Original Article

Effect of ADSCs on Th17/Treg and T-bet/GATA-3 in model mice with primary immune thrombocytopenia

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Article Info

Abstract

We aimed to observe the effects of adipose-derived mesenchymal stem cells (ADSCs) on T helper 17 (Th17)/regulatory T cells (Treg) and T-box transcription factor (T-bet)/GATA-binding protein 3 (GATA-3) in model mice with primary immune thrombocytopenia (ITP). 32 BALB/C mice were selected. ADSCs were isolated from 2 mice and cultured. The other 30 mice were randomly divided into the normal control group, the ITP model control group, and the ITP experimental group. Platelet count (PLT), Th17/Treg cells, related serum cytokines [interleukin-6 (IL-6), IL-17A, IL-10, and transforming growth factor β1 (TGF-β1)], T-bet and GATA-3 mRNA levels in peripheral blood mononuclear cells (PBMCs) in the 3 groups were detected. PLT and Treg in the ITP experimental group were significantly lower than those in the normal control group (P<0.05), but significantly higher than those in the ITP model control group (P<0.05). Th17 and Th17/Treg in the ITP experimental group were significantly higher than those in the normal control group (P<0.05), but significantly lower than those in the ITP model control group (P<0.05). Serum IL-6 and IL-17A levels, and T-bet mRNA level in the ITP experimental group were significantly higher than those in the normal control group (P<0.05), but significantly lower than those in the ITP model control group (P<0.05). Serum IL-10 and TGF-β levels, and GATA-3 mRNA level in the ITP experimental group were significantly lower than those in the normal control group (P<0.05), but significantly higher than those in the ITP model control group (P<0.05). ADSCs can effectively regulate Th17/Treg balance and improve T-bet/GATA-3 mRNA expression levels in ITP model mice.

Keywords: Primary immune thrombocytopenia; Adipose-derived mesenchymal stem cells; Th17/Treg cells; T-box transcription factor; GATA-binding protein 3

1. Introduction

Primary immune thrombocytopenia (ITP) is a blood disease caused by an autoimmune system disorder, resulting in a decreased number of platelets in the blood of patients. Platelets are key components of the blood, responsible for helping clotting and stopping bleeding. In ITP, the patient’s immune system mistakenly recognizes and attacks its own platelets, resulting in a decrease in their number, which increases the risk of bleeding. ITP is a heterogeneous disease, meaning that its presentation and severity in different patients can vary considerably. Some patients may experience only mild symptoms, while others may suffer more severe bleeding problems. The diversity of symptoms also means that clinical treatment will have different effects, and treatment plans need to be tailored to the specific circumstances of each patient. Among all hemorrhagic diseases, ITP accounted for a relatively high proportion, about 30%. This suggests that it is a relatively common hemorrhagic disorder that requires attention from both medical professionals and the public.

One of the most serious complications of ITP is visceral bleeding, which can occur in any visceral organ, such as the gastrointestinal tract or the brain. Visceral hemorrhage is a serious medical emergency that may endanger the life of the patient and requires immediate medical intervention [1]. At present, the conventional treatment of ITP mainly includes splenectomy, hormone drugs and immunosuppressants, etc. However, the long-term efficacy is not ideal and there are many adverse reactions. Moreover, there is no ideal method to prevent and treat refractory ITP (ITP with no obvious effect of splenectomy and hormone drugs) [2,3]. It is very important to find new prevention and treatment methods based on the pathogenesis of ITP. In terms of clinical treatment concept of immune diseases, mesenchymal stem cells have been developed rapidly and are now considered as a new treatment method. As for the source and type of stem cells, compared with other types of mesenchymal stem cells such as bone marrow, adipose-derived mesenchymal stem cells (ADSCs) show more advantages, such as sufficient donor, low immunogenicity, easy acquisition and culture, etc., showing great application prospects and value [4]. Whether ADSCs can be used in the clinical treatment of ITP and achieve good results needs to be supported by more experimental evidence. At

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present, the detailed pathogenesis of ITP has not been fully clarified. Recent studies have shown that the occurrence of ITP is closely related to the immune state of T cells in the body, especially the immune balance between helper T cells (Th17) and regulatory T cells (Treg) plays an important role, and is closely related to the severity of ITP and the prognosis of patients [5]. Many previous studies have found that Th1/Th2 is unbalanced in ITP patients and shows Th1 bias advantage [6,7]. However, the mRNA levels of GATA-3 and T-box transcription factor (T-bet) can sensitively reflect the imbalance of Th1/Th2 [8]. At present, there are few animal experiments on the effect of ADSCs on Th17/Treg in ITP. In this paper, we explored the effects of Th17/Treg and T-bet/GATA-3 on ADSCs in ITP model mice, hoping to provide a basis for ADSCs for the treatment of ITP.

2. Materials and methods

2.1. Materials

Experimental animals: Thirty-two BALB/C mice were selected, all female specific pathogen free (SPF), 6 weeks old, body weight range 18-26 g, animal license number is SCXK (Beijing) > 2009-0004, the control feeding environment is: The air circulation was maintained, the light temperature was about 21 ~ 25℃. After regular disinfection, the experiment began after 1 week of feeding. The study was approved by the ethics committee.

Main drugs, reagents and instruments: Phosphate buffer solution (PBS), MWReg30 monoclonal antibody, TRIzol reagent, Alexa Fluor® 647 interleukin 17 (IL-17), PE-Foxp3 reagent, polymerase chain reaction (PCR) kit, enzyme-linked immunosorbent assay (ELISA) kit, constant temperature shaker, centrifuge, automatic blood cell analyzer, flow cytometry.

2.2. Isolation and culture of ADSCs

Two mice were sacrificed, and 75% ethanol was used for disinfection. About 1g of inguinal adipose tissue was selected, rinsed through PBS, and then cut into pieces to control the size of tissue about 1 mm³. The supernatant was discarded and placed in DMEM medium (containing 15% fetal bovine serum), and the cell suspension concentration was controlled to 1×10⁶/mL, and put into the culture dish. After 24 hours, the culture medium needed to be changed, and the adherent cells were subcultured until the growth showed 80% confluence. Finally, the third generation ADSCs were used for the experiment.

2.3. Grouping, modeling, and processing

Thirty BALB/C mice were randomly divided into normal control group, ITP model control group and ITP experimental group, with 10 mice in each group. The latter model was established according to ITP model establishment method. MWReg30 monoclonal antibody was diluted with PBS 200 µL and injected intraperitoneally every day. The dose of D1 and D2 was controlled at 68 µg/kg, D3 was controlled at 102 µg/kg, and D4-D8 was controlled at 136 µg/kg. Not restricting their activities and drinking water; The success of modeling was determined as following: on D2, ecchymosis began to occur, and the subcutaneous bleeding spots were displayed, most of which were near the injection point. Venous blood samples were collected for platelet count (PLT) determination, and it was found that the degree of reduction was more than 50%. The normal control group was not given any treatment. The ITP model control group was infused with PBS (200 µL) through the tail vein. In ITP group, ADSCs (1×10⁶ /mL) were infused via tail vein (200 µL). The indexes were detected on the 7th day after treatment.

2.4. Detection of PLT and Th17/Treg cell

About 100 µL of tail vein blood was collected, mixed with anticoagulant, and PLT was detected by automatic blood cell analyzer.

The suspension of peripheral blood mononuclear cells (PBMC) was controlled to 1×10⁶ /mL, and then 100 µL suspension was treated with phorbol ester and ionomycin for 4 h, followed by protein transport inhibitor (PPI). The final concentrations of these three agents were 100 ng/mL, 500 ng/mL and 1 µL/mL, respectively. The cell surface was stained with 10 µL CD4 and CD25 antibodies (labeled with fluorescein isothiocyanate), then incubated in the dark for 0.5 h, washed, incubated in 2 mL of fixative solution for 20 min, and then incubated in 2 mL of membrane disruption solution for 0.5 h. After washing, the cells were incubated with 10 µL Alexa Fluor® 647 IL-17 and PE-Foxp3 reagent for 20 min, followed by washing, and the Th17/Treg ratio was determined by flow cytometry.

2.5. Detection of Serum related cytokines

The venous blood was centrifuged (at a rate of 1200× g) for 10min to obtain the upper serum. Interleukin-6 (IL-6), interleukin-17A (IL-17A), interleukin-10 (IL-10) and transforming growth factor-β1 (TGF-β1) were measured by enzyme-linked immunosorbent assay (ELISA) kit. T-bet and GATA-3 mRNA levels were measured by real-time fluorescent quantitative PCR (RT-PCR) for mRNA determination, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference, and the primer sequences are detailed in Table 1. Samples were mixed in TRIzol solution, total RNA was obtained according to the instructions of the kit, dissolved in diethyl pyrocarbonate (DEPC) water, and then 4.0 µl RNA template was used for reverse transcription reaction. The related system was as follows: RNA template volume 4.0

<table>
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<tr>
<th>Variables</th>
<th>Primer sequences</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-bet</td>
<td>Upstream</td>
<td>5'-GCCAGGGAAACCCTATTATG-3'</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>5'-GACGATCTGGTTCACATTGTG-3'</td>
</tr>
<tr>
<td>GATA-3</td>
<td>Upstream</td>
<td>5'-GAGGTGGACGTACTTTTTAACATCG-3'</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>5'-GCCATACCTTGCTCCGT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Upstream</td>
<td>5'-ACCACACTCATGCCATCAC-3'</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>5'-TCCACACCTGGTGTGTA-3'</td>
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</tbody>
</table>
μL, 4.0 μL reverse transcription buffer, DEPC water volume 10.5 μL, 10 mmol/L deoxyribonucleoside triphosphate (dNTPs), 10 pmol/μL downstream primers, 200 U/μL reverse transcriptase (MMLV) 0.5 μL, respectively, a total of 20.0 μL. RT-PCR related reaction system is as follows: 10 μL of 5×SYBR Green I buffer, 3 U/μL of Taq enzyme, 10 pmol/μL of upstream primers, 10 mmol/L of dNTPs, 10 pmol/μL of downstream primers, 31.0 μL of deionized water (ddH2O), and 31.0 μL of DDH2O. 5.0 μL complementary DNA (cDNA) for a total of 50.0 μL. The PCR reaction conditions were as follows: treatment at 93°C for 3 min, 55°C for 15 s, and 72°C for 30 s, for a total of 40 cycles.

2.6. Statistical analysis
Data were entered into Statistic Package for Social Science (SPSS) 25.0 software for analysis (IBM, Armonk, NY, USA), measurement data were described in the form of (±s), multiple groups were tested by one-way analysis of variance, and multiple group comparison was tested by SNK-q method. The test level was 0.05 (two-sided).

3. Results
3.1. Comparison of PLT among the three groups
Refer to Table 2. The platelet count in ITP experimental group was significantly lower than that in normal control group (P < 0.05), and was significantly higher than that in ITP model control group (P < 0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>PLT (×10⁹/L)</th>
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</thead>
<tbody>
<tr>
<td>ITP experimental group</td>
<td>10</td>
<td>335.14±74.85*</td>
</tr>
<tr>
<td>ITP model control group</td>
<td>10</td>
<td>158.97±26.38*</td>
</tr>
<tr>
<td>Normal control group</td>
<td>10</td>
<td>405.23±74.67</td>
</tr>
<tr>
<td>*F</td>
<td></td>
<td>40.674</td>
</tr>
<tr>
<td>*P</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Compared with normal control group, *P < 0.05; Compared with ITP model control group, #P < 0.05.

3.2. Comparison of Th17/Treg cells among the three groups
Refer to Table 3. The levels of Th17 and Th17/Treg in ITP experimental group were significantly higher than those in normal control group (P < 0.05), but significantly lower than those in ITP model control group (P < 0.05). Treg in ITP experimental group was significantly lower than that in normal control group (P < 0.05), but significantly higher than that in ITP model control group (P < 0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Th17 (%)</th>
<th>Treg (%)</th>
<th>Th17/Treg</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITP experimental group</td>
<td>10</td>
<td>2.23±0.40*</td>
<td>5.03±0.09*</td>
<td>0.44±0.07*</td>
</tr>
<tr>
<td>ITP model control group</td>
<td>10</td>
<td>4.23±0.81*</td>
<td>3.72±0.59*</td>
<td>1.14±0.20*</td>
</tr>
<tr>
<td>Normal control group</td>
<td>10</td>
<td>1.55±0.32</td>
<td>6.08±0.12</td>
<td>0.25±0.05</td>
</tr>
<tr>
<td>*F</td>
<td></td>
<td>63.390</td>
<td>113.171</td>
<td>139.051</td>
</tr>
<tr>
<td>*P</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Compared with normal control group, *P < 0.05; Compared with ITP model control group, #P < 0.05.

3.3. Comparison of serum related cytokines among the three groups
Refer to Table 4. The serum levels of IL-6 and IL-17A in ITP experimental group were significantly higher than those in normal control group (P < 0.05), but significantly lower than those in ITP model control group (P < 0.05). The serum levels of IL-10 and TGF-β1 in ITP experimental group were significantly lower than those in normal control group (P < 0.05), and were significantly higher than those in ITP model control group (P < 0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>IL-6 (ng/L)</th>
<th>IL-17A (ng/L)</th>
<th>IL-10 (ng/L)</th>
<th>TGF-β1 (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITP experimental group</td>
<td>10</td>
<td>25.62±4.37*</td>
<td>24.73±4.18*</td>
<td>32.65±6.01*</td>
<td>83.67±15.39*</td>
</tr>
<tr>
<td>ITP model control group</td>
<td>10</td>
<td>39.64±5.92*</td>
<td>62.35±11.74*</td>
<td>20.13±3.82*</td>
<td>40.05±7.63*</td>
</tr>
<tr>
<td>Normal control group</td>
<td>10</td>
<td>18.87±3.46</td>
<td>11.05±1.98</td>
<td>41.37±7.92</td>
<td>99.18±17.65</td>
</tr>
<tr>
<td>*F</td>
<td></td>
<td>50.935</td>
<td>132.963</td>
<td>30.145</td>
<td>46.486</td>
</tr>
<tr>
<td>*P</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Compared with normal control group, *P < 0.05; Compared with ITP model control group, #P < 0.05.
than that in normal control group (P < 0.05), but significantly higher than that in ITP model control group (P < 0.05).

4. Discussion

ITP is an immune comprehensive disease, which is also a hemorrhagic disease. It is common in clinical practice, mainly characterized by the block of new platelet formation and the continuous reduction of the number of existing platelets, which can occur in people of any age [9]. The pathogenesis of ITP is still not completely clear in clinical practice, so there are few effective treatment methods for the etiology, which seriously affects the quality of life of patients. At present, in the clinical treatment of autoimmune diseases, the use of stem cells for biological immunotherapy is a new method, especially in the improvement of Th1/Th2 immune imbalance, and mesenchymal stem cells have made some progress in the treatment of autoimmune diseases [10]. With the emergence of this biological treatment idea, it effectively solves the problems of drug side effects and application safety in basic treatment (such as hormone drugs and immunosuppressants), and has good application prospects and value. ADSCs are mainly isolated from adipose tissue and have multi-directional differentiation potential. They show low immunogenicity among biological individuals and can inhibit excessive cellular immunity, improve immune disorders, induce immune tolerance, and reduce damage caused by inflammation [11,12].

Previous studies have shown that T lymphocyte subsets play an important role in inducing immune responses in ITP. Induction promotes the activation of T lymphocytes, which can damage normal immune cells [13]. Th17 cells belong to the CD4+T cell subtype. The synthesis of IL-17 is a pro-inflammatory factor, which can exert effects on neutrophils, increase the levels of various inflammatory factors, and participate in the formation of autoimmune diseases and the process of collective defense response. Treg cells are mainly produced by natural thymic cells, which can inhibit the proliferation of Th1 and Th17, and prevent autoimmune damage by protecting peripheral immune tolerance. Relevant reports indicate that there is an antagonistic relationship between Th17 and Treg in terms of differentiation and function, and the balance of Th17/Treg is conducive to protecting the normal immune response process and reducing the risk of autoimmune diseases [14]. This study showed that compared with the normal control group, Th17 and Th17/Treg in the ITP experimental group were significantly increased, while Treg was significantly decreased. After ADSCs treatment, Th17 and Th17/Treg in the ITP experimental group were significantly decreased compared with the ITP model control group, and Treg was significantly increased. ADSCs play an important role in improving the balance of Th17/Treg in ITP mouse models. Th17 cells can synthesize specific inflammatory factors, including IL-6 and IL-17, which are the initiating factors of inflammatory response and play an inflammatory condition. Treg cells can synthesize inhibitory factors, including IL-10 and TGF-β1, which can inhibit autoreactive T cells, thus participating in the process of immune tolerance, maintaining immune homeostasis and establishing tolerance, and avoiding immune inflammation [15]. In this study, the levels of IL-6 and IL-17A in ITP experimental group were significantly lower than those in ITP model control group, and the levels of IL-10 and TGF-β1 were significantly higher than those in ITP model control group, indicating that ADSCs could effectively regulate Th17/Treg related cytokines in ITP mice, which further confirmed that ADSCs could regulate Th17/Treg. It can reduce immune inflammatory injury and maintain immune stability. T-bet, an important member of the T-box gene family, is also a Th1 transcription factor (with specificity), which can be specifically synthesized and expressed in Th1 cells [16-18]. GATA-3, a zinc finger protein, is also a Th2 transcription factor, which can be specifically synthesized by Th2 and has the function of regulating all important Th2 cytokines (such as IL-4, L-5 and IL-13) [19]. This study found that compared with the ITP model control group, the ITP experimental group had a significant decrease in T-bet mRNA level and a significant increase in GATA-3 mRNA level, which was consistent with the previous experiment [20], suggesting that ADSCs treatment could correct Th1 shift in ITP mice and promote the recovery of Th1/Th2 balance. This study showed that PLT in ITP experimental group was significantly higher than that in ITP model control group, but significantly lower than that in normal control group, indicating that ADSCs could effectively improve the condition of ITP model mice. The mechanism may be related to the regulation of Th17/Treg and Th1/Th2 balance by ADSCs, down-regulating the expression of T-bet mRNA and up-regulating the expression of GATA-3 mRNA.

In conclusion, ADSCs may regulate Th1/Th2 balance by improving the balance of Th17/Treg cells and the expression of related cytokines, up-regulating the expression of GATA-3 mRNA and down-regulating the expression of T-bet mRNA, and thus alleviate the condition of ITP mice.

Conflict of Interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.
Ethics approval and consent to participate
This study was approved by the Animal Ethics Committee of PLA Rocket Force Characteristic Medical Center Animal Center.

Informed Consent
The authors declare not used any patients in this research.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Haijiao Dong, Zhaqi Ren: Conceptualization, methodology, writing original draft preparation. Wen Shao, Guanghua Duan, Aicui Du: Investigation, software, statistical analysis. Bin Du: Reviewing and editing, funding acquisition, supervision. All authors read and approved the final manuscript.

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References