Alteration in Frequency and Function of CD4⁺CD25⁺FOXP3⁺ Regulatory T cells in Patients with Immune Thrombocytopenic Purpura

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ABSTRACT

Immune thrombocytopenic purpura (ITP) is an autoimmune bleeding disorder characterized by production of auto-antibodies against platelet antigens. It is obvious that regulatory T cells (Tregs) have a major role in controlling immune homeostasis and preventing autoimmunity.

To investigate the frequency and functions of Tregs, twenty ITP patients and twenty age- and sex-matched healthy controls were recruited. The peripheral blood mononuclear cells were isolated and the proportion of Tregs was defined by flow cytometry method. The expression of immune-regulatory markers, cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and glucocorticoid induced tumor necrosis factor receptor (GITR) were also assessed by quantitative Real-time polymerase chain reaction TaqMan method. For evaluation of Treg function, Tregs were enriched and their ability to inhibit proliferation of T cells was measured and levels of immune-regulatory cytokines IL-10 and Transforming growth factor beta (TGF-β) were also measured. Results showed that the frequency of Tregs and the mean fluorescence intensity of forkhead box P3 (FOXP3) protein significantly decreased in ITP patients compared to those in healthy controls. In addition, there was a significant reduction in relative expression of both CTLA-4 and GITR mRNA in ITP patients (p=0.02 and p=0.006, respectively). The suppressive function of Tregs also diminished in ITP patients compared to controls. Both IL-10 and TGF-β cytokines were produced in lower amounts in ITP patients than controls.

It could be concluded that alteration in Treg frequency and functional characteristics might be responsible for loss of self-tolerance and subsequently destructive immune responses observed in ITP patients.

Keywords: Immune thrombocytopenic purpura (ITP); Regulatory T cells (Tregs); Suppressive function
INTRODUCTION

To prevent autoimmune diseases, the immune system has evolved different mechanisms for maintaining self-tolerance known as “recessive and dominant tolerance”.1,2 The “recessive tolerance” is performed by negative selection of high affinity clones in thymus while “dominant tolerance” is mediated by specialized cells in periphery called “regulatory T cells (Tregs)”.1,2 Several subtypes of Tregs have been identified among them the CD4+CD25+FOXP3+ Tregs; also called natural Tregs (nTregs); have taken more attention. The nTregs develop in thymus and have a central role in regulation of immune response in various conditions like allergic diseases, human malignancies, infections and autoimmune disorders.1,3,4 Several regulatory mechanisms by Tregs have been introduced in which both direct cell-cell contact as well as production of soluble cytokines have been highlighted.1,3,4 The role of Tregs in pathogenesis of autoimmune diseases has been investigated in some studies and its association with clinical parameters has also been shown.5-10

Immune thrombocytopenic purpura (ITP) is an organ specific autoimmune disease characterized by production of autoantibodies against platelet antigens, which might result in destruction of platelets by reticuloendothelial system (RES).11-13 Although presence of autoreactive B cells producing autoantibodies against platelets has been considered as the hallmark of the disease, defective cellular immune response has also been known to contribute in the pathophysiology of the ITP.14-16 In this regard, platelet-specific autoreactive T cells predominantly with Th1 phenotype have been recognized in ITP patients.17-19 Therefore, break-down of self-tolerance might account for pathogenic immune responses in ITP patients. There are few reports describing defective Treg frequency and functions in ITP patients and its correlation with clinical phenotype like disease severity.20-22

In this study, the frequency and function of Tregs in ITP patients were evaluated. In addition, the mRNA expression of Treg surface markers, cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and glucocorticoid induced tumor necrosis factor receptor (GITR) and the levels of immune-regulatory cytokines, IL-10 and Transforming growth factor beta (TGF-β), which are associated with Treg inhibitory functions, were assessed in ITP patients.

PATIENTS AND METHODS

Subjects
Twenty ITP patients (11 males and 9 females, with mean age of 16.2±9.09 years) who referred to Hematology Department of Emam Khomeini Hospital were enrolled. The ITP was diagnosed based on the clinical evaluation, platelet count <100×10^9/L, normal bone marrow megakaryocytes without any morphological evidence of dysplasia and no secondary disease that could be associated with thrombocytopenia. Among patients, 12 had chronic ITP and 8 patients had acute disease. None of patients had history of splenectomy.

Twenty age- and sex-matched healthy volunteers (mean age 19.35±7.7 years who had no history of autoimmune diseases and/or malignancies were included as control group. The study was approved by the ethics committee of Tehran University of Medical Sciences and written informed consent was obtained from all participants before sampling.

Isolation of Peripheral Blood Mononuclear Cells
The blood samples were collected in Ethylene diamine tetra acetic acid (EDTA) containing tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque (Lymphoflot, BIO-RAD, Germany). Cells were washed once with RPMI 1640 (Sigma, Germany) and prepared for surface staining.

Immunofluorescent Staining of the Cells
Cells (1×10^5) were resuspended in 100 µl flow cytometry staining buffer (eBioscience, San Diego, CA, USA) and incubated with fluorescein isothiocyanate (FITC) labeled anti-CD4 (clone RPA-T4, eBioscience) and Phycoerythrin (PE) labeled anti-CD25 (clone BC96, eBioscience) antibodies for 30 min at 4 °C in the dark. After permeabilization with Fixation/Permeabilization buffer (eBioscience), cells were incubated with PE/Cy5-conjugated mouse IgG1 and PE-Cy5 conjugated rat IgG2a antibodies were used. Using a Partec flow cytometer (Partec PAS, Germany), lymphocytes were first gated based on their forward
and side scatters. Then, the percentage of Tregs was defined by calculating the CD25+ FOXP3+ double positive populations within CD4+ gate (Figure 1). Data were analyzed with FlowMax software (Partec PAS, Germany).

**Quantitative Real-Time PCR Analysis**

Total RNA was extracted using QIAzol lysis reagent (Qiagen GmbH, Hilden, Germany) followed by cDNA synthesis with M-MuLV reverse transcriptase enzyme (Fermentase Life Science, EU). Quantitative Real-time PCR was performed using TaqMan Premix Ex Taq™ (Perfect Real-Time) master mix (Takara, Japan). For increasing the validation of the test, two housekeeping genes were selected; TBP (TATA binding protein) and YWHAZ (a signal transducer molecule that binds to phosphoserine-containing proteins). All PCR primer pairs and probes are shown in table 1. The Real-Time PCR was carried out in duplicate wells using ABI 7500 machine (Applied Biosystems) and the Ct values for target and housekeeping genes were calculated. The expression levels were normalized to both TBP and YWHAZ housekeeping genes and the fold changes in expression of CTLA-4 and GITR genes in ITP patients compared to controls were calculated with REST 2009 software.

**Cell Purification and Functional Assays**

Tregs were enriched from PBMCs by magnetic cell sorting (MACS) method using human CD4+CD25+ regulatory T cell isolation kit (Miltenyi Biotec, Germany). First, CD4+ T cells were isolated by negative selection of CD4+ T cells followed by positive selection of CD25+ T cells from CD4+ population. The purity of isolated cells was determined by flow cytometry which was >96%. The separated CD4+CD25+ and CD4+CD25- populations were used as Tregs and responder T cells (Tres), respectively. MACS isolated Tres cells (1×10⁴ cells) were cultured with Tregs (1×10⁴ cells) in ratio of 1:1 or incubated alone in flat bottom 96-well plates (Nunc, Denmark) containing 200µl RPMI 1640 supplemented with 10% FBS, 100U/mL penicillin, 100 µg/mL streptomycin. For stimulation of cell proliferations, cells were treated with 5µg/ml soluble functional grade purified anti-CD3 (OKT3) (Clone UCHT1, eBioscience) and 5µg/ml anti-CD28 (Clone 28.2, eBioscience) antibodies in the presence of 2×10⁴ autologous irradiated PBMCs (3000 rad) as antigen presenting cells (APCs). Cell proliferation was assessed with BrdU cell incorporation ELISA kit (BrdU Proliferation ELISA Kit, Roche Applied Biosystems, Germany) in triplicate wells. After 5 days of culture, 20 µl BrdU was added to all wells and cells were cultured for the next 16 hours. The percentage of suppression was calculated with following formula:

\[1-\text{OD of Treg-Tres co-culture/OD of Tres cells alone}] \times 100\%

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer and probe sequences</th>
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<tbody>
<tr>
<td><strong>CTLA-4</strong></td>
<td>5′-CATGGACACGGGACTCTACAT-3′</td>
</tr>
<tr>
<td></td>
<td>5′-GCACGGTTCTGGATCAATTACATA-3′</td>
</tr>
<tr>
<td></td>
<td>5′-FAM-TGCAAGTTGGAGCTCATGTACCC-TAMRA-3′</td>
</tr>
<tr>
<td><strong>GITR</strong></td>
<td>5′-TGCAAACCTTGAGACAGACTGC-3′</td>
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<td></td>
<td>5′-ACAGCGTGTGTTGGTCTTGGTC-3′</td>
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<td>5′-FAM-CCAGTTCCGGTTTTGCTCAGTGTCC-TAMRA-3′</td>
</tr>
<tr>
<td><strong>YWHAZ</strong></td>
<td>5′-AAAGTGCTGGTACCCCATAATGCTT-3′</td>
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<tr>
<td></td>
<td>5′-GTCGTAGAGATGTGTTGGTGTC-3′</td>
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<td></td>
<td>5′-FAM-TATGTTGTGTTGACACTGATGCAATCCC-TAMRA-3′</td>
</tr>
<tr>
<td><strong>TBP</strong></td>
<td>5′-TTCCGGAGTGTTCTGGATTTGGA-3′</td>
</tr>
<tr>
<td></td>
<td>5′-TGGACGTCTCTTTCACCTGTGGGC-3′</td>
</tr>
<tr>
<td></td>
<td>5′-FAM-CCGTGGTTCTGGGCTTATCCTCA-TAMRA-3′</td>
</tr>
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Measurement of Cytokines

The supernatants of Treg/Tres co-culture were collected at day 5 of culture before adding BrdU. The levels of IL-10 and TGF-β were measured with human IL-10 and TGF-β ELISA kits (eBiosciences) according to the manufacturer’s instructions.

Statistical Analysis

Comparison between patients and healthy controls was carried out with student pair t-test and for more than two groups, ANOVA test was used. For correlation analysis, Pearson correlation test was performed. P-values less than 0.05 were considered significant. Statistical analysis was performed using a commercially available software package (SPSS Statistics 17.0.0, SPSS, Chicago, Illinois).

RESULTS

Frequency of Tregs in ITP Patients and Controls

The frequency of CD4^+CD25^+FOXP3^+ Tregs was determined in both patient and control groups. The results showed that the frequency of Tregs significantly decreased in ITP patients compared to those in healthy controls (1.6±0.6 vs. 3.57±1.07, p<0.001) (Figure 2). Then, the expression of FOXP3 protein was evaluated based on its mean fluorescence intensity (MFI). It was observed that in addition to Treg proportion, the MFI of FOXP3 protein was also reduced in ITP patients (2.6±0.57 vs. 3.83±0.98, p<0.001) (Figure 3). Moreover, there was a positive correlation between the Treg frequency and MFI of FOXP3 protein based on intragroup analysis (r=0.49, p=0.02). No significant correlation was seen between the frequency of Tregs and platelete count in ITP patients (r=0.44, p=0.07).

Figure 1. Flow cytometric analysis of Tregs. The lymphocytes were first selected based on their side and forward scatters. Then, the CD25^+FOXP3^+ double positive cells were defined in CD4 gate (R2) and considered the Treg populations (Q2); “a” is representative of an ITP patient and “b” is representative of a healthy control.

Figure 2. The frequency of Tregs in ITP patients and healthy controls. The horizontal line indicates the mean frequency of Tregs.

Figure 3. The expression of FOXP3 (MFI) in ITP patients and healthy controls. The horizontal line indicates the mean MFI of FOXP3.
Expression of CTLA-4 and GITR mRNA in ITP Patients and Controls

The relative expression of CTLA-4 and GITR mRNA was quantified in patients in relation to healthy controls and the fold changes in their mRNA were quantified. There was a significant reduction in relative expression of both CTLA-4 (3.4 fold) and GITR (4 fold) mRNA in ITP patients ($p=0.02$ and $p=0.006$, respectively) (Figure 4).

Treg Function in ITP Patients and Healthy Controls

The suppressive function of Tregs was evaluated by the ability of Tregs to suppress the proliferation of Tres in Treg-Tres co-culture. The results demonstrated that suppressive function of Tregs was markedly diminished in ITP patients compared to that in controls (20.1±2.6 vs. 40.8±13.8, $p<0.001$) (Figure 5). To define the possible mechanisms involving in Treg-mediated suppression, the levels of immune-regulatory cytokines, IL-10 and TGF-β, produced by Tregs were evaluated in Treg-Tres co-culture. The results showed that both IL-10 and TGF-β cytokines were produced in lower amounts in ITP patients compared to controls (113.8±56.7 vs. 355.1±286.2, $p=0.02$ for IL-10 and 46.11±10.38 vs. 85.5±36.9, $p=0.01$ for TGF-β; Figure 6). No correlation was seen between Treg suppressive function and the levels of IL-10 and TGF-β cytokines ($r=0.6$, $p=0.13$, and $r=0.18$, $p=0.68$, respectively).

DISCUSSION

In this study, the frequency and functions of Tregs were evaluated in ITP patients. The results showed that the frequency of Tregs and expression of Treg-lineage specific marker FOXP3 were diminished in ITP patients. Consistent with our results, there are some reports describing reduced numbers of Tregs and FOXP3 protein at mRNA level in ITP patients.20-22 Alterations in thymic output, half-life of Tregs in circulation and tissue distribution of Tregs, all can affect the number of Tregs in blood.23 In addition, since FOXP3 protein is used as a specific marker for identification of Treg subsets,13,14 it is reasonable that the reduction in Treg counts may be relevant to decrease in FOXP3 protein expression. In addition, it has been shown that as a consequence of FOXP3 down-regulation, some CD4+CD25+FOXP3- Tregs may convert into CD4+CD25-FOXP3+ T cells which
have effector T cells rather than Treg functional characteristics, which might be accompanied with increased risk of autoimmunity.\textsuperscript{24,25}

Our results also showed that both CTLA-4 and GITR mRNA levels were decreased in ITP patients. To the best knowledge of the authors, this is the first time that CTLA-4 and GITR genes were evaluated in ITP patients. Immune suppression via the surface receptors like CTLA-4 and GITR are known as main mechanism of Tregs regulatory functions.\textsuperscript{1,3,4} Having high affinity for CD80/CD86 receptors, the CTLA-4 compete with CD28 markers on effector T cells for binding to these ligands on the surface of APCs, thus limiting the over-activation of effector T cells.\textsuperscript{1,26} CTLA-4 can also contribute in generation of tolergenic dendritic cells.\textsuperscript{1} Like CTLA-4, the GITR molecule is constitutively expressed at high levels on Tregs and participates in activation and proliferation of Tregs during the effector phase of the immune responses.\textsuperscript{28,29}

Analysis of the function of Tregs indicated that in addition to their frequency, the function of Tregs was also decreased in ITP patients. Few studied have assessed the function of Tregs in ITP patients which reported the reduction of their function in these patients.\textsuperscript{20,22} To define the possible role of immune-regulatory cytokines, IL-10 and TGF-β in Treg-mediated suppressive function, the levels of these cytokines were evaluated for the first time in ITP patients. Our findings indicated that both IL-10 and TGF-β cytokines were markedly produced in lower levels in ITP patients than healthy controls. Previous studies have shown that the levels of IL-10 and TGF-β cytokines as well as percent of IL-10-producing Tregs were reduced in ITP patients.\textsuperscript{17,19,30,31}

IL-10 is a regulatory cytokine involved in immune tolerance and IL-10-secreting regulatory T cells like CD4\textsuperscript{+}CD25\textsuperscript{+}FOXP3\textsuperscript{+} Tregs and Tr1 cells are considered as an additional mechanism responsible for peripheral tolerance.\textsuperscript{17,32} TGF-β was found to be an important inhibitor of B-cell proliferation and autoantibody production.\textsuperscript{33} It also suppresses some Th1 and Th2 cell-mediated autoimmune disease.\textsuperscript{34} Accordingly, it seems that abnormal production of these cytokines by Tregs may provide additional mechanisms responsible for deleterious immune reactions occurring in ITP patients.

Taken together, the results of this study showed that both Treg frequency and function were defective in ITP patients. In addition, it was demonstrated for the first time that the mRNA levels of CTLA-4 and GITR surface markers as well as the concentration of regulatory cytokines IL-10 and TGF-β produced by Tregs, all were disturbed in ITP patients. Therefore, it could be concluded that alteration in Treg frequency and functions might be responsible for loss of self-tolerance and consequently destructive immune responses observed in ITP patients. Evaluation of Th17 cells or other helper T cell subtypes could give a better understanding of involved mechanism in pathogenesis of ITP.

**ACKNOWLEDGEMENTS**

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