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Cytokine changes in response to TPO receptor agonist treatment in primary immune thrombocytopenia

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ABSTRACT

Thrombopoietin receptor agonists (TPO-RAs) have been clinically used in primary immune thrombocytopenia (ITP) with favorable outcomes, while their effect on cytokine regulation in ITP remains unknown. In the present study, plasma and mRNA expression levels of interleukin (IL)-2, interferon gamma (IFN-γ), IL-4, IL-17A, and transforming growth factor-β1 (TGF-β1) were determined by ELISA and real-time quantitative PCR in 26 corticosteroid-resistant/relapsed ITP patients receiving eltrombopag or rhTPO therapy and 15 healthy controls (HCs). Results showed that plasma and mRNA levels of IL-2, IFN-γ, IL-4, and IL-17A in ITP patients did not change significantly after TPO-RA treatment, whereas TGF-β1 levels increased remarkably. The pre- and post-treatment plasma and mRNA levels of IFN-γ and IL-2 were significantly higher, while the pre- and post-treatment IL-4 levels as well as the pre-treatment TGF-β1 levels were remarkably lower in ITP patients compared with HCs. There was no significant difference in TGF-β1 levels between TPO-RA-treated ITP patients and HCs. No statistical difference was found in plasma levels of IL-17A between ITP patients before or after treatment and HCs. However, the pre- and post-treatment mRNA expression of IL-17A and retinoic orphan receptor (ROR) γt in ITP patients were higher than that in HCs. Overall, these findings indicated that TPO-RA treatment could promote the secretion of TGF-β1, while it could not correct the Th1 and Th17 polarization in ITP patients. This study might improve our understanding of the mechanism of action of TPO-RAs and provide important information for optimizing therapeutic strategies for ITP.

1. Background

Primary immune thrombocytopenia (ITP) is an acquired immune-mediated bleeding disorder characterized by autoantibody-mediated platelet destruction and impaired megakaryocyte maturation with reduced platelet production [1]. More recently, it has become obvious that ITP is a more complex disorder in which T cell abnormalities play important roles in platelet destruction [2–4]. Antiplatelet autoantibody production is under the control of T helper (Th) cells, and elevated antiplatelet T-cell reactivity in ITP has been observed [1,5–8]. Th cell polarization in ITP has been attributed to increased Th1 and Th17 cells, decreased Th2 cells, and reduced or impaired $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) [9–11]. Moreover, enhanced cytotoxic T lymphocyte-mediated platelet destruction has been reported in ITP [5,12–14]. The precise reason for these abnormalities remains to be clarified.

Thrombopoietin (TPO) is the principal hematopoietic cytokine that stimulates thrombopoiesis by activating the cell through TPO-receptor (TPO-R), c-MPL [15–17]. As insufficient TPO has been found to contribute to decreased platelet production in ITP [18,19], a series of thrombopoietic agents have been developed. Two types of TPO receptor agonists (TPO-RAs), eltrombopag and romiplostim, have been approved by the US Food and Drug Administration as

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second-line options for the management of ITP [20–22]. In addition, recombinant human TPO (rhTPO) has been used for the treatment of corticosteroid-resistant and relapsed ITP patients in China [23,24]. A rapid response can often be achieved during TPO-RA treatment. Nevertheless, platelet counts usually return to pre-treatment levels once the regimen is withdrawn, making it difficult to achieve a long-term drug-independent remission.

TPO-R is mainly expressed by $CD34^+$ cells, megakaryocytes, platelets, and several kinds of tumor cells. It has been demonstrated that peripheral blood lymphocytes do not express TPO-R [25]. Nevertheless, TPO-RAs have showed additional effect on regulation of different lymphocyte subsets in ITP. Bao et al. observed that improved activity of Tregs coincided with a remarkable decrease in effector T cell function in TPO-RA-treated ITP patients [26]. Number of regulatory B cells (Bregs) was also increased after TPO-RA treatment in ITP patients [27]. In addition, our recently published paper showed that the recovery of platelet counts after TPO-RA administration in ITP was associated with the restoration of FcγR balance toward the inhibitory FcγRIIb on monocytes [28]. These data indicate that TPO-RAs have profound effect on immune modulation besides their direct role in stimulating platelet production from megakaryocytes.

Cytokine-mediated immunity plays an important role in the pathogenesis of various autoimmune disorders [29–32]. Aberrant cytokine profiles have been correlated with the loss of immune tolerance in ITP patients [8,33]. Moreover, response to different therapeutic strategies, such as high-dose dexamethasone, splenectomy, rituximab, and *Helicobacter pylori* eradication, is often associated with correction of the cytokine abnormalities in ITP [6,34–38]. Even though TPO-RAs have been used to treat ITP patients for several years, their roles in cytokine modulation remain largely unknown.

To evaluate the effect of TPO-RAs on cytokine modulation in ITP, a total of 26 corticosteroid-resistant/relapsed chronic ITP patients receiving eltrombopag or rhTPO treatment were enrolled in the present study. Plasma concentrations and mRNA expression of interleukin (IL)-2, interferon gamma (IFN-γ), IL-4, IL-17A, and transforming growth factor β1 (TGF-β1), as well as retinoic orphan receptor (ROR) γt mRNA levels in peripheral blood mononuclear cells (PBMCs) were determined, which might deepen our understanding about the mechanism of TPO-RAs in the treatment of ITP.

2. Materials and methods

2.1. Patients and controls

A total of 26 corticosteroid-resistant/relapsed chronic ITP patients (15 females and 11 males; 22–84 years of age, median 51 years) were enrolled in this study between June 2013 and March 2015 at the Department of Hematology, Qilu Hospital, Shandong University, Jinan, China. The duration of ITP from the time of diagnosis ranged from 13 to 433 months. Patients were diagnosed according to the practice guidelines of the International Working Group on ITP [39]. All patients had a pre-treatment platelet count below 30×10^9/L (baseline platelet count ranged from 4 to 25×10^9/L, median 13×10^9/L; Table 1), and had relapsed or had been unresponsive to glucocorticosteroid therapy. Previous therapies, including rescue therapy, must have been completed at least 6 weeks prior to enrollment. Cases complicated with any of the following conditions were excluded: (1) thrombosis or use of thrombopoietic agents, (2) liver, kidney, cardiac, or pulmonary dysfunction, or (3) a clinical history of hepatitis B/C virus or human immunodeficiency virus infection. Pregnant or nursing female subjects were also excluded.

Fifteen healthy adult volunteers (9 females and 6 males; 20–45 years of age, median 30 years) were recruited as controls. Platelet counts ranged from 165 to 325×10^9/L, with the median count of 209×10^9/L.

This study was approved by the Medical Ethical Committee of Qilu Hospital, Shandong University. Informed consent was obtained from all patients and controls before enrollment in accordance with the Declaration of Helsinki.

2.2. Treatment regimen

Eltrombopag (GlaxoSmithKline, Ware, UK) was administered orally with an initial dose of 25 mg once daily for 6 weeks. The dose could be increased to 50 or 75 mg once daily to maintain platelet counts $\geqslant 50 \times 10^9$/L. rhTPO (3SBIO, Shenyang, China) was administered subcutaneously at a daily dose of 300 U/kg initially. The dose frequencies could be adjusted to every other day once platelet counts ascended above 100×10^9/L. If response was not achieved after 2 weeks of rhTPO administration, patients were designated as non-responders and treatment was discontinued. To reduce the risk of thrombocytosis, eltrombopag or rhTPO therapy was stopped once platelet counts exceeded 250×10^9/L. The response was evaluated according to the following criteria: complete response (CR) was defined as a platelet count $\geqslant 100 \times 10^9$/L without bleeding; response (R) was defined as a platelet count between 30 and 100×10^9/L without bleeding, at least a doubling of the baseline counts; no response (NR) was defined as either a platelet count $<30 \times 10^9$/L, less than doubling of the baseline platelet count, or bleeding.

2.3. Plasma and PBMC preparation

Peripheral blood samples from all patients prior to TPO-RA therapy and healthy controls were obtained. For patients who achieved R, blood samples were collected 6 weeks after TPO-RA treatment. With regard to the non-responders, blood samples were obtained on the day of treatment discontinuation. Plasma was obtained by centrifugation and stored at −80 °C. PBMCs were isolated from the peripheral blood by gradient centrifugation (400*g*, 20 min) on Ficoll-Paque (HaoYang Biological Manufacture, Tianjin, China) and were stored in aliquots at −80 °C until RNA extraction.

2.4. IFN-γ, IL-2, IL-4, IL-17A and TGF-β1 enzyme-linked immunosorbent assay

Plasma levels of IL-2, IL-17A and TGF-β1 were measured by commercialized enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's protocols (R&D systems, Minneapolis, MN, USA). The lower detection limits for IL-2, IL-17A and TGF-β1 were 7, 15 and 15.4 pg/mL, respectively. Plasma IFN-γ and IL-4 levels were also determined using a commercial ELISA (Sizhengbai, Beijing, China) according to manufacturer's instructions. The lower detection limits for these 2 cytokines were both 2 pg/mL.

2.5. RNA isolation and quantitative real-time polymerase chain reaction analysis

Total RNA was extracted from PBMCs by TRIzol (Takara Biotechnology, Inc, Japan). The amount of RNA was determined using the Nanophotometer P-class (Implen, Germany). cDNA was synthesized using the PrimeScript RT reagent kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. mRNA expression of IL-2, IFN-γ, IL-4, IL-17A, TGF-β1, RORγt, and GAPDH (endogenous control) were quantified by real-time PCR using SYBR Green (Toy-