT and BT type show a small number of bacteria that are included in paucibacillary type with a strong cell-mediated immunity (CMI) response while BB, BL, and LL type shows a high number of bacteria or is included in multibacillary type with poor CMI response. Base on its pathogenesis there are 2 types of leprosy reactions; type 1 leprosy reaction called RR, which occurs in BT, BB, and BL types, and type 2 leprosy reactions called ENL which mainly occur in lepromatous types (BL and LL type) [7].

Several studies show different results related to IL-4 levels in lepers, where there are higher IL-4 levels in LL type compared to the other types [8]. While other study shows IL-4 levels are almost the same in all types of leprosy [9]. In Indonesia alone, there has been no publication on IL-4 studies in leprosy.

Based on the information above, which is high number of new cases of leprosy in the Province of South Sumatra, followed by stagnant level -II disability rates and the pros and cons of the results on IL-4 levels in leprosy, as well as

the absence of IL-4 research studies in Indonesia, we are interested to examine IL-4 levels as its role in the immune response and determinants of leprosy clinical types.

Material and method

Materials
Needle Vacutainer, Red lid vacutainer tube, Holder, Tourniquet, Alcohol swabs, Eppendorf tube, Gloves, Human Interleukin 4 Standard, Human Interleukin 4 Standard Diluent, HRP Conjugate Reagent, Sample Diluent, Chromogen Solution A, Chromogen Solution B, Wash Solution, Stop Solution, ELISA kit, all materials were obtained from sigma Aldrich.

Types of research
This research is a case-control study that aimed to compare serum IL-4 levels between lepers and non-lepers in Dr. Mohammad Hoesin Palembang General Hospital.

Place and time of research
This research is conducted from January to February 2019 at Dr. Mohammad Hoesin Palembang General hospital, while IL-4 examination is carried out at the Medical Biology Laboratory, Faculty of Medicine, University of Sriwijaya Palembang.

Research population
The case group population are all lepers treated at Dr. Muhammad Hoesin General Hospital Palembang from January to February 2019. The control group populations are medical staff and paramedics who work at Dr. Muhammad Hoesin Palembang General Hospital and the patient's family and all the subject were agreed to be a part of this research.

Research samples and ethical clearance
The study sample is the total population (lepers who were treated at Dr. Muhammad Hoesin General Hospital Palembang from January to February 2019). University of Sriwijaya ethics committee was screening this research and approved the ethical clearance on January 2019.

Dependent variable
Level of interleukin 4.

Independent variable
Status of respondents and clinical types of leprosy

Procedures
- Blood collection from all case and control groups, 40 patients each.
- Blood is inserted into the vacutainer tube
- Blood is centrifuged at 2000 rpm for 20 minutes.
- The serum is transferred to the Eppendorf tube (RNA Nuclease-Free).
- The serum is stored in -20^{0}C until it is examined in the Biomolecular laboratory of Sriwijaya University.
- Then, sample wells are prepared.
- Diluent samples were added as much as 40 ul into each sample well.
- 10 ul of each sample is added to the sample well according to the work map. Then the sample mixture is homogenized.
- The remaining solution in well 2 (standard 2) is removed 50 ul.
- Wells that contained standard series 1 to 8 are homogenized.
- 40 ul diluent samples are added into each sample well.
- Each 10 ul of sample is added to the sample well according to the work map. The mixture is homogenized.
- The well is closed with a seal plate and incubated for 30 minutes at 37^{0}C.
- The solution is removed and washed 5 times with a wash solution. It is washed by filling each well with wash solution using an auto washer. Finally, clean the remaining wash solution with aspiration or decanting method. Turn the dish over and clean it with a tissue.
- Conjugate reagent HRP is added 50 ul to each well, except blank wells, then it is homogenized.
- The well is closed with a new seal plate and incubated for 30 minutes at 37^{0}C.
- The solution is discarded. Washing is repeated.
- Chromogen solution A and B are added as much as 50 ul to each well, and then homogenized.
- The well is closed with a new seal plate and incubated for 15 minutes at 37^{0}C and should be protected from light.
- Stop solution is added 50 ul to each well (the color changes from blue to yellow).
- Standard optical density and samples are read by ELISA reader at a wavelength of 450 nm.
- Levels of IL 4 are calculated based on optical density values using the line equation formula.

Result and discussion

Characteristics of respondents
The general characteristics of the research subjects included age, sex, ethnicity, education, occupation, duration of leprosy, and length of drug consumption. Nominal data is calculated on average and the standard deviation for normal distribution data according to Saphiro-Wilk normality test. The median and minimum-maximum values are tested for

data not distributed normally. Categorical data is calculated by number and percentage. Data are also calculated for the P-value in both lepers and non-lepers. Data can be seen in table 1.

Table 1. Characteristic distribution of respondents

Respondent characteristics	Leprosy patient (40)	Non-leprosy patient (40)	*P Value*
Age :			
Average(year)			
Group of age :	35	36.5	0.584 *
18-40	24	26	
41-60	14	12	0.890**
> 60	2	2	
Gender :			
Male	27	16	0.014**
Female	13	24	
Ethnic:			
Malay	32	19	0.029***
Batak	1	2	
Jawa	6	15	
Bugis	0	2	
Chinese	1	2	
Education :			
None	0	1	0.001***
Elementary school	12	6	
Junior high school	3	3	
Senior high school	21	8	
Diploma	1	2	
Bachelor	3	18	
Magister	0	2	
Occupation :			
None	4	0	0.001***
Teacher	1	0	
Employee	16	2	
House wife	11	9	
Farmer	1	5	
Driver	2	0	
Laborer	5	2	
Medic	0	18	
Paramedic	0	4	
Duration :			
0 – 5 years	35		
6- 10 years	4		
> 10 years	1		
Duration of MDT use:			
None	2		
PB 0 – 6 months	3		
PB > 6 months	0		
MB 0 – 12 months	19		
MB > 12 months	9		
ROM	7		

Description :
* = T-independent test
** = Pearson Chi Square test
*** = Kolmogorov Smirnov test

Distribution of Leprosy and Non-Leprosy Patients based on Clinical Type

Characteristics of people affected by leprosy are classified according to the clinical type of leprosy. The clinical type group is divided into two criteria, WHO and Ridley Jopling criteria. WHO criteria are divided into two, paucibacillary (PB) and multibacillary (MB). Ridley Jopling criteria are divided into 5 criteria namely tuberculoid (TT), borderline tuberculoid (BT), borderline (BB), borderline lepromatous (BL), and lepromatous (LL). Distribution of respondents based on these criteria is shown in figure 1.

Serum IL-4 levels in lepers and non-lepers

In this study, the median value of IL-4 level in leprosy patients is higher (209.63 pg/ml) compared to non-leprosy patients (63.38 pg/ml) with SD values of 25.229 and 28.882 in lepers and non-lepers respectively. The highest level of IL-4 in lepers are 243 pg/ml, and the lowest level is 103 pg/ml. While the highest levels of IL-4 in non- lepers are 125 pg/ml and the lowest level are 32 pg/ml. According to the statistical test, there is a significant difference between IL-4 levels in lepers and non-lepers with a p-value of 0.000 as shown in Figure 2.

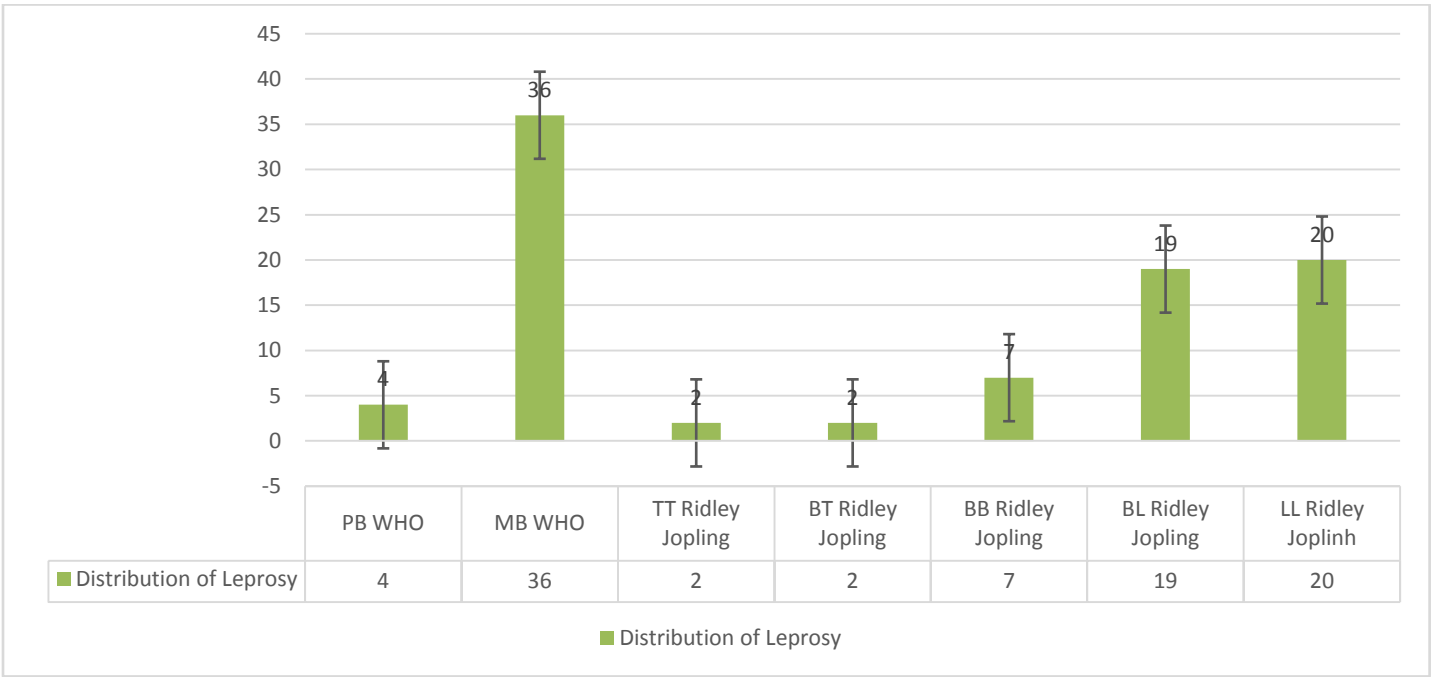

Figure 1. Distribution of leprosy patients based on clinical types of WHO criteria and Ridley Jopling.

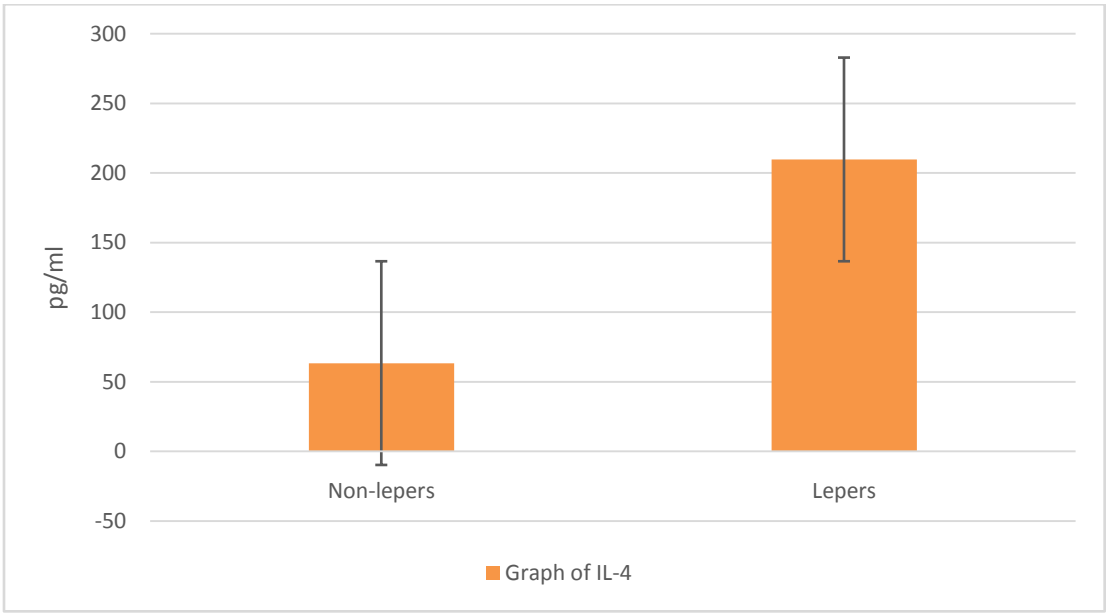

Figure 2. Graph of IL-4 levels in lepers and non-lepers.

Serum IL-4 levels in leprosy patients are based on clinical types of WHO criteria and Ridley Jopling

In Figure 3, it can be seen that in WHO criteria, the highest IL-4 level is in MB patients (214.63 pg/ml) and the lowest level is in PB patients (207.13 pg/ml). Statistical tests show that it was no significant differences of IL-4 levels in PB and MB patients with p-value 0.511; 0.05. Whereas in Ridley Jopling criteria, leprosy patients that obtained the highest median value of IL-4 level are LL type (219.63 pg/ml), and the lowest median value of IL-4 level is BT and BB types (207.13 pg/ml). The statistical test found that there is no significant difference between IL-4 levels in each type of leprosy (TT, BT, BB, BL, and LL) with p-value 0.915; 0.05.

Serum IL-4 levels in lepers and non-lepers are based on Cut-Off Point

In this study, grouping IL-4 levels are based on the cut-off point. IL-4 level cut-off points are obtained through the application of medical statistics as shown in the graph (IL-4 levels 124.63). This shows that IL-4 levels are categorized as; increase" when it is higher that 124.63 pg/ml and do not increase when it is lower or equal to 124.63 pg/ml. In leprosy patients, there are 39 persons who have IL-4 levels higher than 124.63 and only 1 person who does not. On the contrary, all of the non-leprosy patients do not experience increased levels of IL-4 (IL-4 ≤124.63 pg/ml) as shown in figure 4.

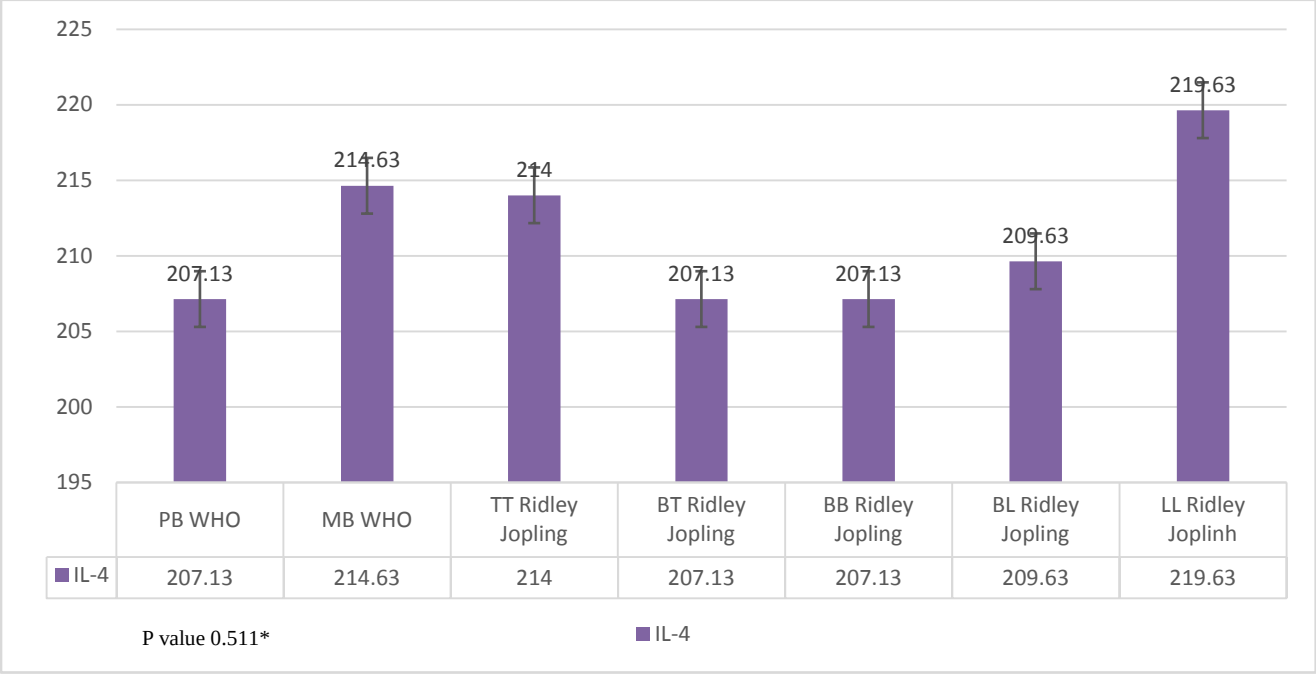

Figure 3. Graph of median IL-4 levels in leprosy patients based on the clinical type of WHO criteria, Ridley Jopling and leprosy reactions.

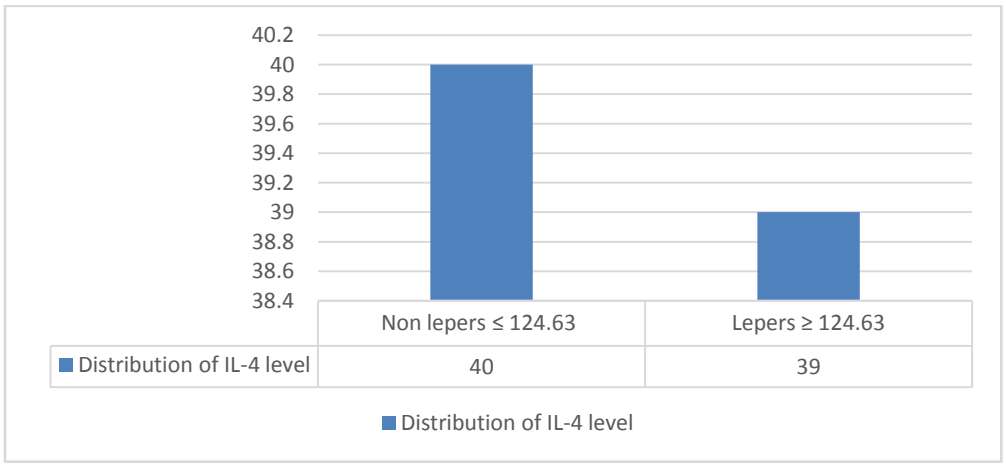

Figure 4. Distribution of IL-4 levels based on the cut-off point value.

Based on gender characteristics, the majority of leprosy patients are male (27 or 67.5%), and there is a relationship between gender and leprosy patients. The incidence and prevalence of leprosy in male according to WHO is indeed higher compared to female. Another study from Manyulleii [8] states that there is a relationship between gender and the incidence of leprosy, where the percentage of leprosy is more common in male (60.8%). Similar results are also found, the research in Cairo show that the percentage of male patients is 62.8% compared to female, 37.2% [8]. However, research by Enas [10] found that the number of males affected by leprosy is higher than female, which is 62.2% (115 people) of the total population (185 respondents). The high number of prevalence of leprosy in men is associated with the production of androgen hormones and 5ά hormones dihydrotestosterone that can decrease cellular immune responses and reduce the expression of MHC II also suppresses the function of macrophages and lymphocytes. Conversely, female have estrogen which can be a protective factor against infection [12].

In this study, according to WHO criteria, MB type is the highest number of patients (36 patients or 90%) while the least is PB type (4 patients or 10%). This is similar to WHO [11] which states that the proportion of MB patients in the world is higher (60.6%) compared to PB type patients. Research from Verma [12] in the north region of India also found a higher percentage of MB patients (80%) compared to PB patients. The same results are found in the study at Sanglah, Denpasar where the percentage of MB patients is 88.8% compare to PB patients, 11.2%. The clinical type of leprosy depends on the immune system in each person, if the cellular immune system is strong then the clinical type will lead towards PB type, but if the cellular immune system is weak, the clinical type will lead to MB type [15].

In addition to this, there are genetic factors associated with HLA that also influence the direction of the clinical type of leprosy, as well as the presence of polymorphisms and 28 genes that affect leprosy type susceptibility and polarization. Besides that, from various studies, it was found that male sex is more often affected by MB type of leprosy compared to women and the latter age factor also contributed in determining the direction of the clinical type. Older age will lead to a clinical type of leprosy toward MB type [16].

According to Ridley Jopling's criteria, it can be seen that the most type of leprosy is BL type (19 patients or 47.5%). This is in line with research by Rinnovi [15] who examined the comparison of ML Flow test and Slit Skin Smear in Morbus Hansen patients in Palembang General Hospital who also found that the highest number of leprosy patients is BL type (50 patients or 79.4%). Other studies in Makassar also received that the largest distribution of subjects with leprosy is BL type, which is 78 out of 131 patients [18].

Referring to WHO criteria, based on the clinical contact relation, in non-lepers, the highest number of carrier is in PB/MB type, which is 55%. This is in line with non-leprosy subjects since some of them are medical personnel or paramedics who work in M. Hosein General Hospital, especially in the skin department, who has contact with the patient almost every day whether with PB or MB type. In a previous study, an analysis of cytokines in leprosy in Brazil found that there are 53% of MB type carrier and 47% for PB type [19].

Another study in Rio de Janeiro also shows a higher number of the MB type carrier with a percentage of 62% compared to the PB type [20]. This is in accordance with the proportion of MB type which has more prevalence in the world compared to the PB type so that more transmission will happen to the relatives from MB patients. Most of the carrier are medical personnel who had contact with two types of leprosy that it becomes one of the risk factors for leprosy transmission.

In this study, the median value of IL-4 level in leprosy patients is higher (209.63 pg/ml) compared to non-leprosy patients (63.38 pg/ml). Through statistical tests, it is also found that there were significant differences between IL-4 levels in lepers and non-lepers. Other studies on estimation of serum IL-4 and IL-17 to determine its immunopathogenesis in leprosy patients in Cairo also shows the same result. The median value of IL-4 in leprosy and non-leprosy patients are 2.31 pg/ml and 2.02 pg/ml respectively [8].

Similar results are obtained in the Poovama [19] and Verma Anurag [20] studies. They found that IL-4 levels in lepers are higher (37.83 and 35.20 pg/ml) compared to non-lepers (6.16 and 5.52 pg/ml). High level of serum IL-4 in lepers is closely related to the immune system balance mechanism activity against M. Leprae infection, antigenic properties of bacteria, genetics (HLA-DQ1), and also the type of leprosy where IL-4 levels will be higher MB type (BL, LL) than in PB type (TT, BT) [17].

In leprosy patients based on WHO criteria, the highest median value of IL-4 level is found in MB type (214.63 pg/ml), and the lowest is in the PB type (207.13 pg/ml). Through statistical tests, it is also found that there is no significant difference between IL-4 levels in PB type and MB type. Research from Verma [20] about serum IL-4 level in leprosy patients in North India also stated the same where IL-4 levels are significantly higher in MB type (37.49 pg/ml) compared to PB type (26.40 pg/ml). Another study in Cairo also found that IL-4 levels in MB type (2.42 pg/ml) are higher than those in PB type (2.24 pg/ml) [8].

In MB type, a large number of bacteria is accompanied by weak cellular immune system, Th1 along with proinflammatory cytokines, which will be suppressed by cytokines produced by Th2 such as IL-4. As a result of the low cellular immune system in MB type, bacterial growth cannot be restricted and thus increase antibodies that stimulated by IL-4 in the humoral immune system [24]. In PB type, the cellular immune system plays a significant role, where Th1 stimulate bactericidal mechanism of

macrophages for the destruction of M. leprae bacteria and is related to regulation and balance of Th1 and Th2. Th1 will suppress cytokine production from Th2 so that the presence of IL-4 in PB type is not significantly increase that the level is lower than MB type [23].

Based on Ridley Jopling's criteria, IL-4 levels are higher in LL type (219.63 pg/ml) compared to others. The same results are also found in studies from Abdallah [6] and Poovama [19] who received the highest IL-4 levels in lepers are from LL type. Other studies on the description of serum cytokine levels in leprosy patients who received treatment found that IL-4 levels are higher in BL types compared to other types of leprosy [18].

High level of serum IL-4 level in lepromatous type is typical. Leprosy clinical spectrum from tuberculoid type with a strong cellular immune system shift lepromatous type where the cellular immune system is weak but the humoral immune system is strong. As it is known that strong humoral immune system in lepromatous type will not be able to kill bacteria. The humoral immune system in lepromatous type is related to ENL reaction, where the immune complex occurs [25].

In patients with LL type, the main characteristic is the formation of a swollen granuloma that contains more than 300 M. Leprae inside the cytoplasmic vacuole, resulting in balloon-like Virchow cells formation. In the next phase, other macrophages will phagocyte Virchow cells which will provide neoanthigenic information expressed through MHC class II, stimulate APC and secrete IL-4, and finally, IL-4 will stimulate humoral immunity. This stimulation of Virchow cells also causes high levels of IL-4 in LL type lepers. In contrast, IL-4 levels are low in the tuberculoid type because of strong cellular immune system and cytokines from Th1. This regulation makes the cytokines from Th2 lower than Th1 [26].

Based on the cut-off point value of IL-4 levels, there were 39 patients who have an increased of IL-4 levels and only 1 person who does not. Meanwhile, there is no increase of IL-4 level from all non-leprosy patients. This shows that, according to the cut-off point, almost all lepers experienced elevated levels of IL-4. Whereas non-lepers do not experience any elevated levels of IL-4. The presence of 1 leprosy patients whose IL-4 levels do not increase can be attributed to improvement and treatment so that IL-4 levels over time will decrease following the regulation of the Th1, Th2 and T regulator immune systems.

But keep in mind that all subjects affected by leprosy are carriers who have been in contact with lepers for more than 2 years. The normal value of interleukin levels is not known. Therefore the increase of IL-4 levels in 1 leper and no IL-4 increase in all non-lepers cannot be said to be normal or abnormal because we still need to consider other factors and need further examination. As is known, a single cytokine cannot be used as a reference to determine identification or severity in a bacterial infection because all cytokines work continuously together [27].

The comparison of serum IL-4 levels in leprosy and non-leprosy patients have a significant difference between the median value IL-4 level with p-value/sig 2 tailed $0.000<0.05$. The median value of IL-4 level in leprosy patients is significantly higher (209.63 pg/ml) compared to non-leprosy patients (63.38 pg/ml). Compared with control groups, IL-4 levels are significantly higher in LL type (219.63 pg/ml) and in BT, BB, and MB types (207.13 pg/ml). This is consistent with research conducted by Abdallah et al. (2013 and 2014) stating that IL-4 levels are significantly higher than controls, where IL-4 levels are significantly higher in LL and TT type compared to controls [8].

Similar results are also found in studies in India where IL-4 are significantly higher in LL type compared with controls [21]. A recent study by Verma [20] also obtained the same results that IL-4 levels were higher in MB patients and in leprosy with ENL reactions compared with controls.

The first factor affecting high levels of IL-4 in lepers is genetic factors such as HLA DQ1 which will lead to the humoral immune system. It is known that HLA plays a role in MHC in presenting antigens from APC to T lymphocyte cells that will initiate a series of immune responses. The presence of HLA DQ 1 will trigger humoral immunity lead the disease into lepromatous type [28]. Another factor that affects high IL-4 levels is the conjunction between M. Leprae, macrophages and Schwann cells via PGL 1 in bacteria mediated by the expression of CD209, whereas IL-4 itself is also known to increase CD209 expression in Schwann cells that magnify the induction of IL-4 levels in lepers [29].

In leprosy patients, especially LL or MB type, the number of bacteria is high due to the weakening of cellular immune system, followed by high levels of IL-4 serum which indicates significant response of humoral immune system, yet this mechanism is not capable of killing all of the bacteria [25]. Virchow cells formed in the lepromatous type that contain M. Leprae antigene will stimulate APC through MHC II that stimulate T-helper 2 to secrete IL-4, and finally, IL-4 will stimulate humoral immunity which makes IL-4 levels high in LL or MB type [26].

Also, dead bacteria due to the destruction of the cellular immune system or medical treatment are also still antigenic because of the capsules they have. IgM Pgl-1 and IgG Pgl-1 antibodies are formed due to stimulation of antigens involved IL-4 that induces B cells into plasma cells and produces antibodies. The conjunction between the Pgl-1 antigen in bacteria and the IgM Pgl-1 and IgG Pgl-1 antibodies will activate the complement system and form an immune complex consisting of antigens, antibodies, and complement [23]. This immune complex will circulate in the body, then colonized on organs such as the skin and nerves.

It triggers an ENL reaction through humoral immune response from the antigen-antibody complex [30].

Besides, the process of cytokines regulation in the body has an important role in the immunological system. If pro-inflammatory cytokines in Th1 rise too high, Th2 cytokines will suppress the levels of each cytokine in the body. Inhibition of IL-4 in Th1 cytokines occurs through various methods such as TLRs macrophage resistance that bacteria PAMP will not bind to macrophages. Thus, cytokine induction will not occur via MHC. Another pathway is through inhibition of pro-inflammatory cytokines by inhibiting IL-12 which plays a role in TCD4^{+}-Th1 cells differentiations. Besides, IL-4 can produce IL-1R and TNF-ά soluble receptors that can inhibit the function of IL-1 and TNF-ά [31].

The majority of leprosy patients received MDT drugs treatment and the other received ROM. In previous studies, it shows that there is an increase in IL-4 levels in lepers after 12 months taking 3 months of ROM medication [24]. IL-4 levels also increase in patients with MDT; this is related to the destruction of the bacillus due to the effects of the treatment that triggers humoral response to eliminate antibody antigens immune complexes. Clofazimine and dapsone in MDT act as an anti-inflammatory agent against pro-inflammatory cytokines, but not on IL-4. Thus, the levels of IL-4 in leprosy patients continue to rise even during the treatment period [31].

In non-leprosy patients with a history of contact, the immune response to the M. Leprae when the first time exposed will begin to react, starting from non-specific or natural immune system to the cellular immune system. This process occurs for years until clinical symptoms arise, but if a serological examination is performed, it will give a positive result or often called subclinical leprosy.

Conclusions

It can be concluded that IL-4 was higher in leprosy patients than non leprosy patients. And also the distribution of leprosy respondents varies throughout its clinical types. According to WHO criteria, MB type has a higher frequency (36 people or 90%) compared to PB type (4 people 10%). While according to Ridley Jopling criteria, the highest type is BL (19 people or 47.5%), LL (10 people or 25%) and the least is BT and TT type (2 people or 5%). The median value of IL-4 levels in non-leprosy patients is 63.38 pg/ml. The median of IL-4 level in leprosy patients is 209.63 pg/ml. Base on WHO clinical type, the highest median value of IL-4 level is in MB type (214.63 pg/ml) and the lowest is in PB type (207.13 pg/ml). Base on Ridley Jopling criteria, the highest median value of IL-4 level is LL type (219.63 pg/ml), and the lowest is BT and BB types (207.13 pg/ml).

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