Mechanism study of the effect of Ningxue Shengban decoction on regulating the ratio of Treg/Th17 cells and downstream inflammatory factors in immune thrombocytopenia model mice

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Abstract

Background: To explore the role and mechanism of T cell imbalance in the process of immune thrombocytopenic purpura (ITP) and the efficacy of the Ningxue Shengban decoction in treating this disease. Methods: Passive immune ITP mouse model was established by injecting CD41 monoclonal antibody into BALB/c mice. The mice were divided into different groups for intervention and drug administration for 9 days. The therapeutic effects (blood cell count, bone marrow morphology) were observed. The changes in the number and proportion of regulatory T/Th17 cells in each group, as well as the expression of key transcription proteins and genes (Foxp3, RORγt) in mouse spleen, and the secretion of related inflammatory factors (interleukin-17, TGF-β, interleukin-21, interleukin-10) in serum were detected. Results: Ningxue Shengban decoction significantly increased the peripheral blood platelet count in ITP mice, improved bone marrow morphology, restored the imbalance of regulatory T/Th17 ratio, and exerted a positive regulatory effect on target proteins and downstream inflammatory factor secretion. Conclusion: Ningxue Shengban decoction may exert its therapeutic effect in treating ITP by regulating T cells and exerting immune regulatory function.

Keywords: immune thrombocytopenic purpura; immunologic balance; traditional Chinese medicine; Ningxue Shengban decoction
Introduction

Immune thrombocytopenia (ITP) is a common immune-mediated bleeding disorder characterized by skin and mucosal bleeding. Severe cases can involve visceral or even intracranial bleeding, posing a life-threatening risk. The incidence rate in adults is 5–10 per 100,000. Currently, commonly used treatment options include glucocorticoids and intravenous immunoglobulin infusion. However, the treatment efficacy varies greatly among individuals, and approximately 25% of ITP patients eventually progress to chronic ITP, often requiring splenectomy as a treatment option, which is not always satisfactory.

Traditional Chinese medicine has shown good therapeutic effects in the treatment of chronic ITP. Our previous research has found that the Ningxue Shengbun decoction (NXXSB) has a significant improvement effect on peripheral blood platelet reduction in an ITP mouse model. The NXXSB consists of water buffalo horn, Rehmanniae Radix, Paeoniae Alba Radix, and Moutan Cortex, combined with Ligustri Lucidi Fructus, Eclipse Herba, Angelicae Sinensis Radix, and Astragali Radix, among other herbs, and has the functions of clearing heat, nourishing Yin (in Chinese philosophy, the female, latent, passive principle, characterized by dark, cold, wetness, passivity, disintegration, etc.), promoting blood circulation, and resolving stasis.

In recent years, it has been found that T cell differentiation has an important role in this disease [1]. Regulatory T (Treg) cells, as a subpopulation of functionally mature T cells, inhibit the immune response and maintain immune tolerance and immunomodulation; while Th17 cells, which mainly secrete interleukin-17 (IL-17), have the main function of recruiting inflammatory cells and causing inflammatory cell infiltration and tissue destruction in autoimmune diseases. The differentiation balance of Th17 cells and Treg cells plays an important role in the development of autoimmune diseases, and the two have opposite effects on inflammation and immunomodulation in the body, checking and balancing each other to maintain the immune balance of the body. In this study, we established a mouse model of ITP and used different doses of NXXSB to investigate the changes in the ratio and number of Th17/Treg cells in the process of ITP and the effects on the release of downstream inflammatory factors, hoping to provide useful information on the treatment of this disease with NXXSB. It is hoped that it will provide a useful discussion on the treatment of this disease with the NXXSB.

Materials and methods

Experimental reagents and apparatus

MWR30g (CD41) was purchased from ebioscience (San Diego, CA, USA); anti-mouse CD25-APC antibody, anti-mouse FoxP3-PE antibody, anti-mouse CD4-FITC antibody; anti-mouse IL-17-APC antibody, anti-mouse CD3-APC antibody and the corresponding counterpart control antibodies, anti-mouse related orphan receptor (ROR) Gamma t antibody, anti-mouse FoxP3 antibody were purchased from Abcam (Waltham, MA, USA); enzyme-linked immunosorbent assay (ELISA) kits: Mouse IL-17 APlatinum ELISA kit, Mouse interleukin-21 (IL-21) Platinum ELISA kit, Mouse TGF-beta1 Platinum ELISA kit, and Mouse interleukin-10 (IL-10) Platinum ELISA kit were purchased from R&D Systems, Inc. (Minneapolis, MN, USA); flow cytometer from Beckman Coulter, Inc. (Indianapolis, IN, USA); and XN-1000V blood cell analyzer (Sysmex, Tokyo, Japan).

Animals and prescription administration

BALB/c mice, male half and male female, aged 6–8 weeks, weighing 20 g ± 2 g, a total of 60 mice (SCXK (Jing) 2016-0006). Purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were housed in clean-grade cages in the Laboratory Animal Room of the Institute of Radiology, Chinese Academy of Medical Sciences, regularly disinfected, with adequate air circulation, suitable temperature and humidity, and normal light-dark cycle. The mice were acclimated for 1 week.

Using the method described in the literature, CD41 (MWR30g) exogenous antibody was injected to induce thrombocytopenia in mice [2]. The specific steps are as follows: dissolve 2 μg of MWR30 monocolonal antibody in 200 μl of phosphate buffer saline (PBS) and store at 4 °C for later use. From day 1 to day 7 of the experiment, inject 200 μl of prepared MWR30 solution into the peritoneal cavity of each mouse daily. Evaluate the model based on the degree of peripheral blood platelet decrease and bone marrow changes. All animal procedures performed in this study were initially conducted by the National Institutes of Health Guide for Care Use of Laboratory Animals and approved by the Animal Care Ethics Committee of Tianjin Medical University (approval no. 2019-0004).

Grouping and intervention

A total of 60 BALB/c mice were randomly divided into six groups (n = 10): the control, model, prednisone, and three NXXSB groups with varying concentrations (10.725 g/kg, 21.5 g/kg, 42.9 g/kg). The 21.5 g/kg concentration was equivalent to the clinical dosage in adults. Control group and model group were given 0.2 mL of distilled water daily for 9 days. Prednisone groups were given gastric perfusion (0.0026 mg/kg) once a day for 9 days. NXXSB groups were given gastric perfusion once a day for 9 days. The dose was calculated by referring to the equivalent measurement method for conversion of human and animal surface areas in Methodology on Chinese medicinal pharmacology (2006) edited by Chen Q [3]. The low, medium and high dose groups were equivalent to 0.5:1:2 times the clinical dose, respectively. The solvent of all administration groups was distilled water, and the administration volume was adjusted to 0.2 mL. Intervention for 9 days. 24 hours after the last administration, each group of mice were decapitated and executed, blood was taken, materials were taken, and organ samples were taken and frozen at a temperature of minus 80 °C.

Mouse platelet count and bone marrow morphology observation

Before each administration, blood samples were taken from each group of mice by collecting 20 μl of blood from the medial canthus vein using a capillary glass pipette. The samples were collected alternately from the left and right eyes and placed in Eppendorf tubes treated with 10% ethylene diamine tetraacetic acid. The samples were then diluted in a 1:5 ratio, mixed thoroughly, and the blood cell count was measured using an automated hematology analyzer.

24 hours after the last administration, the mice were euthanized by cervical dislocation. The sternum and bilateral femurs were collected and washed with 500 μl of bovine serum using a syringe. The flushed bone marrow was centrifuged at 125 g for 15 minutes, and the supernatant was discarded after centrifugation. 20 μl of serum was used to resuspend the bone marrow for smear preparation. The bone marrow was fixed with 10% neutral formalin and stained with Wright-Giemsa stain. Bone marrow cell classification was performed under an optical microscope. The optimal dosage of traditional Chinese medicine was selected based on the blood count and bone marrow examination results for subsequent mechanistic experiments.

Detection of the expression of FoxP3 and RORγt proteins in mouse spleen by immunoblotting

After the last administration for 24 hours, mice were euthanized by cervical dislocation. The spleen was isolated, ground on ice, and passed through a 200-mesh sieve. The separated liquid was collected and the supernatant was discarded after centrifugation at 250 g for 5 minutes. Then, 5 mL of lysis buffer was added and incubated at 37 °C for 5 minutes. After centrifugation at 250 g for 5 minutes, the supernatant was discarded. Bicinchoninic acid protein quantification was performed. 4 × buffer was added and the samples were denatured at 100 °C. Equal amounts of protein samples were subjected to SDS-PAGE gel electrophoresis and transferred onto a membrane. The membrane was blocked with skim milk powder and incubated with primary antibodies against FoxP3 and RORγt at a concentration of 1:1000. The secondary antibody used was horseradish peroxidase-labeled IgG. Electrochemiluminescence detection was performed and the grayscale values of the hybridization bands were submitted.
Detection of peripheral blood Treg and Th17 cell counts by flow cytometry

24 hours after the last administration, the posterior vena cava of mice was collected with 200 μl of peripheral blood, anticoagulated with heparin, and tested within 4 hours. Treg cells: each group was divided into two tubes (experimental tube, control tube). 2 μl of CD3-APC and CD4-FITC were added to the experimental tube, and the corresponding isotype controls were added to the control tube. Incubate at 4 °C in the dark for 20 minutes, wash with PBS, centrifuge at 400 g for 5 minutes, discard the supernatant, add 1 ml of Fix & Perm permeabilization solution, incubate at 4 °C in the dark for 30 minutes, wash with PBS, add 2 ml of Fix & Perm fixation solution, wash with PBS, add 2.5 μl of FoxP3-PE to the experimental tube, add IgG2a-PE to the control tube, incubate in the dark for 30 minutes, wash, and detect using flow cytometry. Th17 cells: 2 μl of CD3-APC and CD4-FITC were added to the experimental tube, and the corresponding isotype controls were added to the control tube. Incubate at 4 °C in the dark for 20 minutes, wash with 2 ml of PBS, centrifuge at 400 g for 5 minutes, discard the supernatant, add 2 ml of Fix & Perm fixation solution, wash with PBS, add 2 ml of Fix & Perm fixation solution, wash with PBS, add 2.5 μl of IL-17-APC to the experimental tube, add IgG2a-APC to the control tube, incubate in the dark for 30 minutes, wash, and detect using flow cytometry.

Expression levels of cytokines in serum

After the last administration of the drug for 24 hours, peripheral blood was collected from the posterior vena cava of mice in each group. After 2 hours of static incubation, the blood was centrifuged at 2500 g for 15 minutes to obtain the upper serum layer, which was stored at -80°C for subsequent measurements. ELISA was used to measure IL-17, IL-21, IL-10, and TGF-β in the serum, following the instructions.

Statistical methods

SPSS 20.0 statistical software was used to analyze the data. Normality test was conducted first. Continuous data were presented as mean ± standard deviation (± s). Independent samples t-test was used to compare the means of two independent samples. One-way analysis of variance was used to compare multiple groups of data. When the variances were equal, pairwise comparisons between groups were conducted using the least significant differences test. When the variances were unequal, Dunnett’s T3 test was used. P < 0.05 denoted statistical significance.

Result

Improvement of peripheral blood and bone marrow morphology in ITP mice by the combined prescription of NXSBD

The results of the blood routine test showed no significant difference in platelet counts among the groups of mice before the experiment. After the injection of exogenous antibody CD41, the model group showed a significant decrease in peripheral blood platelets on the 4th day of the experiment. Throughout the subsequent administration period, the platelet count remained at a low level (always below 50% of the pre-modeling level), with a significant statistical difference compared to the control group (P < 0.01), indicating the successful establishment of the ITP model. Compared to the model group, the high-dose group of NXSBD and the prednisone group showed a more gradual decline in platelets. At the end of the experiment (day 9), there was a statistically significant difference in platelet counts between the high-dose group of NXSBD and the prednisone group compared to the model group (P < 0.05) (Figure 1). Megakaryocyte maturation disorder in the bone marrow is a characteristic pathological change of ITP. As shown in Figure 2, mature platelet-producing megakaryocytes can be observed in the bone marrow of mice in the normal control group: the cytoplasm is uniformly pink-purple, filled with purple-red granules and platelets of varying sizes, and the cell membrane appears pseudopod-like with platelet aggregation on the inner and outer sides. In the model group, a large number of immature megakaryocytes can be seen in the bone marrow, showing naked nuclei and small megakaryocytes: the nuclei are irregular, overlapping, and twisted, with coarse granular chromatin, dense arrangement, visible nucleoli or no nucleoli, and stained blue or light blue, with a few azure granules near the nucleus. The number of megakaryocytes that do not produce platelets and immature megakaryocytes in the bone marrow of the treatment groups mice were reduced. Based on the results of peripheral blood and bone marrow smears, the high-dose group of NXSBD showed the best improvement in blood and bone marrow smears.

Effects of NXSBD on the expression of Treg differentiation pathway-related proteins FoxP3 and RORγt in ITP model

This study detected the expression of Treg cell-related transcription factors in the spleen. The results showed that the expression level of FoxP3 protein in the spleen of the model group mice was significantly lower than that of the normal group, with significant statistical significance (P < 0.01), while the expression of RORγt was significantly increased, with significant statistical significance (P < 0.01). After treatment, the expression level of FoxP3 protein in the prednisone group and NXSBD group was significantly increased (P < 0.01), while the expression levels of RORγt proteins were significantly downregulated, with significant statistical significance (P < 0.01). However, there was no statistical significance between the Western medicine group and the herbal medicine group (Figure 3). According to the above research results, the optimal improvement was observed in the medium-dose group of NXSBD. Therefore, in the subsequent mechanism exploration, the medium-dose group of NXSBD will be used as the representative of the Chinese medicine group for experimentation.

Figure 1 The effect of NXSBD upon peripheral platelet function in a rat model. (A) The effect of NXSBD on peripheral blood platelets in ITP model mice (a line graph showing the changes of peripheral blood PLT in mice of each group over time; (B) histogram showing the PLT situation of mice in each group on the 9th day of the experiment). *(P < 0.05 compared with the control group; **P < 0.01 compared with the control group; ***P < 0.05 compared with the model group; ****P < 0.01 compared with the model group). ITP, Immune thrombocytopenia; NXSBD, Ningxue Shengban decoction; PLT, platelet.
The effect of NXSBD on the changes in the number and proportion of Treg/Th17 cells in the peripheral blood of ITP mice

The results of the immunofluorescence-labeled flow cytometry showed that compared with the control group, the number and ratio of Th17 cells in the peripheral blood of the model group mice increased significantly, with statistical significance ($P < 0.05$), while the number of Treg cells decreased significantly, with statistical significance ($P < 0.05$). This result is consistent with the research results of ITP patients, suggesting that this model conforms to the pathogenesis of immune imbalance in ITP. After treatment, the number of Th17 cells decreased and the number of Treg cells increased in the prednisone group and the NXSBD group, with statistical significance compared to the model group ($P < 0.05$). In the combined formula medium-dose group, the number of Th17 cells decreased and the number of Treg cells increased compared to the model group, with statistical significance ($P < 0.05$). This suggests that the immune imbalance state in ITP mice is alleviated to varying degrees after treatment (Table 1, Figure 4).

Effects of NXSBD on the secretion of T cell-related cytokines IL-17, IL-21, TGF-β, and IL-10 in the peripheral blood serum of ITP model mice

In the serum, the expression of Th17-related pro-inflammatory factors IL-17 and IL-21 in the model group mice was significantly increased compared to the normal group, with significant statistical differences ($P < 0.01$). However, the expression of Treg-related factors TGF-β and IL-10 was significantly down-regulated compared to the normal group, with significant statistical differences ($P < 0.01$). In the treatment group, the expression of IL-17 and IL-21 in the serum of the NXSBD group and the prednisone group was significantly decreased compared to the model group, while the expression of TGF-β and IL-10 was significantly increased compared to the model group, with significant statistical differences ($P < 0.01$) (Figure 5).
Table 1 Statistical results of the proportion of Th17/Treg cells in peripheral blood of mice in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Th17 (CD3⁺CD4⁺IL17⁺)</th>
<th>Treg (CD4⁺CD25⁺FoxP3⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.97% ± 2.30</td>
<td>1.63% ± 1.19</td>
</tr>
<tr>
<td>Model</td>
<td>20.33% ± 3.51*</td>
<td>1.02% ± 0.28*</td>
</tr>
<tr>
<td>Prednisone</td>
<td>12.23% ± 2.21†</td>
<td>2.01% ± 0.28§</td>
</tr>
<tr>
<td>NXSBD</td>
<td>11.97% ± 2.30</td>
<td>2.63% ± 1.19*</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with the control group; †P < 0.05 compared with the model group; §P < 0.01 compared with the model group. NXSBD, Ningxue Shengban decoction; Treg, regulatory T; IL-17, interleukin-17.

Discussion

Under the guidance of the basic theory of traditional Chinese medicine, NXSBD combines traditional Chinese medicines such as clearing heat, nourishing Yin, promoting blood circulation, removing stasis, and has achieved good curative effects in the clinical treatment of patients with ITP. Modern pharmacological research shows that water buffalo horn has effects such as strengthening the heart, shortening blood clotting time, and reducing total white blood cell count [4]. *Rehmanniae Radix* can regulate immune function, dilate blood vessels, reduce inflammation, strengthen the heart, promote...
diuresis, and nourish the blood [5]. *Moutan Cortex* has the functions of reducing fever and pain, regulating immunity, and preventing blood clot formation [6]. *Ligustri Lucidi Fructus* has functions such as antioxidant, immune regulation, and reducing hypercholesterolemia [7]. The main components of *Eclipta Herb* are eclipalactone, dehydro with anolide, and dehydro with anolide B, which can shorten prothrombin time and partial thromboplastin time, increase platelet count and fibrinogen content, and achieve hemostatic effect [8]. *Aragralus polysaccharides* have immunomodulatory effects on various immune cells, promoting the activity of macrophages, natural killer cells, dendritic cells, T lymphocytes, B lymphocytes, and microglial cells, and inducing the expression of various cytokines and chemokines [9]. *Paoniae Alba Radix* is used in traditional medicine to treat autoimmune diseases related to inflammation. Its main active ingredient, paeoniflorin, has immunomodulatory, anti-inflammatory, and analgesic effects, and has been widely used in the treatment of autoimmune diseases [10]. As a natural polysaccharide, Angelica polysaccharide has hematopoietic activity, promotes immunity, has anti-tumor, anti-inflammatory, and antioxidant effects [11].

The autoimmune response in ITP involves both the innate and adaptive immune systems, comprising both humoral and cell-mediated immune responses [12]. Multiple mechanisms in the body contribute to the development of ITP disease, including monocyte-macrophage proliferation leading to excessive platelet consumption in the spleen, reactive B cell activation resulting in the production of autoantibodies [13], and cytotoxic T cell-mediated direct platelet destruction. Regulatory T cells, on the other hand, can help maintain immune homeostasis by modulating various immune processes through cytokines [14]. Regulatory T cells mainly consist of CD4+ T cells, including Th1, Th2, and the recently discovered Th17 and Treg subgroups [15]. Th17 cells and Treg cells are highly correlated in differentiation and functionally antagonistic. Researchers have found that patients with primary immune thrombocytopenia may have a high expression of IL-17A and Th17/Treg [16]. Researchers have found that in vitro cultured peripheral blood mononuclear cells from ITP patients exhibit increased expression of Th17 cells, IL-21, and RORγt mRNA, while Treg cells and Foxp3 mRNA expression are decreased [17].

Th17 is a subset of CD4+ T cells characterized by the secretion of IL-17. Th17 differentiation is driven by IL-6, IL-21, TGF-β, and IL-1β. Additionally, Th17 cells can secrete IL-21, IL-22, TNF, and other cytokines, which can upregulate immune response. These cytokines play important pathogenic roles in various autoimmune diseases such as rheumatoid arthritis and autoimmune encephalomyelitis [18, 19]. The key transcription factor RORγt is involved in the transcriptional regulation of Th17 cell differentiation. RORγt, as the key transcription factor of Th17, is induced by IL-6 and TGF-β in vitro. In the immune mechanism, Th17 differentiation and the production and secretion of IL-17A, IL-17F, and IL-22 genes are driven by the expression of RORγt. Th17 is its main effector molecule, which can promote the recruitment and activation of neutrophils through chemokines, thereby exacerbating the degree of inflammation [20]. Some research results have shown that the proportion of Th17 cells is significantly increased in the peripheral blood of ITP patients, especially in severe ITP patients. The secretion of inflammatory factors such as IL-17A is significantly increased, but it can be alleviated to varying degrees after treatment [21], indicating that the upregulation of Th17 cells may be associated with the occurrence of ITP.

Treg are a functionally mature subset of T cells that can be generated in the thymus and periphery. FoxP3, a transcriptional regulator, is highly expressed in Treg cells and serves as a specific marker for Tregs. It actively regulates the generation and function of Treg cells. TGF-β secreted by Tregs binds to TGF-βR on target cells, reducing T cell proliferation and suppressing the production of large amounts of immunoglobulin by B cells. IL-10 downregulates the production of INF-γ, TNF-α, and IL-12, leading to TGF-β amplification and promoting the aforementioned responses. IL-10 also confers regulatory properties to tolerogenic dendritic cells [22]. In patients with ITP, the number and proportion of Treg cells among CD4+ T cells are significantly decreased. However, treatment with glucocorticoids such as dexamethasone and thrombopoietin can increase the number and activity of Treg cells in patients who respond to the treatment. This indicates that Treg cells play a certain regulatory role in modulating the immune response and restoring the self-immune imbalance in ITP.

Traditional Chinese medicine in the treatment of this disease has been long-term clinical experience effects and fewer side effects [23]. In this study, the high, medium, and low dose groups of NXSSB have positive therapeutic effects on the peripheral blood profile of ITP mice, increasing platelet count and improving the morphology of bone marrow megakaryocytes. Among them, the medium-dose group has the best therapeutic effect and is selected as the representative of the NXSSB group for subsequent mechanistic experiments. The results of the study on T cell differentiation, key transcription protein expression, and downstream target effectors in the ITP pathological process showed that the quantity and proportion of CD4+ CD25+ Treg cells, the expression of Foxp3 protein expression in the spleen, and the levels of cytokines IL-10 and TGF-β were significantly decreased in the ITP model group. After treatment, the expression levels of CD4+ CD25+ Treg cells, Foxp3, and IL-10 in the medium dose group of NXSSB were significantly increased compared to the model group, indicating that NXSSB intervenes in the important regulatory process of Treg cell differentiation, up-regulates the expression of Foxp3 protein, spleen, and decreases the expression of related downstream cytokines TGF-β and IL-10, and further promotes the formation of a benign cycle of regulatory T cell differentiation. It reduces the quantity and proportion of Th17 cells, decreases the expression of key transcription proteins of RORγt, reduces the release of downstream inflammatory factors IL-17 and IL-21, plays a role in immune tolerance, corrects the imbalance of T cell differentiation in the pathological process of ITP, and restores immune homeostasis.

**Conclusion**

NXSSB has significant therapeutic effects on ITP in clinical practice. In this experimental study, it was found that its mechanism of action may be related to the regulation of T cell differentiation, increasing the proportion of Treg cells, upregulating the expression of the key transcription protein Foxp3, downregulating the number and proportion of Th17 cells, reducing the expression of key transcription proteins RORγt, and exerting immunomodulatory effects by regulating the release of downstream effector factors. This provides strong laboratory evidence for the treatment of this disease with NXSSB.

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Integrative Medicine Discovery 2023;7:e23036. https://doi.org/10.53388/IMD202307036

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