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Malfunctioning T-regulatory cells and Th_1/Th_2 associated cytokines' disturbances in Iraqi patients with chronic immune thrombocytopenic purpura

Yusur F. Faraj*, Majed M. Mahmood and Zeyad A. Shabeeb

Dep. of Biology, College of Science, Al-Musatnsirihya University, Baghdad, Iraq.
*Corresponding author: m12_moon@yahoo.com

Abstract

Immune thrombocytopenic purpura (ITP) is an autoimmune bleeding disorder characterized by thrombocytopenia with platelet counts less than $100x10^9$/L due to platelet destruction and/or suppression of platelet production. Th_1/Th_2 cytokine secretion profile were measured using enzyme-linked immunosorbent assay technique (ELISA) and the frequency of regulatory T cells (CD4+CD25+CD127-) was investigated by flowcytometry (FCM) in a sample of (50) Iraqi ITP patients (15 newly diagnosed and 35 on-treatment) in the National Center of Hematology, Al Mustansiriyah University, Baghdad and Oncology Teaching Hospital, Medical City,Baghdad during the period from July 2015 to February 2016. In addition, 20 apparently healthy subjects were included in the study as a control group. Although there was a significant reduction in Tregs number in ITP patients compared with the control individuals ($p<0.001$), the effect of treatment has shown a restored count of Tregs in comparison to the newly diagnosed ones ($p=0.002$).Assessment of cytokine serum levels revealed that IL-10 have a significant ($p<0.001$) lower levels in ITP patients compared to controls. Although the levels of this cytokine in the on-treatment group of patients (IL-10=4.43±0.42 pg/ml) was significantly increased in comparison to the non-treated group of patients (IL-10= 2.39±0.25 pg/ml, $p=0.001$), it was still significantly less than the healthy individuals. In contrast, the level of IL-18 was elevated in the sera of ITP patients compared to the controls (220.42 ± 14.5 pg/µl Vs. 123.82 ± 3.0 pg/µl, $P<0.0001$). Those who were on treatment showed a significant reduction in IL-18 levels (192.10±14.6 pg/µl) compared to the newly diagnosed patients (290.43±28.0 pg/µl), though, there is still a significant difference with the healthy control group. A strong negative correlation was reported between the level of IL-18 and the patients' platelet counts in both patients' groups (non-treated patents: $p=0.01$, $r=-0.75$) and (the on treatment patients: $p=<0.001$, $r=-0.67$).The present study showed that the derangement of cellular immunity is important in the pathogenesis of adult ITP which support the concept that chronic adult ITP is the manifestation of a Th_1 polarized immune response.

Keywords: Immune thrombocytopenic purpura, T-regulatory cells, Th_1/Th_2, Iraqi patients.

Introduction

ITP is an immune mediated bleeding disorder, in which platelets are opsonized by autoantibodies directed against platelet surface membrane glycoproteins and prematurely cleared by Fc-receptors on the surface of macrophages in the reticuloendothelial system. It characterized by a diminished peripheral platelet counts ($<100\times10^9$/L) due to destruction and/or reduction its production in the bone marrow (Liu *et al.,* 2011; Kuwana, 2014). Although clinical presentation of ITP may be insidious with mild or no symptoms, clinical features of ITP include skin petechiae and bleeding in the mucous membranes or internal organs are easily manifested if the platelet count falls below (50×10^9/L) and counts below (20×10^9/L) increase the risk of spontaneous bleeding. The diagnosis of ITP depends on clinical characteristics and the laboratory examinations conducted, as well as the ability of excluding other agents associated with thrombocytopenia (Keith, 2011; Warrier and Chauhan, 2012; Kayal *et al*., 2014). Increasing evidence suggests that the mechanism is multifactorial. Despite the presence or absence of autoantibodies, T cell abnormalities, and particularly those related to pro-inflammatory cytokines, are suspected to be central in the pathogenesis of ITP (Semple and Provan, 2012). In addition, it has been found that the loss of tolerance resulting from a decreased number and defective function of regulatory T cells (Tregs) play an important role in the progression of the disease

because dysregulation in the expression of IL-2, IL-18 and IFN-γ oriented T helper toward (Th_1). The high level expression of the CD4 and CD25 surface markers of Tregs would suppress proliferation of many immune cell types including T and B cells, either directly through cell contact or indirectly through secretion of cytokines, thereby dampening inappropriate immuneactivation and autoreactivity (Sakaguchi *et al.,* 2010). Meanwhile, IL-10 as an immunoregolatory cytokine is required in combination with different co-stimulatory molecules for the differentiation and maintenance of Treg cells and this could be relevant to the clinical diversity of ITP and it might predict long-term outcome in ITP patients (Panitsas *et al.,* 2004). Because the expression of IL-18 has been correlated with several autoimmune and inflammatory disorders, its serum concentration in ITP patients is up regulated. As a result, the elevated levels of IL-18 might enhance Th_1 immune responses, including excessive IFN-γ and TNF-α production leading to exacerbation of ITP(Shan *et al.,* 2008). Therefore, the first line of treatment of this disease is by administration of high-dose dexamethasone to down regulate IL-18 expression (Shan *et al.,* 2009b), but those who do not respond to glucocorticoid treatment or who continue to require glucocorticoids to sustain a safe platelet count would be treated by means of splenectomy as a second line of treatment (Cines and Blanchette, 2002; British Society for Haematology, 2003). In order to evaluate the role of immune cells and their cytokines in the pathogenesis of ITP, this study was designed to investigate the level of CD4+, CD25+, and CD127⁻ profile markers to determine the percentage of regulatory T-lymphocyte (Treg) by using FCM technique. Moreover, to detect the interleukin-18 and interleukin-10 to determine the level of Th_1 and Th_2 cells respectively, by using ELISA assay.

Materials and Methods

Control-base study has been designed upon Iraqi patients with ITP in the National Center of Hematology, Al-Mustansiriyah University, Baghdad and Oncology Teaching Hospital-Medical city, Baghdad during the period from July 2015 to February 2016. Subjects involved in this study include 50 patients (39 females and 11 males) at age range 15-70. 35 of the patients were diagnosed as Chronic ITP by specialists, with a history of disease from few months to several years, and 15 of them were newly diagnosed. However, 20 normal subjects with gender and age range corresponding to that of patients were involved and considered as a control group.

Parameters of study: The current study investigate complete blood picture and blood film, Immnophenotyping profile of Treg cells (CD4+CD25+CD127-) by (FCM)technique as well as serum levels of IL-10 and IL-18 by means of ELISA.

Sample Collection: Blood samples were collected from all subjects (healthy controls and patients).About 5-10 ml of blood were aspirated by using peripheral vein punctures and divided into 2 aliquots; the first one is transferred into EDTA tube for direct examination of CD markers, complete blood picture and blood film. The second was dispensed in a non-heparinized plain tube and left for 15 minutes at 4^0C to clot. Then, it was centrifuged at 3000 rpm for 10 minutes to collect serum which stored in -80^0C until be used for determination of Cytokines.

Immunophenotyping: In this study, Immuno-phenotyping CD4+, CD25+ and CD127- expression were investigated by using fully equipped desktop four –color (Ibrahim *et al*., 2001) Flow Cytometry (FCM) CyFlow Cube features a modular optical concept. This allows using different lasers as light sources. The CyFlow Cube allows easy optimization of the optics for any application by simple exchange of optical filters and mirrors.

Antibody labeling: One hundred µl of whole blood or isolated leukocytes was mixed with 10µl of conjugated antibodies in a test tube, mixed thoroughly then, incubated for 15min in the dark at room temperature.

Leukocytes fixation: From reagent A, 100µl were mixed and incubated for 10min in the dark at room temperature.

Erythrocyte lysis: From reagent B, 2.5ml were added and shaked gently and incubated for 20min in dark at room temperature.

The sample then analyzed by Flow Cytometer.

Calculation of results: Data acquisition, instrument control, and data analysis are controlled and performed by the CyView software.

Assessment of cytokine serum levels:

Estimation of Interleukin 18 level: It was performed according to IL-18 ELISA technique (MyBioSource, USA, product kit code:# MBS824968).

Estimation of IL-10 level: It was performed according to IL-10 ELISA technique(Kombiotech, Korea, product kit code: K0331123).

Results and Discussion

Three immunological parameters were investigated in all healthy and patient subjects; the immunophynotypic profile of Treg cells, IL-10 and IL-18. Changes in concentration of these parameters in different settings and their

significance were recorded and shown in (Table 1).

To reveal whether or not inefficient production of Tregs contributes to loss of tolerance among patients with chronic ITP, immunophenotyping profile represented by circulating CD4+CD25+CD127- cells were investigated, and the results came to state that the frequency of Tregswere diminished significantly in ITP patients compared to their counterparts of control group (0.92 ± 0.1 vs. 4.80 ± 0.3 %, p<0.001). Blood samples obtained from the on-treatment patients have shown a significant higher Treg percentages (1.24±0.2 %) in comparison to the non-treated levels (0.23±0.1 %, p=0.002)(Figure 1).

The correlation between Tregs and the platelet counts was examined and the result showed a positive non-significant relationship in the control (P=0.13, r= +0.35) and patient group (P=0.58, r=+0.08) (Figure 2).

The serum level of two cytokines (IL-10 and IL-18) were investigated in subjects of all groups to evaluate the role of imbalance Th_1/Th_2 ratio in the pathogenesis of ITP.

The presented results indicated that IL-10 levels(3.80 ± 0.3 vs. 9.16 ± 0.3)pg/ml were significantly decreased in ITP patients compared to the control values (P< 0.001) which could not be achieved even in treated patients (4.43±0.4pg/ml), in spite of the significant increase (p=0.001) in their levels in comparison to the non-treated patients (2.39±0.3 pg/ml) as shown in (Figure 3).

The results revealed a positive significant association in the concentration of IL-10 and platelet counts in ITP patients (p=0.01, r=0.34), while this relation was non-significant in the normal control group (p=0.82, r=0.054) as shown in (Figure 4).

In the current study, in contrast to IL-10, the level of IL-18 was elevated in the sera of ITP patients compared to the controls (220.42 ± 14.5 pg/µl Vs. 123.82 ± 3.0 pg/µl, P<0.001).

To determine whether IL-18 level is affected by the treatment with dexamethasone, its level was compared between a group of newly diagnosed ITP patients (before they went on treatment)and the second group who had been already on treatment as shown in (Figure 5). The on treatment group showed a significant reduction in IL-18 levels (192.10±14.6pg/µl) compared with the newly diagnosed patients (290.43±28.0pg/µl),though, there is still a significant difference with the healthy control group.

The present study showed that the increased levels of IL-18 are often associated with the disease activity. In other word, the level of IL-18 is negatively correlated with the platelet counts in peripheral blood (Figure 6) demonstrated the significant negative correlation between the concentration of IL-18 and the patients' platelet counts in both patient's groups (non-treated patents: p=0.01, r=-0.75) and (the on treatment patients: p=<0.001, r=-0.67). It is worthy to be mentioned that this strong negative relations in patient's groups was significant positive in healthy subjects (p=0.01, r=0.57).

Table(1): Evaluation of immunological parameters (Tregs, IL-10 and IL-18) in all patient groups and controls.

Parameters	G_0 Controls (n=20)	G_1 Non-treated p. (n=15)	G_2 p. on treatment (n=35)	G_3 Total p. (n=50)	Significance
Tregs (%) M ± SE	4.80±0.3	0.23±0.1	1.24±0.2	0.92 ± 0.1	$G_{0.\,vs}\ G_{3.}$ (S) G_1. vs G_2 (S) G_1. vs G_3.(S) G_2. vs G_3.(S)
IL-10 (pg/µl) M ± SE	9.16±0.3	2.39±0.3	4.43±0.4	3.80 ± 0.3	$G_{0.\,vs}\ G_{3.}$ (S) G_1. vs G_2 (S) G_1. vs G_3.(S) G_2. vs G_3.(S)
IL-18 (pg/µl) M ± SE	123.82±3.0	290.43±28.0	192.10±14.6	220.42 ± 14.5	$G_{0.\,vs}\ G_{3.}$ (S) G_1. vs G_2 (S) G_1. vs G_3.(S) G_2. vs G_3.(S)

(S) = significant differences; (NS) = non-significant differences
(p)= patients (G)= group
(M)= mean (SE)= standard error

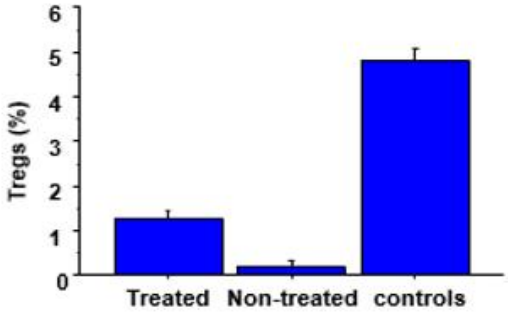
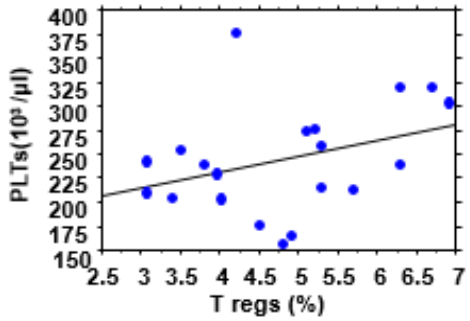


Figure (1): Percentages of Treg in treated and non-treated groups of patient compared to healthy control group.

A



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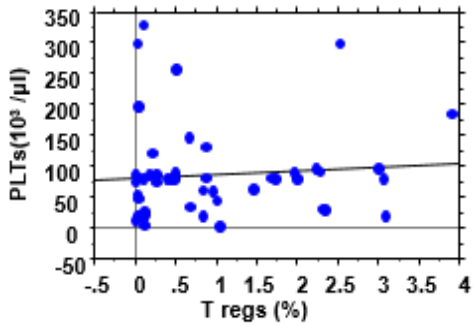
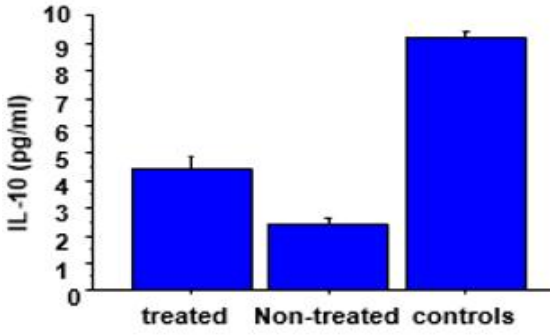
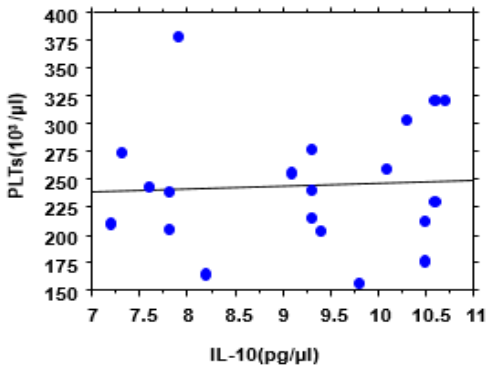


Figure (2): correlative analysis of Tregs percentage and platelet counts (A) in controls group (B) in patients group



Figure(3): Levels of of IL-10 in treated and non-treated groups of patients compared to healthy control group.

A



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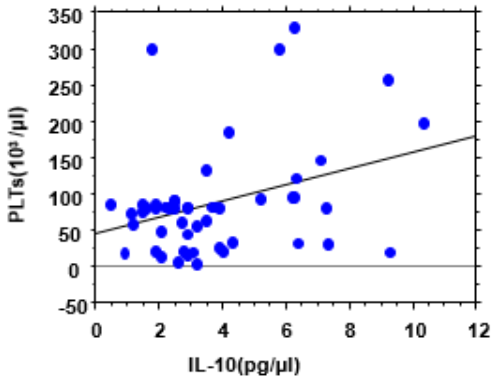

Figure (4): correlative analysis of IL-10 levels and platelet counts (A) in control group (B) in patients group.

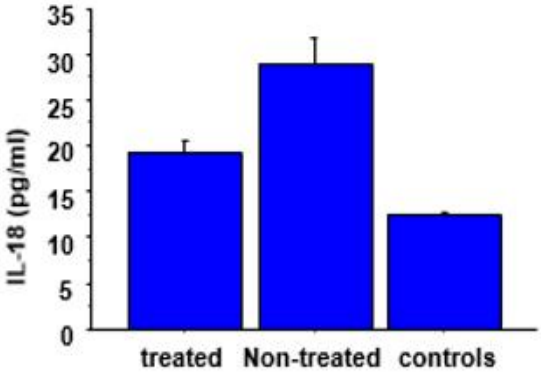

Figure (5): levels of IL-18 in treated and non-treated groups of patient compared to healthy control group.

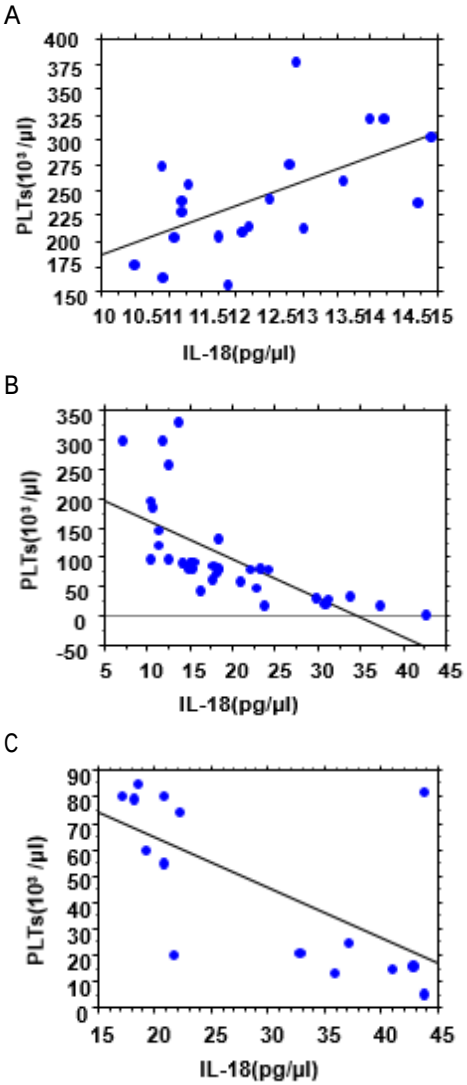

Figure (6): correlative analysis of IL-18 levels and platelet counts (A) in control group (B) in non-treated patients (C) in the on-treatment group of patients.

The exact mechanism of the immune dysfunction in ITP is generally not well known, but a number of T-cell abnormalities has been demonstrated in patients with ITP, these T cell abnormalities may characterized by abnormal numbers and functions of Tregs (Ling *et al*., 2007; Aslam*et al*., 2012). Tregs were expressing a panel of CD4+CD25+CD127⁻ and they secrete regulatory cytokines like IL-10 and TGF-β1 to induce hemostasis and maintain peripheral immune tolerance (Shi *et al.,* 2015).

In the current study, the results have demonstrated that Tregs were reduced in number in ITP patients compared to healthy individuals, while the on-treatment group of patients have shown a higher levels in comparison to the non-treated (newly diagnosed) subjects. Consistent with these results, there are many reports describing reduced numbers of Tregs in adult ITP patients (Liu *et al.,* 2007; Ling *et al*., 2007; Nishimoto *et al.,* 2013). Also many studies reported a highly significant decrease in the percentage of Tregs in children with acute ITP compared with controls (Zahran and Elsayh, 2014;Bakara *et al*., 2014). Possible reasons for decreased Treg numbers can be due to impaired development, survival, proliferation and/or stability of Tregs (Yazadanbakhsh *et al.,* 2013). While Wu *et al* study has shown that the percentages of CD4+CD25+CD127- cells were almost stable when determined by flow cytometry between ITP patients and healthy controls (Wu *et al*., 2015). Moreover, Yu *et al* also found a comparable frequency of circulating CD4+CD25+Foxp3+ Tregs between the patients and the controls, so they suggested that functional defects, not the frequency, in Tregs contribute to breakdown of self-tolerance in patients with chronic ITP(Yu *et al*., 2008). The defects in Tregs function may be explained by failed cell contact dependent suppression or reduced secretion of cytokines including IL-10, TGF-β1 or IL-35 that mediate suppression(Buckner, 2010).Reduced Treg activity may also be due to increased resistance of effector T cells to suppression(Yu *et al*., 2008). Whereas many other studiesconcluded that alteration in both Tregs frequency and functional characteristics were defective in ITP patients and this might be responsible for lossof self-tolerance and subsequently destructive immune responses observed in ITP patients (Liu *et al*., 2007; Arandi *et al.,* 2014). Meanwhile, Yazdanbakhsh *et al*. (2013) mentioned that impaired regulatory compartment, including Tregs and Bregs, have been reported leading to immune dysregulation in ITP patients. In response to Dexamethasone therapy, similar increasing in Treg cell percentages was highlighted byHuang *et al*(Huang*et al.,* 2015). The same treatment induced up-regulation was found by Chunyan*et al*., (2010) but with a higher level than the healthy controls, whereas by using another protocol of treatment (Ritoximab), Stasi *et al* recorded that the elevation was not significantly different between patients in remission and controls (Stasi *et al.,* 2008). All these results were contradicted with what was studied by Wu *et al* (2015) who didn't reach to a significant change among the three groups (pre-treatment patients, post-treatment patients and healthy control).

As the platelet counts reflect the disease activity and it is closely related to platelet destruction, the correlation between Tregs and the platelet counts was examined. The result showed a positive non-

significant relationship between the healthy subjects and patient group. In contrast to this result, Bakara *et al* (2014) have found a significant positive correlation between Treg percentages and platelet counts in acute ITP patients indicating a close association between Treg cells percentage and the parameters known to reflect the degree of platelet destruction. Meanwhile, defective Treg function and number may be explained by reduced secretion of cytokines that mediate suppression including IL-10, TGF-β or IL-35 (Buckner, 2010). These findings raise the possibility that Treg cells may regulate the disease phenotype particularly in relation to the degree of thrombocytopenia. Also, Zhang *et al* (2009) found that the percentage of circulating Tregs may be decreased during active disease and the extent of this decrease correlates with the severity of the disease.

Levels of two cytokines (IL-10 and IL-18) were assessed in sera of ITP patients and healthy controls. IL-10 is a regulatory cytokine involved in immune tolerance and mediate functional IL-10-secreting regulatory T cells like CD4+CD25+FOXP3+ Tregs (Catani *et al*., 2013). To estimate the possible role of immune regulatory cytokine, IL-10, the concentration of this cytokine was estimated in the sera of ITP patients as well as in healthy controls. The presented results indicated that IL-10 levels were significantly reduced in ITP patients compared to the control values. These results may suggest that IL-10 levels might be inversely related with the disease progression, and its protective effects against ITP development cannot be ignored, this might give a hope for a new strategy in the ITP treatment since it has no cure, and relapses may occur years after seemingly successful medical or surgical management.

These findings were in concordance with several previous reports which have shown that the levels of IL-10 was reduced in ITP patients (Shevach and Stephens, 2006; Guo *et al*., 2007; Ma *et al.,* 2014). However, an Egyptian study on acute and chronic ITP in children elucidated a contrast result when showed that the level of IL-10 was elevated significantly in ITP patients (Talaat *et al*., 2014), they suggested that this increase could be related to the activation of macrophages, which have been reported to be stimulated in ITP patients by platelet autoantigen leading to activation of T cells. In respect to the response to treatment, treated patients showed a significant higher levels of IL-10 compared to non-treated ones, yet they were significantly lower than the healthy controls. Moreover, Li *et al*., have stated that the treatment induced increment was significantly higher than the healthy group(Li *et al*., 2016). Concerning IL-10 levels, chronic ITP patients responded significantly to treatment as stated by Guo*et* with no significant difference in comparison to the control group (Guo*et al.,* 2007; Li *et al*., 2016).

Whereas Culic´*et al*. (2006) revealed that there were no significant correlation between platelet counts and the level of IL-10 In adult ITP patients. In addition to the Treg cells, Immune tolerance is also thought to be maintained via a balance between Th_1 and Th_2, and since IL-18 promotes Th_1 responses by stimulating the synthesis of IFN-γ, while IL-10 produced from Th_2 and some other cell types, they play an important role in the Th_1/Th_2 balance to sustain immunological homeostasis (Nakanishi *et al.,* 2010). A number of studies suggest a Th_1 bias, compared with Th_2, in adults with chronic ITP (Yazadanbakhsh *et al.,* 2013). Results of this study observed a significant up-regulation in the serum levels of IL-18 of ITP patients compared to healthy individuals, this was consistent with what was reported by Shan *et al* who showed that the serum IL-18 concentrations in ITP patients were higher compared with normal controls, which confirmed that IL-18 is involved in ITP pathogenesis (Shan *et al*., 2009b) and this shed a light on the presence of shifting toward Th_1 in this disease, meanwhile Shaheen *et al* (204) have pointed that serum IL-18 level was not significantly different among cases and controls in pediatric age groups. Extremely different result was stated by Ma *et al* who found a Th_2 polarization withan elevated IL-10 levels and down regulated Th_1 cytokine IFN-γ in adult ITP. This could be attributed to (1) the pathophysiology of ITP is heterogeneous and complex. (2) Ethnic differences in the studied populations. (3) Low number of patients and controls in the study (Ma *et al.,* 2014).

The significant reduction in IL-18 level after treatment was also reported by Shan *et al* but with no significant difference between the patients in remission and the normal individuals. Previous data demonstrated that IL-18 and IL-18 binding protein (IL-18BP) are expressed in humans (Shan *et al.*, 2009b). Regulating the balance of IL-18 and IL-18BP might provide a reasonable therapeutic strategy for ITP as IL-18BP has been shown to bind IL-18 with high affinity and effectively inhibit its biological activities by reducing induction of IFN-γ mediated responses (Hurgin *et al.,* 2002). Administration of dexamethasone to ITP patients results in elevation of IL-18BP plasma levels thereby IL-18 plasma levels steadily declined(Shan *et al.,* 2009b).

The usual upregulated levels of IL-18 in correlation with the disease activity, were reported in various experimental and human autoimmune

disorders (Boraschi and Dinarello, 2006). While Shan *et al* (2008) recorded no correlation between IL-18 levels and the platelet counts in their study on ITP patients. Taking these results together may give strong evidence which supports the role of T cells and its cytokines in the pathogenesis of ITP, abnormal Treg cell counts together with polarization between Th_1 and Th_2 cytokines both affect the peripheral tolerance and have been therefore regarded as important targets for investigations in ITP patients. In addition, cytokine levels targeted drugs can bring hope to patients with refractory ITP.

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