Low IL-2 Expressing T Cells in Thalassemia Major Patients: Is It Immune Aging

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Abstract Several studies have demonstrated T cell alteration and some features of immunosenescence in thalassemia major. Repeated alloimmunization converts naïve T-cells to memory cells and iron overload causes oxidative stress accelerating immune aging. To determine whether the alteration of T-cell cytokine is matched with early immune aging, the quantity of cytokine expressing T cells and their correlation to some immune aging markers were investigated. The proportion of IL-2- and IFN γ expressing CD4+ and CD8+ T-cells was measured in 27 hepatitis B, C and HIV negative B-thalassemia patients and a control group aged 10–30 years, following stimulation for 6 h with streptococcus enterotoxin B and intracellular cytokine staining. This proportion then were analyzed versus the percentage of the T-cells expressing each phenotyping marker, CD27, CD28, CD57 and CCR7. CD4+ and CD8+ positive T cells expressing IL-2 were significantly lower in β -thalassemia major compared to matched controls, but not T cells expressing IFN γ . No significant difference was observed between splenectomized and non-splenectomized patients in cytokine expressing T cells. A negative

correlation was noted between the percentage of T cells expressing IFN γ and T-cells expressing CD-27, but not other markers. Lower T cells expressing IL-2 may reveal the decline of naïve and central memory T cells and is likely to be a feature of early immune aging. Decreased antigenic stimulation and iron overload may help to prevent this phenomenon.

Keywords Thalassemia major · Immunosenescence · Cytokine · Intracellular · T lymphocyte

Introduction

Thalassemia major, as an inherited disorder is frequent in the Middle East including Iran and known as the most common single gene abnormality with the prevalence of up to 10% [1]. Repeated blood transfusion, RBC hemolysis and iron overload causes oxidative stress in patients leading to reduced GSH level and plasma anti-oxidants activity and increased lipid peroxidation [2–4].

The immune alterations in thalassemia major are related to both the innate and adoptive immune system. Decreased CD4+/CD8+ ratio, impaired neutrophil and macrophages phagocytosis and chemotaxis and compromised natural killer cell function have been reported [5]. Several researches have demonstrated cytokine disturbance in thalassemia major. Meanwhile some features of immune system aging have been reported in these patients. Mean value of IFN γ has been found significantly higher in thalassemia compared to control in some studies, but lower in others [6, 7].

Immunosenescence is a complex process that affects the improvement of the immune system and lymphocytes' function. This process occurs naturally in the elderly, but

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could develop earlier in young patients due to chronic infections, antigenic stimulation and neoplastic conditions [8]. Aging is associated with a number of phenotyping and functional alteration in the T cell compartment. Accumulation of T cells with memory phenotype and impairment of in vitro functional response to pathogens have been reported [9, 10]. Oxidative damage is one of the factors accelerating immunosenescence and numerous researches have confirmed the oxidative stress in thalassemia major which could lead to early immunosenescence [11]. Repeated blood transfusion resulting in immunosuppression and transmission of viruses leads to accelerated immunosenescence [12]. Splenectomy brings about some changes in lymphocytes which lead to intensifying the effect of allogeneic stimulation on the immune system [13]. In vitro alloimmunization has been resulted in conversion of naïve responder cells to central memory CD4+ cells and effector CD8+ cells [14]. CD4+ and CD8+ naïve T cells showed a fourfold and twofold to threefold reduction in very old subjects compared to young ones [15]. It is believed that type I IFN γ signaling thresholds in naïve CD4+ T cells is up-regulated after activation to respond to environmental signals, however older adults have defects in this threshold regulation [16].

To investigate whether the alterations of cytokine profile in thalassemia patients suited with early immunosenescence, the proportion of cytokine expressing T cells were compared to healthy matching volunteers and their correlation with some aging phenotyping markers were investigated.

Materials and Methods

Patients and Controls

27 patients with beta-thalassemia major referring to Hajar Hospital of Shahrekord, Iran between October 2012 and December 2013 were enrolled to the study. The mean age of patients was 17.07 ± 5.02 (range: 10–30) years and 63% of them were male. A control group of 26 healthy subjects (with mean age of 19.6 ± 6.1 years) matched by age, were recruited from local volunteers of whom 57.7% were male. Patients were 10–30 years old and had diagnostic criteria of beta thalassemia major. Ethical permission for the study was obtained from the Ethics Committee of the Shahrekord University of Medical Sciences. After informed consent was obtained, venous blood was collected in three tubes containing EDTA, sodium heparin, and no anti-coagulant.

Hepatitis B and C and HIV positivity, chelation therapy except desferrioxamine, infection within the past 3 weeks, pregnancy and taking vitamin C or folic acid within the

past 48 h were the exclusion criteria. Patients' information consisting of age, gender, the time of disease diagnosis, hemoglobin and ferritin levels, records number, splenectomy, and treatment with desferrioxamine, total number of blood transfusion, transfusion intervals, volume of transfused blood in each referring were collected. Ferritin level was measured using ELISA technique (Orgentec Kit, Germany).

Measurements of CD3+, CD4+ and CD8+ T Cell Counts

T cell counts were determined by the blood count obtained from a blood counter in combination with peripheral blood smear examination for exclusion of NRBCs and then flowcytometric analysis. The antibodies anti-CD3PE, anti-CD4FITC and anti-CD8APC were used on blood samples and the percentage of each cell population was obtained by flow cytometry. These percentage was used for calculation of the cell numbers.

Assessment of IL-2 and IFN γ Expressing CD4+ and CD8+ T-Cells

The frequency of CD4+ and CD8+ T-cells producing IL-2 or IFN γ was determined according to the previously described method [17]. Briefly, sodium-heparinized whole blood was aliquoted into 15 ml tubes. Co-stimulatory monoclonal antibody, anti-CD28 was added to the samples at 1 mg/ml final concentration. Patient samples were divided into no treatment (unstimulated) and SEB (Sigma-Aldrich, Germany) treated. The culture tubes were incubated in a humidified 5% CO₂ atmosphere at 37 °C for a total of 6 h in the presence of 10 mg/mL of brefeldin A, a cytokine secretion inhibitor, (Sigma-Aldrich), for the last 4 h of culture. EDTA (Sigma-Aldrich) was then added to a final concentration of 2 mM for 15 min at room temperature. The RBCs were lysed and leukocytes were fixed with FACS lysing solution (BD Biosciences) for 10 min at room temperature. The cells were washed in wash buffer, PBS containing 0.5% BSA (Sigma-Aldrich) and 0.1% sodium azide and then permeabilized by incubation with FACS permeabilization buffer (BD Biosciences) for 10 min at room temperature. The cells then were washed in wash buffer and stained with directly conjugated mAbs for 30 min at room temperature in the dark. After staining, the cells were washed and fixed in 1% paraformaldehyde in PBS and stored at 4 °C in the dark until flow cytometric analysis. Four-colour flow cytometric analysis was performed (PARTEC, Germany) and data was analyzed using flowmax 2.4 software. Lymphocytes were gated by FSC versus SSC, then CD4+/CD8+ versus CD3+ and displayed as dot plots of cytokine (IL-2/IFN γ) vs CD69.

Spontaneous cytokine production from unstimulated PBMC was then subtracted from SEB stimulated for actual frequency of cytokine positive T cells.

Investigation of Phenotyping Markers CD57, CD28, CD27 and CCR7

Specified number of the cells were incubated with directly conjugated monoclonal antibodies in 4 °C and darkness. Each combination included anti-CD3PE, anti-CD4APC or anti-CD8 PE-CY5 and one of the target antibodies; anti-CD27, anti-CD28, anti-CD57 and anti-CCR7 FITC. The cells were washed and fixed in paraformaldehyde after incubation time, then kept at 4 °C and darkness until flow cytometric analysis. 2×10^4 – 5×10^4 cells were counted, and lymphocytes were gated using FSC versus SSC. The CD3 + cells were then gated versus SSC. Data were analyzed using flowmax 2.4 software and displayed as dot plots of CD4 or CD8 versus other phenotyping markers. The expression of each marker was analyzed versus CD4 or CD8. The absolute number was calculated using flow cytometric data and the cell counts.

Monoclonal Antibodies

The following monoclonal antibodies were purchased from Beckton-Dickinson Immunocytometry System, USA and used in the study: Anti-CD28 (pure), anti-CD57FITC, anti-CD3PE, anti-CD8PE-Cy5, anti-CD4APC, Anti-CD4FITC, anti-CD8 APC, anti-CD69 PC5, Anti-IL-2 FITC, anti-IFN γ FITC, anti-CCR7FITC, anti-CD28FITC, anti-CD27FITC, and mouse IgG1 and IgG2a.

Statistical Analysis

The data on complete blood count, flow cytometric data, and the data obtained from other laboratory tests and clinical findings were analyzed by SPSS version 18 using Mann–Whitney and Spearman's correlation coefficient test. *P* value less than 0.05 was considered significant.

Results

Patients, General Characteristics

27 patients had inclusion criteria of the study of whom 18 were male. 12 patients including two females were splenectomized. The average patient age was 17.7 ± 5.02 at the time of study with no significant difference between males and females. Table 1 shows general characteristics

Table 1 Patient clinical characteristics

Characteristics	Mean $\pm$ SD
Sex: number (%)	
Male 18 (66.6)	
Female 9 (33.3)	
Ages (year)	17.7 ± 5.02
Transfusion times (total)	315.75 ± 121.1
Ferritin level (ng/dL)	1529.3 ± 954.75
Desferioxamine consumption (nights/week)	3.18 ± 0.31

of the patients. The transfusion intervals was 3.18 ± 0.61 weeks and deferoxamine consumption 5.16 ± 0.76 nights/week. All patients were under chelation therapy with deferoxamine. Patients' hematological values were detected at the time of sample collection and summarized in Table 2. Lymphocyte count was significantly higher in patients compared to control (7984.22 ± 620.42 compared to 2122.21 ± 543.71 , $P < 0.005$), whereas WBC count was not statistically different.

CD3+, CD4+ and CD8+ T Cells Did Not Increase in Patients with β -Thalassemia Major

The number of CD3+, CD3+ CD4+ and CD3+ CD8+ lymphocytes was firstly determined in thalassemia patients and controls. Absolute lymphocytosis was observed in the patients ($P < 0.001$) (data not shown), however, no significant difference was observed between patients and controls in the CD3+, CD4+ and CD8+ T cell counts (average of 1064.88 versus 1237, 639.0 versus 679.07, and 414.83 versus 440.60 respectively) (Fig. 1).

CD4+ and CD8+ T Cells in Thalassemia Patients Show Impaired Secretion of IL-2

IL-2 and IFN γ producing T cells were identified following SEB stimulation and intracellular cytokine detection in a panel of thalassemia patients and controls. Unstimulated sample was used as negative control in all experiments and slight cytokine secretion was observed ($< 0.09\%$ in Fig. 2). The frequency of IL-2 producing CD4+ T cells was about half in patients averaged at 4.03 (0.01–14.0)% versus 8.14 (0.82–19.05)% in controls with a significant difference ($P = 0.006$, Table 3). In CD8+ subset, the frequency of IL-2 expressing cells in patients was significantly lower than control with a mean of $2.23 \pm 2.1\%$ compared to $3.80 \pm 3.11\%$ ($P = 0.04$).

Table 2 Patient hematological values at the time of sample collection

Value	Ranges (mean $\pm$ SD)
Hemoglobin (gr/dL)	7.1–9.8 (8.4 $\pm$ 0.66)
Hematocrit (%)	22.2–32 (26.6 $\pm$ 2.01)
MCV (fL)	74.2–88.8 (82.4 $\pm$ 3.3)
MCH (pg)	23.6–28.1 (25.9 $\pm$ 1.03)
MCHC (gr/dL)	29.4–33.2 (31.5 $\pm$ 1.02)
Platelet count/mm ³	14.3 $\times$ 10 ⁴ –12.79 $\times$ 10 ⁵ (46.97 $\times$ 10 ⁴ $\pm$ 30.1 $\times$ 10 ⁴)
WBC count/mm ³	2785–46,842 (8722.5 $\pm$ 8594/10)
Lymphocyte count/mm ³	1166–42,157 (7984.2 $\pm$ 620.42)

Fig. 1 CD3+, CD4+ and CD8 positive T cell counts in controls and thalassemia patients. Comparable numbers of T cell subpopulations were observed between two groups. *NS* not significant

CD4+ and CD8+ T Cells from Thalassemia Patients and Controls Show Comparable IFN γ Response to SEB

The proportion of IFN γ producing CD4+ T cells was not significantly different between patients and controls averaged at 3.52 versus 3.70 respectively. Comparative values of IFN γ secreting CD8+ T cells were observed with the mean of 2.23 ± 2.13 and 2.84 ± 2.78 in the patients and control group respectively ($P = 0.5$) (Table 3). No significant reduction in the ratio of the frequency of IL-2/IFN γ secreting T cells was observed in thalassemia patients (Table 4).

No Significant Difference was Observed Between Splenectomized and Non-splenectomized Patients in Cytokine Secretion After SEB Stimulation

Splenectomized patients represented significant increase in the number of leukocytes and lymphocytes but decrease of T cell counts compared to control ($P < 0.001$ and < 0.05 respectively) (Fig. 3). Table 5 compared the frequency of IFN γ and IL-2 producing CD4+ and CD8+ T cells from splenectomized (no = 12) and non-splenectomized patients. CD4+ IFN γ + and CD8+ IFN γ + T cells averaged at 4.43 versus 2.79 and 2.31 versus 2.16 in splenectomized and non-splenectomized patients respectively. IL-2 expression followed the similar patterns in two groups and no significant difference was observed between them ($P > 0.05$).

The Frequency of IFN γ Secreting T Cells Negatively Correlated with CD27 Expressing T Cells

The cells stained with anti-CD-27, anti-CD28, anti-CD57 and anti-CCR7 monoclonal antibodies and the frequency of CD4+ and CD8+ T cells expressing each of the above marker was investigated. The frequency of CD4+ T cells secreting IFN γ was inversely correlated with CD3+ CD4+ CD27+ T-cells ($P = 0.03$), while there was no correlation between this cell population and the frequency of CD4+ T cells expressing CD28, CD57 and CCR7 (Table 6). The frequency of CD8+ T cells producing IFN γ + was also inversely correlated with CD3+ CD8+ CD27+ T cells ($P = 0.02$), however there was no correlation between this population and other investigated markers. No correlation was observed between the frequencies of IL-2 expressing CD4+ or CD8+ T cells expressing any of the above mentioned markers.

Fig. 2 The frequency of IFN γ and IL-2 expressing CD4 and CD8 T cells from a beta-thalassemia patient. The cells were stimulated with SEB and then stained according to the method described in materials and methods. The cells were gated on CD3 and then CD4 or CD8 cells and the proportion of the cells expressing IFN γ or IL-2 was investigated

SEB Stimulated cells

Discussion

Some diseases including thalassemia lead to more disturbances in the immune function predisposing patients to more infections. Moreover patients with thalassemia are at the risk of blood borne viruses; hepatitis B and C, HIV and rarely HTLV [18, 19]. For the immune system perturbation

in thalassemia major several fundamental concerns can be addressed: pathophysiology of the disease by itself, repeated blood transfusion, iron overload and the role of spleen [11].

Here, patients with thalassemia represented significantly more lymphocyte count compared to controls compatible with previous studies, however this increased number is

Table 3 Comparative analysis of the cytokine expressing CD4+ and CD8+ T cells between control group and the patients following SEB stimulation

Parameters	Patients Ranges (mean $\pm$ SD) (%)	Controls	<i>P</i> value
On CD4+ T cells			
IL-2	0.10–14 (4.03 $\pm$ 3.68)	0.82–19.05 (8.14 $\pm$ 5.60)	0.006
IFN γ	0.07–25.88 (3.52 $\pm$ 5.20)	0.27–15.10 (3.70 $\pm$ 3.75)	0.60
On CD8+ T cells			
IL-2	0.04–10.65 (2.23 $\pm$ 2.66)	0.4–10.79 (3.80 $\pm$ 3.11)	0.047
IFN γ	0.01–7.73 (2.23 $\pm$ 2.13)	0.08–10.16 (2.84 $\pm$ 2.78)	0.51

Table 4 Ratio of IL-2/IFN γ producing T cells

	Patients	Controls	<i>P</i> value
On CD4+ T cells			
Median	1.42	2.43	0.28
Mean	3.78	6.62	
SD	7.30	8.23	
On CD8+ T cells			
Median	0.76	2.14	0.06
Mean	4.14	3.06	
SD	9.28	3.62	

apparently related to B cells as data demonstrated close number of CD3+ cells. B-cell lymphocytosis has already been reported in other cohorts [6, 20] which could be related to repeated blood transfusion.

Some features of immunosenescence have already been noted in these patients [21, 22]. The aged immune system is not as well as the young immune system to cope with infections. Immunosenescence is accompanied with a profound decline of naïve T cells. As cytokine profile is changed along with alteration of T cell compartments, the present study focused on the IL-2 and IFN γ response. A significantly lower frequency of IL-2 producing CD4 and CD8+ T cells compared to healthy matched-controls, but

Fig. 3 Comparison of WBC, lymphocyte and T cell sub-populations between splenectomized and non-splenectomized patients. **a** Significant increase was observed in the number of WBC and lymphocytes in splenectomized compared to non-splenectomized patients

($P < 0.001$). **b** Significant decrease in the number of CD3 + T cells ($P < 0.05$) but similar CD4 and CD8 T cell counts were observed in splenectomized compared to non-splenectomized patients. *NS* not significant, *SPL* splenectomized

Table 5 Comparative analysis of the cytokine expressing CD4+ and CD8+ T cells between splenectomized and non-splenectomized patients following SEB stimulation

Parameter	Mean $\pm$ SD		<i>P</i> value
	Splenectomized (No = 12)	Non-splenectomized (N = 15)	
CD4+IFN γ + T Cells	4.43 $\pm$ 7.44	2.79 $\pm$ 2.34	0.87
CD4+IL-2+ T Cells	4.64 $\pm$ 4.69	3.86 $\pm$ 2.59	0.72
CD8+IFN γ + T Cells	2.31 $\pm$ 2.59	2.16 $\pm$ 1.77	0.69
CD8+IL-2+ T Cells	1.76 $\pm$ 1.21	2.63 $\pm$ 3.45	0.76

Table 6 Correlation between IFN γ or IL-2 expressing T cells and T cell subtypes

Type of lymphocyte	CD27	CD28	CD57	CCR7
On CD3+CD4+ T cells				
CD4+IFN γ +				
Correlation coefficient	– 0.66	0.08	0.08	0.33
P value	0.03	NS	NS	NS
CD4+IL-2+				
Correlation coefficient	– 0.60	– 0.15	– 0.23	– 0.16
P value	0.05	NS	NS	NS
On CD3+CD8+ T cells				
CD8+IFN γ +				
Correlation coefficient	– 0.68	0.06	– 0.012	0.06
P value	0.02	NS	NS	NS
CD8+IL-2+				
Correlation coefficient	– 0.44	0.08	– 0.23	– 0.06
P value	NS	NS	NS	NS

NS not significant

Bold indicates significant

comparable IFN γ response were found. IL-2 is supposed to be involved in inducing or repressing transcription factors which are fundamental in the differentiation and effector function of memory T cells. It is believed that central memory T cells produce more IL-2 and higher proliferative potential upon antigen stimulation; effector memory T cells secrete higher IFN γ , but little or no IL-2 [23]. Cytokine alterations in thalassemia have already been reported in different studies, however almost all of them detected plasma level or supernatant of PBMC. Significant reduction of IL-2 and IFN γ by PHA-activated lymphocytes from patients with thalassemia has been reported in Gharagozloo et al. study [6]. This inconsistency in the IFN γ secretion may reflect the monocyte function rather than lymphocytes as they detected the secretions of activated PBMC.

Increased plasma level of IFN γ were found in alloimmunized thalassemia and SCD patients compared to not alloimmunized group, but higher frequency of IFN γ + producing CD4+ T cells has not been detected in any of them compared to controls [24].

The various lymphocyte functional alterations could be related to the iron overload, because of their minimal detoxifying capabilities compared to monocytes or macrophages. High iron level may have more significant consequences on T cell functional activities than cell numbers. Stimulated lymphocytes from thalassemia patients with higher serum ferritin level have shown to produce significantly less IL-2 in response to PHA [6]. Moreover, Bozdogan et al. [25] have reported the increase of both CD4+CD25(bright) and CD4+CD25+FoxP3+Treg cells in beta thalassemia major. This phenomenon can

negatively impact IL-2 production as it has been noted in Trzonkowski et al. [26] study. They found that the frequency of Treg cells is inversely correlated with the production of IL-2 by autologous CD4+ T cells. As Treg cells accumulates with ageing or medical conditions, IL-2 production is impaired. IL-2 signaling is critical for the later stages of T cell response, in particular for the effector function of Antigen-specific T cells during a secondary response [23].

Al-Ofairi and colleges found a significantly reduced plasma level of IFN γ in thalassemia patients with chronic hepatitis C compared to healthy controls [27], however our patients were hepatitis B and C negative. In addition, we compared in vitro stimulated IFN γ producing CD4+ and CD8+ positive numbers. Ineffective erythropoiesis and impaired reticulocyte maturation in these patients has been found to be related to cytokine profile including the level of IFN γ as erythropoietic inhibitory mediator [28], however as the reported plasma level of IFN γ is controversial, more investigation may be required.

A significant correlation between CD27 expressing CD8+ T cells and the number of transfusion and ferritin level in thalassemia major has already been reported [22]. Here we demonstrated the correlation between the number of IFN γ and CD27 expressing T cells. Effector memory T cells have been known to lose CD27 expression and in aged volunteers the decrease of CD27 expression on T cell surface has been noted [29]. Both groups of effector memory cells (CD45RO+ and RA+) have defective expression of CD27 [30]. The loss of CD27 on the surface of IFN γ positive T cells could confirm that these cells are likely to be effector rather than central memory T cells. Meanwhile chronic contact with antigen has been reported to negatively impact CD27 expressing T lymphocytes in different studies [31, 32] and thalassemia patients are exposed to repeated antigenic stimulation.

The frequency of IL-2 and IFN γ expressing T cells were not different between two groups which is in consistent with the previous report finding similar pattern of plasma level of IL-2 and TNF between splenectomized and non-splenectomized thalassemia patients [13].

Overall, β -thalassemia major is associated with the alteration of global T-cells. This feature may impair the ability of the immune response to heterogeneous antigens, here SEB. The significantly lower IL-2 producing T-cells may reflect decline of naïve or central memory T-cells. Since these patients were hepatitis B and C and HIV negative, other chronic stimulations like blood cell antigens or iron overload can be suspected. This phenomenon may be a feature of early immunosenescence and is likely to be somehow prevented by decrease of required transfusions and consequently less iron overload using fresh blood. For more comprehensive results other variables such

as different chelation therapies, severity of the disease and younger or older cohorts should be further studied. Moreover, investigation of other features of immune aging could be beneficial.

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Compliance with Ethical Standards

Conflict of interest The authors declared no competing interests.

Ethics Committee Ethical Approval for the study was obtained from the Shahrekord University of Medical Sciences Ethics Committee. Informed consent was obtained from all individual participants included in the study.

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