Characterization of Th1- and Th2-associated Chemokine Receptor Expression in Spleens of Patients with Immune Thrombocytopenia

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Abstract

Purpose In view of the numerous clinical observations and laboratory studies that suggest a critical role for the spleen in immune thrombocytopenia (ITP) pathophysiology, we aimed to characterize Th1-associated chemokine receptors CXCR3 and CCR5 and Th2-associated chemokine receptor CCR3 in spleens of ITP patients and assess the significance of their differential expression in the clinical setting.

Methods The histopathology of spleens was observed using hematoxylin-eosin staining (HE), and the positive rate of CXCR3, CCR5 and CCR3 expression in spleens of 24 ITP patients and 12 patients with traumatic splenic rupture as normal controls was detected by immunohistochemistry using the SP method. CXCR3, CCR5 and CCR3 protein expression was analyzed by Western blot and mRNA levels were investigated by real-time polymerase chain reaction (RT-PCR).

Results Reactive hyperplasia could be seen in follicles of the white pulp, the germinal central zone was enlarged and the marginal zone was thickened, the central arteries were thickened and fibrotic, and the density of the capillary vessel was increased in ITP patients. ITP group displayed a higher rate of expression of Th1-associated chemokine receptors CXCR3 and CCR5 (83.3 % vs. 75 %, 100 % vs. 83.3 %) but lower rate of expression of Th2-associated chemokine receptor CCR3 (50 % vs. 66.7 %) compared with the controls (P < 0.05, respectively). Western blot analysis revealed that CXCR3 and CCR5 protein expression was significantly increased in ITP patients while CCR3 was significantly reduced (P < 0.05, respectively). Meanwhile, ITP patients displayed increased mRNA levels of CXCR3 and CCR5 but decreased gene expression of CCR3 (P < 0.05, respectively).

Conclusion The data suggested that the abnormal expression of Th1/Th2 chemokine receptors may participate in splenic immune disorder in patients with ITP. Using corresponding inhibitors may inhibit Th1-dominant expression and mitigate the progress of the disease.

Keywords Immune thrombocytopenia · Th cell · chemokine receptor · spleen

Introduction

Immune thrombocytopenia (ITP) is an acquired organ-specific autoimmune disorder characterized by a low peripheral blood platelet count. The pathogenesis of thrombocytopenia in ITP is complicated, involving not only impaired platelet production but also T cell-mediated immunity.
disorder [1, 2]. The abnormal Th cell could direct autoreactive B cells to differentiate and secrete IgG autoantibodies [3], resulting in platelet destruction in the reticuloendothelial system. The spleen, serving as the largest lymphoid organ, is the main site of autoreactive B cell activation, antiplatelet antibody production and platelet destruction. Splenectomy is an effective treatment in ITP with a success rate of about 66 % [1, 4]. More interestingly, ITP is now considered a Th1 disease, characterized by the oligoclonal accumulation of Th cells [5]. Given their different effector functions, Th1 and Th2 are differentially recruited to peripheral sites of inflammation [6]. It has been shown that Th1, but not Th2, expresses a functional ligand for P- and E-selectin and therefore is selectively recruited to sites where Th1 immune responses occur [7]. In summary, Th cell migration is a complicated process in which a series of cytokines are involved.

Chemokines are small chemotactic cytokines that play a role in a myriad of immune functions such as angiogenesis, hematopoiesis, mast cell degranulation, T cell differentiation and leukocyte trafficking. The diverse biological effects of chemokine-induced signaling are communicated through seven trans-membrane G-protein-coupled receptors. Chemokines and their receptors are essential elements that regulate the positioning of T cells and their partners for priming and Th1- or Th2-mediated responses. More than 50 chemokines have been described to date, and been classified into four major subfamilies according to their structure, namely CC, CXC, CX3C and XC chemokines [8]. However, only two of these groups have been extensively characterized, namely, the CC and CXC chemokines [9]. Chemokine receptors are selectively expressed on T cell surfaces, depending on their antigenic experience and the type of polarization. CXC chemokine receptor CXCR3 and CC chemokine receptor CCR5 are expressed on activated T cells, especially the Th1 subset [10], and are thus associated with a Th1 phenotype [11–13]. In contrast, CCR3 is expressed mainly on Th2 cells [14]. Even though these chemokine receptors are not exclusive for these cell types, they can be used as markers to differentiate Th1/Th2 cells from CD4 cells [15]. Chemokines of the CC subfamily are generally chemoattractant for T lymphocytes, monocytes and natural killer (NK) cells while CXC chemokines attract neutrophils and promote their adherence to endothelial cells [16–18]. In addition to their role in cell recruitment, chemokines have been reported to play an essential part in immunoregulatory activities, such as cytokine production and Th1/Th2 cell induction [19]. The strong chemoattractant activity of chemokine CCL10, along with its receptor CXCR3, has been reported in several autoimmune diseases, such as rheumatoid arthritis (RA) [20], systemic lupus erythematosus (SLE) [21], systemic sclerosis (SS) [22], multiple sclerosis (MS) [23], type 1 diabetes mellitus (T1D) [24], autoimmune thyroiditis and Graves’ disease [25].

However, the status of these chemokines and chemokine receptors in spleens of ITP patients remains unknown. In this study, as well as detected the basic pathological changes, we further analyzed CXCR3, CCR5 and CCR3 protein and gene expression through immunohistochemistry, western blotting and quantitative real-time polymerase chain reaction to analyze the significance of chemokine receptor abnormalities in the spleens of ITP patients.

Methods

Patients

Twenty-four patients with active ITP in the Second Hospital of Shandong University from July 2011 to June 2012 were enrolled. All cases met the diagnostic criteria of ITP as described in the international consensus report in 2010 [1]. The median age was 27.5 years with a range of 12–48 years, and 18 patients were female. The clinical features of the patients are shown in Table I. All patients had been treated with glucocorticoid before splenectomy. The platelet counts ranged between 4 and 24×10⁹/L, with a median count of 17×10⁹/L. Spleens removed from 12 patients with traumatic splenic rupture (nine males and three females; median age 25.5 years with a range of 16–39 years) were studied as controls. Written informed consent was signed by all participants and the study obtained the approval of the ethics committee of the Second Hospital of Shandong University in accordance with the Helsinki declaration.

Preparation for the Staining Protocol

Spleen specimens were fixed in 10 % formalin, paraffin-embedded and cut into 4-μm thick sections, and after mounting on slides, were heated at 60 °C for 60 min. Before proceeding with the staining protocol, the slides were immersed into two changes of xylene for 5 min each and rehydrated in decreasing concentrations (100 %, 95 %, 85 %, 70 %) of ethanol for 3 min each. Then the slides were kept in water until the following hematoxylin-eosin staining or immunohistochemistry.

Hematoxylin-eosin Staining of the Spleens

Hematoxylin-eosin staining was carried out to observe the histopathology of the spleens. The above slides were stained with hematoxylin for 10 min. After washing with distilled water for 3 min, the slides were differentiated in 0.3 % acid alcohol and then rinsed in water to blue up. After staining with eosin for 2 min, the slides were washed in tap water.
and dehydrated in increasing concentrations (70 %, 85 %, 95 %, and 100 %) of ethanol. The slides were cleared and mounted in neutral resin, followed by observation under the light microscope.

Immunohistochemistry SP Method

After heat-induced epitope retrieval using a microwave oven, the slides were incubated with 3 % hydrogen peroxide for 10 min at room temperature to block endogenous peroxidase and rinsed three times in TBS plus 0.025 % Triton X-100 (TBST) with gentle agitation for 10 min each. Then the slides were incubated overnight at 4 °C with primary antibody diluted in PBS. The slides were then washed in TBST and subsequently incubated with Polymer Helper for 60 min. The slides were washed in TBST again, followed by 90-min incubation in poly-HRP anti-Rabbit IgG. After rinsing in TBST three times, the slides were immersed in the enzyme substrate DAB solution. After that, the slides were counterstained with hematoxylin and dehydrated, cleared and mounted with cover slips. Polink-2 plus(R) Polymer HRP Detection System for Rabbit Primary Antibody was purchased from ZSJQ Bio, Beijing, China. The primary antibodies diluted in this process included CXCR3 (ab71864) (1:150), CCR5 (ab65850) (1:100) and CCR3 (ab36827) (1:100). The role of nonspecific staining of primary antibody was analyzed by substitution of the primary antibody with PBS.

Western Blot Analysis

Total proteins were extracted from the spleens using a Total Protein Extraction Kit DBI-1017(DBI Bioscience), and the concentration was tested using the BCA Protein Assay Kit. The proteins were mixed with electrophoresis sample buffer and then boiled for 10 min, and subsequently detected by SDS-PAGE, and then transferred to a nitrocellulose membrane (Boster) using a DYCZ-24D blotting apparatus. After blocking with 5 % non-fat milk for 60 min, the membrane was incubated overnight at 4 °C with primary antibody diluted in Primary Antibody Dilution Buffer (Beyotime). The antibodies involved in this process were CXCR3 (ab71864) (abcam), CCR5 (ab65850) (abcam), CCR3 (ab36827) (abcam) and GAPDH (Beyotime). The dilution

### Table 1 Clinical characteristics of patients with active ITP before splenectomy

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PT petechiae, EC ecchymoses, EP epistaxis, GUH genitourinary hemorrhage, GIH gingival hemorrhage, GIH gastrointestinal hemorrhage
radio was 1:500 for the CCR5 antibody, and 1:1000 for the others. After rinsing with TBST three times for 10 min per wash, the nitrocellulose membrane was immersed in dilute HRP-conjugated goat anti-rabbit IgG (1:5000, ZSJQ Bio, Beijing, China) at room temperature for 90 min. The proteins were visualized using ECL Western blotting detection reagents (Millipore) and exposed to clear-blue X-ray film. Protein expression in patients and controls was quantified using Image J.

Quantitative Real-time Polymerase Chain Reaction Analysis

Total RNA of the spleens was extracted using TRizol reagent (Invitrogen Life Technologies). Amplification and the reverse transcription reactions were performed using an Eppendorf Realplex2 PCR Detection System according to the manufacturer’s instructions. The reagents were the RT reagent kit and SYBR Premix Ex Taq (Takara). RT-PCR primer sequences were as follows: CXCR3 forward: 5′-CCTACTGTATGGCCACATCT-3′, reverse: 5′-AAAGCGCGAGGTCC ATGAG-3′; CCR5 forward: 5′-GAAGAAGCTGTAGACATCCGTTCC-3′, reverse: 5′-ACACCCAGTGAGTAGAGCGGAG -3′; CCR3 forward: 5′-TGCTGAGTTGATT GGAGAAGTG-3′, reverse: 5′-AGTGAAACCACGGAGGTACAG-3′ and β-actin internal control forward: 5′-CCTCTTTCCA GCCCTCCTTCC-3′, reverse: 5′-AGGTCTTTTGCGGATGTCCTCCAC-3′. The PCR reaction parameters were set as follows: 95 °C for 30s followed by 40 cycles of PCR reacting at 95 °C for 15 s, and 60 °C for 30s. The relative quantification (RQ) of gene expression was calculated using the following equation: RQ = 2^{ΔΔct}.

Statistical Analysis

Values are presented as means ± SD. The data on chemokine receptor protein levels and mRNA levels in ITP groups and controls were normally distributed. Thus the independent-samples t test was performed to compare chemokine receptor protein levels and mRNA levels and 2-tailed Fisher exact test was used to compare the positive rate of expression between two groups using the Statistical Package for the Social Sciences version 17.0 (SPSS Inc., Chicago, IL). Values of p<0.05 were considered statistically significant.

Results

The Morphological Characteristics of Spleens in ITP Patients

The surface of the spleen in ITP was smooth, without thickness in capsule, and the splenic nodule was clear, without hemorrhage or necrosis. Characteristics under the light microscope were as follows: the reactive hyperplasia could be seen in follicles of the white pulp, and the germinal central zone was enlarged and the marginal zone was thickened. The splenic histopathology showed dilatation and congestion of the splenic sinusoid, infiltration of neutrophilic leukocytes in the red pulp, and macrophage, red cell and platelet accumulation in the splenic cord. The central arteries were thickened and fibrotic and the density of the capillary vessels was increased (as shown in Fig. 1).

Immunohistological Changes in CXCR3, CCR5 and CCR3 in ITP

The positive rate of CXCR3 expression in spleens of ITP patients was increased and that of CCR3 was decreased compared with trauma patients (as shown in Fig. 2). The positive rate of CXCR3 expression in spleens of ITP patients was 83.3 %, which was higher than that in controls (75 %, p<0.05), and the positive rate of CCR5 was also higher in ITP patients (100 % vs. 83.3 %) (p<0.05). However, the positive rate of CCR3 was lower in ITP patients than that in controls (50 % vs. 66.7 %) (p<0.05).

Fig. 1 The histopathology of the spleens (HE×200). Reactive hyperplasia could be seen in follicles of the white pulp. The germinal central zone was enlarged and the marginal zone was thickened. The central arteries were thickened and fibrotic, and the density of the capillary vessel was increased. b The morphology of spleens from trauma patients is presented in Fig. 1b
Variation in Protein Expression of the Chemokine Receptors in ITP

Th1-associated chemokine receptors CXCR3 and CCR5 expression levels were increased in spleens of ITP patients, while Th2-associated chemokine receptor CCR3 expression was decreased (as shown in Fig. 3). ITP patients displayed increased expression of CXCR3 (1.74±0.18) compared with controls (0.58±0.07, \( p < 0.05 \)). CCR5 expression in spleens was also significantly higher in ITP patients (0.93±0.09) than that in controls (0.79±0.06, \( p < 0.05 \)). However, Th2-associated chemokine receptor CCR3 expression level was significantly lower (0.33±0.04, \( p < 0.05 \)) in spleens of ITP patients compared with that in the trauma patients (1.42±0.11, \( p < 0.05 \)).

Relative Quantification of Chemokine mRNA in ITP

As shown in Fig. 4, mRNA levels of Th1-associated chemokine receptors CXCR3 and CCR5 were increased in spleens from ITP patients while Th2-associated chemokine receptor CCR3 was significantly downregulated. CXCR3 mRNA expression was about 4-fold higher than that in controls (3.85±0.26 vs. 1.09±0.21, \( p < 0.05 \)) and CCR5 mRNA level was also increased in spleens of ITP patients (1.71±0.03) compared with that in controls (1.01±0.01, \( p < 0.05 \)) while CCR3 mRNA level was lower (0.75±0.03) in patients than that in normal controls (1.01±0.01, \( p < 0.05 \)).

Discussion

The study of chemokine receptors and their ligands is a new research focus in the immunologic field. In various human autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, glomerulonephritis, inflammatory bowel disease and solid organ allograft rejection, the increased expression of CXCR3 and CCR5 ligands is followed by the infiltration of CXCR3+ and CCR5+ T cells, suggesting a critical role for these chemokine receptors in T cell-induced tissue damage. T cell-induced immune disorder together with platelet destruction plays an important role in the pathogenesis of ITP,
and platelets are mainly destroyed in the spleen in immune thrombocytopenia. However, CXCR3, CCR5 and CCR3 expression has yet to be characterized in spleens of patients with ITP. Although the polarized immune activity detected in PBMC was thought to reflect the nature of the localized autoimmune process taking place in the target organs of ITP [26], this should be confirmed in the spleen.

In this study, reactive hyperplasia in follicles of the white pulp, enlargement of the germinal central zone together with the thickness of the marginal zone underlined the key role of B cells, as well as Th cells, in ITP pathogenesis. Furthermore, the infiltration of neutrophilic granulocytes in the red pulp, and accumulation of macrophage, red blood cell and platelet in the splenic cord as well as the thickness and fibrosis of central arteries and the increase in density of the capillary vessels indicated an inflammatory response in spleens of ITP. In addition to platelet destruction in the spleen, we found that the spleen of ITP patients presented a higher positive rate of Th1-associated chemokine receptor CXCR3 and CCR5 expression but a lower positive rate of Th2-associated chemokine receptor CCR3 expression, according to immunohistochemistry using the SP method. Meanwhile, CXCR3, CCR5 and CCR3 expression was quantified by RT-PCR and western blot analysis, and the results showed that spleens from the ITP group had CXCR3 and CCR5 were significantly upregulated whereas Th2-associated chemokine receptor CCR3 was downregulated in spleens from ITP patients. a Representative Western blots of CXCR3, CCR5 and CCR3 in spleens from ITP and controls. b CXCR3 expression was about 3-fold higher than that in traumatic spleens. Expression of CCR5 was about 20 % higher, but CCR3 expression was 92.5 % lower (* \( P < 0.05 \))

Fig. 3 The protein expression of CXCR3, CCR5 and CCR3 in ITP. The balance of Th1/Th2 chemokine receptor protein expression in ITP was disrupted. Total proteins were extracted from the spleens using a Total Protein Extraction Kit, and western blot was performed using anti-CXCR3, anti-CCR5 and anti-CCR3. GAPDH was used as an internal control. Bands were scanned, and volumes were calculated by densitometry. Quantification was based on the ratio between chemokine receptors and GAPDH. Th1-associated chemokine receptors

Fig. 4 The relative quantification of CXCR3, CCR5 and CCR3 gene expression in spleens. Total RNA of the spleens was extracted using TRIzol reagent and RT-PCR was performed to analyze mRNA of CXCR3, CCR5 and CCR3. \( \beta \)-actin served as the internal control. The relative quantification (RQ) of gene expression was calculated by the following equation: \( RQ = 2^{-\Delta\Delta C_{T}} \). Spleens from ITP patients displayed significantly enhanced gene expression of Th1-associated chemokine receptors CXCR3 and CCR5 and reduced mRNA expression of CCR3. mRNA of CXCR3 in ITP patients was about 4-fold higher than that in controls. CCR5 expression increased by 70 %, but the relative quantification of CCR3 gene expression decreased by 26.1 % (* \( P < 0.05 \))
significantly higher CXCR3 and CCR5 expression compared to that in the control group, but significantly lower CCR3 expression.

By interacting with their chemotactic ligands, chemokine receptors play significant parts in the leukocyte migration process. Chemokine receptor expression is an essential and critical factor in determining how chemokines work. Generally speaking, chemokine receptors combine with more than one ligand. For example, CXC receptors bind CXC chemokines, while CC receptors exclusively combine with CC chemokines. Chemokine receptors are selectively expressed on Th1 and Th2 effector cells, leading to the distribution of these cells in their particular tissue environments. The expression of chemokine receptors is not only significant in T cell migration but also in T cell proliferation and differentiation, directly or indirectly. Increasing evidence demonstrates that T cell homing potentials and effector functions are coordinately regulated during the differentiation process [27]. Naive T cells, when stimulated by specific antigens and cytokines, enhance the expression of adhesion molecules as well as chemokine receptors on their membrane, which helps them to infiltrate inflamed tissues. CXCR3 and CCR3 are preferentially induced in Th1 and Th2 priming conditions, respectively [11, 28]. For example, developing Th1 cells acquire the ability to express chemokine receptors such as CXCR3, CCR5 and CXCR6 as well as IFN-γ, which are required to migrate to sites of delayed-type hypersensitivity reactions. Conversely, developing Th2 cells acquire the capacity to produce IL-4 and express CCR3, CCR4 and CCR8 and the prostaglandin D2 receptor CRTH2, and to transfer them to sites of allergic reactions [28–30]. Changes in chemokine receptor quantity or type are key factors in controlling Th1 and Th2 cell activation and polarization. CXCR3 and CCR5 have been found predominantly expressed on Th1 cells secreting IFN-γ [9], while CCR3 is expressed on Th2 cells producing IL-4 [13]. In addition, cytokine production by T cells is regulated by the chemokine receptors expressed on their surface [31]. The abnormality in expression of these three chemokine receptors may result in changes in type and/or quantity of T cell accumulation in the spleen tissue and the peripheral blood, and disturb the balance of Th1/Th2 cells, which can cause a cascade of Th1 or Th2 cells, leading to Th1-dominant disease or Th2-dominant disorder. A growing number of studies have demonstrated the importance of CXCR3 and CCR5 receptor-ligand interaction in Th1-dominant autoimmune diseases [32]. For instance, in patients with rheumatoid arthritis and multiple sclerosis, the levels of the CXCR3 ligands CXCL9 and CXCL10 [33] and the CCR5 ligands CCL3, CCL4 and CCL5 [33–35] are significantly higher than those observed in healthy controls, accordingly leading to an accumulation of CXCR3⁺CCR5⁺ T cells [32]. More interestingly, CCR3 expression in the mouse model of spontaneous abortion was significantly lower than that in the normal pregnant model, suggesting that lower expression of CCR3 may cause impairment of Th2 cell dominance, and may be linked to embryo loss [36].

In our study, we found a Th1 polarized phenomenon in spleens of ITP patients. The higher expression of CXCR3 and CCR5 together with the relatively lower level of CCR3 in ITP patients may raise the possibility that the abnormality in chemokine receptor expression may be one of the key factors in the pathogenesis of ITP. The upregulated expression of CXCR3 and CCR5 promotes the proliferation of a large number of Th0 cells into Th1 cells and the recruitment of more Th1 cells to immune response sites. Meanwhile, the reduced expression of CCR3 suggests lower production of Th2 cells. Subsequently, the balance between Th1 and Th2 cells is disturbed, resulting in the accumulation of Th1 cells, more type 1 cytokines, and ultimately ITP. That is to say, the high level of CXCR3 and CCR5 may contribute to Th1 bias, and lower expression of CCR3 may weaken Th2 cell dominance. Moreover, our results are in accordance with the consensus that ITP is a kind of Th1-dominant disease.

Targeting of the Th1-associated chemokine receptors CXCR3 and CCR5 as a therapeutic strategy has been studied in a variety of human and experimental autoimmune diseases [32]. Based on the altered expression of Th1/Th2 chemokine receptors in spleens and the critical role of the spleen in ITP pathophysiology, pharmacological interventions targeting CXCR3 and CCR5 alongside the use of neutralizing antibodies or antagonists may effectively prevent Th1 cell migration in the spleen and impair Th1 polarization. Importantly, these results indicate that splenectomy may be an effective treatment of ITP for correction of the imbalance between Th1 and Th2 chemokine receptors, thus normalizing the Th1/Th2 ratios, which are commonly increased in ITP patients [2, 5, 26, 37].

In summary, the data suggest that the abnormal expression of Th1/Th2 chemokine receptors may participate in splenic immune disorder in patients with ITP and that using corresponding inhibitors may inhibit Th1-dominant expression and mitigate the progress of the disease.

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Competing Interests The authors have declared that no competing interests exist.

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