Conference Paper

Increased Iron in Pediatric β-Thalassaemia Major Associates with CD3+, Not γδ Lymphocytes

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Abstract

Iron in β-thalassaemia major can act as a double-edged sword. Inefficient erythropoiesis and repeated blood transfusion therapy undertaken by these patients contribute to accumulation of iron, where at the cellular level may cause danger since the rising rate of iron in its free form was very toxic to the cells and tissues. Severe infectious disease is the 2nd major cause of death in children with this genetic inheritance disease. Such a complication can be happened when impaired immune cells, burdened with siderophilic bacterial infection which still prevalence in Indonesia, can make β-thalassaemia major susceptible to the pathogen invasion. The γδ T cells and their Vδ2+ subset functioned as innate and adaptive immune also specifically recognizes pathogen. The purpose of this study was to characterize γδ T cells and Vδ2+ subset also investigate the correlation between iron level and percentage of lymphocyte, CD3+ T cells, γδ T cells, and Vδ2+ subset then expression of T-cell receptors, they are CD3 and γδ in pediatric β-thalassaemia major. Flow cytometry was used to characterized and measure the cells parameters. Cross sectional study was done involving 51 pediatric β-Thalassaemia major patients who visit thalassemia clinic. Respectively, there was significant positive correlation between increased serum iron and ferritin level and number of lymphocyte (r = 0.15, P = 0.005) also between number of CD3+ T cells subset and ferritin level (r = 0.42, P = 0.002). In conclusion, iron overload is related to alteration of lymphocyte population and CD3+ T cells subset in pediatric β-thalassaemia major patients.

Keywords: Iron, lymphocyte, γδ T cell, Vδ2+ T cell, thalassemia.

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1. Introduction

Iron as a reactive metal which had pivotal role in human body metabolism, however, it could be inevitable when their equilibrium was disturbed causing iron overload complication. At the cellular setting, it may cause danger since the rising rate of iron in its free form was very toxic to the cells and tissues. Particularly in immune cells, this condition could impair immune response to pathogen invasion therefore vulnerable to infection [1].

Red blood cell contained almost 85% of total body iron [2]. Early degradation of this cells is the principal pathogenesis of β-thalassaemia major. According to World Health Organization (WHO) 2008, this genetically inherited disease prevalence approximately 97,360 [3]. Premature hemolysis of erythrocyte accompanied by repeatedly blood transfusion caused iron overload as one of the most common complication in β-thalassaemia major [4]. Infection is the 2^{nd} most cause of mortality among thalassemia patients with the reason of this predisposition is still questioned. According to that, previous studies showed the alteration of immune system both innate and adaptive in β-thalassaemia major, with alteration can be happened to be changing in number and function of innate and adaptive immune cells [5, 6]. Several bacteria for example *Mycobacterium tuberculosis* (Mtb), *Eschericia coli* (E. coli), and *Salmonella sp.* are iron loving microorganisms, which their infectious disease still prevalence in Indonesia. Consequently, combination of mention facts above made β-thalassaemia major patients who lived in the are made them made them vulnerable to partake the infection.

The γδ T cells, one of immune cell subset in lymphocyte population, acted as innate and adaptive immune cells along with its unique characteristic. Orchestra between γδ T-cell receptor and CD3 co-receptor in the T cells membrane determine their function in bridging the innate to adaptive immune system as response to pathogen invasion [7]. The γδ T cells had many variants, the most widely found was Vγ9Vδ2 (Vδ2+) subset [8]. Their known main roles in responding infection are eliminating pathogen also activate adaptive immune system [9]. These responses can be characterized by applying flow cytometry measuring the number of lymphocytes, γδ T cells subset, Vδ2+ subset and expression of CD3 and γδ receptor in γδ T cell subset. The aim of our present study was to investigate also correlate number of lymphocyte, CD3+ T cells, γδ and Vδ2+ T cells subset also expression of γδ and CD3 receptors with iron excessive indicators such as serum iron, ferritin, and Transferrin Iron Binding Capacity (TIBC).

2. Material and Methods

This study was designed as a cross sectional analytical study involving pediatric β-thalassaemia major having excessive iron level. Subject recruited by simple random selection was begun from October 17, 2016 until November 15, 2016 in Dr. Hasan Sadikin General Hospital Bandung. This study was approved by Health Research Ethics Committee of Faculty Medicine, Universitas Padjadjaran Bandung with approval number 74/UN6.C1.3.2/KEPK/PN/2016 along with approval from the Ethics Committee of Dr. Hasan Sadikin General Hospital Bandung with approval number LB.02.01/C02/15691/XI/2016. Written informed consent was obtained from all participants.

The selected patients were those who had already diagnosed β-Thalassaemia major through clinical, physical, laboratory and supporting examination, had been transfused at least more than two years, and aged less than 15 years old. Patient with past history tuberculosis, cancer, diabetes, autoimmune, chronic infection such as HBV, HIV, patients in immunomodulatory therapy, and in unhealthy condition were excluded.

After written informed consent was obtained from the parents of all subjects, blood specimens were acquired by venipuncture for routine and complete blood examinations just before blood transfusion. Blood profiles such as hemoglobin (Hb), leukocyte, thrombocyte, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), as well as iron status including serum iron, ferritin, and TIBC were performed. This procedure continued by collecting blood sample using Vacutainer containing heparin (Becton Dickinson, Franklin Lakes, New Jersey, USA) for lymphocyte and T cell subset characterization measurement using flow cytometry.

2.1. γδ and Vδ2+ T Cells Characterization and T-Cell Receptor Measurement

Samples for flow cytometry procedure were kept on room temperature and the measurement was started within 1 hour of blood collection. In short, according their phenotypic marker of CD3+, γδ+, and Vδ2+, γδ and Vδ2+ T cell subsets were characterized using a Becton Dickinson FACSCalibur flow cytometer from pre-gated lymphocyte population of whole blood by positive gating. The expression CD3 and γδ receptor were measured as median fluorescence intensity (MFI) of each receptor in γδ T cell subset. Heparinized blood as much as 200 µl were added to 2000 µl FACS Buffer Lysing Solution. After vortex and centrifugation at 1500 rpm for 5 minutes, the supernatant formed was discarded. Monoclonal antibodies consist of CD3 AlexaFlour 488 (Biolegend, San

Figure 1: Expression of phenotypic marker on peripheral blood.

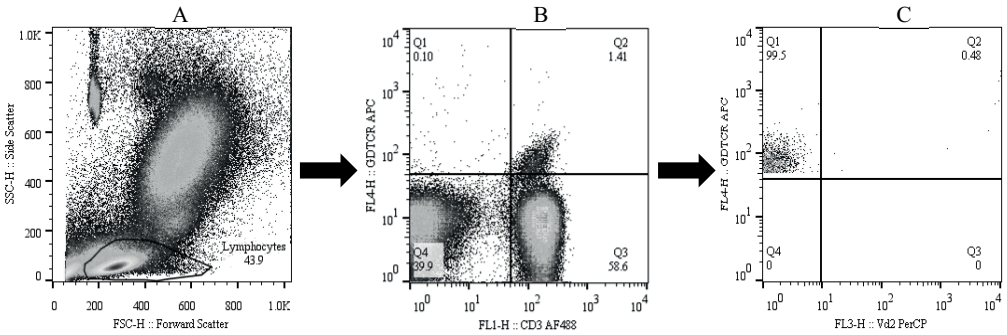

Figure 1: Expression of phenotypic marker on peripheral blood showing percentage of each lymphocyte subset. **A**: Based on forward and side scattered beam, lymphocyte population was identified by applying gate and showed as percentage in whole blood. **B**: Expression of phenotypic marker CD3 and γδ T cells. Region Q2 is γδ T cells population marked by CD3+, γδ+ and presented as percentage. Region Q3 is CD3+ T cells population marked by CD3+, γδ- and presented as percentage. C: Expression of phenotypic marker Vδ2+ subset of γδ T cells. Region Q2 is Vδ2+ subset population marked by CD3+, γδ+, Vδ2+ and presented as percentage.

Diego, CA, US), γδ TCR APC (Biolegend, San Diego, CA, US), and Vδ2 PerCP (Biolegend, San Diego, CA, USA were added and vortexed to cell suspension then incubated for 20 minutes at cold temperature (2 - 4°C) and covered by aluminum foil. Ten-times diluted red cell lysing buffer (Biolegend, San Diego, CA, USA) were added to stained cells and incubated for exactly 12 minutes. Before reading in flow cytometer, lysed cell suspension was vortexed and washed two times using 2000 µl 1% PBA, then cells were suspended using 200 µl 1% PBA. Cells were read according to their phenotypic marker by BD Cell Quest Pro Software (Biosciences, San Jose, CA, US) for 500,000 events, then the FCM output files were analyzed using FlowJo 10 (Tree star). Gating steps and expression of phenotypic marker of cell analysis using flow cytometry is shown in Figure 1.

The number of cells were presented as percentage resulted from the proportion of count designated cells and whole blood or cell population. The proportion of characterized cells in this study were as follows:

- Lymphocyte: proportion of cells gated according to forward and side scattered beam and whole cells event
- CD3+ T cells: proportion of CD3+ lymphocyte in lymphocyte population
- γδ T cells: proportion of cells gated with CD3+, γδ+ and number of lymphocyte
- Vδ2+ subset of γδ T cells: proportion of cells in γδ T cells population

Figure 2: Correlation between Iron and Cell Percentage.

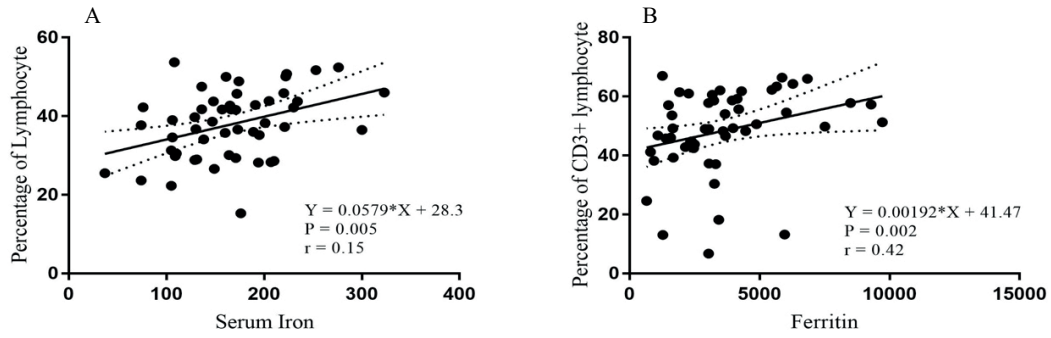

Figure 2: A: Correlation between serum iron level and percentage of lymphocyte population. B: Correlation between ferritin level and percentage of CD3+ T cell subset.

2.2. Statistical Analysis

Non-normally distributed data are expressed as median with interquartile range (IQR); normally distributed data as mean with standard deviation (SD). Correlation between parameters were tested using Spearman correlation coefficient for non-normally distributed data, and Pearson correlation coefficient for normally distributed data. All analyses were performed with Graphpad PRISM version 7.0 (Graphpad Software, Inc., La Jolla, California, USA). $P < 0.05$ were considered statistically significant.

3. Results

A total number of 51 pediatric β-Thalassaemia major patients were enrolled in this study. Characteristic of study participants are presented in Table. 1. All participants who have iron overload status were proved by median of ferritin and mean iron serum more than normal values. The correlation between iron status indicators level with number of cells and expression of receptors is depicted in Table. 2. There was significant positive correlations between number of lymphocyte and serum iron level also between number of CD3+ T cells and ferritin level as depicted in Figure 2.

4. Discussion

Better quality management along with good access to treatment for regular blood transfusion and iron chelation in thalassemia have increased β-thalassemia major patients' welfare. However, iron dysregulation therefore causing exceeded iron level, still to be the most complication which burdened their quality of life [4]. At the cellular

Table 1: Subjects Characteristics.

	Patients' value	Normal value
Gender		
· Male, n (%)	25 (49)	Not Define
· Female, n (%)	26 (51)	Not Define
Mean age (SD), year	9 (2.7)	Not Define
Hematological indicators		
· Mean Hb (SD), g/dL	6.3 (1.1)	10.9-14.9
· Median leucocyte (IQR), /mm3	6000 (4000-8100)	4500-14500
· Median thrombocyte (IQR), 103/mm3	209.9 (127-328)	150-300
· Mean MCV (SD), fl	74.7 (4.7)	79-98
· Mean MCH (SD), pg/cell	25.7 (2.4)	25-33
· Mean MCHC (SD), g/dL	34.4 (1.7)	32-36
Iron status indicators		
· Median ferritin (IQR), µg/L	3235 (1923-4460)	< 1000
· Mean serum iron (SD), µg/dL	166.4 (57.9)	35-150
· Median TIBC (SD), µg/dL	197 (156-711)	240-474
Cell characteristics		
· Mean lymphocyte (SD), (%)	37.9 (8.7)	25-54
· Median CD3+ lymphocyte (IQR), (%)	49.3 (42.9-52.7)	Not Define
· Median γδ T cells (IQR), (%)	0.61 (0.34-1.25)	Not Define
· Median Vδ2 subset (IQR), (%)	2.1 (0.59-4.91)	Not Define
· Median MFI of CD3 receptor (IQR)	122 (105-138)	Not Define
· Median MFI of γδ receptor (IQR)	67.3 (63.2-77.7)	Not Define

level, particularly in immune cells environment, this condition caused alteration in the number and function of the cells, involving lymphocyte, therefore β-thalassemia major patients were susceptible to infection [6]. We aimed to characterized, using flow cytometry, γδ T cells and Vδ2 variant as one of the lymphocyte subset also their T cell receptors expression, which had essential role in innate and adaptive immune system in iron overloaded pediatric β-thalassemia major patients. We analyzed the number of lymphocyte, CD3+ cell, γδ T cell, and Vδ2+ variant of γδ T cell and their T-cells receptor expression (CD3 and γδ receptors) correlated with iron status indicators.

Table 2: Spearman Correlation between Iron Level with Number of Cells and MFI of T-cell receptors.

Parameter		Correlation value	
Lymphocyte	Ferritin	r	0.16
		P	0.27
	Serum iron	r	0.15
		P	0.005*#
	TIBC	r	0.11
		P	0.44
CD3+ lymphocyte	Ferritin	r	0.42
		P	0.002*
	Serum iron	r	0.21
		P	0.14
	TIBC	r	0.05
		P	0.44
γδ T cells	Ferritin	r	-0.08
		P	0.75
	Serum iron	r	-0.14
		P	0.31
	TIBC	r	0.02
		P	0.889
Vδ2 subset	Ferritin	r	0.04
		P	0.79
	Serum iron	r	-0.03
		P	0.86
	TIBC	r	-0.05
		P	0.71
MFI of CD3 T-cell receptor	Ferritin	r	-0.01
		P	0.92
	Serum iron	r	-0.2
		P	0.19
	TIBC	r	0.1
		P	0.45
MFI of γδ T-cell receptor	Ferritin	r	-0.09
		P	0.55
	Serum iron	r	-0.06
		P	0.67
	TIBC	r	-0.06
		P	0.7

*: Statisticall significant; #: Pearson correlation

Respectively, the increased serum iron and ferritin level was found significantly have positive correlation with lymphocyte number and CD3+ cells.

Our present investigation showed iron overload with higher serum iron and ferritin level. Double burden including regular blood transfusion and ineffective erythropoiesis contributed to increased iron store and trafficking in form of, respectively, ferritin and serum iron [2]. This regular treatment causing a reduction to the detoxification tolerance limit and iron storage in the form of ferritin. At the same time, transferrin becomes saturated. The final phase is the rising rate of iron in its free form that is very toxic to the cells and tissues, especially red blood cells. Mentioned unbalanced iron homeostasis was complicated by iron absorption in enteric cells and iron recycle in macrophage therefore increased serum iron level [10]. Free iron can catalyze the production of radical OH- substances from peroxide molecules, known as the Fenton reaction, endangered cell function by damaging their biomolecules.

Existence of iron in β-thalassemia major like a double-edged sword. Yet, this essential can be toxic, for instance at the cellular level, iron overload can affect number and function of immune cells then impaired the harmony of innate and adaptive immune response to pathogen. Siderophilic bacteria may compete with host cells for iron in order to survive. Therefore, excess iron in thalassemia patients may weaken the host immune system so it is more susceptible to infection [11]. Previous studies showed changing of cellular immune system, particularly lymphocyte related to iron overload in β-thalassemia major [6, 12]. A significant positive correlation between higher serum iron level and number of lymphocytes we found in our study consistent with their findings serving fundamental evidence that iron overload can affect cellular immune system in since pediatric life phase, particularly lymphocyte.

According to their co-receptor of signaling protein attached in the cell membrane, T cells are distributed into CD3+, CD4+, and CD8+ T cell. These co-receptors, structurally, act as an important dimer to maintain the conformational binding of T cell receptor, then facilitate phosphorylation process and signal cascade in T cell activation producing cytokine as a response [13]. Previous study by Pourgheysari, in β-thalassemia major patients there was decreased CD3+ T cells related to their iron overload status related to age and frequency of blood transfusion [6]. In contrast to their finding, in our pediatric patients we found significant positive correlation between higher ferritin level and number of CD3+ T cells subset. This inconsistency is likely because of age of the patients at inclusion.

The γδ T cells composed about 1-5% of the total lymphocytes in healthy human body [9]. Most of the cells found in epidermal tissues such as skin and respiratory

tract. Although part of adaptive immune cells, γδ T cells also acted as innate immune cell, directly activated without Antigen Presenting Cell (APC). This unique characteristic related to absence of CD4+ and CD8+ expression in γδ T cells, or double negative γδ T cells, which showed low reactivity towards the ligands that were associated with Major Histocompatibility Complex (MHC) [7, 9]. The γδ T cells had many variants and the most common variant was Vδ2+ T cell. Activation of Vδ2+ T cells directly by phospoantigen such as HMBPP (*Hydroxy-Methyl-Butyl-Pyrophosphate)*, which can be found, for instance, in *Mycobacterium tuberculosis* lipid structure [14]. In contrast to the hypothesis that higher iron status associated with population of γδ T cell, and Vδ2+ variant of γδ T cell and expression of CD3 and γδ T-cell receptor, we found no significant correlation between those parameters. This discrepant result may have been due to the success of iron chelation therapy in our population. Iron chelation therapy is used to remove excess iron in blood, and is generally required in thalassemia patients receiving regular transfusions. This therapy is fundamental in the strategy to prevent and overcome iron overload and susceptible for infection, particularly tuberculosis, since Indonesia is included as one of the high tuberculosis prevalence. Limitation of our study that there is no control group without thalassemia to compare cellular parameter with. Further research should be conducted to reduce limitations and to investigate the function of activated Vδ2+ and γδ T cells applying whole blood stimulation assays in pediatric β-thalassaemia major with iron overload.

5. Conclusion

Iron overload condition in pediatric β-thalassaemia major associates with the alteration number of lymphocyte population and CD3+ T cells subset. Further research is imperative to be done in studying other T cell subsets with CD3+, such as MAIT and NK-T cell, which have important role in innate besides adaptive immune response, particularly in high prevalence and morbidity infectious disease in Indonesia, such tuberculosis.

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References

[1] Porter, J.B. and M. Garbowski, The pathophysiology of transfusional iron overload. Hematol Oncol Clin North Am, 2014. 28(4): p. 683-701.

[2] Leecharoenkiat, K., et al., Iron dysregulation in beta-thalassemia. Asian Pac J Trop Med, 2016. 9(11): p. 1035-1043.

[3] Modella, B. and M. Darlisona, Global epidemiology of haemoglobin disorders and derived service indicators. Bulletin World Health Organization, 2008. 86: p. 480-87.

[4] Origa, R. and R. Galanello, Pathophysiology of beta thalassaemia. Pediatr Endocrinol Rev, 2011. 8 Suppl 2: p. 263-70.

[5] Asadov, C.D., Immunologic Abnormalities in β-Thalassemia. Journal of Blood Disorders Transfusion, 2014. 5(7): p. 1-5.

[6] Pourgheysari, B., L. Karimi, and P. Beshkar, Alteration of T Cell Subtypes in Beta-Thalassaemia Major: Impact of Ferritin Level. J Clin Diagn Res, 2016. 10(2): p. 14-8.

[7] Adams, E.J., S. Gu, and A.M. Luoma, Human gamma delta T cells: Evolution and ligand recognition. Cell Immunol, 2015. 296(1): p. 31-40.

[8] Pang, D.J., et al., Understanding the complexity of gammadelta T-cell subsets in mouse and human. Immunology, 2012. 136(3): p. 283-90.

[9] Zheng, J., et al., gammadelta-T cells: an unpolished sword in human anti-infection immunity. Cell Mol Immunol, 2013. 10(1): p. 50-7.

[10] Steinbicker, A.U. and M.U. Muckenthaler, Out of balance–systemic iron homeostasis in iron-related disorders. Nutrients, 2013. 5(8): p. 3034-61.

[11] Nairz, M., et al., 'Ride on the ferrous wheel'–the cycle of iron in macrophages in health and disease. Immunobiology, 2015. 220(2): p. 280-94.

[12] Sari, T.T., et al., Immune response of thalassemia major patients in Indonesia with and without splenectomy. Acta Med Indones, 2014. 46(3): p. 217-25.

[13] Wucherpfennig, K.W., et al., Structural biology of the T-cell receptor: insights into receptor assembly, ligand recognition, and initiation of signaling. Cold Spring Harb Perspect Biol, 2010. 2(4): p. 1-14.

[14] Meraviglia, S., et al., gammadelta T cells cross-link innate and adaptive immunity in Mycobacterium tuberculosis infection. Clin Dev Immunol, 2011. 2011: p. 1-11.