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Higher CD56+ or CD2+ lymphocyte percentage predicts poor steroid response in patients with immune thrombocytopenia

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ABSTRACT

Introduction: Immune thrombocytopenia (ITP) is known as an immune-mediated disease and often evolves to chronic type in adult. Corticosteroids only work in around 60% of patients. This study evaluated the roles of subgroup lymphocytes from peripheral blood in ITP adults with different treatment response.

Methods: Between October 2009 and March 2017, 37 adults were newly diagnosed as ITP requiring treatment. The patients were separated into two groups: 23 patients with platelet count < 50,000/μL with corticosteroid dependence or second-line treatment (Poor-responder Group), and 14 patients with platelet counts < 50,000/μL with standard steroid treatment, which stopped within three months (Good-responder Group). Subgroup lymphocyte percentages of peripheral blood were determined through flow cytometry before treatment. Data analysis with Mann–Whitney test and receiver operating characteristic curves were performed using GraphPad Prism (Version 7). A p-value of < 0.05 was considered significant.

Results: Lymphocyte percentage was significantly lower in Poor-responder Group than in Good-responder Group ($p = 0.008$). In subgroup lymphocytes, higher percentages of CD19+ B lymphocytes were found in Good-responder Group ($p = 0.03$). In Poor-responder Group, a higher CD2+ and CD56+ lymphocytes were observed ($p = 0.02$ and 0.03). By the cut-off value of percentage of CD56+ lymphocytes with 24.5% or CD2+ lymphocytes with 85.7%, the specificity showed 92.86%.

Conclusions: This study found that newly diagnosed ITP patients with increased percentages of CD56+ or CD2+ lymphocytes in peripheral blood associated with a poorer response to steroid treatment.

1. Introduction

Immune thrombocytopenia (ITP) is a disorder characterized by low platelet count and mucocutaneous bleeding affecting both children and adults [1,2]. ITP is an acquired immune disorder that results from antiplatelet antibodies [3], impaired megakaryocytopoiesis [4], and T-cell-mediated destruction of platelets [5]. The annual incidence of new adult patients is 50–100 patients per million populations per year [6]. Clinical manifestations vary from asymptomatic to intracranial hemorrhage. Only 60%–70% of patients with ITP have platelet-specific immunoglobulin G antibodies [7], and the diagnosis of ITP still employs exclusion criteria [1]. Some patients who do not have antiplatelet antibodies have abnormal T cells, which result in platelet destruction [8]. Cytotoxic T-lymphocytes, which induce platelet lysis by CD8+ T cells, have been demonstrated to be involved in ITP pathogenesis [9]. Natural killer (NK) cell numbers and function also play crucial roles in patients with ITP [10]. There is no standard test to further confirm the pathophysiology and predict treatment response in each patient.

Corticosteroids are the first-line treatment for ITP, but 30%–40% of patients require additional therapy because of refractoriness or dependence on high-dose corticosteroids [11]. Long term or high dose steroid may be accompanied with various side effects, such as fluid retention, weight gain, mood swings, hypertension, worsen diabetes, cataracts, osteoporosis, increase risks of infection … etc. Unfortunately, we remain unable to predict which patients will respond to steroids at diagnosis. Proper prediction factor might provide a precise choice of

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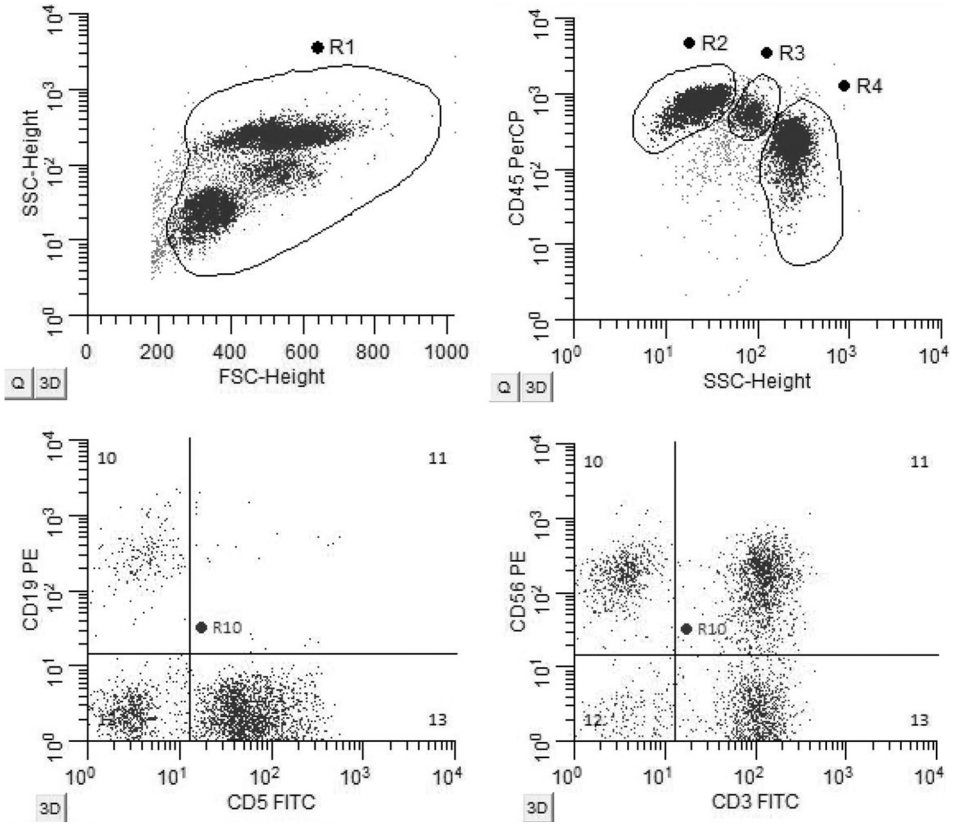

Fig. 1. Manual gating of the lymphocyte population.
Gating was performed in Winlist. R1 shows the total nucleus cells. Lymphocytes (R2), monocytes (R3), and neutrophils (R4) were gated using SSC and CD45 level. Two-color antibodies were used to detect subgroup lymphocytes.

treatment and avoid potential drugs side effects in these patients. This study evaluated the roles of subgroup lymphocyte from peripheral blood in ITP adults with different response to corticosteroids.

2. Materials and methods

2.1. Patients

We conducted a retrospective study for newly diagnosed ITP patients. Patients newly diagnosed as ITP who required steroid treatment and with available flow cytometry analysis results were eligible for this study. Between October 2009 and March 2017, 37 adults were diagnosed with primary ITP and need steroid treatment at Buddhist Tzu Chi General Hospital in Hualien, Taiwan. The diagnosis of ITP was by "The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia" [12]. Primary ITP was defined by the International Working Group (IWG) as a platelet count of < 100,000/μL in the absence of other causes or disorders that may be associated with thrombocytopenia. In our study, because we only enrolled the patients who need steroid treatment, all patients had a platelet count of < 50,000/μL. Flow cytometry analysis from peripheral blood was provided before any treatment, included medications or transfusion, and all the patients received only prednisolone 0.5 to 2 mg/kg per day for ITP initial treatment.

We reviewed medical records, including age, sex, and initial complete blood count (white blood count with the percentage of the differential count, hemoglobin, and platelet count obtained using Sysmex XE-5000 hematology analyzers). Flow cytometry analysis of peripheral blood was used to exclude lymphoproliferative neoplasm for the patients with ITP in our hospital.

2.2. Definition of treatment response

An IWG consensus panel of adult experts in ITP recently guided terminology, definitions, and outcome criteria for this disorder [1]. We separated our patients into two groups according to treatment outcome.

Poor-responder Group (steroid dependence/refractory) comprised 23 patients who received standard steroid treatment with prednisolone and required ongoing or repeated administration of corticosteroid or second-line treatments, including azathioprine, danazol, splenectomy, thrombopoietin-receptor agonists, and cyclosporine, to maintain a platelet count over 30,000/μL or to avoid bleeding.

Good-responder Group (steroid responsive) comprised 14 patients with platelet counts < 50,000/μL at diagnosis who received standard steroid treatment with prednisolone 0.5–2 mg/kg. The steroid treatment was stopped within three months due to the improvement of platelet counts.

2.3. Flow cytometry

All patients underwent a flow cytometry analysis of peripheral blood before treatment. Specimens were analyzed on a FACSCalibur (Becton Dickinson BioSciences, San Jose, CA, USA) flow cytometer. Monoclonal antibodies with a standardized panel of lymphoid lineages included peridinin–chlorophyll-a-conjugated CD45; fluorescein–isothiocyanate-conjugated CD2, CD3, CD5, CD7, CD8; and phycoerythrin-conjugated CD4, CD5, CD19, CD23, CD56 from Becton Dickinson. In a standard three-color immunofluorescence protocol, forward (FSC) and right-angle light scatter (SSC) were recorded along with three-color antibody combinations to generate five characteristics per cellular event. Cell population percentages were assessed through CD45 staining and SSC based on nucleated, nonerythroid cells after

NH4CL lysis. Cell differential was obtained either through gating directly on discrete populations identified by CD45 and SSC or backgating using two-color antibody combinations to identify cells of interest. Data analysis was performed using Winlist software (Verity House, Topsham, ME) [13].

Gating was performed in Winlist on the lymphocytes group (R2 area in Fig. 1). Two-color antibodies and light scattering properties were used to detect CD19/CD5, CD5/CD7, CD23/CD2, CD4/CD8, and CD56/CD3. Data of total nucleus cells were acquired from 10,000 events (R1 area). Each lymphocyte subset was calculated as its percentage multiplied by the total number of lymphocytes in peripheral blood.

2.4. Ethics approval

This retrospective study was approved by the institutional review board of Buddhist Tzu Chi General Hospital (IRB106-167-B) and conducted according to the Declaration of Helsinki. Informed written consent was waived because the study was a retrospective data analysis of medical records.

2.5. Statistical analysis

Data analysis was performed using GraphPad Prism (Version 7). Data were summarized using descriptive statistics (samples size [n] and median). Nonparametric analyses with the Mann–Whitney test were used for analysis. A p-value of < 0.05 was considered significant. The receiver operating characteristic (ROC) curves by GraphPad Prism were also analyzed to evaluate the steroid response of ITP patients by subgroup lymphocyte percentage. Two groups with ROC curve analysis were used to calculate the Youden index J (J = sensitivity + specificity − 1).

3. Results

3.1. Patient characteristics

The demographic, laboratory characteristics and underlying diseases of patients are listed in Table 1. The median ages were 62 and 51.5 years for Poor-responder and Good-responder Groups, respectively. There were no significant differences in white blood, and platelet counts between the two groups. Lymphocyte percentage in total white blood counts by Sysmex XE-5000 hematology analyzers was significantly lower in Poor-responder Group than in Good-responder Group (19.6% vs. 27%, and, $p = 0.008$). We also analyzed the lymphocyte percentage by flow cytometry, and the results also showed lower in Poor-responder Group (14.1% vs. 33.15%, $p = 0.0006$). The hemoglobin level was significantly lower in Poor-responder Group (13.1 g/dL vs 13.9 g/dL, $p = 0.02$), too. The Good-responder Group had higher hemoglobin and lymphocyte percentage. About the underlying diseases, by hypertension, diabetes, Hepatitis B, Hepatitis C infection, or autoimmune disease, there is no significant difference between the two groups. The habits of smoking and alcohol between the two groups did not also have a difference.

Table 1
Demographic, laboratory characteristics, and underlying diseases of patients.

	Poor-responder group		Good-responder group		p-Value[†]
	Median	Mean ± SD	Median	Mean ± SD	
Numbers	23		14		
Male:female	11:12		6:8		0.776
Age (y/o)	62	58.7 ± 19.9	51.5	48.1 ± 19.0	0.12
WBC (/μL)	7470	8068 ± 2817	5325	6409 ± 3104	0.07
Hb (g/dL)	13.1	13.0 ± 1.7	13.9	14.4 ± 1.2	0.02*
Platelet (*1000/μL)	11	17.3 ± 16.2	11.5	14.2 ± 10.5	0.93
Neutrophil (%)	65	64.7 ± 13.6	59.5	56.8 ± 10.2	0.09
Lymphocyte (%)	19.6	22.1 ± 12.0	27	33.1 ± 9.2	0.008*
Monocyte (%)	6	6.2 ± 3.2	6.4	6.8 ± 2.4	0.33
Underlying disease					
Hypertension	11/23 (47.8%)		6/14 (42.9%)		0.776
Diabetes	8/23 (34.8%)		2/14 (14.3%)		0.183
Hepatitis B	1/23 (4.3%)		2/14 (14.3%)		0.296
Hepatitis C	1/23 (4.3%)		1/14 (7.1%)		0.725
Autoimmune disease	0 (0%)		0 (0%)		–
Smoking	6/23 (26.1%)		4/14 (28.6%)		0.873
Alcohol	8/23 (34.8%)		4/14 (28.6%)		0.705

[†] Mann Whitney test (p-value).
* $p < 0.05$.

3.2. In-subgroup lymphocyte percentage

For the subgroup lymphocyte percentages detected through flow cytometry, a higher percentage of CD19+ B lymphocytes were found in Good-responder Group (18.65% vs. 10.4%, $p = 0.03$). The patients with ITP who were steroid good-responsive had higher CD19+ B lymphocyte percentages.

In Poor-responder Group, a higher percentage of CD2+ lymphocytes was observed (87.1% vs 79.65%, $p = 0.02$). For CD56+ lymphocytes, Poor-responder Group was also higher (34.5% vs 17.3%, $p = 0.03$). In Poor-responder Group (steroid-dependent or refractory group), higher CD2+ and CD56+ lymphocyte percentages were noted. No other significant differences in CD3+, CD5+, CD7+, CD23+, CD4+, and CD8+ were observed among the two groups (Table 2).

3.3. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) under ROC curve analysis

The ROC curve was established based on CD56+ and CD2+ lymphocytes percentage between the patients with steroid-responsive and steroid dependence/refractory. For CD56+ lymphocytes, the area under the curve (AUC) showed 0.7795, and p-value was 0.0048. If we set the cut-off at 24.5%, the sensitivity was 39.13%, and specificity was 92.86%. PPV was 90%, NPV was 48.1%, and the odds ratio was 8.36. For CD2+ lymphocytes, the AUC showed 0.7407, and p-value was 0.0152. The best critical point was selected at 85.7%. The sensitivity was 56.52%, and the specificity was 92.86%. PPV was 92.9%, NPV was 56.5%, and the odds ratio was 16.9. (Table 3, Fig. 2).

4. Discussion

Corticosteroids are the first-line treatment for ITP patients, but 30%–40% of patients require additional therapy because of refractoriness or dependence on high-dose corticosteroids [11]. Understanding the predictive factors of treatment response may be helpful in clinical treatment and lead to a new understanding of the pathophysiology of ITP. There are many hypotheses about the pathophysiology of ITP; most suggest the immune-mediated process.

Corticosteroids are pharmacological derivatives of the glucocorticoid steroid hormones, and they bind cytosolic receptors and modulate a large variety of genes, triggering many physiological changes. High-dose dexamethasone or low-dose prednisone in patients with chronic ITP was shown to modulate T cells by increasing the number of peripheral Tregs, restoring the Th1/Th2 ratio with an increase of IL-10 and TGF-β. It also modulates B cell activation via a decrease of BAFF (BlyS) and modulates DCs [14]. Both T and B lymphocytes will play an important role in steroid treatment for ITP patients. We hypothesize that the different subgroup lymphocyte percentages may have different responses to steroid in patients with ITP.

In our study, the patients who responded to steroid had significantly higher lymphocyte percentages. Fahim and Monir et al. reported a significant increase in white blood count and lymphocytes in acute ITP

Table 2
Statistical analysis of subgroup lymphocyte percentages between studied groups.

Marker	Cell type as well as surface markers	Poor-responder group		Good-responder group		*p*-Value[†]
		Median	Mean ± SD	Median	Mean ± SD	
Numbers		23		14		
CD19+%	B cells	10.4	12.0 ± 8.7	18.65	18.4 ± 9.3	0.03*
CD5+%	T cells	69	66.7 ± 12.2	64.7	65.4 ± 11.2	0.61
CD2+%	T and NK cell	87.1	84.7 ± 9.8	79.65	77.3 ± 9.9	0.02*
CD7+%	Mature T cells	71.7	67.8 ± 12.9	70.5	64.4 ± 12.3	0.54
CD4+%	T helper cells, monocytes, macrophages, and dendritic cells	39.35	42.9 ± 14.4	40.1	40.0 ± 11.6	0.70
CD8+%	Cytotoxic T cells, NK cells, and dendritic cells	29.95	33.1 ± 11.4	27	27.4 ± 8.7	0.21
CD4/CD8 ratio		1.6	1.5 ± 0.8	1.35	1.6 ± 0.8	0.53
CD23+%	Mature B cells, activated macrophages, eosinophils, follicular dendritic cells, and platelets	8.6	10.3 ± 9.4	12.05	13.7 ± 10.1	0.22
CD3+%	T cell	68.85	67.2 ± 12.8	65.6	66.1 ± 13.4	0.72
CD56+%	NK cells, gamma delta (γδ) T cells, activated CD8+ T cells, and dendritic cells	34.5	32.1 ± 9.8	17.3	22.0 ± 13.9	0.03*
CD3+/CD56+%		8.1	13.3 ± 9.4	7.1	8.1 ± 6.3	0.08
CD3−/CD56+%		14.0	18.7 ± 11.3	10.3	14.0 ± 12.1	0.23

[†] Mann Whitney test (*p*-value).
* $p < 0.05$.

Table 3
Analysis of subgroup lymphocyte percentage.

	AUC[a]	95% CI	*p*-Value	Cut-off	Sensitivity%	Specificity%
CD56+	0.7795	0.6223–0.9367	0.0048*	> 24.5	39.13	92.86
CD2+	0.7407	0.5758–0.9055	0.0152*	> 85.7	56.52	92.86

	Likelihood ratio (highest)	PPV%[b]	NPV%[c]	Odds ratio
CD56+	5.478	90	48.1	8.36
CD2+	7.913	92.9	56.5	16.9

[a] AUC, area under curve.
[b] PPV, positive predictive value.
[c] NPV, negative predictive value.
* $p < 0.05$.

caused by a preceding viral infection [15], but they only studied a pediatric group. Our result might imply these steroid-responsive patients resembled with pediatric patients in pathophysiology.

CD19 is expressed in all B lineage cells. CD19+ acts as an adaptor protein to recruit cytoplasmic signaling proteins to the membrane, and it works within the CD19/CD21 complex to reduce the threshold for B-cell receptor signaling pathways [16]. The role of B cells in autoimmune diseases involves different cellular functions, including the well-established secretion of autoantibodies, autoantigen presentation and ensuing reciprocal interactions with T cells, secretion of inflammatory cytokines, and the generation of ectopic germinal centers. In autoimmune diseases, B cells are both antibody-mediated, and T-cell mediated [17]. ITP is also an autoimmune disease. Gözmen S et al. reported that B-cell-activating factor is influential in the pathogenesis of newly diagnosed childhood ITP [18]. In our results, higher percentages of CD19+ B lymphocytes were found in the steroid-responsive group, indicating that B lymphocytes played more crucial roles in this group. Steroid treatment is also vital for autoantibodies.

Studies on the roles of NK cells in ITP remain unclear. It is suggested that NK cells may play an immunoregulatory role in disease pathology [19]. NK cell activity was studied in 17 patients with primary chronic idiopathic immune thrombocytopenic purpura. Patients with ITP were found to have a functional defect in NK cytolytic activity [20]. Patients with ITP were also found with decreased quantities of NK cells, impaired total NK function, and insufficient suppression of autoreactive T and B cells [21]. In our study, we found that abnormal CD56+ lymphocytes may have crucial roles in steroid treatment. Increasing the percentage of CD56+ lymphocytes causes patients with ITP to become refractory to steroid treatment. CD56+ lymphocytes play an important role in thrombocyte cytotoxicity in the pathogenesis of ITP. Garcia-

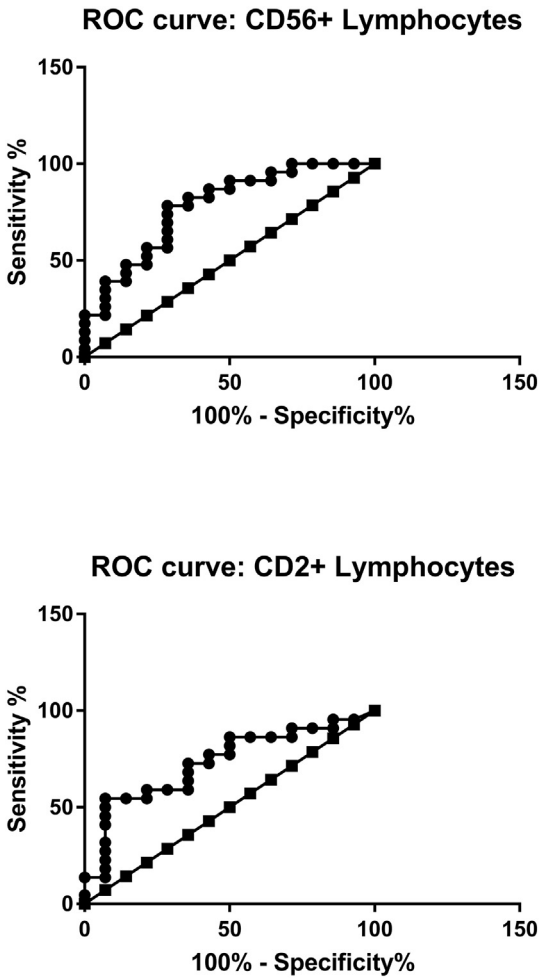

Fig. 2. ROC curve based on CD56+ and CD2+ lymphocytes percentage. The area under the curve (AUC) of CD56+ lymphocytes showed 0.7795, and *p*-value was 0.0048 (upper). The AUC of CD2+ lymphocytes showed 0.7321, and *p*-value was 0.0203.

Suarez et al. observed a significant increase in CD56+ cells in patients who failed to respond to corticosteroid treatment and splenectomy [22]. Different autoimmune diseases are characterized by abnormalities in both the NK and T-lymphocyte compartments, which indicate a pathogenic role for the autoantibodies produced by the B-cell

compartment. In 1996, Singh et al. established that in vivo changes in ratios of lymphocyte–phenotype subsets are altered by glucocorticoid administration in healthy men. CD8+ and CD56+ were higher with steroid than with placebo in pre-exercise and postexercise groups [23]. By the result of Singh et al., it also found that steroid increased CD56+ lymphocytes. From our study results, we hypothesize that the steroid-refractory patients with ITP already have higher percentages of CD56+ lymphocytes; therefore, the steroid does not increase the percentage of CD56+ lymphocytes, and these patients respond more poorly to steroid treatment.

CD2 (cluster of differentiation 2) is a cell adhesion molecule found on the surface of T cells and NK cells [24]. Its major function is interaction with other adhesion molecules, including lymphocyte-function-associated antigen-3 (LFA-3/CD58), which is expressed on the surfaces of other cells [25]. CD2 is also as a costimulatory molecule on T and NK cells [26]. No more data have been reported for the steroid-related effects of CD2+ lymphocytes. A previous report also showed the proliferative response of CD2+ lymphocytes in patients with ITP, but this was not different between acute and chronic groups [22]. In our study, we also found that increasing CD2+ lymphocytes were associated with patients with ITP who were refractory to steroid treatment, but the cause remains unclear. It may be related to stimulation of T and NK cell to generate the same effect as increasing CD56+ lymphocytes.

By the paper of Zhang Y et al. [27], the result showed B cells were increased in the response group (R) but decreased in the non-response group (NR) compared with the normal group ($p = 0.0017$). In the ratio of CD3+ CD4+/CD3+ CD8+, NR group was higher than normal group ($p = 0.031$), but there was no difference between these four groups. The authors did not provide more detail data about the difference between response patients, included a complete response (CR) and response group (R), and NR groups. In the other hand, in our study's lymphocyte panel, we did not have data of CD3+ CD4+ and CD3+ CD8+. Because of the above, we cannot compare our results with the above paper. About the paper of Zhao Z et al. [28], the patients were treated with intravenous immunoglobulin (IVIG) plus corticosteroids, and therapeutic responses were evaluated. In our study, all enrolled patients received the treatment with steroid only. If the patients received IVIG, they were excluded. T cell abnormalities have been reported to play an important role in the pathogenesis of immune thrombocytopenia (ITP) besides specific autoantibodies towards platelet. This study aimed to explore the clinical importance of T lymphocyte subsets in adult patients with newly diagnosed ITP before and after first-line treatment. Elderly ITP patients were also studied, and we tried to analyze the relationships between these items and therapeutic outcomes. Above study suggest that ITP patients usually had fewer numbers of peripheral lymphocytes and patients with higher levels of CD8+ cells or lower levels of CD4+/CD8+ cell ratio were less likely to respond to first-line treatment. In our results, no difference with CD8+ or CD4+/CD8+ ratio was found between good and poor response group. This result may be caused by a small group in our study.

There were some limitations to our study. It was limited by the small number of studied patients. The patients, whose flow cytometry data should be available before any treatment for ITP, were enrolled. If the patients who got the flow data after steroid treatment, they were excluded. So not all newly-diagnosis ITP patients in our hospital were enrolled in this study, and that will have some selection bias. Large-scale studies focusing on different subgroup lymphocyte percentages in ITP may be required to confirm our findings.

In conclusion, this study found that newly diagnosed ITP patients with increased percentages of CD56+ or CD2+ lymphocytes in peripheral blood were associated with poor response to steroid treatment. By our results of ROC curve analysis, under high specificity, if CD56+ lymphocyte is > 24.5% or CD2+ lymphocytes is > 85.7%, these patients will have the high possibility with corticosteroids dependence/refractory. The steroid may not be the best choice of first-line treatment. This result may help to choose frontline therapy for different ITP patients. This may provide insight into the prediction of steroid response in patients with ITP.

Authors' contributions

YW, the conception and design of the study, analysis and interpretation of data; WH, MG, and SL, performed the experiments; WH, SC, and TW, acquisition of data, CL, final approval of the version to be submitted.

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Declaration of competing interest

The authors declare that they have no relevant conflicts of interest.

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