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Evaluation of Interleukin-17 and Gamma Interferon Levels in Primary Immune and Borderline Thrombocytopenia

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ABSTRACT

Background: Few studies have evaluated the Th17 cell associated cytokine in primary autoimmune thrombocytopenia (ITP); all of them included only Chinese populations with little agreement between their results. Monoclonal antibodies against interleukin 17 (IL-17) or its receptor have been developed for clinical application, so, further studies should determine if these inhibitors are clinically useful in the treatment of ITP.

Aims: The aim of this study was to explore the clinical significance of change in the level of IL-17 and gamma interferon (IFNγ) in peripheral blood mononuclear cells of newly diagnosed adult ITP patients before and one month following steroid treatment. We studied also IL-17 and IFNγ levels in borderline thrombocytopenia (healthy individuals with incidentally discovered platelet count between 100 and $150x10^9$/L).

Patients and Methods: Thirty three adult patients with thrombocytopenia and 11 healthy controls were enrolled. Patients were divided into two groups based on their platelet count at the time of the study: (i) Twenty newly diagnosed ITP patients (group 1) and (ii) Thirteen-borderline thrombocytopenia patients (group 2). Patients with newly diagnosed thrombocytopenia (n=20) were subdivided into 2 groups according to their response to steroid therapy for one month. Level of T-helper 1 (IFNγ), and T-helper 17 (IL-17) cytokines in peripheral blood mononuclear cells were investigated by enzyme-linked immunosorbentassay.

Results: The level of IL-17 and IFNγ was increased in patients with untreated ITP and borderline thrombocytopenia (p=0.0001 for IL-17 and p=0.0001 for IFNγ) when compared withcontrols. Furthermore, no statistically significant differencewas present in IL-17 and IFNγ level when comparing untreated ITP and borderline thrombocytopenia group. There was a significant positive correlationbetween IL-17 and IFNγ levels in ITP patients (r=0.621, p=0.003). There was statistically significant reduction in the level of IL-17 in responder patients (p=0.0001) while IL-17 level was insignificantly changed in non-responder patients (p=0.394).

Conclusion: Elevation of the level of IL-17 and IFNγ may be an important dysregulation factor of cellular immunity in ITP patients. Follow-up of persons with borderline thrombocytopenia is mandatory for early detection of future autoimmune abnormality in this group of persons.

Key Words: *ITP – IL-17 – IFN-gamma – ELISA – Autoimmunity.*

INTRODUCTION

Adult idiopathic thrombocytopenic purpura (ITP) is a chronic acquired organ-specific autoimmune hemorrhagic disease characterized by the production of antibodies against antigens on the membrane of platelets, resulting in enhanced Fc-mediated destruction of the platelets by macrophages in the reticuloendothelial system **[1]**.

Some patients have either no symptoms or minimal bruising, while others are at a risk of serious bleeding, which may include fatal intracranial hemorrhage, gastrointestinal hemorrhage or extensive skin and mucosal hemorrhage. The severity of thrombocytopenia correlates, to some extent, with the bleeding risk. Concepts surrounding the mechanisms of thrombocytopenia in ITP have shifted from the traditional view of increased platelet destruction mediated by autoantibodies to mechanisms in which both impaired platelet production and T cell-mediated effects play a role **[2]**.

T helper (Th)1/Th2 balance is essential in regulating immune system under normal conditions and is known to be dysregulated in many autoimmune diseases. The polarization of the

immune system towards either Th1 or Th2 immunity is dependent on the level of cytokines **[3]**.

Adult chronic primary ITP patients have high Th1/Th2 ("helper" CD4+ cells) ratio and high Tc1/Tc2 ("cytotoxic" CD8+ cells) ratio. Furthermore, the Th1/Th2 ratio imbalance is inversely correlated with disease severity, meaning the higher the Th1/Th2 ratio, the lower the platelet count. ITP patients also exhibit decreased numbers of CD4+CD25+ T-regulatory (T-regs) cells, which function to down-regulate T-cell responses. Not surprisingly, the degree of decrease in numbers of T-regs is associated with more severe disease in ITP. In addition to these changes, the total CD4:CD8 ratio is also observed to be diminished in ITP and improves with disease remission **[4]**.

Recently, a novel subset of CD4+ T cells, distinct fromTh1 and Th2, was identified. It is characterized by the production of interleukin 17 (IL-17) and, therefore, designated as Th17 cells. Th17 has been shown to play a crucial role in the induction of autoimmune diseases-including rheumatoid arthritis and experimental autoimmune encephalomyelitis which previously were considered to be mainly associated with dysregulated Th1 cell and IFNγ. It has been demonstrated that Th17 cells are more potent than Th1 cells in inducing autoimmune diseases **[3]**.

There are no data to date about Th17 levels in patients with ITP **[3]**. Few studies have evaluated the Th17 cell associated cytokines in ITP, all of them included only Chinese populations and there is little agreement between results **[5]**.

With advancements in the genetic manipulation of specific antigenic epitopes associated with the pathogenesis of ITP, the scope for a clearer understanding of the mechanisms contributing towards the pathophysiology of ITP will undoubtedly create a greater means by which potential therapies to manage ITP can be developed **[6]**. Because monoclonal antibodies against IL-17 or its receptor and a soluble IL-17 receptor have been developed for clinical application, further studies should determine whether these inhibitors are clinically useful in the treatment of chronic ITP **[7]**. The aim of this study was to explore the clinical significance of change in the level of IL-17 and IFNγ in peripheral blood mononuclear cells of newly diagnosed adult patients with primary immune thrombocytopenia before treatment and one month following steroid treatment.We studied also IL-17 and IFNγ levels in patients with borderline thrombocytopenia.

PATIENTS AND METHODS

Twenty adult patients with newly diagnosed ITP (group 1) according to the ITP diagnosis criteria proposed by an international working group (IWG) **[8]** were enrolled in this study. Six patients were females and fourteen were males. Their age ranged from 21-33 with a median of 25 years. They were randomly selected from outpatient clinics from Alexandria and Cairo Universities. All patients were symptomatic and their platelet counts were less than $30x10^9$/L. These patients were given treatment in the form of first-line corticosteroids according to international consensus **[8]** due to clinically significant bleeding and/or extremely low platelet count. One month after the initial treatment, response to therapy was validated. Eleven patients responded while nine patients did not respond to steroid treatment.

Thirteen patients with borderline thrombocytopenia (healthy individuals with incidentally discovered platelet count between 100 and $150x10^9$/L) **[9]** were considered group 2. Eleven healthy volunteers of matched age and sex were taken as normal controls (group 3).

Secondary ITP, pregnant patients, patients contraindicated to steroid therapy, patients who had received previous treatment for ITP, including immunosuppressive agents, or who had undergone splenectomy,were excluded[8].Newly diagnosed primary ITP was defined by the IWG as a platelet count less than $100x10^9$/L up to 3 months from diagnosis in the absence of other causes or disorders that may be associated with thrombocytopenia **[8]**.

Patients with ITP received treatment in the form of prednisone 1mg/kg orally for 21 days then tapered off. Response is defined as a platelet count ≥30 but $<100x10^9$/L and a doubling from baseline. Complete response is consistent with the new diagnostic threshold of $>100x10^9$/L **[8]**.

All participants in this study were subjected to:

- Thorough history-taking and clinical examination with special stress on evidence of bleeding, duration of ITP, previous treatment received for ITP and excluding other causes of thrombocytopenia including hepatitis C virus infection and helicobacter pylori infection (secondary ITP).
- Complete blood picture **[10]**.
- Bone marrow aspiration (for patients only) to exclude secondary causes of thrombocytopenia **[10]**.
- Hepatic and renal function tests **[11]**.
- Platelet specific antibody by modified antigen-capture ELISA.
- *Immunological tests:*

i- Quantitative estimation of IL-17 and IFNγ after mitogen; phytohemagglutinin (PHA) stimulated whole peripheral venous heparinized blood using commercial enzyme-linked immunosorbent assay (ELISA) kits. "RayBio® Human IL-17 ELISA USA" **[12]**.

ii- Estimation of IFN-γ levels were done by isolation of peripheral blood mononuclear cells from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Sigma Aldrich) and cultured at $2x10^5$ cells per 500µL in Roswell Park Memorial Institute medium (RPMI) 1640(Sigma Aldrich) supplemented with antibiotics and 5% fetal calf serum (Sigma Aldrich). For stimulation, 5µg/µl PHA mitogen (Wellcome Diagnostics) was used. Incubation of cultures was performed at 37ºC in a humidified atmosphere of 5% CO_2. After 48 hours incubation, culture supernatants were collected from each tube and stored at –20ºC to be assayed using commercial ELISA kits (Ray Biohuman IFNγ). We used a standard curve to detect IFNγ at the sub-nanogram level (≥100pg) **[13,14]**.

Statistical analysis:

Data were analyzed using SPSS program version 10. Data were expressed as mean±standard deviation (SD). Qualitative variables were compared using chi square (X^2) while one-way ANOVA test was done for normally distributed quantitative data. Between groups further analysis of ANOVA was done using least significant difference (LSD). Paired *t*-test was used to compare data before and following steroid treatment in group 1. Correlation between variables was assessed by Pearson's correlation (*r*). The level of significance was chosenas $p \leq 0.05$.

RESULTS

Table (1) shows clinical and laboratory criteria of the three studied groups. Patients' mean (±SD) platelet count was 22.15 (±5.87) $x10^9$/L. The duration of the disease was less than 3 months in all the studied patients in group 1.

Table (1): Clinical and laboratory parameters of the studied groups.

Parameter	Group 1 (n=20)	Group 2 (n=13)	Group 3 (n=11)	*p* value	LSD
Age (years)	21-33 25.3±3.06	14-34 25.54±6.996	19-30 22.91±3.33	0.311	
Gender:					
Male	14	9	5	0.35	
Female	6	4	6		
Platelets: ($x10^9$/L)	13-29 22.15±5.87	103-143 124±11.45	170-244 192.27±19.91	0.0001	1.2 1.3 2.3
Presence of platelet antibody	13	–	–		
IL-17 (pg/mL)	54-118 91.55±19.59	72-93 83.44±8.40	50-94 59.91±12.79	0.0001	1.3 2.3
IFNγ (pg/ml)	453-660 513.2±52.82	460-660 531.85±65.27	300-485 381.64±71.12	0.0001	1.3 2.3

Group 1: Immune thrombocytopenia.
Group 2: Border line thrombocytopenia.
Group 3: Normal Controls.
Values are expressed as range, mean ±SD.
LSD : Least significant difference, denote significant difference between groups
p : Is significant at the 0.05 level.

Table (2) shows comparison between age, platelet count and level of IL17 and IFNγ before and after steroid therapy in ITP patients responding (n=11) and non-responding (n=9) to steroid treatment.

Table (3) shows correlation between age, platelet count and level of IL17 and IFNγ in ITP patients (group 1). No correlation was detected between the level of IL17 and IFNγ and either age or platelets count in the three studied groups. Statistically significant direct correlation was present between IL17 level and IFNγ level in ITP group (r=0.621, p=0.003) (Fig. 1), borderline thrombocytopenia group (r=0.745, p=0.003) and controls (r=0.765, p=0.006).

Table (2): Comparison between responders and non-responders immune thrombocytopenic group as regards platelet count and cytokines level pre and poststeroid treatment.

Parameter	Responders (n=11)		*p* value	Non responders (n=9)		*p* value
	Before	After		Before	After	
Platelets:	13-29	200-375	0.0001	14-29	20-29	0.287
($x10^9$/L)	22.09±6.25	266.09±47.23		22.22±5.74	24.44±3.43	
IL-17 (pg/mL)	54-118	55-70	0.0001	67-116	61.4-107.8	0.394
	100.64±19.15	61.0±5.35		80.44±14.17	76.01±15.78	
IFNγ (pg/ml)	460-660	288-481	0.0001	453-512	430-485	0.121
	542.27±53.84	366.82±73.31		477.67±20.48	462.44±17.27	

Values are expressed as range, mean ±SD. *p* : Is significant at the 0.05 level.

Table (3): Correlation between age, platelet count and cytokines' level in twenty immune thrombocytopenic patients (group 1)

Parameter	Age		Platelets		IL-17		IFN-γ	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Age	–	–	–0.003	0.991	–0.252	0.284	–0.251	0.287
Platelets	–0.003	0.991	–	–	0.067	0.779	–0.094	0.692
IL-17	–0.251	0.287	0.067	0.779	–	–	0.621	0.003
IFN-γ	–0.251	0.287	–0.094	0.692	0.621	0.003	–	–

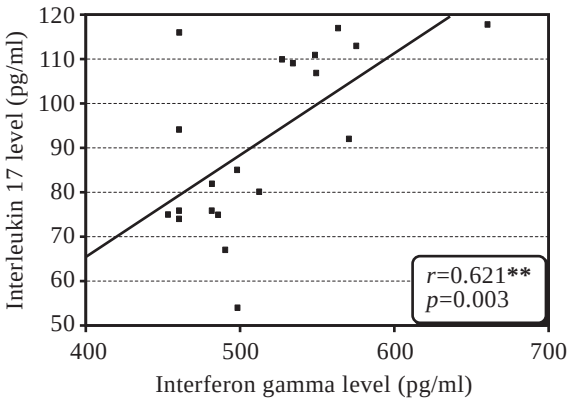

Fig. (1): Correlation between level of interleukin 17 and gamma interferon in immune thrombocytopenic patients (group 1).

DISCUSSION

Several studies have suggested that Th17 T cells may be the major cell type involved in orchestrating tissue inflammation and autoimmunity. Specifically, Th17 cells have been shown to play a crucial role in the induction of rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus (SLE) and psoriasis **[15]**. Semple et al., **[16]** first reported that T cells had reactivity against platelets, which initiated the investigation on T cell disorders in ITP.

Whether Th17 cells play a role in the pathogenesis of ITP remains undetermined because

several investigations on Th17 in ITP indicated contradictory conclusions **[17]**. While some authors demonstrated increased percentages of Th17 cells in the peripheral blood of ITP, Guo et al., **[18]** found comparable frequency of circulating Th17 cells (flow cytometry analysis) and comparable expression of IL-17 transcripts (RT-PCR evaluation) in patients and controls. In these studies Th17 cells were enumerated after stimulation of mononuclear cells with various molecules (phorbolmyristate acetate and ionomycin) and, therefore, not under physiological conditions.

IL-17 has potent immunogenic properties. It induces the release of colony stimulating factors, chemokines, metalloproteinases, tumor necrosis factor alpha, and IL-6. Moreover, IL-17 mobilizes and activates neutrophils **[19]**.

In our study,we detected statistically significant high mean value of IL-17 level in newly diagnosed immune thrombocytopenic group before treatment and in borderline thrombocytopenia group as compared to controls. Also, there was statistically insignificant difference between IL-17 levels when immune thrombocytopenia group was compared with borderline thrombocytopenia group. However, no correlation was detected between the level of IL17 and either age or platelet count in the three studied groups.

One possible link has been proposed by-Doreau et al., **[20]** who demonstrated that IL-17 alone or in combination with B-cell activating factor (BAFF) protects B cells from apoptosis, promotes B-cell proliferation and drives plasma cell differentiation, probably playing a role in the pathogenesis of SLE. Similar mechanisms might be involved in the pathogenesis of ITP, a disease also marked by a loss of B-cell tolerance, abnormal production of auto-antibody and high serum levels of BAFF **[21]**.

In 2009, Zhang and colleagues **[22]** first described up-regulation of Th17 cells along with Th1 in patients with ITP and suggested that Th17 cytokines promoted an imbalance favoring a more Th1-type immune response in ITP.Furthermore, in the natural course of experimental autoimmune encephalitis, an antigen-specific effector T cell secreting both IL-17 and IFNγ has consistently been found in vivo in the inflamed central nervous system **[7]**. Since ITP is also an autoimmune disorder, a search for a similar mechanism might be considered.

In our patients groups, we detected statistically significant high IFNγ level in both untreated newly diagnosed immune thrombocytopenia group and borderline thrombocytopenia group as compared to controls with statistically insignificant difference between IFNγ level in immune thrombocytopenia and borderline thrombocytopenia. There was also a significant positive correlation between IFNγ and IL-17 levels in the three studied groups. Thus, the most likely scenario is that both Th1 and Th17 cell types are involved.

In our study, statistically significant difference was detected in IL-17 level in immune thrombocytopenic patients responding to steroid therapy before and after treatment while there was no significant difference in IL-17 level between non responder immune thrombocytopenic patients before and after steroid treatment. This may reflect a role for IL17 in steroid responsiveness as reported by Hamid and colleagues **[23]** using both bronchial biopsies and primary bronchial epithelial cells in asthmatic patients. They concluded that IL-17, the signature cytokine of Th17 cells, can influence glucocorticoid receptor (GR) signaling capacity in asthmatic patients by reducing GRα levels and enhancing the expression of GRß which leads to further modulation of GRα function **[24]**. Reduced expression of GRα is also recently reported to be associated with glucocorticoid resistance in adult ITP patients **[25]**.

Based on the results of Stasi et al., **[9]**, healthy individuals with incidentally discovered platelet count between 100 and $150x10^9$/L have a 10-year probability of developing persistent thrombocytopenia of only 6.9% and of developing autoimmune disorders other than ITP of 12%. However, this criterion has not been formally validated yet. In our study, non-statistically significant difference was detected between the level of both IL17 and IFNγ in ITP patients in comparison with patients with borderline thrombocytopenia which confirm this observation and makes follow-up of these persons mandatory for early detection of future autoimmune abnormality.

In a previous study, patients with borderline thrombocytopenia have enhanced levels of pro-inflammatory cytokines linked to Th1 and Th17 cell response. They are also more frequently carriers of polymorphisms in genes that encode cytokines involved in the commitment of Th1 and Th17 immune response. This is similar to that observed in patients with chronic ITP, which points to the need of a search for pathogenic mechanisms associated with this condition **[26]**.

In conclusion, elevation of IL-17 and IFNγ may be an important dysregulation factor of cellular immunity in ITP patients. Follow-up of persons with borderline thrombocytopenia is mandatory for early detection of future autoimmune abnormality in this group of persons.

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