

Original Research

Fcγ receptor III expression and morphological maturity on neutrophil are associated with higher iron level of major beta-thalassemia

Mohammad Ghozali^{1*}, Tiwi Harjanti Cakranita², Adi Imam Tjahjadi¹, Lelani Reniarti³, Reni Ghrahani³, MRAA Syamsunarno¹, Budi Setiabudiawan³, Ramdan Panigoro¹

¹Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Indonesia

²Faculty of Medicine, Universitas Padjadjaran, Indonesia

³Department of Pediatrics, Faculty of Medicine, Universitas Padjadjaran, Indonesia

Correspondence to: moh.ghozali@unpad.ac.id

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Abstract: Lifetime blood transfusion experienced by major β -thalassemia patients complicated with iron overload, therefore, may lead to their tissue injury. Ultimately, free toxic iron may alter immune response via dysregulation of immune cell activity producing prolonged effector reaction. Neutrophil as one of the vital innate immune cell despite serves as the first line of defense resulting acute inflammation has a pivotal role in chronic inflammation while releasing the toxic substance that interferes biological processes. This process is initiated by one of them by activation of Fc γ Receptor III (CD16), a neutrophil membrane-bound protein. A cross-sectional laboratory study involving lysed-erythrocyte heparinized whole blood of fifty pediatric major β -thalassemia patients treated with monoclonal antibodies i.e. CD16, CD14, and HLA-DR, dissected into CD16+ and CD16++ population using flow cytometry. Expression of Fc γ Receptor III was measured as Median Fluorescent Intensity (MFI). Hematology and iron status were measured. A correlation analysis was done. MFI of CD16 neutrophil [509.5 (371 – 796.5)] and ferritin level [(3209 μ g/L, 1862 – 4564)] was positively correlated ($r = 0.4$, $P = 0.007$). Respectively, ferritin and serum iron were found negatively correlated with segmented neutrophils ($r = -0.3$, $P = 0.02$; $r = -0.3$, $P = 0.02$). Change in CD16 expression may implicate preliminarily neutrophil activation as a response of iron-overloaded tissue and result in chronic inflammation in β -thalassemia patients. However, the maturity of this cell may be altered. Future study in the understanding of neutrophil-mediated inflammation, particularly related to immune complexes and functionality, is imperative to be explored.

Key words: Neutrophil; Fc γ Receptor III; Iron; Thalassemia.

Introduction

Neutrophils, vital innate immune cells fundamentally known to be widely contributed to the first line battling cell as a response to tissue injury, whilst the pivotal player in the chronic inflammatory condition of a variety of clinical disorders (1). Despite that thalassemia patient clinically suffered from chronic anemia, they are chronically immuno-stimulated by premature hemolysis and ineffective erythropoiesis. Ultimately, while iron overload condition complicated them because of regular blood transfusion as their definitive lifetime therapy in overcoming the anemia, produced free-form iron acts as free radical causing injury and without resolution (2, 3).

Prolonged aforementioned impairing exposure to neutrophils may alter their immune response, while their optimal function is determined by their structure and maturity in the blood (4, 5). Abundant evidence both in the animal model and human patients had been linked iron-mediated chronic inflammation to compromised neutrophil effector function (6-8), however, evidence showing the protein interaction involved in neutrophil activation related to signaling system related to body iron status is still limited.

Fc γ Receptor III (Fc γ R III), also known as CD16, is a glycosyl phosphatidylinositol-anchored (GPI) protein that acts as a receptor for the Fc region of immunoglobulin gamma. Specifically expressed by human neutrophil, despite this protein is used to dissect neutrophil from whole blood, also used in studying the neutrophil capacity(9). During inflammation, Fc γ R III has been previously described as an essential contributor in neutrophil effector function related with phagocytosis of opsonized and certain non-classically opsonized particles, a release of inflammatory mediators, and antibody-dependent cellular cytotoxicity (10, 11). The present investigation found an association between activated neutrophils based on Fc γ R III expression with the iron status in pediatric major β -thalassemia patients.

Materials and Methods

Patients and study design

Fifty patients involved in this study were selected from population who had already diagnosed as major beta-thalassemia through clinical, physical, and laboratory examination, having an excessive iron level (ferritin > 1000 μ g/L), had received a routine blood transfusion at least more than two years, and aged less than 15 years old. Patient with past history such as tuberculosis, diabetes, cancer, autoimmune, HBV infection, HIV infection, having immunomodulatory therapy, and unhealthy condition were excluded. A cross-sectional laboratory analytical study design was applied in this study. Sub-

jects were recruited by simple random selection from major beta-thalassemia patients who routinely visit thalassemia clinic of Dr. Hasan Sadikin General Hospital Bandung, which is the main tertiary academic referral center for West Java, taking care of more than 150 major β-thalassemia patients daily, and begin from October until November 2016.

Written informed consent was obtained from the parents of all subjects. Blood specimens were acquired by venipuncture just before blood transfusion. Hematology profile measurement, serum iron status including ferritin, serum iron, and Total Iron Binding Capacity (TIBC), as well as neutrophil characterization were performed from collected blood samples, respectively, using EDTA-contained, plain, and Heparin-contained tubes.

Ethics

All procedures were conducted in accordance with policies of the Faculty of Medicine, Universitas Padjadjaran and Dr. Hasan Sadikin General Hospital, Bandung, West Java, Indonesia. This study was approved by Health Research Ethics Committee of Faculty of 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.

Laboratory procedures

Characterization of Neutrophil

Peripheral venous blood was collected in Vacutainer tube containing lithium and sodium heparin (Becton Dickinson, Franklin Lakes, New Jersey, USA). Samples were kept at room temperature and the measurement was started within 1 hour of blood collection. Panel of monoclonal antibodies consists of CD14 Alexa-Flour 488 (BioLegend, San Diego, CA, USA), CD16 PE (BioLegend, San Diego, CA, USA), and HLA-DR PerCP (BioLegend, San Diego, CA, USA) were used to dissect neutrophils by employing multi-color flow cytometry as previously described (12).

As much as 2000 μL PBA 0.5% was added to 200 μL heparinized blood and continued with vortex and centrifugation at 1500 rpm for 5 minutes without break. The cell suspension was collected. A mixture of mentioned monoclonal antibodies was added and vortexed to cell suspension in FACS buffer diluted solution then proceeded with incubation for 20 minutes at cold tem-

perature (2-8^oC) covered by aluminum foil. Ten-time diluted red cell lysing buffer (BioLegend, San Diego, CA, USA) was added to stained cells and incubated for exactly 12 minutes. Just before reading in the flow cytometer (FACSCalibur, Becton Dickinson, USA) lysed cell suspension was vortexed and washed two times using 2000 μL 0.5% PBA, then cells were suspended using 200 μL 0.5% PBA. Cells were read according to their phenotypic marker by BD Cell Quest Pro Software (Biosciences, San Jose, CA, USA) for 500,000 events, then the FCM output files were analyzed using FlowJo 10 (Tree star, USA). Steps of positive gating strategy in flow cytometry were employed to identify neutrophil according to the phenotypic marker expressed by CD14, HLA-DR, and CD16.

Employing a positive-negative gating strategy, neutrophils were properly identified from lysed-erythrocyte blood (Figure 1) as modified from previously described flow cytometry gating methods (9, 13). In short, selection of these cells began from gating on granulocytes population successively based on forward and side scattered signal then continued with assort them based on CD14 negative and HLA-DR negative population. Assorted cells then dissected according to CD16 positive signal which defined as “true” neutrophils.

The population of true neutrophil was presented as a percentage which defined as the proportion of true neutrophils and negative CD14 and HLA-DR. The expression of Fcγ R III was expressed by Median Fluorescence Intensity (MFI) of designated protein on true neutrophil.

Hematology profile assessment

Vacutainer tube containing potassium EDTA (Becton Dickinson, Franklin Lakes, New Jersey, USA) was used to collect peripheral venous blood for hematology profiling. Automatic hematology analyzer (Sysmex Corp., Japan) was employed to measure hemoglobin (Hb) level, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), as well as leukocyte count also percentage of the band and segmented neutrophil.

Body iron status measurement

Collected sera from plain Vacutainer were centrifuged to be used for iron status measurement included serum iron and ferritin. The Elecsys ferritin immunoassay kit (Roche, Switzerland) was applied to measure serum ferritin, while serum iron assay kit (Merck, Singapore) was used to measure serum iron.

Statistical analysis

Non-normally distributed data are presented as median with interquartile range (IQR), while normally distributed data as mean with standard deviation (SD). Correlation between parameters was 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, CA, USA). *P* < 0.05 is considered statistically significant.

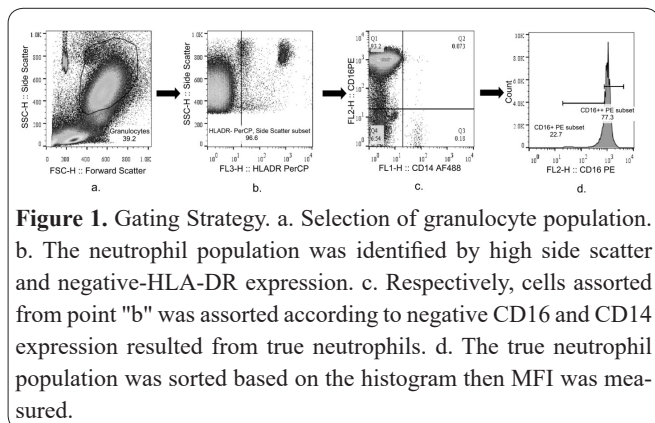


Table 1. Clinical and cellular characteristics.

	Patient's Value	Normal Value
Gender		
Male (n)%	50 (25)	Not defined
Female (n)%	50 (25)	Not defined
Mean Age (SD), year	8 (2.9)	Not defined
Hematological Indicators		
Mean Hb (SD), g/dL	6.4 (1.1)	10.9 – 14.9
Mean MCV (SD), fl	74.29 (5.536)	79 – 98
Mean MCH (SD), pg/cell	25.6 (2.697)	25 – 33
Mean MCHC (SD), g/dL	34.4 (1.664)	32 – 36
Iron Status Indicators		
Med. Ferritin (IQR), μg/L	3209 (1862 – 4564)	<1000
Mean serum iron (SD), μg/dL	157.9(64.08)	35 – 150
Cell Characteristic		
Mean Neutrophil (SD), (%)	89.35 (15.94)	40 – 80
Mean Seg. Neutrophil (SD), (%)	0.4 (0.7)	Not defined
Mean Band Neutrophil (SD), (%)	51.5 (9.2)	Not defined
Med. true Neutrophil pop. (IQR), (%)	93.7 (90.5–95.4)	Not defined
Med. MFI CD16 (IQR)	509.5 (371–796.5)	Not defined

*Med.: median; Seg.: segmented; pop.: Population.

Results

Clinical and cellular characteristics

The characteristics of study participants are presented in Table. 1. All participants who have iron overload status were indicated by a median of ferritin and mean iron serum more than normal values.

Correlation result

The results summarized in table 2, indicated a significant positive correlation between Fcγ R III expressions and serum ferritin level. However, a negative correlation found between iron overload condition, indicated by serum ferritin and iron level, and segmented neutrophil percentage population.

Discussion

This study finding initially corroborates that chronically immuno-stimulated major β-thalassemia experiences altered immune response as the complication of iron overload, which mediate chronic inflammation and unusual neutrophil effector function including its maturity and character, therefore they are susceptible to infection (8). Fcγ Receptor III (Fcγ R III) acts as one of the tissue injury response initiating signal transduction of neutrophil effector, continued with its phagocytic and antibody-dependent cellular cytotoxicity event (1, 5,

9). Flowcytometrically, alongside that this membrane-bound protein commonly used to identify neutrophil, integrated with clinical characteristics it also used to evaluate neutrophil activity. The Fcγ R III expression was positively correlated with higher ferritin and serum iron level, while inversely association between segmented neutrophil percentage population and higher iron body status was found in this study.

Through hematopoiesis, neutrophils are produced within the bone marrow then released neutrophil may have two nuclear morphology views; band neutrophil and segmented neutrophil. Newly released neutrophils usually have band morphology while aged and most functional neutrophils have segmented morphology (1). The correlation result of this study found a significant negative correlation between segmented neutrophil population and iron status in β-thalassemia patient which usually undergo chronic inflammation. It may suggest that neutrophil condition in β-thalassemia patient may undergo a left shift in their composition. On the other hand, this result also implies that there is increasing neutrophil recruitment and altered maturation process because of the persistent inflammation in β-thalassemia patient.

Immature neutrophils, mostly found in altered immune response condition such as sepsis, are able to mediate innate immune function although less proficient than the mature neutrophils (13). A study also has revealed that the sequence for the functional differentiation of neutrophil is begun with Fc receptors followed by immune phagocytosis, complement receptors, oxygen-independent microbial killing, oxygen-dependent microbial killing, and the last is chemotaxis (14). Despite the facts of these immature neutrophil population, surprisingly band neutrophil may suggest a pro-inflammatory phenotype since they have higher basal intracellular tumor necrosis factor-α/interleukin-10 ratio than that of mature neutrophils (13, 14). Major β-thalassemia complicated with iron overload as the impact of their routine blood

Table 2. Significant correlation result.

Parameter		Correlation value	Correlation
MFI CD16	Ferritin	r 0.36	Positive Correlation
	Ferritin	P 0.007	
Segmented Neutrophil	Ferritin	r -0.30	Negative Correlation
		P 0.02	
	Serum Iron	r -0.30	
		P 0.02	

transfusion has been known to have an alteration in the immune response.

The iron overload condition produces iron in a free form which acts as free radical inside the human body. These free radicals yet toxic may interact with a various chemical composition in the human body including chemical component during neutrophil's production, differentiation, and maturation. There is also a phagocytosis defect in individual who has increased iron and ferritin level, therefore iron radical might alter phagocytosis through peroxidation of neutrophil membrane lipids (13, 15). This phenomenon may explain the cause of susceptibility of β -thalassemia patients to infection while the pro-inflammatory phenotype may contribute to persistent prolonged acute inflammation which leads to a chronic-like inflammation appearance. Our present study makes it more pronounced.

This study provides preliminary data on neutrophil activity applying flow cytometry for robust neutrophil identification based on their granularity and negative expression of respective membrane protein i.e. lipopolysaccharide receptor (CD14) and HLA-DR, while positively expressed Fcγ R III (CD16). Fcγ R III is initially expressed at low levels, then progressively increased during the developmental and differentiation process, particularly in the last two stages of neutrophil differentiation (9). Fcγ R III serves mainly as an immune complex receptor and gives a response to activation of neutrophil while cell degranulation and releasing proteolytic enzymes proceed with phagocytosis. This study revealed activated neutrophil by expressing the Fcγ R III which their expression is positively correlated with higher ferritin. This finding might suggest that there is an amplification of neutrophil function particularly via Fcγ R III signaling related to iron overload in major beta-thalassemia patients.

Infection is one of the leading etiology of mortality in β -thalassemia patient after tissue injury lead to organ failure, while at the cellular level, particularly involving immune cells, the damage is unpreventable (8, 16). Injured tissue condition triggered by the rich free radical environment such as high-intensity exercise, which also happened in iron overload (6), change the Fcγ R III expression of neutrophils and lead to their degranulation and respiratory burst activity (17). Major β -thalassemia patient's neutrophils may undergo another functional maturity changes related to stimulation of iron overload condition. The fundamental notion mechanism connecting the neutrophil dysfunction contributed to the susceptibility of major beta-thalassemia patients to infection has already discussed (6, 8). While positive correlation between Fcγ R III of neutrophils and iron overload condition indicated by higher ferritin pronounced by this study may become an underlying possibility for early neutrophil dysfunction in β -thalassemia patient. Supported by the pro-inflammatory phenotype of band morphology, activated neutrophil identified Fcγ R III expression, and pathological condition of iron in patient's body may trigger prolonged-acute inflammation which destroys surrounding tissue. Limitation of our study that there is no control group without thalassemia to compare cellular parameter with.

The positive association between neutrophils' Fcγ R III expression and higher ferritin may implicate the pre-

liminary neutrophils dysfunction started with their activation via this protein and degranulation in an iron overload condition. Ultimately, chronic immune-stimulated inflammatory condition resulted in major β -thalassemia patients, even maturity of neutrophil may be altered. Future study in the understanding of neutrophil-mediated inflammation, particularly related to immune complexes and degranulation, is imperative to be explored. This study also implies that in the future β -thalassemia patient management regarding iron chelation to prevent immature neutrophil activation.

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Interest conflict

The authors declare that there is no conflict of interest.

Authors' contribution

M. Ghozali, Tiwi Harjanti Cakranita, Adi Imam Tjahjadi, Lelani Reniarti, Reni Ghrahani, MRAA. Syamsunarno, Budi Setiabudiawan, and Ramdan Panigoro conceived the study and participated in the design and data analyses. M. Ghozali, Tiwi Harjanti Cakranita, and Adi Imam Tjahjadi were involved in data acquisition and laboratory work. All authors contributed towards drafting and agree to be accountable for all respects of the work. Lelani Reniarti, Reni Ghrahani, MRAA. Syamsunarno, Budi Setiabudiawan, and Ramdan Panigoro critically reviewed the manuscript. All the authors read and approved the manuscript.

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