Thrombosis Research 181 (2019) 10–16

Contents lists available at ScienceDirect

Thrombosis Research

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

Full Length Article

The prediction value of Treg cell subtype alterations for glucocorticoid treatment in newly diagnosed primary immune thrombocytopenia patients

Luya Cheng[a,1], Chanjuan Liu[a,1], Feng Li[a,b], Boting Wu[c], Zhihui Min[d,e], Pu Chen[a], Yanxia Zhan[a], Yang Ke[a], Fanli Hua[b], Ling Yuan[a], Lihua Sun[b], Hao Chen[e], Lili Ji[a,*], Yunfeng Cheng[a,b,d,f,**]

[a] Department of Hematology, Zhongshan Hospital Fudan University, Shanghai 200032, China
[b] Department of Hematology, Zhongshan Hospital Qingpu Branch, Fudan Universiy, Shanghai 201700, China
[c] Department of Transfusion Medicine, Zhongshan Hospital Fudan University, Shanghai 200032, China
[d] Institute of Clinical Science, Zhongshan Hospital, Fudan University, Shanghai 200032, China
[e] Department of Thoracic Surgery, Zhongshan Hospital Xuhui Branch, Fudan University, Shanghai 200031, China
[f] Center for Tumor Diagnosis & Therapy, Jinshan Hospital, Fudan University, Shanghai, 201500, China

ARTICLE INFO

Keywords:
Immune thrombocytopenia
Tregs subtypes
CD45
IL-10

ABSTRACT

Background: Primary immune thrombocytopenia (ITP) is an autoimmune heterogeneous disorder of which Treg cells are numerically or functionally deficient. It is known that human FoxP3$^+$CD4$^+$ T cells were composed of 3 phenotypically and functionally distinct subpopulations (resting Treg, rTreg; activated Treg, aTreg; and non-suppressive Treg, n-sTreg). The current study was aimed to determine whether these Treg subtypes are altered in ITP patients and the related potential clinical applications.

Method: Normal control volunteers and newly diagnosed ITP patients were enrolled in the study. The percentage of Treg cells' subtypes in peripheral blood was examined by flow cytometry before and after the glucocorticoid treatment. The IL-10 production by Treg subtypes was also examined.

Results: Treg cell subtypes of aTreg increased, rTreg decreased, and n-s Treg increased in newly diagnosed ITP patients' peripheral blood. The IL-10 production by respective Treg subtype didn't change after the treatment, and aTreg cells had the highest IL-10 yield. Patients who gained remission during follow-up had a higher aTreg cells' percentage than those who did not at the disease diagnosis.

Conclusion: Tregs cell subtypes percentage was altered when ITP occurred. The increased aTreg cells at disease diagnosis might predict a better glucocorticoid treatment efficacy.

1. Background

Primary immune thrombocytopenia (ITP) is an autoimmune heterogeneous disorder presenting with decreased platelet count and increased bleeding risk. Both impaired platelet production and increased platelet destruction mediated by T cells play roles in the pathogenesis of ITP [1]. As Tregs play a fundamental role in the maintenance of immune tolerance, they have been found numerically or functionally deficient in ITP patients [2–6]. CD4$^+$ CD25^{hi} Foxp3$^+$ Treg cells are produced in the thymus, or generates from conventional CD4$^+$ T cells in peripheral sites. They shed suppression on the activation and proliferation of Teffs by cell-to-cell contact and by secreting cytokines such as interleukin-10 (IL-10) and tumor growth factor-β (TGF-β) [7,8]. Previously we found that insufficient secretion of IL-10 by Treg cells compromised its control on over-activated CD4$^+$ T effector cells in ITP patients [3].

Human Foxp3$^+$CD4$^+$ T cells are composed of 3 phenotypically and functionally distinct subpopulations [9–13]: CD45RA$^+$Foxp3^{lo} resting Treg cells (rTreg cells, Group I), CD45RA$^-$Foxp3^{hi} activated Treg cells (aTreg cells, Group II, most of which originated from rTreg cells), both

* Corresponding author at: Department of Hematology, Zhongshan Hospital Fudan University, Shanghai 200032, China
** Correspondence to: Y. Cheng, Institute of Clinical Science and Department of Hematology, Zhongshan Hospital, Fudan University, 180 Fenglin Rd, Shanghai 200032, China.
E-mail addresses: cheng.luya@zs-hospital.sh.cn (L. Cheng), 15111210015@fudan.edu.cn (C. Liu), li.feng@zs-hospital.sh.cn (F. Li), wu.boting@zs-hospital.sh.cn (B. Wu), min.zhihui@zs-hospital.sh.cn (Z. Min), chen.pu@zs-hospital.sh.cn (P. Chen), zhan.yanxia@zs-hospital.sh.cn (Y. Zhan), ke.yang@zs-hospital.sh.cn (Y. Ke), hua_fanli@fudan.edu.cn (F. Hua), yuan.ling@zs-hospital.sh.cn (L. Yuan), h.chen@fudan.edu.cn (H. Chen), ji.lili@zs-hospital.sh.cn (L. Ji), yfcheng@fudan.edu.cn (Y. Cheng).
[1] Drs. Luya Cheng and Chanjuan Liu contributed equally to this work.

https://doi.org/10.1016/j.thromres.2019.07.001
Received 18 February 2019; Received in revised form 18 June 2019; Accepted 1 July 2019
Available online 02 July 2019

Table 1
Clinical characteristics of ITP patients.

Patient no.	Sex	Age (years)	Platelet counts ($\times 10^9$/L)		Treatment efficacy
			Pre-treatment	Post-treatment	
ITP1*	M	59	22	444	CR
ITP2*	F	65	4	163	CR
ITP3*	F	58	5	103	CR
ITP4*	F	44	18	109	CR
ITP5*	F	69	1	165	CR
ITP6*	F	63	8	276	CR
ITP7*	M	19	8	79	PR
ITP8	F	71	4	94	PR
ITP9	F	68	9	166	CR
ITP10	M	32	16	224	CR
ITP11	M	68	9	102	CR
ITP12	M	29	11	183	CR
ITP13	F	43	23	103	CR
ITP14	F	55	1	139	CR
ITP15	F	64	2	327	CR
ITP16	F	53	24	112	CR
ITP17	F	46	1	226	CR
ITP18	F	61	17	238	CR
ITP19	F	25	2	101	CR
ITP20	F	43	2	220	CR
ITP21	M	39	15	216	CR
ITP22*	F	79	12	10	NR
ITP23*	M	25	7	5	NR
ITP24	M	18	7	12	NR
ITP25	M	59	9	7	NR
ITP26	M	68	9	2	NR
ITP27	F	56	20	25	NR
ITP28	F	60	9	8	NR
ITP29	M	31	2	4	NR
ITP30	F	66	13	25	NR
ITP31	F	53	1	2	NR
	M:F = 11:20	56(18–79)	9(1–24)	103(2–444)	

* These 9 patients' treg cells were cultured for IL-10 production analysis.

of which are suppressive in vitro, and $CD45RA^-Foxp3^{lo}$ non-suppressive T cells (n-s Treg cells, Group III), which might represent those activation-induced Foxp3-expressing cells that transiently express Foxp3 in vitro [9,14,15]. The dynamic differentiation and fate of the various Foxp3-expressing human peripheral $CD4^+$T cells was underscored [16].

Detailed analysis of Treg subpopulations would be more informative than total Treg cells in investigating the mechanisms of autoimmune disease. Several studies have reported that Treg subtypes changed in different autoimmune disease, e.g., aTreg cells decreased in systemic lupus erythematosus (SLE) patients [9,17], impaired numbers of functional $CD45RA^+Foxp3^{low}$ naive Treg was found in SLE patients [18], $CD45RO^+$ aTreg cells increased in type 1 diabetes patients [19] while $CD39^+CD45RA^+$ rTreg cells were decreased [20], rheumatoid arthritis, and Behcet's disease patients' aTreg cells were decreased compared to health controls [21]. However, it remains unclear regarding Treg cell subtypes changes in ITP. The present study was aimed to examine whether the Treg subtypes numerically and functionally changed in ITP patients with correlated treatment and prognosis.

2. Materials and methods

2.1. Patients and healthy controls

From Jan. 2015 to Mar. 2017, patients with newly diagnosed ITP according to the ITP diagnosis criteria proposed by an international working group [1] were enrolled in this study. All of these patients requiring therapy for their platelet counts were $< 30 \times 10^9$/L. Secondary ITP and pregnant patients were excluded. All patients received first-line corticosteroids treatment according to an international consensus [1,22]. In detail, the patients received oral 1 mg/kg prednisone for 4 weeks and then tapered off. One month after the initial glucocorticoid treatment, the therapeutic efficacies were validated in accordance with the Vicenza Consensus Conference [1]. Nineteen healthy adult volunteers were recruited as normal control (NC). Twenty mL venous blood samples were collected for each of the patients and health controls upon enrollment and 1mo post initial treatment (only the patients' blood were collected at this time) for further examination.

The study was approved by the institutional review board of Zhongshan Hospital, Fudan University. Written informed consent was obtained from each patient and volunteer before enrollment.

2.2. Sample preparation

Venous blood samples were collected in ethylenediaminetetraacetic acid-treated tubes and diluted 1:2 with Hanks balanced salt solution (HBSS) before Ficoll-Hypaque gradient centrifugation (2200 rpm at room temperature for 15 min). Washed and re-suspended, isolated peripheral blood mononuclear cells (PBMCs) were cryopreserved in fetal bovine serum containing 10% dimethyl sufloxide (DMSO), and stored in liquid nitrogen for future studies.

2.3. Cell purification and culture

Cryopreserved PBMCs were thawed at 37 °C, washed twice with HBSS. Before cell purification, 1×10^6 PBMCs were allocated to determine the number of Tregs by flow cytometic (FCM) analysis. Dead cells were removed by dead cell removal kit (Miltenyi Biotec, Auburn, California, USA, Cat# 130-090-101). Tregs and Teffs were isolated from PBMC using the $CD4^+CD25^+$Treg cells isolation kit (Miltenyi Biotec, Cat# 130-091-301) per manufacturer's instruction.

Before culture, Teffs were CFDA-SE (CFSE)-labeled (Invitrogen,

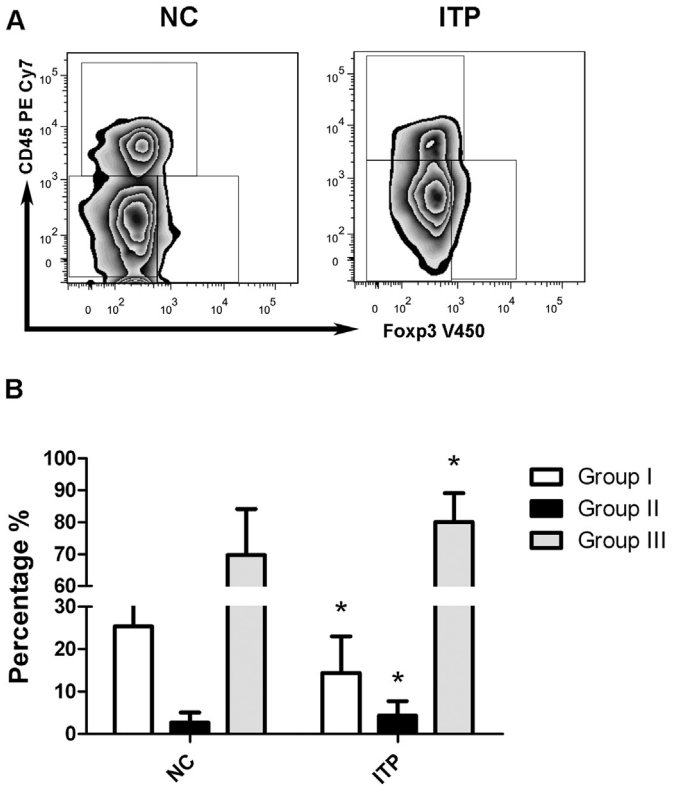

Fig. 1. Group I decreased, Group II increased, and Group III increased in ITP patients.
A. Representative zebra figure of $CD4^+CD25^{hi}Foxp3^+$ Treg cells in NC and ITP groups. B. The mean ± SD of the percentage of Group I, Group II, and Group III Treg subtypes of two groups.
NC, normal control group; ITP, ITP patients group; Group I, $CD45RA^+Foxp3^{lo}$ resting Treg cells; Group II, $CD45RA^-Foxp3^{hi}$ activated Treg cells; Group III, $CD45RA^-Foxp3^{lo}$ non-suppressive T cells.
*$p < 0.05$ when compared with the counterpart of NC group.

Carlsbad, California, USA, Cat# C34554) with in PBS staining of 0.8 μM at 37 °C. Teffs were cultured with Tregs at 4:1 ratio, adjusted concentration at 1×10^6/ml in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 200 U/ml penicillin, and 100 μg/ml streptomycin and 200 ng/ml interleukin-2 (IL-2; Biolegend, Cat# 589102). The cells were cultured in 200 μl per well in round-bottom 96-well plates, which were pre-incubated with anti-CD3 (10 μg/ml; Biolegend, Cat# 300414), and stimulated with anti-CD28 (10 μg/ml, Biolegend, Cat# 302914). After culturing for 5 days, cells were collected for FCM analysis.

2.4. Flow cytometry analysis

For Treg subtypes test, 1×10^6 PBMCs were stained with CD4 FITC (eBioscience, San Diego, California, USA, Cat# 11-0048-42), CD25 APC-H7 (BD Bioscience, Cat# 506225), CD45 PE-Cy7 (BD Bioscience, Cat# 560675) and Foxp3 V450 (BD Bioscience, Cat# 560459). To detect IL-10 production by Treg subtypes, 1×10^6 cultured $CD4^+$T cells were adjusted concentration as 5×10^5/ml in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 200 U/ml penicillin, and 100 μg/ml streptomycin. The $CD4^+$T cells were incubated for 4 h with 50 ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich, St. Louis, Missouri, USA, Cat# P8139) and 500 ng/ml ionomycin (Sigma-Aldrich, Cat# I9657). After 2 h of incubation, 1 μl/ml brefeldin A solution (BFA, Biolegend, Cat# 420602) was added into the culture system. Then $CD4^+$T cells were stained with CD25 APC-H7, CD45 PE-Cy7, Foxp3 V450, and IL-10 APC (BD Bioscience, Cat# 554707) according to the manufacturer's protocol. Stained cells were tested on a FACS Aria II flow cytometer (BD biosciences) and then analyzed using Flowjo software version 7.6.1 (Tritar Inc., San Carlos, California, USA).

2.5. Statistical methods

All analyses were performed with STATA 7.0 software (StataCorp LP, College Station, Texas, USA). Data were expressed as mean ± SD. Normality was assessed by Shapiro-Wilk W test. In pairwise comparison, Student *t*-test and Wilcoxon rank-sum (Mann-Whitney) test were used for data fulfilled normal distribution and for those did not, respectively. When multiple groups were compared, One Way ANOVA and Kruskal Wallis test were used for data fulfilled normal distribution and for those did not, respectively. Pearson or spearman analysis was used to evaluate the correlation between groups when the data fulfilled or not fulfilled normal distribution. For all tests, two-sided *p* values < 0.05 were considered statistically significant.

3. Results

Thirty-one adult patients (20 females and 11 males, age range 18–79 years, median age 56 years) with newly diagnosed ITP who meet aforementioned criteria were enrolled. Nineteen healthy adult human volunteers were recruited as control (NC, 12 females and 7 males, age range 22–75 years, median age 57 years). The age and sex between ITP group and NC group was comparable ($p > 0.05$). Detailed characteristics of these patients were listed in Table 1.

3.1. aTreg subtype increased while rTreg subtype decreased in newly diagnosed ITP patients' peripheral blood

We compared the composition of Treg subtypes in the peripheral blood of newly diagnosed ITP patients and health volunteers (the composition of each subgroup was showed as percentage account in the total Treg cells). The results showed that the $CD45RA^-Foxp3^{hi}$ activated Treg cells' percentage (aTreg cells, Group II) increased in ITP patients ((4.33 ± 3.45) % vs NC (2.72 ± 2.38) %, $p = 0.0376$). On the other hand, the $CD45RA^+Foxp3^{lo}$ resting Treg cells' percentage (rTreg cells, Group I) decreased in ITP patients ((14.33 ± 8.66) % vs NC (25.38 ± 13.39) %, $p = 0.0030$). The $CD45RA^-Foxp3^{lo}$ non-suppressive T cells' percentage (Group III) was higher in the ITP patients when compared with NC volunteers ((80.01 ± 9.05) % vs (69.69 ± 14.42) %, $p = 0.0044$) (Fig. 1). We also compared the immune-suppressive fraction (Group I + Group II) of Trges between ITP patients and health controls, less suppressive fraction of Tregs was found in ITP patients ($p = 0.0050$).

3.2. The production pattern of IL-10 by Tregs subtypes didn't change in ITP patients

The purified $CD4^+CD25^-$T effector cells and $CD4^+CD25^+$Treg cells were co-cultured with focus on the IL-10 production of each subtype of Treg cells. The co-cultured Tregs' composition altered similarly to the peripheral blood. Group I decreased ((8.94 ± 8.38) % vs NC (21.67 ± 10.71) %, $p = 0.0222$), Group II increased ((13.89 ± 7.13) % vs NC (7.94 ± 3.49) %, $p = 0.0327$), and Group III increased ((77.17 ± 9.31) % vs NC (59.90 ± 14.37) %, $p = 0.0330$) (Fig. 2A, B). The mean fluorescence intensity (MFI) of IL-10 APC was taken as a marker of the production of IL-10 by Treg cells. For health controls, the MFI of 3 subtypes of Treg cells were statistically different ((Group I (636.50 ± 378.85) vs Group II (3012.00 ± 3165.25) vs Group III (834.32 ± 1365.40), $p = 0.0463$)). However, The IL-10 production pattern among the subtypes remained unchanged in ITP patients ((Group I (385.49 ± 416.89) vs Group II (3934.56 ± 3633.33) vs Group III (556.00 ± 416.63), $p = 0.0007$)).

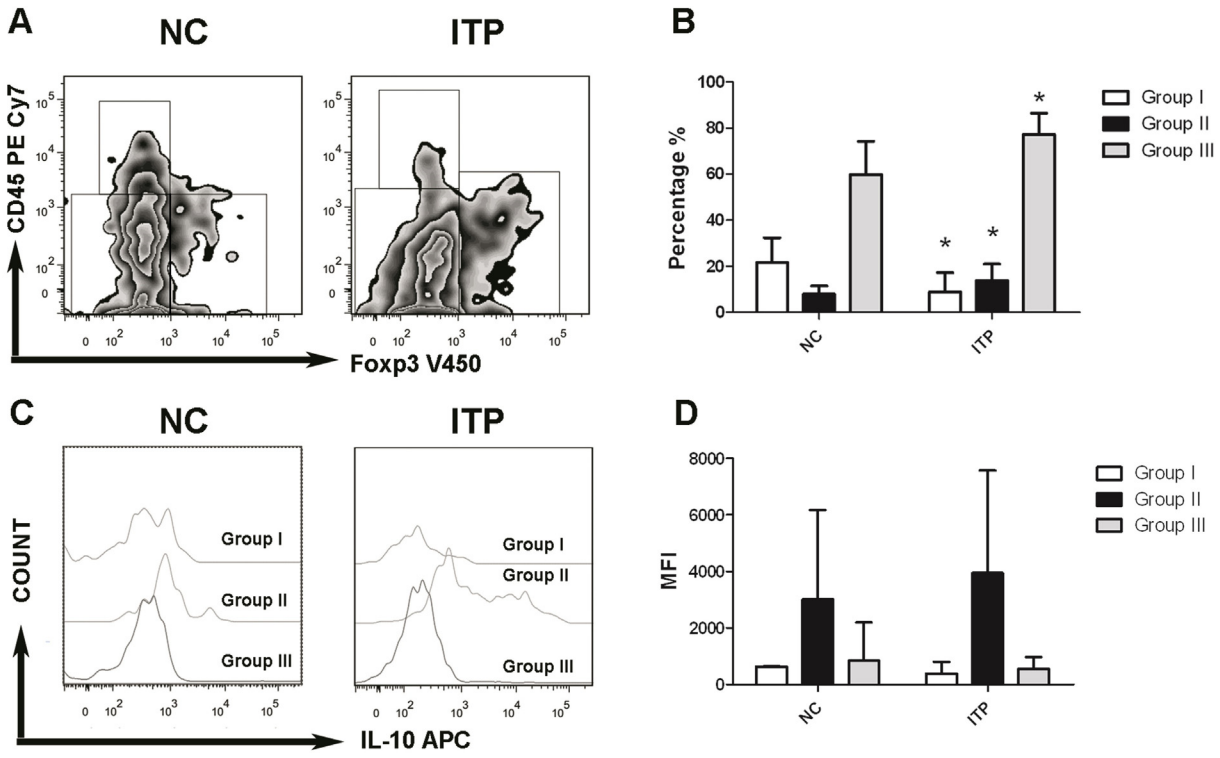

Fig. 2. The subtypes changed same in the culture condition. The IL-10 production by Tregs subtypes didn't change in ITP patients.
A. Representative zebra figure of $CD4^+CD25^{hi}Foxp3^+$ Treg cells in NC and ITP groups in the same culture condition. B. The mean ± SD of the percentage of Group I, Group II, and Group III Treg subtypes of two groups in the same culture condition. C. Representative histogram of IL-10 MFI of Treg subtypes in NC and ITP groups in the same culture condition. D. The mean ± SD of IL-10 MFI of Group I, Group II, and Group III Treg subtypes of two groups in the same culture condition.
NC, normal control group; ITP, ITP patients group; Group I, $CD45RA^+Foxp3^{lo}$ resting Treg cells; Group II, $CD45RA^-Foxp3^{hi}$ activated Treg cells; Group III, $CD45RA^-Foxp3^{lo}$ non-suppressive T cells.
*$p < 0.05$ when compared with the counterpart of NC group.

No significance was found between NC and ITP on the MFI of each Treg cells' subtype (Fig. 2C, D).

3.3. The Treg subtypes' change according to the treatment efficacy

ITP patients were followed up for 1 month after the initiation of glucocorticoid treatment. According to the Vicenza Consensus Conference [1], 21 patients were complete remission (CR) or partial remission (PR), they were named as ITP-R group. The other 10 patients remained no remission (NR, ITP-NR group). Data showed that before the treatment (Pre), rTreg cells were decreased in both of the response groups when compared with NC (ITP-R pre (15.52 ± 9.75) %, $p = 0.0032$; ITP-NR pre (11.82 ± 5.32) %, $p = 0.0008$). However for aTreg cells, the change was different: the aTreg fraction increased in ITP-R pre ((5.39 ± 3.73) %, $p = 0.0038$), remained unchanged in ITP-NR pre ((2.10 ± 0.76) %, $p = 0.7121$). Group III Treg cells in ITP-R pre patients were comparable to NC group (ITP-R pre (77.51 ± 9.33) %, $p = 0.0562$), while that component of ITP-NR pre increased ((85.26 ± 5.85) %) when compared with NC group ($p = 0.0013$), or with ITP-R pre ($p = 0.0230$) (Fig. 3A, B).

After treatment, the three subtypes of Treg cells of ITP-R patients (ITP-R post) were changed as compared with NC group, respectively: Group I Treg cells were decreased, ((13.50 ± 8.62) %, $p = 0.0008$); Group II Treg cells were increased ((5.72 ± 5.20) %, $p = 0.0051$); Group III Treg cells increased ((78.36 ± 7.64) %, $p = 0.0122$). There was no statistic difference between the ITP-R pre and the ITP-R post (Fig. 3C & D). For ITP-NR patients, Group I Treg cells were decreased ((11.63 ± 5.84) %, $p = 0.0006$; Group II Treg cells were at normal level ((2.24 ± 1.82) %, $p = 0.7480$); Group III Treg cells were increased ((85.44 ± 5.13) %, $p = 0.0002$), as compared to NC group respectively. No statistic difference was found between the ITP-NR pre and the ITP-NR post (Fig. 3C & D).

As it was mentioned, age could potentially affect the Treg subtypes. Age distribution was comparable in ITP-R patients and ITP-NR patients ($p = 0.9572$). Pearson or spearman analysis showed that age was not correlated with Group I Treg cells ($p = 0.7722$), Group II Treg cells ($p = 0.7036$), or Group III Treg cells ($p = 0.9287$).

Higher Group II Treg cells percentage at diagnosis might predict greater responsiveness to corticosteroids.

We compared each group of Treg cells at diagnosis of ITP-R patients with ITP-NR patients. The p value of Group I was 0.4098, which meant that no difference was found in this group of Treg cells between ITP-R patients and ITP-NR patients. As for Group II Treg cells, higher percentage of aTreg cells at diagnosis was found in ITP-R patients ($p = 0.0007$), as compared to that of ITP-NR patients. Taking Difference (Plt) (platelet count post-treatment minus platelet count pre-treatment, Diff (Plt)) as a parameter, the Spearman's Rho between the aTreg cells percentage and the Diff (Plt) was 0.3698 ($p = 0.0406$). This result suggested that higher aTreg cells percentage correlate with higher Diff (Plt) (Fig. 4), in other words, a higher aTreg cells might correlate with good response to glucocorticoids treatment.

4. Discussion

ITP is an autoimmune disease, abnormal Treg cells' number and function takes an important role in its pathogenesis. Recently, Treg's heterogeneity has been highlighted [23–25]. There are 3 different subtypes of Treg cells, with different surface markers and different immune-function [9]. The role of the different circulating regulatory T-cells (Treg) subsets, as well as their relations with clinical outcome of

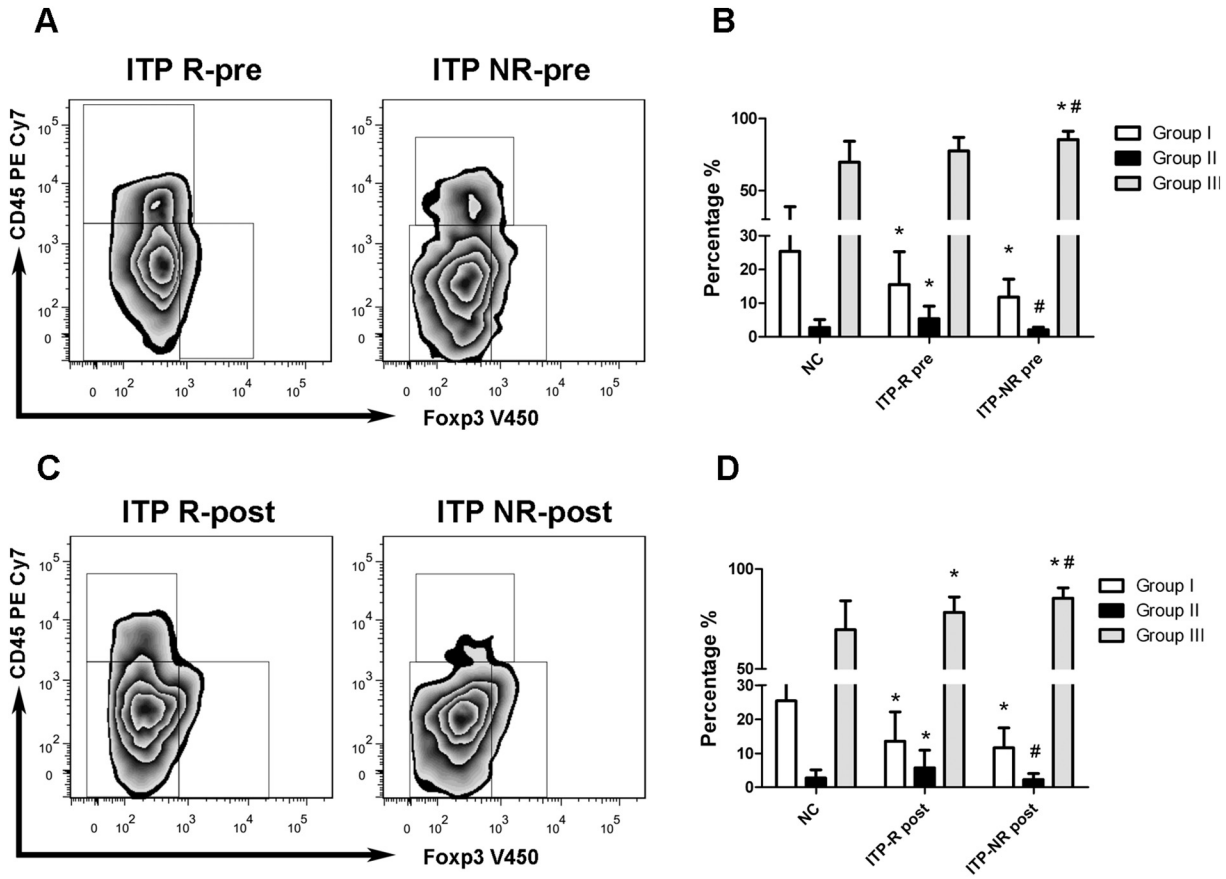

Fig. 3. The Treg subtypes' alteration according to the treatment efficacy.
A. Representative zebra figure of $CD4^+CD25^{hi}Foxp3^+$ Treg cells in ITP-R pre and ITP-NR pre groups. B. The mean ± SD of the percentage of Group I, Group II, and Group III Treg subtypes of NC, ITP-R pre, ITP-NR pre groups. C. Representative zebra figure of $CD4^+CD25^{hi}Foxp3^+$ Treg cells in ITP-R-post and ITP-NR post groups. D. The mean ± SD of the percentage of Group I, Group II, and Group III Treg subtypes of NC, ITP-R post, ITP-NR post groups.
NC, normal control group; ITP-R pre, ITP patients who later got remission, before treatment; ITP-NR pre, ITP patients who later remained no remission, before treatment; ITP-Rpost, ITP patients who later got remission, after treatment; ITP-NR post, ITP patients who later remained no remission, after treatment; Group I, $CD45RA^+Foxp3^{lo}$ resting Treg cells; Group II, $CD45RA^-Foxp3^{hi}$ activated Treg cells; Group III, $CD45RA^-Foxp3^{lo}$ non-suppressive T cells.
*$p < 0.05$ when compared with the counterpart of NC group.
#$p < 0.05$ when compared with the counterpart of ITP-R pre or ITP-R post.

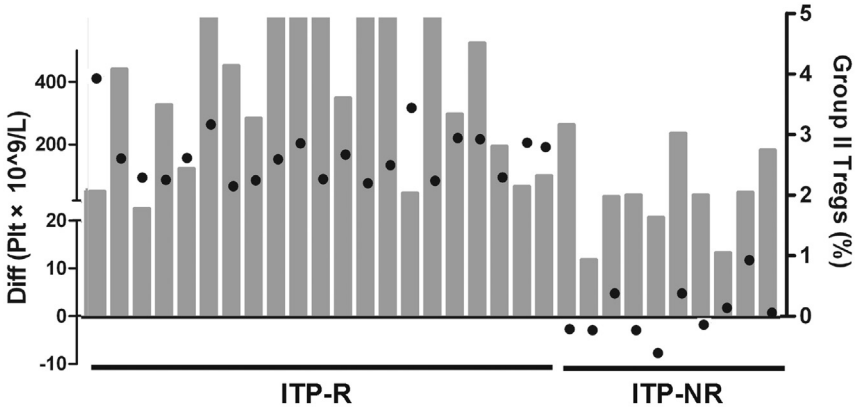

Fig. 4. The correlation between the aTreg cells percentage and treatment efficacy.
Higher aTreg cells percentage was seen in ITP-R patients at diagnosis. ITP-R, ITP patients who later got remission; ITP-NR, ITP patients who later remained no remission; Diff (Plt), platelet count post-treatment minus platelet count pre-treatment.

ITP patients is poorly understood. The present study showed that the composition of Treg subtypes was changed in ITP patients comparing to health controls. The non-immuno-suppressive fraction (Group III) of Treg cells was increased at the occurrence of the disease while the immune-suppressive fraction of Treg cells was decreased. This finding, together with our previous report that the sum of Treg cells is decreased in ITP patients [4], supports a conclusion that the immunosuppressive Treg cells are compromised in ITP.

It is known that rTreg cells could be activated and turned to aTreg cells when stimulated by antigens, and the proliferation of aTreg could

generate a negative feedback to stop the rTreg cells from converting into aTreg cells. On the other hand, aTreg cells die shortly after activation, and this would boost the rTreg cells' turnover into aTreg cells [9,16]. In the current study, aTreg cells (Group II) were found increased, while the rTreg cells (Group I) were decreased in ITP patients. We speculate that a high turnover rate from rTreg to aTreg cells resulted in this phenomenon. However, it is not known how rTreg cells turn into aTreg cells, so this speculation needs further validation. Besides, it is unclear whether there is a life-long thymic output of $CD45RA^+$ rTreg cells or they are self-maintained. So it also remains unsolved whether the decrease of rTreg cells is caused by exhaustion or by lower output of thymus [26]. To address these issues, we put the same amount of $CD4^+CD25^+$ T cells in the culture systems. After 5-days' stimulation of CD3/CD28 antibodies, Group II Treg cells of ITP group were numerically much more than that of NC group. This result supported our hypothesis that the turnover rate from Group I to Group II is higher in ITP patients than in health controls.

The treatment efficacy evaluation during follow up found that remission patients had more aTreg cells (Group II) at diagnosis. Furthermore, the aTreg cells' percentage was correlated with the Diff (Plt) after corticosteroids treatment. Both results could support the argument that a higher aTreg cells' percentage at the diagnosis might predict a greater responsiveness to corticosteroids in ITP patients. In the in vitro culture study, we showed that higher Group II Treg cells was probably caused by higher turnover rate from Group I to Group II in ITP patients. Thus, the higher turnover might be helpful in the recovery of thrombocytopenia. If this is true, then the treatment efficacy of ITP could be improved by targeting the turnover rate.

When evaluating suppressive Tregs (Groups I and II) as a whole, it showed that the suppressive Treg cells were decreased in ITP patients. It has been proved that Treg cells might become unstable under certain inflammatory conditions [27,28]. According to our previous report, the $CD4^+$ effector cells are over-activated [3], which could create an inflammatory condition and consequently cause the decrease of suppressive Treg cells in ITP patients, suggesting another novel therapeutic target for ITP.

The co-culture experiment testified that aTreg cells produced most of the IL-10 among the three subtypes of Treg cells, no matter in health controls, or in ITP patients. The comparison between NC and ITP group showed that the capacity of producing IL-10 from each subgroup did not change in ITP patients. However, as it is the abnormality of secretion, not the production of IL-10, causes the dysfunction of Treg cells, this finding does not hamper the possibility that the rTreg or/and aTreg cells are dysfunctional in ITP, which deserves further investigations.

5. Conclusion

Treg cells subtype percentage alters in ITP patients. A higher group II aTreg cells percentage at diagnosis might predict a better glucocorticoid treatment efficacy. The investigations on how to adjust the plasticity of Treg cells might be helpful to improve the treatment efficacy of ITP.

Ethics approval and consent to participate

The study was in accordance with the ethical standards formulated in the Helsinki Declaration and was approved by the respective local Medical Ethics Committees of Zhongshan Hospital of Fudan University. Written informed consent was obtained from each patient before being included in the study.

Consent for publication

Not applicable.

Availability of data and materials

Data sharing: no additional data available.

Funding

This work was supported by grants from National Natural Science Foundation of China (81870098, 81600090, 81500090, 81470282, 81300381), the Science and Technology Commission of Shanghai Municipality (18ZR1407200), and Shanghai Municipal Health Bureau Funding (201840351, 20134y117 and 20144Y0194). All authors obtain permission to acknowledge from all those mentioned in the Acknowledgements.

Authors' contributions

LC, LJ and YC conceived the study; LC, MT, YZ, LC, FL, BW, LJ and YC performed the literature review, drafted and revised the manuscript; LJ, FL and YC contributed to the critical revision of the manuscript; FH, ZM, PC, YK, CL, LS, HC and LC performed the experiments, analyzed data. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

The authors thank Jianjun Jin and Bijun Zhu for their technical support.

References

[1] F. Rodeghiero, R. Stasi, T. Gernsheimer, M. Michel, D. Provan, D.M. Arnold, J.B. Bussel, D.B. Cines, B.H. Chong, N. Cooper, B. Godeau, K. Lechner, M.G. Mazzucconi, R. McMillan, M.A. Sanz, P. Imbach, V. Blanchette, T. Kuhne, M. Ruggeri, J.N. George, Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group, Blood 113 (11) (2009) 2386–2393.

[2] M. Perera, T. Garrido, Advances in the pathophysiology of primary immune thrombocytopenia, Hematology 22 (1) (2017) 41–53.

[3] F. Li, L. Ji, W. Wang, F. Hua, Y. Zhan, S. Zou, L. Yuan, Y. Ke, Z. Min, D. Song, L. Sun, H. Chen, Y. Cheng, Insufficient secretion of IL-10 by Tregs compromised its control on over-activated $CD4^+$ T effector cells in newly diagnosed adult immune thrombocytopenia patients, Immunol. Res. 61 (3) (2015) 269–280.

[4] L. Ji, Y. Zhan, F. Hua, F. Li, S. Zou, W. Wang, D. Song, Z. Min, H. Chen, Y. Cheng, The ratio of Treg/Th17 cells correlates with the disease activity of primary immune thrombocytopenia, PLoS One 7 (12) (2012) e50909.

[5] J. Yu, S. Heck, V. Patel, J. Levan, Y. Yu, J.B. Bussel, K. Yazdanbakhsh, Defective circulating CD25 regulatory T cells in patients with chronic immune thrombocytopenic purpura, Blood 112 (4) (2008) 1325–1328.

[6] R. Stasi, N. Cooper, P.G. Del, E. Stipa, E.M. Laura, E. Abruzzese, S. Amadori, Analysis of regulatory T-cell changes in patients with idiopathic thrombocytopenic purpura receiving B cell-depleting therapy with rituximab, Blood 112 (4) (2008) 1147–1150.

[7] E.M. Shevach, Mechanisms of foxp3+ T regulatory cell-mediated suppression, Immunity 30 (5) (2009) 636–645.

[8] M. Miyara, G. Gorochov, M. Ehrenstein, L. Musset, S. Sakaguchi, Z. Amoura, Human $FoxP3^+$ regulatory T cells in systemic autoimmune diseases, Autoimmun. Rev. 10 (12) (2011) 744–755.

[9] M. Miyara, Y. Yoshioka, A. Kitoh, T. Shima, K. Wing, A. Niwa, C. Parizot, C. Taflin, T. Heike, D. Valeyre, A. Mathian, T. Nakahata, T. Yamaguchi, T. Nomura, M. Ono, Z. Amoura, G. Gorochov, S. Sakaguchi, Functional delineation and differentiation dynamics of human $CD4^+$ T cells expressing the FoxP3 transcription factor, Immunity 30 (6) (2009) 899–911.

[10] J.N. Wang, X.X. Cao, A.L. Zhao, H. Cai, X. Wang, J. Li, Increased activated regulatory T cell subsets and aging Treg-like cells in multiple myeloma and monoclonal gammopathy of undetermined significance: a case control study, Cancer Cell Int. 18 (2018) 187.

[11] S. Kohler, T.O.P. Keil, S. Hoffmann, M. Swierzy, M. Ismail, J.C. Ruckert, T. Alexander, A. Meisel, $CD4^+$ $FoxP3^+$ T regulatory cell subsets in myasthenia gravis patients, Clin. Immunol. 179 (2017) 40–46.

[12] H. Fujii, J. Josse, M. Tanioka, Y. Miyachi, Regulatory T Cells in Melanoma Revisited by a Computational Clustering of $FOXP3^+$ T Cell Subpopulations, 196(6) (2016), pp. 2885–2892.

[13] T. Viisanen, A.M. Gazali, E.L. Ihantola, I. Ekman, K. Nanto-Salonen, R. Veijola, J. Toppari, M. Knip, J. Ilonen, T. Kinnunen, FOXP3$^+$ regulatory T cell compartment is altered in children with newly diagnosed type 1 diabetes but not in autoantibody-positive at-risk children, Front. Immunol. 10 (2019) 19.
[14] S.E. Allan, S.Q. Crome, N.K. Crellin, L. Passerini, T.S. Steiner, R. Bacchetta, M.G. Roncarolo, M.K. Levings, Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production, Int. Immunol. 19 (4) (2007) 345–354.
[15] D.Q. Tran, H. Ramsey, E.M. Shevach, Induction of FOXP3 expression in naive human CD4$^+$FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype, Blood 110 (8) (2007) 2983–2990.
[16] M. Battaglia, M.G. Roncarolo, The fate of human Treg cells, Immunity 30 (6) (2009) 763–765.
[17] M. Barreto, R.C. Ferreira, L. Lourenco, M.F. Moraes-Fontes, E. Santos, M. Alves, C. Carvalho, B. Martins, R. Andreia, J.F. Viana, C. Vasconcelos, L. Mota-Vieira, C. Ferreira, J. Demengeot, A.M. Vicente, Low frequency of CD4$^+$CD25$^+$ Treg in SLE patients: a heritable trait associated with CTLA4 and TGFbeta gene variants, BMC Immunol. 10 (2009) 5.
[18] X. Pan, X. Yuan, Y. Zheng, W. Wang, J. Shan, F. Lin, G. Jiang, Y.H. Yang, D. Wang, D. Xu, L. Shen, Increased CD45RA$^+$ FoxP3(low) regulatory T cells with impaired suppressive function in patients with systemic lupus erythematosus, PLoS One 7 (4) (2012) e34662.
[19] W. Du, Y.W. Shen, W.H. Lee, D. Wang, S. Paz, F. Kandeel, C.P. Liu, Foxp3$^+$ Treg expanded from patients with established diabetes reduce Helios expression while retaining normal function compared to healthy individuals, PLoS One 8 (2) (2013) e56209.
[20] K. Akesson, A. Tompa, A. Ryden, M. Faresjo, Low expression of CD39$^+$/CD45RA$^+$ on regulatory T cells (Treg) cells in type 1 diabetic children in contrast to high expression of CD101$^+$/CD129$^+$ on Treg cells in children with coeliac disease, Clin. Exp. Immunol. 180 (1) (2015) 70–82.
[21] J.R. Kim, J.N. Chae, S.H. Kim, J.S. Ha, Subpopulations of regulatory T cells in rheumatoid arthritis, systemic lupus erythematosus, and Behcet's disease, J. Korean Med. Sci. 27 (9) (2012) 1009–1013.
[22] C. Neunert, W. Lim, M. Crowther, A. Cohen, L. Solberg Jr., M.A. Crowther, The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia, Blood 117 (16) (2011) 4190–4207.
[23] D.V. Sawant, D.A. Vignali, Once a Treg, always a Treg? Immunol. Rev. 259 (1) (2014) 173–191.
[24] S. Hori, Lineage stability and phenotypic plasticity of Foxp3$^+$ regulatory T cells, Immunol. Rev. 259 (1) (2014) 159–172.
[25] H. Morikawa, S. Sakaguchi, Genetic and epigenetic basis of Treg cell development and function: from a FoxP3-centered view to an epigenome-defined view of natural Treg cells, Immunol. Rev. 259 (1) (2014) 192–205.
[26] R. Aslam, Y. Hu, S. Gebremeskel, G.B. Segel, E.R. Speck, L. Guo, M. Kim, H. Ni, J. Freedman, J.W. Semple, Thymic retention of CD4$^+$CD25$^+$FoxP3$^+$ T regulatory cells is associated with their peripheral deficiency and thrombocytopenia in a murine model of immune thrombocytopenia, Blood 120 (10) (2012) 2127–2132.
[27] S. Sakaguchi, D.A. Vignali, A.Y. Rudensky, R.E. Niec, H. Waldmann, The plasticity and stability of regulatory T cells, Nat. Rev. Immunol. 13 (6) (2013) 461–467.
[28] T.M. Carr, J.D. Wheaton, G.M. Houtz, M. Ciofani, JunB promotes Th17 cell identity and restrains alternative CD4$^+$ T-cell programs during inflammation, Nat. Commun. 8 (1) (2017) 301.