Chinese Journal of Integrative Medicine
Available online at link.springer.com/journal/11655
Journal homepage: www.cjim.cn/zxyjhen/zxyjhen/ch/index.aspx
E-mail: cjim_en@cjim.cn

Feature Article

Research Progress on Chinese Medicine Immunomodulatory Intervention for Chronic Primary Immune Thrombocytopenia: Targeting Cellular Immunity*

LIU Wen-bin, LI Sai, YU Xiao-ling, DAI Tie-ying, and GAO Rui-lan

Prof. GAO Rui-lan

ABSTRACT Chronic primary immune thrombocytopenia (CITP) is the most common acquired autoimmune disease that seriously threaten the physical and mental health of patients. Compared with Western medicine treatment, the intervention and treatment of Chinese medicine (CM) has shown certain therapeutic advantages. This paper reviewed the new pathogenesis progress on T cell immune abnormality in CITP, and CM studies on interferes effects of cellular immune regulation of CITP in recent years. Qi deficiency failing to control blood and internal obstruction of blood stasis are the two common types of CM syndromes in CITP patients, the corresponding treatments include invigorating Pi (Spleen), supplementing qi, activating blood, as well as tonifying qi and activating yang, regulating Gan (Liver) to invigorate Pi. The authors also mentioned the problems in the research field of CM for CTIP treatment, and put forward new ideas for the research in the future.

KEYWORDS chronic immune thrombocytopenia, cellular immunity, Chinese medicine

Primary immune thrombocytopenia (ITP) is the most common acquired autoimmune disease in clinic, accounting for about 30% of hemorrhagic illness, and the morbidity is approximately 50–100 new cases per million population per year.[1] ITP was termed as "purpura disease" in Chinese medicine (CM) in 2008, which belongs to the category of "blood syndrome". The American Society of Hematology (ASH) changed its name to "primary immune thrombocytopenia" since 2011. Chronic primary immune thrombocytopenia (CITP) refers to ITP lasting for more than 12 months,[2] including some refractory ITP (RITP) and severe ITP, which are the key and difficult points of ITP treatment, and also good points for CM intervention in ITP therapy.

Since 1951, researchers had found that ITP was caused by anti-platelet antibodies, it is gradually recognized that abnormal humoral immune, and immune intolerance are the core pathogenesis of ITP.[3] As early as 1951, glucocorticoid therapy for ITP was initiated to inhibit antibody production and combine reaction of antigen-antibody. In 1981, immunoglobulin was applied to neutralize and eliminate anti-platelet antibodies. The response to these treatments has not been always satisfactory, besides they may cause serious side effects. Clinical application of anti-CD20 monoclonal antibody (rituximab) had been carried out since 2001. In 2015, Miyakawa, et al[4] applied rituximab to treat CITP patients for 6 months, and the total response rate was only 30.8%. A retrospective study from Wang, et al[5] showed that the total response rate was 37.9% in CITP. Compared with Western medicine treatment, the intervention and treatment of CM has shown certain therapeutic advantages for patients with CITP, which are safer, more effective with lower costs.

The studies abovementioned indicate that abnormal humoral immune is unable to cover the whole pathogenesis of CITP, in addition, anti-platelet antibodies cannot be detected in about 40% of ITP patients. Current study has suggested that T cells immune abnormality is

*Supported by the National Natural Science Foundation of China (No. 81774068), National Major Project for the Innovative New Drugs of "the 13th Five-year Plan" (No. 2016ZX09101071)
Institute of Hematology Research, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou (310006), China
Correspondence to: Prof. GAO Rui-lan, Tel: 86-571-87911040, E-mail: gaoruilan@126.com.com
DOI: https://doi.org/10.1007/s11655-019-3031-9

one of the key factors in the pathogenesis of CITP.[6]

T Cell Immune Abnormality: A New Understanding on Pathogenesis of CITP

Failure of immune tolerance to platelet autoantigen mediated by T cells is an important pathogenesis of CITP, the main manifestations are excessive activation and proliferation of T cells in response to platelet autoantigen, abnormal number and function of regulatory T cells (Treg), as well as helper T cells (Th). Therefore, T cell subsets and their secreted cytokines play an important role in the pathogenesis of ITP, and also become potential therapeutic targets and evaluation index for therapeutic effect.

Normally, the ratio of Th1/Th2 is dynamically balance to maintain a relatively stable state of our body. It was found that patients with CTIP presented Thl polarization, and the level of related cytokines IFN-γ significantly increased, while Th2 polarizing was observed during remission stage. GATA binding protein 3 (GATA-3)/ T-bet plays as the transcription factors to modulate specifical differentiation of Thl/Th2. T-bet promotes Th0 cells differentiation into Th1 cells, meanwhile, it inhibits polarization of Th2 cells.[7] The transcription factor GATA-3 plays a core role in promoting formation of Th2 cells, and inhibiting generation of Th1 cells. The research result showed that mRNA level of GATA-3/T-bet was significantly increased in CITP patients, while decreased after granalooyte colony-stimulating factore treatment.[8]

Treg cells as a special T cells subsets possess immunomodulatory function *in vivo*, which play an important role in maintaining the immune balance of the body.[9] According to the source and function, Treg cells can be classified into 2 types: natural Treg (nTreg) and induced Treg (iTreg), which play an important role in immune tolerance and immune regulation by secreting cytokines interleukin (IL)-10 and transforming growth factor-beta (TGF-β). For CITP patients, the number of Treg cells is decreased and their function is reduced. The mRNA expression levels of IL-10 and forkhead box P3 (Foxp3) transcription factor are decreased, resulting in weakening the ability of Treg which inhibit the activation of self-reactive T cells and maintain autoimmune balance, therefore, it may be an important factor in the pathogenesis of CITP.[10] Th17 cells are a class of effector $CD4^+$ T cells, activated $CD4^+$ T cells activate lonely nuclear receptor (RORγt) through signal transduction pathway of transcriptional factor signal transducers and activators of transcription 3 (STAT3), which promote differentiation into Th17 cells to secret IL-17 and the other cytokines. Our earlier investigation also confirmed that Th17 and $CD4^+$ T cells play an important role in the pathogenesis of CITP,[11,12] and Th17 cells in the CITP patients showed up-regulating mRNA expression on IL-17A and RORγt.[13,14]

Recent studies have shown that imbalance of Treg/ Th17 cells ratio is also one of important pathogenesis in ITP.[15-17] IL-17A and IL-21 enhance Th17 cell formation, while inhibit Treg cell generation through Th17 cell-related signaling pathways.[18] IL-35 inhibits the proliferation of $CD4^+$ and $CD8^+$ T cells, and promotes the proliferation of Treg cells.[19] $CD16^+$ monocytes promote the proliferation of Th1 cells and negatively regulate the proliferation of Treg and Th17 cells. These results indicated that the regulation balance of Th17/Treg cells ratio might be a potential target for the treatment of CITP.

Follicular helper T lymphocyte (TFH) is a newly discovered $CD4^+$ T cell subgroup with independent cell phenotype as $CD4^+CD57^+$ C-X-C motif chemokine receptor type 5^+ ($CXCR5^+$), which express specific CD40, inducible T cell costimulator (ICOS), CXCR5, B cell lymphoma-6 (Bcl-6), and secrete IL-21. By expressing $CD40L^{HI}$ and $ICOS^{HI}$, TFH cells are respectively bound to CD40 and ICOS ligand on surface of B cells. By the germination center, TFH cells promote B cells transform towards to memory B cells and plasma cells,[20,21] which is crucial for maintaining immune tolerance and preventing the occurrence of autoimmune diseases. The studies indicated that the proliferation of TFH cells is inversely proportional to the count of peripheral blood platelet in CITP patients and the mRNA expression of Bcl-6 and c-maf bzip transcription factor are significantly increased, while the expression of prephenate dehydratase-1 (PD-1) is decreased.[22] TFH cells promote the differentiation of B cells, and increase the production of anti-platelet antibodies.[23] After treatment with rituximab, the number of TFH cells and the expression of C-X-C motif chemokine receptor type 13 (CXCL13) are significantly reduced in CITP patients.[24]

Other T cells subsets, such as Th22 cells, mainly secreting IL-22, are a new Th cell subgroup. A study has confirmed that CITP patients have higher proportion of Th22 cells and IL-22 level, which are positively

correlated with Th17 and Th1.[25] Cytotoxic T cell (CTL)-mediated platelet lysis or apoptosis has been proved to be an important mechanism of platelet destruction in CITP,[26] therefore inhibition cytotoxic effects of CTL may become a new strategy for CITP treatment.

Taken together, the pathogenesis of CITP involves complex immune disorders, but T cell subsets and related cytokines play a leading role in its immune abnormalities. It also provides accurate therapeutic targets and observation indicators for CM therapy with immunoregulation, so as to restore its immune balance status in CTIP.

CM Interferes and Modulates with Cellular Immunity in CITP: From Basic to Clinical

The blood syndrome chapter of *Jingyue's Collected Works* (Jing Yue Quan Shu) summarized the pathogenesis of blood syndrome in the outline of strong fire and qi impairment. Modern medical experts gradually realize the relationship between blood stasis and blood syndrome. They believe that if blood stasis exists, fresh blood would not come into being. The pathogenesis include the three parts of "heat, deficiency and stasis". In 1987, CM syndromes of ITP were classified into 4 types in the National Symposium on Emergency of CM, including blood-heat bleeding, yin deficiency and blood heat, qi deficiency failing to control blood and internal obstruction of blood stasis. Studies have shown that ITP patients with different CM syndromes appeared as different degrees imbalance on Th17/Treg cells ratio.[27] CM syndromes of patients with CITP are more common in the latter two types: both qi deficiency failing to control blood and internal obstruction of blood stasis. In order to cure the disease, it is necessary to use the product of nourishing qi to invigorate Pi (Spleen), as well as the product of regulating qi to disperse stagnation, so as to eliminate the evil of deficiency and excess.

Invigorating Pi and Tonifying Qi, Governing and Stopping Bleeding

The book of *Synopsis of the Golden Chamber* (Jin Gui Yao Lue) notes that blood of internal organs in human body, depends entirely on the control of the Pi qi, which provides a evidence for treatment of blood syndrome. Modern medical experts also believe that deficiency of Pi and qi failing to control blood are regarded as the key pathogenesis of CITP.[28-30] Domestic scholars analyzed the distribution rule of CM syndromes and found that incidence rate of qi deficiency syndrome was up to 80% in patients with CITP.

Basic Research and Clinical Application of Effective Components of CM on Cellular Immunity Regulation in CITP

Chinese Ginseng herb is praised as the first effective medicine for invigorating qi, which can replenish qi and nourish blood, tranquilize the mind, increase intelligence and invigorate qi to assist yang. Since 1992, our study group has found and confirmed that panaxadiol saponins possessed both hematopoiesis promotion and immune regulation effects.[30] It can effectively induce proliferation, differentiation and maturation of megakaryocytes. Animal experiments indicated that it was efficacious for treating mouse models with ITP, which could restore the abnormality of T cell subsets by increasing the number of $CD4^+$ T and $CD4^+CD25^+$ T cells in peripheral blood, and the ratio of $CD4^+/CD8^+$ cells. Meanwhile, it stimulated the proliferation of marrow megakaryocyte hematopoietic progenitor cells, promoted the maturation of protokaryotic megakaryocytes, and effectively increased the peripheral blood platelet count in animal models with ITP.[32,33] The new Chinese patent medicine of Pai-neng-da Capsule (派能达胶囊) was successfully approved by State Food and Drug Administration (SFDA) of China to conduct clinical trials. The result from the phase Ⅰ clinical trial had demonstrated that average peripheral blood platelet count was significantly higher than placebo-controlled group, and good response rate was improved by 40.0% after treated with Pai-neng-da Capsule (240 mg/d) alone for 8 weeks in CITP patients with sydrome of deficiency of qi and blood.[34,35] The CITP patients were treated with Pai-neng-da Capsule combined with Ammonia peptide hormones, the results showed that peripheral blood platelet count was effectively increased with high response rate of 64.5%.[36] Besides, Pai-neng-da Capsule can significantly improve CM deficiency syndrome and patients' quality of life without obvious side effects.[34-36] Recently, the phase Ⅱb clinical trial of multicenter, randomized and double-blind in the treatment of CITP is under way.

Cellular Immunomodulatory Effect Initiated by CM Decoction on CITP

Modern pharmacological and clinical research have confirmed that the CM compound preparations

of reinforcing qi and nourishing blood, which are rich in *Astragalus mongholicus* and Ginseng herbs, possess a good clinical effect on patients with CITP. Guipi Decoction (归脾汤) was first dscribed in the book of *Prescriptions to Aid the Living* (Ji Sheng Fang). After continuous improvement by medical experts of later generations, the clinical application indication of Guipi Decoction has been expanded. It could inhibit humoral and cellular immunity, reduce the generation of antibodies and lymphoid cytokines, and suppress abnormal hyperactive immunity. Guipi Decoction was used to treat ITP model mice with type of Pi failing in governing blood, through reducing $CD8^+$ T cells to normal level, restoring the ratio of $CD4^+/CD8^+$ T cells, and increasing the peripheral blood platelet count. A randomized, parallel controlled clinical research showed that the total effective rate was 88.57% after Guipi Decoction treatment for 4 weeks in CITP patients, which was significantly higher than the control group.[37] Modified Guipi Decoction (加味归脾汤) can regulate abnormal T cell immune function by increasing the numbers of Treg cells, and enhance the immune suppression function on Treg cells, thereby playing a therapeutic role for CITP patients through reducing the generation of autoantibodies.[38] Yang, et al[39] also deemed that qi failing to control blood due to Pi-qi deficiency, is the key pathogenesis of CITP, and involves the course of CITP. She proposed that invigorating qi and strengthening Pi for hemostasis was the main method, and was good at using Guipi Decoction for the treatment CITP patients and achieve its efficacy.

The hospital preparation Shengxueling Granules (生血灵颗粒) developed by Zhou, et al[40] could restore the balance of Th1/Th2 cells ratio by up-regulating the expression levels of IL-2 and IL-10 in CITP patients, and regulating the ratio of glucocorticoid receptors (GR) α/GR β, and their protein expression levels, so as to eliminate antigens and achieve the therapeutic effect by reverting hormone resistance and activating the body's autoimmune function.[41,42] Based on Sijunzi Decoction (四君子汤), Prof. Chen added hemostatic CM to form the "Pi-invigorating, qi-replenishing and blood-containing formula", which has a good hemostatic effect on zebrafish bleeding model induced by simvastatin.[43,44] By down-regulating β-endorphin (β-EP) and up-regulating vasoactive intestinal peptides (VIP) and salivary IgA protein levels, it can increase count of peripheral blood platelets and the amount of hemoglobin in the model mice.[45,46] Meanwhile, it can significantly improve the symptoms of Pi qi deficiency in ITP mice. Result from a randomized controlled, multi-center clinical trial showed that the sydrome of ITP patients with Pi qi deficiency were significantly improved, and the ratio of $CD4^+/CD8^+$ cells was decreased. The effects of abovementioned prescription include regulating immune function and increasing peptide neurotransmitters VIP, improving the clinical symptoms, alleviating bleeding tendency, and increasing the peripheral blood platelet count.[47,48]

Supplementing Qi and Activating Blood, Eliminating Stasis and Stopping Bleeding

Blood stasis is a key pathological factor of CITP, and involves in the whole onset process of CITP.[49] Enriching qi and activating blood is the way to treat CITP patients with type of blood stasis obstructing vessels. Qilong Granules (芪龙颗粒) as a CM compound of benefiting qi and activating blood, can effectively improve the clinical symptoms of ITP model mice with type of qi deficiency and blood stasis. Through increasing peripheral blood $CD3^+/CD4^+$ T cell ratio and reducing levels of serum IL-2, IL-6 in model mice with ITP, it can promote bone marrow megakaryocyte differentiation, maturation, and recover peripheral blood platelet count. In a randomized, double-blind and placebo control trial, combined Qilong Granules with Western medicine treatment for 3 months, the CM symptom score of qi deficiency and blood stasis was decreased, and the bleeding symptom significantly improved. Treatment with Gilong Granule alone, the total effective rate was 70% in CITP patients, which was significantly higher than 31.6% in the placebo control group. The study also found that CM therapy can significantly increase the number of megakaryocytes which produces platelets in bone marrow, and increase platelet count in peripheral blood.[50]

The extracts Madder and shiver can promote hemostasis in ITP mice model, and significantly increase platelet count by promoting the maturation and differentiation of megakaryocytes, regulating $CD3^+$ T cells subsets, and increasing the proportion of $CD4^+CD25^+$ T cells.[50] Animal experiments showed that Ziqian Capsule (紫茜胶囊) could reduce the damage of platelet by suppression of platelet-related antibodies such as PAIgG, PAIgA and PAIgM in ITP model mice, and raise the platelet count by increasing the number

of megakaryocytes that produce platelets. The results of clinical research indicated that Ziqian Capsule could significantly increase platelet count and decrease recurrence rate of CITP through down-regulating the expression of Fas ligand (FasL), IL-2 R, IL-4, IL-6 and tumor necrosis factor-alpha (TNF-α) and up-regulating the expression levels of tumor necrosis factor related apoptosis inducing ligand (TRAIL) and IL-2, and promoting T lymphocyte apoptosis in CITP patients.[51]

Tonifying Qi and Activating Yang, Regulating Gan (Liver) to Invigorate Pi

Prof. MA Rou considered that CITP is caused by qi impairment. Qi deficiency is the main performance in the early stage, as the disease drags on, it will lead to yang qi deficiency. Study result revealed that Yiqi Tongyang Decoction (益气通阳汤) can increase the platelet count and megakaryocyte population as well as recover T lymphocyte subset balance by increasing $CD3^+$, $CD4^+$ and ratio of $CD4^+$/ $CD8^+$ T cells in ITP model mice.[52] The clinical study has also achieved satisfactory therapeutic effect.[53]

Research found that T lymphocyte subset such as $CD3^+$ human leukocyte antigen-DR^+ ($HLA\text{-}DR^+$) T, $CD3^+CD25^+$T, $CD4^+\gamma\delta$ T, $CD8^+\gamma\delta$ T, $CD8^+$NKT, Thl and Treg cells were significantly increased in CTIP patient, but the population of $CD4^+$NKT, Th17 cells were significantly decreased, and the expression of T-bet and GATA-3 mRNA were significantly reduced. It suggests that prescription of tonifying qi and activating yang can restore the balance of cellular immune by regulating ratio of Thl/Th2 and Th17/Treg in CTIP patients.[54] According to the theory of "yang fluidizing qi and yin forming shape", Prof. Hu considers that megakaryocyte and platelet are tangible substances that belong to yin, megakaryocyte possess the ability to mature, differentiate and generate platelets, which belongs to yang. Shen is the foundation of constitution, acted as hiding genuine yin and cultivating original yang, storing essence of life and charging the bone and marrow. If congenital inadequacy or acquired essence lose nurture, it results in Shen yin deficientcy and bleeding. The method of invigorating yang and tonifying qi is effective in treating this type of CITP patients.[55] Li, et al[56] believe that the pathogenesis of CITP is Gan losing to act freely and Pi failing to control blood. He explored the method of soothing Gan and invigorating Pi for CITP patients and also achieve good curative effect.

Problems and Perspectives

The application of CM therapy for CITP has shown certain advantages and curative effects in both basic and clinical research, but some problems remain unsolved. On one hand, some experts of CM have slightly different opinions regarding the etiology, pathogenesis, treatment and prescription of CITP. On the other hand, systematic research about blood syndrome theory of CM are still insufficiency, furthermore, in-depth research on immune regulation intervention mechanism are needed. Overall speaking, there is little research on the development of new Chinese medicine for CITP in China. Lack of sufficient clinical studies on multi-center and large population of CM treatment for CITP, and evaluation criteria for efficacy and side effects of CM are still a major concern.

Taken together, CITP is a complex heterogeneous autoimmune disease, multiple mechanisms are involved in the pathophysiological process, immune-intolerance is one of the most fundamental pathogenic factors, T cell immune-intolerance locates at the upstream center link of CITP pathogenesis, which play a key role in the production of autoantibodies and CTL against platelets. Therefore, it is crucial to restore T cell immune tolerance in the treatment of CTIP. In cellular immunity, T cells induce and restrict each other to form T cell networks. The effector lymphocytes interact with each other by secreting cytokines, which is a central link for maintain the stability of T cell network. The state of immune balance between cells determines the occurrence and development of immune response, which is coincides with the theory of yin-yang of CM. The imbalance between yin and yang is the basic pathogenesis, therefore, the immune regulation effects of CM can be utilized to regulate the yin-yang balance of the body, and directly or indirectly intervene with the immune internal environment. Giving full play to the unique advantages of CM, it provides a new therapeutic strategy and thoughts in the treatment of CITP.

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(Accepted October 8, 2018)
Edited by TIAN Lin