*Original Research*

# Imbalance of follicular regulatory T (Tfr) cells/follicular helper T (Tfh) cells in adult patients with primary immune thrombocytopenia

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### Impact statement

This study investigated the role of the imbalance of Tfr cells/Tfh cells in adult patients with primary immune thrombocytopenia (ITP). The results demonstrate that Tfr cell proportion in peripheral blood is decreased and Tfh cell proportion is increased, leading to unbalanced Tfr/Tfh ratio in ITP patients pre-therapy. The imbalance of Tfr/Tfh is recovered post-therapy, suggesting that the Tfr and Tfh cells may be involved in ITP pathogenesis. Our findings may provide more treatment options in clinic.

### Abstract

This study is to investigate the role of follicular regulatory T (Tfr) cells/follicular helper T (Tfh) cells imbalance in adult patients with primary immune thrombocytopenia (ITP). Totally, 40 cases of primary ITP patients and 30 healthy controls were enrolled. Blood samples were collected from ITP patients (pre- and post-therapy) and controls. Flow cytometry was used to detect the proportion of Tfr and Tfh cells in peripheral blood. Real-time quantitative polymerase chain reaction (PCR) was performed to detect the mRNA expression levels of *FOXP3*, *BCL-6*, and *BLIMP-1*. Enzyme-linked immunosorbent assay (ELISA) was conducted to detect interleukin (IL)-10 and IL-21 levels. Spearman's correlation was used for correlation analysis. Compared with control, Tfr cell proportion, *FOXP3* mRNA, and IL-10 were significantly decreased in the pre-therapy ITP group, but were significantly increased post-therapy. Tfh cell proportion, *BCL-6* mRNA, and IL-21 were increased, while *BLIMP-1* mRNA was decreased, in the pre-therapy ITP group than the control group. These effects were reversed in the post-therapy ITP group. Moreover, the Tfr/Tfh ratio was decreased in the pre-therapy ITP group than control group, whereas was increased in the post-therapy ITP group than the pre-therapy ITP group. Furthermore, Tfr cell proportion, *FOXP3* mRNA, IL-10, and Tfr/Tfh ratio were positively correlated with the platelet count (PLT) in the ITP pre-therapy group. In addition, Tfh cell proportion, *BCL-6* mRNA, and IL-21 were negatively correlated with the PLT, while *BLIMP-1* mRNA was positively correlated with the PLT. Conclusively, Tfr cell proportion in peripheral blood is decreased and Tfh cell proportion is increased, leading to unbalanced Tfr/Tfh ratio in ITP patients pre-therapy. The imbalance of Tfr/Tfh is recovered post-therapy, suggesting that the Tfr and Tfh cells may be involved in ITP pathogenesis. The abnormal expression of *FOXP3*, *BCL-6*, and *BLIMP-1* mRNA and the changes in IL-10 and IL-21 levels may be related to the imbalance of Tfr/Tfh.

**Keywords:** Primary immune thrombocytopenia, follicular regulatory T (Tfr) cells, follicular helper T (Tfh) cells, transcription factors, cytokines

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## Introduction

Primary immune thrombocytopenia (ITP) is an autoimmune disease characterized by thrombocytopenia, with the incidence of $10/10^5$/year in adults.[1] Skin and mucous membrane hemorrhage is observed in 2/3 of ITP cases.[1] Moreover, about 80% of the adult ITP patients have chronic disease course,[2] which significantly declines their quality of life. At present, there is no absolute cure for ITP. High-dose dexamethasone therapy still represents the first choice for treatment of ITP.[3] However, in some patients, the side effects cannot be tolerated. Therefore, it is particularly important to further study the pathogenesis of ITP and provide more treatment options in clinic.

T lymphocytes are reported to be involved in ITP pathogenesis.[1] The follicular helper T (Tfh) cells are the new subset of CD4+ T cells, with the surface markers of CXC chemokine receptor 5 (CXCR5), inducible costimulator (ICOS), programmed cell death 1, and so on. The main transcription factor of Tfh cells is B-cell lymphoma 6 (Bcl-6),

**Table 1.** General information of the subjects.

| | Healthy control ($n=30$) | ITP pre-therapy ($n=40$) | ITP post-therapy ($n=30$) | $P$ |
|---|---|---|---|---|
| Sex, female/male | 24/6 | 28/12 | 22/8 | >0.05 |
| Age, years | 26–50 | 18–59 | 18–40 | >0.05 |
| Median age, years | 40 | 45 | 42 | – |
| Platelet count ($10^9$/L) | 236.00 ± 52.78 | 14.00 ± 7.92 | 123.00 ± 73.31 | – |
| White blood cell ($10^9$/L) | 7.75 (4.94–11.65) | 6.51 (5.17–11.85) | 8.83 (6.83–13.75) | >0.05 |
| Hemoglobin (g/L) | 136 ± 9.09 | 128 ± 10.26 | 133.43 ± 11.06 | >0.05 |

while interleukin (IL)-21 is the main cytokine. Tfh cells play important roles in the proliferation and differentiation of B cells, the production of specific antibodies, and the transformation of immunoglobulins, therefore representing an essential part of humoral immunity.[4] Tfh cells can mediate the occurrence and development of autoimmune diseases by secreting IL-21 and regulating antibody production by B cells.[5–7] Under normal circumstances, the antibodies produced by the GC (germinal center) reaction can precisely target foreign pathogens, limiting autoimmunity and excessive inflammation. If the Tfh-mediated B-cell reaction is too strong and excessive antibodies are produced, the autoimmune diseases, chronic diseases, inflammation, allergic reactions, or B-cell-related tumors may be induced.[6,8] Studies have shown that in children and adult ITP patients,[9,10] the proportion of Tfh cells is increased significantly, but little is known about the causes of excessive activation of Tfh cells. Therefore, negative regulation in GC is also very important. Follicular regulatory T (Tfr) cells are a subgroup of Treg cells. Tfr cells express forkhead transcription factor P3 (FoxP3) and cytotoxic T lymphocyte-associated antigen 4, and so on and secrete IL-10, exerting inhibitory effects on the response of Tfh-mediated B cells.[11–13] Tfr cells can inhibit the secretion of IL-21 by Tfh cells, and meanwhile inhibit the transformation of B cells and antibody production.[14,15] Tfr and Tfh cells in peripheral blood have the same immunophenotype as in GC and are related to the occurrence and development of immune diseases.[16–18] An imbalance of the Tfr/Tfh ratio has been found in the peripheral blood of patients with autoimmune diseases, such as systemic lupus erythematosus (SLE), systemic sclerosis, and ulcerative colitis (UC), and this imbalance is related to the disease activity and inflammatory indicators.[19–21] However, there are few reports concerning the changes of Tfr cells and Tfh cells as well as their transcription factors and cytokine levels in the peripheral blood of adult ITP patients, before and post-therapy.

In this study, the roles of the imbalance of Tfr/Tfh ratio, as well as the changes in transcription factors and cytokine levels, in the pathogenesis of ITP, were explored. The proportions of Tfr cells and Tfh cells in the peripheral blood in the control group and the ITP groups (before and post-therapy) were detected. Moreover, the mRNA expression levels of *FOXP3*, *BCL-6*, and *BLIMP-1* were analyzed, and the levels of IL-10 and IL-21 cytokines were investigated. Furthermore, the relationship of these factors with the platelet count (PLT) was studied. Our findings may clarify the possible role of Tfr/Tfh imbalance in the pathogenesis of ITP and provide more ideas for the clinical treatment of ITP.

## Materials and methods

### Study subjects

Totally, 40 patients with primary ITP, who were hospitalized in the Blood Disease Center of the First Affiliated Hospital of Xinjiang Medical University from January 2019 to June 2020, were enrolled. These patients were diagnosed with primary ITP for the first time. They were with therapeutic indications (PLT $< 30 \times 10^9$/L). The diagnosis of primary ITP met the diagnostic criteria of the Chinese Expert Consensus on the Diagnosis and Treatment of Adult Primary Immune Thrombocytopenia (2016 Edition). In these enrolled patients, all Han Chinese, there were 28 females and 12 males, with a median age of 45 years (18–59 years). Exclusion criteria were as follows: (1) subjects with abnormal liver and kidney function (serum transaminase elevation ⩾2.0 times of the upper limit or creatinine ⩾132.6 μmol/L); (2) subjects with a history of thrombosis; and (3) subjects with pregnancy or lactation. For control, we enrolled 30 age- and sex-matched individuals (24 females and 6 males) with a median age of 40 years (26–50 years), as the control group, from a contemporaneous physical examination center during the same period. The basic information of all subjects was shown in Table 1. The study was approved by the Ethics Committee of the Medical Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (approval no. K202105-14). All subjects signed the written informed consent.

### Therapeutic regimen and treatment efficacy evaluation

Dexamethasone tablets (40 mg/day) were given to ITP patients orally for four consecutive days. The treatment efficacy was evaluated based on the Chinese Expert Consensus on the Diagnosis and Treatment of Primary Immune Thrombocytopenia in Adults (2016 Edition), as follows: (1) complete response (CR): PLT was $\geqslant 100 \times 10^9$/L post-therapy, and there was no bleeding; (2) response (R): PLT was $>30 \times 10^9$/L post-therapy and was at least a twofold increase of the baseline PLT (platelet count before treatment), and there was no bleeding; and (3) none response (NR): PLT was $<30 \times 10^9$/L post-therapy, or PLT increase was less than two times of the baseline PLT before treatment, and there was bleeding.

### Sample collection

The peripheral blood samples were collected from the 30 control subjects, the 40 ITP patients pre-therapy, and 30 ITP

patients with responses (i.e. CR + R) post-therapy. After centrifugation at 300*g* for 10 min, the serum was isolated.

### Flow cytometry analysis

Totally, 100 µL anticoagulated blood samples were added with FITC antihuman CD4 antibody (Biolegend, Beijing, China) and PE antihuman CD185 (CXCR5) antibody (BioLegend, San Diego, CA, USA) and incubated at room temperature in dark for 15–30 min. The 1× TF Fix/prem Working Solution was used to fix cells at 2–8°C in dark for 40–50 min. After lysing the red blood cells, the 1× Perm/Wash Working Solution (Shanghai Chaoyan Biotechnology Co., Ltd., Shanghai, China) was added to fix cells. After centrifugation, the samples were incubated with the FoxP3 antibody (BB700 mouse antihuman FoxP3 clone 236A/E7; BD Biosciences, San Jose, CA, USA) in dark for 40–50 min. Finally, the samples were analyzed for Tfr and Tfh cell proportion on the DxFlex Clinical Flow Cytometer (Beckman, Brea, CA, USA). The Tfr cells were defined as CD4 + CXCR5 + FoxP3+ T cells, and the Tfh cells were defined as CD4 + CXCR5+ T cells.

### Quantitative real-time polymerase chain reaction

Total RNA was extracted from peripheral blood mononuclear cells and the complementary DNA (cDNA) synthesis was conducted. For the quantitative real-time polymerase chain reaction (PCR), the primers were synthesized by the Shanghai OE Biotech Co., Ltd (China). The primer sequences were shown in Table 2. The 10-µL reaction system consisted of 0.4 µL primer each, 0.2 µL ROX Reference Dye II (50×), 5 µL TB Green Premix Ex Taq II (2×) (Shanghai Chaoyan Biotechnology Co., Ltd.), 1 µL cDNA sample, and 3 µL dd$H_2O$. Reaction conditions were as follows: 95°C for 30 s; 95°C for 5 s; and 60°C for 34 s, for totally 40 cycles; 95°C for 15 s; 60°C for 1 min; 95°C for 15 s; and 60°C for 15 s. Experiment was performed in triplicates. Relative expression levels of target genes were calculated with the $2^{-\Delta\Delta^{CT}}$ method. β-actin was used as the internal reference.

### Enzyme-linked immunosorbent assay

The serum levels of IL-21 and IL-10 cytokines were detected with human IL-21/IL-10 enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Lianke Biological Co., Ltd., Shanghai, China), according to the manufacturer's instructions. Briefly, 50 µL diluted standard sample, standard diluent, serum sample, and biotin-labeled antibody against IL-21 and IL-10 were added in sequence into each well and incubated at 37°C for 1 h. Following washing for three times, totally 80 µL affinity streptomycin-HRP was added into each well and incubated at 37°C for 30 min. After washing for three times, 50 µL substrates A and B, respectively, were added into each well and incubated at 37°C in dark for 10 min. After adding 50 µL stop solution, the optical density values at 450 nm were read with the microplate reader. The concentrations of IL-10 and IL-21 were calculated according to the reference standard curve.

### Statistical analysis

The SPSS19.0 software was used for statistical analysis. Data were expressed as mean ± SD. The differences among the groups were compared by the *t*-test of independent samples. Spearman's correlation was used for correlation analysis. The GraphPad Prism V8.0 software was used to plot the graphs. $P < 0.05$ was considered as statistically significant.

**Table 2.** Gene primer sequences for *BCL-6*, *BLIMP-1*, *FOXP3*, and β-*actin* for quantitative real-time PCR.

| Gene | Primer sequences (5'-3') | Length (bp) |
|---|---|---|
| *BCL-6* | F:5'-GCCCTATCCCTGTGAAATCTG-3'<br>R:5'-GACGAAAGCATCAACACTCCA-3' | 66 |
| *BLIMP-1* | F:5'-TCCAGCACTGTGAGGTTTCA-3'<br>R:5'-TCAAACTCAGCCTCTGTCCA-3' | 57 |
| *FOXP3* | F:5'-GTGGCCCGGATGTGAGAAG-3'<br>R:5'-GGAGCCCTTGTCGGATGATG-3' | 61 |
| β-*actin* | F:5'-AGTTGCGTTACACCCTTTCTTG-3'<br>R:5'-TCACCTTCACCGTTCCAGTTT-3' | 60 |

PCR: polymerase chain reaction.

## Results

### Analysis of Tfr and Tfh cell proportions and Tfr/Tfh ratio in peripheral blood

Tfr and Tfh cell proportions in peripheral blood were measured with flow cytometry. Our results showed that, pre-therapy, the proportion of Tfr cells in the peripheral blood of the ITP group was decreased compared with the control group (2.13 ± 1.04 vs 4.73 ± 1.26; $P < 0.001$) (Figure 1(A)). However, the proportion of Tfr cells post-therapy was significantly increased, compared with pre-therapy (3.09 ± 1.53 vs 2.13 ± 1.04; $P = 0.003$) (Figure 1(A)). Moreover, compared with the control group, the proportion of Tfh cells in the peripheral blood in the ITP pre-therapy group was significantly increased (26.03 ± 5.57 vs 17.00 ± 2.09; $P < 0.001$) (Figure 1(B)). The proportion of Tfh cells post-therapy was significantly decreased than pre-therapy (22.13 ± 1.82 vs 26.03 ± 5.57; $P < 0.001$) (Figure 1(B)). Furthermore, the Tfr/Tfh ratio in the ITP pre-therapy group was significantly decreased than control group (0.08 ± 0.04 vs 0.28 ± 0.09; $P < 0.001$) (Figure 1(C)). In addition, the Tfr/Tfh ratio post-therapy was significantly increased compared with pre-therapy (0.14 ± 0.07 vs 0.08 ± 0.04; $P < 0.001$) (Figure 1(C)). These results suggest that Tfr/Tfh ratio is decreased in ITP group before treatment, but it is increased after effective treatment.

### Analysis of *FOXP3*, *BLIMP-1*, and *BCL-6* mRNA expression

The mRNA level was assessed with quantitative real-time PCR. The results showed that, compared with the control group, the mRNA expression levels of *FOXP3* in the ITP pre-therapy group was significantly reduced (2.17 ± 1.76 vs 7.55 ± 0.70; $P < 0.001$) (Figure 2(A)). The *FOXP3* mRNA expression level was significantly increased post-therapy compared with pre-therapy (3.44 ± 1.54 vs 2.17 ± 1.76; $P = 0.003$). Moreover, the mRNA expression level of *BLIMP-1* in the ITP pre-therapy group was significantly decreased than control group (1.83 ± 1.35 vs 8.45 ± 3.93; $P < 0.001$) (Figure 2(B)). Furthermore, compared with pre-therapy, the *BLIMP-1* mRNA expression level was significantly increased

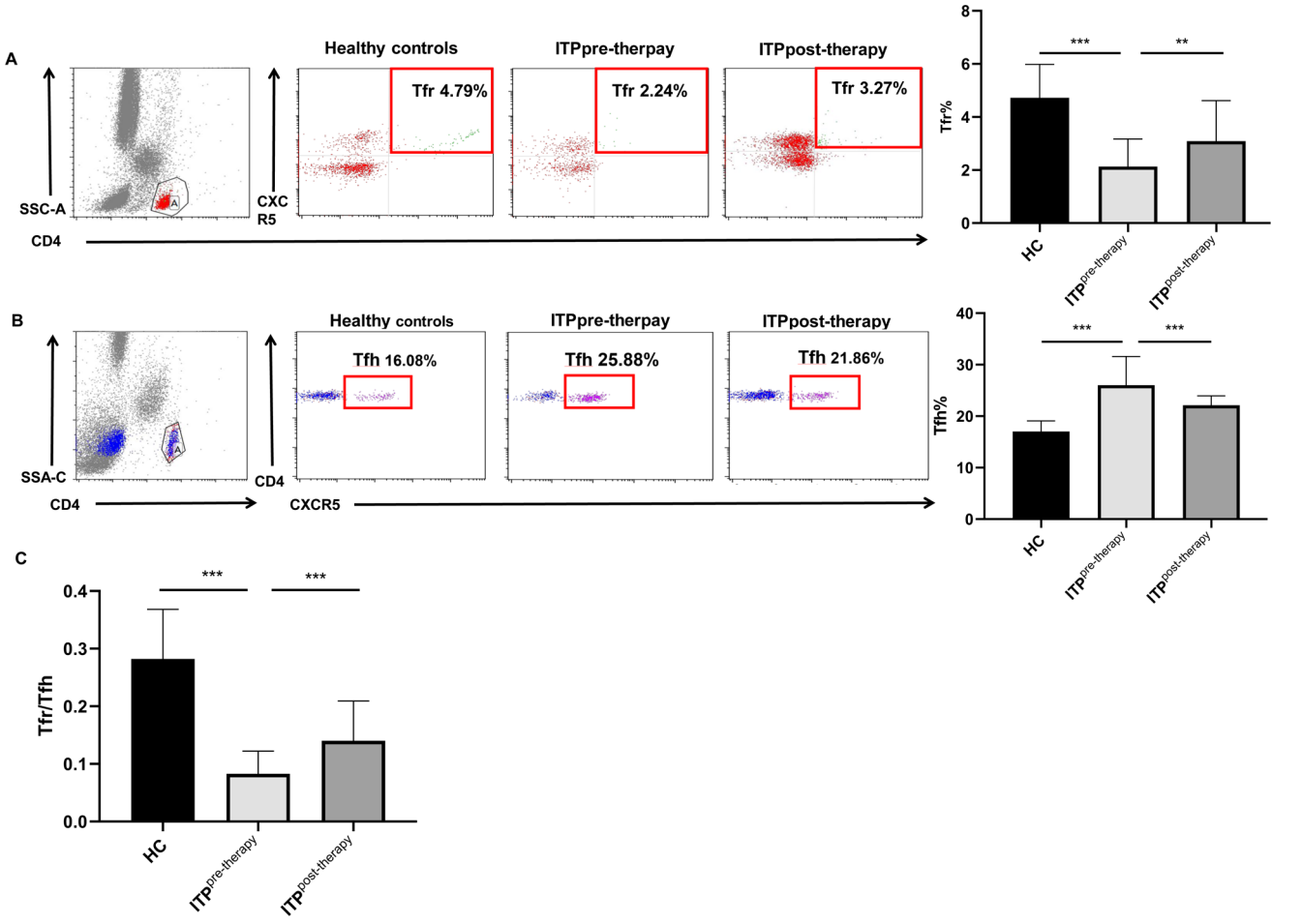


**Figure 1.** Analysis of Tfr and Tfh cell proportion. Flow cytometry was performed to detect proportions of Tfr (A) and Tfh (B) cells in ITP patients (pre- and post-therapy) and control subjects. Representative and quantitative flow cytometry results were shown. Gating strategy: lymphocytes were first gating using FSC and SSC. Then, CD4+ T cells were gated in lymphocytes, and on CD4+ T cells, Tfr cells (CD4 + CXCR5 + FoxP3+ T cells) and Tfh cells (CD4 + CXCR5+ T cells) were further gated. (C) The Tfr/Tfh ratio in ITP patients (pre- and post-therapy) and control subjects. $^{**}P < 0.05$; $^{***}P < 0.005$.

post-therapy ($3.97 \pm 1.93$ vs $1.83 \pm 1.35$; $P < 0.001$). In addition, compared with the control group, the expression of *BCL-6* mRNA in the ITP pre-therapy group was significantly increased ($4.00 \pm 1.90$ vs $0.87 \pm 0.42$; $P < 0.001$) (Figure 2(C)). The *BCL-6* mRNA expression level post-therapy was significantly decreased than pre-therapy ($1.55 \pm 0.97$ vs $4.00 \pm 1.90$; $P < 0.001$). There results suggest that, the mRNA expression levels of *FOXP3* and *BLIMP-1* in ITP group are decreased before treatment, while the mRNA expression levels of *BCL-6* are increased. However, after effective treatment, the changes of these mRNA expression levels are reversed.

## Analysis of IL-10 and IL-21 cytokine levels in serum

ELISA measured the serum levels of IL-10 and IL-21. As shown in Figure 3(A), the serum level of IL-10 in the ITP pre-therapy group was significantly lower than the control group ($258.14 \pm 72.88$ vs $320.15 \pm 74.49$ pg/mL) ($P = 0.001$). The serum IL-10 level in the ITP post-therapy group was significantly higher than that in the ITP pre-therapy group ($298.19 \pm 72.94$ vs $258.14 \pm 72.88$ pg/mL) ($P = 0.02$). However, as shown in Figure 3(B), the serum level of IL-21 in the ITP

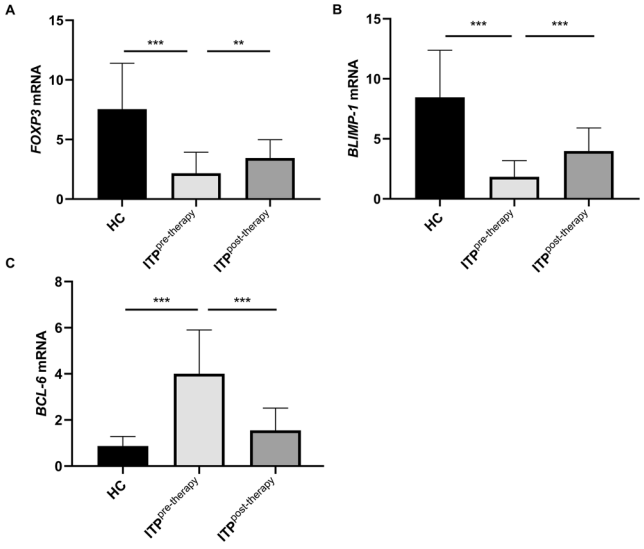


**Figure 2.** Analysis of *FOXP3*, *BLIMP-1*, and *BCL-6* mRNA levels. Expression of *FOXP3* (A), *BLIMP-1* (B), and *BCL-6* mRNA (C) in ITP patients (pre- and post-therapy) and control subjects were analyzed with quantitative real-time PCR. $^{**}P < 0.05$; $^{***}P < 0.005$.

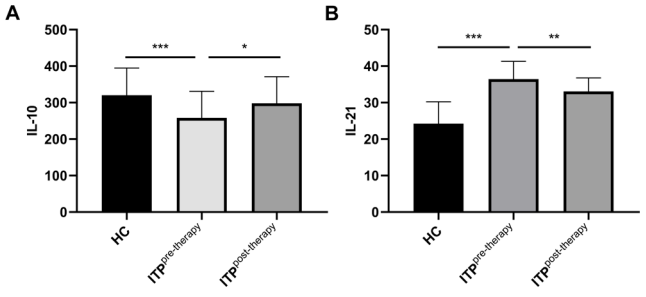


**Figure 3.** Analysis of serum IL-10 and IL-21 levels. ELISA measured the serum level of IL-10 (A) and IL-21 (B) in ITP patients (pre- and post-therapy) and control subjects. $^{**}P < 0.05$; $^{***}P < 0.005$.

pre-therapy group was significantly higher than the control group ($36.43 \pm 4.86$ vs $24.21 \pm 6.00$ pg/mL) ($P < 0.001$). However, the serum IL-21 level post-therapy was significantly lower than pre-therapy ($33.07 \pm 3.70$ vs $36.43 \pm 4.86$) ($P = 0.002$). These results suggest that the IL-10 levels are decreased and IL-21 levels are increased in ITP patients before treatment. However, IL-10 levels are increased and IL-21 levels are decreased after effective treatment.

### Spearman's rank correlation analysis

The correlations of Tfr proportion, *FOXP3* mRNA, IL-10, and Tfr/Tfh ratio with PLTs, as well as the correlations of Tfh proportion, *BLIMP-1* mRNA, *BCL-6* mRNA, and IL-21 with PLTs, in the ITP pre-therapy group were investigated. Our results showed that PLTs were positively correlated with Tfr proportion, *FOXP3* mRNA, and IL-10 ($r = 0.392$, $P = 0.026$; $r = 0.681$, $P < 0.001$; and $r = 0.596$, $P < 0.001$) ($\rho = 0.537$, $P < 0.005$; $\rho = 0.772$, $P < 0.001$; $\rho = 0.360$, $P = 0.023$), respectively (Figure 4(A) to (C)). Moreover, PLTs were positively correlated with the Tfr/Tfh ratio ($r = 0.471$, $P = 0.006$) ($\rho = 0.484$, $P = 0.002$) (Figure 4(D)). In addition, PLTs were negatively correlated with Tfh proportion (Figure 4(E)), *BCL-6* mRNA (Figure 4(F)), and IL-21 (Figure 4(G)) ($r = -0.629$, $P < 0.001$; $\rho = -0.542$, $P < 0.005$; $\rho = -0.612$, $P < 0.001$; $\rho = -0.857$, $P < 0.001$); but positively correlated with *BLIMP-1* mRNA ($r = 0.588$, $P < 0.001$) ($\rho = 0.612$, $P < 0.001$) (Figure 4(H)). These results suggest that Tfr, *FOXP3* mRNA, IL-10, *BLIMP-1*, and Tfr/Tfh ratio are positively correlated with PLT. The Tfh, *BCL-6*, and IL-21 are negatively correlated with PLT.

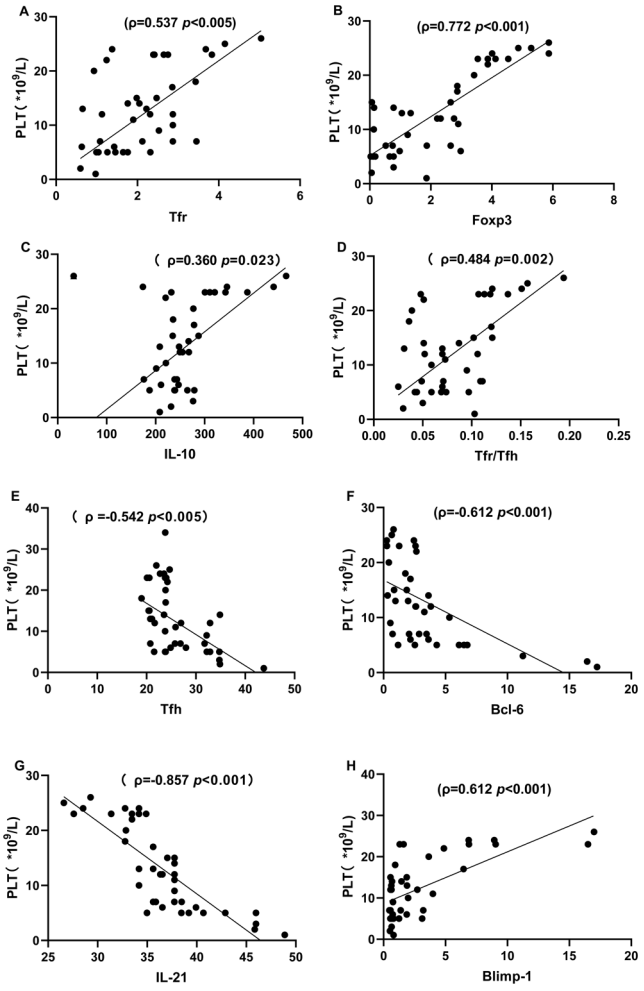


**Figure 4.** Correlation analysis. Spearman's correlation was performed. (A) The correlation between the proportion of Tfr cells and PLT. (B) The correlation between the expression of *FOXP3* mRNA and PLT. (C) The correlation between the level of IL-10 and PLT. (D) The correlation between the Tfr/Tfh ratio and PLT. (E) The correlation between the proportion of Tfh and PLT. (F) The correlation between the expression of *BCL-6* mRNA and PLT. (G) The correlation between the level of IL-21 and PLT. (H) The correlation between the expression of *BLIMP-1* mRNA and PLT.

## Discussion

ITP is a complex, acquired autoimmune hemorrhagic disease. The pathogenesis of ITP is complex and diverse, involving the humoral immune disorders, cellular immune disorders, abnormal cytokine secretion, PLT apoptosis, and genetic and environmental factors.[1,22] In recent years, more and more studies have shown that the abnormal immune regulation mediated by CD4+ T cells may be the initiating factor in the pathogenesis of ITP.[23,24] The imbalance of Tfr/Tfh ratio is closely related to the occurrence and development of autoimmune diseases.[19,21,25] For example, in the peripheral blood of SLE patients, the proportion of CXCR5 + ICOS+ Tfh cells was significantly increased, and it was positively correlated with the activity index of SLE and autogenic antibodies. In the peripheral blood of UC patients, the proportion of Tfr cells decreased while that of Tfh cells increased, leading to unbalanced Tfr/Tfh ratio, which was positively correlated with the disease severity in UC patients.

Similar to the findings in SLE[26] and UC[27] patients, this study showed Tfr/Tfh imbalance in untreated ITP patients. This is also consistent with the results by Chen *et al.*[28] This imbalance manifests as an increase in Tfh cells and may further promote the Tfh-mediated B-cell response,[29,30] leading to the production of excessive antibodies, mediating the destruction of PLT, and possibly inducing or promoting the occurrence of ITP. However, the above studies did not further explore the imbalance of Tfr/Tfh cells in peripheral blood of ITP patients after treatment. In this study, we collected peripheral blood samples from patients with ITP after effective treatment and found that the Tfr/Tfh imbalance was recovered in ITP patients after treatment. The restoration of this imbalance may be conducive to the recovery of PLTs of ITP patient. Further correlation analysis showed that the Tfr cells and Tfr/Tfh ratio were positively correlated with the PLTs, whereas the Tfh cells were negatively correlated with

the PLTs. These results suggest that the imbalance of Tfr/Tfh ratio may be involved in the pathogenesis of ITP and mediate the occurrence of PLT reduction in patients with ITP.

Bcl-6 is the main transcription factor that induces the differentiation of Tfh cells. If Bcl-6 is continuously expressed, it will enhance the differentiation of CD4+ T cells into Tfh cells, indicating that Bcl-6 is a necessary and sufficient factor for Tfh cell differentiation.[22,31] The high expression of Blimp-1 would block the expression of Bcl-6 and the differentiation of Tfh cells, which would decrease the production of specific antibodies and reduce the occurrence of autoimmune diseases.[32,33] The CD4+ T cells lacking Blimp-1 are more likely to differentiate into Tfh cells, suggesting that Bcl-6 and Blimp-1 have diametrically opposite effects on the differentiation of Tfh cells, and they are the key transcription and regulatory factors regulating the differentiation of Tfh cells 32. In this study, our results showed that *BCL-6* mRNA expression level increased and *BLIMP-1* mRNA expression level decreased in ITP patients, suggesting that the changes of *BCL-6* and *BLIMP-1* mRNA levels may be involved in the pathogenesis of ITP. This is consistent with previous studies.[28,34,35] Under this condition, more CD4+ T cells will be induced to differentiate into Tfh cells, thus leading to the increase of Tfh cells. The transcription factor FoxP3 has been considered to be the main factor that performs the suppressive function of Tfr cells.[36,37] Loss of FoxP3 would lead to multiorgan autoimmune diseases in mice, and result in human immune disorders, polykeratinopathy, enteropathy, and X-linked syndrome.[31,32] In this study, we found that the expression level of *FOXP3* mRNA in the ITP group before treatment was significantly lower than that in the control group. We speculate that the low expression of *FOXP3* may weaken the inhibitory effect of Tfr cells on abnormal immune response, thus indirectly mediating the pathogenesis of ITP. However, in patients after effective treatment, *FOXP3* mRNA expression increased, suggesting that the increase in *FOXP3* mRNA level is conducive to the recovery of ITP patients.

IL-10 is the main cytokine secreted by the Tfr cells and one of the main executors of the suppressive function of Tfr cells.[38] The study of Li *et al*.[39] showed that insufficient secretion of IL-10 by Tregs compromised its control on over-activated CD4+ T effector cells in newly diagnosed adult patients with ITP. Our results showed that there was a decrease in the expression level of IL-10 in ITP patients before treatment. This is consistent with the findings by Liu and Liu,[40] and Gudbrandsdottir *et al*.[41] suggesting that Tfr cells have weakened inhibitory effects on abnormal immunity. IL-10 levels were significantly elevated in ITP patients after effective treatment. We suppose that, with the IL-10 level recovery, the inhibitory effect of Tfr cells on abnormal immune response would be gradually recovered. IL-21 is the main cytokine secreted by Tfh cells. In the process of Tfh cell promoting B-cell proliferation and mediating the production of specific antibodies, IL-21 plays an important role.[42] Our results showed that in untreated ITP patients, there was an increase in the level of IL-21, which was consistent with previous studies.[9,34] It is suggested that the high levels of IL-21 may further promote the proliferation of B cells and eventually lead to the production of more specific antibodies in plasma cells, which may be involved in the progression of ITP.

In summary, our results showed that there was an imbalance of Tfr/Tfh ratio in patients with ITP, at the cellular level, transcription factor level, and cytokine level, exhibiting the Tfh cellular immune deviation. Meanwhile, we also observed the changes of these indexes in ITP patients after effective treatment. Specifically, in patients with effective response, with the increase in PLT, the imbalance of Tfr/Tfh ratio recovered. These results suggest that the imbalance of Tfr/Tfh ratio participates in the pathogenesis of ITP. Of course, further in-depth studies are still needed to investigate the detailed underlying mechanism. Our findings may provide new ideas for the clinical treatment of ITP patients.

## AUTHORS' CONTRIBUTIONS

MS and XG designed the study. MS, XW, NZ, LW, XW, WF, QL, and MS conducted the experiments and collected the data. MS and XW participated in the analysis of the data. MS, WF, and LW participated in the interpretation of the studies. MS, YL, XW, and XG prepared the manuscript. QL, YL, and MS searched the literature. XG collected the funds. All authors have read and approved the submitted manuscript.

## DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## ETHICAL APPROVAL

The study was approved by the Ethics Committee of the Medical Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (approval no. K202105-14). All subjects signed the written informed consent.

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## REFERENCES

1. Audia S, Mahevas M, Samson M, Godeau B, Bonnotte B. Pathogenesis of immune thrombocytopenia. *Autoimmun Rev* 2017;**16**:620–32
2. Provan D, Arnold DM, Bussel JB, Chong BH, Cooper N, Gernsheimer T, Ghanima W, Godeau B, Gonzalez-Lopez TJ, Grainger J, Hou M, Kruse C, McDonald V, Michel M, Newland AC, Pavord S, Rodeghiero F, Scully M, Tomiyama Y, Wong RS, Zaja F, Kuter DJ. Updated international consensus report on the investigation and management of primary immune thrombocytopenia. *Blood Adv* 2019;**3**:3780–817
3. Neunert C, Terrell DR, Arnold DM, Buchanan G, Cines DB, Cooper N, Cuker A, Despotovic JM, George JN, Grace RF, Kuhne T, Kuter DJ, Lim W, McCrae KR, Pruitt B, Shimanek H, Vesely SK. American Society of Hematology 2019 guidelines for immune thrombocytopenia. *Blood Adv* 2019;**3**:3829–66
4. Audia S, Rossato M, Santegoets K, Spijkers S, Wichers C, Bekker C, Bloem A, Boon L, Flinsenberg T, Compeer E, van den Broek T, Facy O, Ortega-Deballon P, Berthier S, Leguy-Seguin V, Martin L, Ciudad M, Samson M, Trad M, Lorcerie B, Janikashvili N, Saas P, Bonnotte B, Radstake TR. Splenic TFH expansion participates in B-cell differentiation and antiplatelet-antibody production during immune thrombocytopenia. *Blood* 2014;**124**:2858–66

5. Choi JY, Seth A, Kashgarian M, Terrillon S, Fung E, Huang L, Wang LC, Craft J. Disruption of pathogenic cellular networks by IL-21 blockade leads to disease amelioration in murine lupus. *J Immunol* 2017;**198**:2578–88
6. Alahgholi-Hajibehzad M, Durmuş H, Aysal F, Gülşen-Parman Y, Oflazer P, Deymeer F, Saruhan-Direskeneli G. The effect of interleukin (IL)-21 and CD4+CD25++ T cells on cytokine production of CD4+ responder T cells in patients with myasthenia gravis. *Clin Exp Immunol* 2017;**190**:201–7
7. Huang X, Wu H, Qiu H, Yang H, Deng Y, Zhao M, Luo H, Zhou X, Xie Y, Chan V, Lau CS, Lu Q. The expression of Bcl-6 in circulating follicular helper-like T cells positively correlates with the disease activity in systemic lupus erythematosus. *Clin Immunol* 2016;**173**:161–70
8. Sage PT, Sharpe AH. T follicular regulatory cells. *Immunol Rev* 2016;**271**: 246–59
9. Yao X, Li C, Yang J, Wang G, Li C, Xia Y. Differences in frequency and regulation of T follicular helper cells between newly diagnosed and chronic pediatric immune thrombocytopenia. *Blood Cells Mol Dis* 2016;**61**:26–36
10. Xie J, Cui D, Liu Y, Jin J, Tong H, Wang L, Ruan G, Lu Y, Yuan H. Changes in follicular helper T cells in idiopathic thrombocytopenic purpura patients. *Int J Biol Sci* 2015;**11**:220–9
11. Sage PT, Sharpe AH. T follicular regulatory cells in the regulation of B cell responses. *Trends Immunol* 2015;**36**:410–8
12. Zhu Y, Zou L, Liu YC. T follicular helper cells, T follicular regulatory cells and autoimmunity. *Int Immunol* 2016;**28**:173–9
13. Paterson AM, Lovitch SB, Sage PT, Juneja VR, Lee Y, Trombley JD, Arancibia-Carcamo CV, Sobel RA, Rudensky AY, Kuchroo VK, Freeman GJ, Sharpe AH. Deletion of CTLA-4 on regulatory T cells during adulthood leads to resistance to autoimmunity. *J Exp Med* 2015; **212**:1603–21
14. Sage PT, Tan CL, Freeman GJ, Haigis M, Sharpe AH. Defective TFH cell function and increased TFR cells contribute to defective antibody production in aging. *Cell Rep* 2015;**12**:163–71
15. McHeyzer-Williams LJ, Milpied PJ, Okitsu SL, McHeyzer-Williams MG. Class-switched memory B cells remodel BCRs within secondary germinal centers. *Nat Immunol* 2015;**16**:296–305
16. Stebegg M, Kumar SD, Silva-Cayetano A, Fonseca VR, Linterman MA, Graca L. Regulation of the germinal center response. *Front Immunol* 2018;**9**:2469
17. Wang X, Yang C, Xu F, Qi L, Wang J, Yang P. Imbalance of circulating Tfr/Tfh ratio in patients with rheumatoid arthritis. *Clin Exp Med* 2019; **19**:55–64
18. Zheng J, Wang T, Zhang L, Cui L. Dysregulation of circulating Tfr/Tfh ratio in primary biliary cholangitis. *Scand J Immunol* 2017;**86**:452–61
19. Choi JY, Ho JH, Pasoto SG, Bunin V, Kim ST, Carrasco S, Borba EF, Gonçalves CR, Costa PR, Kallas EG, Bonfa E, Craft J. Circulating follicular helper-like T cells in systemic lupus erythematosus: association with disease activity. *Arthritis Rheumatol* 2015;**67**:988–99
20. Ricard L, Jachiet V, Malard F, Ye Y, Stocker N, Rivière S, Senet P, Monfort JB, Fain O, Mohty M, Gaugler B, Mekinian A. Circulating follicular helper T cells are increased in systemic sclerosis and promote plasmablast differentiation through the IL-21 pathway which can be inhibited by ruxolitinib. *Ann Rheum Dis* 2019;**78**:539–50
21. Wang X, Zhu Y, Zhang M, Hou J, Wang H, Jiang Y, Wang H, Gao P. The shifted balance between circulating follicular regulatory T cells and follicular helper T cells in patients with ulcerative colitis. *Clin Sci* 2017;**131**:2933–45
22. Miltiadous O, Hou M, Bussel JB. Identifying and treating refractory ITP: difficulty in diagnosis and role of combination treatment. *Blood* 2020;**135**:472–90
23. Kostic M, Zivkovic N, Cvetanovic A, Marjanović G. CD4(+) T cell phenotypes in the pathogenesis of immune thrombocytopenia. *Cell Immunol* 2020;**351**:104096
24. Wang Q, Li J, Yu TS, Liu Y, Li K, Liu S, Liu Y, Feng Q, Zhang L, Li GS, Shao LL, Peng J, Hou M, Liu XG. Disrupted balance of CD4(+) T-cell subsets in bone marrow of patients with primary immune thrombocytopenia. *Int J Biol Sci* 2019;**15**:2798–814
25. Xu H, Liu J, Cui X, Zuo Y, Zhang Z, Li Y, Tao R, Li Y, Pang J. Increased frequency of circulating follicular helper T cells in lupus patients is associated with autoantibody production in a CD40L-dependent manner. *Cell Immunol* 2015;**295**:46–51
26. Jin X, Chen J, Wu J, Lu Y, Li B, Fu W, Wang W, Cui D. Aberrant expansion of follicular helper T cell subsets in patients with systemic lupus erythematosus. *Front Immunol* 2022;**13**:928359
27. Long Y, Xia C, Xu L, Liu C, Fan C, Bao H, Zhao X, Liu C. The imbalance of circulating follicular helper T cells and follicular regulatory T cells is associated with disease activity in patients with ulcerative colitis. *Front Immunol* 2020;**11**:104
28. Chen Y, Hu J, Chen Y. Platelet desialylation and TFH cells-the novel pathway of immune thrombocytopenia. *Exp Hematol Oncol* 2021;**10**:21
29. Chen Y, Luo L, Zheng Y, Zheng Q, Zhang N, Gan D, Yirga SK, Lin Z, Shi Q, Fu L, Hu J, Chen Y. Association of platelet desialylation and circulating follicular helper T cells in patients with thrombocytopenia. *Front Immunol* 2022;**13**:810620
30. Audia S, Rossato M, Trad M, Samson M, Santegoets K, Gautheron A, Bekker C, Facy O, Cheynel N, Ortega-Deballon P, Boulin M, Berthier S, Leguy-Seguin V, Martin L, Ciudad M, Janikashvili N, Saas P, Radstake T, Bonnotte B. B cell depleting therapy regulates splenic and circulating T follicular helper cells in immune thrombocytopenia. *J Autoimmun* 2017;**77**:89–95
31. Vacchio MS, Ciucci T, Gao Y, Watanabe M, Balmaceno-Criss M, McGinty MT, Huang A, Xiao Q, McConkey C, Zhao Y, Shetty J, Tran B, Pepper M, Vahedi G, Jenkins MK, McGavern DB, Bosselut R. A Thpok-directed transcriptional circuitry promotes Bcl6 and Maf expression to orchestrate T follicular helper differentiation. *Immunity* 2019;**51**:465.e6–78.e6
32. Fu SH, Yeh LT, Chu CC, Yen BL, Sytwu HK. New insights into Blimp-1 in T lymphocytes: a divergent regulator of cell destiny and effector function. *J Biomed Sci* 2017;**24**:49
33. Yang M, Long D, Hu L, Zhao Z, Li Q, Guo Y, He Z, Zhao M, Lu L, Li F, Long H, Wu H, Lu Q. AIM2 deficiency in B cells ameliorates systemic lupus erythematosus by regulating Blimp-1-Bcl-6 axis-mediated B-cell differentiation. *Signal Transduct Target Ther* 2021;**6**:341
34. Yang Y, Lv X, Zhan L, Chen L, Jin H, Tang X, Shi Q, Zou Q, Xiang J, Zhang W, Zeng Z, Jiang H, Lv X. Case report: IL-21 and Bcl-6 regulate the proliferation and secretion of Tfh and Tfr cells in the intestinal germinal center of patients with inflammatory bowel disease. *Front Pharmacol* 2020;**11**:587445
35. Dai L, He L, Wang Z, Bai X, He Y, Cao L, Zhu M, Ruan C. Altered circulating T follicular helper cells in patients with chronic immune thrombocytopenia. *Exp Ther Med* 2018;**16**:2471–7
36. Zhang D, Zhang X, Li H, Xue F, Zhang L, Yang R. Association of FOXP3 gene polymorphisms with chronic immune thrombocytopenia in a Chinese Han population. *Int J Lab Hematol* 2021;**43**:1104–9
37. Hou S, Clement RL, Diallo A, Blazar BR, Rudensky AY, Sharpe AH, Sage PT. FoxP3 and Ezh2 regulate Tfr cell suppressive function and transcriptional program. *J Exp Med* 2019;**216**:605–20
38. Canete PF, Sweet RA, Gonzalez-Figueroa P, Papa I, Ohkura N, Bolton H, Roco JA, Cuenca M, Bassett KJ, Sayin I, Barry E, Lopez A, Canaday DH, Meyer-Hermann M, Doglioni C, Fazekas de St Groth B, Sakaguchi S, Cook MC, Vinuesa CG. Regulatory roles of IL-10-producing human follicular T cells. *J Exp Med* 2019;**216**:1843–56
39. Li F, Ji L, Wang W, Hua F, Zhan Y, Zou S, Yuan L, Ke Y, Min Z, Song D, Sun L, Chen H, Cheng Y. Insufficient secretion of IL-10 by Tregs compromised its control on over-activated CD4+ T effector cells in newly diagnosed adult immune thrombocytopenia patients. *Immunol Res* 2015;**61**:269–80
40. Liu Q, Liu Y. Role of IL-10 and IL-22 cytokines in patients with primary immune thrombocytopenia and their clinical significance. *J Clin Lab Anal* 2022;**36**:e24573
41. Gudbrandsdottir S, Brimnes M, Køllgaard T, Hasselbalch HC, Nielsen CH. Effects of rituximab and dexamethasone on regulatory and proinflammatory B-cell subsets in patients with primary immune thrombocytopenia. *Eur J Haematol* 2018;**100**:45–52
42. Long D, Chen Y, Wu H, Zhao M, Lu Q. Clinical significance and immunobiology of IL-21 in autoimmunity. *J Autoimmun* 2019;**99**:1–14


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