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The cytokines (IFN-γ, IL-2, IL-4, IL-10, IL-17) and Treg cytokine (TGF-β1) levels in adults with immune thrombocytopenia

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Previous studies have indicated that autoimmune diseases might be caused by an imbalance of T helper cells (Th), cytokines, and regulatory T cells(Treg) cytokines. We measured the plasma concentrations of Th1-associated cytokines (IFN-γ, IL-2), Th2 -associated cytokines (IL-4, IL-10), Th17-associated cytokine (IL-17) and Treg -associated cytokine (TGF-β1) in adult patients with immune thrombocytopenia (ITP) and evaluated their clinical relevance. Plasma IFN-γ, IL-2, IL-4, IL-10, IL-17 and TGF-β1 concentrations of 52 ITP patients and 30 age- and sex-matched healthy controls were measured by enzyme-linked immunosorbent assay method (ELISA). Concentration of Th2 cytokines (IL-4 and IL-10) were significantly higher in ITP patients compared to controls ($P < 0.05$). However, concentrations of Th1 cytokines (IFN-γ, IL-2), Th17 cytokine (IL-17) and Treg cytokine (TGF-β1) were lower in ITP patients ($P < 0.05$). Concentration of IL-17 was significantly higher in chronic ITP patients compared to severe ITP patients ($P < 0.05$), and no significant difference of cytokine concentration among the other subgroups in ITP patients was found. Among the ITP patients, concentration of IFN-γ correlated positively and significantly with PAIgG ($r = 0.48$, $P = 0.02$). A significant correlation was neither found between other cytokine levels and platelet count, nor between cytokine levels and megakaryocytes number, nor between cytokines levels and PAIgG or GPIIb/IIIa and/or GPIb/IX autoantibodies. The present study demonstrates that an imbalance of Th and Treg cytokines may mediate the pathogenesis of ITP.

1. Introduction

Adult immune thrombocytopenia (ITP) is an acquired organ-specific autoimmune hemorrhagic disease characterized by autoantibodies against platelet surface glycoproteins (GP) (particularly IIb/IIIa), resulting in enhanced platelet elimination macrophages (Chu et al. 2000). Auto reactive B-lymphocytes secreting antiplatelet antibodies are considered as the primary immunologic defect in ITP. There is increasing evidence that a disturbed T-cell homeostasis plays a critical role in the development of ITP (Zhang et al. 2009; Panitsas et al. 2004; Ma et al. 2008; Andersson et al. 2000). The main T-cell subsets which are pivotal for this T-cell balance consist of T helper (Th) cells and regulatory T (Treg) cells. Abnormal Th cells, cytokines, and Treg cells cytokines have been shown to be involved in the pathogenesis of autoimmune diseases, such as systemic lupus erythromatosus (SLE) (Wong et al. 2000), and ITP (Ma et al. 2008; Andersson et al. 2000).

T helper cells are defined as Th1-, Th2- or Th17-cells characterized by differential expression of certain cytokines. Th1 cells produce both interferon-γ (IFN-γ) and interleukin-2 (IL-2) and mainly induce cellular immunity. In contrast, Th2 cells induce antibody production through synthesis of cytokines, IL-4, IL-5, IL-6, IL-10 and IL-13. So far, adult chronic ITP was accepted mostly as the manifestation of a Th1 polarized immune response (Zhang et al. 2009; Panitsas et al. 2004; Semple et al. 1996; Lazarus et al. 2000). However, some other studies suggested that a less prominent Th1 response was involved (Ma et al. 2008; Andersson et al. 2000; Ogawara et al. 2003) or even a Th2 response was present in patients with chronic ITP (Webber et al. 2001). Th17 cells, a more recently described Th cell subset, are characterized by their ability to produce IL-17, and shown to play a crucial role in the induction of autoimmune diseases, including rheumatoid arthritis and allergen-specific responses (Weaver et al. 2006). Treg cells are characterized by the expression of CD4 and CD25, and the production of transforming growth factor-β1 (TGF-β1) and IL-35 to mainly induce immune tolerance. Regarding autoimmune diseases, the data on Treg cells was limited and controversial. Some studies indicated that Treg cells were decreased and TGF-β1 level was less than normal in ITP patients (Andersson et al. 2000; Ling et al. 2007), whilst others suggested an increase (Sakamaki et al. 1999) or no difference in Treg cells (Panitsas et al. 2004; Sakakura et al. 2007) in ITP patients compared to healthy controls. Th cytokines and Treg cytokines response in ITP requires further investigation.

To further investigate the possible role and imbalance of Th cells and Treg cells in the pathogenesis of ITP, we measured the plasma concentrations of Th1-associated cytokines (IFN-γ, IL-2), Th2 -associated cytokines (IL-4, IL-10), Th17-associated

Table 1: Cytokine concentrations in ITP versus controls (pg/ml, mean ± SD)

	IFN-γ	IL-2	IL-4	IL-10	TGF-β1	IL-17
Patients (n = 52)	25.54 ± 7.97*	27.63 ± 16.54*	102.49 ± 13.17*	65.52 ± 21.99*	605.72 ± 143.4*	10.33 ± 4.69*
Controls (n = 30)	39.59 ± 4.34	55.33 ± 9.17	84.67 ± 7.12	27.94 ± 7.74	861.62 ± 83.44	15.55 ± 2.91

* $P < 0.05$, VS. normal controls.

cytokine (IL-17) and Treg -associated cytokine (TGF-β1) simultaneously and evaluated their clinical relevance.

2. Investigations and results

2.1. Cytokine concentrations in ITP versus controls

There was a maximum of TGF-β1 observed in both ITP patients and controls with concentrations of 605.72 ± 143.4 pg/ml and 861.62 ± 83.44 pg/ml respectively, followed by IL-4 (Table 1). In contrast, there was a minimum of IL-17 present in both ITP patients and controls at 10.33 ± 4.69 pg/ml and 15.55 ± 2.91 pg/ml respectively. Compared to healthy controls, Th2 cytokines (IL-4 and IL-10) were significantly higher in patients with ITP, with an IL-4 concentration of 102.49 ± 13.17 pg/ml and an IL-10 concentration of 65.52 ± 21.99 pg/ml ($P < 0.05$). Other cytokines, including Th1 cytokines (IFN-γ and IL-2), Th17 cytokine (IL-17) and Treg cytokine (TGF-β1) were significantly lower in patients with ITP ($P < 0.05$).

2.2. Cytokine concentrations in subgroups of patients with ITP

Cytokine concentrations in plasma were also analyzed by patient subgroups according to gender, age, newly diagnosed or persistent stage of disease and platelet count. Only plasma IL-17 was significantly higher in chronic ITP patients than severe ITP patients ($P < 0.05$), while there was no significant difference between cytokine concentration among the other paired subgroups (Table 2).

2.3. Correlation between cytokine concentrations and laboratory parameters in ITP

There was a significantly positive correlation between platelet associated IgG (PAIgG) and IFN-γ (r = 0.48, $P = 0.02$), while no correlation was found between PAIgG and other cytokines (Table 3). In addition, no significant correlations between cytokine concentrations with platelet count or megakaryocytes number or glycoprotein (GP) IIb/IIIa and/or GPIb/IX autoantibodies were found in ITP patients.

3. Discussion

Imbalance of circulating Th cells and an impairment of Treg cells have been suggested to be involved in the pathogenesis of many autoimmune diseases. In ITP patients, some previous studies have indicated that it was the manifestation of type-1 polarized immune responses (Zhang et al. 2009; Panitsas et al. 2004; Semple et al. 1996; Lazarus et al. 2000), whereas others yielded inconsistent or even opposing results (Ma et al. 2008; Andersson et al. 2000; Ogawara et al. 2003; Webber et al. 2001). Our study found that plasma concentrations of Th2 cytokines IL-4 and IL-10 were elevated, while Th1 cytokines IL-2 and IFN-γ decreased in adult ITP. IL-4 is mainly secreted by Th2 lymphocytes, and essential for T-dependent B-cell differentiation and isotype switching to several IgG isotypes including antiplatelet antibodies (Hamidpaur et al. 2006). IL-10 is produced not only by Th2 cells, but also by monocytes, NK cells and B lymphocytes.IL-10 can suppress macrophages and decrease antigen presenting cells to present antigens to Th1 cells, consequently inhibiting Th1 cytokine production and weakening cellular immunity (Krakauer 1995). This is presented as decreased plasma

Table 2: Cytokine concentrations in ITP subgroups (pg/ml, mean ± SD)

Subgroups	IFN-γ	IL-2	IL-4	IL-10	TGF-β1	IL-17
Gender						
Male (n = 18)	26.56 ± 9.26	32.57 ± 17.87	100.20 ± 15.46	59.68 ± 24.43	655.38 ± 132.90	10.89 ± 4.50
Female (n = 34)	25.01 ± 7.29	25.02 ± 15.43	103.71 ± 11.86	68.62 ± 20.28	579.44 ± 143.62	10.03 ± 4.82
P-value	0.53	0.14	0.41	0.19	0.06	0.53
Age						
≤ 60 yrs(n = 41)	26.05 ± 7.91	27.34 ± 16.11	101.37 ± 13.01	64.15 ± 22.55	611.41 ± 148.23	10.86 ± 4.66
> 60 yrs (n = 11)	23.65 ± 8.29	28.71 ± 18.87	106.68 ± 13.57	70.63 ± 19.90	584.54 ± 127.94	8.36 ± 4.45
P-value	0.65	0.83	0.26	0.37	0.56	0.12
Disease status						
Newly diagnosed (n = 43)	24.89 ± 7.15	27.12 ± 14.69	103.88 ± 13.41	67.11 ± 22.10	595.66 ± 133.51	10.12 ± 4.71
Persistent (n = 9)	28.69 ± 11.09	30.06 ± 14.53	95.89 ± 10.10	57.92 ± 18.01	653.81 ± 185.41	11.33 ± 4.68
P-value	0.20	0.63	0.10	0.26	0.27	0.50
Stages						
Severe (n = 35)	25.19 ± 7.18	26.95 ± 15.02	103.87 ± 14.07	66.46 ± 21.91	592.47 ± 134.79	9.40 ± 4.57
Chronic (n = 17)	26.28 ± 9.60	29.04 ± 19.73	99.65 ± 10.94	63.59 ± 22.71	633.01 ± 160.52	12.20 ± 4.49
P-value	0.65	0.20	0.24	0.67	0.38	**0.04***
PLT($\times 10^9$/L)						
> 10 (n = 26)	24.76 ± 6.75	23.91 ± 14.28	102.11 ± 12.35	69.09 ± 21.97	592.20 ± 132.87	10.51 ± 4.34
≤ 10 (n = 26)	26.33 ± 9.10	31.36 ± 18.04	102.87 ± 14.19	61.96 ± 21.85	619.24 ± 154.65	10.15 ± 5.09
P-value	0.48	0.11	0.84	0.25	0.50	0.79

Significant difference of IL-17 level between severe ITP patients and chronic ITP patients (* $P = 0.04$).

Table 3: Correlations between cytokines and laboratory parameters in patients with ITP

Cytokines	PLT n = 52	Megakaryocytes n = 45	PAIgG n = 23	GPIIb/IIIa and/or GPIb/IX antibodies n = 30
IFN-γ	−0.13[1] (0.35)[2]	−0.02 (0.92)	0.48 (0.02*)	0.10 (0.62)
IL-2	−0.17 (0.22)	0.15 (0.34)	0.24 (0.28)	0.15 (0.34)
IL-4	−0.4 (0.80)	−0.08 (0.60)	0.04 (0.87)	0.21 (0.15)
IL-10	0.15 (0.29)	−0.03 (0.84)	−0.22 (0.31)	0.13 (0.64)
TGF-β1	−0.07 (0.62)	0.03 (0.85)	0.23 (0.29)	0.05 (0.68)
IL-17	0.01 (0.95)	−0.05 (0.77)	0.35 (0.11)	−0.07 (0.27)

[1] Correlation coefficient; [2] *P* value. * A positive correlation between PAIgG and IFN-γ ($P < 0.05$) in ITP patients.

concentrations of both IL-2 and IFN-γ in ITP patients in our study. Our result differs from some previous reports, which may be partly explained by the following: (1) The pathophysiology of ITP is heterogeneous and complex. (2) Ethnic differences in the studied populations. (3) Low number of patients and controls in the study. (4) Differences in the methods employed to evaluate the cytokines (ELISA x intracellular cytokines). Therefore, more sensitive and accurate method should to be used, different methods (e.g. ELISA, flow cytometry and molecular biology) need to be combined to analysis in future study.

Analyzing by subgroups of ITP patients, no statically significant difference was observed in Th1 and Th2 cytokine concentrations in each paired subgroup. Ma et al. (2008) also did not find any significant difference in plasma IFN-γ in either acute or chronic ITP patients or healthy controls, but found IFN-γ was significantly higher in male patients than female patients. Panitsas et al. (2004) reported that mRNA expression of Th1 cytokines was significantly higher in chronic patients compared to acute patients with ITP. They suggested that the levels of Th1 and Th2 cytokines maybe varied with gender, disease status, stage, and severity in ITP. There was no statistical difference in our study that might be due to the smaller number of ITP patients in some subtype group. Further studies on a greater number of patients are necessary to examine these issues.

Recently, a novel subset of Th cells was identified, characterized by producing a distinct profile of effector cytokines, IL-17 and IL-6, probably enhancing host clearance of a range of pathogens distinct from those targeted by Th1 and Th2. The IL-17 receptor is distributed ubiquitously in various tissues, and its engagement activates both transcription factor NF-κB and kinase Jnk pathways (Park et al. 2005; Bettelli et al. 2007; Moseley et al. 2003; Schwandner et al. 2000). IL-17 has been associated with many autoimmune diseases such as rheumatoid arthritis, asthma and SLE (Moseley et al. 2003; Aggarwal et al. 2002, Kolls and Linden 2005), although its regulation and function have remained unclear. In ITP patients, some studies suggested a higher Th17 response (Zhang et al. 2009; Rocha et al. 2011; Hu et al. 2011) but some investigations found no difference compared to controls (Ma et al. 2008; Guo et al. 2009). In our study, IL-17 was significantly lower in ITP patients compared to controls, and significantly lower in severe patients than in chronic patients with ITP. We conjectured that decreased IL-17 in ITP patients might be related to lower TGF-β1, causing a failure of a cooperative function of TGF-β1 and IL-6 to induce Th17 cell proliferation (Bettelli et al. 2007).

Treg cells are featured by expression of CD4 and CD25, functioning in induction and maintenance of peripheral self-tolerance. The elimination or inactivation of Treg cells is known to result in various severe autoimmune diseases.TGF-β1 is mainly produced by Treg cells and induces thrombopoietin secretion from bone marrow stromal cells (Sakamaki et al. 1999). We found that TGF-β1 was significantly lower in ITP patients compared to healthy controls which is in line with several reports (Mouzaki et al. 2002; Andersson et al. 2002). This suggested that down-regulation of Treg cells may lead to activation of auto reactive T cells and suppression of thrombopoietin production from bone marrow, which is involved in the immunopathology of ITP.

Circulating anti-platelet autoantibody was frequently detected in ITP patients. The most specific targets of anti-platelet antibody is the GPIIb/IIIa complex and GPIb/IX. The most sensitive target of anti-platelet antibody is PAIgG. We analyzed the correlations between cytokines, platelet count, megakaryocytes number, PAIgG and anti-platelet GPIIb/IIIa and/or GPIb/IX autoantibodies, finding a significantly positive correlation between PAIgG and IFN-γ concentration ($r = 0.48$, $P = 0.02$). The reason may be that IFN-γ can induce B lymphocytes into plasma cells and help produce antibodies against platelets.

In conclusion, in our study we demonstrated that ITP is related to the imbalance of subsets of Th cells and Treg cells, presenting as increasing Th2 cytokines (IL-4, IL-10), reducing Th1 cytokines (IFN-γ, IL-2), Th17 cytokine (IL-17) and Treg cytokine (TGF-β1). Further investigation is required for elucidating the detail of *in vitro* production mechanism and the role of IFN-γ, IL-2, IL-4, IL-10, IL-12, IL-17and TGF-β1 in the pathogenesis of ITP.

4. Experimental

4.1. Subjects and study design

A total of 52 Chinese patients with ITP and 30 age-and sex- matched healthy volunteers were recruited from the First Affiliated Hospital of Dalian Medical University, Dalian, China, between October 2010 and September 2012 (Table 4). All of the patients met the diagnostic criteria for ITP (Rodeghiero et al. 2009). Patients with hypertension, diabetes, cardiovascular diseases, who are pregnant, with acute or chronic infections, or connective tissue diseases, such as SLE, were excluded. There was no history of glucocorticoids and immunosuppressive agents use by any of the patients and healthy volunteers two weeks prior to the study. Five milliliters of heparinized venous blood were taken from each patient at diagnosis before treatment and control subject at 8 AM. Plasma was isolated by centrifugation (1000 x g, 10 min). All plasma samples were stored at -80 °C until the determination

Table 4: Clinical characteristics of ITP subjects

	Case#	M:F[1]	Age (years)	PLT[2] (x 10^9/L)	Megakaryocytes	PAIgG[3] (ng/10^7 pl)
Patients	52	18:34	43[4] (16−69)[5]	9.4 (1−50)	185 (6−1932)	146 (29.1−3200)
Controls	30	11:19	36 (19−52)	152(93−321)	n/a	n/a

[1] Male/female ratio; [2] Platelet count; [3] Normal range of PAIgG is 0−108 ng/10^7 pl in our laboratory [4] Median; [5] Range.

of cytokines. The study was approved by the Medical Ethical Committee of the First Affiliated Hospital of Dalian Medical University (Dalian, China) and informed written consent was obtained from all patients.

4.2. Cytokines quantification

Quantitative analysis of IFN-γ, IL-2, IL-4, IL-10, IL-17 and TGF-β1 levels in plasma were performed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (KeyGEN Co. Ltd, Nanjing, China). The assay was performed in triplicate and the cytokine concentration was calculated based on a standard curve constructed using recombinant cytokine standards.

4.3. Observation of megakaryocytes

Bone marrow aspirates were obtained from the posterior superior iliac spine of patients during diagnostic procedures. Bone marrow aspirate (10 μl) was taken to make a smear (slide size: 1.5 cm × 3.0 cm). The morphology and number of megakaryocytes were analyzed on each smear under microscopy.

4.4. Anti-platelet autoantibody determination

According to Tsubakio et al. (1981), PAIgG levels were measured through a competitive enzyme immunoassay by using alkaline phosphatase-conjugated anti-human IgG (Sun Co. Ltd, Shanghai, China). The specific anti-platelet glycoprotein (GP) GPIIb/IIIa and/or GPIb/IX autoantibodies were measured by modified monoclonal antibody-specific immobilization of platelet antigens (MAIPA), which was carried out as described elsewhere (Hou et al.2003).

4.5. Statistical analysis

All the data were expressed as mean ± SD. The difference between the two groups was assessed using the unpaired Student's t-test. Spearman's test was used for correlation analysis of non-parametric, and Pearson's test was employed for correlation analysis of parameters. All tests were performed by SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA).A value of $P < 0.05$ was considered to be significant.

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