Type 1 and Type 2 T-cell profiles in idiopathic thrombocytopenic purpura

Tingting Wang
Hui Zhao
He Ren
Jianhai Guo
Maoqiang Xu
Renchi Yang
Zhong Chao Han

Background and Objectives. Adult idiopathic thrombocytopenic purpura (ITP) is a chronic acquired organ-specific autoimmune hemorrhagic disease characterized by the production of antibodies against antigens on the membranes of platelet, resulting in enhanced Fc-mediated destruction of the platelets by macrophages in the reticuloendothelial system. Dysfunctional cellular immunity is considered important in the pathophysiology of ITP. The aim of this study was to explore the profile of type1 and type2 T cells in chronic ITP patients.

Design and Methods. The balance of Th1/Th2 and Tc1/Tc2 was studied by simultaneous analysis of intracellular cytokines of peripheral blood mononuclear cells and splenocytes in short-term cultures activated with PMA/ionomycin as well as mRNA expression of T-bet and GATA-3 in peripheral blood mononuclear cells and splenocytes using real-time polymerase chain reaction.

Results. Patients with active disease but not patients in remission had significant higher Th1/Th2 (p<0.01) and Tc1/Tc2 (p<0.01) ratios in peripheral blood (PB) and significant higher Th1/Th2 ratio in splenocytes (p<0.01) than those in the control group. The Tc1/Tc2 ratio in splenocytes in ITP patients was higher than that in control, but did not reach significant difference (p=0.082). GATA-3 mRNA expression in ITP patients was significantly lower both in PB (p<0.01) and in splenocytes (p<0.01) than in corresponding samples from controls while there was no difference in T-bet expression.

Interpretation and Conclusions. Our data indicate that ITP is a T1 cell (Th1 and Tc1) predominant disease although the precise mechanisms await further functional assay. The T-bet/GATA-3 ratio may provide a surrogate marker of T1/T2 cytokine balance. Shifting the cytokine patterns from T1 to T2 might be a potential immunotherapy for ITP.

Key words: idiopathic thrombocytopenic purpura, Th1, Th2, Tc1,Tc2.

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From State Key Laboratory of Experimental Hematology (TW, HZ, HR, RY, ZCH); Institute of Hematology and Blood Diseases Hospital (TW, HZ, HR, JG, MX, RY, ZCH), Chinese Academy of Medical Sciences and Peking Union Medical College, 288 Nanjing Road, Tianjin 300020, PR China.

Correspondence:
Dr. Renchi Yang, M.D., State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, CAMS and PUMC, 288 Nanjing Road, Tianjin 300020, PR China.
E-mail: yangrenchi@hotmail.com

Adult idiopathic thrombocytopenic purpura (ITP) is a chronic acquired organ-specific autoimmune hemorrhagic disease characterized by the production of antibodies against antigens on the membranes of platelet, resulting in enhanced Fc-mediated destruction of the platelets by macrophages in the reticuloendothelial system.[1,2] Although autoreactive B lymphocytes secreting antiplatelet antibodies are considered as the primary immunologic defect in ITP, dysfunctional cellular immunity is considered important in the pathophysiology of ITP.[3] The latter includes the presence of activated platelet-specific autoreactive T cells that recognize and respond to autologous platelet antigens and drive the generation of platelet reactive autoantibodies by B cells as well as the presence of T-cell mediated cytotoxicity and complement mediated lysis of platelet in ITP patients.[4-6] Both $CD4^+$(Th) and $CD8^+$(Tc) T lymphocytes can be divided into type 1 (IFN-γ, IL-2, TNF-β) and type 2 (IL-4, IL-5, IL-6, IL-10 and IL-13) subsets on the basis of their cytokine secretion profile. Type 1 (T1) cells secrete interferon (IFN)-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-β whereas type 2 (T299 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13.[7-11] However, human T1 and T2 subsets are usually defined according to IFN-γ/IL-4 production because the synthesis of IL-2, IL-6, and IL-10 is not stringently restricted to a single subset.[12] T1 and T2 cells play important immunoregulatory roles. Cytokines produced by distinct T helper and T cytotoxic subsets are critical to the effectiveness of immune response. The dysregulated expansion of one of these subsets may contribute to the impaired T-cell-mediated response in clinical states

such as infections, autoimmunity or cancer.[13] T-box expressed in T cells (T-bet) and GATA- binding protein 3 (GATA-3) are two major T transcription factors that regulate the expression of T1 or T2 cytokine genes and play a crucial role in T-cell differentiation.[14-19]

Several studies have documented the Th cell profile in ITP patients *in vitro*.[20-26] Until now, there had been no study on the profile of Tc cells in ITP and on the expression of the two important transcription factors: T-bet and GATA-3. To the best of our knowledge, this is the first study to examine simultaneously both Th1/Th2 balance and Tc1/Tc2 balance by analysis of intracellular cytokines and to examine the mRNA expression of T-bet and GATA-3 in peripheral blood mononuclear cells (PBMC) and splenocytes in ITP. In addition, this study revealed T1 and T2 profiles in different clinical phases of ITP.

Design and Methods

Patients

After approval by institutional review board, 30 adults with chronic ITP (22 women and 8 men; mean age 34 years) were studied. Informed consent was obtained from each participating patients. The diagnosis of chronic ITP was based on the criteria reported previously which are consistent with the ASH guidelines[27] except that thrombocytopenia was defined as a platelet count less then 100×10^9/L,[28] i.e. persistent thrombocytopenia (platelet counts < 100×10^9/L) (The normal range of platelet count of Chinese is 100-300×10^9/L)[29] for at least 6 months, no hepatosplenomegaly or lymphadenopathy, normal or increased numbers of megakaryocytes in bone marrow aspirates, negative antinuclear antibody and HIV serological test, and absence of underlying conditions such as systemic lupus erythromatosus (SLE), lymphoproliferative disorders, and congenital or hereditary thrombocytopenia. The clinical and laboratory findings of those subjects are summarized in Table 1. At the time of blood sample collection, 20 of the 30 patients had active disease and 10 were in remission (platelet count >100×10^9/L). Ten of 30 patients were receiving prednisolone, two were receiving intravenous immunoglobulin G (IVIG) and one both IVIG and prednisolone. The control group consisted of 20 adult healthy volunteers (13 women and 7 men; mean age 31 years). At the time of blood sample collection, the platelet count of patients with active disease ($29.6\pm3.5\times10^9$/L) was significantly lower than that of patients in remission ($186.5\pm18.7\times10^9$/L) ($p<0.01$) or of the controls ($209.0\pm15.4\times10^9$/L)($p<0.01$). The CD4/CD8 ratio of patients with active disease (1.32±0.15) was also significantly lower than that of patients in remission (1.75±0.14) ($p<0.05$) or of the controls (1.68±0.10) ($p<0.05$).

Table 1. Clinical and laboratory parameters of ITP patients

Patients	*Sex*	*Age (year)*	*PLT (10^9/L)*	*CD4/CD8 ratio*	*Therapy*
PB					
Active patients					
P1	M	46	22	2.86	Pred
P2	M	52	26	0.80	–
P3	M	42	13	1.28	Pred
P4	M	32	14	1.31	Pred
P5	M	41	9	1.40	Pred
P6	F	51	12	0.34	Pred
P7	F	36	21	1.69	–
P8	F	25	39	0.55	–
P9	F	35	24	0.97	Pred
P10	F	16	45	2.60	IVIG
P11	F	51	41	1.00	–
P12	F	18	37	0.71	–
P13	F	19	25	1.86	–
P14	F	16	18	1.05	Pred+ IVIG
P15	F	21	21	1.00	Pred
P16	F	24	37	1.72	–
P17	F	48	27	1.76	–
P18	F	20	45	0.80	–
P19	F	54	56	0.76	IVIG
P20	F	35	39	1.89	–
Patients in remission					
P21	M	17	187	2.50	–
P22	M	21	210	1.60	–
P23	M	19	130	2.01	Pred
P24	F	21	123	1.69	Pred
P25	F	40	145	1.21	–
P26	F	41	169	1.52	–
P27	F	58	230	2.19	–
P28	F	55	321	1.73	–
P29	F	43	149	2.07	Pred
P30	F	48	201	1.00	–
SP					
P31	M	42	30	–	Pred
P32	M	27	12	–	Pred+ IVIG
P33	M	31	45	–	Pred
P34	F	41	50	–	Pred
P35	F	33	21	–	Pred+ IVIG
P36	F	47	11	–	IVIG
P37	F	39	23	–	Pred+ IVIG
P38	F	26	49	–	Pred

CD4/CD8 ratio was analysis by flow cytometry, which gated on CD3+ cells and measured CD3+CD4+ cells and CD3+CD8+ cells. PB: peripheral blood; SP: splenocytes.

Spleen tissue was obtained from splenectomy performed for clinical indications in eight ITP patients (five women and three men; mean age, 30 years) and six patients with hereditary spherocytosis (HS) (three women and three men; mean age 35 years). Splenocytes were prepared from about 10g of the spleen tissue. The splenocytes from HS patients were considered as control cells. The platelet count of these ITP patients ($30.1\pm5.7\times10^9$/L) was significantly lower than that of patients with HS (212.5±

25.6×10^9/L) on admission. After short-term therapy with prednisolone and/or (IVIG), the mean platelet count of ITP patients had reached 71×10^9/L prior splenectomy.

Preparation of peripheral blood mononuclear cells (PBMC) and splenocytes

PBMC were isolated from heparinized venous blood using Lymphoprep density gradient centrifugation. Splenocytes were prepared as previously described.[30] Briefly, sterile spleen tissue in RPMI 1640 (JR Scientific Inc., Wooldland, CA, USA) was washed twice to remove peripheral blood and crushed with a syringe plunger. After all the cells had been dissociated, the cell suspension was filtered through a nylon mesh and subjected to Lymphoprep density gradient centrifugation. The recovered cells suspended in complete culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mM), penicillin (100U/mL), streptomycin (100 μg/mL) were used as splenocytes.

Intracellular cytokine analysis

Intracellular cytokines were studied by flow cytometry as described previously.[31-32] Briefly, heparinized peripheral whole blood (500 μL) with an equal volume of RPMI 1640 medium or about 1×10^6 splenocytes in complete culture medium were incubated in flat-bottomed 24-well tissue culture plates (B-D PharMingen, San Diego, CA, USA) for 4h at 37°C, 5% CO_2 in the presence of 25 ng/mL of PMA, 1 μg/mL of ionomycin and 10 ug/mL of BFA (Sigma, St Louis, MO, USA). After two washes with wash buffer (phosphate-buffered saline containing 0.5% bovine serum albumin and 0.1% sodium azide), the cells were stained with PreCP-conjugated anti-CD4 or anti-CD8 monoclonal antibodies (B-D PharMingen) at room temperature in the dark for 15 min. After washing with 2 mL wash buffer, erythrocytes were lysed with FACSTM Lysing solution (B-D PharMingen), and the remaining cells were permeabilized using FACSTM Permeabilizing solution (B-D PharMingen). After another wash with wash buffer, the cells were stained with FITC-conjugated anti- IFN-γ and PE-conjugated anti-IL-4 monoclonal antibodies (B-D PharMingen) at room temperature in the dark for 30 min. As the last step, the cells were washed with wash buffer and resuspended in 1% paraformaldehyde for analysis by three-color flow cytometry (B-D PharMingen). Analysis gates were set on lymphocytes according to forward and side scatter properties. All cells gated as lymphocytes were on $CD4^+$ or $CD8^+$ cells according to Becher *et al.*[32] At least 10 000 $CD4^+$ or $CD8^+$ cells were collected and analyzed. Data were analyzed using CellQuest software and display as dot plots of IFN-γFITC vs IL-4 PE. IgG1 pre-CP, IgG2b FITC and IgG1 PE (B-D PharMingen) were used as isotype controls. We defined the cell populations as follows: $CD4^+$ cell population- Th1, IFN-γ positive and IL-4 negative; Th2, IFN-γ negative and IL-4 positive; $CD8^+$ cell population: Tc1, IFN-γ positive and IL-4 negative; Tc2, IFN-γ negative and IL-4 positive.

Isolation of total RNA from PBMC and splenocytes and reverse transcription

Total RNA of the PBMC and splenocytes (1×10^6) was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, USA). The reverse transcription reactions were done using the Superscript First-Strand Synthesis System (Invitrogen Life Technologies) following the manufacturer's procedure.

Measurement of T-bet and GATA-3 mRNA expression by real-time quantitative PCR

Real-time PCR was performed using an ABI 7000 Sequence Detection System (PE Applied-Biosystems) in the presence of SYBR-green. The QuantiTect™ SYBR® Green RT-PCR Kit was purchased from Qiagen (Qiagen, Hilden, Germany). Amplification was performed in a total volume of 25 μL containing 12.5 μL of kit-supplied QuantiTectTM SYBR® Green RT-PCR Master mix (including HotStar Taq DNA polymerase, reaction buffer, dNTP mix, and SYBR dye), 0.5 μl of each primer (50 ng/μL), 2ul of cDNA (100 ng RNA). PCR primer sequences are as follows: β-actin internal control - forward: 5'-GTGACGAGGCCCAGAGCAAGAG-3', reverse: 5'- ACGCAGCTCATTGTAGAAGGTGTGG-3'. T-bet mRNA – forward: 5'- TGGGTGCAGTGTGGAAAGGC-3', reverse: 5'- ACTGGAGCACAATCATCTGGG-3'; GATA-3 mRNA – forward: 5'-AGGACGAGAAAGAGTGCC-3', reverse: 5'-GAAGAGTCCGGAGCTGTAC -3'. The PCR cycling parameters were set as follows: 95°C for 15 min followed by 40 cycles of PCR reacting at 94°C for 15 s, 60°C for 1 min and 72°C for 45 s. All measurements were conducted in triplicate wells. After PCR, a melting curve was obtained by increasing the temperature from 60°C to 95°C with a temperature transition rate of 0.10C/s. The melting curve of all final PCR products allowed us to distinguish genuine products from non-specific products, and primer dimers. To ensure that the correct product was amplified in the reaction, all samples were also visualized on ethidium bromide-stained 2.5% agarose. T-bet, GATA-3 and β-actin mRNA expression cDNA fragments were detected at 175bp, 170bp, and 123bp. The quantity of the T-bet and GATA-3 mRNA expression was therefore calculated assuming 100% efficient PCR in which each C_T value of the reactions was normalized to β-actin mRNA expression, as shown by the equation

$$2 -\{[Ct1\ (target)-Ct1\ (\beta\text{-actin})]- [Ct2\ (target)-Ct2\ (\beta\text{-actin})]\}$$

The Ct1 (target) and Ct2 (target) represent the Ct value for the target gene expression (T-bet or GATA-3) in the group of ITP patients (in active or remission state) and control group sample, respectively. Ct1 (β-actin) and Ct2 (β-actin) represent the Ct value for the β-actin gene expression in in the group of ITP patients (in active or remission state) and control group sample, respectively.

Statistical analysis

Differences in the cytokine levels and mRNA expression between the two groups were analyzed by Student's T test. A p value of <0.05 was considered to be statistically significant.

Results

Intracellular cytokines of CD4⁺ T cells in peripheral blood (PB)

The percentage of Th1 cells was not significantly different in patients with active ITP (mean 16.90, 95% CI 6.90-25.98) and in the control group (mean 17.50, 95% CI 13.92-21.07) (p=0.113) based on cytokine patterns after *in vitro* activation by PMA/ionomycin in short-term cultures. The percentages of Th1 cells in patients in remission state (mean 13.34, 95% CI 4.04-22.63) and the control group were similar (p=0.381).

The percentage of Th2 cells (mean 1.33, 95% CI 0.27-2.09) in patients with active ITP was significantly lower than that in the control group (mean 2.29, 95% CI 1.76-2.82) (p<0.001). In contrast, the percentage of Th2 cells was not significantly different between patients in remission state (mean 2.89, 95% CI 0.43-5.64) and the control group (p=0.98).

The intracellular IFN-γ/IL-4 ratio in the CD4⁺ T cells (Th1/Th2) was significantly higher in patients with active ITP (mean 25.62, 95% CI 15.05-35.94) than in the control group (mean 9.86, 95% CI 6.11-13.60) (p=0.002) while there was no significantly difference between the IFN-γ/IL-4 ratio of patients in remission (mean 8.29, 95% CI 2.75-14.23) and that in the control group (p=0.823) (Figures 1-3,Table 2).

Intracellular cytokines of CD8⁺ T cells in PB

The percentage of Tc1 cells was not significantly different between patients with active ITP (mean 26.86, 95% CI 14.33-36.82) and the control group (mean 22.91, 95% CI 17.99-27.83) (p=0.654) based on cytokines pattern after *in vitro* activation by PMA/ionomycin in short-term cultures. Similarly, there was no significant difference in Tc1 cells percentage between patients in remission state (mean 14.83, 95% CI 2.38 -22.31) and the control group (p=0.182).

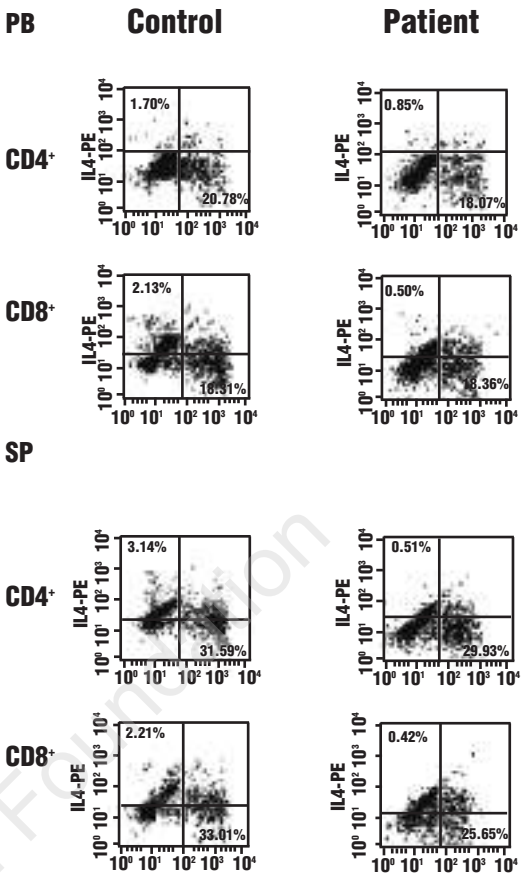

Figure 1. Dot plot of the intracellular cytokine analysis by flow cytometry PB: peripheral blood SP: splenocytes.

The percentage of Tc2 cells was significantly lower in the patients with active ITP (mean 0.84, 95% CI 0.61-1.09) than in the control group (mean 2.30, 95% CI 1.55-3.06) (p<0.001). In contrast, the percentage of Tc2 cells was not significantly different in patients in remission (mean 1.42, 95% CI 0.74-2.40) and in the control group (p=0.258).

The intracellular IFN-γ/IL-4 ratio in the CD8⁺ T cells (Tc1/Tc2) was significantly higher in patients with active ITP (mean 30.23, 95% CI 18.49-37.57) than that in the control group (mean 12.58, 95% CI 9.40-15.76) (P=0.004) while there was no significant difference between that in remission patients (mean 10.10, 95% CI 3.52-16.68) and that in the control group (p=0.498) (Figures 1-3,Table 2).

Intracellular cytokines of CD4⁺ T cells and CD8⁺ T cells in splenocytes

The percentage of Th1 cells in splenocytes based on cytokine pattern after *in vitro* activation by PMA/ionomycin in short-term cultures was not sig-

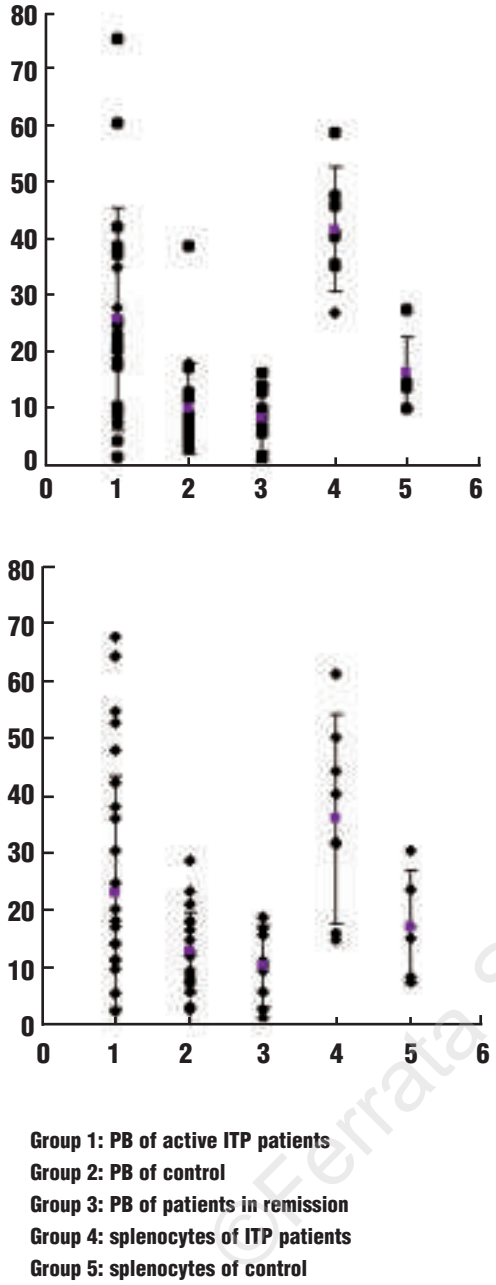

Figure 2. Scatter plot of the intracellular cytokine analysis by flow cytometry

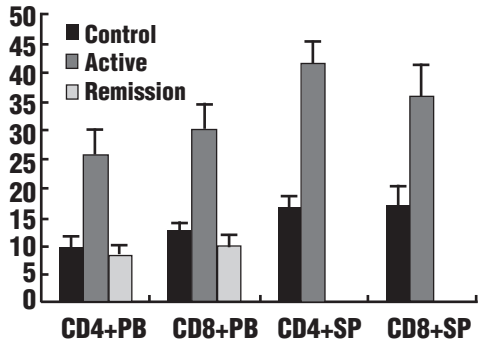

Figure 3. Th1/Th2 and Tc1/Tc2 ratio in PB and SP according the intracellular cytokine analysis by flow cytometry

Table 2. Results of the intracellular cytokine analysis.

	Active patients	*Patients in remission*	*Control*
PB			
Th1	16.90 (6.90-25.98)	13.34 (4.04-22.63)	17.50 (13.92-21.07)
Th2	1.33 (0.27-2.09)°	2.89 (0.43-5.64)	2.29 (1.76-2.82)
Th1/Th2	25.62 (15.05-35.94)°	9.86 (6.11-13.60)	8.29 (2.75-14.23)
Tc1	26.86 (14.33-36.82)	14.83 (2.38 -22.31)	22.91 (17.99-27.83)
Tc2	0.84 (0.61-1.09)°	1.42 (0.74-2.40)	2.30 (1.55-3.06)
Tc1/Tc2	30.23(18.49-37.57)°	10.10 (3.52-16.68)	12.58 (9.40-15.76)
SP			
Th1	24.69 (2.55-46.82)	–	19.91(3.87-35.95)
Th2	0.59 (0.05-1.12)	–	1.33 (0.15-2.51)
Th1/Th2	41.46 (29.89-53.02)°	–	16.30(8.48-24.13)
Tc1	23.11 (1.16-45.07)	–	32.66(9.53-55.78)
Tc2	0.99 (0.18-2.17)	–	2.23(1.16-3.30)
Tc1/Tc2	35.80 (16.75-54.85)	–	16.88(4.62-29.14)

*Data were shown as mean (95% CI).*p<0.05 ; °<0.01 compared with control; PB: peripheral blood; SP: splenocytes.*

nificantly different between ITP patients (mean 24.69, 95% CI2.55-46.82) and the control group (mean 19.91, 95% CI 3.87-35.95) (p=1.000). The percentage of Th2 cells was lower in patients with ITP (mean 0.59, 95% CI 0.05-1.12) compared to that in the control group (mean 1.33, 95% CI 0.15-2.51) but did not reach significant difference (p=0.247). The intracellular IFN-γ/IL-4 ratio in the $CD4^+$ T cells (Th1/Th2) was significantly higher in patients with ITP (mean 41.46, 95% CI 29.89-53.02) than that in the control group (mean 16.30, 95% CI 8.48-24.13) (p=0.009) (Figures 1-3,Table 2).

The percentage of Tc1 cells was not significantly different between patients with ITP (mean 23.11, 95% CI 1.16-45.07) and the control group (mean 32.66, 95% CI 9.53-55.78) (p=0.537). The percentage of Tc2 cells (mean 0.99, 95% CI 0.18-2.17) was lower in patients with ITP compared to that in the control group (mean 2.23, 95% CI 1.16-3.30) but it did not reached significant difference (p=0.177). The intracellular IFN-γ/IL-4 ratio in the $CD8^+$ T cells (Tc1/Tc2) was higher in patients with ITP (mean 35.80, 95% CI 16.75-54.85) than that in the control group (mean 16.88, 95% CI 4.62-29.14) but it did not reached significant difference (p=0.082) (Figures 1-3,Table 2).

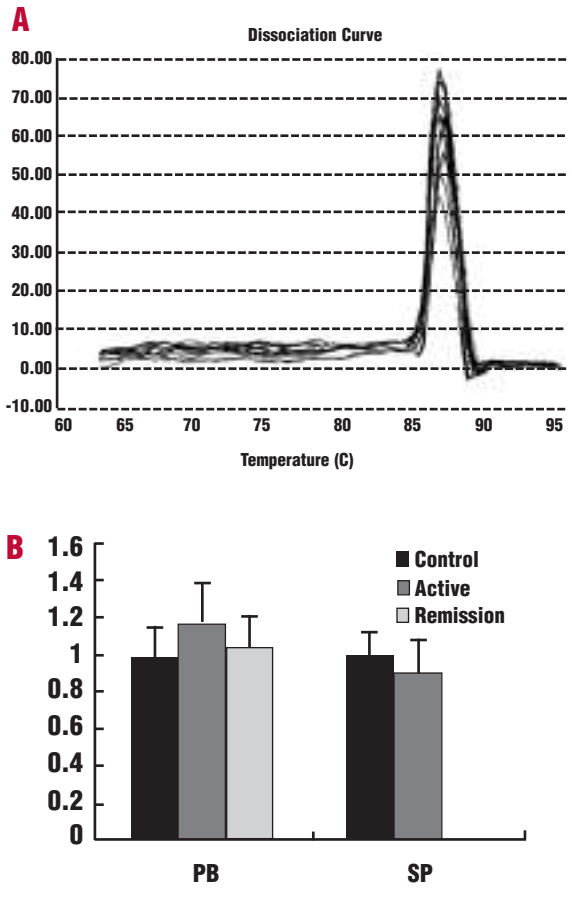

Figure 4. Results of T-bet mRNA expression in real-time PCR. PB: peripheral blood; SP: splenocytes. Results were expressed as mean±standard error of the mean.

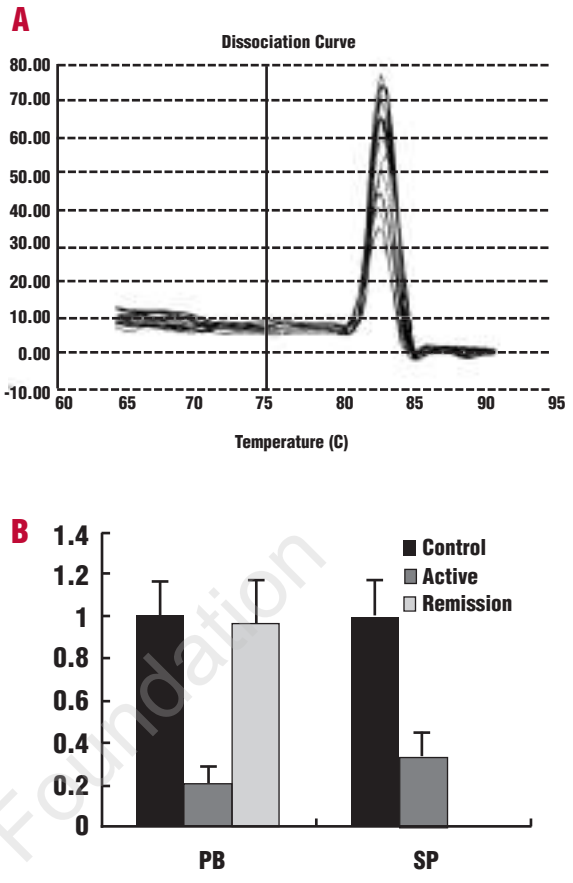

Figure 5. Results of GATA-3 mRNA expression in real-time PCR. PB: peripheral blood; SP: splenocytes. Results were expressed as mean±standard error of the mean.

Quantitative analysis of T-bet mRNA expression in PB and splenocytes

Relative quantitative analysis of T-bet mRNA expression by real-time PCR showed that in PB, there was no significant difference between active ITP patients and control group (mean 1.17 folds, range 0.61-2.23, p=0.21), and between patients in remission state and the control group (mean 1.04 fold range 0.71-2.04, p=0.42) (Figure 4). Similarly, there was no significant difference between T-bet mRNA expression in splenocytes from ITP patients and that from control group (mean 0.91 fold, range 0.52-1.58 p=0.19). The melting curve was shown in the Figure 4.

Quantitative analysis of GATA-3 mRNA expression in PB and splenocytes

GATA-3 mRNA expression by relative quantitative analysis by real-time PCR was significantly lower in PB of active ITP patients compared to that of the control group (mean 0.20 fold, range 0.11-0.36, p=0.003) but with no difference between patients in remission state and the control group (mean 0.97fold, range 0.66-2.09, p=0.34). Similarly, there was significantly lower GATA-3 mRNA expression in splenocytes from ITP patients compared to that from the control group (mean0.34 fold, range 0.23-0.61 p=0.007) (Figure 5). The lower GATA-3 level was consistent with the results described above that active ITP patients had lower IL-4 level and higher T1/T2 ratio.

The T-bet/GATA-3 ratio according to the results of real-time PCR

As shown in Figure 6, the mean T-bet/GATA-3 ratio was significantly increased in PB of active patients and in splenocytes of ITP patients (mean 5.85 and 2.68 fold, respectively, p<0.001) while the ratios in PB of remission patients and in the control were similar (mean 1.07 fold, p=0.39).

Discussion

Th1 cells are responsible for cell-mediated immune defense against intracellular pathogens. They also

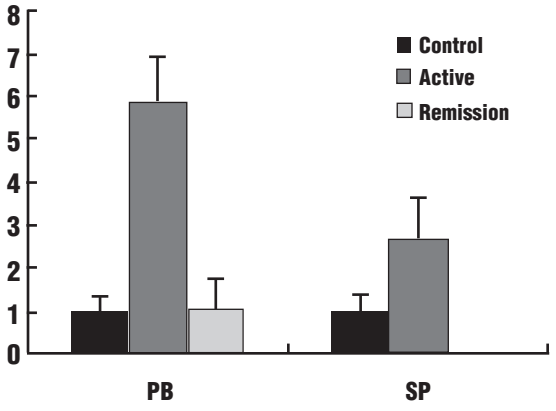

Figure 6. The T-bet/GATA-3 ratio in real-time PCR. PB: peripheral blood. SP: splenocytes. Results were expressed as mean±standard error of the mean.

play a central role in immunopathology of organ-specific autoimmune diseases.[33-38] Th2 cells defend the host against extracellular parasites such as helminthes and take part in atopic and allergic reaction.[39] The Th1/Th2 balance is well known to regulate the immune system under normal condition, and is known to be impaired in many autoimmune diseases. Several studies have found evidence supporting a Th1 polarization of the immune response in ITP in vitro,[20-24] whereas others have yielded inconsistent[25] or opposing[26] results. This study employed flow cytometry to assess intracellular IL-4 and IFN-γ production at single cell level. PMA/ionomycin were used for the induction of cytokine genes studied because they induce the expression of cytokine genes, not randomly, but according to the differentiation program of each Th phenotype.[40] Our study suggested that active ITP patients had significant higher Th1/Th2 ratio after *in vitro* activation by PMA/ionomycin in short-term cultures because of a decrease in the number of Th2 cells. In addition, our finding showed that Th1/Th2 ratio approached normal range when the disease was in remission.

In addition to their classic role in the killing of infected cells, $CD8^+$ T cells play a role in the regulation of $CD4^+$ cells. This regulation could be mediated through secreted products (cytokines, chemokines) or by cell-cell interaction. $CD8^+$ T cells alter the balance of Th1/Th2 responses *in vivo*[41-42] by influencing the development of IL-4 -or IFN-γ-secreting $CD4^+$ cells.[43-44] In addition, $CD8^+$ T cells appear to play a role in the development of $CD4^+$ T cells perforin-mediated cytoxicity[43] and also have been reported to suppress $CD4^+$ T cells proliferative responses[45-46] through the inhibition of costimulatory interactions. $CD8^+$ T cells are also capable of influencing other components of the immune response, such as regulation of antibody production by B cells[47-51] and activation of macrophages.[52]

The importance of distinct subsets of $CD4^+$ T lymphocytes in the etiology of a variety of immune-mediated diseases has become clear in the past 10 years. However, the role of Tc subsets has been less well defined. Vukmanovic-Stejic *et al.*[12] found Tc1 clones favored the development of CD4 effectors that were Th1-biased, whereas Tc2 clones had the opposite effect. They also demonstrated that Tc2 clones not only could promote Th2 effectors but also could efficiently suppressed the development of Th1 cells. It seems likely that Tc1 and Tc2 cells develop and function under the influence of a particular microenvironment, probably alongside Th1 and Th2 response respectively.

The profile of Tc1/Tc2 has been studied in other autoimmune disease.[31,53-55] Our results suggested that in PB, Tc1 cell response was predominant in active ITP patients based on cytokine pattern after in vitro activation by PMA/ionomycin in short-term cultures, which returned to normal when disease remitted. Several factors determined the fate of activated T cells, including antigen form, dose, type of antigen presenting cells, co-stimulatory molecules, chromatin structure, and most importantly, cytokines present in the local environment of the cell at the time of stimulation.[19] The development of primary CD8 T cell responses is thought to be assisted directly by $CD4^+$ T cells in two ways: (i) the activation of professional APC, which increase their ability to drive costimulation to Ag-specific naïve CD8 T cells and (ii) the secretion of cytokines that facilitate CTL expansion and activity.[56] A cross-regulation between these T cells subsets can occur, so that production of Th1/Tc1-type cytokines inhibits the development and activation of Th2/Tc2 cells and vice versa.[57-58] It may be that the predominant Tc1 cells develop and function under the influence of a particular microenvironment, probably alongside Th1 response. Although the precise mechanisms await further functional assay, $CD8^+$T cells may play a dual role as effectors and modulator cells in patients with active ITP.

The spleen is thought to play an important role in the pathogenesis of ITP, because about 80% of adult ITP patients achieve a stable increased platelet count after splenectomy.[59] In chronic ITP, spleen is considered to be the primary site of both platelet destruction and anti-platelet antibodies production.[60] Kuwana *et al.* reported that spleen was a primary site for activation of platelet-reactive T and B cells in patients with ITP.[30] Although Panitsas *et al.*[23] thought the polarized immune activity detected in PBMCs could reflect the nature of the localized autoimmune process taking place in the target organs of ITP such

as spleen, we think it is necessary to confirm this in the splenocytes directly. In our study, we found a Th1 polarized immune response in splenocytes of ITP patients after in vitro activation by PMA/ionomycin in short-term cultures, which was in accordance with that found in PB. The Tc1 cell was higher than that in the controls, but it did not reached significant difference. This result may be a reflection of the adjunctive role of the change in Tc subset. However, we cannot exclude the limitation relating to the small size of the sample and the bias of the control selection.

T-bet is a member of the T-box family of transcription factors, whose express is primarily limited to the immune system. T-bet is rapidly induced in the early developing Th1 cells but is absent in the developing Th2 cells.[14-15] Introduction of T-bet into polarized CD4 Th2 cells and their CD8 counterparts, Tc2 primary cells, has been found to result in the conversion of these cells respectively into Th1 and Tc1 cells, as evidenced by their production of IFN-γ and repression of IL-4 and IL-5 production.[14] In these studies, T-bet appeared to simultaneously induce Th1 and Tc1 differentiation and inhibited Th2 and Tc2 differentiation although it is not required for IFN-γ gene transcription in the CD8 T cell lineage.[61] In our study, we investigated the T-bet expression in mRNA level in ITP patients and in normal controls to determine if it played a role in the immune imbalance of ITP. We found no significant difference between our study groups.

GATA-3 is a member of the GATA family of zinc finger proteins, and plays a pivotal role in the development of the Th2 phenotype while inhibiting Th1 cells.[16-17]GATA-3 significantly down-regulates IFN-γ production from developing Th1 cells in addition to inducing IL-4 and IL-5 levels.[62-64] In fact, GATA-3 is expressed throughout T cell development, from common lymphoid precursors in the bone marrow and the earliest DN1 precursors in the fetal thymus[65-66] to mature SP thymocytes.[67] Hernandez-Hoyos *et al.*[68] found that the level of GATA-3 activity could play an important role as a negative regulator of the CD8 differentiation program and simultaneously as a positive regulator of CD4 development. GATA-3 therefore has reciprocal roles in the generation of CD4 and CD8 mature T cells, favoring the former and inhibiting the latter. The reciprocal effect on CD4/CD8 ratios indicates that regulation of CD4/CD8 differentiation is likely to be a normal function of GATA-3 in vivo. Nartijn *et al.*[67] also found that peripheral $CD8^+$ T cells from CD2-GATA-transgenic mice manifested functional defects in IL-2 and IFN-γ production and thus assumed a parallel role for GATA-3 in Tc1/Tc2 development. As reported by others,[69-71] we also found that in patients with ITP, a reduced ratio of CD4/CD8 was associated with active disease, with an improvement of this ratio in remission in our study (Table 1). In our study, GATA-3 mRNA expression was significant lower in patients with active disease, which corroborates our results described above that active ITP patients had lower IL-4 level, lower CD4/CD8 ratio and higher T1/T2 ratio. Our data suggested that GATA-3 might play an important role in ITP.

Chakir *et al.*[19] suggested that T-bet and GATA-3 were up-regulated in several cells that produce T1 and T2 cytokines and could be analyzed readily by RT-PCR using total RNA isolated from mixed cell populations or cultured splenocytes thereby providing a surrogate marker of T1/T2 cytokine balance under a variety of conditions. In comparison to methods which require long isolation procedures for preparation of cells, RT-PCR measurement of T-bet and GATA-3 in freshly isolated tissue might provide an immediate snapshot of T1/T2 cytokine status reflecting the in vivo biology. They also found the T-bet/GATA-3 ratio in mRNA level could reflect T(h)1 and T(h)2 cytokine gene and protein expression in mixed population of cells. They suggested that GATA-3 gene expression was more responsive than T-bet under both T(h)1 and T(h)2 condition. It was the relative expression of T-bet and GATA-3, rather than the expression of either transcription factor, that was more representative of T(h)1/T(h)2 cytokine balance. In our study, we also found the change in GATA-3 expression was proportionally greater than that in T-bet, and this was reflected in the T-bet/GATA-3 ratio which was more than five fold in PB of active patients compared to that of controls (Figure 6). In conclusion, our present study indicates that ITP is a T1 cells (Th1 and Tc1) predominate disease though the precise mechanisms await further functional assay. The T1 arm is thought to be required for effective clearance of appropriate effector cells and isotype switching to opsonizing or complement-fixing antibodies.[57] Defects in T1 immunity result in clinically significant immunodeficiency syndromes.[72-73] In contrast, unregulated T1 response may cause organ-associated immunopathology of autoimmune reactions.[74] Based on these observations, a potential immunotherapy for autoimmunity is to shift the cytokine patterns from T1 to T2. This approach has been successful in many experimental models of autoimmune diseases.[75-76] The assessment of T1/T2 balance in ITP may contribute to further understanding of the mechanism of ITP. GATA-3 may provide a new target for therapy.

TW did the experiments including preparation of PBMCs and splenocytes, intracellular cytokine analysis, isolation of total RNA from PBMCs and splenocytes, reverse transcription and real-time PCR. HZ participated in the intracellular cytokine analysis; HR participated in the real-time PCR. JG and MX contributed to the sample collection and data analysis. RY and ZCH contributed to the concept and design of the study and its revision, gave final approval, obtained funding and provided administrative support. All authors were involved in the preparation and revision of the manuscript and have approved the final version of the manuscript.

The authors are listed in an order based on the contributions they made to the experiments. The authors declare that they have no potential conflicts of interests. The authors would also like to thank Prof. Man-Chiu Poon (University of Calgary, Canada) for critical review of the manuscript.

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