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Evaluation of IVIG response in relation to Th1/Th2 cytokines in pediatricm immune thrombocytopenia

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ABSTRACT

Immune thrombocytopenia (ITP) is a common immune-mediated bleeding disorder in children, and intravenous immunoglobulin (IVIG) is widely used as the initial therapy of ITP. Effective predictive factors of response to IVIG in ITP are important for guiding the treatment decisions. A retrospective study was performed on 197 Chinese ITP patients, and the data of their serum interleukin (IL)-2, IL-4, IL-6, IL-10, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) levels, age at onset, duration of disease, white blood cell count (WBC), platelet count, and gender ratio were collected. Our results showed that ITP patients had higher IL-2, IL-6, IL-10, and IFN-γ levels than healthy children. Moreover, lower IL-4 level (< 3.5 pg/ml), higher WBC (> $6.37*10^9$/L), and higher platelet count (> $12*10^9$/L) at diagnosis were favorable predictive factors for IVIG response in the newly diagnosed ITP. In addition, ITP patients with lower IL-10 level (< 3.7 pg/ml) and older onset age (> 2.84 years) were more resistant to therapy and developed to chronic ITP more easily. These findings may help guide the treatment decisions making for ITP patients.

1. Introduction

Immune thrombocytopenia (ITP), previously named as immune thrombocytopenic purpura, is an immune-mediated bleeding disorder in children, with increased platelet destruction and decreased platelet production (less than 100×10^9 platelets/L). ITP is classified into newly diagnosed (less than 3 months), persistent (3–12 months) or chronic (more than 12 months) ITP [1], with approximately 20% of patients developing into chronic ITP [2].

At presentation, clinical biomarkers that can separate the newly diagnosed, persistent and chronic ITP are partially lacking. Serum cytokine levels were known to be different in acute and chronic ITP. Semple et al. found that ITP patients who had higher interleukin (IL)-2, interferon-γ (IFN-γ), and/or IL-10 levels were more resistant to therapy and developed to chronic ITP more easily [3]. Vecchio et al. found that ITP patients with higher level of IL-10 at the onset of disease would obtain disease remission in less than 1 year [4]. Jernas et al. showed that the relative expressions of IL-4 in newly diagnosed and chronic ITP were 2.8 ± 0.30 pg/ml and 6.4 ± 0.40 pg/ml, respectively ($P < 0.001$), which indicated that chronic ITP patients had higher IL-4 levels [5].

Intravenous immunoglobulin (IVIG) was first used for ITP in the early 1980s [6]. Until now, IVIG is still widely used as the initial therapy of ITP, especially in young patients [7–11]. However, the mechanism of IVIG resistance in ITP is currently unresolved. Researchers found that cytokines were related to IVIG responses in ITP. Mouzaki et al. reported that ITP patients with higher expression of IFN-γ at presentation had either transient responses or were refractory to IVIG [12]. Beside cytokines, there were some other factors also related to IVIG responses in ITP. Morimoto et al. reported that white blood cell count (WBC) lower than $7.0*10^9$/L at diagnosis of ITP was an unfavorable predictive factor of IVIG response [13]. Older age at diagnosis [14,15], lower platelet count (< $9.0*10^9$/L) [14], insidious onset of symptoms [16], high level of lactic dehydrogenase (LDH) [17], and slow reduction of LDH [18] were all associated with poorer response to IVIG in ITP.

In this study, we tested IL-2, IL-4, IL-6, IL-10, tumor necrosis factor-α (TNF-α), IFN-γ levels, age at onset, duration of disease, WBC, platelet

Abbreviations: ITP, immune thrombocytopenia; IVIG, intravenous immunoglobulin; IL-2, interleukin-2; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; WBC, white blood cell count; CR, complete response; PR, partial response; NR, no response; HLH, haemophagocytic lymphohistiocytosis; Th, T-helper; ROC, receiver operating characteristic; TGF-β1, transforming growth factor β1; CX3CL1, chemokine (C-X3-C motif) ligand 1; ROS, reactive oxygen species; FcγRIIB, Fcγ receptor IIB; FcRn, Fc receptor; CSF-1, colony stimulating factor-1; GSH/GSSG, reduced to oxidized glutathione ratio; PPAR-γ, peroxisome proliferator-activated receptor-γ; IDO, indoleamine 2, 3-dioxygenase

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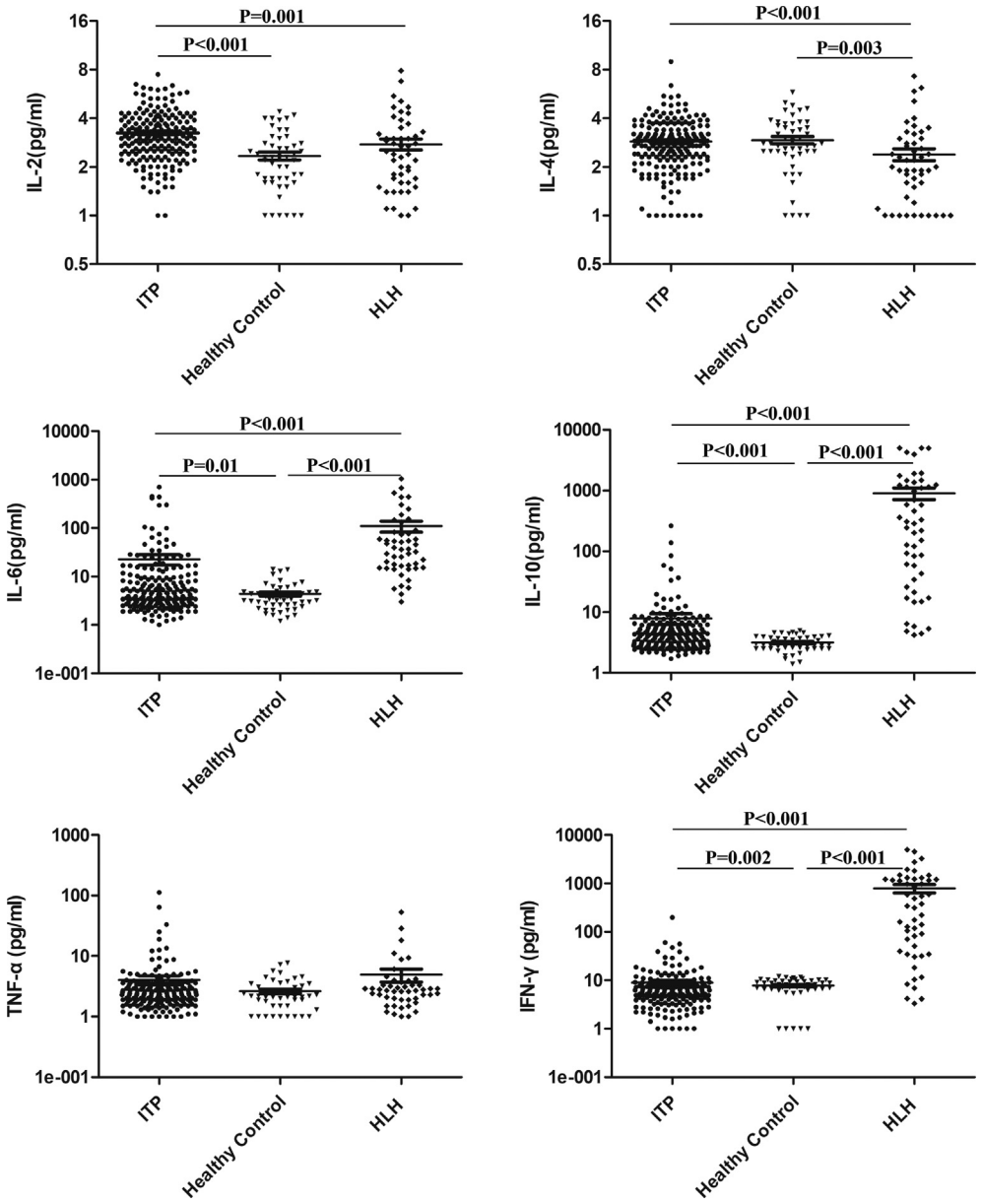

Fig. 1. Comparisons of serum Th1/Th2 cytokine concentrations among ITP patients, healthy control, and HLH control. The center horizontal line and whiskers were Mean ± the standard error of the mean (SEM). The minimum and maximum limits of detection for all six cytokines were 1 and 5000 pg/ml, respectively.

count, and gender ratio of all our ITP patients, and assessed their effects on IVIG response. The aims of this study were to evaluate whether these cytokines could be used as indicators to differentiate newly diagnosed, persistent and chronic pediatric ITP, as well as predictors for IVIG response in ITP patients.

2. Methods

2.1. Participants

The diagnoses of all our ITP children and their responses to IVIG were evaluated according to the International Consensus Report [19] and American Society of Hematology 2011 [20].

The original IVIG dose of 1 g/kg daily was given on consecutive days, varied from one to three days. Without any other treatment, complete response (CR) was defined as platelet count $\geq 100.0 * 10^9$/L, partial response (PR) as platelet count $\geq 30.0 * 10^9$/L, and no response (NR) as platelet count $< 30.0 * 10^9$/L.

A cohort of 197 hospitalized ITP patients were enrolled in the study, which included 121 males and 76 females with a male-to-female ratio of 1.59:1, and a mean age of 3.84 years with a range of 4 days through 14.25 years. Among the 197 ITP patients, there were 156 newly diagnosed, 18 persistent, and 23 chronic ITP cases. The 156 newly diagnosed ITP cases included 62 CR, 53 PR, 21 NR cases, and 20 ITP patients without IVIG treatments. Patients who had been treated by other treatments except IVIG before referral to our hospital were excluded from the newly diagnosed ITP group. A total of 50 unrelated healthy individuals and 50 unrelated haemophagocytic lymphohistiocytosis (HLH) were recruited as healthy and HLH controls, as HLH is a disease characterized with ineffective immune response to antigens and had a specific T-helper (Th) 1/Th2 cytokine pattern [21]. All the patients and controls were from our hospital between February 2014 and September 2018.

The study protocol was reviewed and approved by the Ethics Committee at Children's Hospital Zhejiang University School of Medicine and the written informed consents were obtained from all

participants' parents or guardians before this study.

2.2. Measurement of Th1/Th2 cytokines

All ITP patients and controls were tested for the six cytokines including IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ. Peripheral blood samples were collected, transferred to a serum separating tube and centrifuged at 1000*g* at 20 °C for 20 min after clotting. The serum was carefully harvested, and the determination of the cytokines was performed immediately, or if the situation was not so urgent, the aliquot was temporarily stored at 2–8 °C until analysis (usually within 12 h). Concentrations of the six cytokines aforementioned were quantitatively determined using the CBA Human Th1/Th2 Cytokine Kit II (BD Biosciences, San Jose, California) as described in our previous study [22]. Briefly, the CBA technique was based on 6 bead populations, each with a distinct fluorescence intensity, that had been coated with capture antibodies specific for IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ proteins. Following the acquisition of sample data on a FACSCalibur flow cytometer (Becton Dickinson), the sample results were generated in graphic and tabular format using BD Biosciences CBA software. The minimum and maximum limits of detection for all six cytokines were 1 and 5000 pg/ml, respectively.

2.3. Statistical analysis

Serum concentrations of individual cytokines were compared between groups using the Mann-Whitney *U* test. Chi-square tests were used to assess ratio differences between groups. Receiver operating characteristic (ROC) curves were derived from the cytokine levels, age, WBC, and platelets for all ITP patients. In ROC curves, the sensitivity and specificity of IL-10 and age were calculated for the differentiation among the newly diagnosed, persistent, and chronic ITP. At the same time, the sensitivity and specificity of IL-4, WBC, and platelets were calculated for the IVIG response to the newly diagnosed ITP. All statistical analyses were performed using SPSS 20.0 software (SPSS Inc, Chicago, Illinois). A two-sided P-value < 0.05 was considered to be statistically significant.

3. Results

3.1. IL-2, IL-6, IL-10, and IFN-γ levels were higher in ITP patients than in healthy controls

IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ levels, and IFN-γ/IL-4 ratio were tested in ITP patients, healthy controls, and HLH subjects (Fig. 1 and Supplementary Table 1). Compared to the healthy controls, ITP patients had higher IL-2, IL-6, IL-10, and IFN-γ levels, and there were no significant differences of IL-4, TNF-α, and IFN-γ/IL-4 ratio between these two groups. When comparing to the HLH patients, ITP patients had higher IL-2 and IL-4 levels, but lower IL-6, IL-10, IFN-γ levels, and IFN-γ/IL-4 ratio.

ROC curves of IL-2, IL-6, IL-10, and IFN-γ levels showed that $>$ 2.9 pg/ml, $>$ 5.1 pg/ml, $>$ 4.8 pg/ml, and $>$ 11.8 pg/ml had sensitivity of 59.9%, 44.2%, 34.5%, and 13.7%, while specificity of 76.0%, 78.0%, 98.0% and 98.0%, respectively (Supplementary Table 4), which we picked as their best cutoff values to differentiate ITP patients from the healthy controls.

3.2. Decreased IL-10 levels in chronic ITP patients compared to the newly diagnosed ITP patients

IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ levels, and IFN-γ/IL-4 ratio were tested in the newly diagnosed, persistent, and chronic ITP patients (Fig. 2 and Supplementary Table 2). Among all six cytokines, only IL-10 showed a significant difference between the newly diagnosed and chronic ITP ($P = 0.047$), indicating that the chronic ITP patients had lower IL-10 levels.

ROC curves of IL-10 levels showed that $<$ 2.5 pg/ml, $<$ 3.7 pg/ml, and $<$ 7.2 pg/ml had sensitivity of 90.4%, 59.6%, and 17.3%, while specificity of 17.4%, 69.6% and 87.0%, respectively (Supplementary Table 5). We picked IL-10 level $<$ 3.7 pg/ml as its best cutoff value to differentiate chronic from the newly diagnosed ITP patients.

3.3. Lower IL-4 levels indicated better IVIG response in the newly diagnosed ITP patients

IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ levels, and IFN-γ/IL-4 ratio were tested in the newly diagnosed ITP patients, included CR, PR, NR, and ITP patients without IVIG treatments (Fig. 3 and Supplementary Table 3). No difference was found between the CR and PR subgroups. Compared to IVIG non-responder group, the IVIG responder group (CR + PR) demonstrated a decreased IL-4 level ($P = 0.009$).

ROC curves of IL-4 levels showed that $<$ 2.1 pg/ml, $<$ 3.5 pg/ml, and $<$ 4.4 pg/ml had sensitivity of 95.2%, 57.1%, and 14.3%, while specificity of 17.4%, 79.1%, and 93.9%, respectively (Supplementary Table 6). We picked IL-4 level $<$ 3.5 pg/ml as its best cutoff value to differentiate the IVIG responder from IVIG non-responder in the newly diagnosed ITP patients.

3.4. Older onset age was an unfavorable factor of outcome in ITP patients

Age at onset, duration of disease, WBC, platelets, and gender ratio were tested in the newly diagnosed, persistent, and chronic ITP patients (Table 1). Compared to the newly diagnosed and chronic ITP patients, the persistent group had a higher level of platelets. Compared to the persistent and chronic ITP patients, those in the newly diagnosed group had a higher male-to-female ratio. Meanwhile, comparisons between the newly diagnosed group and chronic group showed that ITP patients with older onset age developed into chronic ITP more easily ($P < 0.001$), indicating that older onset age was an unfavorable factor of outcome in ITP patients.

ROC curves of onset age showed that $>$ 1.96 years, $>$ 2.84 years, and $>$ 8.13 years had sensitivity of 82.6%, 78.3%, and 17.4%, while specificity of 56.4%, 66.7%, and 88.5%, respectively (Supplementary Table 5). We picked onset age $>$ 2.84 years as its best cutoff value to differentiate chronic from the newly diagnosed ITP patients.

3.5. Higher WBC and platelet counts indicated better IVIG response in the newly diagnosed ITP patients

Age at onset, duration of disease, WBC, platelets, post-WBC (three days after the first dose of IVIG treatment), post-platelets (three days after the first dose of IVIG treatment), and gender ratio were tested in the newly diagnosed ITP patients, included CR, PR, NR, and ITP patients without IVIG treatments (Table 2). When comparing to the PR subgroup, the patients in the CR subgroup showed a younger onset age ($P = 0.001$) and a lower male-to-female ratio ($P = 0.01$). Compared to the IVIG non-responder group, the IVIG responder group (CR + PR) demonstrated a higher WBC ($P = 0.004$) and a higher platelet count ($P = 0.001$).

ROC curves of WBC levels showed that $> 5.05 * 10^9$/L, $> 6.37 * 10^9$/L, and $> 8.54 * 10^9$/L had sensitivity of 90.4%, 74.8%, and 39.1%, while specificity of 28.6%, 61.9%, and 76.2%, respectively (Supplementary Table 6). ROC curves of platelet counts showed that $> 5 * 10^9$/L, $> 9 * 10^9$/L, $> 12 * 10^9$/L, and $> 21 * 10^9$/L had sensitivity of 92.2%, 72.2%, 59.1%, and 32.2%, while specificity of 19.0%, 57.1%, 81.0%, and 95.2%, respectively (Table 5). We picked WBC count $> 6.37 * 10^9$/L and platelet count $> 12 * 10^9$/L as their best cutoff values to differentiate the IVIG responder from IVIG non-responder in the newly diagnosed ITP patients.

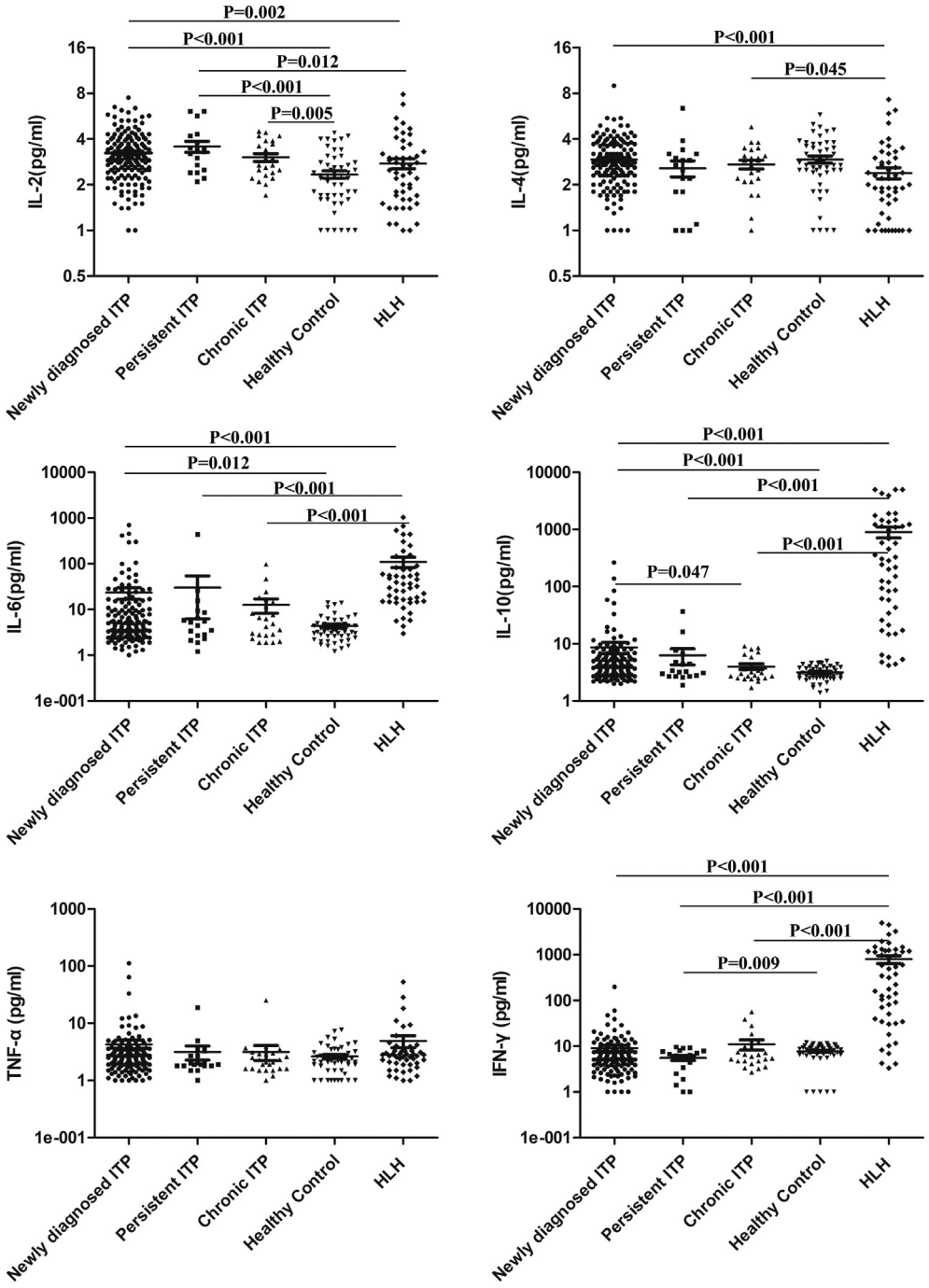

Fig. 2. Comparisons of serum Th1/Th2 cytokine concentrations among the newly diagnosed ITP patients, persistent ITP patients, chronic ITP patients, healthy control, and HLH control. The center horizontal line and whiskers were Mean ± SEM. The minimum and maximum limits of detection for all six cytokines were 1 and 5000 pg/ml, respectively.

4. Discussion

Previous studies have shown that Th cells (e.g. Th1, Th2, and Th17 cells) and their secreted cytokines were involved in pediatric ITP and in IVIG response, indicating that cytokines were related to the mechanisms of pediatric ITP and IVIG function [23,24]. Th17 cells were elevated in ITP patients, and produced large amounts of IL-17A, IL-17F, IL-21, and IL-22 [25]. Zhang et al. reported that the circulating levels of Th17 cells were increased in CR ITP patients but decreased in PR and NR ITP patients [26]. IL-17A and IL-21 induced Th17 and inhibited regulatory T cells re-differentiation via Th17-associated signaling pathway in ITP patients *in vitro*, which highlighted the potential values of IL-17A and IL-21 in the pathogenesis of ITP [27]. Besides Th1, Th2, and Th17 cells, there were many other pro-inflammatory cytokines involved in ITP. Shan et al. showed that IL-18 level was significantly increased in patients with active ITP as compared to those in control subjects. Jernas et al. reported that children with ITP had lower plasma levels of transforming growth factor β1 (TGF-β1) and elevated levels of chemokine (C-X3-C motif) ligand 1 (CX3CL1) and IL-22 when comparing with healthy children [28]. Liu et al. reported that active ITP patients had decreased level of IL-27 [29], and the mechanism was that IL-27 inhibits cytotoxic T-lymphocyte-mediated platelet destruction in ITP [30]. Sun et al. reported that IL-35 level was decreased in patients with ITP, and found that IL-35 promoted the secretion of IL-10 and

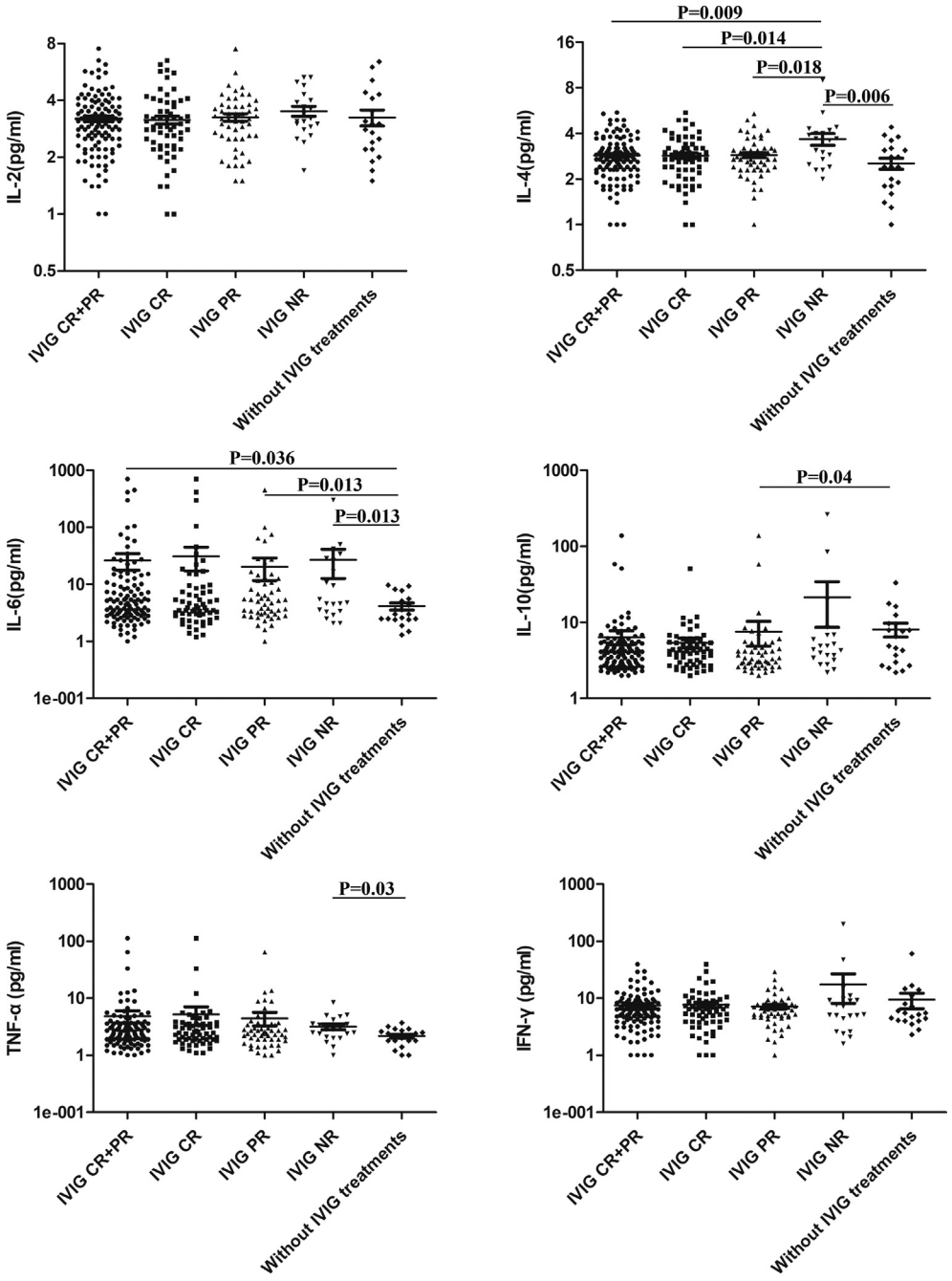

Fig. 3. Comparisons of serum Th1/Th2 cytokine concentrations among the IVIG complete responders + partial responders (IVIG CR + PR), IVIG complete responders (IVIG CR), IVIG partial responders (IVIG PR), IVIG non-responders (IVIG NR), and ITP patients without IVIG treatments. The center horizontal line and whiskers were Mean ± SEM. The minimum and maximum limits of detection for all six cytokines were 1 and 5000 pg/ml, respectively.

Table 1
Clinical information of the newly diagnosed, persistent and chronic ITP patients.

Clinical information	Newly diagnosed ITP (n = 156)	Persistent ITP (n = 18)	Chronic ITP (n = 23)
Age at onset (year)	3.03 (0.01–14.25)★	4.45 (0.04–12.75)	5.27 (0.42–11.08)
Duration of disease (day)	6.9 (0.5–60.0)☆★	161.7 (90.0–300.0)★	1128.3 (360.0–3600.0)
WBC (*10^9/L)	7.85 (2.60–20.32)	7.98 (4.26–22.90)	8.10 (2.88–13.71)
Platelets (*10^9/L)	17 (2–95)☆	31 (11–90)★	21 (4–64)
Gender (Male/Female)	102/54☆★	7/11	12/11

Values are the median (range).
WBC, white blood cells.
☆ $P < 0.05$ versus persistent ITP patients.
★ $P < 0.05$ versus chronic ITP patients.

Table 2
Clinical information of the newly diagnosed ITP patients who were IVIG responders, IVIG nonresponders, and without IVIG treatments.

Information	IVIG CR + PR (n = 115)	IVIG CR (n = 62)	IVIG PR (n = 53)	IVIG NR (n = 21)	Without IVIG treatments (n = 20)
Age at onset (year)	2.89 (0.11–13.58)	2.02 (0.11–10.75)#	3.91 (0.12–13.58)	3.51 (0.01–11.58)	3.35 (0.15–14.25)
Duration (day)	6.4 (0.5–60.0)	4.8 (0.5–30.0)	8.3 (0.5–60.0)	5.2 (0.5–20.0)	11.3 (0.5–60.0)
WBC (*10^9/L)	8.17 (3.70–20.32)●	7.85 (3.70–14.72)●	8.56 (4.00–20.32)●	6.80 (3.09–19.10)	7.07 (2.60–12.13)
Platelets (*10^9/L)	15 (2–9)●○	14 (3–29)●○	15 (2–29)●○	8 (2–20)	39 (7–95)●
Post-WBC (*10^9/L)	7.30 (2.66–19.29)●○	7.22 (3.20–19.29)●○	7.38 (2.66–14.07)●○	6.20 (1.89–18.93)	9.47 (3.40–23.07)●
Post-Platelets(*10^9/L)	119 (37–297)●	158 (100–297)#●○	73 (37–99)●○	17 (4–29)	129 (31–347)●
Gender (Male/Female)	75/40	36/36#	39/14	10/11	17/3●

Values are the median (range).
CR, complete response; PR, partial response; NR, no response.
WBC, white blood cells.
# P < 0.05 versus IVIG PR.
● P < 0.05 versus IVIG NR.
○ P < 0.05 versus without IVIG treatments.

TGF-β1 but reduced the levels of IFN-γ and IL-17A [31].

In this study, we focused on IFN-γ and IL-2 cytokines (secreted by Th1 cells), IL-4, IL-6 and IL-10 cytokines (secreted by Th2 cells), and TNF-α (produced by both T cells and macrophages) [32]. Compared to the healthy controls, ITP patients had higher IL-2, IL-6, IL-10, and IFN-γ levels, which were consistent with results from other researchers [33–35]. As for IL-4, TNF-α, and IFN-γ/IL-4 ratio, there were no significant differences found between healthy controls and ITP patients in our study, yet lower [33] or higher IL-4 level [34], decreased [36] or elevated level of TNF-α [34], lower [42] or higher IFN-γ/IL-4 ratio at diagnosis [37–41] were reported by different studies. Compared the newly diagnosed ITP patients to the chronic ITP patients in our cohort, except IL-10, all the other five cytokines did not show any difference. However, Tag et al. demonstrated that IL-2 was significantly increased in chronic ITP when comparing to acute ITP [43]. Based on the results from our cohort, we speculated that the reason for different statistical conclusions from different researches originated from their different sample sizes, especially those with small-sample size studies.

In the next step, we tested the cytokine levels in the newly diagnosed ITP patients to explore their effects on IVIG response. The conventional dosage of IVIG is 1 g/kg for two days or 2 g/kg for a single day, with a total dose of 2 g/kg body weight. Kovaleva et al. reported that the IVIG dosage in their trials was 1 g/kg body weight per day over two consecutive days or administered at 400 mg/kg body weight per day over a period of five consecutive days [44]. Zhou et al. reported that they applied 200 mg/kg, 300 mg/kg, and 400 mg/kg for five consecutive days in their Chinese ITP patients [8]. In our clinical center, we administered IVIG at 1 g/kg body weight per day varied from one to three days depending on the patient's response. If the ITP patient reached complete or partial response after one or two dosages, we then did watchful waiting. However, if the patient showed no response, we would add a third dosage of IVIG before we applied other treatments. Compared to IVIG non-responder group, the IVIG responder group (CR + PR) demonstrated a decreased IL-4 level, and a cutoff value of IL-4 < 3.5 pg/ml would give a sensitivity of 57.1% and a specificity of 79.1%, respectively. Some published data supported a role for IL-4 in the mechanism of IVIG action [45,46], while others declared that IL-4 was not involved in the mechanism of IVIG action to ameliorate ITP [43,47]. Based on our results, we speculated a possible role of IL-4 in the ITP amelioration. Furthermore, we checked onset age, WBC, and platelet counts as predictive factors of the response to IVIG, and our results were consistent with Morimoto et al. [13] and Higashide et al. [14], showing that lower WBC and lower platelet count were unfavorable factors for IVIG response. Functions of IFN-γ [48,49] and TNF-α [50,51] to IVIG therapy were previously discussed, however, we could not confirm that IFN-γ and TNF-α affect IVIG response in ITP patients.

IVIG is an effective therapy in pediatric ITP, yet its mechanism is not well understood. In the past, there were three possible mechanisms for the action of IVIG suggested: Fcγ receptor IIB (FcγRIIB), sialylation of IgG within IVIG, and neonatal Fc receptor (FcRn) theories. Samuelsson et al. showed that IVIG increased the expressions of the inhibitory FcγRIIB on splenic macrophages and reduced their phagocytic capabilities [52], while Bruhns et al. added that a two-step model for IVIG protection led to the induction of FcγRIIB on colony stimulating factor-1 (CSF-1)-independent "effector" [53], and Schwab et al. indicated that IVIG activity was strictly dependent on the presence of terminal sialic acid residues and the FcγRIIB [54]. Kaneko et al. suggested that sialylation of some IgG molecules within IVIG was critical for the product's ability to raise platelet counts in ITP [55]. Hansen et al. suggested that action of IVIG was mediated through the neonatal FcRn. However, these three mentioned mechanisms were all questioned by other studies, as Leontyev et al. questioned FcγRIIB [56] and IgG sialylation theories [57], while Crow et al. questioned FcRn theory [58].

Concerning the mechanism of cytokines related to the IVIG responses in ITP, one hypothesis was this: triggers of ITP (like infection) released reactive oxygen species (ROS) and caused a decrease in reduced to oxidized glutathione ratio (GSH/GSSG), which activate the peroxisome proliferator-activated receptor-γ (PPARγ) and resulted in cytokine secretion, implicating an oxidative stress pathway in the pathogenesis of chronic pediatric ITP [2]. Furthermore, Cetindere et al. showed that indoleamine 2,3-dioxygenase (IDO) acted through consumption of ROS [59], and Loubaki et al. proposed a TNF-α/TGF-β/IDO axis hypothesis of IVIG's action [51]. Platelet apoptosis [60] and platelet homeostasis [61] may also played roles in ITP.

Our study has some limitations. First, most of the persistent and chronic ITP patients were not diagnosed in our hospital, and we could not do follow-ups of their cytokine patterns. Second, this study was based on a single-centered retrospective study with relatively small number of patients. Third, we did not check the cytokines at mRNA level and whether they have similar response as proteins in level were not known.

In summary, ITP patients had higher IL-2, IL-6, IL-10, and IFN-γ levels than healthy children, while lower IL-4 level (< 3.5 pg/ml), higher WBC (> 6.37 * 10^9/L), and higher platelet count (> 12 * 10^9/L) at diagnosis were favorable predictive factors for IVIG response in the newly diagnosed ITP. Furthermore, ITP patients with lower IL-10 level (< 3.7 pg/ml) and older onset age (> 2.84 years) were more resistant to therapy and developed to chronic ITP more easily. These findings may help guide the treatment decisions making for ITP patients.

Author contributions

YYC and YMT designed the study, YYC, YQZ, NZ, and YZ did the acquisition and analysis of data, YYC and YQZ drafted the manuscript, YMT and WQX revised the manuscript. All authors approved the final

version to be published.

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Declaration of Competing Interest

All authors declare that they have no competing interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.05.014.

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