Immune Response of Thalassemia Major Patients in Indonesia with and without Splenectomy

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ABSTRAK

Tujuan: *mengevaluasi profil respons imun pasien talasemia mayor di Indonesia yang menjalani splenektomi dan tanpa splenektomi.* ***Metode:*** *penelitian dilakukan di Pusat Thalassaemia, Rumah Sakit Cipto Mangunkusumo Jakarta pada bulan September 2013 – Februari 2014. Metode belah lintang komparatif dilakukan pada pasien talasemia mayor sehat berusia >12 tahun dan HIV negatif. Kelompok non-splenektomi terbentuk setelah melakukan matching usia dan jenis kelamin pada kelompok pasca-splenektomi. Analisis terdiri dari respons imun non-spesifik (jumlah dan fagositosis neutrofil) dan respons imun spesifik (jumlah dan fungsi imunitas selular). Episode infeksi dianalisis sebagai parameter respons imun in vivo.* ***Hasil:*** *kelompok pasca-splenektomi mempunyai jumlah neutrofil lebih tinggi dan fagositosis neutrofil lebih rendah dibanding non-splenektomi. Kelompok pasca-splenektomi mempunyai jumlah limfosit total, jumlah limfosit T, jumlah limfosit T CD4+ dan CD8+ yang lebih tinggi dibanding non-splenektomi ($p<0,05$). Rasio CD4+/CD8+ sama antara kedua kelompok. Tidak ada perbedaan respons imun spesifik kualitatif (fungsi limfosit T CD4+ dan CD8+) antara kedua kelompok. Median infeksi ringan kelompok pasca-splenektomi adalah 2,02 (kisaran 0 sampai 12) kali dan kelompok non-splenektomi adalah 0,81 (kisaran 0 sampai 8) kali ($p=0,004$). Infeksi berat kelompok pasca-splenektomi adalah sepsis selama 2 minggu dan diare selama 1 minggu. Sedangkan infeksi berat kelompok non-splenektomi adalah demam tifoid selama 4 hari.* ***Kesimpulan:*** *terdapat perbedaan bermakna respons imun diantara pasien talasemia mayor. Kelompok pasca-splenektomi menunjukkan kerentanan terhadap infeksi lebih besar dibanding non-splenektomi.*

Kata kunci: *respons imun, talasemia mayor, splenektomi.*

ABSTRACT

Aim: *to describe non-spesific and specific immune response profile in Indonesian thalassemia major with and without splenectomy.* ***Methods:*** *this study was held at Thalassaemia Centre, Cipto Mangunkusumo Hospital Jakarta on September 2013 – February 2014. A comparative cross sectional study was conducted in healthy, thalassemia major aged more than 12 year and seronegative HIV. They were matched in age and sex for splenectomised and*

non-splenectomised groups, analysing the non-spesific immune response (neutrophil count and phagocytosis) and specific immune response (count and function of cellular immunity). Infection episodes were also analized as immune response in vivo parameter. ***Results:*** *splenectomised thalassemia major showed increased neutrophil count but significantly decreased non-spesific immune response (neutrophil phagocytosis). Spesific immune response of splenectomised group presented significantly higher absolute lymphocyte, lymphocyte T, CD4+ and CD8+ counts compared to non-splenectomised thalassemia major ($p<0.05$). Ratio CD4+/CD8+ were similar in these groups. Serum marker of activated cellular imunity function (IL-2 and TNF-α) were similar among two groups. Mild infection episodes on splenectomised and non-splenectomised group were 2.02 (ranged 0 to 12) times and 0.81 (ranged 0 to 8) times ($p=0.004$), respectively. Severe infection on splenectomised group were sepsis for 2 weeks and diarrhea for 1 week, whereas on non-splenectomised group was typhoid fever for 4 days.* ***Conclusion:*** *there were significant differences on immune response among thalassemia major patients. Splenectomised thalassemia major showed a greater degree of susceptibility to infections than non-splenectomised thalassemia major.*

Key words: *immune response, thalassemia major, splenectomy.*

INTRODUCTION

Most of the thalassemia patient's morbidity and mortality are caused by heart failure or infection.[1-3] In Italy, infection was the second cause of death after heart failure whereas in Thailand, infection was the most common cause of morbidity and mortality.[4,5] In Thalassaemia Center, Cipto Mangunkusumo Hospital, Jakarta, infection (34%) was the second mortality cause after heart failure (46%).[6] Moreover, post-splenectomy infection has higher mortality and morbidity.[7] A review of Bisharat[7] showed that 3.2% of 19,680 splenectomy patients had invasive infection with mortality rate 1.4%. Whereas, an observation at Thalassaemia Centre, Cipto Mangunkusumo Hospital in 2011 found that number of invasive infection among post-splenectomy patient was higher than non-splenectomy patient ($p<0.001$). However, there was no significant difference between type of thalassemia and number of infection ($p>0.05$). The severe infections were pneumonia, sepsis, liver abscess and tuberculosis.[6] In addition, pneumonia was one of the important causes of infection following splenectomy in thalassemia major patients.[8,9]

The mechanisms underlying this increased susceptibility to infections in thalassemia are abnormal immune response due to either pathophysiology of the disease, blood transfusion, iron overload, splenectomy or zinc deficiency.[2,4,10,11] Several studies also showed that abnormal immune response was associated with chelation therapy.[12-14]

The results of immune response study among thalassemia patients varied. Research of innate immunity on thalassemia patients showed disturbance of macrophage phagocytes function[2,4] whilst Shaiegan et al.[15] and Pattanapanyasat et al.[16] revealed no differences on neutrophil phagocytosis between thalassemia patients and normal person. Furthermore, a research on cellular imunity on thalassemia patients showed increased amount and activity of suppressor cell CD8+, decreased ratio of CD4+/CD8+, decreased of T lymphocyte proliferation and increase T lymphocyte activation[2,4] whereas humoral immunity research showed similar result between thalassemia and normal person.[17-19] A research conducted by Vergin et al.[19] showed no difference on immunoglobin level between β-thalassemia post-splenectomy and non-splenectomy. Until now, non-spesific and specific immune response in thalassemia in Indonesia are still unknown. Therefore, the aim of this study is to describe non-spesific and spesific immune response between post-splenectomy and non-splenectomy thalassemia patients. Furthermore, it is important for internist to know about this study since most of the splenectomy patients in Indonesia were already at adult stage.

METHODS

Patients

A comparative cross sectional study was held at Thalassaemia Centre, Cipto Mangunkusumo

Hospital Jakarta on September 2013 – February 2014. This present study enrolled 116 healthy thalassemia major patients, aged 12 years or older (median age 21 years; ranged 12 to 38 years). Patients were excluded from this study if they had HIV positive. Blood was taken just before a scheduled transfusion. The protocol for the present study was approved by the Ethics Committee of Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia. Informed consent was obtained from all subjects before participated in this study.

Clinical Laboratory Parameters

Clinical laboratory examinations included hemoglobin, hematocrit, differential leukocyte count and peripheral blood morphology was carried out. Blood smear were performed in order to rule out the nucleated RBCs. Levels of serum ferritin and serum transferrin saturation were also determined. Serum transferrin saturations were calculated according to ratio serum iron to total iron binding capacity (TIBC) multiplied by 100.

Thalassemia Major Immune Response

This study evaluated non-spesific and spesific immune response. Non-spesific immune response consisted of neutrophil count and phagocytosis. The quantitative specific immune response evaluated the lymphocyte count, the subsets CD4+ and CD8+ T lymphocyte and ratio CD4+/CD8+. Moreover, qualitative specific immune response test evaluated T lymphocyte function in order to assess the ability to produce IL-2 and TNF-α after PHA stimulation.

Analysis of Lymphocyte Surface Markers

It used 50 μL fresh heparinized blood. The principle is evaluate the positive result after adding CD45+ (to differentiate lymphocytes group), CD3+ (to differentiate B and T lymphocyte), CD4+ (to differentiate T helper cell), and CD8+ (to differentiate T cytotoxic cell) markers. T helper cell is CD45+/CD3+/CD4+ and T cytotoxic cell is CD45+/CD3+/CD8+. The measurement of positive result was using flow cytometry BD FACS CaliburTM .

Analysis of T Cell Activation Markers

The function of T lymphocyte was evaluated by measuring the expression of IL-2 and TNF-α on the unstimulated CD4+ and CD8+ T lymphocyte before and after PHA stimulation. The lymphocyte was stimulated for 4-6 hours followed by FITC IL-2 and FITC TNF-α specific staining (BD Biosciences). The flow cytometry BD FACS CaliburTM will calculate the proportion of IL-2 and TNF-α expression that produce by T lymphocyte.

Analysis of Neutrophil Phagocytosis

The Neutrophil Phagocytosis examination was measured using Phagotest kit (Glycotope Biotechnology). This test will count the percentage of phagocyte actively eliminate the bacteria. The examination is consisting of dispensing, activation, incubation, quenching, washing, lysing and fixation, washing, DNA staining and reading. The neutrophil count used 10000-15000 cells per sample. Then the percentage of bacteria contained neutrophil labelled by fluorescence and counted using flow cytometry BD FACS CaliburTM. Neutrophil was identified as high side light scatter (SSC-H) and large forward light scatter (FSC-H) distribution.

Statistical Analysis

Analysis of the data was done using Statistical Package for Social Sciences (SPSS) version 20.0. The categoric variable consisted of sex, type of thalassaemia, nutrition status, type of blood transfusion, frequency of transfusion, iron chelation and hepatitis status. The numeric variables were age, anemia degree, neutrophil count and phagocytosis, total lymphocyte count, T lymphocyte count, CD4+/CD8+ ratio, activated T lymphocyte with IL-2 and TNF-α markers, ferritin, and transferin saturation. The immune response parameter was analysed by paired t-test for the normal distribution and wilcoxon signed ranks for the not normal one.

RESULTS

From 67 post-splenectomy medical records, only 58 subjects were eligible (6 subjects declined, 2 subjects were still under tuberculosis treatment and 1 subject was unable to be contacted). The 58 non-splenectomy thalassemia subjects were matched by age and sex with post-splenectomy thalassemia subjects. Twenty-two

out of 58 (37.9%) non-splenectomy subjects were on hypersplenism status. The median age was 21 years old (range 12-38 years) and more than half of the subjects were female (68 subjects, 58.6%). (**Table 1)**

Table 1. Subjects characteristics

Parameter	Post-splenectomy (n = 58)	Non-splenectomy (n = 58)
Type of thalassemia		
- β	29	26
- β-HbE	29	31
- Alpha	0	1
Nutritional status		
- Obese	0	1
- Overweight	0	2
- Normal	25	28
- Underweight	20	23
- Severe malnourished	13	4
Blood transfusion frequency		
- Rarely (≤2 times/ year)	1	0
- Sometimes (3-4 times/ year)	7	0
- Frequent (>4 times/ year)	50	58
Blood transfusion type		
- PRC	5	9
- Leucodepleted PRC	21	37
- Washed PRC	32	12
Ferritin, ng/mL	6,585.50 (645 to 21,835)	4,744 (1,381 to 14,554)
Transferrin saturation, %	100 (26 to 118)	97.07 (9 to 100)
Iron chelation		
- Desferoxamin (DFO)	8	8
- Deferiprone (DFP)	32	32
- Deferasirox (DFX)	13	12
- Combination DFO + DFP	5	6
Hepatitis marker		
- Hepatitis B	13	13
- Hepatitis C	22	21

The most common type of thalassemia during this study were β and β-HbE on both groups. Almost half of the subjects (45.7%) had good nutritional status followed by underweight (37.1%), severe malnourished (14.7%), overweight (1.7%) and obesity (0.8%). Although nutritional status were equally distributed between both groups (p=0.086), the mean calorie intake of post-splenectomy group (1,943.29 (SD 334.43) kcal) were lower than non-splenectomy group (2,102.45 (SD 388.85) kcal) (p=0.019). Therefore, calorie intake and nutritional status had moderate correlation on both, post-splenectomy and non-splenectomy (r=0.421, p<0.001), (r=0.561, p<0.001), respectively.

Eight out of 58 post-splenectomy subjects had less than 4 times per year transfusion compared to non-splenectomy groups who had more frequent transfusion (p=0.014). Therefore, it was no significant difference on mean haemoglobin level between post-splenectomy (7.50 (SD 0.87) g/dL) and non-splenectomy (7.22 (SD 0.87) g/dL) (p=0.088). Blood transfusion frequency within a year on post-splenectomy groups had median 8 (0 to 24) times compared to non-splenectomy groups with median 12 (6 to 36) times (p=0.005). Post-splenectomy groups used more washed PRC than non-splenectomy. Moreover, severe iron overload was found on post-splenectomy group that showed by higher ferritin and transferrin saturation significantly compared to non-splenectomy group. In addition, deferiprone is the most common iron chelation therapy on both groups.

Hepatitis is an important infection that can affect the immune response parameter. The total subjects who were positively infected by hepatitis B or C was similar between both groups (p>0.05). Respiratory infection was the most common mild infection found on post-splenectomy and non-splenectomy groups within a year. The median of mild infection episodes within one year on post-splenectomy groups were 2.02 (0 to 12) times with duration of 4.5 (0 to 60) days whilst non-splenectomy groups was 0.81 (0 to 8) times with duration 0 (0 to 100) days, (p=0.004, p=0.001 respectively). Severe infections on post-splenectomy group were sepsis for 2 weeks and diarrhea for 1 weeks whereas on non-splenectomy

was typhoid fever for 4 days. Eventually, post-splenectomy groups were often infected with longer duration of infection.

Thalassemia Major Non-specific Immune Response

Neutrophil count on post-splenectomy had median 8,846 (2,720 to 67,150) sel/µL whereas non-splenectomy had 3,211 (1,130 to 7,360) sel/µL ($p<0.001$). Neutrophil phagocytosis of post-splenectomy group had median 29.79 (4 to 81) percent whilst non-splenectomy group had 55.83 (2 to 133) percent ($p<0.001$). Therefore, the ability of neutrophil phagocytosis on post-splenectomy group was significantly lower than non-splenectomy group. Hypersplenism is one of indication to perform splenectomy on thalassemia patients. The differences of neutrophil phagocytosis between hypersplenism and non-hypersplenism of non-splenectomy group are shown in **Figure 1**.

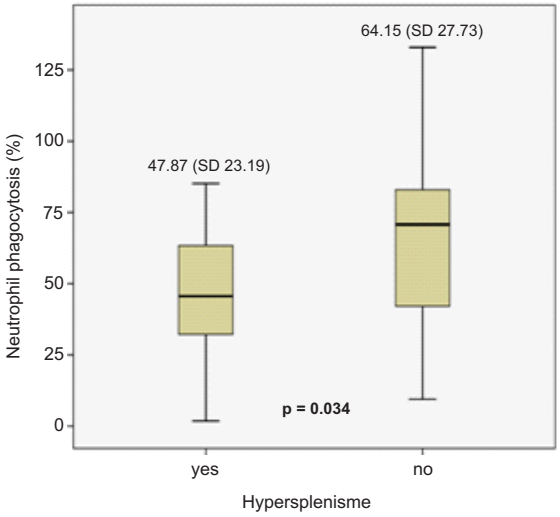

Figure 1. Neutrophil phagocytosis function differences among non-splenectomy group with and without hypersplenism

Thalassemia Major Specific Immune Response

The quantitative tests for thalassemia major specific immune response were higher in all parameters on post-splenectomy group compared to non-splenectomy group. Most of the parameters except CD4+/CD8+ ratio showed significant differences. The total lymphocyte count and total T lymphocyte count was significantly increased on post-splenectomy group, as a consequence, the T lymphocyte CD4+ and CD8+ was also significantly accelerated. Therefore, CD4+/CD8+ ratio was stable. There were no differences from all immune response parameter among non-splenectomy group with and without hipersplenism.

Table 2. Spesific immune response of thalassemia major, quantitative tests

Parameter	Post-splenectomy (n = 58)	Non-splenectomy (n = 58)#	p*
Total lymphocyte count, cell/µL	6,754 (2,080 to 50,000)	1,583 (370 to 4,010)	<0.001
Total T lymphocyte, cell/µL	4,387.50 (1,288 to 45,699)	1,269 (264 to 3,127)	<0.001
Total T lymphocyte CD4+, cell/µL	1,845.50 (363 to 21,177)	532 (89 to 1,272)	<0.001
Total T lymphocyte CD8+, cell/µL	1,720.50 (652 to 5,038)	554.50 (128 to 1,543)	<0.001
CD4+/CD8+ ratio	0.98 (0.3 to 2.4)	0.97 (0.5 to 2)	0.700

*Wilcoxon Signed Ranks test, # median (range)

The production of IL-2 and TNF-α was used in order to measure function of T lymphocyte activation. **Table 3** showed there were no differences on T lymphocyte CD4+ and CD8+ cytokine production (IL-2 and TNF-α) between both groups.

DISCUSSION

Thalassemia major patients in Indonesia has unique characteristics due to insufficient

Table 3. Spesific immune response of thalassemia major, qualitative tests

Parameter	Post-splenectomy (n = 58)#	Non-splenectomy (n = 58)#	p*
T lymphocyte CD4+ producing cytokine, %			
- IL-2	13.99 (1 to 44)	11.13 (2 to 49)	0.523
- TNF-α	23.83 (1 to 63)	23.4 (2 to 80)	0.513
T lymphocyte CD8+ producing cytokine, %			
- IL-2	10.47 (1 to 34)	7.37 (1 to 40)	0.917
- TNF-α	21.70 (1 to 59)	17.09 (3 to 61)	0.538

*Wilcoxon Signed Ranks test, # median (range)

blood transfusion and inadequate use of iron chelation. The blood transfusion was improperly scheduled due to financial constrainst for visiting the hospital. Besides, these patients showed low compliance on iron chelating resulted in severe iron overload. Moreover, splenectomized thalassemia major patients usually has history of hypersplenism and varied indication of splenectomy. The other limitation of this study was improper documentation of infection episodes on medical record and insufficient total subject for neutrophil phagocytosis examination.

More than half of subjects were β-HbE thalassemia (51.7%) followed by β-thalassemia (47.4%) and α-thalassemia (0.9%). These might be due to the place of study was a refferal hospital and located at Java. The patients who visited Thalassaemia Center, Cipto Mangunkusumo Hospital comes from Jakarta and surrounding area. According to Lanni[20], β-thalassemia trait was 3.2% and HbE thalassemia trait was 4.8% in Java island. Moreover, Eijkman Molecular Biology Institute reported that α-thalassemia comes from Chinese (69%) and Javanese (31%) ethnic.[21] Eventually, the place of thalassemia center and ethnicity surrounding the center will influence variety of thalassemia type.

In this study, good nutritional status was more common on non-splenectomy group. On the other hand, severe malnourished was more common on post-splenectomy group. These were due to less calorie intake on post-splenectomy group. It is supported by moderate correlation and significant result between nutritional status and calorie intake on both group of thalassemia major. Severe malnourished was more common on post-splenectomy group because this group had lesser blood transfusion compared to non-splenectomy group. Fung et al.[22] reported the relationship between nutritional status with hemoglobin level and blood transfusion frequency. Subjects who received routine blood transfusion has higher body fat mass and body fat percentage compared to subjects who received less blood transfusion. Fung et al[22], also stated that hemoglobin level has positive correlation with lean mass (p=0.001). Additionally, the body weight of thalassemia major patient might not be the actual body weight since the presence of spleen enlargement. Therefore, upper arm circumference should be used instead of body weight on thalassemia major children patients. Beside upper arm circumference, calliper could be an option in order to measure muscle thickness for the adult thalassemia major patient. Moreover, it is important to consider body fat in thalassemia major patients since adequate body fat was needed for optimal growth.[22] In relation of blood transfusion type, most of post-splenectomy subjects received washed PRC due to incompatibility history.

In this study, subject with positive hepatitis B and C was distributed equally on both groups (p>0.05). These markers are important for thalassemia immune response evaluation as hepatitis can lead to liver damage which correlated to immune abnormality.[23] Monitoring and good management such as Hepatitis B immunization were needed to prevent liver damage.

Previous study reported that main predictor for infection in thalassemia major was duration of thalassemia since the patient diagnosed, frequency of blood transfusion, splenectomy and iron chelation.[24] It is similar with this study that episode of infection was more common on post-splenectomy than non-splenectomy subjects. The higher ferritin level found on post-splenectomy subjects affected immune response, which resulted in higher susceptible to infection. Spleen is an important source of immunocompetent lymphocyte.[4] Splenectomy and aged more than 10 years are the precipitating factors for severe infection on thalassemia major.[25]

In normal people, the neutrophil phagocytosis function is 96.8–99.59%. This study found that neutrophil phagocytosis on thalassemia major patients was lower compared to normal. It decreased further after splenectomy (29.79%). Other study reported there was no difference of neutrophil phagocytosis in thalassemia compared to normal subjects.[16] We found distruption on neutrophil phagocytosis in thalassemia major especially post-splenectomy subjects. These conditions might be due to research methodology differences. The lower innate imunity on thalassemia major might be correlated to higher level of iron overlod as reported by Cantinieaux

et al.[26]

Iron overload indicated by ferritin and transferrin saturation was higher on post-splenectomy group than non-splenectomy group. This condition was due to post-splenectomy patients rarely visiting the hospital to get iron chelation. Thus, it will affect the immune system of post-splenectomy subjects. In this study, there were differences on total lymphocyte count, total T lymphocyte, CD4+ and CD8+ T lymphocyte between post-splenectomy and non-splenectomy groups ($p<0.001$). These results were similar to the previous study and correlated to amount of blood transfusion and ferritin level.[1,12,14,27] Iron is important to regulate marker expression in surface T lymphocyte and expansion of T lymphocyte subsets in vivo and in vitro.[23,28] In addition, iron chelation has a greater effect for influencing thalassemia patients's immune response.[13] Deferiprone was the most common chelation that used (55.2%) since it is the cheapest oral iron chelation in the world[29], including Indonesia.

Increased T lymphocyte count on post-splenectomy might be associated with antigen could not effectively filtered by spleen. This suggests that spleen could play some part in the regulation of lymphocyte counts and act as a reservoir for lymphocytes produced in the body.[27] Moreover, CD4+ and CD8+ T lymphocyte were higher on post-splenectomy groups compared to non-splenectomy and control group in the previous study.[27] These conditions showed an activated immune response although it is not visible clinically. The CD4+/CD8+ ratio showed no significant differences on both groups. However, the CD4+/CD8+ ratio is lower than the control group in the previous study.[27] Reduced CD4+/CD8+ ratio is associated to the amount of blood transfusion and has negative correlation with ferritin level.[1]

Meanwhile, there were no differences on proportion of activated lymphocyte producing cytokine (IL-2 and TNF-α) on both groups. It was proved that the ability of CD4+ and CD8+ T lymphocyte in producing cytokine was not affected even after splenectomy. Until now, we could not find any study that measured proportion of lymphocyte in producing cytokine after mitogen exposure. Interleukin-2 (IL-2) is the most important cytokine in activation and proliferation of T lymphocyte especially T reg.[30,31] Gharagozloo et al[27], showed decreased IFN-γ and IL-2 on thalassemia as a result of immunosupression due to iron overload. Whilst, enhancement of TNF-α on post-splenectomy thalassemia major subjects was a result of poor antigen filtration which resulted in enhanced inflammatory. Moreover, T lymphocytes express activated phenotype in multitransfused thalassemia major patients. In contrast, T-cell proliferation and effector function are significantly suppressed.[27] These conditions are due to increased immune activation and constant T-cell turnover could generate the premature aging of the immune system and immune resources exhaustion.[27]

Multitransfusion leads to a functional status of partial immunodeficiency, which, nevertheless seems to have no substantial clinical relevance.[32] Blood transfusion and activation of chronic immune might induce the T reg cell which suppresses the T cell effector functions. The association between expression marker of activated T lymphocyte and multitransfusion was indicated that chronic immunologic stimulation is presence. Contaminating leukocyte in PRC might be a source of imunization to histocompatibility antigen in thalassemia patient. Although the absence of donor leukocytes on PRC are presented in the major histocompatibility complex class I pathway of recipient antigen-presenting cell (APC), which result in activation and expansion of recipient CD8+ T cells specific for donor minor histocompatibility antigens.[27]

Overall, immune response profile of hypersplenism was in between post-splenectomy and non-splenectomy. Neutrophil count was higher and phagocytosis of hypersplenism was lower than non-hypersplenism. Although, cellular immunity of hypersplenism had no significant changes, clinically hypersplenism state was important because non-specific immune response were already reduced. On the other hand, pancytopenia and massive splenomegaly were also important to consider. Therefore, this study showed that splenectomy need a better preparation and consideration. It is

recommended to give higher blood transfusion volume and effective iron chelation for several months in order to reduced hypersplenism state.

CONCLUSION

There were significant differences on immune responses among post-splenectomy and non-splenectomy groups. The differences were on non-specific (neutrophil count and phagocytosis) and quantitative specific immune response (total lymphocyte count, CD4+ and CD8+ T lymphocyte). Splenectomised thalassemia major showed a greater degree of susceptibility to infections than non-splenectomised thalassemia major. Improvement of immune response could be achieved by consuming antioxidant vitamin, and trace elements (e.g zinc, selenium). They provide tissue protection from damaging agents such as reactive oxygen through transcription factor reaction also cytokine and prostaglandin protection. Furthermore, vitamin and trace elements boost the improvement of immune response through Th1 and inhibit the conversion of Th1 into Th2.

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