Reduced CD4⁺CD25⁺ T cells in patients with idiopathic thrombocytopenic purpura

Miho Sakakura⁠a, Hideo Wada⁠b,⁎, Isao Tawara⁠a, Tsutomu Nobori⁠b, Takashi Sugiyama⁠c, Norimasa Sagawad, Hiroshi Shiku⁠a

⁎ Corresponding author. Department of Laboratory Medicine, Mie University Graduate School of Medicine, 2–174 Edobashi, Tsu, Mie 514–8507, Japan. Tel.: +81 59 232 1111; fax: +81 59 231 5204.
E-mail address: wadahide@clin.medic.mie-u.ac.jp (H. Wada).

Abstract Immunoregulatory CD4⁺CD25⁺ T cells play an important role in the induction and maintenance of peripheral self-tolerance. These professional regulatory cells prevent the activation and proliferation of potentially autoreactive T cells that have escaped thymic deletion. Therefore, CD4⁺CD25⁺ T cells are believed to possibly play an important role in pathogenic autoimmune diseases. We measured the count of CD4⁺CD25⁺ T cells in 44 patients with idiopathic thrombocytopenic purpura (ITP), and the number of CD4⁺CD25⁺ T cells and clinical features were then analyzed. By using a flow cytometric analysis, the number of CD4⁺CD25⁺ T cells in the patients with ITP showed a very wide distribution in comparison to healthy volunteers. The number of CD4⁺CD25⁺ T cells was significantly lower in the ITP patients in the severe phase, and in patients positive for anti-glycoprotein IIb-IIIa antibody. However, the number of those cells increased in the patients at the complete remission phase, especially after a splenectomy. The Foxp3 mRNA levels of peripheral blood mononuclear cells (PBMC) of ITP patients were higher with an improved platelet count than in those with a low platelet count. In addition, the Foxp3 mRNA levels closely correlated with the number of CD4⁺CD25⁺ cells. These mechanisms remain to be fully elucidated, however, the count of CD4⁺CD25⁺ T cells is considered to possibly be related to the severity of ITP.

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Foxp3;
Platelet count

Introduction

Some autoimmune diseases such as Grave’s disease, myasthenia gravis and immune cytopenias have...
been recognized to be mediated by pathogenic autoantibodies. It is evident that the autoantibody production by B cells requires the presence of autoantigens-specific T cells [1]. Indeed, autoreactive T cells have been identified in patients with various systemic or organ-specific autoimmune diseases [2-4]. On the other hand, autoreactive T cells are also present in peripheral blood (PB) of a healthy individual without any evidence of autoimmune disease [5]. These findings thus suggest that some mechanisms exist which regulate these autoreactive T cells in order to thereby prevent autoimmune disease.

Naturally arising CD4+CD25+ T cells, which regulate autoreactive T cells, have been described in rodent models [6-8]. These professional regulatory cells prevent the activation and proliferation of potentially autoreactive T cells that have escaped thymic deletion. In animal models, the elimination or inactivation of CD4+CD25+ T cells is known to result in various severe autoimmune diseases [8,9]. In humans, CD4+CD25+ T cells have also been reported to have a similar regulatory function to that of the murine population as they were anergic to stimulation by T cell receptor cross-linking (TCR) in the absence of exogenous IL-2 and also regarding their ability to suppress the activation of other T cells in a cell-contact dependent manner that could not be inhibited by blocking such cytokines as IL-10. An increased number of CD4+CD25+ cells was found in patients with autoimmunity, cancer, or chronic infection [10-13]. It has recently been shown that the effector function of CD4+CD25+ had a marked decrease in multiple sclerosis (MS) [14].

Idiopathic thrombocytopenic purpura (ITP) is one of the autoimmune diseases characterized by an increased platelet clearance caused by anti-platelet autoantibodies. Although Kuwana et al. [15] reported that autoreactive CD4+ T cells to glycoprotein on platelet membrane (GpIb-IIIa, GpIIb) mediate anti-platelet autoantibody production in patients with ITP, the role of CD4+CD25+ Tcells in ITP remain to be elucidated.

In this study, we defined regulatory CD4 T cells as high CD4+CD25+ T cells, and we studied the number of CD4+CD25+ T cells in patients with ITP, and analyzed the relationship of the regulatory cells and clinical features.

Materials and methods

Patients

ITP was defined as thrombocytopenia (platelet count <100 ×10^9/L), normal or increased bone marrow (BM) megakaryocytes without any morphological evidence for dysplasia, and no secondary immune or nonimmune diseases that account for the thrombocytopenic state. In this study, the PBMC from 44 patients with ITP and 21 healthy volunteers was examined. The study protocol was approved by the Human Ethics Review Committees of Mie University Graduate School of Medicine and a signed consent form was obtained from each subject. Among these ITP patients, 7 were newly diagnosed while 37 were in the chronic phase (CP) when this study was done. The patients with ITP were classified into three groups according to their platelet count, as follows: Group I; platelet <50 ×10^9/L, Group II; platelet 5-10 ×10^9/L, Group III; platelet >10 ×10^9/L.

Flow cytometry

PBMC were isolated by Ficoll density gradient centrifugation, then the CD4+CD25+ T cell population was measured using a flow cytometric analysis. The cells were washed with PBS with 2% FCS, and then were stained for 20 min at 4 °C with an optimal dilution of each antibody for Cy-Chrome labeled anti-CD4 and FITC labeled anti-CD25 (BD Biosciences). The cells were washed again and analyzed by flow cytometry (FACScalibur™ and CELLQuest™ software; Becton Dickinson).

ELISA assay for anti-platelet antibodies

PAIgG was measured using alkaline phosphatase-conjugated anti-human IgG (Sigma Co, St Louis, Missouri, USA) by a competitive enzyme immunoassay. Antibody to platelet glycoproteins (GP) I Ib-IIIa was detected using a platelet antibody screening kit (GTI PAKPLUS, Wisconsin, USA). The serum was incubated in microtiter wells which had been pre-coated with platelet GPIb-IIIa. An alkaline phosphatase labeled anti-human globulin reagent was added to the wells. After a brief incubation period, the microtiter wells were washed, and the enzyme substrate PNPP (p-nitrophenyl phosphate) was added. The optical density of the color produced was measured in an ELISA reader at a wavelength of 405 or 410 nm.

Quantitative real-time polymerase chain reaction analysis

The mRNA was extracted using an MACS mRNA Isolation Kit (Miltenyi Biotec, Auburn, California, USA) according to the manufacturer’s instructions, and cDNA was prepared with 2.5 μM random hexamers.
(Applied Biosystems Inc., Foster City, California, USA). The mRNA level was quantified by real-time PCR using the iCycler iQ Real-Time PCR Detection System (Applied Bio-Rad Laboratories Inc.). Amplification was carried out in a total volume of 20 μl for 50 cycles of 15 s at 95 °C, 1 min at 60 °C, and the product was detected using SYBR Green I dye (Molecular Probes Inc., Eugene, Oregon, USA). Samples were run in triplicate, and their relative expression was determined by normalizing the expression of each target to GAPDH, and then comparing this normalized value to the normalized expression in a reference sample to calculate a fold-change value. The primers were designed so that amplicons spanned intron/exon boundaries to minimize the amplification of genomic DNA. The primer sequences were as follows: GAPDH: sense, 5′-CCC ATG TTC GTC ATG GGT GT-3′ and anti-sense, 5′-TGG TCA TGA GTC CTT CCA CGA TA-3′; Foxp3: sense, 5′-CCC AGG AAG GAC AGC ACC CTT-3′ and anti-sense, 5′-TTC TCA CAT CCG GGC CAC TTG-3′.

**Statistical analysis**

All data were expressed as the mean ± SD or median values. Differences between the groups were examined for statistical significance using the Mann-Whitney’s U test while the correlation between two variables was tested by Pearson’s correlation analysis. A P value of less than 0.05 denoted the presence of a statistically significant difference.

**Results**

**Patients**

In this study, 44 cases with ITP were studied. Among these patients, the median age at examination was 48 years (range, 20-80) consisting of 16 males and 28 females. Median platelet count at this study was 56 × 10^9/L (range, 2-403 × 10^9/L). Thirty patients had been taking various medications for ITP until this examination, specifically, 23 patients had been taking prednisolone, 5 patients had been taking immunosuppressive therapy, and 2 patients had been taking other drugs. A splenectomy was performed in 8 cases for the treatment of ITP (Table 1).

### Table 1 Character of patients with ITP

<table>
<thead>
<tr>
<th>Character</th>
<th>ITP (n=44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>16 Male, 28 Female</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>48 (20-80)</td>
</tr>
<tr>
<td>Median platelet count (×10^9/L)</td>
<td>56 (2-403)</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
</tr>
<tr>
<td>Prednisolone</td>
<td>14</td>
</tr>
<tr>
<td>Immune suppressive therapy</td>
<td>23</td>
</tr>
<tr>
<td>Others</td>
<td>5</td>
</tr>
<tr>
<td>Splenectomy</td>
<td>2</td>
</tr>
<tr>
<td>(+)</td>
<td>8</td>
</tr>
<tr>
<td>(−)</td>
<td>36</td>
</tr>
</tbody>
</table>

**Number of CD4+CD25+ cells in the patients with ITP**

In each case, the percentage of CD4+CD25+ cells in the PBMC was examined by flow cytometry, and then the number of CD4+CD25+ cells was calculated. The mean number of CD4+CD25+ cells in healthy volunteers was 69.2 ± 13.6 × 10^6/L and that of 44 patients with ITP was 73.8 ± 38.3 × 10^6/L. In comparison to the healthy volunteers, the number of CD4+CD25+ cells in the patients with ITP showed a very wide distribution (Fig. 1) (to analyze the relationship between the platelet count and the number of CD4+CD25+ cells, ITP were classified into three groups according to their platelet count; Group I <50 × 10^9/L, 50 × 10^9/L ≤ Group II ≤ 100 × 10^9/L, Group III >100 × 10^9/L). All patients in Group III were treated by some type of medication and their platelet count thus improved. As shown in Fig. 2, the number of CD4+CD25+ cells of the patients in Group I (59.7 ± 27.5 × 10^6/L) were closely similar to those of the healthy volunteers. On the other hand, the number of CD4+CD25+ cells of the patients in Group III (100.8 ± 37.3 × 10^6/L) was

![Figure 1](https://example.com/figure1.png)
significantly higher than that of healthy volunteers. All together, the number of the CD4+CD25+ cells in the patients before treatment was similar to that of the healthy volunteers (data not shown).

Association between the number of CD4+CD25+ cells and a splenectomy

A splenectomy is one of the effective treatments for ITP, therefore, the number of CD4+CD25+ cells was investigated in the ITP patients who had undergone a splenectomy (Fig. 3). In 8 patients treated with a splenectomy, the platelet counts improved in 5 patients. It is very interesting that the number of CD4+CD25+ cells in the patients who had undergone a splenectomy was significantly higher (108.2 ± 33.4 × 10⁶/L) than in either those without a splenectomy (P=0.038) or healthy volunteers (data not shown). Furthermore, 8 patients who had their

![Figure 2](image2.png)

**Figure 2** The number of CD4+CD25+ cells in patients with ITP for various platelet counts. I; less than 50×10⁹/L of platelet count, II; 50-100×10⁹/L of platelet count, III; more than 100×10⁹/L of platelet count. *P=0.018.

![Figure 3](image3.png)

**Figure 3** The number of CD4+CD25+ cells in the ITP patients with or without a splenectomy. The number of CD4+CD25+ cells was 66.2 ± 35.3 × 10⁶/L in the patients without a splenectomy and 108.2 ± 33.4 × 10⁶/L in those with a splenectomy. *P=0.038.

![Figure 4](image4.png)

**Figure 4** Relationship between the effect of splenectomy and the number of CD4+CD25+ cells. Eight patients with ITP treated splenectomy were classified into two groups according to the effect of a splenectomy. In low responders, the increase in the platelet count was less than 10×10⁹/L after a splenectomy. In high responders, the increase in the platelet count was more than 10×10⁹/L after a splenectomy.

![Figure 5](image5.png)

**Figure 5** Relationship of between the anti-platelet antibody and the number of CD4+CD25+ cells.
spleens removed were classified into two groups according to their effect; namely low responders in which the platelet count did not change or changed by less than $100 \times 10^9/L$ after a splenectomy, and high responders which showed an improvement in their platelet count of more than $100 \times 10^9/L$ after a splenectomy. As shown in Fig. 4, the number of CD4+CD25+ cells was significantly higher in the high responders than that in the low responders ($P=0.0006$).

**Relationship with the number of CD4+CD25+ cells and the anti-platelet antibodies**

In many ITP patients various anti-platelet antibodies which recognize such platelet membrane glycoproteins as GPIIb-IIIa, GPlla-IIa, GPll/IX, and so on, have been reported [16-18]. The most common target recognized by anti-platelet antibodies in ITP patients is GPIIb-IIIa. Antibodies against platelet membrane GPIIb-IIIa were detected in 6 of 33 patients (18.2%). Although no statistical significance was observed, the number of CD4+CD25+ cells tended to be low in the patients who were positive for antibody against GPIIb-IIIa ($P=0.078$) (Fig. 5).

**Expression mRNA levels of Foxp3 in PBMC from patients with ITP**

Since CD25 is also expressed on activated T cells. It is necessary to identify a unique cell marker which is expressed by regulatory T cells. Foxp3 is found in regulatory T cells and cells transfected with Foxp3 are known to have a regulatory function. Therefore, the expression of Foxp3 is considered as a specific marker for regulatory cells. Finally, the expression levels of Foxp3 in PBMC from ITP were investigated using real-time RT-PCR. As shown in Fig. 6, the expression mRNA level of Foxp3 in PBMC was significantly higher in the patients with more than $50 \times 10^9/L$ of platelet count than in those with less than $50 \times 10^9/L$ ($P=0.05$). All together, the expression levels of Foxp3 correlated well with the number of CD4+CD25+ cells.

**Discussion**

The mechanisms to maintain immunological self-tolerance in the peripheral blood and thymus, as the clonal deletion of self reactive T cells in the thymus (negative selection) and anergy in peripheral blood (passive tolerance), are present. Nevertheless, potentially pathogenic autoreactive T cells which avoid these mechanisms are present in the peripheral blood of healthy individuals. The dominant mechanisms to regulate autoreactive T cells and prevent autoimmune disease exist. Recently, some investigators have described the role of CD4+CD25+ T cells in the regulation self-reactive T cells in the murine models.

Chronic immune thrombocytopenic purpura (ITP) is an autoimmune disease characterized by an increased platelet clearance caused by anti-platelet autoantibodies, which bind to circulating platelets, thus resulting in the destruction of platelet autoantibody complex by the reticuloendothelial system [19,20]. The major targets of anti-platelet antibodies have been reported to be platelet membrane GPs, including GPIIb-IIIa, GPIIb-IX and GPlV [21], however, the mechanisms of onset and maintenance of the disease are still unclear. In 1998, Kuwana et al. reported that CD4+ T cells to GPIIb-IIIa are involved in production of anti-platelet autoantibody in ITP patients and are related to the pathogenic process in chronic ITP [15]. From these findings, it is speculated that a disorder of the regulatory T cells which regulate autoreactive T cells thus exists regarding the onset and maintenance of disease in ITP as other autoimmune diseases.

In this study, the numbers of CD4+CD25+ in the patients with ITP showed a very wide distribution. However, no reduction in the number of CD4+CD25+ cells was found in the group with a low platelet count including the onset cases, thus suggesting that the number of CD4+CD25+ T cells are not related with the onset of ITP. However, the number of CD4+CD25+ cells was significantly high in the cases with a platelet count of more than $100 \times 10^9/L$. In all such cases, the recovery of the platelet count occurred after various types of medication, thus suggesting that CD4+CD25+
T cells may be related to the effect of these medications. However, the number of CD4+CD25+ T cells was not significantly high in the patients not demonstrating a recovery of the platelet count. These findings suggest that CD4+CD25+ T cells play an important role in the recovery of the platelet count.

It is interesting that the number of CD4+CD25+ cells was found to be significantly high in the patients treated with a splenectomy [22]. Although the detailed mechanisms for the increase of CD4+CD25+ cells after a splenectomy remain to be elucidated, our results suggest that an increased number of CD4+CD25+ T cells may possibly have an immunosuppressive effect on ITP.

Foxp3, which encodes a forkhead/winged-helix transcription factor designated as Scurfin [23-26]. IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is characterized by multi-organ autoimmune diseases, allergy and IBD, and XLAAD (X-linked autoimmunity-allergic dysregulation syndrome) in human, or scurfy, a mouse mutant strain which succumbs to X-linked recessive autoimmune/inflammatory disease, are caused by a mutation of Foxp3 [27]. Recently, Foxp3 has been shown to be specifically expressed in CD4+CD25+ T cells in thymus and peripheral blood of normal mice, and it is a key regulatory gene for the development of regulatory T cells [28].

In this study, the expression levels of Foxp3 were significantly high in cases with improved platelet counts. In this investigation, CD4+CD25+ cells closely correlated with Foxp3. These results also suggested that regulatory T cells were thus related to the activity of ITP.

ITP is a very heterogenous disease, which has various clinical features and responses to different therapies. Therefore, further studies are needed to elucidate the role of regulatory T cells on ITP.

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Reference


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