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The relationship between different interleukins and T helper cells count in patients with immune thrombocytopenia

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ABSTRACT

Immune thrombocytopenia is the most common autoimmune disorder involving blood types. In several studies, the role of T CD4+ cells in patients with immune thrombocytopenia has been associated with different results. Therefore, in this study, with the aim of applied research in the pathogenesis of immune thrombocytopenia, the relationship was investigated between the number of T CD4+ cells, serum levels of IL-11 and IL-17 cytokines, and platelet count. In this regard, 100 patients with immune thrombocytopenia and 100 healthy individuals were included in the study. The T CD4+ cell counts were examined by flow cytometry and in addition, serum levels of interleukins 11 and 17 were measured by ELISA. The results of this study showed that the number of T CD4+ cells and plasma level of IL-17 were not significantly different between the two groups, but plasma levels of IL-11 in the patient group were significantly higher than the control group (P = 0.286). Overall, in this study, the level of cytokine IL-11 was significantly increased in comparison with IL-17 and T CD4+ cells in patients with immune thrombocytopenia, so it is suggested that measurement of cytokine IL-11 level in these patients could be considered as a critical diagnostic marker and indicator in the stages of disease progression.

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Introduction

Immune thrombocytopenia (ITP) is an autoimmune hemorrhagic disease in which autoantibodies are produced against platelet membrane glycoproteins, leading to their destruction and clearance by macrophages of the reticuloendothelial system (1). The annual incidence of this disease is about one in ten thousand children, and according to research, it becomes chronic between 10 and 25% of cases (2). Unlike the acute form of the disease, which is usually benign and spontaneously limited, the chronic condition presents persistent thrombocytopenia (3). In less than one-third of chronic cases, spontaneous recovery occurs over months and years to come. The pathophysiology of ITP is complex and unknown (4). Although lymphocytes that produce autoantibodies to antiplatelet cells are considered to be primary immune-deficiencies, research has shown that dysfunction in cellular immunity also plays a vital role in the pathogenesis of ITP (5).

The T CD4+ lymphocytes are effective cells in the immune system that play an essential role in producing autoreactive antibodies and the replacement of the antibody class (6, 7). There are different types of these cells, so T helper lymphocytes 1 and 2 (Th1, Th2) are classic types of T CD4+ cells, which Th1 cells have an essential role in activating macrophages by secreting interferon-gamma, and Th2 cells are involved in inducing antibody production and allergic reactions (8, 9). The balance between the two subgroups Th1 and Th2 is affected by several immune responses and is impaired in various autoimmune diseases. Some of these studies have reported lower Th1 cell responses or even Th2 cell responses in chronic patients with ITP, but most studies have shown high Th1 responses (10).

Recently, a new subset of T CD4+ helper cells has been identified, a distinct class of Th1 and Th2 cells, characterized by the production of interleukin-17 (IL-17) and is considered Th17 lymphocytes (11, 12). Studies have shown that Th17 cells play an essential role in inducing autoimmune diseases such as systemic lupus erythematosus, autoimmune encephalomyelitis, and myasthenia gravis (13, 14). In addition, the role of these cells in ITP has been

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investigated with conflicting results. IL-11 is also a cytokine that has been implicated in platelet synthesis (15). Unfortunately, few studies have been done on the role of IL-11 in ITP, which have yielded conflicting results (13). Therefore, in this study, to evaluate the molecular mechanisms, serum levels of IL-11, IL-17, and T $CD4^+$ cells in chronic ITP patients were studied.

Materials and methods

Demographic information of the patients

In a case-control study, 100 patients with chronic ITP including 61 women and 39 men with a mean age of 43.9±16.1 and 100 healthy individuals including 60 women and 40 men with a mean age of 42.8±15.4 were selected in the control group. The diagnosis was made by hematology and oncology specialist according to clinical symptoms and complete blood and platelet count. Exclusion criteria were history of any drug use and also the history of autoimmune diseases. The inclusion criteria were a decrease in platelet count for unknown reasons based on the diagnostic criteria. The mean platelet count in the case group was $95.1 \times 10^3 \pm 41.7 \times 10^3$ and in the control group was $273 \times 10^3 \pm 43.6 \times 10^3$ (Table 1).

Table 1. The demographic information of the control group and case group

Variable	Control Group (n=100)	Case Group (n=100)
Age (year)	42.8±15.4	43.9±16.1
Gender		
Man	40 (40%)	39 (39%)
Woman	60 (60%)	61 (61%)
History of autoimmune disease	Not Having	Not Having
History of drug use	Not Having	Not Having
The onset time of the disease (year)	-	3.8±1.5
Platelet count	$273 \times 10^3 \pm 43.6 \times 10^3$	$95.1 \times 10^3 \pm 41.7 \times 10^3$

Cell count and serum level determination

After informing about the present study and after obtaining informed written consent, 3-5 ml of blood was collected from the subjects. To perform flow cytometry to determine the number of T $CD4^+$ cells, we isolated peripheral blood mononuclear cells (PBMC) by Ficoll Solution (Sigma-Aldrich, USA). The cells were then washed with phosphate buffer saline (PBS) containing 2% FCS. Then they were exposed to an Anti-CD4 antibody for 20 minutes at 4°C and finally, these cells were analyzed with a PAS II flow cytometer (Partec GmbH, Munster, Germany) (16). Serum levels of IL-11 and IL-17 were determined by ELISA using ELISA kits protocol (Bender MedSystems GmbH, Vienna, Austria) (17).

Construction of PCAT1 and ZNF217 shRNA vectors and cell transfection

The PCAT1 shRNA targeting 5 -GAACCTAACTGGACTTTAA-3, ZNF217 shRNA targeting 5'-GCTCACGCCTGTAATCTCA-3' and negative control (NC) shRNA plasmid targeting 5'-TTCTCCGAACGTGTCACGT-3' were constructed (Sangon Biotech) and ligated to the pcDNA3.1 plasmid to construct pcDNA3.1-shPCAT1 and pcDNA3.1-shZNF217 vectors, respectively. For transfection, 1 ×105 cells were plated in six-well plates and transfected with different vectors using Lipofectamine 2000. The cells with stable PCAT1 and ZNF217 knockdown were selected for a future experiment.

Statistical analysis

Data were analyzed in SPSS software version 16. Comparison of quantitative variables performed with Mann-Whitney u test and Student's t-test. Spearman test was also used for the correlation of quantitative variables. $P < 0.05$ was considered significant.

Results and discussion

Comparing the serum level of IL-11 in the two groups, the result was that the mean serum level in control subjects was 226.82 ± 19.7 and in patients was 315.91 ± 23.8. The results of this study showed that the serum level of IL-11 was statistically significant between the patient and control groups ($P = 0.035$) (Table2). The mean serum level of IL-17 in both groups was 13.1 ± 7.9 in the control group and 16.87 ± 3.6 in the patient group. The levels of cytokine IL-17 in the statistical study did not show a significant difference between the two groups ($P = 0.286$).

Also, the mean number of T $CD4^+$ cells in the control group was 857.4 ± 19.7 and in patients was

919.2 ± 32.7 per microliter, which was not statistically (Table 1, Fig. 1).

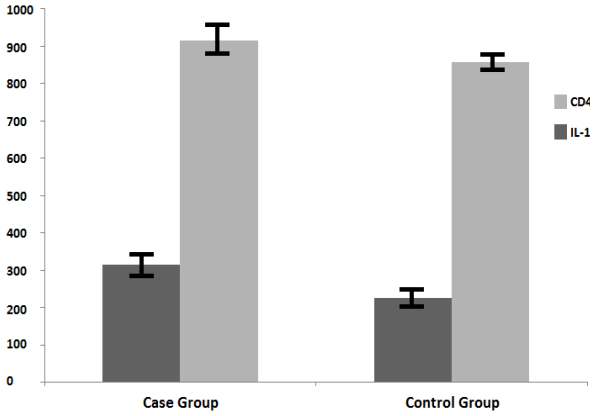

Figure 1. Mean number of T CD4⁺ cells and IL-11 plasma concentration in case and control groups.

In the study of the relationship between IL-17, IL-11, T CD4⁺ cell, and platelet count, there was a positive correlation between T CD4⁺ cells and platelets (P = 0.029) (Fig 2). In addition, a negative correlation was observed between IL-11 and age in both case and control groups. Other factors in the groups were not significantly related to each other. Considering the relationship between IL-11 and age, the independent effect of IL-11 between the control and patient groups was investigated. The result obtained by logistic regression showed that IL-11 had a significant increase in patients (P = 0.0001).

Table 2. Comparison of the mean of the studied quantitative variables in the control and case groups; Platelet count (A), IL-11 Serum Level (B), IL-17 Serum Level (C), Number of CD4⁺ Cells (D)

Variable	Control Group	Case Group	P-value
A	$273 \times 10^3 \pm 43.6 \times 10^3$	$95.1 \times 10^3 \pm 41.7 \times 10^3$	0.0001
B	226.82 ± 19.7	315.91 ± 23.8	0.035
C	13.1 ± 7.9	16.87 ± 3.6	0.286
D	857.4 ± 19.7	919.2 ± 32.7	0.412

Significant between the control and control (*P* = 0.412)

This study examined T CD4⁺ lymphocytes as cells involved in antibody production and inflammation in patients with immune thrombocytopenia. The cytokines IL-11, IL-17 (as an indicator of Th17 cells), and platelet count were also evaluated. Since the pathophysiology of ITP is complex and unknown, several studies have been conducted to understand the mechanism of ITP (18). In this study, we measured the number of T CD4⁺ cells in case and control groups. Although the mean of these cells was higher in the patient group, there was no statistically significant difference between them (P=0.412).

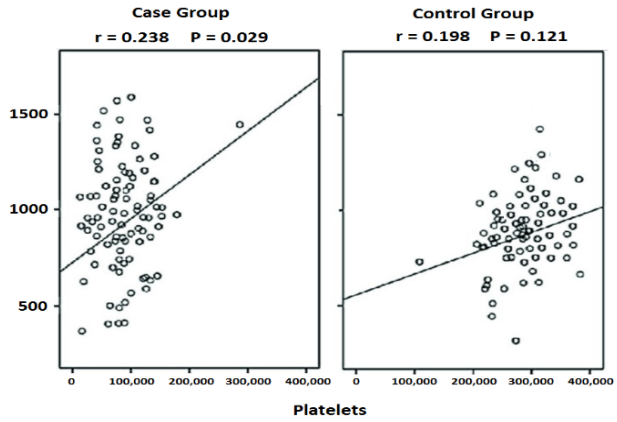

Figure 2. Relationship between T CD4⁺ cell number and severity of platelet decline in patients with ITP and control group.

Several studies on the role of T helper cells in ITP patients showed that the ratio of Th1/Th2 and Th17 cells had increased significantly (19, 20). In some studies, such as the Ma *et al*. study (21), there was no significant relationship between the two groups in cytokines related to Th1 cells. But the researcher's opinion on this contradiction was that the measurement method was not sensitive enough compared to other studies. Given that most studies have reported Th1/Th2 imbalance as the predominance of Th1 cells in many autoimmune diseases, including ITP, it can be said that the lack of T CD4⁺ cells in both groups may be due to this (19). The general level of these cells in ITP patients does not change much, and only the balance between the T helper subgroups is disturbed (22).

On the other hand, we measured only the T CD4⁺ marker in this study, while there are several types of T CD4⁺ cells. According to previous studies, some of them, such as Th1 and Th22 cells, increased in ITP and some, such as Th2 and T regulatory, decreased, so it is suggested that other subsets of T CD4⁺ cells be evaluated separately to determine the role of these cells more accurately.

In this study, we measured the relationship between T CD4⁺ cells and platelets in the control and patient groups. In the control group, although there was a

positive correlation between them, this correlation was not statistically significant (P = 0.121), but the patient group had a significant positive correlation between T $CD4^+$ cells and platelets (P = 0.029).

In the Gu *et al*. study (19), the relationship between platelets and Th1 cells was investigated, and these two variables were significantly negatively correlated in patients. Also, in the Sakakura *et al*. study (23), the relationship between regulatory T cells and platelets in ITP patients was investigated, and in this study, there was a positive correlation between them. Therefore, because T $CD4^+$ cells were not isolated in the present study, it was impossible to determine which of these cell types had a positive association with platelet levels. Based on previous studies, it is expected that with an increase in platelets in ITP patients, the level of T-helper2 and T regulatory cells will increase (3).

Although Th1 cells decrease with platelet proliferation in these patients (24), in our study, the overall level of T$CD4^+$ cells increased with platelet proliferation, which may indicate that the majority of these cells are likely to be Th2 and T regulatory. Interleukin 11 is one of the cytokines involved in platelet synthesis. In our study, we measured the level of IL-11 in two groups that IL-11 in the patient group was significantly higher than the control group (P = 0.0001). In examining the relationship between IL-11 level and platelet count in patients, there was a negative correlation between these two variables, i.e. with decreasing platelet level, IL-11 level increased but this correlation was not statistically significant (P = 0.121).

In the Ling *et al*. study (25), serum IL-11 levels were measured in patients with ITP, which increased significantly. In another study by Hong *et al*. (26), patients with refractory ITP were injected with recombinant human IL-11, which significantly increased platelet counts after injection. The present study, along with some studies, has increased the level of IL-11 in the patient group compared to the control group, which seems to be due to the decrease in platelet count in patients with the disease. In addition, the increase in platelet levels in patients can be due to increased secretion of interleukin-11. Although IL-11 had a negative relationship with age in both control and patient groups, i.e., its level decreased with age, using statistical methods, measuring the independent effect of IL-11 between the two groups was also significant.

Another cytokine that has been shown to play an influential role in ITP is IL-17, which indirectly indicates the T helper 17 cells in these patients (27). In the present study, we measured the level of IL-17 between the control and patient groups, which was higher in the patient group. Still, there was no statistically significant difference between the two groups. In the study of the relationship between IL-17 level and platelet count, there was a negative correlation between the two variables in the patient group that was not significant. Although the result we obtained in this study was inconsistent with several studies such as Zhu *et al*. (28) and Hu *et al*. (29), it was consistent with some studies such as Ma *et al*. (21). Ma *et al*. pointed out that one of the reasons for the lack of IL-17 levels between the control and patient groups could be the difference between the cytokine assays in the study population (21).

Finally, it can be concluded that there is no significant relationship between the total level of T helper $CD4^+$ cells and IL-17 level with ITP disease. Still, there was a significant relationship between IL-11 level and disease. In addition, the mechanism of ITP is unknown and complex. More molecular studies should be performed to truly understand the role of T $CD4^+$ cells and related cytokine levels in ITP patients. It is hoped that by identifying the cells and cytokines involved in the pathogenesis of ITP and suppressing or replacing them in these patients, more effective treatment strategies will be provided to minimize the complications and costs of existing treatments. This study showed that IL-11 is one of the effective molecules in platelet production. Therefore, its measurement can be an essential diagnostic and therapeutic indicator in preventing and identifying patients.

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