Hyperferritinemia Correlated with Activated Population of Natural Killer Cells in Pediatric Major β-Thalassemia Patients

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Abstract
Natural killer (NK) cells act both as cytotoxic and cytokine producers in the innate immune response. Hyperferritinemia resulting from a routine blood transfusion as a specific treatment in major β-thalassemia patients may disturb the cellular immune system’s harmony. This study aims to investigate the correlation between hyperferritinemia and the NK cell subsets in major β-thalassemia settings. Pediatric major β-thalassemia patients who routinely received a blood transfusion at Dr. Hasan Sadikin General Hospital in 2016 were included in this cross-sectional study. Blood samples were treated with the monoclonal antibody of CD3, CD56, and CD16 to count the NK cells subsets as CD56<sup>bright</sup>, CD56<sup>dim</sup>, and CD16<sup>+</sup> using flowcytometry. CD69<sup>+</sup> used as an activation marker. The median fluorescence intensity (MFI) of CD56, CD16, and CD69 was measured. Total iron-binding capacity (TIBC), ferritin, and serum iron level examined as iron status. A Spearman correlation test was used for statistical analysis. Fifty-five blood samples were obtained for analysis. This study reveals that the percentage of CD3<sup>−</sup> lymphocyte population was correlated with the ferritin levels (r=−0.45, p=0.0009). Positive correlation was revealed between activated population (CD69<sup>+</sup>) of CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cell subsets and hyperferritinemia [(r=0.353, p=0.008) and (r=0.355, p=0.008)]. The activated CD56<sup>bright</sup> cells was associated with ferritin level (r=0.353, p=0.008) and TIBC (r=0.334, p=0.018). Hyperferritinemia in pediatric major β-thalassemia patients may influence NK cell subsets’ balance population, particularly the CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cell subsets, then alter their immune response to pathogens.

Key words: Hyperferritinemia, major β-thalassemia, NK cells

Korelasi antara Hiperferitinemia dan Sel Natural Killer Teraktivasi pada Anak dengan Talasemia Beta Mayor

Abstrak
Sel-sel natural killer (NK) telah diketahui memiliki peran sitotoksik dan dalam produksi sitokin pada respons imun bawaan. Hiperferitinemia merupakan hasil dari transfusi darah rutin yang dijalani sebagai terapi utama pada talasemia mayor. Penelitian ini bertujuan untuk meneliti hubungan hiperferitinemia dan sel NK pada talasemia beta mayor. Penelitian ini melibatkan anak dengan talasemia beta mayor yang secara rutin menerima transfusi darah di RSUP Dr. Hasan Sadikin selama tahun 2016. Sampel darah diberi marker CD3, CD56 dan CD16 untuk menghitung subset sel NK sebagai CD56<sup>bright</sup>, CD56<sup>dim</sup>, dan CD16<sup>+</sup> menggunakan flowcytometry. CD69<sup>+</sup> digunakan sebagai penanda aktivasi. Median fluorescence intensity (MFI) CD56, CD16, dan CD69 diukur. Kadar TIBC, ferritin, dan Fe serum diperiksa sebagai status besi. Uji korelasi Spearman digunakan pada analisis statistik. Analisis dilakukan terhadap 55 sampel darah anak dengan talasemia. Penelitian ini mendapatkan bahwa populasi limfosit CD3 berkorrelasi dengan kadar feritin (r=−0.45, p=0.0009). Korelasi positif didapatkan pada populasi teraktivasi (CD69<sup>+</sup>) dari subset sel CD56<sup>bright</sup> dan CD56<sup>dim</sup> NK dan hiperferritinemia [(r=0.353, p=0.008) dan (r=0.355, p=0.008)]. Sel CD56<sup>bright</sup> teraktivasi berkorrelasi dengan kadar feritin (r=0.353, p=0.008) dan TIBC (r=0.334, p=0.018). Hiperferitinemia pada anak dengan talasemia mayor dapat memengaruhi populasi sel NK, khususnya pada subset CD56<sup>bright</sup> dan CD56<sup>dim</sup> sehingga berpengaruh pada respons imun terhadap patogen.

Kata kunci: Hiperferitinemia, sel NK, talasemia beta mayor
Introduction

Major β-thalassaemia, a familial blood disorder, characterized by defects of hemoglobin (Hb) beta-chain synthesis, resulting in severe anemia and treated with a routine blood transfusion as its definite therapy. Iron overload often comes up in major β-thalassemia due to multiple blood transfusions, increased destruction of red blood cells, and increased gastrointestinal iron. Excess iron stored in the reticuloendothelial system as ferritin and secreted gradually in a small amount to the blood. Therefore, iron overload presented in increased ferritin levels, also known as hyperferritinemia.

Excessive iron is highly harmful to all cells, including the innate immune cells, by reducing the number of cells population or the number of cell subsets, potentially as an immune response to pathogens. Therefore, major β-thalassemia patients may be more susceptible to infection. Furthermore, immune cell alteration might increase infectious disease management challenges in this population to another level, including the prevention, diagnostic effort, and treatment. An earlier report showed a decrease in NK cell activity in major thalassemia patients, and this might occur due to iron overload.

NK cells are prominent cells of innate immune defense, filling out approximately 10 to 20% of the lymphocytes population in normal peripheral blood. NK cells are initially known for their ability to lyse tumor cells without prior activation. NK cells are also known for their cell-kill function and cytokine production, such as interferon (IFN)-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF). Therefore, their innate immune defense role becomes more critical to counter a considerable number of viral, bacterial, and parasitic pathogens. In addition to their critical role in innate immune response, NK cells also play a role in the adaptive immune response through direct interaction with dendritic cells, which provide negative or positive dendritic cell activity regulation.

NK cells were identified by the presence of CD56 and the absence of T-cell receptor (TCR) and CD3. Subsets of NK cells classified by the expression of CD56 and the presence of CD16. CD56 expression of NK cells related to cells’ ability to secrete cytokines. At the same time, CD16 is related to cell cytotoxicity function. CD56-CD16- (CD56bright) subset is the most abundant subsets comprising approximately 90% of the NK cells population. They have the most effective cytotoxic function, with less ability to secrete cytokine. CD56+CD16- (CD56bright) subset comprises 10% NK cells population, known as cytokine producers subsets. They can secrete many cytokines, including IFN-γ, tumor necrosis factor (TNF)-β, IL-10, IL-13, TNF-α, and GM-CSF, but have less cytotoxicity function. However, the cytotoxic ability of CD56brightCD16- subset strengthened after activation by IL-2 or IL-12. CD56-CD16+ (CD56dim) subset is another mature subset, minor in number, and have impaired effector function in both cytotoxic and cytokine production. The previous study found that the CD56dimCD16+ subset increased in HIV-1, HCV, and hantavirus infection.

Activated NK cells displayed by expression of CD69 and HLA-DR, IFN-γ secretion, and enhancement of cytotoxic function. CD69 is an early membrane receptor expressed right after activation of lymphocytes, including NK cells. Not detected in resting NK cells, CD69 rapidly induced in NK cells shortly after activation.

Iron overload in major β-thalassemia might affect the NK cells’ functions and population number and might alter the immune response in time. Therefore, we examine the NK cell subsets characterization in pediatric major β-thalassemia major with iron overload and the correlation between NK cell subsets and hyperferritinemia.

Methods

A cross-sectional study was conducted from October 17th–November 15th, 2016, in Thalassemia Outpatient Clinic, Dr. Hasan Sadikin General Hospital Bandung. Pediatric major β-thalassemia patients with iron overload recruited using simple random selection.

Our study included 55 pediatric major β-thalassemia patients with multiple blood transfusions and aged less than 15 years old. All subjects had been diagnosed with major β-thalassemia through a clinical examination, confirmed by a positive result on hemoglobin electrophoresis for β-thalassemia, and had undergone a routine blood transfusion for at least two years. All patients with signs of acute infections, chronic infections such as HBV, HIV, and unhealthy conditions were excluded.

Informed consent was obtained from the parents of all subjects. Blood specimens were
acquired by venipuncture before blood transfusion and collected in three tubes (heparin-contained, EDTA-contained, and plain). Anti-coagulated blood was used to perform flow cytometry of NK cells and hematology assessment, while non-anti-coagulant blood was processed into the serum to measure iron status.

Peripheral venous blood collected in vacutainer tubes containing lithium and sodium heparin (Becton Dickinson, Franklin Lakes, NJ, USA). Immunophenotyping NK cells’ subsets were done employing multicolor flow cytometry, started within 1 hour after blood collection.

Utilizing a Becton Dickinson FACS™ Calibur™ flow cytometer, according to their phenotypic marker of CD3⁺, CD56⁺, and CD16⁺, NK cells were grouped into three functional subsets based on stain index guided manual gating of NK cells population of whole blood. The activation marker of CD69⁺ was used to measure the activated cells. Afterward, the fluorescence intensity of CD56, CD16, and CD69 was measured as markers of each protein expression at every subset.

For preparation, 2,000 µL PBA 0.5% added to 200 µL heparinized blood. After vortex and centrifugation at 1,500 rpm for 5 minutes, the cell suspension was formed, and the supernatant was then discarded. Monoclonal antibodies mixture of CD3 AlexaFlour 488 (Biolegend, San Diego, CA, USA), CD16 PE (Biolegend, San Diego, CA, USA), CD56 PerCP (Biolegend, San Diego, CA, USA), and CD69 APC (Biolegend, San Diego, CA, USA) were added and vortexed to cell suspension in FACS buffer diluted solution. The antibody-cell suspension was then covered by aluminum foil and incubated for 20 minutes at 2–8°C. Ten-time diluted red cell lysing buffer (Biolegend, San Diego, CA, USA) was added to stain cells, and the cells were then re-incubated for 12 minutes. The lysed cell suspension was then vortexed and washed two times using a 2,000 µL 0.5% PBA solution, and then cells were resuspended using 200 µL of the same 0.5% PBA solution. Cells were read according to their phenotypic marker by BD Cell Quest Pro Software (Biosciences, San Jose, CA, US) for 500,000 events, and then the FCM output files were analyzed using FlowJo 10 (Tree star). The CD3 monoclonal antibody (mAb) is used to separate NK cells as CD3⁺ cells. CD56 and CD16 mAb were used to differentiate NK cell subsets as CD56⁺CD16− (CD56dim), CD56⁺CD16⁺ (CD56⩾bright), and CD56−CD16⁺ (CD56neg). CD69 mAb was used to define the activated cells as CD69⁺.

The population of NK cells subsets delivered as a percentage, which defined the proportion of NK cells count based on CD56bright, CD56dim, and CD56neg NK cells. Activated cells of each subset also delivered as a percentage of cells expressing CD69⁺. The expression of the surface protein (CD56, CD16, and CD69) showed by median fluorescence intensity (MFI) of each monocyte subsets’ designated protein.

Hematology assessment performed using peripheral venous blood samples collected in a potassium EDTA tube (Becton Dickinson, Franklin Lakes, New Jersey, USA). An automatic hematology analyzer (Sysmex Corp., Japan) was used to measure hematology parameters.

Collected sera from plain Vacutainer centrifuged for iron status measurement, including serum iron and ferritin. Serum ferritin was measured using the Elecsys ferritin immunoassay kit (Roche, Switzerland), while the serum iron assay kit (Merck, Singapore) was used to measure serum iron.

Non-normally distributed data presented as median with interquartile range (IQR); normally distributed data presented as mean with standard deviation (SD). Correlation between parameters tested using Spearman correlation coefficient for non-normally distributed data, and the Pearson correlation coefficient for normally distributed data. All analyses were performed with GraphPad PRISM version 6.0 (Graphpad Software, Inc., La Jolla, California, USA). The result with a p-value<0.05 is considered statistically significant. Linear regression analysis was performed following a significant correlation result.

All procedures were conducted in conformity with the Faculty of Medicine policies, Universitas Padjadjaran and Dr. Hasan Sadikin General Hospital, Bandung, West Java, Indonesia. This study was approved by the Health Research Ethics Committee of Faculty of Medicine, Universitas Padjadjaran Bandung, with approval number 74/UN6.C1.3.2/KEPK/PN/2016 and the Ethics Committee of Dr. Hasan Sadikin General Hospital Bandung with approval number LB.02.01/C02/15691/XI/2016.

Results

Characteristics of the study participants are presented in Table 1. Their mean age is 8 (±2.9) years old with an equal proportion of
gender. Hemolytic anemia [Hb=6.3 (±1.1) g/dL, MCV=74.7 (±4.7) fL], as experienced in major thalassemia, prove the clinical setting of this study. The leucocyte profile showed, respectively, normal total leucocyte count and differential lymphocyte count. All participants have iron overload status, proved by a high iron serum level [157 (±63.9) µg/dL], and a very high ferritin level [3,183 (1,678–4,460) µg/dL]. Utilizing a negative-gating strategy to separate

| Table 1 Demographic and Clinical Characteristics of Study Participants |
|-----------------------------|------------------|------------------|
| Characteristics             | Patients’ Value  | Normal Value     |
| Gender {n (%)}              |                  |                  |
| Male                        | 28 (51)          | Not define       |
| Female                      | 27 (49)          | Not define       |
| Age, year {mean(SD)}        | 8 (2.9)          | Not define       |
| Hematological indicators    |                  |                  |
| Hb, g/dL {mean (SD)}        | 6.3 (1.1)        | 10.9–14.9        |
| Leucocyte, /mm³ {median (IQR)} | 6,000 (4,000–8,100) | 4,500–14,500   |
| Thrombocyte, 10⁹/mm³ {median (IQR)} | 209.9 (127–328) | 150–300         |
| MCV, fl {mean (SD)}         | 74.7 (4.7)       | 79–98           |
| MCH, pg/cell {mean (SD)}    | 25.7 (2.4)       | 25–33           |
| MCHC, g/dL {mean (SD)}      | 34.4 (1.7)       | 32–36           |
| Iron status indicators (µg/L) |                |                  |
| Ferritin {median (IQR)}     | 3,183 (1,678–4,460) | <1,000         |
| Serum iron {mean (SD)}      | 157 (63.9)       | 35–150          |
| TIBC {median (IQR)}         | 197 (156–219)    | 240–474         |
| Cell characteristics (%)    |                  |                  |
| Lymphocyte {mean (SD)}      | 33.81 (10.32)    | 25–54           |
| CD3⁻ lymphocyte {median (IQR)} | 46 (37.8–53.5)  | Not defined     |
| CD56bright NK cells {median (IQR)} | 3.1 (1.59–4.96) | Not defined     |
| CD56dim NK cells {median (IQR)} | 2.49 (0.74–5.95) | Not defined    |
| CD56neg NK cells {median (IQR)} | 7.04 (1.75–9.77) | Not defined    |
| CD69⁺ of CD56bright NK cells {mean (SD)} | 36.23 (9.56) | Not defined |
| CD69⁺ of CD56dim NK cells {mean (SD)} | 35.96 (13.03) | Not defined |
| CD69⁺ of CD56neg NK cells {mean (SD)} | 28.95 (10.33) | Not defined |

Note: Hb (hemoglobin), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), and MCHC (mean corpuscular hemoglobin concentration) were presented as mean with SD (standard deviation); TIBC (total iron-binding capacity) presented as median with IQR (interquartile range); CD (cluster of differentiation) and NK (natural killer) cell are in percentage and presented as mean with SD or median with IQR

| Table 2 Correlation between Activated NK Cells and Ferritin Level |
|-----------------------------|------------------|------------------|
| Parameter                  | TiBC r | TiBC p | Ferritin r | Ferritin p |
| (%)                        |        |        |            |            |
| CD3⁻ cells                 | −0.11  | 0.461  | −0.45      | 0.0009 †    |
| CD56bright                 | −0.246 | 0.089  | 0.029      | 0.837       |
| CD56dim                    | −0.139 | 0.357  | −0.087     | 0.55        |
| CD56neg                    | −0.135 | 0.359  | −0.017     | 0.903       |
| CD69⁺ on CD56bright        | 0.334  | 0.018 †| 0.353      | 0.008 †     |
| CD69⁺ on CD56dim           | 0.136  | 0.375  | 0.355      | 0.008 †     |
| CD69⁺ on CD56neg           | 0.062  | 0.614  | 0.240      | 0.08        |

Note: Spearman’s correlation, statistically significant if p<0.05
cells with CD3\(^{-}\) and followed by using CD56 and CD 16 as markers in cell sorting, NK cells subsets adequately identified from lysed-erythrocyte blood (Figure 1).

NK cells’ selection began with gating on lymphocyte subpopulation successively continued with assort them based on the CD3 negative population. The selected cells were then classified into three distinct NK cell subsets, CD56\(^{\text{bright}}\), CD56\(^{\text{dim}}\), and CD56\(^{\text{neg}}\) subsets, based on CD56 and CD16 surface expression (Figure 1). The median of CD56\(^{\text{bright}}\), CD56\(^{\text{dim}}\), and CD56\(^{\text{neg}}\) subsets are respectively 3.1\%, 2.49\%, and 7.04\% (Table 1). The mean of activated cells of CD56\(^{\text{bright}}\), CD56\(^{\text{dim}}\), and CD56\(^{\text{neg}}\) subsets are respectively 36.23\%, 35.96\%, and 28.95\% (Table 1).

This study showed that the percentage of CD3\(^{-}\) cells populations in thalassemia patients negatively correlated with the ferritin levels (r=-0.45, p=0.0009). The higher ferritin level on

**Figure 1 Identification of Blood NK Cells Subset Applying Multicolor Flow Cytometry**

Identification of blood NK cells subset applying multicolor flow cytometry. Gating strategy for NK cells subsets identification presenting successive inclusion of lymphocyte population. (A) Identification of lymphocyte subpopulation in blood. (B) Selection for NK cells by gating on the CD3 negative lymphocyte population. (C) The selected population then differentiated on CD16 vs CD56 scatterplot to give three NK cells subsets. (D–F) Further cell sorting on each subset against CD69 to obtain the activated NK cells. (G) Median and IQR of each subsets population. (H) Median and IQR of activated NK cells in each subset.
blood, the lower CD3⁻ cells population becomes. Other findings we have are a positive correlation between activated cells (CD69⁺) on CD56bright and CD56dim towards ferritin level, and also activated CD56bright NK subsets against TIBC. This figure shows a positive correlation between ferritin level and the number of activated CD56bright and CD56dim subsets, and also between TIBC level and the number of activated CD56bright subsets. Meanwhile, the ferritin level shows a negative correlation with the number of CD3⁻ lymphocyte cells.

Discussion

Natural killer (NK) cells are unquestionably a substantial part of the innate immune defense. Having been known for decades as the relatively primitive killer, their role in early host defense against a variety of pathogens (viruses, bacteria, and parasites) become more crucial coincide with increasing knowledge of NK cell biology. NK cells can also produce cytokines, i.e., IFN-γ, TNF-β, IL-10, IL-13, TNF-α, and GM-CSF, as a response to stimulation from monocyte-derived cytokines (monokines), i.e., IL-2, IL-12, and IL-18. NK cells are one of the key sources of IFN-γ. We sharply differentiate NK cells subsets as CD56bright (CD56brightCD16⁺/−), CD56dim (CD56dimCD16⁺), and CD56neg (CD56negCD16⁺) subsets as shown in Figure 1. In normal conditions, CD56dim subsets fill up to 90% of NK cells, and CD56bright comprise up to 80% of NK cells. In this study, we focus on CD56bright NK cells as they play a crucial role in innate immunity.

Figure 2  Linear Regression Analysis of CD3⁻ Lymphocyte Cells

Linear regression analysis of CD3⁻ lymphocyte cells (A), activated CD56bright NK subsets (B), and CD56dim NK subsets (C) against ferritin level, and also activated CD56bright NK subsets against TIBC. This figure shows a positive correlation between ferritin level and the number of activated CD56bright and CD56dim subsets, and also between TIBC level and the number of activated CD56bright subsets. Meanwhile, the ferritin level shows a negative correlation with the number of CD3⁻ lymphocyte cells.

![Figure 2](image-url)
to 10% NK cells population. Interestingly, our study finds that the proportion between the three subsets is barely equal, with a slightly higher of the CD56\textsuperscript{neg} population. Unfortunately, our study does not have healthy control to be compared.

These subsets have distinct roles and function in innate immune defense. CD56\textsuperscript{dim} NK cells have a lot higher cytotoxic ability than CD56\textsuperscript{bright} cells, and they have more granymes, perforin, and other cytolytic granules. Higher CD16 expression of CD56\textsuperscript{dim} NK cells makes them efficient mediators of antibody-dependent cellular cytotoxicity (ADCC). Stimulation with cytokines such as IL-2 or IL-12 makes the cytotoxic ability of all NK cell subsets significantly augmented, but the cytokine-producing ability decreased. On the other hand, CD56\textsuperscript{bright} NK cells are the most efficient cytokine producers. The cytokines secreted by CD56\textsuperscript{bright} NK cells are IFN-γ, TNF-β, IL-10, IL-13, TNF-α, and GM-CSF, depending on the precise conditions of stimulation. Stimulation with IL-2 and IL-18 will increase IFN-γ production.

CD56\textsuperscript{neg} NK cells are functional and mature NK cells subset with less cytotoxic and cytokine-producing ability. The previous study found that the CD56\textsuperscript{neg} NK cells population expanded in HIV-1 infection and HCV infection.

Regarding the CD56\textsuperscript{bright} NK cells as abundant cytokine producers, particularly in IFN-γ production, alteration of this subset might lead to an inadequate innate immune response in eliminating pathogens, and contribute to diagnostic laboratory misinterpretation, such as tuberculin skin test (TST) and interferon gamma release assay (IGRA) in diagnosing tuberculosis.

We distinguished NK cells in an active state by using CD69 as an early activation marker, which rapidly expressed during activation of NK cells. As shown in Table 2 and Figure 2, we convey in our study that hyperferritinemia correlated with the number of CD3- lymphocytes, and the number of activated cells of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK subsets. Considering that hyperferritinemia is the most common complication in pediatric major β-thalassaemia major, these findings explain why major β-thalassemia patients are more susceptible to infections.

**Conclusions**

Our study finds that hyperferritinemia correlated with the number of CD3- lymphocytes, and the number of activated cells of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK subsets. Considering that hyperferritinemia is the most common complication in pediatric major β-thalassaemia major, these findings explain why major β-thalassemia patients are more susceptible to infections.

**Conflict of Interest**

All authors declare that they have no competing interests.

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