RESEARCH ARTICLE

DOI: 10.4274/tjh.galenos.2025.2025.0106
Turk J Hematol 2025;42:306-314

Evaluation of Immune Functions in Transfusion-Dependent Thalassemia Patients with Alloimmunization

Alloimmünizasyon Gelişmiş Talasemi Hastalarının İmmün Fonksiyonlarının Değerlendirilmesi

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Abstract

Objective: Regular erythrocyte suspension transfusions are still performed for most patients with beta-thalassemia major to prevent anemia. In recent years, it has been observed that patients are exposed to multiple allogeneic antigens and this leads to changes in the immune system. Understanding the immune regulators responsible for alloantibody development in thalassemia patients will provide appropriate data for the reduction and/or prevention of alloimmunization. We aimed to evaluate the association of alloimmunization and immune functions in these patients.

Materials and Methods: Fifty-four patients with thalassemia between the ages of 1 and 24 years were retrospectively analyzed. The frequency and types of alloantibodies and the immune functions and demographic characteristics that affected their formation were examined in these patients.

Results: The rate of alloantibody detection was 29.6%. There was a median interval of 13.7 years from the start of transfusions to alloantibody development. The age at initiation of regular transfusions was significantly higher in patients with alloantibody development. We found strong relationships between alloantibody development and both direct Coombs positivity and low $C4^+$ and low $CD19^+$ B-cell numbers. However, no significant difference was found between the groups in terms of serum immunoglobulin (Ig) G, IgA, IgM, and C3 levels; total lymphocyte count; or $CD3^+$, $CD4^+$, $CD8^+$, and natural killer cell counts.

Conclusion: Studies at the molecular level should be increased and research should be conducted with larger numbers of patients to clarify the immune pathogenesis of alloimmunization and determine the markers that will enable early recognition.

Keywords: Beta-thalassemia major, Alloimmunization, Alloantibody

Öz

Amaç: Düzenli eritrosit süspansiyon transfüzyonları, anemiyi önlemek için hala çoğu hastada uygulanmaktadır. Son yıllarda, hastaların çok sayıda allojenik antijene maruz kaldığı ve bunun immün sisteminde değişikliklere yol açtığı belirtilmiştir. Talasemi hastalarında alloantikor gelişiminden sorumlu immün düzenleyicilerini anlamak, alloimmünizasyon oranının azaltılması ve/veya önlenmesi için uygun veriler sağlayacaktır. Bu çalışmada, alloimmünizasyon ile immün fonksiyonları arasındaki ilişkiyi değerlendirmeyi amaçladık.

Gereç ve Yöntemler: Bir-24 yaş arasındaki 54 talasemi hastası retrospektif olarak analiz edilmiştir. Bu hastalarda alloantikorların sıklığı ve türleri ile bunların oluşumunu etkileyen immün fonksiyonları ve demografik özellikler incelenmiştir.

Bulgular: Alloantikor tespit oranı %29,6 olarak bulundu. Transfüzyon başlangıcından alloantikor gelişimine kadar geçen süre median 13,7 yıl olarak bulundu. Alloantikor gelişen hastalarda düzenli transfüzyona başlama yaşı belirgin olarak geç bulundu. Alloantikor gelişimi ile direkt Coombs pozitifliği, düşük $C4^+$ ve düşük $CD19^+$ B-hücre sayıları arasında güçlü bir ilişki bulunmuştur. Ancak, serum immünoglobulin (Ig) G, IgA, IgM ve C3 düzeyleri, toplam lenfosit sayısı, $CD3^+$, $CD4^+$, $CD8^+$ ve NK hücre sayıları açısından gruplar arasında anlamlı bir fark bulunmamıştır.

Sonuç: Alloimmünizasyonun immün patogenezini aydınlatmak ve erken tanıyı sağlayacak belirteçleri belirlemek için moleküler düzeyde çalışmaların artırılması ve daha büyük hasta grupları ile çalışmalar yapılması gerektiğine inanıyoruz.

Anahtar Sözcükler: Beta talasemi majör, Aloimmünizasyon, Aloantikor

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Received/Geliş tarihi: March 19, 2025
Accepted/Kabul tarihi: August 15, 2025
Epub: August 18, 2025

Introduction

Although allogeneic stem cell transplantation is an increasingly used treatment method in patients with beta-thalassemia major, regular erythrocyte suspension transfusions are still performed for most patients to prevent anemia. Efforts are made to prevent complications due to both anemia and recurrent transfusions, targeting the maintenance of normal growth and development. In recent years, it has been noted that patients are exposed to multiple allogeneic antigens, leading to changes in the immune system and iron accumulation with repeated transfusions. Alloimmunization resulting from the development of one or more specific erythrocyte antibodies is one of these complications. From a clinical perspective, erythrocyte alloantibodies cause hemolysis, difficulty in the cross-matching of blood, and an increased frequency of transfusions [1,2]. The factors causing alloimmunization are complex and still poorly understood. However, antigenic differences in erythrocytes between the blood donor and recipient, the recipient's immunity status, and the immunomodulating effect of repeated allogenic blood transfusions on the recipient's immune system are thought to play roles in its pathophysiology [1]. Some studies have evaluated red blood cell alloimmunization in thalassemia patients [3,4]. However, there is limited research addressing the immune functions of these alloimmunized patients.

An understanding of the immune mechanisms responsible for the development of alloantibodies in thalassemia patients will provide the necessary data for limiting and/or preventing alloimmunization. In the present study, we aimed to evaluate the association of alloimmunization and immune mechanisms in transfusion-dependent patients with thalassemia.

Materials and Methods

This study included 54 patients with beta-thalassemia major aged between 1 and 24 years who were regularly followed in the Thalassemia Unit of University of Health Sciences Türkiye, Dr. Behçet Uz Children's Training and Research Hospital between January 2010 and December 2019. Patients who had missing medical records or who were unwilling to participate in the study were excluded. The study data were obtained from the hospital's electronic database and the thalassemia unit's patient follow-up files and were retrospectively examined. The patients' demographic information (sex, age, date of start and duration of regular transfusions, transfusion frequency, and splenectomy status), iron chelator use and dosage, laboratory results, development of autoantibodies, and time from the start of regular transfusions to the development of alloantibodies were recorded.

Laboratory data included complete blood count, lymphocyte panel, and serum C3, C4, immunoglobulin (Ig) G, IgA, IgM, and ferritin levels. Ig levels were evaluated according to age groups based on the results obtained in the study by Aksu et al. [5]. Lymphocyte subgroups (CD3, CD4, CD8, CD16, CD19, CD45, CD56, and anti-HLA-DR) were studied using flow cytometry (FACS CANTO II device, Becton Dickinson, Franklin Lakes, NJ, USA). Lymphocyte subpopulation levels were evaluated according to age groups based on the results of Comans-Bitter et al. [6]. According to percentile values for age, levels below the 5th percentile were considered low, those between the 5th and 95th percentiles were considered normal, and those above the 95th percentile were considered high. In addition, the absolute values of lymphocyte subgroups were recorded.

In our hospital, blood products (with pre-storage leukocytes reduced) are supplied by the Turkish Red Crescent Society. When our patients are to be transfused, appropriate blood products are determined in our hospital's transfusion center by checking the ABO blood group, Rh factor, and subgroup compatibilities and cross-matching of the blood. Antibody screens and identification tests are carried out for every patient before each transfusion. When alloantibodies are detected, transfusions are continued with blood products that do not contain the detected antigen.

Antibody scanning and identification tests were carried out in this study with the microcolumn agglutination method to identify alloantibodies. Commercial cell sets prepared from erythrocytes obtained from O group donors with known antigenic properties were studied in gel-containing microcolumns (Across Gel, Dia Pro, Gebze, Türkiye). Donor erythrocytes used in antibody identification test panels with different antigen combinations with various cell lines were used to observe reactions occurring with antibodies against blood group RHD/RHCE, Kell, Kidd, Lewis, Lutheran, Duffy, MNS, and P erythrocyte antigens. For patients with indirect Coombs positivity, if the antigen equivalent to the identified antibody was absent, it was considered an alloantibody. For the investigation of C, c, E, e, Cw, and K antigens, specific antisera-containing microcolumns were used (Across Gel, Dia Pro). The detection of Lua and Kpa antigens was performed using monoclonal antisera (Lorne Laboratories, Reading, UK) in neutral gel microcolumns (Across Gel, Dia Pro).

This study was reviewed and approved by the Local Ethics Committee of Dr. Behçet Uz Children's Research and Training Hospital with approval number 2019/17-13, protocol number 2019/362, and date 19.12.2019.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics 25.0 for Windows (IBM Corp., Armonk, NY, USA). The results were presented as mean, standard deviation, median, absolute number, or percentile values. Categorical variables were compared with the chi-square test and the Fisher test. The Mann-Whitney U test and Kruskal-Wallis test were used to compare two and three groups, respectively, in evaluations of intergroup differences of

continuous variables. Statistical significance was set at p<0.05.

Results

Twenty-seven of the analyzed patients (50%) were male and 27 (50%) were female. The median follow-up duration was 14.6 years, ranging from a minimum of 6 months to a maximum of 23.1 years. The median age was 15.8 (1.1-24) years. The distribution of age groups is shown in Table 1. The median age at the time of starting regular transfusions was 1 year, ranging from a minimum of 4 months to a maximum of 4.5 years. The median time from the start of regular transfusions to the time of the study day was 14.3 years, ranging from 4 months to 22.4 years. Five of the 54 patients had undergone splenectomy at the ages of 3, 4, 11, 13, and 15 years. None of them developed alloantibodies during follow-up. The absolute numbers of lymphocyte subgroups were compared between patients with and without splenectomy, and we found that absolute total lymphocyte, CD8, CD19, and natural killer (NK) cell numbers were significantly higher in the splenectomized patients (p=0.047, p=0.020, p=0.013, and p=0.034, respectively). These findings are shown in Table 1.

Forty-eight (88.8%) of 54 patients were using deferasirox as an iron chelator and 6 (11.2%) were using a combination of deferasirox and deferiprone. When the patients were grouped by blood group, 20 (37%) patients belonged to blood group A, 8 (14.8%) belonged to blood group B, 21 (38.9%) belonged to blood group O, and 5 (9.3%) belonged to blood group AB.

Findings in Patients with Alloantibodies

While 38 (70.4%) patients had no alloantibodies, 16 (29.6%) patients had alloantibodies, and of the latter group, 7 (13%) patients had a single alloantibody and 9 (16.6%) patients had 2 or more antibodies. The median time from starting transfusions to alloantibody development was 13.7 years, ranging from 1 month to 22 years. Eleven (68.8%) of the 16 patients with alloantibodies were female and 5 (31.2%) were male. Although there was no statistically significant difference between the sexes regarding the presence of alloantibodies (p=0.074), the rate was numerically three times higher in female patients than male patients. The patients without alloantibodies had a median age of 14.8 (1.6-24) years, and those with alloantibodies had a median age of 16.6 (1.1-23.9) years. The distribution of the presence of alloantibodies by age group is shown in Table 2.

A total of 30 alloantibodies were detected in 16 patients. The most common alloantibody was anti-E (8 patients), followed by anti-K (5 patients). Anti-C and anti-CW (4 patients each) were the third most common alloantibodies, while the remainder were anti-e (3 patients) and anti-c, anti-Kpa, and anti-Lua (2 patients each). Of 9 patients with multiple antibodies, 4 had dual alloantibodies (anti-C and anti-Lua; anti-E and anti-K; anti-E and anti-K; anti-E and anti-C). The remaining 5 patients had triple alloantibodies (anti-C, anti-K, and anti-e; anti-E, anti-K, and anti-c; anti-c, anti-e, and anti-Lua; anti-E, anti-K, and anti-Cw; anti-C, anti-Cw, and anti-e). When the blood groups of the patients with alloantibodies were examined, 8 (50%) of 16 patients belonged to blood group O, 3 (18.8%) belonged to blood group B, and 5 (31.2%) belonged to blood group A; none belonged to blood group AB. No significant correlation was found between the ABO blood group system and the presence of alloantibodies (p=0.35).

An analysis of the relationship between the age at starting transfusions and the development of alloantibodies showed

Table 1. Differences in lymphocyte subgroups between patients with and without splenectomy.

Cell counts (/mm^3)	Total patients (n=54)	Patients with splenectomy (n=5)	Patients without splenectomy (n=49)	p
Lymphocyte count Mean±SD Median (range)	2671.53±1691.59 2165 (780-9500)	4168±1542.76 4760 (1540-5240)	2518±1562.34 2130 (780-9500)	**0.047**
CD3$^+$ count Mean±SD Median (range)	1888.37±1086 1611.5 (478-5948)	2774.8±1115.92 2878 (1044-3812)	1797.91±1053.16 1595 (478-5948)	0.063
CD4$^+$ count Mean±SD Median (range)	1037.75±567.6 908 (196-2948)	1465.8±625.8 1806 (463-1995)	994.08±549.6 901 (196-2948)	0.089
CD8$^+$ count Mean±SD Median (range)	757.96±531.82 617 (213-3307)	1150.2±463.75 959 (613-1660)	717.93±526.03 612 (213-3307)	**0.02**
CD19$^+$ count Mean±SD Median (range)	490.87±455.38 339.5 (90-2965)	863.4±341.49 986 (296-1122)	452.85±450.92 333 (90-2965)	**0.013**
NK cell count Mean±SD Median (range)	235.12±161.56 176.5 (55-753)	423±248.58 506 (165-753)	215.95±140.07 170 (55-716)	**0.034**

SD: Standard deviation; NK: natural killer.

that the 38 patients without alloantibodies started transfusions at a median age of 11.5 (4-32) months. In contrast, those with alloantibodies started transfusions at a median age of 15 (6-54) months. The age at the time of starting transfusions was significantly higher in the patients with alloimmunization (p=0.03).

An analysis of antibodies by the frequency of transfusion revealed that 5 (31.3%) of 16 patients with alloantibodies were transfused more often than every 3 weeks and 11 (68.7%) were transfused every 3 weeks. None of the patients who were transfused every 4 weeks had alloantibodies. Among 38 patients with no alloantibodies, 28 (73.7%) were transfused every 3 weeks and 10 (26.3%) every 4 weeks. An increased need for transfusion and thus the administration of more frequent transfusions were significantly more common in patients with alloantibodies (p<0.001).

Eight patients with alloantibodies had direct Coombs positivity. Five of 38 patients without alloantibodies had direct Coombs positivity; there was a significant correlation between the presence of alloantibodies and direct Coombs positivity (p=0.01). Direct Coombs positivity was 6.6 times more common in patients with alloantibodies. The patients with direct Coombs positivity plus alloantibody positivity and those with direct Coombs negativity plus alloantibody positivity were compared in terms of lymphocyte subgroups. However, the two groups did not differ significantly concerning total lymphocyte count and $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, or NK cell absolute numbers (p=0.959, p=0.798, p=1, p=0.078, p=1.05, and p=0.878, respectively). These findings are presented in Table 3.

When the relationship between serum ferritin level and the development of alloantibodies was examined, alloantibodies were present in 1 (6.3%) of 3 patients with a ferritin level below 500 µg/L, in 5 (31.3%) of 20 patients with levels of 500-1000

Table 2. Distribution of the presence of alloantibodies by age groups.

	Alloantibody status		Total
	Present	Absent	
Age range	**n (%)**	**n (%)**	**n (%)**
1-5 years old	3 (5.4%)	2 (3.6%)	5 (9%)
6-10 years old	0 (0%)	11 (20%)	11 (20%)
11-15 years old	4 (7.5%)	5 (9.5%)	9 (17%)
16-18 years old	3 (5.4%)	8 (16.6%)	11 (20%)
19-24 years old	6 (11.3%)	12 (22.7%)	18 (34%)
Total	16 (29.6%)	38 (70.4%)	54 (100%)

n: Number of patients.

Table 3. Differences in lymphocyte subgroups between patients with alloantibodies and direct Coombs positivity and negativity.

Cell counts (/mm^3)	Total patients with alloantibodies (n=16)	Patients with direct Coombs positivity (n=8)	Patients with direct Coombs negativity (n=8)	p
Lymphocyte count Mean±SD Median (range)	2583.12±2089.33 2125 (780-9500)	3017.5±2817.15 2210 (780-9500)	2148.75±993.28 2125 (970-4230)	0.959
$CD3^+$ count Mean±SD Median (range)	1832.12±1315.59 1607.5 (478-5948)	2005±1768.83 1569.5 (478-5948)	1659.25±715.36 1720 (637-3014)	0.798
$CD4^+$ count Mean±SD Median (range)	978.87±548.13 936 (196-2271)	1007.5±694.67 908 (196-2271)	950.25±399.21 998.5 (303-1642)	1
$CD8^+$ count Mean±SD Median (range)	754.25±733.71 617 (213-3307)	871.37±1014.47 617 (213-3307)	637.12±305.08 612 (266-1185)	0.878
$CD19^+$ count Mean±SD Median (range)	510.12±711.07 267 (90-2965)	742.62±954.7 368 (90-2965)	277.62±220.14 212 (93-787)	1.05
NK count Mean±SD Median (range)	179.62±106.23 147 (80-456)	193±131.72 129 (96-456)	166.25±80.15 159 (80-343)	0.878

SD: Standard deviation; NK: natural killer.

µg/L, in 6 (37.5%) of 17 patients with levels of 1000-1500 µg/L, and in 4 (25%) of 14 patients with levels above 1500 µg/L. No significant correlation was found between the development of alloantibodies and serum ferritin level (p=0.920).

Splenectomy was needed in 5 (9%) of our cases, but none of these patients developed alloantibodies during the course of the study.

All of the patients used oral iron chelators due to ease of use. No comparison was made to establish a relationship between alloimmunization and iron chelator type.

Serum C3, C4, IgG, IgA, and IgM levels and lymphocyte panel results were studied for an evaluation of immune functions. Table 4 shows differences in C3, C4, IgG, IgA, and IgM levels between groups. Serum C4 level was low in 8 (14.8%) of 54 patients; of these patients, 6 had alloantibodies. Serum C4 level was significantly lower in patients with alloantibodies (p=0.006).

Table 5 shows the differences in total lymphocyte subgroup absolute cell numbers and percentiles between groups. For patients whose lymphocyte panels were examined, the two groups did not significantly differ concerning total lymphocyte count or $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and NK cell absolute numbers (p=0.586, p=0.703, p=0.755, p=0.397, p=0.076, and p=0.11, respectively). When lymphocyte subgroup percentiles were evaluated according to age, the presence of alloantibodies was found to correlate with $CD19^+$ B-cell percentage (p=0.023).

Discussion

In this study, alloantibodies were detected in 16 (29.6%) patients. The alloimmunization rate in thalassemia patients has been reported to range between 4% and 45% in various centers [1,2,7,8,9]. The genetic homogeneity of blood donors in a given country, the age at which regular transfusions start, and the cross-matching techniques and protocols of different centers for blood group phenotyping are factors responsible for these wide variations in alloimmunization prevalence [7]. We found a median interval of 13.7 years from the start of transfusions to alloantibody development. This was in line with previous reports in the literature, with intervals ranging from 1.5 to 14 years [8]. We found alloantibody prevalence rates of 18.5% in male patients and 40.7% in female patients. Thus, alloantibodies were three times more common in female patients, but the difference between the sexes was not statistically significant. Some studies on the presence of alloantibodies in patients with thalassemia have failed to establish any correlation between sex and alloantibody development [2,9,10]. However, other studies have recently reported that alloantibodies are more prevalent in female patients [7]. Although researchers have attributed this sex-based difference to the exposure of fetal erythrocytes to antigenic stimulation during pregnancy, the fact that our patients were in the pediatric age group and had no history of pregnancy suggests that other unexplained factors may exist [7].

A total of 30 alloantibodies were detected in 16 patients, most commonly including alloantibodies developed against the Rh system (70%). Similarly, the most common antibodies identified by previous studies were reported against Rh and Kell antigens [2,7,8,9,10,11,12,13].

Thirteen of 16 patients with alloantibodies in this study were older than 10 years old. This finding suggests that the rate of alloimmunization increases with age. While some studies have reported a correlation between alloimmunization and age, some others have not [2,7,8,9,10,11,12,13]. The age at which patients started regular transfusions was significantly higher among patients with alloantibodies. While some studies have reported

Table 4. Differences in C3, C4, IgG, IgA, and IgM levels between groups.

		Presence of alloantibodies				
		Low	Normal	High	Total	p
		n (%)	n (%)	n (%)	n (%)	
C3	No	7 (18.4%)	31 (81.6%)	0 (0%)	38 (100%)	**1.00**
	Yes	3 (18.8%)	13 (29.5%)	0 (0%)	16 (100%)	
C4	No	2 (5.3%)	36 (94.7%)	0 (0%)	38 (100%)	**0.006**
	Yes	6 (37.5%)	10 (62.5%)	0 (0%)	16 (100%)	
IgG	No	2 (5.3%)	34 (89.5%)	2 (5.3%)	38 (100%)	**0.403**
	Yes	0 (0%)	16 (100%)	0 (0%)	16 (100%)	
IgA	No	0 (0%)	28 (73.7%)	10 (26.3%)	38 (100%)	**0.747**
	Yes	0 (0%)	11 (68.8%)	5 (31.2%)	16 (100%)	
IgM	No	0 (0%)	34 (89.5%)	4 (10.5%)	38 (100%)	**0.272**
	Yes	1 (6.3%)	14 (87.5%)	1 (6.3%)	16 (100%)	

n: Number of patients; Ig: immunoglobulin.

Table 5. Differences in lymphocyte subgroups between groups.

Cell counts (/mm³) and percentages (%) according to age	Total patients (n=54)	Alloantibody-positive patients (n=16)	Alloantibody-negative patients (n=38)	p
Lymphocyte count				
Mean±SD	2671.53±1691.59	2583.12±2089.33	2708.76±1408.15	0.58
Median (range)	2165 (780-9500)	2125 (780-9500)	2235 (980-7070)	
Lymphocyte percentage				
Low	2	1	1	
Normal	47	15	32	0.27
High	5	0	5	
CD3⁺ count				
Mean±SD	1888.37±1086	1832.12±1315.59	1912.05±993.45	0.70
Median (range)	1611.5 (478-5948)	1607.5 (478-5948)	1611.5 (614-5020)	
CD3⁺ percentage				
Low	3	2	1	
Normal	45	13	32	0.29
High	6	1	5	
CD4⁺ count				
Mean±SD	1037.75±567.6	978.87±548.13	1062.55±580.99	0.75
Median (range)	908 (196-2948)	936 (196-2271)	887.5 (365-2948)	
CD4⁺ percentage				
Low	4	3	1	
Normal	45	13	32	0.05
High	0	0	5	
CD8⁺ count				
Mean±SD	757.96±531.82	754.25±733.71	759.52±432.30	0.39
Median (range)	617 (213-3307)	617 (213-3307)	624 (249-2185)	
CD8⁺ percentage				
Low	0	0	0	
Normal	46	14	32	1.00
High	8	2	6	
CD19⁺ count				
Mean±SD	490.87±455.38	510.12±711.07	482.76±303.06	0.076
Median (range)	339.5 (90-2965)	267 (90-2965)	399.5 (114-1160)	
CD19⁺ percentage				
Low	5	4	1	
Normal	43	11	32	**0.023**
High	6	1	5	
NK count				
Mean±SD	235.12±161.56	179.62±106.23	258±175.85	0.11
Median (range)	176.5 (55-753)	147 (80-456)	188.5 (55-753)	
NK percentage				
Low	4	2	2	
Normal	49	14	35	0.537
High	1	0	1	

SD: Standard deviation; NK: natural killer.

a correlation between age at the time of starting transfusions and alloimmunization, some others have not [2,7,8,9,10]. It has been suggested that such findings may be due to a resistance to alloimmunization, particularly when the immune system's antibody production is still immature [2].

In this study, none of the patients who were transfused once every 4 weeks had alloantibodies. The need for transfusion and thus the administration of more frequent transfusions were significantly increased in patients with alloantibodies. This finding was in line with previous observations in the literature [1,2,8,9,10,11].

Iron overload, splenectomy, and deferoxamine use are implicated as the leading causes of immunological problems in patients with thalassemia, attributed to the toxic effect of high iron levels on lymphocyte functions [14,15,16]. The present study did not find any correlation between serum ferritin level and alloimmunization. Since no patient used deferoxamine, it could not be evaluated in this study as a potential risk factor for the

development of alloimmunization. Many studies to date have shown that splenectomy causes alloimmunization [1,2,7,8,9]. An increased prevalence of alloimmunization is a problem explained by the inability to remove antigens and damaged erythrocytes after the removal of the spleen, resulting in the development of alloantibodies and autoantibodies [1,2]. Although splenectomy was needed in 5 of our cases, none of those patients developed alloantibodies during the subsequent follow-up period. Thus, our findings indicate that splenectomy does not increase the risk of alloimmunization in thalassemia. Our results are similar to those reported by Lv et al. [17], who found that total lymphocyte counts and percentages of B lymphocytes in peripheral blood were increased after splenectomy compared to pre-splenectomy values in patients with cirrhotic portal hypertension. Although our patients were diagnosed with thalassemia, the absolute total lymphocyte, CD8, CD19, and NK cell numbers were significantly higher in patients who underwent splenectomy compared to patients who did not.

A correlation was found between the presence of alloantibodies and direct Coombs positivity. However, among patients who developed alloantibodies, no significant difference was found when patients with positive and negative direct Coombs results were compared in terms of lymphocyte subgroup numbers. We detected alloantibodies in these patients but we do not know whether there were autoantibodies in patients with direct Coombs positivity because we did not conduct elution/absorption tests. Studies investigating the pathophysiology of the coexistence of autoantibodies and alloantibodies have primarily been conducted in patients with sickle cell anemia. The proposed mechanisms suggest that alloantibodies binding to transfused erythrocytes may cause conformational changes in antigenic epitopes, which in turn may stimulate autoantibody production, particularly in splenectomized patients. Another explanation, as discussed by Ofosu et al. [18] in their study on the major histocompatibility complex in patients with sickle cell anemia, is that genetic determination may be involved in autoantibody development. The presence of autoantibodies in alloimmunized patients has a concealed effect on alloantibodies in pretransfusion tests, which may fail to detect alloantibodies [7,18,19]. The mechanism of autoimmunization in chronically transfused patients is still incompletely understood, especially in patients with thalassemia.

Patients with thalassemia have impaired distribution and function of T and B lymphocytes, impaired production of Ig, and suppression of the complement system [20,21]. While some studies on this subject have reported normal levels of Ig and complement system components in these patients, others have reported reduced levels [22,23,24,25,26]. In the present study, we did not find any significant difference between patients with and without alloimmunization regarding serum IgG, IgA, IgM, and C3 levels. However, serum C4 levels were low in 8 of 54 patients, of whom 6 had alloantibodies. Patients with alloimmunization had significantly lower C4 levels, and the likelihood of having a low C4 level was increased 10-fold by the presence of alloantibodies. Decreased levels of C3 and C4 have been reported in thalassemia patients; however, it was not specified how many of these patients were alloimmunized [3]. We did not find decreased levels of C3. The role of the complement system in the alloantibody-induced hemolysis process remains unclear. Furthermore, the role of complements in B-cell biology has generally been studied following exposure to infectious organisms and the role of complements in immune responses to alloantigens remains unclear [27]. Our study failed to demonstrate any significant difference between the two groups in terms of total lymphocyte, $CD3^+$, $CD4^+$, $CD8^+$, or NK cell counts. However, $CD19^+$ B-cell counts were lower in the alloimmunized group. This decrease was not statistically significant according to absolute B-cell count, but it was substantial according to age percentile evaluations. The role of B lymphocytes in the production of alloantibodies against transfused erythrocytes and humoral immunity is of critical importance in patients with β-thalassemia major. It has been reported that patients with thalassemia major have increased percentages of B lymphocytes compared to healthy control groups [3,28]. However, Bozdogan et al. [24] found normal $CD19^+$ B-cells in patients with thalassemia. While previous studies evaluated the percentage of B lymphocytes, they did not specify how many patients were alloimmunized. In this study, we found a decrease in the number of $CD19^+$ cells and serum C4 levels in patients with alloimmunization. Recent research suggests that neonatal fragment crystallizable receptor (FcRn) has a role in antigen presentation and binding of immune complexes; thus, FcRn is a central molecule in both homeostasis and immune responses [29]. Studies are now being conducted on the use of FcRn inhibitors in the treatment of alloimmunization [30]. The standard explanation for the B-cell results and low C4 levels obtained in the present study may be FcRn functions. However, further studies are needed to confirm this.

Study Limitations

The limited number of patients was a restricting factor in this study. In the laboratory, plasma cell markers such as CD38 could not be included in the immunophenotyping panel. In addition, the lack of elution and absorption procedures for autoantibody identification in patients with direct Coombs positivity is a shortcoming of this study.

Conclusion

This study showed that age at the time of starting regular transfusions was significantly higher in patients with alloantibodies. Furthermore, the presence of alloantibodies had a strong correlation with direct Coombs positivity, low C4

level, and CD19⁺ B-cell percentage. Alloimmunization occurs as a result of the evolution of one or more specific erythrocyte antibodies in patients undergoing repeated transfusions and it is an important complication of thalassemia treatment. From a clinical perspective, these alloantibodies cause hemolysis, difficulties in cross-matching blood, an increased frequency of transfusions, and a frequent need for immunosuppressive treatment. Therefore, it is essential to prevent the development of alloimmunization. Antibody screens and identification tests are carried out for every patient before each transfusion for this reason. When alloantibodies are detected, transfusions should be continued with blood products that do not contain the detected antigen. However, the factors causing alloimmunization to occur are complex and still poorly understood. Further studies are needed to explain them.

Ethics

Ethics Committee Approval: This study was reviewed and approved by the Local Ethics Committee of Dr. Behçet Uz Children's Research and Training Hospital with approval number 2019/17-13, protocol number 2019/362, and date 19.12.2019.

Informed Consent: Written informed consent was obtained from participants' parents for children to participate in the study.

Footnotes

Authorship Contributions

Surgical and Medical Practices: S.O.A., N.G., Y.O.; Concept: T.H.K.; Design: T.H.K.; Data Collection or Processing: N.Ö.Ö., S.O.A., Y.A., Y.O.; Analysis or Interpretation: T.H.K., N.G., F.G.; Literature Search: N.Ö.Ö.; Writing: N.Ö.Ö.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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