Positive Correlation between Ferritin and Activated Monocyte in Iron Overloaded Major β-thalassemia Patients

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ABSTRACT

Introduction: Regular blood transfusion for ß-thalassemia patients is a life-saving therapy, hence, it results in iron overload lead to immune dysregulation triggered by chronic activation of immune system. This fundamental notion contributes to their morbidity and mortality. Monocyte plays a critical role in regulating and bridging innate to adaptive immunity. Our pilot study analyzed the presence of activation markers, CD14 and CD69, on monocyte of major ß-thalassemia patients associated with their iron status. **Method:** Fifty pediatric ß-thalassemia patients routinely visited thalassemia clinic for clinical examination and blood transfusion were involved in this cross-sectional study. Flow cytometry applying antibody of CD14, HLA-DR, CD69 was used to dissect CD14+CD69+ monocytes from lysed-erythrocyte heparinized whole blood and defined as cell percentage also median fluorescent intensity (MFI) of CD69 of CD14+CD69+ monocytes. Iron status was indicated by ferritin and serum iron level. A correlation study was done. **Results :** We found 87.4% (76.1 − 91.4) CD14+CD69+ of dissected monocytes from iron overloaded pediatric ß-thalassemia patients (Ferritin level: 3118 µg/L, 1675 − 9718). Positive correlation was found between percentage of CD14+CD69+ monocytes and ferritin level (r = 0.3, P = 0.04). **Conclusion:** Considering the function of CD14 and CD69 on monocyte and the iron accumulation, our result may implicate that pediatric major ß-thalassemia patients have a tendency towards chronic inflammation. Future direction for research of our study aimed at discovering collateral activation of immune cells via monocyte to explain organ damage caused by iron overload is imperative.

Keywords: Monocyte, CD14, CD69, iron, β-thalassemia

INTRODUCTION

Beta (ß)-thalassemia is a blood genetic disease caused by the alteration of beta chains of hemoglobin protein synthesis distributed worldwide according to thalassemia belt where Indonesia was included [1]. The major type of this disease depended on regular blood transfusion to overcome the anemia caused by accelerated hemolysis and ineffective erythropoiesis [1]. Nowadays, major ß-thalassemia patients have a convenient access to the definite therapy, therefore increased their life expectancy. However this fact resulted into an inevitable iron overload condition which is related to persistent inflammation and increased risk for infection [2, 3].

Monocyte is a professional innate sentinel cell that participates in various inflammatory conditions through activation and recruitment in response to many cytokines. In addition, this cell bridges innate and adaptive immunities that also play a major role in iron regulation [4]. These integrated activities of monocytes can be studied by identifying and measuring their membrane protein. Previous study has showed that monocyte of thalassemia patients underwent hyperplasia and is hyperactive however demonstrate the low phagocytic activity in inflammation insult [3], however how monocyte was activated and therefore started the inflammation cascade in thalassemia patients need to be further elaborated.

CD69 is a C-type transmembrane lectin which is constitutively expressed in monocytes. Increase of which has been dubbed as an activation indicator of this cell [5-7]. All these studies lead us to think that there is an immune alteration caused by the constant state of iron overload. We hypothesize that monocytes are affected by the

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constant state of iron overload and activating the monocyte via this protein.

METHODS

Study Design, participants, and procedure

A study implementing cross sectional and analytic design involving 50 pediatric major ß-thalassemia patients that routinely visited the Thalassemia Clinic of Hasan Sadikin General Hospital, Bandung, West Java was done. Inclusion criteria were ß-thalassemia sufferers aged under 15 confirmed by genetic examination and had been transfused at least 2 years or more. Exclusion criteria were diabetes, autoimmune, cancer, tuberculosis, chronic infections such as HBV and HIV, and immunomodulatory treated patients.

Ethics

Ethics of this study was made in accordance to with Faculty of Medicine Universitas Padjadjaran and Hasan Sadikin General Hospital. The study was approved under the approval number of 74/UN6.C1.3.2/KEPK/PN/2016 and LB.02.01/C02/15691/XI/2016 for Faculty of Medicine and ethics committee of Dr. Hasan Sadikin Hospital respectively.

Laboratory Procedures

Characterization of CD69 monocytes

Blood was drawn from 50 patients through venipuncture and placed in vacutainer with lithium and sodium heparin. The blood was stored for 1 hour in room temperature before being analyzed. The tube that contains 200µL of heparinized blood was treated with 2000µL of PBA 0.5% and then vortexed/centrifuged for 5 minutes without break at 1500 rpm. The suspension is vortexed in FACS buffer with the antibodies, incubated for 20 minutes at 2-8oC, covered by aluminum foil, given ten –time diluted red cell lysing buffer, incubated again for 12 minutes, and lastly washed with 2000µL PBA 0.5% and suspended with 200µL PBA 0.5%.

After preparations were done, the samples were put into flow cytometer. The antibodies used were HLA-DR, CD14, and CD69 antibodies (All from BioLegend, San Diego, CA, USA). 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). The monocytes were distinguished from granulocytes and lymphocytes with forward and side scatter, HLA-DR, and CD16 subsequently. The gated result was analyzed with CD14/CD69. The percentage that positively express CD69 and

the mean fluorescent intensity (MFI) was recorded.

Hematology assessment: Complete Blood Count (CBC)

Vacutainer containing potassium EDTA (Becton Dickinson, Franklin Lakes, New Jersey, USA) was used for CBC. Automatic hematology analyzer (Sysmex Corp., Japan) was employed to measure hemoglobin (Hb), leukocyte, monocyte, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

Iron status measurement

Sera were extracted were analyzed for iron status. Serum iron and ferritin kit, The Elecsys ferritin immunoassay kit (Roche, Switzerland), was used to get ferritin concentration. All data was analyzed with a correlation study. Serum iron assay kit (Merck, Singapore) was used to measure serum iron.

Statistical analysis

Data with non-normally distribution 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). $P \leq 0.05$ is considered statistically significant.

RESULTS

Identification of CD69 monocytes

The CD14+CD69+ monocyte population result was depicted in figure 1. The selection of monocyte began by distinguishing the monocyte population according to size and granularity. Positive gating of the selected population employing HLA-DR and CD14 on monocyte membrane expression was used to identify true monocytes. Further, HLA-DR+CD14+ monocytes were selected. The final result, CD69+ monocytes of true monocytes were counted also the MFI of CD69 expression was measured as showed in table 1.

Hematologic characteristic

Hematologic data acquired which depicted in Table 2 showed an excess of serum iron and ferritin content in these patients.

Correlation result

Results show ferritin is a significant positively correlated with CD14$^+$CD69$^+$ monocytes of total monocytes and CD14$^+$HLA-DR$^+$ monocytes. These p values are significant ($p<0.05$).
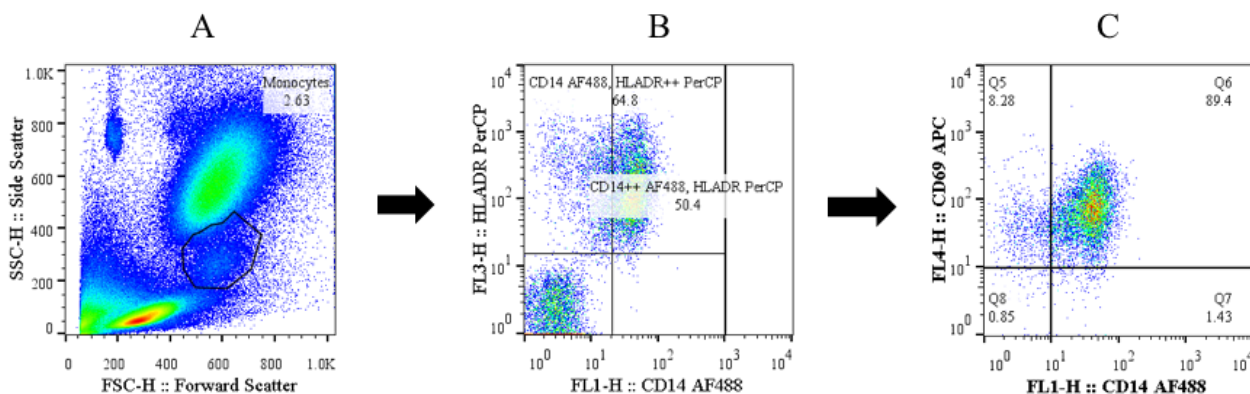

Figure 1. Identification of blood monocyte subset applying multicolor flow cytometry. Gating strategy for monocyte subsets identification presenting successive inclusion of monocytes population. (A.) Identification of monocyte subpopulation in blood. (B.) Selection for "true" monocytes by gating on CD14 positive and HLA-DR positive population. (C.) Remaining population was further discriminated on a CD14 vs. CD69 scatterplot to give CD14+CD69+ monocytes.

Table 1. Population of Monocytes

	Patients value
CD69$^+$CD14$^+$ monocyte (%) (Mean)	39.02
Median MFI of CD69 of CD14$^+$CD69$^+$ monocyte	80.2

Table 2. Correlation of monocyte characteristics in pediatric ß-thalassemia patients

Parameter		Value
Serum iron	CD14+/CD69+ of Total monocytes	r0.121
		p0.404
	CD14+/CD69+ of CD14+/HLA-DR+	r0.044
		p0.759
	Mean MFI for CD69+ of CD14+ /CD69+	r-0.004
		p0.978
	Median MFI for CD14+/CD69+	r-0.021
		p0.887
Ferritin	CD14+/CD69+ of Total monocytes	r0.278
		p0.050
	CD14+/CD69+ of CD14+/HLA-DR+	r0.291
		p0.040
	Mean MFI for CD69+ of CD14+ /CD69+	r0.012
		p0.934
	Median MFI for CD14+/CD69+	r0.015
		p0.916

Table 3. Clinical Characteristics

	Patients' value	Normal value
Gender		
Male, n (%)	25 (50)	Not Defined
Female, n (%)	25 (50)	Not Defined
Mean age (SD), year	8 (3.0)	Not Defined
Hematological assessment		> 29
Mean Hb (SD), g/dL	6.5 (1.2)	
Median leucocyte (IQR), /mm³	6000 (4000-8100)	10.9-14.9
Median monocyte count (IQR), %	6 (5-7)	4500-14500
Median MCV (IQR), fl	75.6 (71.2-78.6)	
Mean MCH (SD), pg/cell	25.5 (2.7)	79-98
Mean MCHC (SD), g/dL	34.3 (1.6)	25-33
Iron status		32-36
Median ferritin (IQR), µg/L	3118 (1675-5009)	
Mean serum iron (SD), µg/dL	163.5 (60.4)	< 1000
		35-150

DISCUSSION

Monocyte has pivotal function in bridging the interplay between innate and adaptive immune cells in the progression of inflammation. Activated blood circulating monocytes are recognized by the expression HLA-DR protein on their cell membrane, while the pro-inflammatory ones are when they are expressing CD14 and CD69 [6, 8-10]. These facts suggested that in major ß-thalassemia patients burdened with iron overload complication the existence of CD14+CD69+ monocyte play important role in the pathogenesis of persistent inflammation happened in major ß-thalassemia.

The results acquired shows that there is a moderate positive correlation between ferritin level and the percentage of CD14+CD69+ monocyte population. Monocyte activation can be shown via the CD69 protein. This protein is encoded in chromosome 12p13. The cross-linking of this protein increases monocyte in producing prostaglandin, leukotriene B4, nitric oxide, and extracellular calcium influx [5-7, 11]. These functions implicate CD69 as a pro-inflammatory protein on monocyte. CD69 has shown increase in persistent inflammatory conditions, such as sarcoidosis and HIV. It is implicated in activation of monocytes, in which it is also tied to an induction of TNF-α release by macrophages [12]. The activation process or status in monocytes has also been related to more release of cytokines but less on phagocytic function. Studies showed a downgrading effect of monocyte activation making phagosomes less efficient [13, 14].

Iron may be significant to monocytes because the life cycle of erythrocytes ends in phagocytosis. Release of iron and storage of iron in the form of ferritin is exactly why monocytes can be affected.

To manage iron content, cells such as monocytes express proteins such as DMT (Nramp2) to internalize iron. Free iron has to be controlled not just because of its dangers but also because of its benefits to microbes. In conditions of iron overload, free iron that is very toxic to the cells and tissues is formed and may continue to rise and catalyze the production of radical OH- substances from peroxide molecules, known as the Fenton reaction. At the final phase, this condition endangered cell function by damaging their biomolecules and inflammation. This study shows excess of iron indicated by higher ferritin and serum iron level.

As iron levels rise in the blood, hepcidin need to create a waste disposal environment rather than keeping the balance. Thus, hepcidin functions to keep the internalizer, which is DMT, but turns off the externalizer, which is ferroportin. These findings in iron overloaded patients is why monocytes can become a reservoir for iron and hence became intoxicated by it. To prevent that, iron has to be contained in its more stable form which is incorporated into ferritin. However, this mechanism most likely to be imbalanced. May be this is why there is a correlation between ferritin content and the CD69 protein. But, since CD69 is indicated by many as an "activation" marker, and a part of the pro-inflammatory mechanism, how is it related to iron?

Our humble suspicion is that the main link between iron and activation in monocytes might be NFκB. A study in kupffer cells in the liver showed that iron in its ferrous form blocks IkBα, increased the number of p65 and p65/p50 binding to DNA, and it is a direct agonist to the IKK. These evidence may be the tying knot between iron and the activation of monocytes because it

shows a preference to the activation of NFκB canonical pathway, and CD69 may be a part of this pathway.

As many substances triggered by the canonical pathway is activated, an inflammatory state is created. The canonical pathway may affect the behavior of the monocytes because of the constant activation. High iron content, and hence the high ferritin content may just be the reason why the canonical pathway is more active and hindering the balance because the alternative pathway is constantly off. The imbalance in immune function may just be the reason why there is an increased susceptibility to infections and hence increased mortality rate in patients with iron overload.

CONCLUSION

Considering the function of CD14 and CD69 on monocyte and the iron accumulation, our result may implicate that pediatric major ß-thalassemia patients have a tendency towards chronic inflammation. Thus CD14$^+$CD69$^+$ monocytes are implicated as marker of chronic inflammation in patients suffering from iron overload and early identification by this study makes it even more pronounced. Many more studies are needed to increase our understanding of the mechanisms with which iron overload causes immune dysregulation. Future direction for research of our study aimed at discovering collateral activation of immune cells via monocyte to explain organ damage caused by iron overload is imperative.

Disclosure

M.Ghozali and M. Fariz Anggia are co-first authors

Competing Interests
Authors declare that they have no competing interests.

Author's Contributions
M. Ghozali, M. Fariz Anggia, 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 and M. Fariz Anggia were involved in data acquisition. All authors contributed towards drafting and agree to be accountable for all respects of the work. M. Ghozali, Adi Imam Tjahjadi, 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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