

# Percentage of Memory B Lymphocytes and Regulatory T Lymphocytes in Peripheral Blood are Low but Not Predictive of Therapy outcomes in Newly Diagnosed Adult Patients with Primary Immune Thrombocytopenia

Mustafa Yilmaz<sup>1</sup> · Semiha Ayhan<sup>2</sup>

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**Abstract** Although changes in the number and function of regulatory T lymphocytes have been reported in primary immune thrombocytopenia (ITP), no study has investigated whether quantification of these cell types in peripheral blood could be used as early predictive marker of treatment outcome. And, it is not clear whether any change occurs in peripheral blood memory B lymphocyte levels in ITP. Hence, the aim of this study was to investigate the percentage of regulatory T lymphocytes and memory B lymphocytes in peripheral blood of ITP patients compared to controls, and also examine whether these levels have any significant predictive value for therapy outcome. A total of 20 newly diagnosed, untreated patients with ITP and 20 healthy controls were included. Flow cytometric analyses of lymphocyte subtypes in the peripheral blood were performed in specimens obtained from patients at the time of diagnosis and one month after the therapy initiation. First line corticosteroid (1 mg/kg/day methylprednisolone) therapy or splenectomy as second line treatment was performed, and patients were followed up for 3 years. Percentage of regulatory T lymphocytes ( $0.25 \pm 0.17\%$  vs.  $1.14 \pm 0.77\%$ ,  $P < 0.0001$ ,  $n = 20$ ) and percentage of memory B lymphocytes ( $1.57 \pm 1.24\%$  vs.  $4.38 \pm 2.41\%$ ,  $P < 0.001$ ,  $n = 20$ ) was significantly lower in ITP patients than healthy controls, at baseline. After one month therapy, the percentage of memory B lymphocytes of ITP patients significantly increased (from  $1.66 \pm 1.31\%$  to  $3.0 \pm 1.7\%$ ,

$P < 0.009$ ,  $n = 17$ ). The initial value of regulatory T ( $0.33 \pm 0.30\%$ ,  $n = 10$  vs.  $0.16 \pm 0.05\%$ ,  $n = 7$ ,  $P > 0.05$ ) and memory B lymphocytes percentages ( $2.1 \pm 1.8\%$ ,  $n = 10$  vs.  $1.1 \pm 0.75\%$ ,  $n = 7$ ,  $P > 0.05$ ) were not significantly different for those who had complete response to first line therapy than those required splenectomy. These results indicate that regulatory T lymphocytes and memory B lymphocytes percentages are not useful for predicting treatment outcome in patients with newly diagnosed adult patients with ITP.

**Keywords** Primary immune thrombocytopenia · Memory B lymphocytes · Regulatory T lymphocytes · Therapy outcome prediction · Peripheral blood

## Introduction

Primary immune thrombocytopenic purpura (ITP) is an autoimmune disorder characterised by decreased platelet production and/or increased platelet destruction due to action of specific immunoglobulin G (IgG) antibodies, produced by B lymphocytes, against platelet membrane glycoprotein GpIIb/IIIa complex. The incidence of ITP in adults is about 16 per 100,000. Persisting thrombocytopenia lasting longer than 12 months is considered as chronic ITP, which mostly affect adults and may require medical treatment. ITP related thrombocytopenia can commonly lead to petechiae, purpura and easy bruises, epistaxis, gingival bleeding and menorrhagia, and less commonly to gastrointestinal (GI) bleeding and gross hematuria, and extremely rarely to death from intracranial hemorrhage. The risk and frequency of GI bleeding and intracranial hemorrhage increases with advancing age and co-morbid conditions.

✉ Mustafa Yilmaz  
myilmaz1971@yahoo.com

<sup>1</sup> Department of Hematology, Faculty of Medicine, Karadeniz Technical University, 61080 Trabzon, Turkey

<sup>2</sup> Department of Internal Medicine, Kanuni Training and Research Hospital, Trabzon, Turkey

CD4<sup>+</sup>CD25<sup>+</sup> T lymphocytes, also known as regulatory T lymphocytes, have been shown to suppress activation and proliferation of potentially autoreactive T lymphocytes that have escaped into peripheral circulation from thymic deletion. Deficiency in regulatory T lymphocytes results in development of some autoimmune disorders. Studies has shown that functional deficiency and decreased percentage of regulatory T lymphocytes in peripheral blood are linked to autoimmune diseases including multiple sclerosis, myasthenia gravis, diabetes mellitus, rheumatoid arthritis (RA) and psoriasis [1, 2]. Peripheral blood B lymphocytes are classified into two main subtypes which includes CD19<sup>+</sup>CD27<sup>+</sup> memory and CD19<sup>+</sup>CD27<sup>-</sup> naive cells. Peripheral blood memory B lymphocyte percentage are reported to remain within normal limits, decreased or increased in autoimmune diseases including multiple sclerosis, RA and systemic lupus erythematosus (SLE) [3].

Generation and proliferation of antiplatelet-antibody-producing B lymphocytes as a result of activation of specific auto reactive T lymphocytes is one of the primary immune defects in ITP. Thus, autoimmune mediated thrombocytopenia and life threatening bleeding can develop, however, in some rare cases. In most adult ITP patients with therapy indications, medical therapy with methylprednisolone is first line and splenectomy is the second line therapy. Sustained response rates of 28–42% to steroid therapy and 60% to splenectomy have been reported [4, 5]. In adult ITP patients, although age and response to intravenous gammaglobulin (IVIG) has been reported to be valuable factors for predicting prognosis and response to subsequent therapy with splenectomy, but more specific disease parameter(s) that can be used in clinical practice are still needed.

In ITP, peripheral blood lymphocyte subtype analysis has been subject to several studies [1–3], both at the time of diagnosis and over the course of treatment, but possible clinical value of changes in regulatory T lymphocyte percentages on outcomes with respect to predicting response to therapy and prognosis are under evaluated, and has not been clarified yet. And, there is only limited data analysing whether any change in peripheral blood memory B lymphocytes of patients with ITP occurs and whether or not this possible changes are significant for the outcome of the disease. So possibilities of use of these parameters to predict whether patients will respond to first line therapy would be of importance in clinical practice.

Hence, the aim of this study was to investigate the percentage of regulatory T lymphocytes and memory B lymphocytes in peripheral blood of ITP patients compared to controls at baseline, and also determine whether these parameters have any significant predictive value in the disease and therapy outcome.

## Patient and Methods

After ethical approval by Institutional Review Board of Karadeniz Technical University School of Medicine (20.06.2008 date and 2008/24 file number) the study was performed in our university hospital setting. A total of 20 newly diagnosed, untreated ITP patients (13 female and 7 male, aged between 17 and 68 years) who was admitted to hematology outpatient clinic of Karadeniz Technical University, Faculty of Medicine Farabi Hospital from December 2008 to December 2010 (study closed for inclusion) were included in this prospective cohort study. Age and gender-matched healthy controls were allocated to the study from hospital workers (13 female and 7 male, aged between 17 and 65 years). Patients were followed-up for three years for response to therapy and course of the disease. The mean of the assessment times was 14 visits.

The criteria for ITP diagnosis establishment were: platelet count of  $< 100.000/\text{mm}^3$ , no typical physical examination sign other than bleeding, no sign other than isolated thrombocytopenia on whole blood count and peripheral smear. The inclusion criteria for patient acceptance were: (1) newly diagnosed untreated ITP patients, (2) no previous (within 6 month) treatment with corticosteroids or immunomodulatory agents for any indication (3) written informed consent, iii) normal levels of thyroid-function test.

Patients were also evaluated for conditions that potentially cause secondary ITP including positivity for hepatitis B surface antigen, hepatitis C virus, human immune deficiency virus and antinuclear antibody. To confirm ITP diagnosis in cases with suspicious findings for ITP or cases over 60 years-age, bone marrow aspiration and biopsy was performed.

## Flow Cytometric Analysis of Peripheral Blood Lymphocyte Subtypes

Blood samples were obtained from ITP patients, first at the time of diagnosis and second 1 month after methylprednisolone therapy, while only one sample being obtained from the control subjects. Flow cytometric (Beckman-Coulter Epics XL-MCL, Nyon, Switzerland) analysis of T regulatory lymphocyte (CD4<sup>+</sup> CD25<sup>+</sup>), T lymphocyte (CD3<sup>+</sup> CD19<sup>-</sup>), T helper (CD3<sup>+</sup>CD4<sup>+</sup>), T suppressor (CD3<sup>+</sup>CD8<sup>+</sup>), B lymphocyte (CD19<sup>+</sup>CD3<sup>-</sup>), memory B lymphocyte (CD19<sup>+</sup>CD27<sup>+</sup>) percentage determination were performed within 2 h of blood sampling. For peripheral blood analysis, peripheral blood cells were stained with monoclonal antibodies including anti CD3-PE (phycoerythrin) for CD3, anti CD4-FITC (fluorescein isothiocyanate) for CD4, anti CD8-FITC for CD8, anti

CD19-PE for CD19, anti CD25-PE for CD25, and CD27-FITC for CD27. Red blood cells were removed by incubating the blood samples with coulter TQ.prep' for 10 min in the presence of lyses buffer. Specific isotopes were used for each sample. Lymphocytes were identified according to described "forward scatter" and "side scatter" profiles and analysed by including in "gates for lymphocytes". Calculations were made by counting a total of 10.000 cells for each analysis. Lymphocytes were identified according to fluorescence characteristics of reacted monoclonal antibody and reported as percentage.

### Statistical Analysis

Parametric data from patient and controls were found to be normally distributed. Student's *t* test was used for statistical comparisons. Paired samples *t* test was used for comparing the baseline and one month treatment results. Parametric data were presented as mean  $\pm$  SD, while countable data being presented as percentage.  $P < 0.05$  was considered as significant.

### Results

At the time of diagnosis, percentage of the T lymphocytes, helper T lymphocytes, regulatory T lymphocytes and memory B lymphocytes were significantly lower in the patients group compared to the controls (Table 1). Suppressor T lymphocytes and B lymphocyte percentage in peripheral blood of patient and control groups was not significantly different (Table 1).

Seventeen out of 20 ITP patients received 1 mg/kg/day methylprednisolone treatment. One month after start of the corticosteroid treatment, the peripheral blood memory B lymphocyte percentage was significantly increased from the values at the time of diagnosis (Table 2). T regulatory cells did not change significantly after the treatment (Table 2).

Patients were followed up for three years from the time of diagnosis. Of the treated patients, sustained response was obtained in 10 out 17, and splenectomy was performed as

second line therapy in the remaining 7 patients with no response to the given steroid therapy or relapsed after the therapy. Percentages of regulatory T lymphocyte and memory B lymphocytes in peripheral blood among responders and non-responder ITP patients to first line therapy, at the time diagnosis and one month after first line therapy, was not significantly different (Tables 3, 4; Fig. 1).

At the time of diagnosis, there was no significant difference on the percentages of regulatory T lymphocytes and memory B lymphocytes among ITP patients with favorable prognosis (those not required therapy or successfully regressed by first line therapy) and poor prognosis (refractory to first line therapy with methylprednisolone or relapsed, requiring splenectomy) (Table 5; Fig. 1).

Data regarding to the other 3 patients with no indication of treatment was not analyzed due to the low number of patients in this group. Gross evaluation of this data revealed no prominent change percentages of regulatory T lymphocyte and memory B lymphocytes in peripheral blood of this group. Percentages of regulatory T lymphocyte and memory B lymphocytes were not evaluated following the splenectomy in the 7 patients.

### Discussion

In the present study it was found that the initial peripheral blood percentages of T lymphocytes, helper T lymphocytes, regulatory T lymphocytes and memory B lymphocytes in ITP patients were significantly lower than controls. This is in accordance with the previous studies which have reported lower levels of T regulatory lymphocytes in patients with chronic ITP compared to healthy controls [6–9]. Furthermore, by investigating the regulatory T lymphocyte levels in adult patients with ITP Teke et al. have reported lower levels of these cells at active stage of the disease [10]. Shu et al. reported lower regulatory T lymphocytes levels in ITP patients at the time of diagnosis as compared with post treatment levels and control values [11], also reporting a significant positive correlation

**Table 1** Peripheral blood lymphocyte subtype percentages of the patients at the time of diagnose, and controls

Lymphocyte subtypes	Patients n = 20	Control n = 20	<i>p</i>
Regulatory T lymphocytes (%)	0.25 $\pm$ 0.17	1.14 $\pm$ 0.77	0.0001
T lymphocytes (%)	67.6 $\pm$ 7.7	73.5 $\pm$ 4.4	0.006
Helper T lymphocytes (%)	40.9 $\pm$ 8.8	49.5 $\pm$ 4.4	0.001
Suppressor T lymphocytes (%)	28.4 $\pm$ 9.7	24.1 $\pm$ 3.4	NS
B lymphocytes (%)	16.1 $\pm$ 5.8	17.7 $\pm$ 4.9	NS
Memory B lymphocytes (%)	1.57 $\pm$ 1.24	4.38 $\pm$ 2.41	0.001

Values are expressed as mean  $\pm$  SD

NS Not significant

**Table 2** Percentages of peripheral blood regulatory T lymphocyte and memory B lymphocyte at the time of diagnose and one-month after therapy in patients with ITP

Lymphocyte subgroups	At the time of diagnose (n = 17)	One month after therapy (n = 17)	<i>p</i>
Regulatory T lymphocytes (%)	0.21 ± 0.15	0.50 ± 0.66	>0.05
Memory B lymphocytes (%)	1.66 ± 1.31	3.0 ± 1.7	<0.008

Values are expressed as mean ± SD

**Table 3** Initial values of regulatory T lymphocyte and memory B lymphocytes in peripheral blood among responders and non-responder ITP patients to the first line therapy

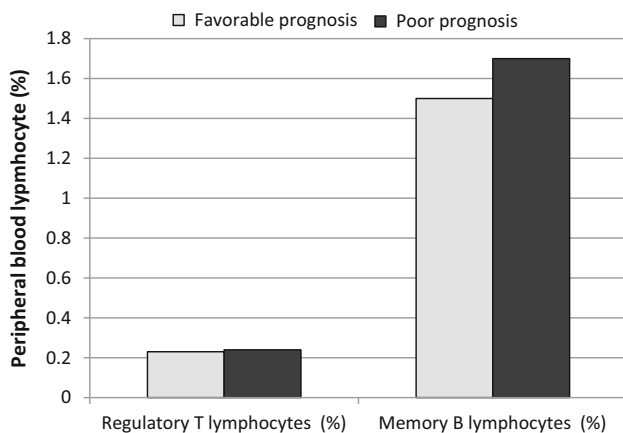
Lymphocyte subgroups	Responder to first line therapy (n = 10)	Non-responder to first line therapy (n = 7)	<i>p</i>
Regulatory T lymphocyte (%)	0.33 ± 0.30	0.16 ± 0.05	>0.05
Memory B lymphocyte (%)	2.1 ± 1.8	1.1 ± 0.75	>0.05

Values are expressed as mean ± SD

**Table 4** Post-methylprednisolone therapy percentages of regulatory T lymphocyte and memory B lymphocytes among responder and non-responder ITP patients

	Responder to first line therapy (n = 10)	Non-responder to first line therapy (n = 7)	<i>p</i>
Regulatory T lymphocyte %	1.75 ± 1.1	0.16 ± 0.05	>0.05
Memory B lymphocyte %	3.7 ± 2.2	4.4 ± 2.1	>0.05

Values are expressed as mean ± SD



**Fig. 1** Levels of peripheral blood regulatory T lymphocyte and memory B lymphocytes of ITP patients with favorable and poor prognosis

between the levels of regulatory T cells and thrombocyte counts.

Although there is evidence suggesting that the levels of regulatory T lymphocytes and memory B lymphocytes in patients with ITP is lower than controls, possible significance of this parameters in predicting disease outcome and

response to therapy remains undefined. We hypothesized that these parameters at the time of diagnosis could be useful in predicting disease course and therapy outcome, and tested this hypothesis in a group of adult patients with newly diagnosed ITP. And, we did not find any significant association between the levels of regulatory T lymphocytes and memory B lymphocytes and disease outcome including response to first line therapy with methylprednisolone, and concluded that regulatory T lymphocytes and memory B lymphocytes percentages are not a valid predictor of outcome in ITP. Yet, changes in memory B lymphocytes in ITP are among novel findings of this study.

Thus, regulatory T lymphocyte percentage was determined to be lower in the patient group. Regulatory T cell percentage was higher in responder versus non responder ITP patients to the steroid therapy, both at the time of diagnosis and at the end of one month therapy. This finding was striking, although statistical significance was not reached, may be due to low number of cases and relatively variability of this parameter among the patients. This implies that there may be a relation between response to first line medical therapy with methylprednisolone and percentage of T regulatory cells in ITP. As suggested by

**Table 5** Percentages of peripheral blood regulatory T lymphocyte and memory B lymphocytes of ITP patients with favorable and poor prognosis

	Favorable prognosis (n = 13)	Poor prognosis (n = 7)	<i>p</i>
Regulatory T lymphocytes %	0.23 ± 0.17	0.24 ± 0.16	>0.05
Memory B lymphocytes %	1.5 ± 1.5	1.7 ± 0.86	>0.05

Values are expressed as mean ± SD

Shu et al. [11], prominent increases in regulatory T lymphocyte percentages may be an early indicator of therapy effectiveness.

The decrease in the percentage of T regulatory lymphocytes may be responsible from the increased level of peripheral blood mononuclear cell proliferation and interleukin 2 secretion and increased production of antiplatelet antibody [12]. Moreover, the decrease in the number of T regulatory cells could be responsible from proinflammatory Th1 cytokine response and Th2 production in ITP patients who entered remission [13]. The regulatory T cells can directly suppress autoreactive lymphocytes by cell-to-cell contact as well as causing their inhibition through stimulation of secretion of cytokines including interleukin 10 and transforming growth factor beta. Although the currently available evidence indicates role of decreased regulatory T lymphocytes in the ITP pathogenesis, the level of decrease in the percentages and functions of these cells and biological basis of these changes needs further clarification.

The major function of B lymphocytes is production of antibodies against specific antigens. When naïve or memory B cells are activated by antigen, they proliferate and differentiate into effector cells. The effector cells produce and secrete antibodies. There are reports about changes of peripheral blood memory B lymphocyte subpopulations in a variety of autoimmune diseases [14–16]. A relation between distribution and activity of peripheral blood memory B cells and disease activity has been reported in SLE [14]. Lower level of peripheral blood memory B cells has been reported in patients with Sjögren's syndrome, and this was suggested to be due to their accumulation in the inflamed salivary glands [15]. However, results in the literature on the peripheral blood memory B lymphocytes in rheumatoid arthritis (RA) are controversial. Yet, Souto-Carneiro et al. [16] reported a significantly lower frequency of peripheral blood memory B cells in RA patients than healthy controls, which was independent of disease duration. In agreement with the reports from patients with Sjögren syndrome and RA [15, 16], we determined lower level of peripheral blood memory B cells in patients with ITP.

Despite a significant number of investigations concerning changes in peripheral blood memory B cells in various autoimmune diseases, there is only limited number of published investigation on disturbances in peripheral blood

memory B cells in ITP. By investigating the effects of splenectomy on circulating memory B cells Martinez-Gamboa et al. [17] determined decreased frequencies of memory B cells in splenectomized ITP patients. Considering the location of memory B lymphocytes, this is an expected finding. In the latest study [17] involving limited number of ITP patients, although no statistical analysis being performed, pre-splenectomy levels of peripheral blood memory B lymphocytes in ITP patients was reported to be lower than controls. Despite their evident role in autoimmunity, the finding of lower rate of peripheral blood memory B lymphocytes could be due to their accumulation in target tissues including spleen, liver and bone marrow for thrombocyte destruction. Thus, at the end of one month first line therapy with prednisolone the peripheral blood memory B cells percentage was significantly increased and reached to comparable levels compared to those of control group, but the percentage of peripheral blood memory B cells was not different among ITP patients with favorable or poor therapy outcomes, in our cohort.

One of the limitation of this report could be the low number of cases involved. But, ITP is a rare disease with an estimated number of new cases of 1.6–3.9/100.000 per year. The study was performed in a university hospital setting. So, mild cases are usually admitted and get treated in primary or secondary care hospitals. And, rather low number of cases allocated to our study. Detailed description of inclusion and exclusion criteria are given in methods section.

In this cohort, by following the ITP patients for three years from the time of diagnosis, we fail to identify any significant impact of the initial peripheral blood regulatory T lymphocytes and memory B lymphocytes levels on disease progress or therapy outcome.

#### Compliance with Ethical Standard

**Conflict of interest** None

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