ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
April 2014; 13(2):85-92.

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

Nargess Arandi[1], Abbas Mirshafiey[1], Mahmood Jeddi-Tehrani[2], Mohammadreza Shaghaghi[3], Bamdad Sadeghi[3], Hassan Abolhassani[3], Ramazan Ali Sharifian[4], Mohammad Saeid Rahiminejad[5], and Asghar Aghamohammadi[3]

[1] Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[2] Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
[3] Research Center for Immunodeficiency, Pediatrics Center of Excellence, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran
[4] Clinic of Hematology and Oncology, Vali-Asr Hospital, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[5] Division of Pediatric Hematology Oncology, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran

Received: 18 January 2013; Received in revised form: 4 February 2013; Accepted: 3 March 2013

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 (Trges); Suppressive function

Corresponding Author: Asghar Aghamohammadi, MD, PhD; Research Center for Immunodeficiency, Pediatrics Center of Excellence, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 6642 8998, Fax: (+98 21) 6692 3054, e-mail: aghamohammadi@tums.ac.ir

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

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 $<100\times10^9$/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 (1×10^6) 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-labeled anti-FOXP3 antibody (clone PCH101, eBioscience) for 30 min at 4˚C in the dark. For isotype control antibodies, FITC and PE-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 (1×10^4 cells) were cultured with Tregs (1×10^4 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 2×10^4 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-OD of Treg-Tres co-culture/OD of Tres cells alone] ×100

Table 1. The sequences of primers and probes used for Real-Time PCR.

Genes	Primer and probe sequences
CTLA-4	5′-CATGGACACGGGACTCTACAT-3′
	5′-GCACGGTTCTGGATCAATTACATA-3′
	5′-FAM-TGCAAGGTGGAGCTCATGTACCCACC-TAMRA-3′
GITR	5′-TGCAAACCTTGGACAGACTGC-3′
	5′-ACAGCGTTGTGGGTCTTGTTC-3′
	5′-FAM-CCAGTTCGGGTTTCTCACTGTGTTCC-TAMRA-3′
YWHAZ	5′-AAGTTCTTGATCCCCAATGCTT-3′
	5′-GTCTGATAGGATGTGTTGG TTGC-3′
	5′-FAM-TATGCTTGTT GTGACTGATCGACAATCCC-TAMRA-3′
TBP	5′-TTCGGAGAGTTCTGGGATTGTA-3′
	5′-TGGACGTTCTTCACTCTTGGC-3′
	5′-FAM-CCGTGGTTCGTGGCTCTCTTATCCTCA-TAMRA-3′

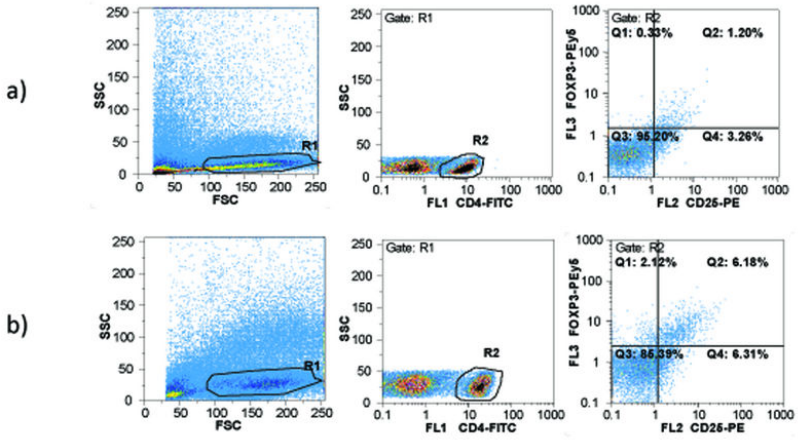

Figure 1. Flow cytometric analysis of Tregs. The lymphocytes were first selected based on their side and forward scatters. Then, the CD25$^+$FOXP3$^+$ double positive cells were defined in CD4 gate (R2) and considered the Treg populations (Q2); "a" is representative of an ITP patient and "b" is representative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

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).

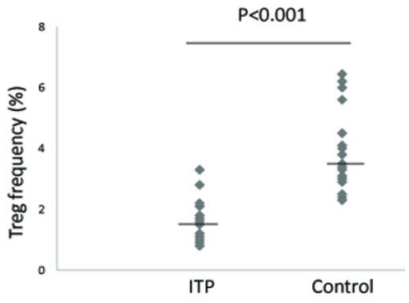

Figure 2. The frequency of Tregs in ITP patients and healthy controls. The horizontal line indicates the mean frequency of Tregs

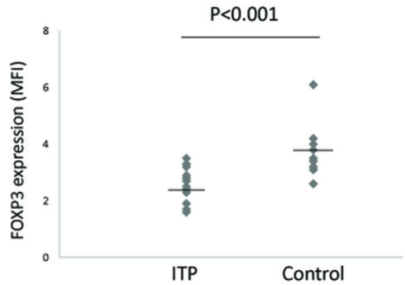

Figure 3. The expression of FOXP3 (MFI) in ITP patients and healthy controls. The horizontal line indicates the mean MFI of FOXP3.

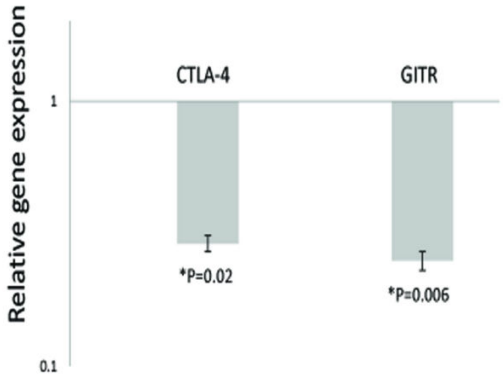

Figure 4. The relative expression of CTLA-4 and GITR genes in ITP patients compared to healthy controls; the mRNA of CTLA-4 and GITR genes are normalized to both housekeeping genes and presented as the relative expression of these genes. Data are presented in logarithmic scale.

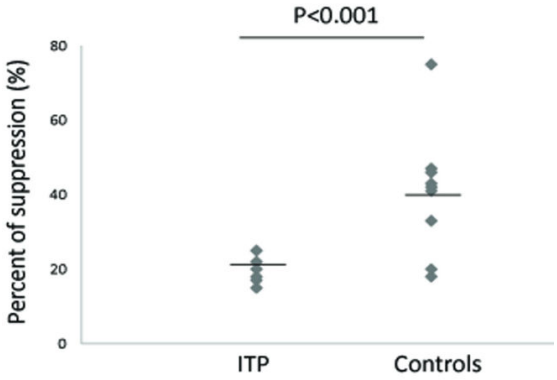

Figure 5. The suppressive function of Tregs in ITP patients and healthy controls

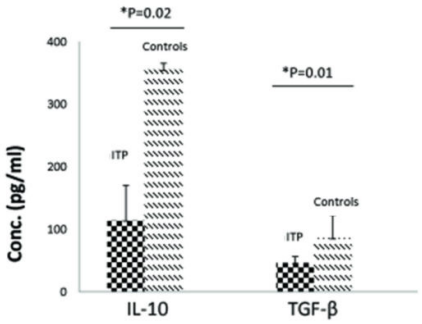

Figure 6. The levels of the IL-10 and TGF-β in ITP patients and healthy controls

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,[1,3,4] 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.[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.[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.[1,26] CTLA-4 can also contribute in generation of tolergenic dendritic cells.[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.[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.[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.[17,19,30,31]

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

This work was extracted from PhD-thesis and supported by a grant (88-04-30-9644) from Tehran University of Medical Sciences.

REFERENCES

1. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. Annu Rev Immunol 2012; 30:531-64.
2. Romagnoli P, van Meerwijk JPM. Thymic Selection and Lineage Commitment of CD4+ Foxp3+ Regulatory T Lymphocytes. Prog Mol Biol Transl Sci 2010; 92:251-77.
3. Mottet C, Golshayan D. CD4+CD25+Foxp3+ regulatory T cells: from basic research to potential therapeutic use. Swiss Med Wkly 2007; 137(45-46):625-34.
4. Toda A, Piccirillo CA. Development and function of naturally occurring CD4+CD25+ regulatory T cells. J Leukoc Biol 2006; 80(3):458-70.
5. Barreto M, Ferreira RC, Lourenco L, Moraes-Fontes MF, Santos E, Alves M, et al. Low frequency of CD4+CD25+ Treg in SLE patients: a heritable trait associated with CTLA4 and TGFbeta gene variants. BMC Immunol 2009; 10:5.
6. Bonelli M, Savitskaya A, von Dalwigk K, Steiner CW, Aletaha D, Smolen JS, et al. Quantitative and qualitative deficiencies of regulatory T cells in patients with systemic lupus erythematosus (SLE). Int Immunol 2008 Jul; 20(7):861-8.
7. Crispin JC, Martinez A, Alcocer-Varela J. Quantification of regulatory T cells in patients with systemic lupus erythematosus. J Autoimmun 2003; 21(3):273-6.
8. Saruta M, Yu QT, Fleshner PR, Mantel PY, Schmidt-Weber CB, Banham AH, et al. Characterization of FOXP3+CD4+ regulatory T cells in Crohn's disease. Clin Immunol 2007; 125(3):281-90.

9. Venigalla RK, Tretter T, Krienke S, Max R, Eckstein V, Blank N, et al. Reduced CD4+,CD25- T cell sensitivity to the suppressive function of CD4+,CD25high,CD127 -/low regulatory T cells in patients with active systemic lupus erythematosus. Arthritis Rheum 2008; 58(7):2120-30.
10. Xu WH, Zhang AM, Ren MS, Zhang XD, Wang F, Xu XC, et al. Changes of Treg-Associated Molecules on CD4(+)CD25 (+)Treg Cells in Myasthenia Gravis and Effects of Immunosuppressants. J Clin Immunol 2012; 32(5):975-83.
11. Cines DB, Bussel JB, Liebman HA, Prak ETL. The ITP syndrome: pathogenic and clinical diversity. Blood 2009; 113(26):6511-21.
12. Provan D, Stasi R, Newland AC, Blanchette VS, Bolton-Maggs P, Bussel JB, et al. International consensus report on the investigation and management of primary immune thrombocytopenia. Blood 2010; 115(2):168-86.
13. Rodeghiero F, Stasi R, Gernsheimer T, Michel M, Provan D, Arnold DM, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood 2009; 113(11):2386-93.
14. Semple JW, Provan D. The immunopathogenesis of immune thrombocytopenia: T cells still take center-stage. Curr Opin Hematol 2012; 19(5):357-62.
15. Zhou B, Zhao H, Yang RC, Han ZC. Multi-dysfunctional pathophysiology in ITP. Crit Rev Oncol Hematol 2005; 54(2):107-16.
16. Ware RE, Howard TA. Phenotypic and clonal analysis of T lymphocytes in childhood immune thrombocytopenic purpura. blood 1993; 82(7):2137-42.
17. Panitsas FP, Theodoropoulou M, Kouraklis A, Karakantza M, Theodorou GL, Zoumbos NC, et al. Adult chronic idiopathic thrombocytopenic purpura (ITP) is the manifestation of a type-1 polarized immune response. blood 2004; 103(7):2645-7.
18. Semple JW, Milev Y, Cosgrave D, Mody M, Hornstein A, Blanchette V, et al. Differences in serum cytokine levels in acute and chronic autoimmune thrombocytopenic purpura: relationship to platelet phenotype and antiplatelet T-cell reactivity. blood 1996; 87(10):4245-54.
19. Wang T, Zhao H, Ren H, Guo J, Xu M, Yang R, et al. Type 1 and type 2 T-cell profiles in idiopathic thrombocytopenic purpura. Haematologica 2005; 90(7):914-23.
20. Liu B, Zhao H, Poon MC, Han Z, Gu D, Xu M, et al. Abnormality of CD4+ CD25+ regulatory T cells in idiopathic thrombocytopenic purpura. Eur J Haematol 2006; 78(2):139-43.
21. Sakakura M, Wada H, Tawara I, Nobori T, Sugiyama T, Sagawa N, et al. Reduced Cd4+ Cd25+ T cells in patients with idiopathic thrombocytopenic purpura. Thromb Res 2007; 120(2):187-93.
22. Yu J, Heck S, Patel V, Levan J, Yu Y, Bussel JB, et al. Defective circulating CD25 regulatory T cells in patients with chronic immune thrombocytopenic purpura. Blood 2008; 112(4):1325-8.
23. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995; 155(3):1151-64.
24. Wei S, Kryczek I, Zou W. Regulatory T-cell compartmentalization and trafficking. Blood 2006; 108(2):426-31.
25. Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature 2007; 445(7129):766-70.
26. Yurchenko E, Shio MT, Huang TC, Da Silva Martins M, Szyf M, Levings MK, et al. Inflammation-driven reprogramming of CD4+ Foxp3+ regulatory T cells into pathogenic Th1/Th17 T effectors is abrogated by mTOR inhibition in vivo. PLoS One 2012; 7(4):e35572.
27. Ermann J, Fathman CG. Costimulatory signals controlling regulatory T cells. Proc Natl Acad Sci U S A 2003; 100(26):15292-3.
28. Andre S, Tough DF, Lacroix-Desmazes S, Kaveri SV, Bayry J. Surveillance of antigen-presenting cells by CD4+ CD25+ regulatory T cells in autoimmunity: immunopathogenesis and therapeutic implications. Am J Pathol 2009; 174(5):1575-87.
29. Nocentini G, Riccardi C. GITR: a modulator of immune response and inflammation. Adv Exp Med Biol 2009; 647:156-73.
30. Shevach EM, Stephens GL. The GITR-GITRL interaction: co-stimulation or contrasuppression of regulatory activity? Nat Rev Immunol 2006; 6(8):613-8.
31. Andersson PO, Olsson A, Wadenvik H. Reduced transforming growth factor-β1 production by mononuclear cells from patients with active chronic idiopathic thrombocytopenic purpura. Br J Haematol 2002; 116(4):862-7.

32. Catani L, Sollazzo D, Trabanelli S, Curti A, Evangelisti C, Polverelli N, et al. Decreased expression of indoleamine 2, 3-dioxygenase 1 in dendritic cells contributes to impaired regulatory T cell development in immune thrombocytopenia. Ann Hematol 2013; 92(1):67-78.
33. Fujio K, Okamura T, Yamamoto K. The Family of IL-10-Secreting CD4+ T Cells. Adv Immunol 2010; 105:99-130.
34. Bridoux F, Badou A, Saoudi A, Bernard I, Druet E, Pasquier R, et al. Transforming Growth Factor β (TGF-β)-dependent Inhibition of T Helper Cell 2 (Th2)-induced Autoimmunity by Self–Major Histocompatibility Complex (MHC) Class II–specific, Regulatory CD4+ T Cell Lines. Journal Exp Med 1997; 185(10):1769-75.
35. Cross D, Cambier JC. Transforming growth factor beta 1 has differential effects on B cell proliferation and activation antigen expression. J Immunol 1990; 144(2):432-9.