Journal of Inflammation Research

Dovepress
Taylor & Francis Group

Open Access Full Text Article

ORIGINAL RESEARCH

Inflammatory Monocyte Subsets Correlation with Iron Levels in Low Vitamin D Pediatric Transfusion-Dependent Thalassemia

Mohammad Ghozali[1], Matahari Matahari[2], Adi Imam Cahyadi[1], Sri Devi Agustini[2], Reni Ghrahani[3], Lelani Reniarti[3], Budi Setiabudiawan[3], Ramdan Panigoro[1]

[1]Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Bandung, West Java, 45363, Indonesia; [2]Faculty of Medicine, Universitas Padjadjaran, Bandung, West Java, 45363, Indonesia; [3]Department of Pediatrics, Faculty of Medicine, Universitas Padjadjaran, Bandung, West Java, 40161, Indonesia

Correspondence: Mohammad Ghozali, Email moh.ghozali@unpad.ac.id

Background: Patients with transfusion-dependent thalassemia experience iron dysregulation, which affects the immune response. Surface proteins such as FcγRIII (CD16), lipopolysaccharide receptor (CD14), and human leukocyte antigen (HLA-DR) on monocytes are crucial for innate and adaptive responses. Blood monocytes, identified by their CD14 and CD16 expression, show functional diversity during injury or inflammation. Considering the mechanisms of vitamin D activation and its potential interaction with monocytes, further investigation of its immunomodulatory role in transfusion-dependent thalassemia is essential.

Purpose: This study evaluated monocyte subsets, population, and surface receptor expression (CD14, CD16, and HLA-DR), and their association with iron status and vitamin D levels in patients with transfusion-dependent thalassemia.

Patients and Methods: Fifty lysed erythrocyte-heparinized whole blood samples from transfusion-dependent thalassemia patients were analyzed by flow cytometry and classified into three monocyte subsets: CD14++CD16- (classical), CD14++CD16+ (intermediate), and CD14+CD16++ (non-classical). Cell percentage referred to the monocyte subset population. Median fluorescence intensity (MFI) indicated surface protein expression. The 25(OH)vitamin D level was used to measure vitamin D levels. Iron status was assessed using ferritin and serum iron levels. A correlational study was performed.

Results: We did not find a correlation between low vitamin D levels (22.9 ng/mL ± 3.9) and monocyte characteristics, iron status, or hematology profile. However, we observed a negative correlation between the percentage of intermediate and non-classical monocytes and hemoglobin and ferritin levels ($P = 0.02$, $r = -0.3$; $P = 0.04$, $r = -0.3$). Additionally, we found a positive correlation between the median fluorescence intensity (MFI) of CD14 in non-classical monocytes and serum iron ($P = 0.04$, $r = 0.3$).

Conclusion: Our findings suggest that iron overload and anemia may influence the function of inflammatory monocyte subsets. Considering the immunomodulatory role of vitamin D through monocyte modulation during pathogen insult, further research utilizing a whole-blood stimulation assay is imperative.

Keywords: ferritin, hemoglobin, iron, monocyte, thalassemia, vitamin D

Introduction

Thalassemia, a type of congenital anemia characterized by insufficient synthesis of one or more globin subunits in normal human hemoglobin, is witnessing a yearly increase in the number of affected patients.[1] Equally affects males and females, thalassemia occurs in approximately 40,000 births each year globally, with the Mediterranean, the Middle East, and Southeast Asia being the regions with the highest prevalence. More than 25.000 people worldwide are diagnosed with β-thalassemia and require holistic lifetime treatment.[2] In Indonesia, the frequency of β-thalassemia genes has been reported to range from 3 to 10%. In 2018, the Indonesian Thalassemia Foundation-Association of Parents of Thalassemia Patients (YTI-POPTI) reported approximately 9000 thalassemia patients in Indonesia. By June 2021, this number had risen to 10,973.[3,4]

Received: 8 September 2024
Accepted: 15 November 2024
Published: 8 January 2025

Routine blood transfusion is the definitive therapy for some thalassemia patients to address anemia; however, this results in the inevitable accumulation of iron. Despite the administration of iron chelation drugs to address iron buildup, iron overload and toxicity repeatedly occur due to premature hemolysis and ineffective erythropoiesis in these patients.[1,5] The free form of iron is very toxic to cells and tissues; therefore, in the cellular setting, it may cause danger. Immune cells can be impaired, thus making transfusion-dependent thalassemia patients vulnerable to infection.[5,6]

Macrophages are crucial for regulating cellular iron by scavenging and recycling degraded erythrocytes to form stored ferritin. Furthermore, they maintain appropriate tissue integrity and a balanced immune response; therefore, their availability and functionality must be sufficient.[6] During inflammation and pathogen challenge, circulatory monocytes are recruited to the injured tissue to supply the macrophage population, thus making them key players in innate effectors that trigger and polarize the adaptive immune response.[6,7] Monocytes in the peripheral blood exist in a heterogeneous population. Three distinct monocyte subsets have been identified and characterized based on their expression of the surface markers CD14 and CD16: classical monocytes (CD14++CD16−), intermediate monocytes (CD14++CD16+), and non-classical monocytes (CD14+CD16++). While the population numbers of each subset have remained stable at a steady state, dynamic changes in the proportional distribution of monocyte subsets and their surface protein expression have yielded different effects under various inflammatory conditions.[8,9] These critical innate sentinel cells anchor HLA-DR molecules to monocytes. These membrane-bound molecules are pivotal for facilitating the transition from innate to adaptive immune responses by presenting antigens to T cells.[10] Previous study showed that the dynamic expression of HLA-DR in monocyte subsets indicates the triumph of an optimal immune response.[11]

Elevated serum ferritin levels are correlated with oxidative stress markers and hematological alterations associated with inflammatory diseases. This phenomenon is particularly pronounced in transfusion-dependent thalassemia and may alter monocyte function. Previous studies have reported reduced phagolysosome formation in monocytes.[12,13] However, it is necessary to further elaborate on the specific monocyte subsets affected and how phenotypic changes are related to iron overload markers.

Vitamin D deficiency is common in patients with transfusion-dependent thalassemia.[14–16] Low vitamin D levels have been linked to cardiac dysfunction, low bone mass, inflammation, impairment of monocyte phagocytosis, and high ferritin levels.[17] Monocytes also harbor a Vitamin D Receptor (VDR) and hydroxylase (CYP27B1 enzyme). Therefore, they can metabolize vitamin D to its active form, thereby modulating monocyte function.[18] Previous studies, including our group examining immune responses in thalassemia, have primarily concentrated on the connection between clinical symptoms, such as anemia and iron overload, and how iron levels affect monocytes in their early cell activation. However, they have yet to incorporate an analysis of vitamin D levels and how they might impact the immune responses of thalassemia patients who received regular blood transfusions.[2,6,12,17,19,20] Our study employed flow cytometry to characterize the monocyte functional subsets of patients with transfusion-dependent thalassemia based on CD14, CD16, and HLA-DR proteins expressed on monocytes. Subsequently, it correlated with vitamin D levels, hematological indicators, and iron status.

This study is part of a more comprehensive investigation to reveal the role of cholecalciferol administration as an immunomodulator in maintenance therapy and vitamin D deficiency in pediatric patients with transfusion-dependent thalassemia. Previous studies in our group revealed activation of immune cells, namely neutrophil,[21] lymphocytes,[22] natural killer cells,[23] and monocyte[20] through their immune receptors, unveiling a chronic inflammatory condition in pediatric patients with transfusion-dependent thalassemia. Notably, in monocytes, our previous study found a positive correlation between CD14 +CD69+ monocytes and ferritin, which may implicate a specific early activation marker for chronic inflammation as a form of immune dysregulation under the same conditions.[20] Hence, this research presents a new methodology for quantifying monocyte subsets to better understand the role of each subset and its relationship with iron levels. Furthermore, we evaluated the correlation between vitamin D levels and monocyte subsets to explore the potential of vitamin D as an immunomodulatory agent.

Materials and Methods

Study Design, Participants, and Procedure

This cross-sectional analytical study involved pediatric patients with transfusion-dependent thalassemia. The study participants were selected through simple random sampling from October 17, 2018, to November 15, 2018. This study

was performed at the Thalassemia Clinic of Hasan Sadikin General Hospital, Bandung, the main tertiary academic referral center for West Java, taking care of more than 150 transfusion-dependent thalassemia patients.

Fifty pediatric patients with an established transfusion-dependent thalassemia diagnosis who regularly visited thalassemia clinics for blood transfusions were selected based on their medical record history, all aged under 15 years. The inclusion criteria were based on clinical diagnoses of transfusion-dependent thalassemia, confirmed by hemoglobin electrophoresis results, and a minimum of two years of transfusion history. Patients with a history of tuberculosis, cancer, diabetes, autoimmune, chronic infections such as HBV, HIV, immunomodulatory therapy, and unhealthy conditions were excluded.

After obtaining written informed consent from the parents of all subjects, blood specimens were collected via venipuncture for routine and complete blood examinations immediately prior to blood transfusion. The samples were then collected in heparin- and EDTA-containing tubes and plain tubes. Anticoagulated blood was used for monocyte characterization and hematology assessment, while non-anticoagulated blood was processed into serum. Subsequently, the collected sera were used to assess the iron status and vitamin D levels.

Ethics

All procedures were conducted according to the policies of the Faculty of Medicine, Universitas Padjadjaran, and Hasan Sadikin General Hospital, Bandung, West Java, Indonesia. This study received approval from the Health Research Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran Bandung (approval number 50/UN6.KEP/EC/2018) and the Ethics Committee of Dr. Hasan Sadikin General Hospital Bandung (approval number LB.02.01/X.2.2.1/1373/2018). The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from the parents or guardians of all participants.

Laboratory Procedures

Efficacy

Peripheral venous blood was collected in Vacutainer tubes containing lithium and sodium heparin (Becton Dickinson, New Jersey, NJ, USA). Monocyte characteristics were measured using multicolor flow cytometry, as previously described.[24] Samples were kept at room temperature, and measurements began within one hour of blood collection. In short, by employing a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, New Jersey, NJ, USA), according to their phenotypic markers, CD14+, CD16+, and HLA-DR+, monocytes were characterized into three functional subsets based on stain index-guidance followed by positive manual gating of the monocyte population of whole blood. Next, the fluorescence intensities of CD14, CD16, and HLA-DR were measured as protein expression markers for each subset.

Up to 2000 µL of 0.5% PBA (phosphate-buffered saline) was added to 200 µL heparinized blood. A cell suspension was formed after mixing and spinning at 1500 rpm for five minutes without breakage, and the supernatant was discarded. A monoclonal antibody mixture consisting of CD14 Alexa Fluor 488 (BioLegend, San Diego, CA, USA), CD16 PE (BioLegend, San Diego, CA, USA), and HLA-DR PerCP (BioLegend, San Diego, CA, USA) was added and mixed to the cell suspension in FACS buffer diluted solution. Then, the antibody cell suspension was covered with aluminum foil and incubated for twenty minutes at cold temperature (2–8°C) covered by aluminum foil was applied to the antibody cell suspension. Ten-fold diluted red cell lysis buffer (BioLegend, San Diego, CA, USA) was added to the stained cells and incubated for twelve minutes. Before reading in the flow cytometer, the lysed cell suspension was mixed and washed twice using 2000 µL of 0.5% PBA, and then the cells were suspended in 200 µL of 0.5% PBA. Cells were read according to their phenotypic markers using BD Cell Quest Pro Software (Biosciences, San Jose, CA, USA) for 500,000 events, and the FCM output files were analyzed using FlowJo 10 (Tree Star, Ashland, OR, USA). The positive gating strategy was employed to identify monocytes yielding three monocyte subsets and the expression of the phenotypic markers (CD14, CD16, and HLA-DR) of each subset using flow cytometry.

The population of activated monocyte subsets, namely, classical, intermediate, and non-classical, was presented as a percentage, which was defined as the proportion of count-designated monocyte subsets and CD14+HLA-DR+

monocytes. The expression of surface proteins such as CD14, CD16, and HLA-DR in each monocyte subset was expressed by the designated protein median fluorescence intensity (MFI).

Hematology Assessment

A Vacutainer tube containing potassium EDTA (Becton Dickinson, New Jersey, USA) was used to collect peripheral venous blood for hematological assessment. An automatic hematology analyzer (Sysmex Corp., Tokyo, Japan) was used to measure hemoglobin (Hb), leukocytes, monocytes, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

Iron Status Measurement

Serum collected from a plain Vacutainer was centrifuged for iron status measurements, including serum iron and ferritin levels. An Elecsys ferritin immunoassay kit (Roche, Rotkreuz, Switzerland) was used to measure serum ferritin levels. In contrast, a serum iron assay kit (Merck, Singapore, Singapore) was used to measure serum iron levels.

Vitamin D Level Measurement

Sera were collected, centrifuged, and stored at –75°C until assayed. The vitamin D concentration was measured based on the level of 25-hydroxyvitamin D (25 (OH) vitamin D) using a human 25(OH)vitamin D ELISA kit (Qayee, Shanghai, China). This assay detects 25(OH)vitamin D using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). Vitamin D deficiency was considered if the 25(OH)vitamin D concentration was < 20 ng/mL, whereas 21–29 ng/mL was deemed insufficient.[25]

Statistical Analysis

Non-normally distributed data are presented as medians with interquartile ranges (IQR), whereas normally distributed data are presented as mean ± standard deviation (SD). The correlation between the parameters was tested using the Spearman correlation coefficient for non-normally distributed data and the Pearson correlation coefficient for normally distributed data. All analyses were performed using GraphPad PRISM version 10 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was set at $P < 0.05$. To achieve eighty percent power for detecting a medium effect, a significance criterion, the determination of minimum sample size required fifty subjects to test the study hypothesis.

Results

Identification of Monocyte Subsets

A positive gating strategy identified monocyte subsets in lysed erythrocyte blood samples. Monocytes were selected by gating monocyte subpopulations and sorting them based on CD14-positive and HLA-DR-positive populations. Boolean NOT gates on a bivariate plot of CD14+HLA-DR+ vs CD14-HLA-DR+ were applied to identify “true” monocytes. Monocytes were categorized into three subsets based on their CD14 and CD16 surface expression. The majority (approximately 85%) belonged to the classical CD14++CD16- subtype, whereas intermediate CD14++CD16+ monocytes accounted for approximately 8%. The remaining 4% consisted of non-classical CD14+CD16++ subtypes, as indicated in Table 1. Correspondingly, the surface expression levels of CD14, CD16, and HLA-DR were quantified in each subset.

Demographic and Clinical Characteristic

The study comprised 50 children with an equal sex ratio, a mean age of 8 years, and a standard deviation of 3 years. Table 2 presents the characteristics of the study participants and laboratory findings.

Higher median ferritin levels characterized all participants with iron overload status and mean serum iron levels compared to the normal range. Following the Endocrine Society Clinician Vitamin D Guideline,[25] our study identified vitamin D insufficiency in pediatric thalassemia patients.

Hematological examination revealed that hemoglobin and MCV values were lower than normal, indicating clinical anemia in patients with transfusion-dependent thalassemia. These data suggest the occurrence of hemolytic anemia in these patients. Leukocyte and monocyte counts were within the normal range.

Table 1 Population of Monocyte Subsets

	Patient's Value
Classical monocytes, (IQR), (%)	85.2 (82.4–90.9)
Intermediate monocytes, (IQR), (%)	8.3 (5.1–12.8)
Non-classical monocytes, (IQR), (%)	4.5 (2.8–7. 5)

Table 2 Demographic and Clinical Characteristics

	Patient's 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
Vitamin D (SD), ng/mL	22.9 (3.9)	> 29
Hematological assessment		
Mean Hb (SD), g/dL	6.5 (1.2)	10.9–14.9
Median leucocyte (IQR), /mm^3	6000 (4000–8100)	4500–14500
Median monocyte count (IQR), %	6 (5–7)	
Median MCV (IQR), fl	75.6 (71.2–78.6)	79–98
Mean MCH (SD), pg/cell	25.5 (2.7)	25–33
Mean MCHC (SD), g/dL	34.3 (1.6)	32–36
Iron status		
Median ferritin (IQR), µg/L	3118 (1675–5009)	<1000
Mean serum iron (SD), µg/dL	163.5 (60.4)	35–150

Correlation Result

Table 3 presents the significant correlations among the variables, including monocyte characteristics, hematological assessment, iron status, and vitamin D concentrations. This study found a significant negative correlation between the population of intermediate monocyte subsets and hemoglobin levels and between the percentage of non-classical monocyte subsets and ferritin levels. A positive correlation was found between the expression of activated monocyte surface markers and iron overload, which was indicated by the positive association between the median fluorescent intensity (MFI) of CD14 in non-classical monocytes and higher serum iron levels.

Discussion

Monocytes are critical sentinel cells that contribute to innate immune responses and serve as intermediaries that bridge the activation of the adaptive immune response during inflammation and pathogenic challenges. This capability is evident in their dynamic functional differentiation, which aids in restoring the disrupted homeostasis. The advent of flow cytometry has allowed for a more robust characterization of blood monocytes by considering the variation in their immune receptor expression. Notably, using the expression of CD14 and CD16, three distinct subsets can be identified, each of which can be further assessed for their functionality based on the expression of surface proteins such as HLA-

Table 3 Correlation of Monocyte Characteristics in Transfusion-Dependent Thalassemia Patients. There is a Weak, Significant Negative Correlation Between Intermediate Monocytes and Hemoglobin and Non-Classical Monocyte Percentages and Ferritin. Conversely, Activated Monocyte Surface Markers, Particularly CD14 Expression in Non-Classical Monocytes, Positively Correlated with Elevated Serum Iron Levels

Parameter	Correlation Value		
% intermediate monocytes	Hemoglobin	r	−0.3
	Hemoglobin	p	0.02
% non-classical monocytes	Ferritin	r	−0.3
	Ferritin	p	0.04
MFI of CD14 of non-classical monocytes	Serum iron	r	0.3
	Serum iron	p	0.04

DR.[26] Mentioned surface proteins are immune receptors, whose activation by ligands or factors in the cell milieu will determine monocyte activity. VDR (Vitamin D receptor) in monocytes also plays a crucial role in modulating monocyte capacity. Our study revealed that the clinical manifestations in pediatric transfusion-dependent thalassemia patients, characterized by anemia and iron overload indicators in the context of low vitamin D levels, were correlated with the distribution of monocyte subsets and expression levels of CD14, CD16, and HLA-DR.

Heterogeneous monocytes of classical, intermediate, and non-classical subset populations were successfully dichotomized from the blood monocytes of pediatric patients with transfusion-dependent thalassemia. This outcome indicates the mobilization of monocytes to sites of inflammation where they accomplish their respective functions in promoting inflammation or facilitating tissue homeostasis.[8,9] All subjects in question exhibited low vitamin D levels accompanied by iron overload, as evidenced by elevated serum ferritin and iron levels. Regular blood transfusion, a definite treatment for anemia caused by premature hemolysis and ineffective erythropoiesis, double-burdened these patients and contributed to elevated iron storage and trafficking, reflected in high ferritin and serum iron levels.[5] Concurrently, transferrin becomes saturated. Even with iron chelation therapy, the detoxification tolerance limits and iron storage in ferritin may be unbalanced. Free iron, which is very toxic to cells and tissues, is formed and may continue to accumulate, catalyzing the production of radical OH- substances from peroxide molecules, known as the Fenton reaction. In the final phase, this condition endangers cell function by damaging biomolecules and causing inflammation.[5]

In transfusion-dependent thalassemia, the activation and recruitment of macrophages are enhanced due to the scavenging of damaged erythrocytes and infections.[6,27] Monocyte recruitment is crucial at sites of infection or sterile injury. Notably, in the second disorder, extravasation of monocytes from the circulatory system into the tissue acknowledges these cells as precursors of macrophages and dendritic cells. They differentiate into professional antigen-presenting cells, which can resolve inflammation and facilitate wound repair at injured sites.[7]

To our knowledge, this study is the first to employ flow cytometry to characterize blood monocytes of transfusion-dependent thalassemia in correlation with hematological assessment, iron status, and vitamin D level. This study characterized activated monocytes, while the leukocyte count and monocyte percentage remained within the normal ranges. Classical monocytes (CD14++CD16-) possess phagocytic function and inflammatory effects, whereas intermediate monocytes (CD14++CD16+) have both phagocytic and anti-inflammatory effects. However, based on cellular function, the intermediate subsets produce higher level proinflammatory cytokines, such as IL-1β and TNF-α at steady state. Moreover, the intermediate monocytes expressed higher levels of HLA-DR under the same conditions.[8,9,11]

A significant negative correlation between the population of intermediate monocytes and hemoglobin levels found in this study suggests that anemia may modulate the balance of intermediate monocyte populations. Anemia persevered in

these patients, most likely resulting from cellular hypoxia exacerbated by inflammation, making blood transfusion an essential and immediate therapy.[5] Hypoxia and iron overload in transfusion-dependent thalassemia are like a double-edged sword, two clinical indicators that require clinician's attention to preserve their quality of life. Therefore, this condition may complicate the immune status of transfusion-dependent thalassemia in persistent inflammation, as previously described.[12,13] Routine blood transfusions, resulting in a higher ferritin level and accumulation of free from toxic iron coupled with hypoxia, intensify cellular damage, leading to modification of the functional monocyte subset proportion distribution. The presence of HLA-DR in the monocyte membrane enables this cell to activate an adaptive immune response.[5,6,28] A higher level of HLA-DR in intermediate monocytes most likely plays an essential role in modulating immune response. Previous studies have shown that the reduced expression of HLA-DR in monocytes indicates an imbalance in the immune response.[29,30] Finally, by integrating the association between intermediate monocytes and knowledge of HLA-DR expressed on these cells, this concept strongly suggests that the potential immunoparalysis risk in transfusion-dependent thalassemia might occur from functional monocyte modulation.

Non-classical monocytes possess immune surveillance capacity. These monocytes can migrate rapidly from the blood vasculature into the tissue following inflammation, thereby facilitating tissue repair.[8,9] Thus, ensuring the appropriate presence of nonclassical monocytes in patients with transfusion-dependent thalassemia is important. This study found a significant inverse correlation between the percentage of non-classical monocyte subsets and ferritin levels, providing a notable explanation regarding the mechanism of ongoing inflammation caused by ferritin through the modification of functional monocyte numbers, which is in line with our previous research.[20] CD14, a glycosylphosphatidylinositol (GPI) anchored predominantly expressed on monocytes, is known as the lipopolysaccharide receptor and an additional protein of toll-like receptor-4 on monocytes. This surface protein initiates various effector functions, including cytokine secretion, proliferation, co-stimulation, and phagocyte maturation.[31] This study found a positive correlation between the MFI of CD14 in non-classical monocytes and serum iron levels; hence, it supports the suggestion that in transfusion-dependent thalassemia, the migration and maturation of non-classical monocytes are predominantly influenced by iron dysregulation, which is particularly pronounced.

Following the discovery of VDR and 25(OH)vitamin D hydroxylating enzymes in monocytes, vitamin D was shown to modulate the monocyte immune response.[17] CD14 is a monocyte protein that is stimulated along with VDR activation.[18] Previous studies have revealed that Vitamin D, through VDR expression, modifies monocyte function. Grubczak et al observed a reduction in CD16-positive subsets and TNF-α secretion when exposed to 1, 25-$(OH)_2D_3$.[32] Recent findings also suggest that Vitamin D predominantly enhances the proapoptotic activity of monocytes and macrophages, exhibits antibiotic properties through autophagy, and suppresses their production of proinflammatory cytokines by promoting anti-inflammatory cytokines such as IL-10. These properties are thought to help to eradicate the immune system and prevent autoimmunity.[17,18,25,32] Concerning previous studies, insufficient vitamin D levels were found in all transfusion-dependent thalassemia patients.[14] However, no significant association was found between the vitamin D levels in this study. One possible explanation might be that vitamin D modulation works under inflammatory conditions, causing pathogen insult, as previously reported.[17,32]

Our study had several limitations, the most significant of which was the absence of control groups without thalassemia, which would have allowed for a comparison of cellular parameters. Furthermore, the absence of an additional control group comprising Transfusion-dependent Thalassemia patients without Vitamin D deficiency prevented us from assessing the specific impact of Vitamin D on modulating macrophage/monocyte responses in this population. We also did not further differentiate the thalassemia variant and its subvariant (ie, patients with normal hemoglobin (AA) plus β-thalassemia or whether they had another structural hemoglobinopathy). Finally, positive manual gating was performed by relying only on dominant surface markers located on monocytes; therefore, cells other than monocytes with the same markers could be selected.

Conclusion

Our findings indicate a disruption in the monocyte population among transfusion-dependent thalassemia patients with low vitamin D levels, attributable to anemia and iron dysregulation affecting the migration and maturation of inflammatory subsets, specifically non-classical monocytes and intermediate monocyte populations. Advancements in

molecular laboratory techniques, quality management, and improved access to treatment, such as regular blood transfusion and iron chelation, have enabled the early diagnosis and therapy of transfusion-dependent thalassemia. Therefore, novel mediation of complications, particularly in the immune response, must be introduced as early as a patient is diagnosed. Integrating the concept of crosstalk between vitamin D and monocyte immune response, there is an imperative need for further research to study the functional monocyte subsets during the inflammatory response to pathogen insult using a whole blood stimulation assay.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

This study was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran Bandung, approval number 50/UN6.KEP/EC/2018, along with approval from the Ethics Committee of Dr. Hasan Sadikin General Hospital Bandung, with approval number 50/UN6.KEP/EC/2018. Written informed consent was obtained from the parents and guardians of all participants.

Acknowledgments

We thank Ms. Fitria Utami, Dwi Febni, Emira Andini, and Anbarunik Putri Danthin, staff of laboratory immunology, Faculty of Medicine, Universitas Padjadjaran, for their fruitful collaboration in laboratory work.

Author Contributions

All authors made a significant contribution to the work reported, whether in the conception, study design, execution, acquisition of data, analysis and interpretation, or all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This study and its publication were funded by the collaborative funding of national competitive research grants, RISBINIPTEKDOK 2016 ("Riset Pembinaan IPTEK Kedokteran 2016") from the Ministry of Health, Republic of Indonesia, and Academic Leadership Grant (ALG), Universitas Padjadjaran Research Grant 2017.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Jaing TH, Chang TY, Chen SH, Lin CW, Wen YC, Chiu CC. Molecular genetics of beta-thalassemia: a narrative review. *Medicine*. 2021;100(45): e27522. doi:10.1097/MD.0000000000027522
2. Kattamis A, Forni GL, Aydinok Y, Viprakasit V. Changing patterns in the epidemiology of beta-thalassemia. *Eur J Haematol*. 2020;105(6):692–703. doi:10.1111/ejh.13512
3. Hernaningsih Y, Syafitri Y, Indrasari YN, et al. Analysis of common beta-thalassemia (β-Thalassemia) mutations in East Java, Indonesia. *Front Pediatr*. 2022;10:925599. doi:10.3389/fped.2022.925599
4. Handayani S, Choiriyah L, Supardi S, Indarwati I. Family emotional support in efforts to improve self-esteem of thalassemia patients in adolescent. *Indones J Glob Health Res*. 2022;4(4):6. doi:10.37287/ijghr.v4i4.1321
5. Coates TD. Iron overload in transfusion-dependent patients. *Hematology Am Soc Hematol Educ Program*. 2019;2019(1):337–344. doi:10.1182/hematology.2019000036
6. Cronin SJF, Woolf CJ, Weiss G, Penninger JM. The role of iron regulation in immunometabolism and immune-related disease. *Front Mol Biosci*. 2019;6:116. doi:10.3389/fmolb.2019.00116
7. Williams H, Mack C, Baraz R, et al. Monocyte differentiation and heterogeneity: inter-subset and interindividual differences. *Int J Mol Sci*. 2023;24:8757. doi:10.3390/ijms24108757
8. Sampath P, Moideen K, Ranganathan UD, Bethunaickan R. Monocyte subsets: phenotypes and function in tuberculosis infection. *Front Immunol*. 2018;9:1726. doi:10.3389/fimmu.2018.01726

9. Abeles D, McPhail MJ, Sowter D, et al. CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by CD14hi/CD16neg monocytes: expansion of CD14hi/CD16pos and contraction of CD14lo/CD16pos monocytes in acute liver failure. *Cytometry Part A*. 2012:13. doi:10.1002/cyto.a.22104
10. Winkler MS, Rissiek A, Priefler M, et al. Human leucocyte antigen (HLA-DR) gene expression is reduced in sepsis and correlates with impaired TNFalpha response: a diagnostic tool for immunosuppression. *PLoS One*. 2017;12(8):188–197. doi:10.1371/journal.pone.0182427
11. Joshi I, Carney WP, Rock EP. Utility of monocyte HLA-DR and rationale for therapeutic GM-CSF in sepsis immunoparalysis. *Front Immunol*. 2023;14:1130214. doi:10.3389/fimmu.2023.1130214
12. Piyajaroenkij T, Tantiworawit A, Khikhuntod J, et al. Alteration of monocyte subsets and their functions in thalassemia patients. *Int J Hematol*. 2023;117(2):188–197. doi:10.1007/s12185-022-03484-9
13. Kernan KF, Carcillo JA. Hyperferritinemia and inflammation. *Int Immunol*. 2017;29(9):401–409. doi:10.1093/intimm/dxx031
14. Manolopoulos PP, Lavranos G, Mamais I, Angouridis A, Giannakou K, Johnson EO. Vitamin D and bone health status in beta thalassemia patients—systematic review. *Osteoporosis Int*. 2021;23(6):401–409. doi:10.1007/s00198-021-05821-w
15. Büyükşimşek M, Başlamışl İF. The relationship between iron accumulation, vitamin D deficiency and bone mineral density in patients with thalassemia major, thalassemia intermedia and sickle cell. *Turk J Osteoporos*. 2020;26:121–126. doi:10.4274/tod.galenos.2020.85520
16. Yu U, Chen L, Wang X, et al. Evaluation of the vitamin D and biomedical statuses of young children with beta-thalassemia major at a single center in southern China. *BMC Pediatr*. 2019;19(1):375. doi:10.1186/s12887-019-1744-8
17. Ao T, Kikuta J, Ishii M. The effects of vitamin D on immune system and inflammatory diseases. *Biomolecules*. 2021;11:1624. doi:10.3390/biom11111624
18. Lin R. Crosstalk between vitamin D metabolism, VDR signalling, and innate immunity. *Biomed Res Int*. 2016;2016:1–5. doi:10.1155/2016/1375858
19. Pourgheysari B, Karimi L, Beshkar P. Alteration of T cell subtypes in beta-thalassaemia major: impact of ferritin level. *J Clin Diagn Res*. 2016;10(2):DC14–8. doi:10.7860/JCDR/2016/16094.7272
20. Ghozali M, Anggia MF, Cahyadi AI, Ghrahani R, Reniarti L, Panigoro R. Positive correlation between ferritin and activated monocyte in iron overloaded major β-thalassemia patients. *Hiroshima J Med Sci*. 2018;67(Special issue):78–82.
21. Ghozali M, Cakranita TH, Tjahjadi AI, et al. Fcγ receptor III expression and morphological maturity on neutrophil are associated with higher iron level of major beta-thalassemia. *Cell Mol Biol*. 2018;64(5):97–101. doi:10.14715/cmb/2018.64.5.16
22. Ghozali M, Panjaitan U, Cahyadi AI, et al. Increased iron in pediatric β-thalassaemia major associates with CD3+, not γδ lymphocytes. *KnE Life Sci*. 2017;2017:450–459. doi:10.18502/kls.v3i6.1154
23. Cahyadi AI, Ghozali M, Ghrahani R, et al. Hyperferritinemia correlated with activated population of natural killer cells in pediatric major β-thalassemia patients. *Glob Med Health Commun*. 2021;9(1):14–22. doi:10.29313/gmhc.v9i1.6346
24. Mukherjee R, Kanti Barman P, Kumar Thatoi P, Tripathy R, Kumar Das B, Ravindran B. Non-classical monocytes display inflammatory features: validation in sepsis and systemic lupus erythematous. *Sci Rep*. 2015;5:13886. doi:10.1038/srep13886
25. Pludowski P, Takacs I, Boyanov M, et al. Clinical practice in the prevention, diagnosis and treatment of vitamin D deficiency: a central and eastern European expert consensus statement. *Nutrients*. 2022;14(7):1483. doi:10.3390/nu14071483
26. Haschka D, Petzer V, Kocher F, et al. Classical and intermediate monocytes scavenge non-transferrin-bound iron and damaged erythrocytes. *JCI Insight*. 2017;4(8):24. doi:10.1172/jci.insight.98867
27. Gluba-Brzozka A, Franczyk B, Rysz-Gorzynska M, Rokicki R, Koziarska-Rosciszewska M, Rysz J. Pathomechanisms of immunological disturbances in beta-thalassemia. *Int J Mol Sci*. 2021;22(18):9677. doi:10.3390/ijms22189677
28. Ni S, Yuan Y, Kuang Y, Li X. Iron metabolism and immune regulation. *Front Immunol*. 2022;13. doi:10.3389/fimmu.2022.816282
29. Ahout IM, Jans J, Haroutiounian L, et al. Reduced expression of HLA-DR on monocytes during severe respiratory syncytial virus infections. *Pediatr Infect Dis J*. 2016;35(3):e89–96. doi:10.1097/INF.0000000000001007
30. Doring M, Cabanillas Stanchi KM, Haufe S, et al. Patterns of monocyte subpopulations and their surface expression of HLA-DR during adverse events after hematopoietic stem cell transplantation. *Ann Hematol*. 2015;94(5):825–836. doi:10.1007/s00277-014-2287-6
31. Ciesielska A, Matyjek M, Kwiatkowska K. TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. *Cell Mol Life Sci*. 2021;78. doi:10.1007/s00018-020-03656-y
32. Grubczak K, Lipinska D, Eljaszewicz A, et al. Vitamin D3 treatment decreases frequencies of CD16-positive and TNF-alpha-secreting monocytes in asthmatic patients. *Int Arch Allergy Immunol*. 2015;166(3):170–176. doi:10.1159/000380882