The Profile of Interferon-γ (IFN-γ) and Interleukin-10 (IL-10) in Pulmonary Tuberculosis Patients

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ABSTRACT

Introduction: Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* and becomes the main health problems in the world and in Indonesia, as well. The microorganism itself is an intracellular pathogen. The control of tuberculosis infection depends on cell-mediated immunity involving activated macrophages, T cells, and cytokines. The balance and dynamic changes between Th1 cytokine and Th2 cytokine reflect the immune response of host and influence the clinical manifestation of the disease.
Objective: This research was designed to study the profile and interaction of IFN-γ (Th1 cytokine) and IL-10 (Th2 cytokine) of pulmonary tuberculosis (PTB) patients in endemic area.
Methods: Peripheral blood mononuclear cells of 23 pulmonary TB patients and 16 healthy persons was cultured and stimulated by phytohaemagglutinin (PHA) to investigate the ability to secrete IFN-γ and IL-10.
Result: The result showed that there was a decreased of IFN-γ response to PHA in PTB patients, suggesting the deficiency of general immune capacity in PTB. In contrast, IFN-γ secreted by specific antigen was higher in PTB patients which minimal lung lesion was higher than moderate-far advanced. It is related to IFN-γ roles as immunomodulator in cellular immunity and immunoprotectant through stimulated antimicrobial capacity in macrophage. In fact, IL-10 response to PHA and *M.tuberculosis* antigen in PTB patients was lower than that of in healthy persons; moderate-far advanced lung lesion was the lowest. It was probably reflecting their poor general conditions. Paired distribution between IFN-γ and IL-10 pointed out the leaning of negative interaction. It reflected the existence of counterpart/cross regulation between IFN-γ (Th1 cytokine) and IL-10 (Th2 cytokine).
Conclusion: In conclusion that specific immune response of PTB is related to the degree of lung lesion. This study also provides the balance of Th1 cytokine and Th2 cytokine in relation to TB.

Key words: tuberculosis, immune response, IFN-γ, IL-10, lung lesion

INTISARI

Pendahuluan: Tuberculosis (TB) merupakan penyakit infeksi yang disebabkan oleh *Mycobacterium tuberculosis* dan menjadi masalah kesehatan utama di dunia dan Indonesia. Mikroorganisme ini merupakan patogen intraseluler. Pengendalian infeksi TB tergantung pada imunitas *cell-mediated* melibatkan makrofag teraktivasi, sel T dan sitokin. Keseimbangan dan perubahan dinamik antara sitokin Th1 dan Th2 menggambarkan respon imun inang dan mempengaruhi manifestasi klinis penyakitnya.
Tujuan: Penelitian ini dirancang untuk mempelajari profil dan interaksi antara IFN-γ (sitokin Th1) dan IL-10 (sitokin Th2) pada pasien tuberculosis paru (TB paru) di daerah endemik.
Metode: Sel mononuclear darah tepi dari 23 pasien TB paru dan 16 orang sehat dikultur dan distimulasi dengan antigen phytohaemagglutinin (PHA) dan *M.tuberculosis* yang disonikasi untuk diteliti kemampuan sekresi IFN-γ dan IL-10.

Hasil: Terdapat penurunan respon IFN-γ terhadap PHA pada pasien TB paru, yang menunjukkan defisiensi kapasitas imun umum pada TB paru. Sebaliknya, IFN-γ yang disekresi oleh pacuan antigen spesifik lebih tinggi pada pasien TB paru yang memiliki lesi paru minimal dibandingkan dengan yang moderat – lanjut sekali. Ini berhubungan dengan peran IFN-γ sebagai imunomodulator pada imunitas seluler dan imunoprotektan melalui jalur stimulasi kapasitas antimikrobia pada makrofag. Respon IL-10 terhadap PHA dan antigen *M.tuberculosis* pada pasien TB paru lebih rendah daripada orang sehat; pasien dengan lesi moderate-lanjut sekali merupakan yang terendah. Hal ini mungkin merefleksikan kondisi umum mereka yang berat. Distribusi berpasangan antara IFN-γ dan IL-10 menunjukkan interaksi negatif berketergantungan. Ini merefleksikan keberadaan *counterpart/cross regulation* antara IFN-γ (sitokin Th1) dan IL-10 (sitokin Th2).
Simpulan: Respon imun spesifik TB paru berkaitan dengan derajat lesi paru. Penelitian ini juga menyajikan tentang kesetimbangan antara sitokin Th1 dan Th2 dalam kaitannya dengan TB paru.

Kata kunci: tuberculosis, respon imun, IFN-γ, IL-10, lesi paru

INTRODUCTION

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis* and a health problem in the world. It is estimated one third of the world population has been infected[1]. Indonesia is ranked third in the world with the approximate number of patients ten percent of the total number of patients in the world[2]. In Yogyakarta in 2005 there were 5,416 suspected tuberculosis patients with the finding of positive acid resistant bacteria in only 488 persons[3].

Tuberculosis can occur in any organ in various clinical manifestation reflecting the balance between the virulence of *M.tuberculosis* and the response of host[4,5]. An obligate aerobic intracellular pathogen with the predilection for oxygen-rich lung tissue[6], phagocytosis on bacilli by alveolar macrophage is the first interaction of the host with the phatogen[7]. Tuberculosis infection particularly depends on cellular immunity, that is the interaction between macrophage, stimulated antigen-specific T-Lymphocyte and secreted cytokine[7,8].

Based on the secreted cytokine, T-Lymphocyte helper is divided into 2 subsets[9], namely Th1 cell having the characteristic of primary production of IL-2 and IFN-γ, also called Th1/type-1 cytokine; and Th2 cell having characteristic of production of IL-4, IL-5 and IL-10, also called Th2/type-2 cytokine[7]. Both subsets develop from naive T cell whose differentiation is influenced by environment. Promotion of Th1 cell is inducted by IL-12 and IFN-γ produced by activated macrophage and dendritic cell, while promotion of Th2 cell is inducted by IL-4 and IL-6[10,11]. Th1 and Th2 cytokines will stimulate each other or inhibit the work each other.

On the infection of mycobacterium, Th1 cytokine is important for protective immunity. Meanwhile, Th2 inhibits *in vitro* production of IFN-γ and activation of macrophage and therefore weaken the defense of the host. Dynamic balance and changes between Th1 and Th2 reflect immunity system and influence clinical manifestation of the disease[5]. Several studies indicates the increase of Th2 cytokine on tuberculosis patients[12], but it is not a consistent finding and contradicts to the finding stating that TB patients produces IFN-γ higher than that of tuberculin-reactive healthy individuals[13]. As a result, relationship between Th1-Th2 and disease vulnerability is still unclear.

This research was aimed at identifying the profile and interaction between IFN-γ (Th1 cytokine) and IL-10 (Th2 cytokine) on pulmonary tuberculosis in endemic areas. In order to identify and examine further about the role of

Th1 and Th2 cytokines on infected lung lesion, mononuclear cells of pulmonary TB patients and healthy persons as the control were cultured and stimulated by antigen to measure the ability to secrete IFN-γ and IL-10. PHA was utilized to identify the immunity capacity of the immunity system and *M.tuberkulosis* sonicate antigen to assess specific immune response related to pathogen.

MATERIALS AND METHODS

Sample collection. Ten ml of venous blood samples of new pulmonary tuberculosis patients were obtained from the Pulmonology of dr. Sardjito Hospital and BP4 Semarang from April until August 2008. Diagnosis of pulmonary tuberculosis was established based on the examination of BTA sputum and radiological description of pulmonary roentgen. Subsequently, based on the width of lung lesion in chest roentgen, samples were classified into minimum lung lesion and moderate-far advance lung lesion. At the same time, samples of healthy persons were used as the controls, interviewed with questionnaires, and given routine physical and blood examination. Within less than 24 hours (fresh whole blood), blood samples had to be isolated in the Tropical Medicine Laboratory, Department of Parasitology, Faculty of Medicine, Universitas Gadjah Mada. In this study, 23 blood samples of pulmonary tuberculosis patients and 16 of healthy persons as the control.Inclusion criteria in this research were pulmonary tuberculosis patients willing to participate in the research, with exclusion criteria of HIV and diabetes mellitus patients. The research subjects received the informed consent explaining the purpose of sampling and obtaining approval to be employed in this research.

Isolation of peripheral blood mononuclear cells from blood samples. Venous blood cells taken using heparin vacutainer were separated from the plasma. Blood samples were put into 50ml conical tube, added with RPMI media (1:1) and slowly shaken. Ficoll-HyPaque gradient centrifugation was put into the tube base with Pasteur pipette (1:1) and then centrifuged at the rate of 1800 rpm for 20 minutes with low braking resulting in the formation of 3 liquid layers ((RPMI, lymphocyte, Ficoll-HyPaque) and erythrocyte sedimentation and granulocyte. Interphase layer (lymphocyte) was moved into 15ml conical tube, centrifuged at the rate of 1800rpm for 10 minutes resulting in the formation of pellet. Supernatant was discarded and cells were resuspended with PBS. It was then centrifuged at the rate of 1200rpm for 10 minutes. Resuspension was repeated 3 times. In the final phase, pellet was resuspended with 1 ml of culture media consisting of AIM V or IMDM containing 10% FCS, 2% Penicillin-Streptomycin, 0.5% Fungizone. The number of cells was calculated and made into suspension with concentration of $1.5x\ 10^6$cell/ml. All steps in this phase were conducted at room temperature.

Mononuclear cell culture and simulation with tuberculosis antigen. A hundred ul suspension containing $1.5x10^5$ mononuclear cells was cultured for 5 days in roundbottorm 96-well culture crucible in the incubator at the temperature of 37^oC and 5% CO2. Cells were stimulated with PHA antigen (final concentration 5ug/ml) and *M.tuberculosis* sonicate antigen (final concentration 10ug/m) as well as culture media as the negative control. Each antigen was made into 3 replications. At the fifth day, the existing supernatant was taken as much as 75-100µl from each well and the cytokine level was measured.

Measurement of IFN-γ level with ELISA method. IFN-γ level was measured using ELISA Kits of U- Cytech, Netherland with antibody coating MD-2, antihuman IFNγ-biotin antibody detector and standard in the form of natural

human IFNγ. The method was used according to manual instruction, that crucible was coated with 100µl of coating antibody overnight at 4°C, then resuspended with PBS/0.05% Tween20 for 5 times. It was then blocked with 100µl of PBS/2% dry skimmed milk for 1 hour at 37°C. The well was emptied and then added with 100µl of standard and sample and incubated overnight. The crucible was resuspended again 5 times and added with 100µl of detector antibody in PBS/1% BSA and incubated for 1 hour at 37°C. After resuspended again 5 times, it was added with 100µl of streptavidin-HRP and incubated for 45 minutes at 37°C. The crucible was resuspended again 10 times and mixed with 100µl of TMB-substrate buffer and incubated until the color of the highest standard concentration did not change any more. The reaction was stopped by 100µl of 1M H_2SO_4. OD was measured at 450nm. OD obtained from standard (10000pg/ml-39pg/ml) was then transfered into standard curve to identify IFN-γ level from the tested supernatant sample.

Measurement of IL-10 level with ELISA method. It utilized ELISA Kits of Biosource with antihuman IL-10 coating antibody materials, antihuman IL-10 biotin detector antibody and standard recombinant human IL-10. It utilized the method in accordance with the enclosing manual instruction. The palate was coated with 100ul coating antibody and then incubated overnight at the temperature of 4°C. It was then emptied and resuspended twice with PBS/Tween 200ul/well. It was blocked with PBS/BSA/Tween 200ul/well for at room temperature. Standard and sample of 50ul/well was put in and added with PBS 50ul/well and detector antibody 50ul/well. It was incubated for 2 hours at room temperature with shaker, resuspended 5 times with wash buffer. Streptavidin-HRP 100ul/well was put in and the crucible was then incubated for 30 minutes with shaker. It was resuspended again 5 times with wash buffer. 100ul/well TMB-substrate buffer was put in and the crucible was incubated for 30 minutes with shaker. 100ul/well 1M H_2SO_4 was put in and the absorbance was at 450nm (double: 650nm) for 30 minutes after the addition OD stoppage. OD obtained from standard (2000pg/ml-31,2pg/ml) was then changed into standard curve in order to identify IL-10 level from the tested sample supernatant.

Data analysis. ELISA result in the form of absorbance value (OD) obtained after reading with the ELISA reader which was then translated into cytokine level with MPM (Multi Crucible Manager) ELISA Software. OD from the standard was transferred into standard curve in order to identify IFN-γ and IL-10 level from the tested samples. Data from the calculation result were analyzed using SPSS 15 program in the form of non-parametric Kruskal-Wallis and Mann-Whitney test and correlation test to identify the relation.

RESULTS AND DISCUSSION

Subject Characteristics. Six out of 23 tuberculosis samples (26%) were obtained from Pulmonology of dr. Sardjito Hospital Yogyakarta and 17 samples (74%) from BP4 Semarang. Based on sex, there were 12 males (52.2%) and 11 females (47.80%) with average age of 38.61 (17-69) years old. Meanwhile, based on the width of lung lesion on breast roentgen, samples were classified into minimum lung lesion (n=10; 43.5%) and moderate-far advanced lung lesion (n=13; 56.5%). Meanwhile, there were 16 samples of healthy persons consisting 8 males (50%) and 8 females with average age of 31.44 (19-50) years old.

Based on the physical and blood examination as shown on the Table 1, although still in the category of normal, the average of body mass index (BMI) of tuberculosis patients was lower than that of healthy persons ((19.61±3.03 kg/

Table 1. Characteristic of tuberculosis patients compared to healthy person

Parameter	Healthy Persons	Tuberculosis Patients	
		Minimum Lesion	Moderate-Far advanced Lesion
Number of Cases	16	10	13
Median of age (range), in year	31.44 (19-50)	42.00 (20-69)	36.00 (17-55)
Number of Male (Percentage)	8 (50)	6 (60)	6 (46,15)
Mean of body mass index (kg/m2)	20.25±3,69	19.79±3.35	19.47±2.89
Mean of Leucocyte number (/mmk)	8757±1412	8258±4395	9808±3626
Mean of Hb level (g/dl)	15.09±1.14	12.06±1.06	12.07±1.37

Table 2. Statistical test of interferon-γ secretion

	Mean of IFN level (pg/1.5x10^{5}sel/ml/5days)		*p*
	Normal (Healthy)	Tuberculosis	
PHA	8117.55±1583,57 (181.11-16000)	2583.27±4881.24 (0-502.24)	0.004[a]
M. tuberculosis Sonicate	223.21±90.36 (0-16000)	435.31±92.81 (0-1338)	0.049[a]

[a] Statistically significant (p<0.05)

m2 vs 20.25±3.69 kg/m2, p=0.575). Meanwhile, although still in the normal limit[14], the average of leucocyte of tuberculosis patients was higher than that of healthy persons (9.079±4.197/mmk vs 8.757±1.412/mmk, p=0,991). In addition, the average of Hb level of tuberculosis patients was lower than that of healthy person (12.06±1.23g/dl vs 15.09±1.14 g/dl, p=0.000, CI 95%). It indicates that anemia often accompanies tuberculosis infection[15].

Secretion of IFN-γ towards PHA and *M.tuberculosis*. The result of Secretion of IFN-γ by PBMC stimulated with PHA on lung tuberculosis-infected patients was lower than that of healthy persons (8117.55±1583.57pg/ml vs 2583.27±4881.24pg/ml, p<0.05, mean ± S.E). It indicates that the capacity of one's immunity decreases in general/not specific. On the contrary, IFN-γ secretion on lyphocyte stimulated by *M.tuberculosis* antigen indicates that secretion of IFN-γ level on tuberculosis patients was higher than that of healthy person (223.21±90.36pg/ml vs 435.31±92.81pg/ml, p<0.05).

Based on the degree of lung lesion, IFN-γ level lowered from minimum lesion, moderate-far advanced lesion and healthy person respectively (624.84±164.19pg/ml vs 289.52±91.16pg/ml vs 223.21±90.36pg/ml). It reflects the protective role of IFN-γ through the increase of antimicrobial capacity on macrophage.

Secretion of IL-10 toward PHA and *M.tuberculosis*. Interleukin-10 is cytokine produced by Th2 cell, dendritic cell and

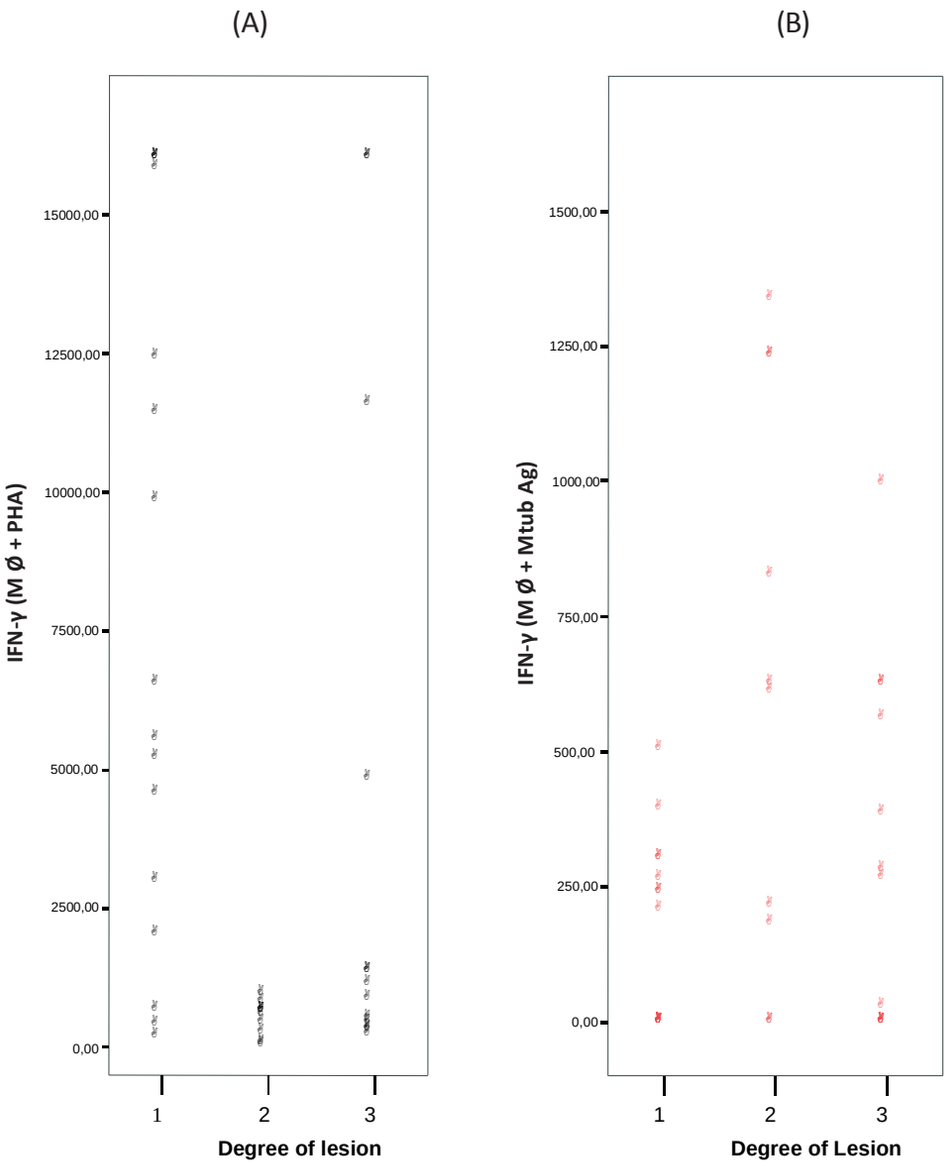

Figure 1. Distribution of IFN-γ secretion (pg/1.5x10^{5}sel/ml/5days) of mononuclear based on the degree of lung lesion. (A) stimulated with PHA. (B) stimulated with *M. tuberculosis* antigen. (1: healthy/normal, 2: minimum lung lesion, 3: moderate-far advance lung lesion)

especially activated macrophage. The research result indicated that mean of IL-10 secretion on healthy persons was higher than that of tuberculosis patients either stimulated with PHA (426.47±79.58pg/ml vs 59.07±25.68pg/ml, p<0.05) or with *M.tuberkulosis* (261.97±68.98pg/ml vs 33.41±20.29pg/ml, p<0.05).

Based on the degree of lung lesion, mean of IL-10 secretion toward *M.tuberkulosis* sonicate antigen, minimum lung lesion was higher than that of moderate-far advance lung lesion (64.41±45.34pg/ml vs 9.57±6.30pg/ml, p<0.05). on all stimulation, moderate-far advance lung lesion secreted cytokine with low level. It is assumed that there is extensive systemic deficiency of immune system resistance.

Table 3. Statistical test of interleukin-10 secretion

	Mean of IL-10 level (pg/1.5x10^{5}sel/ml/5days)		*p*
	Normal (Healthy)	Tuberculosis	
PHA	426.47±79.58	59.07±25.68	0.000[a]
M.tuberkulosis sonicate	261.97±68.98	33.41±20.29	0.000[a]

[a] Statistically significant (p<0.05)

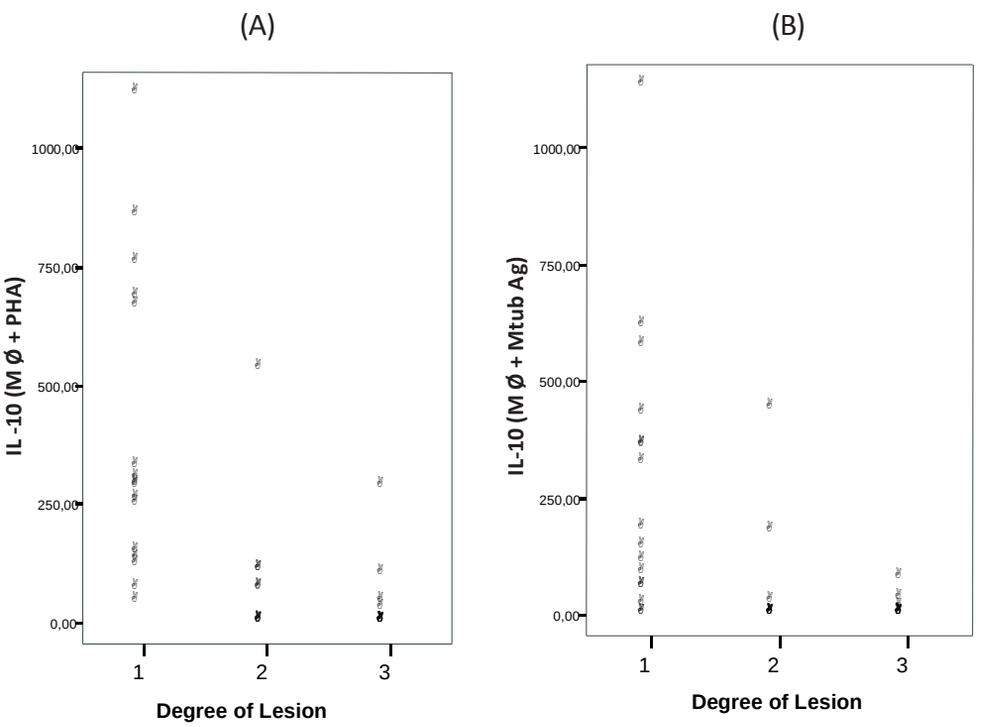

Figure 2. Distribution of IL-10 secretion (pg/1.5x10^{5}sel/ml/5days) of mononuclear cell based on the degree of lung lesion. (A) Stimulated with PHA. (B) Stimulated with *M. tuberculosis* antigen. (1: healthy/normal, 2: minimal lung lesion, 3: moderate-far advance lung lesion).

Relation between IFN-γ (Th1 cytokine) and IL-10 (Th2 cytokine) secretion. In the paired distribution between the secretion of IFN-γ and the secretion of IL-10 on mononuclear cells toward *M.tuberkulosis* specific antigen, it was evident that the linear line between IFN-γ and IL-10 secretion tended to be inclined to the left with a low coefficient regression (R2=0,02; p>0.05, figure 1). This tendency signified the negative complexion between IFN-γ and IL-10.

M. tuberculosis is an intracellular pathogen most commonly found in macrophages. It meant that the control over TB infection depends on the cell-mediated immunity involving activated macrophages mediated by T lymphocytes and cytokines. In the concept of Th1 and Th2 cytokines, the balance between both cytokines reflected the immunity response between the

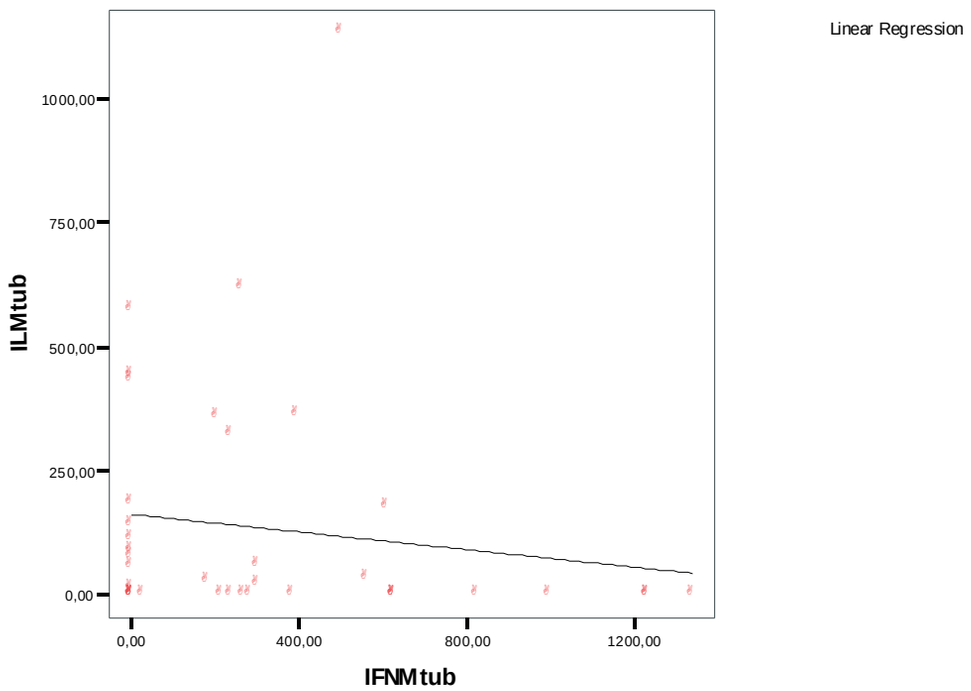

Figure 3. Paired distribution of IFN-γ and IL-10 toward *M.tuberculosis* antigen (pg/1.5x105sel/ml/5days)

strength of the host and the cause of infection. It also influenced the clinical manifestation of the disease[7,16].

Most of the times, patients with active tuberculosis infection suffered from immune system deficiency. It was usually indicated by an anergy skin test and low IFN-γ secretion[13,17,18]. It occurred during the research, that IFN-γ secretion on the stimulation toward PHA in tuberculosis patients was lower than that of healthy persons. It indicated that in general, immunity deficiency in the form of cellular immune response depression occurred, causing the host's failure in resisting mycrobacterium bacilli that managed to reach and nested in the bronchioles and lung alveolus, which would result in the manifestation of tuberculosis infection[5,18].

On the other hand, the higher level of IFN-γ secretion on the mono-nuclear cells of tuberculosis patients was stimulated with the *M. tuberculosis* antigen due to the fact that the antigen represented the specific immunity of the tuberculosis infection. Subsequently, the antigen would evoke the immunity system capable of memory, namely the T cell that had been sensitized by the previous *M.tuberculosis*[19]. Also, as a specific response of meeting a familiar antigen, the T cell of the sensitized individual, or the individual infected with tuberculosis would proceed with producing higher IFN-γ.

Based on the degree of lung lesion, the level of IFN-γ on minimum lesion was higher than that of moderate-far advance lesion. It was allegedly related to the bigger role of IFN-γ as an immunomodulator on cellular immunity and immunoprotectant on the tuberculosis infection by its ability in increasing macrophage antimicrobial capacity[5,20].

Rats whose IFN-γ was damaged and then infected with *M.tuberculosis* was not able to control the infection, allowing the bacilli in the lungs, spleen, and liver to proliferate rapidly[21].

Furthermore, the decrease of IFN-γ secretion by the mononuclear cells on tuberculosis infection could be employed as a marker of severe tuberculosis infection[22].

Interleukin-10 is a cytokine produced by Th2 cells, dendritic cells and especially activated macrophage. Mocellin states that IL-10 carried pleiotropic effects, namely immunosupression and immunostimulatory[23]. In a tuberculosis infection, IL-10 was also known as an anti-inflammation cytokine produced by macrophage that had performed phagocytosis on *M.tuberkulosis* and bound microbacterium LAM5. IL-10 also protected the host from excessive immune response inducted by type-1 cytokine on the intracellular infection, playing a role as response balancer. If IL-10 disappeared, extensive damage would occur on the host[24].

Based on the degree of lung lesion, IL-10 secretion on moderate-far advanced lung lesion was lower than that of minimum lung lesion. The low of the cytokine secretion on all stimulation of moderate-far advance was allegedly related to the deficiency of is extensive systemic deficiency of immunity system resistance.

The tendency of negative characteristic of IFN-γ and IL-10 on the tuberculosis infection indicated the existence of counterpart or cross regulation between them[7]. IFN-γ as Th1 cytokine had an important role in protective immunity towards tuberculosis infection. Along with TNF-α, IFN-γ would activate alveolar macrophage and eliminate intra cellular pathogen through the induction of antimicrobacterial activities such as expression of NOS[27]. IL-10 as Th2 cytokine allegedly antagonized the response of proinflammation cytokine by down-regulating production of IL- macrophage resulting in decreasing production of IFN-γ of T cell and activation of macrophage. However, depression of IFN-γ was only affected by IL-10. Rats whose IL-10 gen was disrupted still had capability of eliminating tuberculosis bacilli well[21].

CONCLUSION

It can be inferred from this research that specific immune response on the lung TB patients is related to the degree of lung lesion. The higher secretion of IL-10 on the lighter degree of lesion accompanied with tendency of negative relation between IFN-γ and IL-10 indicate that the depression of type-1 immunity is related to the increase of type-2 immunity, but not directly correlated with the degree of lung lesion.

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