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Elevated profile of Th17, Th1 and Tc1 cells in patients with immune thrombocytopenic purpura

T-lymphocyte abnormalities are considered important in the pathogenesis of chronic immune thrombocytopenic purpura (ITP). Both CD4⁺ (Th) and CD8⁺ (Tc) T lymphocytes can be functionally divided into type 1 (T1) and type 2 (T2) subsets based on the secretion of cytokines. Since Semple¹ discovered an early Th0 and Th1 cell activation in children with chronic ITP, it has become evident a higher Th1 response was closely related to the etiology and status of chronic ITP.² Until now, there have been few studies on the Tc cell profile in ITP, and we only find one report which suggests that Tc1 cell response was predominant in active ITP patients.³ Th17 cells characterized by the production of IL-17 have recently been identified as a unique subset of Th cells.⁴ Considerable evidence suggests Th17 cells have been linked to the development of autoimmune diseases,^{5,6} so we presume that Th17 cells may be of importance in ITP. To further investigate the role of Th17, Th1 and Tc1 cells in the pathogenesis of ITP, we examined the levels and correlation of Th17, Th1 and Tc1 cells in ITP patients by intracellular cytokine analysis.

Thirty adult chronic ITP patients (16 women and 14 men; mean age 36, range 17-80 years) were enrolled by diagnostic criteria for ITP,⁷ and the platelet count ranged between 1 and 30×10⁹/L, with a median count of 11×10⁹/L. Patients with complications, i.e. viral hepatitis, diabetes, hypertension, cardiovascular diseases, pregnancy, active infection, or connective tissue diseases, were excluded. The control group consisted of 30 adult healthy volunteers matched for sex and age with the study population and platelet counts ranged from 136 to 298×10⁹/L, with the median count of 225×10⁹/L. Informed consent was obtained from each patient and the study was approved by the Medical Ethical Committee of Qilu Hospital of Shandong University.

Intracellular cytokines were studied by flow cytometry to reflex the cytokine-producing cells. Briefly, heparinized peripheral blood (400 µL) with an equal volume of RPMI 1640 medium was incubated for 4 h at 37°C, 5% CO₂ in the presence of 25 ng/mL phorbol myristate acetate (PMA), 1 µg/mL ionomycin, and 1.7 µg/mL Monensin (Alexis Biochemicals, San Diego, CA). After incubation, the cells were stained with PE-Cy5-conjugated anti-CD3 and FITC-conjugated anti-CD8 to delimitate CD4⁺ T cells because CD4 was down-modulated when cells were activated by PMA.⁸ After the sur-

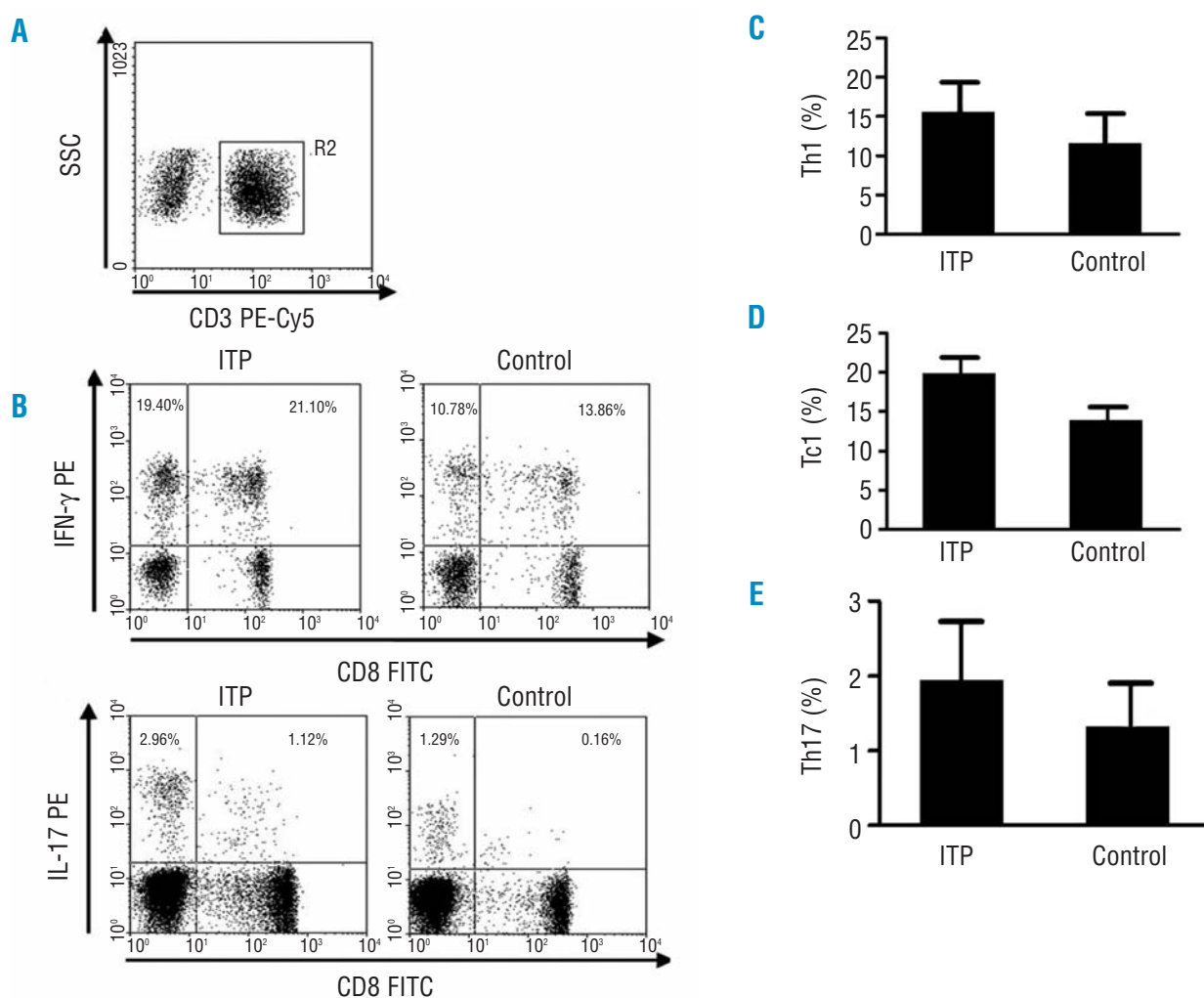


Figure 1. Circulating percentages of Th1, Tc1 and Th17 increased in ITP patients compared with controls. (A) CD3⁺ T subsets were gated by flow cytometry. Plots in intern box represented CD3⁺ T cells. (B) Representative IFN- γ and IL-17 expression in CD3⁺CD8 \neq T subsets (CD4⁺ T subsets) and CD3⁺CD8⁺ T subsets from each group was shown. The percentage of positive cells was shown in each panel. (C) The percentage of circulating Th1 was significantly higher in ITP patients (15.50 \pm 4.20%), as compared with the control group (11.6 \pm 4.23%) (p <0.01). (D) The percentage of circulating Tc1 was significantly higher in ITP patients (19.96 \pm 8.91%) than that in the control group (13.71 \pm 6.77%) (p <0.05). (E) The percentage of circulating Th17 was markedly higher in ITP patients (1.94 \pm 0.93%) than that of normal controls (1.33 \pm 0.64%) (p <0.05).

face staining, the cells were stained with PE-conjugated anti-IL-17A for Th17 detection or PE-conjugated anti-IFN- γ for Th1 detection after fixation and permeabilization. Isotype controls were given to enable correct compensation and confirm antibody specificity. All the antibodies were from eBioscience, San Diego, CA, USA. Stained cells were analyzed by flow cytometric analysis using a FACScan cytometer equipped with CellQuest software (BD Bioscience Pharmingen). Th17 and Th1 cells were identified as those that were CD3⁺CD8 \neq IL-17A⁺ and CD3⁺CD8 \neq IFN- γ ⁺, and Tc1 cells were those that were CD3⁺CD8⁺IFN- γ ⁺. In peripheral blood, the percentages of both Th1 and Tc1 in ITP patients increased significantly compared with controls (p <0.01 for Th1; p <0.05 for Tc1) (Figure 1C and D), and there was a significantly positive correlation between Th1 and Tc1 (r =0.58, p =0.01) in ITP patients (Figure 2A).

More importantly, the percentage of Th17 in patients with ITP was markedly higher than that of normal controls (p <0.05) (Figure 1E). Also, the percentage of Th17 positively correlated with Th1 (r =0.61, p =0.007) while there was no significant correlation between Th17 and Tc1 (r =0.09, p =0.71) (Figure 2B and C). Among the ITP patients, there were no statistical differences of the three kinds of cells tested between primary and recurrent ITP patients (p =0.18 for Th17, p =0.36 for Th1, p =0.35 for Tc1).

Our data strongly supported the hypothesis that the ITP patients were Th1 profile in accordance with previous reports,^{1,2} and demonstrated for the first time that up regulation of Th17 cells may be an important determinant in the evolution of ITP. In addition, the positive correlation of the percentages between Th17 and Th1 cells suggested that Th17 and Th1 cells may play a

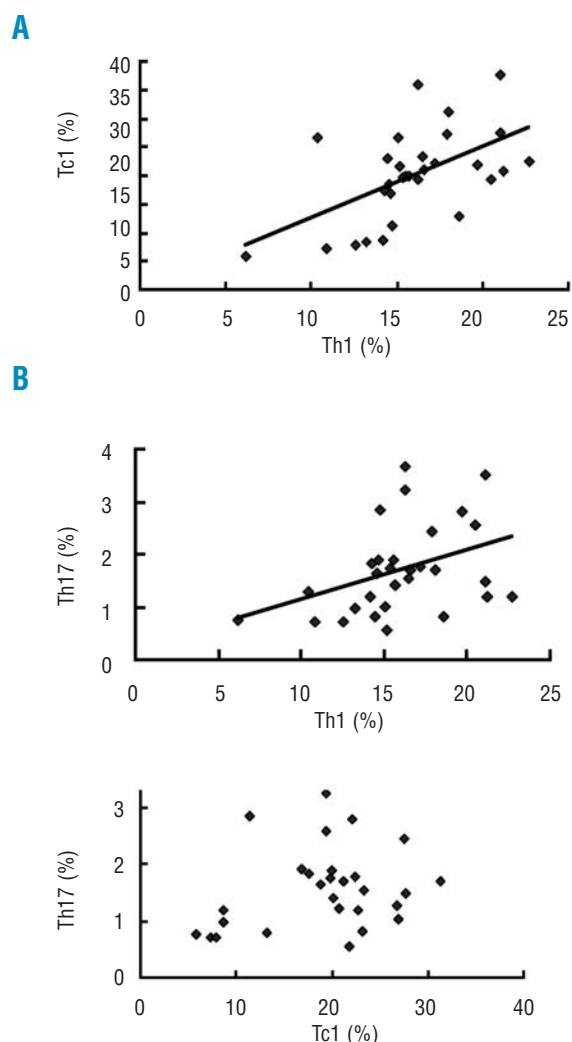


Figure 2. Correlations of Th1/Tc1, Th17/Th1 and Th17/Tc1 in ITP patients. (A) Th1 percentage positively correlated with the percentage of Tc1 ($r=0.58$, $p=0.01$). (B) Th17 percentage positively correlated with the percentage of Th1 ($r=0.61$, $p=0.007$). (C) There was no significant correlation between Th17 and Tc1 ($r=0.09$, $p=0.71$).

cooperative or synergetic function in the pathogenic mechanism of ITP, and the biological effects of Th17 cells by promoting a cytokine imbalance toward a Th1-type immune response may induce ITP. Also, our results suggested that Tc1 cell response was predominant in ITP, and there was a significantly positive correlation between Th1 and Tc1 in ITP. This strongly supported the hypothesis that cytotoxic T lymphocyte-mediated cytotoxicity was an alternative mechanism for platelet destruction in ITP, and demonstrated that the predominant Tc1 cells might develop and function under the influence of a particular microenvironment, probably alongside Th1 response. T-cell function derangement has been demonstrated in ITP with abnormal cytokine profiles correlated to loss of immune tolerance.^{9,10} Studies have suggested functional defects in CD4⁺CD25⁺ regulatory T (Treg) cells contributed to breakdown of self-tolerance in ITP.^{10,11} Therefore, the

imbalance of Th17/Treg in ITP patients needs to be clarified.

In summary, our study demonstrates Th17 may be an important determinant in the evolution of ITP along with Th1 and Tc1, suggesting that blocking the abnormality of Th17 cells is likely to be a promising therapeutic concept for ITP.

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Second bone marrow transplantation for patients with thalassemia: risks and benefits

A recent paper by Angelucci and Baronciani¹ discussed numerous aspects of thalassemia major with special attention to two dilemmas. The first is the choice between transplantation, which is still defined as the only curative treatment but bears chances of severe complications such as chronic extensive graft versus host disease (GVHD), and conservative treatment which will eventually lead to death. The second is the influence of the underdeveloped society setting on treatment possibilities. There is an additional unique group of patients that represent 10-20% of all transplanted patients, who to date have received little consideration in the literature: those who lost their graft. It seems that in these cases, marked erythropoietic hyperplasia contribute more to the graft failure than a robust host immune system. Gaziev *et al.* have shown improved results after a second BMT in thalassaemic patients, with an improved overall survival (OS) of 49% in older series² and 79%³ in a more recent cohort study.

Twenty-seven of our 107 thalassaemic patients transplanted from 1981 to 2008 experienced graft failure. In 18 patients thalassemia recurred; in 10 of these cases autologous back-up stem cell infusion was given due to primary graft failure. Nine patients proceeded to a second allogeneic BMT using the same donor (Table 1). As we did not include routine liver biopsy in our pre-transplant evaluation, we cannot accurately stratify our patients according to the Pesaro risk classification system. However, all of these 9 patients had hepatomegaly and inadequate iron chelation and, therefore, could be stratified to at least class II risk group, while we cannot rule out that some of them belonged to a higher risk category.

Of 18 patients who did not undergo a second transplantation, 2 died: one from veno-occlusive disease, sepsis and disseminated intravascular coagulation (DIC), and one from brain toxoplasmosis; one patient suffered from a severe peritransplant complication requiring frontal lobectomy due to intracranial hemorrhage during post-transplant aplasia. Fourteen patients are alive, transfusion dependent, treated by chelation and in good clinical condition. Nine patients received additional allogeneic hematopoietic stem cell therapy for treatment of graft failure: median follow-up is 24 (2-98) months from the second BMT. Seven BMTs were performed as a second approach to establish normal hematopoiesis; in 2 other cases BMT were performed as an emergency in the context of graft failure. Of these 9, 3 patients died from early complications of the second BMT (aGVHD, DIC, pulmonary hemorrhage). Two of them underwent a second BMT within six months of the first. Six of the 9 patients developed grade II-IV aGVHD, which progressed in 2 cases to extensive chronic GVHD. Two patients of the 6 surviving experienced severe life-threatening events (LTE) (Table 2). One patient experienced thalassemia

Table 1. Transplantation details for the 9 patients receiving a second allogeneic transplant.

	1st BMT	2nd BMT
Donor	Sibling 6 MFD 3	Same 9
Graft, TNC×10 ⁸ (X+SD)	11.3±12.3 BM-8, PBSC-1	14.2±12.7 (ns) BM-6, PBSC-3
Conditioning	TLIBuCy-5 BuCy-2 BuCyTT-1 FluBu(ATG)-1	Cy(HU)-3 TLIBuCy-1 BuCyMit-1 CyMPC1H-1 FluBuTTATG-1 FluMelTTTBI-1 FluTBI-1

BMT: bone marrow transplantation; MFD: matched family donor; BM: bone marrow; PBSC: peripheral blood stem cells; TL: total lymphoid irradiation; Bu: busulfex; Cy: cyclophosphamide; TT: thiotepa; Flu: fludarabine; ATG: anti-thymocyte globulin; HU: hydroxyurea; Mit: mitoxantrone; MP: methylprednisolone; C1H: camptoth-1H; TBI: total body irradiation; Mel: melphalane.

Table 2. Post-bone marrow transplantation course after second allogeneic transplantations.

Case n.	2 nd BMT associated LTE	aGVHD (grade II-IV)	Late consequences	Chimerism (donor)	Transfusion independence	Last follow-up (days)	Outcome
1	Yes ¹	Yes				151	Dead
2	Yes ¹	Yes				107	Dead
3	Yes ¹					68	Dead
4	Yes ²	Yes	Epilepsy, cGVHD	100%	Yes	400	Alive
5	Yes ³	Yes		100%	Yes	1020	Alive
6	No			10%	No	7263	Alive
7	No			40%	Yes (except stress)	1500	Alive
8	No	Yes	cGVHD	100%	Yes	724	Alive
9	No	Yes		100%	Yes	712	Alive

BMT: bone marrow transplantation; GVHD: graft versus host disease; ¹Multiorgan failure; ²Status epilepticus; ³Septic shock, acute respiratory distress syndrome.