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## Increased IL-17 and TGF- $\beta$ serum levels in peripheral blood of patients with $\beta$ -thalassemia major: implication for continual transfusions role in T helper 17-mediated proinflammatory responses

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**Background/aim:** Recent studies have shown that IL-17-producing CD4<sup>+</sup> T helper (Th17) cells play an important role in proinflammatory processes. In this report we analyzed IL-17, IL-21, and TGF- $\beta$  serum levels in the peripheral blood of Iranian beta-thalassemia major patients that clinically exhibited splenectomy and iron overload.

**Materials and methods:** Blood samples were collected from 43 beta-thalassemia patients and 43 healthy individuals with no history of malignancies or autoimmune disorders. Then serum levels of IL-17, IL-21, and TGF- $\beta$  were measured by enzyme linked immunosorbent assay (ELISA).

**Results:** The levels of IL-17 ( $P = 0.005$ ) and TGF- $\beta$  ( $P < 0.001$ ) were significantly higher in the thalassemia patients compared to the healthy control. No significant differences in the level of serum IL-21 was observed between the patients and controls. There were no significant differences in serum levels of IL-17, IL-21, and TGF- $\beta$  between patients with high or low serum levels of ferritin.

**Conclusion:** Multiple blood transfusions cause constant immune stimulation, as a result of repeated exposure to new alloantigens. This might have significant effects on the stimulation of cytokine producing cells in those patients and cytokine profile can be used as a related marker for assessing disease severity and consequently therapeutic intervention.

**Key words:** Beta-thalassemia, interleukin-17, interleukin-21, transforming growth factor beta

### 1. Introduction

Beta-thalassemia is a congenital hemolytic disorder caused by a partial or complete deficiency of  $\beta$ -globin chain synthesis. Patients with beta-thalassemia major suffer from severe anemia and other serious complications from early childhood. The disease is treated by chronic blood transfusion. However, this can cause severe iron overload, resulting in progressive organ failure (1,2).

Infectious complications and immune abnormalities have always been considered as causes of morbidity and mortality in  $\beta$ -thalassemia. A wide range of functional and quantitative immune alterations have been described in  $\beta$ -thalassemia patients with multiple transfusions. These abnormalities seem to be acquired and secondary to allogenic stimulation of the antibody-producing cells by continuous blood transfusions, together with iron overload (3–8).

Two decades ago Mossman and Coffman (for review see 9) proposed that CD4<sup>+</sup> T cells differentiate into two

subsets with reciprocal functions and patterns of cytokine secretion, termed T-helper 1 (Th1) and Th2 (10). This paradigm was maintained until 2005, when a third T-cell subset, known as Th17, was identified (11,12). The main feature of this subset is its release of interleukin 17 (IL-17) (13,14). The role and function of Th17 indicate that this subset of CD4<sup>+</sup> T cells plays a fundamental role in infiltration and recruitment of inflammatory cells against intercellular parasites and fungi (15) and recently in certain Th1 mediated autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (16). Several recent studies demonstrated that transforming growth factor beta (TGF- $\beta$ ) and IL-6, but not IL-23, are critical factors for murine Th17 cell differentiation in vitro (17,18).

It appears that TGF- $\beta$  plays an essential role in dictating whether CD4<sup>+</sup> T cells become Treg cells or Th17 cells. The combination of TGF- $\beta$  and IL-6 promotes the differentiation of Th17 cells and inhibits Treg cell differentiation in mice (17,18), whereas TGF- $\beta$

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plus retinoic acid inhibits Th17 cell differentiation and promotes Treg cells (19,20).

It is shown that IL-21 is another cytokine highly expressed by mouse Th17 cells. IL-21 is induced by IL-6 in activated T cells, a process that is dependent on STAT3 but not ROR- $\gamma$ . IL-21 potently induces Th17 differentiation and suppresses Foxp3 expression, which requires STAT3 and ROR- $\gamma$ , which is encoded by Rorc. IL-21 deficiency impairs the generation of Th17 cells and results in protection against experimental autoimmune diseases (18).

Apart from synthesis by Th17, IL-17 may also be produced by other cells such as CD8<sup>+</sup> T cells, NKT cells, epithelial cells, and cells from the innate immune system (for review see 21). It seems IL-17 acts as an interface between inflammatory response and cell-mediated immunity in the case of cancer and also in infectious diseases. Inflammatory reactions are considered a first line of host immune response against pathogens (22). It seems the role of IL-17 in recruiting inflammatory cells and potentiating of this event is necessary. IL-17 and IL-21 are proinflammatory cytokines whose roles in recruiting inflammatory cells and potentiating of this event should be considered.

Several factors may be significant in triggering Th17 and consequently the synthesis and release of IL-17 such as TGF- $\beta$ , IL-6, prostaglandin E2, IL 21, IL-23, IL-1 $\beta$ , and TNF- $\alpha$  play major roles in the induction of Th17 differentiation (23–25).

Despite the known roles of IL-17 in enhancing inflammatory reactions, reports on the role and involvement of iron overload in Th17 response do not exist. On the other hand, it is shown that iron directs the immune response toward a Th2 response pattern, which is unfavorable for fighting a bacterial or viral infection (3). Gharagozloo et al. found low production of IL-2 and IFN- $\gamma$  by PHA-stimulated peripheral blood mononuclear cells from thalassemia patients with serum ferritin levels higher than 4500 ng/mL (26). Other research has found that thalassemia patients have higher serum levels of mediators of inflammation, such as IL-6, IL-18, IL-1, and TNF- $\alpha$  (27–30).

Therefore, we aimed to study the relation of repeated blood transfusions and high serum ferritin levels on the serum levels of IL-17, IL-21, and TGF- $\beta$  on Th17 cells in the blood of thalassemia major patients in Iran. We also compared the results according to the patients' clinical or pathological status.

## 2. Materials and methods

Forty-three  $\beta$ -thalassemia major patients (17 males and 26 females) of mean age  $15.8 \pm 5.9$  years (range 6–23) with serum ferritin levels 460 to 7200 ng/mL over the previous

6 months (34 children with ferritin <4500 and 9 patients with >4500 ng/mL) participated in this study, of which only eleven were splenectomized (seven males and four females). The patients were referred to the immunology laboratory from the hospitals of Jahrom University of Medical Sciences. The patients were regularly transfused with packed red cells every 3–4 weeks to maintain mean hemoglobin levels above 9.8 g/dL, and were receiving regular iron chelating therapy (deferoxamine 40 mg/kg daily) without ascorbic acid that was similar between all patients. The mean dose of deferoxamine was 40–50 mg/kg per infusion over 8–12 h, 5–6 days per week in all patients. All experiments were performed (once for each patient) before transfusion. Patients were excluded from this study if they had one of the following conditions: poor deferoxamine compliance (those with less than 80% compliance), chronic liver disease such as hepatitis B or C infection, a history of a positive HIV test, chronic renal or heart failure, iron chelation therapy with deferiprone, pregnancy, and infection 2–3 weeks before. Meanwhile, five and two patients were excluded from study because of colds and some technical errors, respectively. Data on age, sex, white blood cell count, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count, splenectomy performance, and deferoxamine treatment were obtained from the hospital records for patients and controls. The control group included 43 subjects matched for age and sex (20 males and 23 females) selected randomly from healthy individuals undergoing a check-up and routine complete blood cell count. The controls were of mean age  $10 \pm 5.8$  years; range 3–20 years. During sample collection, it was ensured that subjects had neither infection nor any acute or chronic disease. Sample characteristics and hematological findings of the  $\beta$ -thalassemia major patients and healthy controls are summarized in Table 1. During the data analyses, six from the 49 samples were excluded. All subjects provided informed consent to participate in the study and to allow their biological samples to be analyzed. Approval for the study was given by the Ethics Committee of the Shiraz University of Medical Sciences (Jahrom, Iran).

### 2.1. Enzyme linked immunosorbent assay (ELISA)

The amounts of IL-17, TGF- $\beta$ , and IL-21 in the patients' and controls' sera were measured at the same time by the same technician, using ELISA kits (eBiosciences, San Diego, CA, USA). Briefly, premixed standards were reconstituted in PBS (pH 7.2), generating a stock concentration of 500, 1000, and 4000 pg/mL for IL-17, TGF- $\beta$ , and IL-21, respectively. Sensitivity for IL-17 was 4 pg/mL and minimal cross-reactivity IL-17 to the recombinant human IL-17AF heterodimer was observed at 0.4%. Sensitivity for

**Table 1.** Descriptive statistics of general characteristics and hematological findings for  $\beta$ -thalassemia major patients and controls.

Characteristics and hematological findings	Patients (n = 43)	Controls (n = 43)	P
Age (years)	15.8 $\pm$ 5.9	10 $\pm$ 5.8	-
Male	17 (39.5%)	28 (65.1%)	0.5
Female	26 (60.5%)	15 (34.9%)	-
White blood count $\times 10^9/L$	9.5 $\pm$ 6.3	9.1 $\pm$ 3.3	0.19
Red blood cell count $\times 10^{12}/L$	3.6 $\pm$ 0.5	4.6 $\pm$ 0.6	<0.0001
Hemoglobin (g/L)	9.8 $\pm$ 1.0	12.8 $\pm$ 1.7	<0.0001
Hematocrit (%)	29.3 $\pm$ 5.2	37.4 $\pm$ 5.0	<0.0001
Mean corpuscular volume (fL)	79.9 $\pm$ 9.7	80.7 $\pm$ 8.5	0.23
Mean corpuscular hemoglobin (pg/cell)	26.5 $\pm$ 2.3	27.9 $\pm$ 3.4	0.0003
Mean corpuscular hemoglobin concentration (27–33.3 g/dL)	32.9 $\pm$ 1.5	34.0 $\pm$ 2.5	0.004
Platelet count ( $150\text{--}400 \times 10^9/L$ )	361.6 $\pm$ 199.5	236.4 $\pm$ 115.5	<0.0001
Splenectomy (%)	28.9	-	-
Deferoxamine treatment (week/ night)	3 $\pm$ 1	-	-
Deferoxamine dosage (mg/kg)	40–50	-	-

both IL-21 and TGF- $\beta$  was 8 pg/mL. The standard stocks were serially diluted in Reagent Diluent to generate 7 points for the standard curves. Diluted Capture Antibody was added to a 96-well, flat-bottomed, polystyrene microtiter plate, at final volume of 100  $\mu$ L. The plates were sealed and incubated overnight at room temperature, then washed with Wash Buffer. Premixed standards or samples (100  $\mu$ L) were added to each well, which covered with an adhesive strip and incubated for overnight at 4  $^{\circ}$ C. After incubation and washing, 100  $\mu$ L of the premixed Detection Antibody was added to each well and the plate was covered with a new adhesive strip and incubated for 2 h at room temperature. After incubation and washing, Streptavidin-HRP was added to each well (100  $\mu$ L). The incubation was terminated after 20 min at room temperature and the plates were kept away from direct light. Then 50  $\mu$ L of Stop Solution was added to each well, and the optical density of each well was immediately determined using a microplate reader set to 450 nm. The results were expressed in pg/mL.

## 2.2. Statistical analysis

The serum levels of IL-17, IL-21, and TGF- $\beta$  in the peripheral blood were compared to the corresponding values from control samples using nonparametric Mann-Whitney tests by SPSS v. 11.5 (SPSS, Chicago, IL, USA). Finally, correlations between different cell populations were evaluated using Spearman correlation coefficients. The variable levels were evaluated by means of Prism

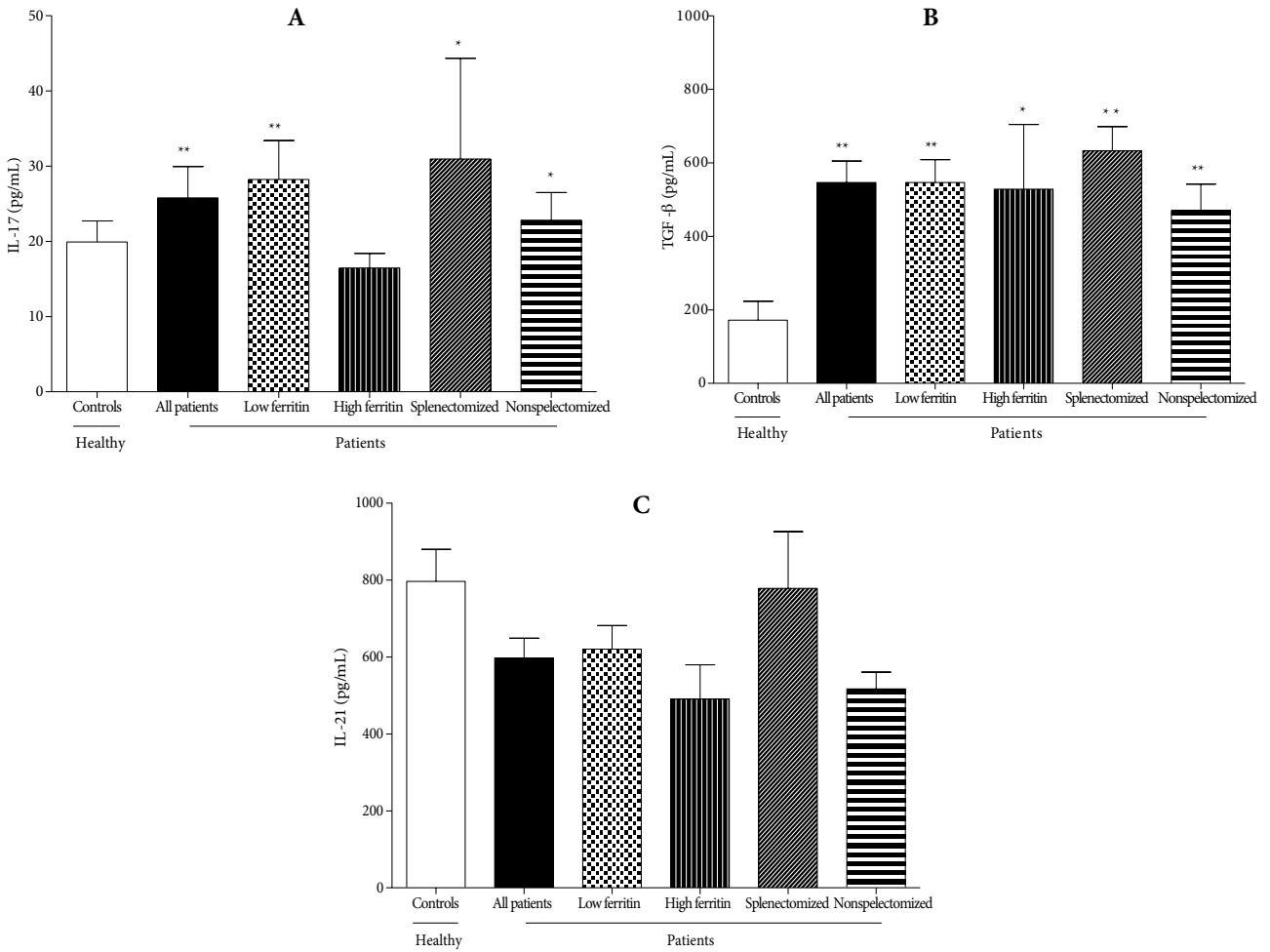
4 software (San Diego CA, USA, 2003).  $P < 0.05$  was regarded as significant in all statistical analyses.

## 3. Results

### 3.1. Cytokine assay in patient and healthy groups

Serum levels of IL-17 among patients were significantly increased compared to the controls (25.76  $\pm$  4.15 vs. 19.91  $\pm$  2.79 pg/mL;  $P = 0.005$ ) (Figure 1A). We noted significant differences in the serum levels of IL-17 in peripheral blood of patients with low ferritin (less than 4500 ng/mL) and splenectomized and nonsplenectomized patients compared to the healthy controls (28.21  $\pm$  5.17, 30.94  $\pm$  13.38, and 22.79  $\pm$  3.69 vs. 19.91  $\pm$  2.79 pg/mL;  $P = 0.004$ , 0.04, and 0.01, respectively). However, there was no significant difference in the serum level of IL-17 between the healthy controls compared to patients with high ferritin (higher than 4500 ng/mL) ( $P > 0.05$ ). In addition, in patients none of the hematological findings showed significant difference in IL-17 serum level (Table 2). The levels of IL-17 in splenectomized and nonsplenectomized patients, and between patients with low ferritin and high ferritin were similar ( $P > 0.05$ ). These results are summarized in Table 3.

Comparison of the findings shows significantly higher levels of TGF- $\beta$  in all patients, patients with low ferritin and high ferritin, and splenectomized and nonsplenectomized



**Figure 1.** Serum level of IL-17, TGF-β, and IL-21 in the peripheral blood of patients with thalassemia patients and normal controls. (A) Significant differences were found in the serum levels of IL-17 in the peripheral blood of all patients, patients with low ferritin, splenectomy, and nonsplenectomy compared to the healthy controls. (B) TGF-β serum levels were significantly higher in the peripheral blood of all patients, patients with low ferritin, high ferritin, splenectomy, and nonsplenectomy compared to the healthy controls. (C) No significant difference was found in the serum levels of IL-21 among patients compared to the healthy controls. Presented data were analyzed with the nonparametric two-tailed Mann–Whitney test and the horizontal lines show the median of the groups. \*P < 0.05 and \*\*P < 0.01.

**Table 2.** Correlations between ferritin, Hb, and HCT with IL-17, TGF-β, and IL-21 serum levels in patients with thalassemia.

		IL-17	TGF-β	IL-21
Ferritin	<i>r</i>	-0.08	-0.48	-0.22
	P	0.59	0.76	0.14
Hb	<i>r</i>	0.10	-0.28	-0.13
	P	0.52	0.06	0.40
HCT	<i>r</i>	-0.13	-0.19	-0.24
	P	0.93	0.21	0.12

Hb; Hemoglobin, HCT; Hematocrit

patients than in healthy volunteers ( $546.35 \pm 58.27$ ,  $546.15 \pm 62.39$ ,  $527.96 \pm 176.26$ ,  $632.74 \pm 65.30$ , and  $469.67 \pm 72.15$  vs.  $171.58 \pm 51.68$  pg/mL;  $P = 0.0002$ ,  $0.001$ ,  $0.01$ ,  $0.005$ , and  $0.007$ , respectively) (Figure 1B). However, there is no significant difference in the serum levels of TGF-β in patients with low ferritin compared to those with high ferritin, or between splenectomized and nonsplenectomized patients ( $P > 0.05$ ) (Table 3). We noted no correlation between TGF-β serum level with ferritin, Hb, or HCT in patients ( $r = -0.48$ ,  $-0.28$ , and  $-0.19$ , respectively;  $P > 0.05$ ) (Table 2).

Serum levels of IL-21 in patients did not differ compared to the control group ( $597.24 \pm 51.37$  vs.  $796.18 \pm 83.71$  pg/mL;  $P = 0.2$ ). Moreover, among the patients with low ferritin and high ferritin, and splenectomized

**Table 3.** The serum concentrations of IL-17, TGF- $\beta$ , and IL-21 in patients with thalassemia in low ferritin, high ferritin, splenectomy, and nonsplenectomy groups. Presented data were analyzed with the nonparametric two-tailed Mann-Whitney test.

	Low ferritin <sup>b</sup>		High ferritin <sup>c</sup>	
	Splenectomy	Nonsplenectomy	Splenectomy	Nonsplenectomy
IL-17 <sup>a</sup>				
Mean $\pm$ SEM	44.19 $\pm$ 15.97	21.63 $\pm$ 2.51	_ <sup>d</sup>	31.86 $\pm$ 14.95
Median	27.05	17.25	_ <sup>d</sup>	13.28
P	n.s.		n.s.	
TGF- $\beta$ <sup>a</sup>				
Mean $\pm$ SEM	490.40 $\pm$ 106.72	593.97 $\pm$ 88.22	_ <sup>d</sup>	494.38 $\pm$ 108.78
Median	671.34	653.88	_ <sup>d</sup>	616.64
P	n.s.		n.s.	
IL-21 <sup>a</sup>				
Mean $\pm$ SEM	565.65 $\pm$ 87.70	585.86 $\pm$ 49.45	_ <sup>d</sup>	639.52 $\pm$ 172.78
Median	490.34	540.55	_ <sup>d</sup>	465.24
P	n.s.		n.s.	

<sup>a</sup> pg/mL, <sup>b</sup> <4500 ng/mL, <sup>c</sup> >4500 ng/mL, <sup>d</sup> there was no value, n.s.: Nonsignificant

and nonsplenectomized patients, the serum levels of IL-21 were not different compared to those of the controls (620.42  $\pm$  61.41, 490.34  $\pm$  89.10, 778.20  $\pm$  147.40, and 517.04  $\pm$  43.26 vs. 796.18  $\pm$  83.71 pg/mL;  $P > 0.05$ ) (Figure 1C). In addition, there was no significant difference in the serum levels of IL-21 in patients with low ferritin compared to those with high ferritin, or between splenectomized with nonsplenectomized patients ( $P > 0.05$ ) (Table 3). Similar to previous cytokines, IL-21 levels in patients did not correlate with level of ferritin, Hb, or HCT ( $r = -0.22, 0.13,$  and  $-0.24,$  respectively;  $P > 0.05$ ) (Table 2).

#### 4. Discussion

In this study, serum levels of IL-17, IL-21, and TGF- $\beta$  in the peripheral blood of patients with  $\beta$ -thalassemia were analyzed. The data indicated higher levels of IL-17 and TGF- $\beta$ , but not IL-21, in the patients compared to the controls, suggesting an inflammatory status associated with the suppression of T cell immune response. This is in agreement with Balouchi et al's study showing increased TGF- $\beta$  and IL-17 in thalassemia patients, which means that T cells show a stimulated phenotype, while their activity has been suppressed (31).

The effects of splenectomy, iron overload, and hematologic factors on the immune response of  $\beta$ -thalassemia major patients were also investigated. In this study, no correlation was observed between ferritin

and the cytokine levels in thalassemia patients, whereas increased IL-17 serum levels in patients with low ferritin, splenectomy, and nonsplenectomy were found. In addition, it was shown that TGF- $\beta$  serum levels in splenectomized and nonsplenectomized, and low ferritin and high ferritin patients were higher than in the healthy controls. Weiss et al. reported that iron directs the immune response toward a Th2 response, which is unfavorable for fighting bacterial or viral infections (3). Gharagozloo et al. also demonstrated a significant decreasing in IL-2, IFN- $\gamma$ , and IL-4 production by activated lymphocytes from patients with  $\beta$ -thalassemia compared to the normal group (26). However, Salasa and Zoumbos showed that stimulated blood mononuclear cells from thalassemia patients produced more IFN- $\gamma$  than their control group. This might be due to infections in  $\beta$ -thalassemia patients (32).

In the present study, serum ferritin levels in ten patients were higher than 4500 ng/mL and those of the rest were less than 4500 ng/mL. Although the mean serum levels of IL-17, IL-21, and TGF- $\beta$  were not different between ferritin subgroups, high serum levels of IL-17 in patients with serum ferritin levels less than 4500 ng/mL compared to those with levels higher than 4500 ng/mL rather than healthy controls were found. This is somewhat in keeping with the findings of Gharagozloo's study, which reported low production of IL-2 and IFN- $\gamma$  in patients with serum ferritin levels higher than 4500 ng/mL in response to PHA

stimulation (26). Accordingly, iron overload may suppress Th1 immunity in thalassemia patients with increased serum ferritin levels (3,33,34).

Cytokine serum levels between splenectomized and nonsplenectomized patients were similar in our study. However, other studies reported that patients with splenectomy have marked absolute lymphocytosis. This suggests that the spleen could play some part in the regulation of lymphocyte counts and act as a reservoir for lymphocytes produced in the body but it could not affect cytokine levels (26).

The effect of Th17 should be considered within the context of Treg function, as the two cell subsets of the immune response have evolved to fine-tune immune suppression versus immune potentiation. In this scenario, our finding of increased expression of IL-17 as proinflammatory cytokine in peripheral blood from a group of patients that contained many children without splenectomy can be interpreted as a reflection of a proinflammatory response. Furthermore, higher levels of TGF- $\beta$  in either the current study or other studies (31,35) in thalassemia patients compared to the control group show TGF- $\beta$  is mainly produced by Treg, which mediates immune suppression to limit immunopathogenesis associated with chronic inflammation and persistent infections. Therefore, increased production of IL-17 and TGF- $\beta$  might contribute to abnormalities in iron metabolism and it is probably due to overstimulation of

Th17. In fact, iron deposition in the reticuloendothelial system such as macrophages and epithelial cells may influence the regulation of Th17 responses in thalassemia patients and result in higher levels of its cytokines in the circulation. On the other hand, multiple blood transfusions may cause the immune system in  $\beta$ -thalassemia patients to be under constant alloantigen stimulation, despite the suppressed immune responses due to iron overload (36).

Taken together, it seems T lymphocytes are activated in multitransfused  $\beta$ -thalassemia major patients, though T-cell suppression due to TGF- $\beta$  is seen too. These observations may have implications for the associations between repeated immune activation and premature aging of the immune system that result in exhaustion of immune resources (37). On the other hand, blood transfusion and chronic immune activation might induce Treg cells, which suppress T-cell effector functions. Finally, this cytokine profile clinically can be used as a related marker for assessing disease severity and an indicator in following the disease and consequently therapeutic intervention.

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