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Granulocyte colony-stimulating factor decreases the Th1/Th2 ratio in peripheral blood mononuclear cells from patients with chronic immune thrombocytopenic purpura in vitro

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ABSTRACT

Chronic immune thrombocytopenia purpura (ITP) is an autoimmune disease that exhibits an abnormally high Th1/Th2 ratio. Granulocyte colony-stimulating factor (G-CSF) has been shown to decrease the Th1/Th2 ratio in healthy donors. In this study, we investigated the effects of G-CSF treatment on the Th1/Th2 cells and the underlying mechanisms in patients with ITP in vitro. Peripheral blood mononuclear cells (PBMCs) isolated from patients with ITP and healthy controls were treated with G-CSF. Expression levels of interferon (IFN)-γ, interleukin (IL)-2, IL-4, and IL-13 in supernatants were measured by enzyme-linked immunosorbent assays. The expression of IFN-γ, IL-4, and G-CSF receptor (G-CSFR) on Th1 and Th2 cells were examined by flow cytometry and confocal microscopy. The mRNA expression of IFN-γ, IL-2, IL-4, IL-13, and T-box expressed in T cells (*T-bet*) and GATA-binding protein 3 (*GATA-3*) in PBMCs was evaluated by reverse transcription polymerase chain reaction. The results showed that G-CSF could significantly reduce the Th1/Th2 ratio in PBMCs from patients with ITP in vitro. As the concentration of G-CSF increased, Th1/Th2 ([IFN-γ + IL-2]/[IL-4 + IL-13]) cytokine ratios and *T-bet/GATA-3* mRNA ratios decreased in a concentration-dependent manner. Th1 cells and Th2 cells both expressed G-CSFR. These results suggest that G-CSF could decrease the Th1/Th2 ratio in the context of ITP, and elucidate the direct and indirect immunomodulatory mechanisms underlying G-CSF functions in Th1/Th2 cells, thus supporting the therapeutic potential of G-CSF in the treatment of patients with ITP.

1. Introduction

Chronic immune thrombocytopenia purpura (ITP) is an autoimmune disorder characterized by a low platelet count and mucocutaneous bleeding [1], with heterogeneous and complex pathophysiology. Macrophages, CD8 + T-cells, and autoantibodies can lyse platelets and, thus, play an important role in the pathogenesis of ITP [2,3]. However, autoantibodies against platelet antigens are considered as the diagnostic hallmark of ITP, and T helper (Th) cells are the major switches mediating B-cell antibody production [4]. ITP is the manifestation of a Th1-polarized immune response [5–8], and the high Th1/Th2 ratio is inversely correlated with platelet counts and disease severity [5,8]. Thus, the reversal of Th1 polarization and recovery of the Th1/Th2 balance may be of fundamental importance to the treatment of patients with ITP [9,10].

Th cells play a critical role in the regulation of the immune system by mediating the secretion of cytokines [4]. The roles of Th1 and Th2 cells differ in terms of their effects on regulation of the immune response. Th1 cells produce IFN-γ and IL-2 and promote the pathogenesis of autoimmune diseases, whereas Th2 cells predominantly secrete IL-4 and IL-13 and show protective effects against autoimmune diseases [11]. In addition, several factors such as the regulatory T cells [12–14], granulocyte-colony stimulating factor (G-CSF) [15,16], T-box expressed in T cells (T-bet) [17], and GATA-binding protein 3 (GATA-3) [18–20] have been proved to influence cytokine secretion by Th1 and Th2 cells. Two major T-cell transcription factors, T-bet and GATA-3, could regulate the expression of Th1 or Th2 cytokine genes and play a crucial role in T-cell differentiation [17–21]. The T-bet/GATA-3 ratio has been shown to reliably reflect the Th1/Th2 status [22].

G-CSF is a key hematopoietic factor of the myeloid lineage and an essential mediator of immune responses. Recent evidence indicates that G-CSF links innate and adaptive immunity and plays an important role in the induction and maintenance of T-cell tolerance [15]. In healthy donors, G-CSF inhibited T-cell allogeneic and mitogenic reactivation [23], reduced interferon (IFN)-γ production, increased interleukin (IL)-4

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production, and decreased the Th1/Th2 ratio [15,16,23–25]. In addition, G-CSF has been shown to be beneficial for the prevention and/or treatment of immune diseases [26–30]. However, the effects of G-CSF on abnormal immunity in patients with ITP have not been reported.

By binding to the G-CSF receptor (G-CSFR; CD114), G-CSF directly mediates diverse biological effects. G-CSFR is expressed by myeloid lineage cells, platelets, endothelial cells, monocytes, B lymphocytes, and other cell types [31]. However, the expression of G-CSFR on T lymphocytes remains controversial. G-CSFR is not expressed on resting T lymphocytes [32], but is inducible in T lymphocytes [33,34]. However, to the best of our knowledge, few studies have examined the expression of G-CSFR in distinct Th cell subsets, such as Th1 and Th2.

We hypothesized that the abnormally high Th1/Th2 ratio in patients with ITP can be decreased by G-CSF treatment. To test this hypothesis, we explored the effects of G-CSF on the Th1/Th2 ratio in patients with ITP. In addition, the expression of G-CSFR on Th1 and Th2 cells in the presence of phytohemagglutinin (PHA) was studied and the *T-bet* and *GATA-3* mRNA expression in G-CSF-treated peripheral blood mononuclear cells (PBMCs) from patients with ITP was investigated. Our results show the therapeutic potential of G-CSF in shifting the Th1/Th2 ratio of patients with ITP, and elucidate the direct and indirect immunomodulatory mechanisms of G-CSF function in Th1/Th2 cells.

2. Methods

2.1. Patients

Twelve patients with chronic ITP (seven women and five men; age range 19–79 years, median 49.5 years) and 12 age- and sex-matched healthy controls were enrolled in this study between September 2013 and December 2015 at the First Affiliated Hospital of Harbin Medical University. ITP was diagnosed based on the International Working Group of ITP guidelines issued in 2009 [35]. Patients with ITP-related diseases were excluded. None of the patients had been treated with glucocorticosteroids before the first sampling. The median platelet count was 10.0×10^9/L (range, 0.4–19.6 $\times 10^9$/L). Among the 12 patients enrolled in this study, 5 (41.7%) had serum anti-platelet antibodies detected using an indirect immunofluorescence method. The clinical characteristics of patients with ITP are shown in Table 1.

Written informed consent was acquired, and the study protocol was approved by the Medical Ethics Committee of the First Affiliated Hospital of Harbin Medical University.

2.2. Isolation, culture, and treatment of PBMCs

Plasma was prepared by centrifugation for 15 min at 1500 ×*g* at room temperature, and stored at −80 °C, plasma samples were thawed in a 37 °C water bath before use. PBMCs were isolated from fresh whole blood by density gradient centrifugation over Ficoll Hypaque (Tbdscience, Tianjin, China). The concentration of PBMCs was adjusted to 1×10^6/mL in RPMI 1640 supplemented with 10% fetal bovine serum, 50 U/mL penicillin G, and 50 μg/mL streptomycin. The cells were then treated with 0, 12.5, 25, 50, or 100 ng/mL recombinant human G-CSF (rhG-CSF; Xiamen Amoytop Biotech Co., Xiamen, China) and incubated for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2, with or without 10 μg/mL PHA (Sigma, St. Louis, MO, USA) added 24 h later to stimulate cytokine production. PBMCs were used immediately after treatment or stored at −80 °C with 10% DMSO (they were thawed in a 37 °C water bath before use). The expression of IFN-γ and IL-4 on CD4+ T cells and G-CSFR on Th1 and Th2 cells was assessed by flow cytometry and confocal microscopy, and quantification of *T-bet* and *GATA-3* mRNA was performed by reverse transcription polymerase chain reaction (RT-PCR).

Table 1
Clinical characteristics of patients with ITP.

Patients no.	Sex (M/F)	Age (years)	Platelets ($\times 10^9$/L)	Hemoglobin (g/L)	WBC ($\times 10^9$/L)	Anti-platelet antibodies (±)
1	M	50	15.2	114	4.5	+
2	M	75	19.6	159	7.4	−
3	F	19	2.4	153	8.8	+
4	M	53	13.6	130	8.4	−
5	F	51	4.6	100	10.7	+
6	M	49	13.4	148	4.8	+
7	F	34	0.5	37	6.6	−
8	F	76	9.3	111	11.4	−
9	F	54	13.3	139	4.7	−
10	M	21	10.7	120	8.9	+
11	F	23	7.7	115	3.7	−
12	F	36	0.4	152	10.5	−

Abbreviation: M, male; F, female, WBC, white blood cell count.

2.3. Determination of cytokine production by ELISA

Levels of G-CSF in plasma and production of IFN-γ, IL-2, IL-4, and IL-13 (e-Bioscience, San Diego, CA, USA) in cell supernatants were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instructions. All determinations were made in duplicate. No significant cross-reactivity was observed with various recombinant cytokines. The limits of detection were 39.1 pg/mL (G-CSF), 0.99 pg/mL (IFN-γ), 9.2 pg/mL (IL-2), 1.3 pg/mL (IL-4), and 0.7 pg/mL (IL-13).

2.4. Immunofluorescence staining for flow cytometry

Flow cytometry was used to examine the phenotypic characteristics of IFN-γ and IL-4 in $CD4^+$ T cells and of G-CSFR on Th1 and Th2 cells. PHA was added after G-CSF (0 or 100 ng/mL) treatment for 24 h. After 72 h of treatment, PBMCs were harvested and then incubated with 20 μL each of CD4-PerCP and CD114-PE antibodies (BD Bioscience, San Jose, CA, USA) for 15 min in the dark. After washing with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3, cells were resuspended in 0.5 mL FACS permeabilizing solution (BD Bioscience) for 10 min at room temperature in the dark and then washed with PBS. Next, 5 μL each of IFN-γ fluorescein isothiocyanate (FITC)-labeled and IL-4 allophyocyanin (APC)-labeled antibodies (both from BD Bioscience) were added for intracellular cytokine staining. Cells were then incubated for 30 min in the dark and analyzed within 3 h by flow cytometry on FASCAria (BD Biosciences). Data were analyzed with FlowJo software (TriStar, Ashland, OR, USA). Isotype controls were included to define the gates for the discrimination of antigen-positive versus -negative events. IFN-γ^+ IL-4^- cells were defined as Th1 cells, and IFN-γ^- IL-4^+ as Th2 cells [7].

2.5. Detection of G-CSFR by confocal microscopy

After 72 h incubation of PBMCs in vitro with or without 100 ng/mL G-CSF, PHA was added after 24 h. Cells were then harvested and incubated with the Pacific Blue-conjugated anti-CD4 (BD Biosciences) and CD114-PE antibodies for 15 min in the dark. Cells were resuspended in 0.5 mL FACS permeabilizing solution for 10 min at room temperature in the dark, washed with PBS, incubated with FITC-conjugated antibodies against IFN-γ or IL-4 (BD Biosciences) for 30 min and washed with PBS

Table 2
Primer sequences for RT-PCR.

Name	Forward primer	Reverse primer
IFN-γ	TTGGCTTAATTCTCTCGGAAACG	CGCTACATCTGAATGACCTGC
IL-2	CACAGCTACAACTGGAGCATTTAC	TGCTGATTAAGTCCCTGGGTC
IL-4	TGCCTCCAAGAACACAACTGA	CCAACGTACTCTGGTTGGCTT
IL-13	CTCCTCAATCCTCTCCTGTT	GTTGAACCGTCCCTGGCGAAA
T-bet	TGGGTGCAGTGTGGAAAGGC	ACTGGAGCACAATCATCTGGG
GATA-3	AGGACGAGAAAGAGTGCC	GAAGAGTCCGGAGCTGTAC
β-actin	TTGCCGACAGGATGCAGAA	GCCGATCCACACGGAGTACT