Elevated profiles of Th22 cells and correlations with Th17 cells in patients with immune thrombocytopenia

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Abstract

T-helper (Th) 22 and Th17 cells are implicated in the pathogenesis of autoimmune diseases. However, the role of Th22 cells in the pathophysiology of immune thrombocytopenia (ITP) remains unclear. Th22, Th17 and Th1 cells in both ITP patients and healthy controls were examined by flow cytometry. Plasma interleukin-22 (IL-22) level was measured by enzyme linked immunosorbent assay (ELISA). Signal transducers and activators of transcription 3 (STAT-3) and transcription factor RAR-related organ receptor C (RORC) messenger RNA (mRNA) expressions were examined by quantitative reverse transcription polymerase chain reaction (RT-PCR). Th22 cells, Th17 cells, Th1 cells and plasma IL-22 were significantly higher in ITP patients than in healthy controls. Moreover, Th22 cells showed a positive correlation with the levels of plasma IL-22 as well as Th17 and Th1 cells in ITP patients. Significant up-regulations of both STAT-3 and RORC transcription factors were also observed. Additionally, the percentage of Th22 cells was higher in autoantibody-negative ITP patients than in autoantibody-positive patients. Our results demonstrate a possible role of Th22 cells in ITP, and thus, the blockade of IL-22 may be a reasonable therapeutic strategy for ITP.

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1. Introduction

ITP is an immune-mediated bleeding disorder in which platelets are opsonized by autoantibodies that are directed against platelet surface membrane glycoproteins (GPs) and prematurely cleared by phagocytic cells in the reticuloendothelial system of the spleen, liver or bone marrow. The pathophysiology of ITP is heterogeneous and complex. In addition to the classical mechanism of GP-specific autoantibody-mediated platelet destruction, several cellular defects pertinent to immune modulation, such as the disturbed balance of Th1/Th2, the decreased number and dysfunction of regulatory T cells and the cytotoxic T lymphocyte (CTL)-mediated platelet destruction, have been described [1–3]. Th17 cells comprise a novel Th cell subset that is characterized by the production of IL-17 [4]. Th17 cells have been shown to play a crucial role in the induction of autoimmune diseases, including rheumatoid arthritis (RA), encephalomyelitis (EAE) and allergen-specific responses [5]. Our previous work demonstrated, for the first time, that Th17 cells are elevated in ITP patients [6,7].

Th22 cells are recently identified inflammatory CD4+ T cells that produce IL-22 but do not express IL-17 or interferon-gamma (IFN-γ) [8–10]. Th22 cells resemble Th17 cells in their expression of chemokine (C-C motif) receptor 6 (CCR6) and CCR4. In contrast to Th17 cells, human Th22 cells express CCR10 but do not express CD161 [8]. In addition to secreting IL-22, Th22 cells produce IL-13 as well as other factors involved in tissue remodeling, such as fibroblast growth factor isoforms, suggesting that Th22 cells might mediate the interaction of the immune system with non-hematopoietic stromal cells. Th22 cells are distinct in the profile of altered genes compared with other T cells, such as Th1, Th2 and Th17, indicating that the Th22 subset has an individual signature [9].

The effector cytokine of Th22 cells is IL-22, which belongs to the IL-10 cytokine family. IL-22 mediates its effects via a heterodimeric transmembrane receptor complex consisting of IL-22R1 and IL-10R2, as well as subsequent Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathways.
including STAT-3, Jak1 and Tyk2 [11]. IL-22 induces STAT activation in several cell lines [12,13]. In a previous study, STAT-1, STAT-3 and STAT-5 showed rapid and robust activation in response to IL-22 [14]. A later study showed that the binding of IL-22 to its surface receptor in the rat hepatoma cell line H4IE induced the rapid activation of JAK1 and Tyk2, leading to the phosphorylation of STAT-1, STAT-3 and STAT-5 [15]. The differentiation of Th17 cells also depends on the activation of STAT-3 and RORC and is regulated by a combination of cytokines, including IL-6, IL-1β, transforming growth factor-β1 (TGF-β1) and IL-23A, as well as the autocrine activity of IL-21 [16–19].

Recent studies have implicated the involvement of IL-22 in the pathogenesis of certain autoimmune diseases in humans, such as RA [20], Crohn’s disease [21], systemic lupus erythematosus (SLE) [22] and psoriasis [23]. To date, no study has reported data regarding pure Th22 cells (CD4+IFN-γ) and psoriasis. To study the roles of Th22 and its association with other Th subsets in ITP patients, we measured Th22 cells, Th17 cells, Th1 cells, plasma IL-22 levels and the relative quantitative mRNA expressions of transcription factors STAT-3 and RORC. We also analyzed their relationships in the pathogenesis of ITP.

2. Materials and methods

2.1. Patients and controls

Thirty-four newly-diagnosed ITP patients (20 females and 14 males; age range, 16–67 years; median age, 43 years) were enrolled in this study. Enrollment occurred between January 2011 and October 2011 in the Department of Hematology of Qilu Hospital (Jinan, China). Our research was approved by the Medical Ethical Committee of Qilu Hospital of Shandong University. The platelet counts ranged from 1 × 10⁹/L to 207 × 10⁹/L, with a median count of 25.12 × 10⁹/L. Patients with diabetes, hypertension, cardiovascular diseases, pregnancy, active or chronic infections, or connective tissue diseases such as SLE were excluded. All patients met the diagnostic criteria of ITP, which have been previously described [24]. Thirty-six healthy controls were included (25 females and 11 males; age range, 21–58 years; median age, 33 years). The platelet counts of healthy controls ranged from 133 × 10⁹/L to 290 × 10⁹/L, with a median count of 207 × 10⁹/L.

2.2. Flow cytometric analysis

Intracellular cytokines were assayed by flow cytometry to identify the cytokine-producing cells. Briefly, heparinized peripheral whole blood (400 μl) with an equal volume of rosewell park memorial institute (RPMI)-1640 medium was incubated for 4 h at 37 °C in 5% CO₂ in the presence of 25 ng/mL of phorbol myristate acetate (PMA), 1 μg/mL of ionomycin, and 1.7 μg/mL of monensin (all from Alexis Biochemicals, San Diego, CA, USA).

PMA and ionomycin are pharmacologic T cell-activating agents that mimic signals generated by the T cell receptor (TCR) complex and have the advantage of stimulating T cells of any antigen specificity. Monensin was used to block the intracellular transport mechanisms, thereby leading to an accumulation of cytokines in the cells. After incubation, the cells were stained with PE-Cy5-conjugated anti-CD4 monoclonal antibodies at room temperature in the dark for 20 min. The cells were next stained with FITC-conjugated anti-IFN-γ monoclonal antibodies, PE-conjugated anti-IL-17 monoclonal antibodies, and APC-conjugated anti-IL-22 monoclonal antibodies after fixation and permeabilization. All antibodies were obtained from eBioscience (San Diego, CA, USA). Isotype controls were utilized to enable correct compensation and to confirm antibody specificity. Stained cells were analyzed by flow cytometric analysis using a FACS Calibur cytometer equipped with CellQuest software (BD Bioscience Pharmingen, San Jose, CA, USA).

2.3. IL-22 enzyme-linked immunosorbent assay (ELISA)

Peripheral blood was collected into heparin-anticoagulant vacuum tubes. Plasma was obtained from all subjects by centrifugation and stored at −80 °C to determine the cytokines. IL-22 levels were determined with a quantitative sandwich enzyme immunoassay technique according to the manufacturer’s recommendations (i.e., a minimum detection limit of 9 pg/mL; eBioscience).

2.4. Quantitative real-time polymerase chain reaction analysis

Total RNA was isolated by Trizol (Invitrogen, USA) according to the manufacturer’s instructions. Approximately, 1 μg of total RNA from each sample was subjected to first-strand cDNA synthesis using RevertAid™ First Strand cDNA Synthesis Kit (MBI, Fermentas, USA). Reverse transcription reaction was done at 42 °C for 1 h, followed by 95 °C for 5 min. Real-time PCR was conducted using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems Foster City, CA, USA) in accordance to the manufacturer’s instructions. The real-time PCR contained, in a final volume of 20 μl, 10 μl of 2 × SYBR Green Real-time PCR Master Mix, 1 μl of cDNA, and 1 μl of the forward and reverse primers. The primers for human STAT-3, RORC and β-actin were as follows: STAT-3 forward, 5'-ACC AGC AGT ATA GCC GCT TC-3' and reverse, 5'-GCC ACA ATC CGG GCA ATC T-3'; RORC forward, 5'-TTT TCC GAG GAT GAG ATT GC-3' and reverse, 5'-CTT TCC ACA TGG CTA CA-3'; β-actin forward, 5'-CTT TCC TGG GCA TGG ACT C-3' and reverse, 5'-GGG GCA ATG ATG TTC ATC TTC-3'. The PCR products were analyzed by melt curve analysis and agarose gel electrophoresis to determine product size and to confirm that no by-products were formed. The relative concentrations of the PCR products derived from the target gene were calculated using Applied Biosystems System software. The results were expressed relative to the number of β-actin transcripts used as an internal control. All experiments were conducted in triplicate.

2.5. Anti-platelet autoantibody determination

All plasma samples were stored at −20 °C prior to use. The specific anti-platelet GPIIb/IIa and/or GPIb/IX autoantibodies were analyzed by modified monoclonal antibody-specific immobilization of platelet antigens (MAIPA), which was carried out as previously described in detail by Hou et al. [25].

2.6. Statistical analysis

The results are expressed as median (range) or mean ± SD. Comparisons between the two groups were assessed using the Wilcoxon rank-sum test, and the Spearman’s test was used for correlation analysis. All statistical tests were performed using SPSS (version 16.0; SPSS Inc., Chicago, IL, USA). P < 0.05 was considered to be statistically significant.

3. Results

3.1. Elevated Th22 cells correlated with increased plasma IL-22 levels in ITP patients

We analyzed the percentage of Th22 cells from the cytokine patterns after in vitro activation by PMA/ionomycin in short-term cultures. As shown in Fig. 1, Th22 was defined as CD4+IFN-γ IL-17 IL-22+ T cells to exclude Th1 or Th17 cells. Compared with
the healthy controls (median, 0.73%; range, 0.24–1.56%; \(N = 23\)), the percentage of Th22 cells was significantly higher in ITP patients (median, 1.19%; range, 0.14–9.51%; \(N = 34\); \(***P < 0.0001; \text{Fig. 2A}\)).

We also observed a statistically significant increase in the plasma IL-22 levels of ITP patients (13.39 ± 8.33 pg/ml; \(N = 34\)) compared with the healthy controls (9.98 ± 1.06 pg/ml; \(N = 36\); ***\(P < 0.0001; \text{Fig. 2B}\)).

More important, a positive correlation was found between the percentage of Th22 cells and the plasma IL-22 concentration in the ITP patients (\(r = 0.8457, P < 0.001; \text{Fig. 3A}\)) whereas no such correlation was observed in the healthy subjects.

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**Fig. 1.** The percentage of Th22 cells in representative patients with immune thrombocytopenia (ITP) and in the healthy controls. Heparinized peripheral whole blood from all subjects was stimulated with phorbol myristate acetate, ionomycin, and monensin for 4 h and then stained with labeled antibodies, as described in the "Section 2" (A) Lymphocytes were gated by flow cytometry. (B, D) The percentage of circulating CD4+IFN-\(\gamma\)/IL-17/IL-22+ cells in ITP patients and controls. (C, E) The percentage of circulating Th22 (CD4+IFN-\(\gamma\)/IL-17+ IL-22+) cells in ITP patients and controls.
Fig. 2. Th22, Th17 and Th1 cells populations as well as the concentration of plasma IL-22 between the ITP patients and the healthy controls. (A) A significantly higher Th22 percentage was observed in the ITP patients (median, 1.19%; range, 0.14–9.51%; N = 34) than in the controls (median, 0.73%; range, 0.24–1.56%; N = 23; **P < 0.0001). (B) The level of IL-22 was significantly higher in the ITP patients (13.39 ± 8.33 pg/ml; N = 34) than in the healthy controls (9.98 ± 1.06 pg/ml; N = 36; **P < 0.0001). (C) A significantly higher Th17 cell percentage was observed in the ITP patients (median, 2.03%; range, 0.50–8.18%; N = 34) than in the healthy controls (median, 1.16%; range, 0.04–2.57%; N = 29; **P < 0.0001). (D) A significantly higher Th1 cell percentage was observed in the ITP patients (median, 12.03%; range, 5.63–30.79%; N = 20) than in the healthy controls (median, 8.27%; range, 5.03–15.1%; N = 17; *P < 0.05).

Fig. 3. Correlations between Th22 cells and Th17, Th1 cells, plasma IL-22 concentrations and autoantibodies in ITP patients. (A, B) A positive correlation was found between Th22 cells and IL-22 (r = 0.8457, P < 0.001; N = 30) in the ITP patients but not in the controls. C. A positive correlation was found between the percentages of Th22 cells and the percentages of Th17 cells in the ITP patients. (r = 0.7678, P < 0.0001; N = 34). (D) A positive correlation was found between Th22 cells and Th1 cells (r = 0.6056, P = 0.0047; N = 20). (E) Th17 percentage was positively correlated with Th1 percentage (r = 0.5344, P = 0.0126; N = 21). (F) Percentages of the Th22 population in anti-platelet GPIIb/IIIa and/or GPIb/IX autoantibody-negative ITP patients (median, 2.35; range, 0.39–9.51; N = 13) were higher than in autoantibody-positive patients (median, 0.83; range, 0.23–1.24; N = 10; P = 0.013).
correlation was found in the controls. \(r = 0.2253\), \(P = 0.3014\); Fig. 2B).

3.2. Elevated Th17 and Th1 cells in ITP patients

Th17 was defined as CD4\(^+\)IL-17\(^+\) T cells. We found that the percentage of Th17 cells was significantly higher in the ITP patients (median, 2.03%; range, 0.50–8.18%; \(N = 34\)) than in the healthy controls (median, 1.16%; range, 0.04–2.57%; \(N = 29\); \(^*\times P < 0.0001\); Fig. 2C). In addition, the frequency of Th1 cells was also significantly higher in the ITP patients (median, 12.03%; range, 5.63–30.79%; \(N = 20\)) than in the healthy controls (median, 8.27%; range, 5.03–15.1%; \(N = 17\); \(P < 0.05\); Fig. 2D).

3.3. Correlations between Th22, Th17 and Th1 cells in ITP patients

In the ITP patients, a positive correlation was found between Th22 cells and Th17 cells \((r = 0.7678, P < 0.0001; N = 34\); Fig. 3C), as well as between Th22 cells and Th1 cells \((r = 0.6056, P = 0.0047; N = 20\); Fig. 3D). Moreover, a significantly positive correlation was found between Th17 and Th1 cells \((r = 0.5344, P = 0.0126; N = 21\) in the ITP patients (Fig. 3E).

3.4. Increased STAT-3 and RORC mRNA in ITP patients

We also detected the related transcriptional factors of Th22 and Th17. The results showed that the STAT-3 mRNA level was higher in the ITP patients (median, 1.644; range, 1.164–2.189; \(N = 15\)) than in the healthy controls (median, 1.584; range, 1.057–1.776; \(N = 13\); \(P < 0.05\); Fig. 4A). Furthermore, the RORC mRNA level was also higher in the ITP patients (median, 2.502; range, 1.885–2.846; \(N = 15\)) than in the healthy controls (median, 2.208; range, 1.381–2.886; \(N = 18\); \(P < 0.05\); Fig. 4B).

3.5. Elevated Th22 in autoantibody-negative ITP patients

Circulating anti-platelet autoantibodies are frequently detected in ITP patients. The most common targets of anti-platelet antibodies are the GPIIb/IIIa and GPIb/IX complexes. We determined the correlation between Th22 and anti-platelet GPIIb/IIIa and/or GPIb/IX autoantibodies in the ITP patients. The number of Th22 cells was higher in patients who had a negative MAIPA test (median, 2.35; range, 0.39–9.51) than in patients with a positive MAIPA test (median, 0.83; range, 0.23–1.24; \(P = 0.013\); Fig. 3F).

3.6. Clinical relevance of Th22 in ITP patients

We analyzed the association between Th22 and platelet counts in the ITP patients. No significant correlation was observed between the number of Th22 cells and the platelet count \((r = −0.0849, P = 0.493)\).

4. Discussion

ITP is an autoimmune disease in which abnormalities in cellular immunity have been clearly demonstrated [26]. Apart from a shift in the Th1/Th2, and Th17/Tregs balances, a new T cell subset (Th22 cells), the primary CD4\(^+\) cells producing IL-22, but not secreting IL-17 or IFN-\(\gamma\), is recently regarded as the principal instigator of autoimmune disorders.

The Th22 subset is a new and unique cell lineage in terms of function and differentiation [8–10]. A considerable amount of data shows that the levels of Th17 and Tc17 cells are increased in ITP patients [27]. However, only a few studies have focused on abnormal Th22 in autoimmune diseases. More recently, studies have shown that the percentage of Th22 cells was abnormal in patients with autoimmune diseases, implicating a potential pathogenic role for Th22.

A recent study reported elevated population of CD4\(^+\)IL-17 \(\cdot\) IL-22\(^+\) cells in ITP patients [28]. However, those CD4\(^+\)IL-17 \(\cdot\) IL-22\(^+\) cells were not true Th22 subset, as they included both Th22 (CD4\(^+\)IFN-\(\gamma\) \(\cdot\) IL-17 \(\cdot\) IL-22\(^+\)) and CD4\(^+\)IFN-\(\gamma\) \(\cdot\) IL-17 \(\cdot\) IL-22\(^+\) cells. The true Th22 cells produced IL-22 only and had low or undetectable expression of the IL-17 and Th1 transcription factors RORC and T-bet [8]. In our study, we focused exactly on the Th22 (CD4\(^+\)IFN-\(\gamma\) \(\cdot\) IL-17 \(\cdot\) IL-22\(^+\)) population, which excluded the Th1 subset. Our results showed that Th22 cell numbers were significantly higher in the peripheral blood of patients with ITP, implicating that Th22 may be involved in the pathogenesis of ITP. The CD4\(^+\)IFN-\(\gamma\) \(\cdot\) IL-17 \(\cdot\) IL-22\(^+\) population was also found higher in ITP than that in healthy controls (data not shown). Our results clarified the profile of Th22 subset in ITP patients which is not clearly defined in former report [28]. We also found Th22 to be positively correlated with elevated levels of Th17 and Th1 cells, which suggest that the differentiation of Th22, Th1 and Th17 cells may be driven in an influential manner in patients with ITP. IL-6 is required for IL-17 induction from naïve T cells [29] and can promote the expression of IL-22 [30]. In addition, IL-23 is essential for human Th17 differentiation [31] while IL-23 treatment can induce IL-22 production [30]. These associations might have contributed to the positive correlation between Th22 cells and Th17 cells in our study. Detailed mechanisms need additional future research.

IL-22 is a member of the IL-10 family of cytokines that is an important effector molecule of activated Th22 cells [10]. IL-22 has been implicated in the pathogenesis of many autoimmune inflammatory diseases, including psoriasis [23], RA [20] and...
Crohn’s disease [21]. IL-22 can activate important kinases, such as ERK1/2 and p38 MAPK, which play important roles in the proliferation of synovial fibroblasts and in the production of MCP-1 [15]. In accord with the increasing percentages of Th22 cells, elevated concentrations of plasma IL-22 were observed in the ITP patients. Th22 cells are the major subsets of T cells that produce IL-22 while accounting for 37% to 63% of all the IL-22-producing T cells, which might have led to the positive correlation between plasma IL-22 and Th22 cell frequency in our study.

Elevated STAT-3 and RORC play important roles in the crossroad of Th17 and the inducible-regulatory T cell differentiation program involving naïve CD4+ T cells [32]. The stimulation of naïve T cells in a TGF-β1-rich environment is reportedly sufficient to induce terminal differentiation into iTreg. This stimulation also induces the production of IL-6 during inflammation, and its activation of STAT-3 results in the up-regulation of ORC and the skewing of the developmental program toward the Th17 lineage [33]. Thus, the STAT-3 and RORC pathways are situated at the juncture where Th17 and iTreg developmental programs diverge [32].难题 helper cells, Th22 cells, which are involved in many autoimmune disorders, are sources of IL-22. Moreover, Th22 mediates the crossways of Th17 cells by secreting IL-22 cytokine. In our study, we looked into the mRNA level on the Th22 and Th17 pathway in ITP patients for the first time. Our data may inspire us more on the mechanism of Th helper cells signal cross-pathway in ITP disorder. The results confirmed that the Th17 transcription factors STAT-3 and RORC were at higher levels in the peripheral blood of ITP patients than controls. This finding indicates that Th22 subset might play a part in the pathogenesis of ITP. We speculate that up-regulated Th22 produced a high level of IL-22, thereby activating the STAT-3 pathway in the differentiation of Th17 cells. Although mRNA expression levels are not an optimal way to study transcription factor activity, the significantly increased expressions of STAT-3 and RORC support the combined roles of IL-22 and Th17 pathways in ITP.

A subset of patients in our study did not respond to antibody reduction or suppression therapy, suggesting the possible involvement of other pathogenic mechanisms, such as antigen shedding, T cell-mediated platelet destruction or marrow suppression [3]. The more obvious elevation of Th22 in antibody negative ITP patients may indicate that the Th22 subset might be involved in the pathogenesis of antibody negative ITP patients.

In conclusion, our study demonstrated that the predominant Th22 population, along with the plasma IL-22 level, was elevated in ITP patients. These novel findings support the hypothesis that Th22 cells may participate in the mechanism of the pathogenesis of ITP, and thus, reducing the abnormally increased Th22 cells may lead to a novel therapeutic strategy for ITP.

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