Thrombosis Research 180 (2019) 1–9

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Excessive expressions of T cell activation markers in pediatric immune thrombocytopenia☆

Yuanyuan Chen, Yanqiong Zhou, Ping Chen, Ping Zhang, Ming Jia, Yongmin Tang*

Division of Hematology-oncology, Children's Hospital of Zhejiang University School of Medicine, #57 Zhuganxiang Road, Hangzhou 310003, PR China

ARTICLE INFO

Keywords:
Immune thrombocytopenia
Children
CD25
HLA-DR
CD69

ABSTRACT

Background: Immune thrombocytopenia (ITP) is an immune-mediated bleeding disorder in children. Activated T cells have been shown to play important roles in ITP. The aims of this study were to evaluate whether these T cell activation markers could be used as indicators to differentiate ITP patients from controls, and to assess whether they could be used as predictors of IVIG response in ITP patients.

Methods: A cohort of 92 hospitalized ITP patients, 49 unrelated healthy children, and 48 thrombocytosis patients were enrolled in this retrospective study between February 2013 and September 2018. Expression of CD25, HLA-DR, and CD69 on the surfaces of CD4+ and CD8+ T cells were detected by flow cytometry. All statistical analyses were performed using SPSS 20.0 software.

Results: Compared to the healthy controls, ITP patients had higher percentages of CD4 + CD25+ T cells, CD4 + HLA-DR+ T cells, CD8 + HLA-DR+ T cells, and CD8 + CD69+ T cells. Compared to the thrombocytosis patients, ITP patients had higher percentages of CD4 + HLA-DR+ T cells and CD8 + HLA-DR+ T cells, and lower CD4 + CD69+ T cells and CD8 + CD69+ T cells. Platelet count at admission had a negative correlation with CD4 + CD25+ T cells in ITP. CD4 + CD69+ T cells were decreased in chronic compared to the newly diagnosed and persistent ITP patients. Activated T cell markers had no predictive value for IVIG response in ITP patients.

Conclusions: T cell activation markers were excessively expressed in pediatric ITP, and those markers had no predictive value for IVIG response in ITP patients.

1. Introduction

Immune thrombocytopenia (ITP) is an acquired immune-mediated bleeding disorders in children (defined as a peripheral blood platelet count $< 100 \times 10^9$/L), classified into three types: newly diagnosed (< 3 months), persistent (3 to 12 months), and chronic (> 12 months) ITP [1]. ITP could occur in isolation (primary) or in association with other disorders (secondary). Secondary causes include autoimmune diseases, infections, bone marrow transplantation side effect, vaccination side effect, and certain drugs [2]. The incidence of ITP may differ in males and females. Options of first line therapy for newly diagnosed ITP included intravenous immunoglobulin (IVIG), anti-D immunoglobulin, and Corticosteroids (prednisone, dexamethasone, and methylprednisolone) [3]. Current guidelines recommend that ITP children with no bleeding or mild bleeding (defined as skin manifestations only, such as bruising and petechiae) be managed with observation alone regardless of platelet count [2].

The mechanisms of thrombocytopenia in ITP are complicated. T cells have been shown to play important roles in the pathogenesis of ITP. Previous studies showed that the percentages of T lymphocytes (CD3+) and CD4+ T cells were increased in ITP patients compared to controls [4], and CD4-CD8+ mucosal-associated invariant T cells in peripheral blood were significantly decreased in patients with ITP [5], while CD4+ T cells were significantly reduced in chronic ITP patients [6,7]. Researchers found that CD8+ T cells participated in thrombocytopenia by increasing platelet apoptosis [8,9], and platelet glycoprotein-reactive CD4+ T cells were essential for the stimulation and maintenance of antiplatelet autoantibody production in chronic ITP [10,11].

Furthermore, activated T cell populations were related to the pathogenesis of ITP. The counts of CD4 + CD25+ T cells in pediatric and adult ITP patients were measured by several studies. CD4 + CD25+ T cells decreased in acute pediatric ITP patients when compared with control children [612], and there was a positive correlation between

☆ We have revised our manuscript according to the reviewers' comments.

* Corresponding author.
E-mail address: y_m_tang@zju.edu.cn (Y. Tang).

https://doi.org/10.1016/j.thromres.2019.05.010
Received 2 January 2019; Received in revised form 10 May 2019; Accepted 14 May 2019
Available online 15 May 2019

CD4 + CD25+ percentage and platelet count in the acute group [6]. Zahran et al. reported that the percentages of CD4 + CD25+ cells were similar in acute pediatric ITP patients and in healthy children, while CD4 + CD25 + ^{High} cells were significantly decreased in ITP children [12]. Likewise, the number of CD4 + CD25+ T cells was significantly lower in adult ITP patients in their severe phase, and increased at the complete remission phase [13,14]. However, associations of other activated T cell populations with the pathogenesis of ITP were partially lacking. Besides CD25, there are several other T cell activation markers, such as CD69 and human leukocyte antigen-DR (HLA-DR). CD69 was considered to be the early activation marker, while HLA-DR was considered to be late activation markers of T cells [15].

The management of ITP patients vary widely. In our clinical center, IVIG and prednisone are used as the main therapies in ITP patients. Intravenous immunoglobulin (IVIG) was first used for ITP in the early 1980s [16]. Until now, IVIG is still widely used as one of the initial therapies in ITP, as IVIG induces a rapid increase of platelets [17]. Researchers found that some aspects were related to IVIG response in ITP, such as lower white blood cell count (WBC) [18], older age at diagnosis [19], lower platelet count [20], or higher interferon-γ(IFN-γ) levels [21]. However, there was no consensus on the predictive factors for response to IVIG, and whether T cell activation markers could be used as indicators for IVIG response in ITP patients were not known.

In this article, expressions of CD69, CD25, and HLA-DR on CD4+ and CD8+ T cells, information of age at admission, gender, days from diagnosis, infections (human immunodeficiency virus, hepatitis C virus, cytomegalovirus, and blood culture), bleeding symptoms, transfusion, treatments, current WBC, current hemoglobin, and current platelet count at admission were collected in our ITP patients and controls. The aims of this study were to evaluate whether these T cell activation markers could be used as indicators to differentiate ITP patients from controls, and to assess whether they could be used as predictors of IVIG response in ITP patients.

2. Methods

2.1. Participants

The diagnoses of all our ITP children and their response to IVIG were evaluated according to the International Consensus Report [3] and American Society of Hematology 2011 [2]. The IVIG administered in our department were 2.5 g per bottle (Jiangxi, Boya See-hot Pharmaceutical Co., Ltd.). 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 [22]. 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 [23]. 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. Without any other treatment, complete response (CR) was defined as platelet count $\geq 100.0 \times {}^{9}$/L, partial response (PR) as platelet count $\geq 30.0 \times 10^9$/L, and no response (NR) as platelet count $< 30.0 \times 10^9$/L. 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.

A cohort of 92 hospitalized ITP patients were enrolled in this retrospective study, which included 54 males and 38 females with a male-to-female ratio of 1.4:1, and a mean age of 4.79 years with a range of 0.51 year through 14 years. Among the 92 ITP patients, there were 59 newly diagnosed, 16 persistent, and 17 chronic ITP cases. The 59 newly diagnosed ITP cases included 13 CR, 26 PR, 7 NR cases, and 13 ITP patients without IVIG treatments (whose parents refused to use IVIG but preferred careful watching or prednisone). 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 49 unrelated healthy individuals and 48 thrombocytosis patients (platelet count $> 450 \times 10^9$/L) [24] were recruited as controls, and the age of all groups was comparable. All the patients and controls were from our hospital between February 2013 and September 2018. The study protocol was reviewed and approved by the Ethics Committee at Children's Hospital Zhejiang University School of Medicine and written informed consents were obtained from all participants' parents or guardians in this study.

2.2. Measurement of activated T cell subsets

Heparinized peripheral blood of all patients (before treatments) and healthy children were acquired in our hospital. Measurements were performed routinely in each patient and thus data was available. However, patients were retrospectively selected if the parents or guardians of these patients agreed to be included in this cohort. The main reagents used in this study were all from BD Biosciences, and showed as follows: CD3-FITC (Number 561807), CD8-PerCP-Cy™5.5 (Number 565310), CD4-APC (Number 555349), CD25-PE (Number 555432), CD69-PE (Number 555531), HLA-DR-PE (Number 555812), FITC Mouse IgG1 κ Isotype Control (Number 555748), PerCP-Cy™5.5 Mouse IgG1 κ Isotype Control (Number 550795), APC Mouse IgG1 κ Isotype Control(Number 555751), PE Mouse IgG1 κ Isotype Control (Number 555749), PE Mouse IgG2a κ Isotype Control (Number 555574), and Lysing Buffer (Number 555899). In brief, 100 μL peripheral blood was mixed and incubated for 30 min in dark at room temperature with one test of antibodies or isotype controls. After 8 min incubation at room temperature in dark with 1 ml Lysing Buffer per sample to lyse red blood cells and rinsing, the samples were fixed with 1% paraformaldehyde and analyzed with flow cytometry. Gating strategies for the identification of T cell activation markers were as following: we first gated CD3 positive cells in the cytogram (R1 gate), then gated CD4 positive lymphocytes (R2 gate) and CD8 positive lymphocytes (R3 gate). Later, CD25, HLA-DR, and CD69 positive populations were gated and calculated. The data were collected on a BD FACS Canto II (BD Biosciences, San Jose, CA, USA) and analyzed with Cell Quest software (BD Biosciences, San Jose, CA, USA).

2.3. Statistical analysis

Expressions of T cell activation markers for 3 (or more) groups were performed with Kruskal-Wallis tests, and comparisons between two groups were performed with the Mann-Whitney *U* test. Pearson correlation analysis were performed between T activation markers and platelets, as well as T activation markers and age at admission. 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. Using activated T cell markers to identify ITP patients from healthy controls and thrombocytosis patients

Results of Kruskal-Wallis tests about T cell activation markers for ITP, healthy controls, and thrombocytosis groups were shown as follows: CD4 + CD25+ T cells ($P = 0.001$), CD4 + HLA-DR+ T cells ($P < 0.001$), CD4 + CD69+ T cells ($P < 0.001$), CD8 + CD25+ T cells ($P = 0.192$), CD8 + HLA-DR+ T cells ($P = 0.001$), and CD8 + CD69+ T cells ($P = 0.001$). Compared to the healthy controls, ITP patients had higher percentages of CD4 + CD25+ T cells, CD4 + HLA-DR+ T cells, CD8 + HLA-DR+ T cells, and CD8 + CD69+ T cells. When comparing to the thrombocytosis patients, ITP patients had higher percentages of CD4 + HLA-DR+ T cells and CD8 + HLA-DR

+ T cells, and lower CD4 + CD69+ T cells and CD8 + CD69+ T cells (Fig. 1). Dot plots of T cell activation populations were demonstrated in Supplementary Fig. 1. Expressions of T cell markers between males and females using the Mann-Whitney *U* test showed no statistical difference (all *P* values > 0.05).

We subsequently performed Pearson correlation analysis between T activation markers and platelet count, as well as T activation markers and age at admission. Correlation with platelet only among ITP patients (n = 92) indicated that platelet count had a negative correlation with CD4 + CD25+ T cells (Pearson correlation coefficient was −0.239, P = 0.022, Fig. 2). If we put all the data of 189 cases together (92 ITP patients, 49 healthy controls, and 48 thrombocytosis patients), the results showed that platelet count had negative correlations with both CD4 + HLA-DR+ T cells and CD8 + HLA-DR+ T cells (Pearson correlation coefficient were both −0.19, P = 0.009, Supplementary Fig. 2). However, there were no correlation with age at admission among ITP patients (Fig. 3) and in whole cohort (Supplementary Fig. 3).

3.2. Decreased CD4 + CD69+ T cells in chronic ITP patients compared to the newly diagnosed and persistent ITP patients

Results of Kruskal-Wallis tests about T cell activation markers for the newly diagnosed, persistent, and chronic groups were shown as

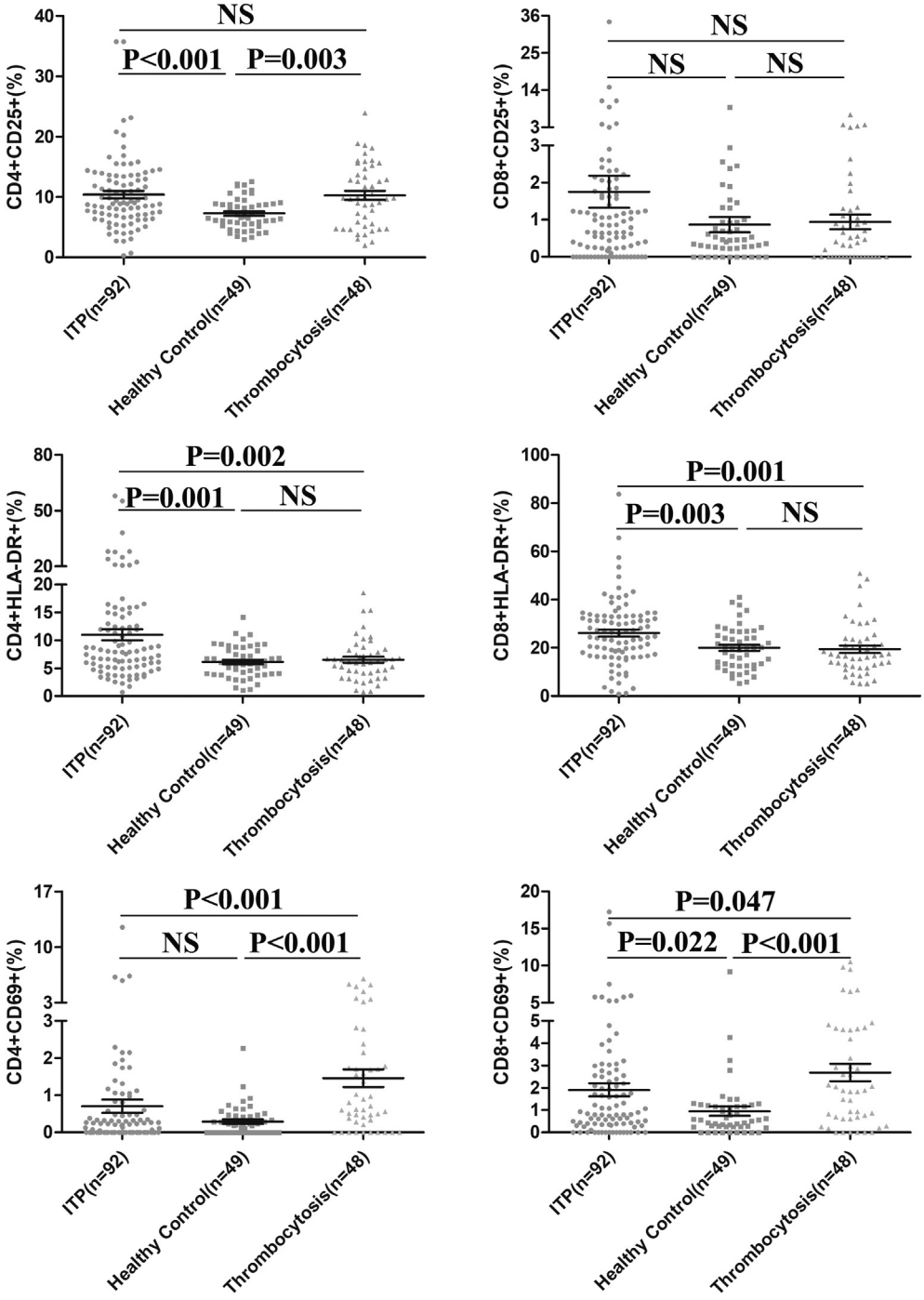

Fig. 1. Percentages of activated T cell subset markers in ITP patients, healthy controls, and thrombocytosis patients. The center horizontal line and whiskers were Mean ± the standard error of the mean (SEM). Comparisons between two groups were performed with the Mann-Whitney *U* test.

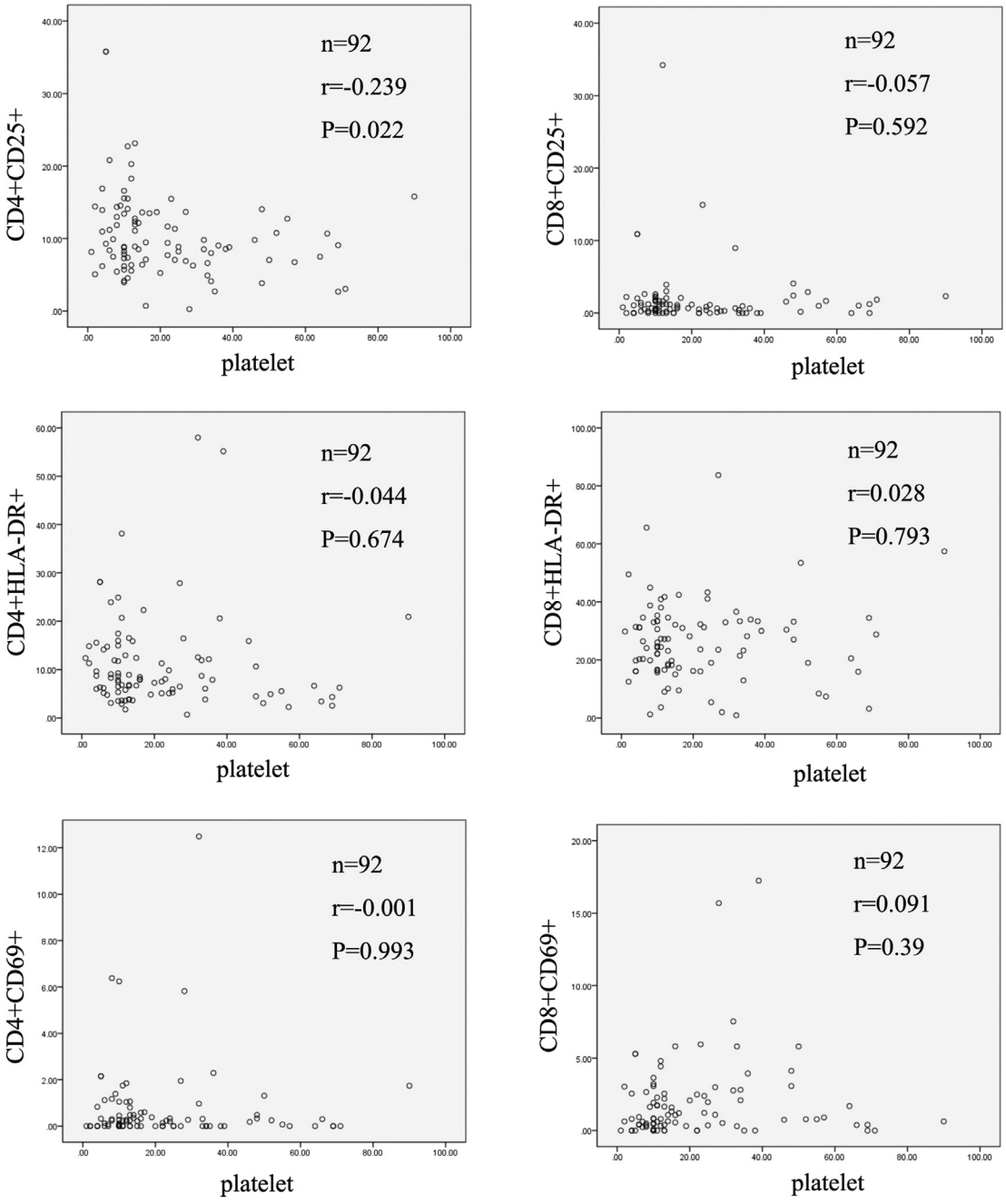

Fig. 2. Pearson correlation analysis of activated T cell markers (%) and the initial platelet counts (10^9/L) in 92 cases of ITP patients.

follows: CD4 + CD25+ T cells ($P = 0.502$), CD4 + HLA-DR+ T cells ($P = 0.196$), CD4 + CD69+ T cells ($P = 0.017$), CD8 + CD25+ T cells ($P = 0.624$), CD8 + HLA-DR+ T cells ($P = 0.576$), and CD8 + CD69+ T cells ($P = 0.448$). When comparing to both the newly diagnosed and persistent ITP groups, the chronic group had lower percentages of CD4 + CD69+ T cells (Fig. 4), as 14 cases of the chronic group had no expression of CD4 + CD69+ T cells.

3.3. Activated T cell markers had no predictive value for IVIG response in ITP patients

Results of Kruskal-Wallis tests among IVIG CR, PR, NR and the Without IVIG treatments groups were shown as follows: CD4 + CD25+ T cells ($P = 0.668$), CD4 + HLA-DR+ T cells ($P = 0.261$), CD4 + CD69+ T cells ($P = 0.052$), CD8 + CD25+ T cells ($P = 0.811$), CD8 + HLA-DR+ T cells ($P = 0.893$), and CD8 + CD69+ T cells ($P = 0.013$). In the next step, we used the Mann-Whitney *U* test to evaluated the percentages of CD4 + CD25+ T cells, CD4 + HLA-DR+ T cells, CD4 + CD69+ T cells, CD8 + CD25+ T cells, CD8 + HLA-DR + T cells, and CD8 + CD69+ T cells between IVIG CR and NR groups, IVIG CR and PR groups, IVIG PR and NR groups, and IVIG CR + PR and NR groups, respectively, and the results showed that there were no statistical differences (Fig. 5). Compared to the Without IVIG treatments group, both PR and NR groups showed lower expressions of CD8 + CD69+ T cells, which resulted to the significant difference in Kruskal-Wallis test. In short, those results indicated that these activated T cell markers had no predictive value for IVIG response in ITP patients.

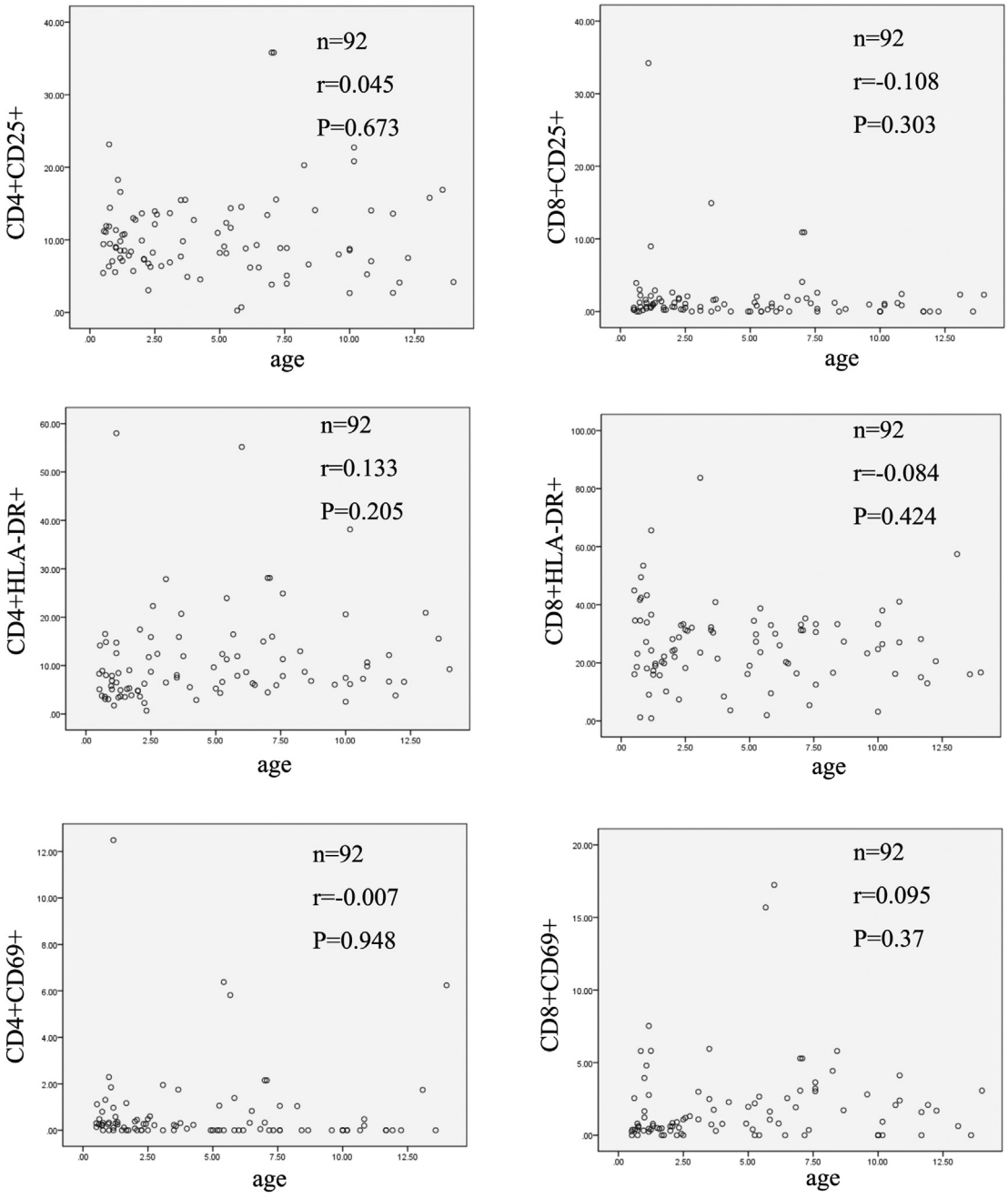

Fig. 3. Pearson correlation analysis of activated T cell markers (%) and age (year) in 92 cases of ITP patients.

3.4. Declined tendencies of CD4 + HLA-DR+ T cells and CD8+ HLA-DR + T cells from newly diagnosed ITP to persistent or chronic ITP

Among the 16 persistent and 17 chronic ITP patients, only one persistent case and one chronic ITP case were first diagnosed in our hospital. The other15 persistent cases and 16 chronic cases were previously diagnosed and treated in their local hospitals, and we could not analyze the activated T cell markers in their newly diagnosed phases. We tested the activated T cell markers of the newly diagnosed phases, persistent phase, or chronic phase in the two cases, and found that both CD4 + HLA-DR+ T cells and CD8 + HLA-DR+ T cells displayed declined tendencies from the newly diagnosed phases to persistent phase or chronic phase (Supplementary Fig. 4).

3.5. Characteristics of ITP patients and controls

Characteristics of ITP patients and controls were shown in Table 1 and Supplementary Table 1. We compared the expressions of T cell markers between males and females using the Mann-Whitney *U* test, and there was no statistical difference (all *P* values > 0.05). Infections (included human immunodeficiency virus, hepatitis C virus, cytomegalovirus, and blood culture) were checked in 36 ITP patients, and only one female in the CR group had Gram-positive bacterial infection, while others all showed negative results. Bleeding symptoms were collected in all ITP patients according to the grading of hemorrhage [25], with 64 cases had skin bleeding, 9 cases had epistaxis, 7 cases had oral bleeding, and 12 cases without any bleeding symptoms. None had life-threatening or fatal bleeding. As for transfusion, only one female in the NR group transfused platelets among those 92 ITP patients.

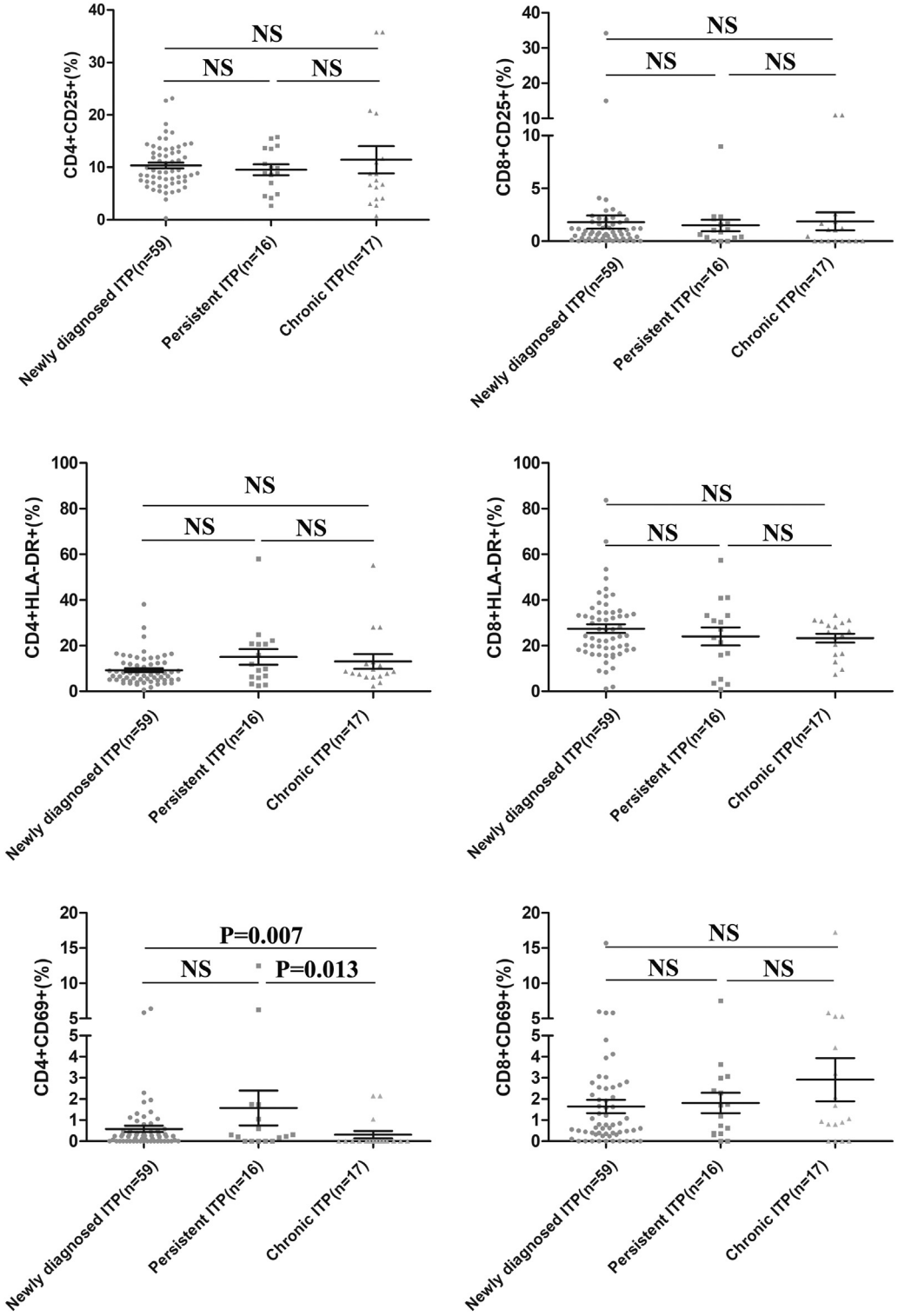

Fig. 4. Percentages of activated T cell subset markers in newly diagnosed, persistent, and chronic ITP patients. The center horizontal line and whiskers were Mean ± SEM. Comparisons between two groups were performed with the Mann-Whitney *U* test.

4. Discussion

Lymphocyte populations and their subsets revealed age-related changes in the human cellular immune system. Lin et al. reported that the percentage of CD4+ T cells remained relatively unchanged from infancy to adolescence, but the percentage of CD8+ T cells was lowest at birth and reached maximal levels in the one to two years period. The percentage of naive T cells declined with time, but the percentage of memory T cells increased with age. These researchers also found that the expressions of the activation markers CD25 and HLA-DR on CD4+ T cells increased with age in a cohort of Chinese children [26]. In our cohort, age had no correlation with the expressions of CD25, HLA-DR, or CD 69.

In the present study, we showed that both CD4 + HLA-DR+ T cells and CD8 + HLA-DR+ T cells were excessively expressed in ITP patients when comparing to healthy children and to thrombocytosis patients,

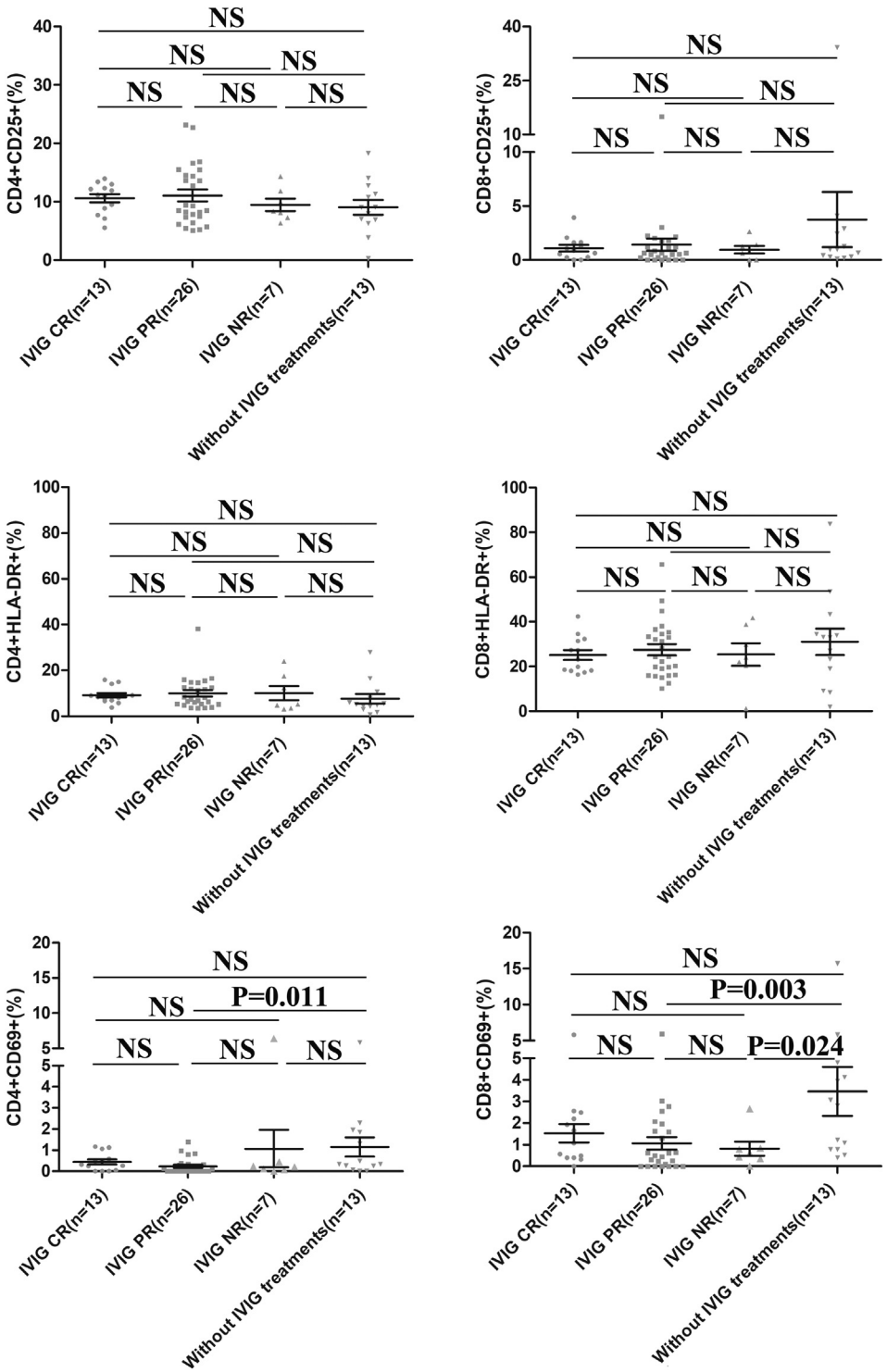

Fig. 5. Percentages of activated T cell subset markers in the newly diagnosed ITP patients who were IVIG responders (CR and PR), IVIG non-responders (NR), and those without IVIG treatments. The center horizontal line and whiskers were Mean ± SEM. Comparisons between two groups were performed with the Mann-Whitney *U* test.

which were consistent with the results from Audia et al., as they showed higher expressions of CD8 + HLA-DR+ T cells in ITP patients than in normal controls [27]. The excessive expression of HLA-DR may due to infections of varied viruses, such as HIV-1 [28,29], hepatitis C virus [30], and Zika virus [31]. However, we did not find any virus infection in our ITP patients. The mechanism of CD4 + HLA-DR+ T cells and CD8 + HLA-DR+ T cells contributing to ITP had not been elucidated.

CD69 was the earliest activation antigen synthesized and expressed during initial T cell activation, and reached a peak level within 18–24 h [32]. CD69 was expressed on infiltrated leukocytes during different human inflammatory conditions [33–36]. However, the expressions of CD69 on T subsets in ITP was not well defined. In our study, the percentages of CD4 + CD69+ T cells and CD8 + CD69+ T cells were analyzed, and we found that ITP patients had higher CD69 expressions than healthy children. What's more, both the newly diagnosed and persistent ITP patients had higher percentages of CD4 + CD69+ T cells

Table 1
Characteristics of ITP patients and controls.

Characteristics	CR (n = 13)	PR (n = 26)	NR (n = 7)	Without IVIG (n = 13)	Persistent ITP (n = 16)	Chronic ITP (n = 17)	Healthy controls (n = 49)	Thrombocytosis (n = 48)
Age(year)	1.25(0.70–3.00)	2.25(1.17–6.67)	2.00(0.74–5.25)	3.08(1.04–6.33)	5.79(3.21–10.00)	7.08(5.63–10.09)	3.75(2.29–5.09)	4.46(2.92–9.05)
CD3 +	42.85(23.35–68.90)	71.10(48.45–73.95)	53.74(22.23–79.50)	74.80(55.59–87.85)	63.56(58.97–77.74)	62.98(50.69–81.45)	69.21(67.25–75.43)	64.64(59.37–71.95)
CD4 +	33.55(27.35–64.30)	28.90(11.49–45.15)	24.18(4.85–35.15)	27.21(18.65–35.75)	27.52(19.39–35.33)	25.83(20.99–34.95)	41.50(37.88–44.60)	33.79(25.93–39.07)
CD8 +	23.35(12.95–41.40)	29.81(23.20–36.24)	23.91(15.56–40.55)	50.35(20.58–52.17)	29.99(18.98–48.83)	31.42(21.76–36.26)	23.90(19.30–28.84)	23.23(19.00–27.28)
CD4 + CD25 +	11.20(8.31–12.67)	8.91(7.13–14.78)	8.38(7.29–11.86)	9.04(6.59–12.05)	9.34(5.45–13.64)	7.51(4.04–15.98)	6.63(5.38–8.92)	9.81(5.22–14.44)
CD4 + HLA-DR +	8.46(6.73–11.60)	7.65(5.22–13.10)	5.17(3.57–17.45)	5.09(3.70–9.27)	10.90(6.08–20.86)	8.65(6.45–12.56)	5.93(3.92–8.44)	6.26(4.10–8.10)
CD4 + CD69 +	0.32(0.03–0.82)	0.06(0.00–0.33)	0.21(0.08–0.45)	0.33(0.16–1.90)	0.27(0.00–1.57)	0.00(0.00–0.00)	0.19(0.00–0.46)	0.76(0.30–2.06)
CD8 + CD25 +	0.86(0.24–1.61)	0.66(0.17–1.79)	0.79(0.00–1.40)	0.96(0.29–2.66)	0.94(0.34–1.99)	0.44(0.00–1.76)	0.46(0.24–0.97)	0.50(0.00–1.21)
CD8 + HLA-DR +	23.14(18.15–31.82)	24.35(16.16–34.75)	24.08(20.33–38.75)	32.94(14.00–38.87)	25.42(8.03–33.33)	26.04(16.36–30.30)	19.64(12.62–26.83)	16.82(12.48–23.82)
CD8 + CD69 +	1.08(0.39–2.34)	0.48(0.00–1.71)	0.61(0.36–0.84)	2.81(0.78–4.46)	1.46(0.37–2.84)	1.07(0.40–4.86)	0.57(0.27–1.19)	1.88(0.63–4.61)

Abbreviations and notes: Data were shown in median (interquartile range, IQR).
CR: complete response of newly diagnosed immune thrombocytopenia (ITP).
PR: partial response of newly diagnosed ITP.
NR: no response of newly diagnosed ITP.
Without IVIG: without IVIG treatments of newly diagnosed ITP.

than the chronic ITP patients. The expressions of CD69 were not correlated with IVIG responses.

CD4 + CD25+ T cells were decreased in acute pediatric ITP patients, and CD4 + CD25 + ^{high} cells played suppression roles in ITP patients [6,12–14]. In our study, the percentages of CD4 + CD25+ T cells in ITP patients were higher than healthy children. Correlation with platelet count among ITP patients indicated that platelet count had a negative correlation with CD4 + CD25+ T cells. CD8 + CD25+ T cells were not analyzed by other studies of ITP, and our demonstrated that the expressions of CD8 + CD25+ T cells had no statistical difference among all ITP groups.

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 a follow-up of their activated T cell subset markers. Second, this study was based on a single-centered retrospective study with relatively small number of patients. Thus, a multi-centered prospective study with large number of cases is warranted to validate the results. Third, this study is of a mostly descriptive nature, and we could not unveil the underlying mechanism by which HLA-DR, CD69, and CD25 contributed to ITP. Fourth, data shown on IVIG response were not adjusted for age in this cohort as we found age had no correlation with the expressions of CD25, HLA-DR, or CD 69. Fifth, we did not gate CD4 + CD25 + ^{high} T cells in this study, and only focused on CD4 + CD25+ T cells.

In summary, when comparing to the healthy controls, ITP patients had higher percentages of CD4 + CD25+ T cells, CD4 + HLA-DR+ T cells, CD8 + HLA-DR+ T cells, and CD8 + CD69+ T cells. Compared to the thrombocytosis patients, ITP patients had higher percentages of CD4 + HLA-DR+ T cells and CD8 + HLA-DR+ T cells, and lower CD4 + CD69+ T cells and CD8 + CD69+ T cells. Correlation with platelet only among ITP patients indicated that platelet count at admission had a negative correlation with CD4 + CD25+ T cells. CD4 + CD69+ T cells were decreased in chronic ITP patients compared to the newly diagnosed and persistent ITP patients. Activated T cell markers had no predictive value for IVIG response in ITP patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.05.010.

Author contributions

YYC and YMT designed the study, YYC, YQZ, PC, and PZ did the acquisition and analysis of data, YYC, YQZ, and MJ drafted the manuscript, YMT and PC revised the manuscript. All authors approved the final version to be published.

Funding

This project was supported by grants from China Postdoctoral Science Foundation (No. 2018M632488), Advanced Programs of Zhejiang Provincial Postdoctoral Science Foundation (No. X81701), and Zhejiang Medical Health Technology Project (No. 2018KY447). The funders did not take part in the study.

Declaration of Competing Interest

All authors declare that they have no competing interest.

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