Article

Polarization and Apoptosis of T Cell Subsets in Idiopathic Thrombocytopenic Purpura

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It is well-known that idiopathic thrombocytopenic purpura (ITP) is an acquired organ-specific autoimmune hemorrhagic disease and dysfunctional cellular immunity is considered important in the pathophysiology of ITP. However, polarization patterns and apoptosis profiles of T lymphocytes remain unclear. In this study, we investigated the polarization of T cell subsets, the expressions of apoptotic proteins Fas/FasL on the subsets and the level of anti-apoptotic gene bcl-2 and bax mRNA. It was demonstrated that the ratios of Th1/Th2 and Tc1/Tc2 in ITP children were increased obviously and that the average percentages were increased clearly for Th1 and Th2, but not for Tc1 and Tc2. In ITP children, the enhancing expressions were detected for FasL on Th1 and Tc1 and for Fas on Th2 and Tc2. With increasing level of bcl-2 mRNA and decreasing expression of bax mRNA in ITP children, the ratio of bcl-2/bax mRNA was improved obviously, which was positive correlated with the ratio of Th1/Th2. Taken together, our findings indicate that ITP is a Th1 predominant disease. This polarization pattern of T cell subsets might be related to the high ratio of bcl-2/bax mRNA and the abnormal expressions of Fas and FasL on T cell subsets. *Cellular & Molecular Immunology*. 2005;2(5):387-392.

Key Words: idiopathic thrombocytopenic purpura, polarization, apoptosis, T cell subset

Introduction

Idiopathic thrombocytopenic purpura (ITP) is an acquired organ-specific autoimmune thrombocytopenic syndrome characterized by the production of autoantibodies against antigens on the membranes of platelet, resulting in enhanced Fc-mediated destruction of the platelets by macrophages in the reticuloendothelial system (1). However, recent researches noticed that lymphocytosis and apoptotic deficiency in active lymphocytes and abnormal polarization between Th1 type cells and Th2 type cells in peripheral blood might play an important role in ITP pathogenesis (2-4), but until now, there is no final viewpoint about the polarization and apoptosis of T cells in ITP (5-7), especially the expressions of apoptotic protein Fas, FasL on Th1 type and Th2 type cells and the polarization of Tc1/Tc2 had not been reported clearly so far. Therefore, in this study it was to determinate the polarization of T cell subsets (Th1/Th2, Tc1/Tc2) and the expressions of apoptotic protein Fas, FasL on T cell subsets by FCM, and the levels of anti-apoptosis gene bcl-2/bax mRNA in T cells by RT-PCR, and to analyze the effects of Fas/FasL on T cell subsets and bcl-2/bax gene on the polarization of T cell subsets, and to clarify the probable role of abnormal polarization of T cell subsets and the disturbed expressions of Fas/FasL, bcl-2/bax mRNA in ITP immunopathogenesis.

Chemoattractant receptor-homologs molecule expressed on Th2 cells (CRTH2) is a novel surface marker expressed on Th2 cells and Tc2 cells, which has been showed to be a second receptor for prostaglandin D_2 (PGD_2) (8). In this study, anti-CRTH2-PE mAb was used to detect Th2 cells and Tc2 cells in purified peripheral blood $CD3^+$ T cells by flow cytometry. That was, $CD4^+CRTH2^-$ T represented for Th1, $CD4^+CRTH2^+$ T for Th2, $CD8^+CRTH2^-$ T for Tc1 and $CD8^+CRTH2^+$ T for Tc2. This is the first report revealing the polarization of Th1/Th2, Tc1/Tc2 in the peripheral blood from ITP children.

Materials and Methods

Patients and normal subjects

Fifty ITP children (24 boys and 26 girls) coming from the No.1, 2 and 3-affiliated hospital of Guangdong Medical College were fulfilled with the diagnostic criteria for ITP (9).

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The patients had an average age of 3.8 years (ranging from 3.0 months to 12.0 years). Anti-coagulated venous blood by heparin was collected from the untreated patients, and 50 healthy children (27 boys and 23 girls, ranging from 0.5 years to 11 years, having an average age of 4.2 years).

Monoclonal antibodies
Cy5 labeled mouse anti-human CD4 mAb, CD8 mAb and mouse IgG1 isotype control were purchased from EB Company. PE labeled mouse anti-human IFN-γ mAb , IL-4 mAb, CRTH2 mAb and mouse IgG1 isotype control were purchased from Miltenyibiotec Company. FITC labeled mouse anti-human Fas mAb, FasL mAb and mouse IgG1 isotype control were purchased from AC Company.

Segregation of peripheral blood T cells
Anti-coagulated venous blood by heparin was collected from idiopathic thrombocytopenic purpura children and healthy children. Lymphocytes were segregated by lymphocytic separating medium (AXIS-SHIELD), then incubated in a glass petri dish at 37°C, so that monocytes were removed out. Erythrocytes were lysed when the samples were incubated at 37°C for 10 min with hemolysin (0.85% NH_4Cl). Finally, T cells were collected when the samples passed through Human $CD3^+$ T Cell Enrichment Column (BD). The purity of T cells was analyzed by immunofluorescent staining and flow cytometry by anti-CD3 mAb (EB), and the activity of T cells was detected by trypan blue staining. The concentration of lymphocytes was adjusted to 2×10^6/ml with RPMI-1640 culture media (containing 20% fetal bovine serum).

Immunofluorescent staining
Purified T cells were added to each tube with the volume of 100 μl. Fluorescence labeled mAbs were added to reasonable tube, and the tubes were shaked to mix the sample and mAbs, incubated in the dark at 37°C for 30 min. Then the samples were washed twice by PBS, and detected by flow cytometry after suspended in 0.5 ml buffer.

Cytokine assays of Th1 and Th2 types by FCM
T cells were resuspended at 2×10^6/ml and restimulated with 50 ng/ml phorbol-myrisate-acetate (PMA) and 500 ng/ml ionomycin for 4 h, and monensin (2 μmol/L) was added 2 h before cell harvest. Cytokine production was assessed by intracellular staining with PE-conjugated mouse anti-human IFN-γ and IL-4 mAbs.

Determination of T cell subsets
T cell subsets were analyzed by immunofluorescent staining and bicolor flow cytometry (Beckman, EPICS-XL) by CD4, CD8, CD4/CRTH2 and CD8/CRTH2 gating.

Determination of apoptotic proteins (Fas, FasL) expressed on T cell subsets
The expressions of Fas and FasL on T cell subsets were detected by immunofluorescent staining and tricolor flow cytometry by Fas/CD4, FasL/CD4, Fas/CD8 and FasL/CD8 gating.

Table 1. The determination of intracellular cytokines

Groups	IFN-γ^+ T (%)	IL-4^+ T (%)	IFN-γ^+/IL-4^+ T
Normal	17.77 ± 2.32	12.55 ± 1.23	1.40 ± 0.18
ITP	26.67 ± 2.82[(a)]	4.55 ± 2.32[(a)]	4.79 ± 0.66[(a)]

Data were presented as mean ± SD. Compared with normal, [(a)]$p < 0.05$.

Analysis of anti-apoptotic gene bcl-2 and bax mRNA expression by RT-PCR
Total RNA of purified T cells was isolated by Trizol reagent according to manufacturer's protocol (Invitrogen). Bcl-2 and bax cDNA fragments were amplified by one step RT-PCR Kit (Invitrogen) using 2 μg of total RNA. The primers are bcl-2: 5'-CAG CTG CAC CTG ACG CCC TT-3' and 5'-GCC TCC GTT ATC CTG GAT CC-3'; bax: 5'-TTT GCT TCA GGG TTT CAT CC-3' and 5'-CGT CCC AAA GTA GGA GAG GGA-3'; β-actin: 5'-AGC GGG AAA ACG TGC GTG AC-3' and 5'-ACT CCT GCT TGC TGA TCC ACA TC-3'. β-actin was used as positive control. The PCR products were analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide and then taken photos under the burdick lamp. The image of amplified strips was analyzed by 2-dimension laser scanner and corrected by β-actin, indicated with the ratio of absorbance.

Statistical analysis
SPSS11.0 software was used for statistical analysis of the results. Data were expressed as the mean ± SD. Statistical differences between the two groups were evaluated by analysis with Student's *t*-test. Analysis of covariance was used to evaluate the variety between Fas and FasL. Values of $p < 0.05$ were accepted as indicating significance.

Results

Increased Th1 type cells in ITP children
Compared with healthy children, in ITP children, the average percentage of IFN-γ^+ T (Th1 type) cells and the ratio of IFN-γ^+/IL-4^+ T (Th1/Th2) was increased significantly ($p < 0.05$). Meanwhile, the average percentage of IL-4^+ T (Th2 type) cells was decreased obviously ($p < 0.05$) (Table 1).

Phenotypic determination of Th1/Th2 and Tc1/Tc2
Compared with healthy children, in ITP children, the average percentages of $CD4^+CRTH2^-$ T (Th1) and $CD4^+CRTH2^+$ T (Th2) cells were increased significantly ($p < 0.05$), and the ratios of $CD4^+CRTH2^-$ T/$CD4^+CRTH2^+$ T and $CD8^+CRTH2^-$ T/$CD8^+$ $CRTH2^+$ T were also increased clearly ($p < 0.05$). Meanwhile, the average percentages of $CD8^+CRTH2^-$ T (Tc1) and $CD8^+$ $CRTH2^+$ T (Tc2) cells did not change obviously ($p > 0.05$) (Table 2).

Expressions of apoptosis protein Fas and FasL on T cell

Table 2. The determination of T cell subsets: Th1/Th2, Tc1/Tc2

Groups	$CD4^+$			$CD8^+$		
	$CRTH2^-$	$CRTH2^+$	$CRTH2^-/CRTH2^+$	$CRTH2^-$	$CRTH2^+$	$CRTH2^-/CRTH2^+$
Normal	21.92 ± 6.42	0.39 ± 0.14	48.76 ± 6.17	28.72 ± 5.20	1.52 ± 0.68	18.90 ± 4.12
ITP	39.01 ± 5.47[(a)]	0.80 ± 0.16[(a)]	56.21 ± 5.95[(a)]	30.95 ± 5.45[(b)]	1.34 ± 0.84[(b)]	23.09 ± 3.31[(a)]

Data were presented as mean ± SD. Compared with normal, [(a)]$p < 0.05$; [(b)]$p > 0.05$.

subsets

Compared with healthy children, the expressions of Fas and FasL on $CD4^+$ T (Th), $CD8^+$ T (Tc), $CD4^+CRTH2^-$ T (Th1), $CD4^+CRTH2^+$ T (Th2), $CD8^+CRTH2^-$ T (Tc1) and $CD8^+$ $CRTH2^+$ T (Tc2) cells were all increased in ITP children. The expressions of FasL on $CD4^+CRTH2^-$ T (Th1) and $CD8^+$ $CRTH2^-$ T (Tc1) were increased sharply than Fas, whereas the expressions of Fas on $CD4^+CRTH2^+$ T (Th2) and $CD8^+$ $CRTH2^+$ T (Tc2) were increased clearly than FasL (Figure 1).

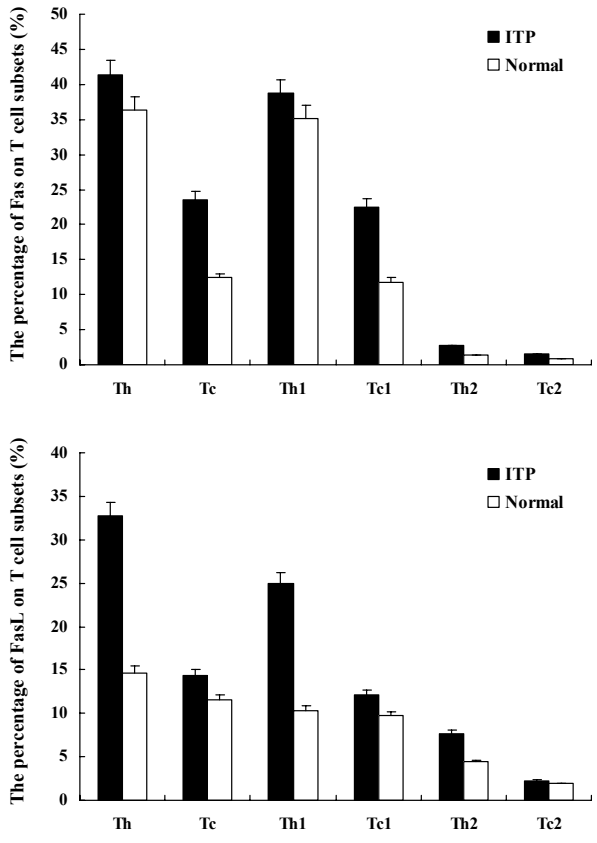

Figure 1. Expressions of Fas and FasL on T cell subsets by FCM. Anti-coagulated venous blood by heparin was collected from healthy children and idiopathic thrombocytopenic purpura children. T cells were segregated by Human $CD3^+$ T Cell Enrichment Column. The concentration of T cells was adjusted to 2×10^6/ml with RPMI-1640 culture media (containing 20% fetal bovine serum). Expressions of Fas and FasL on T cell subsets were detected by FCM as described under "Materials and Methods." Shown were the percentages for Fas and FasL on Th1, Th2, Tc1, Tc2 in healthy children or ITP children.

Expressions of anti-apoptosis gene bcl-2, bax in mRNA level

Compared with healthy children, in ITP children, the expressions of bcl-2 mRNA were increased obviously and the expressions of bax mRNA were decreased ($p < 0.05$), but the ratio of bcl-2/bax mRNA was increased clearly ($p < 0.05$) (Table 3, Figure 2).

Correlation analysis between the ratio of bcl-2/bax mRNA and the ratio of IFN-γ^+/IL-4^+ T

Compared with healthy children, in ITP children, the ratio of bcl-2/bax mRNA was increased significantly ($p < 0.05$), and meanwhile, the ratio of IFN-γ^+/IL-4^+ T (Th1/Th2) also was increased sharply ($p < 0.05$). There was positive correlation between them ($r = 0.873$, $p < 0.05$), but in healthy children, there was no correlation between them ($r = 0.146$, $p > 0.05$) (Figure 3).

Table 3. The expressions of bcl-2 and bax mRNA in T cells in ITP children

Groups	Bcl-2	Bax	Bcl-2/Bax
Normal	0.39 ± 0.13	0.75 ± 0.17	0.52 ± 0.80
ITP	0.82 ± 0.22[(a)]	0.32 ± 0.28[(a)]	2.67 ± 0.78[(a)]

Data were presented mean ± SD. Compared with normal, [(a)]$p < 0.05$.

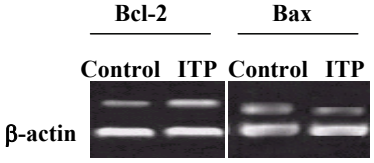

Figure 2. Detection of anti-apoptosis gene bcl-2/bax in mRNA level. Anti-coagulated venous blood by heparin was collected from healthy children and idiopathic thrombocytopenic purpura children. T cells were segregated by Human $CD3^+$ T Cell Enrichment Column. The concentration of T cells was adjusted to 2×10^6/ml with RPMI-1640 culture media (containing 20% fetal bovine serum). Anti-apoptotic gene bcl-2, bax mRNAs were analyzed by RT-PCR as described under "Materials and Methods." Shown were the electrophoregram of RT-PCR products of bcl-2 and bax mRNA in T cells.

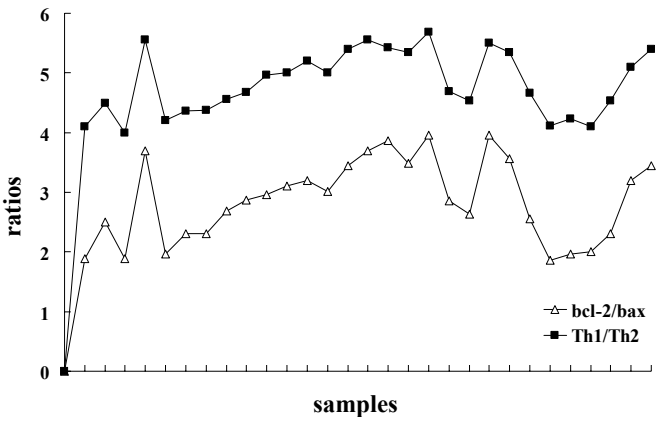

Figure 3. Analysis of correlation between the ratios of bcl-2/bax mRNA and the ratios of IFN-γ^+/IL-4^+ T. According to the analysis of SPSS11.0 software, there were positive correlation between the ratios of bcl-2/bax mRNA and the ratios of IFN-γ^+/IL-4^+ T (Th1 /Th2). As the graph shown, X axis represents the sample numbers (n = 30), Y axis represents the ratios of IFN-γ^+/IL-4^+ T and the ratios of bcl-2/bax mRNA.

Discussion

Autoimmune diseases are kinds of clinical common diseases, but the causes and mechanisms are very complex, and remain unclear now. Maybe there are some kinds of self-immuno-genicity and/or self-tolerance destruction, which brings about continual and over-degree autoimmune responses. Recent studies showed that the equilibration between Th1 cells and Th2 cells might play a major role in the developing procedure of autoimmune diseases (10). Dominguez thought that it was the equilibration between Th1 cells and Th2 cells that was an important regulating site between humoral immunity and cellular immunity (11). Th1 cells can up-regulate cellular immunity reaction. They can produce interleukin-2 (IL-2) and interferon γ (IFN-γ), which induce activation of cytotoxic T (Tc) cells and macrophages, then target cells may be phagocytized and killed. Moreover, Th1 cells can do harm to target cells through secreting tumor necrosis factor alpha (TNF-α) and tumor necrosis factor beta (TNF-β). Th2 cells mainly produce interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-10 (IL-10) to up-regulate humoral immune reaction. Recently, $CD8^+$ cytotoxic T cells had similarly been shown to be divided into Tc1 cells synthesizing IL-2, INF-γ and TNF-β, and Tc2 cells synthesizing IL-4, IL-5, IL-6, IL-10 and IL-13 (12). Furthermore, Mosmsnn classified Th1 cells and Tc1 cells as Th1-like cells while Th2 cells and Tc2 cells as Th2-like cells (13). Chen held the same viewpoint in his report about the relation between T cell subsets and systemic lupus erythematosus (SLE) (14).

To discuss the equilibration between Th1-like cells and Th2-like cells will have an important significance as far as kinds of autoimmune diseases including ITP are researched. But a specific surface marker to delimit Th1-like cells and Th2-like cells cannot be found out for long time. Recent studies regarded that CRTH2 (chemoattractant receptor-homologs molecule expressed on Th2 cells) was the best reliable surface marker on human Th2 cells and Tc2 cells. CRTH2 is a new seven-transmembrane G-protein-couple receptor, which has great homology with C5a receptor and FMLP (formylmethionine-leucine-phenylalanine) receptor. IL-4 can up-regulate its expression whereas IFN-γ can inhibit its expression. At the same time, CRTH2 is a receptor for prostaglandin D2, involved in migration of leukocytes, which may participate in inflammatory reaction, but the precise function remains unclear presently. Cosmi suggested that although not all $CRTH2^+$ T cells produced Th2 type cytokines, $CRTH2^+$ T cells only contained Th2 cells and Tc2 cells, and did not contain Th0 cells or Tc0 cells, and they also did not contain Th1 cells and Tc1 cells (15). Experiments by Tsuda made clear that CRTH2 was expressed on not Th1 cells or Tc1 cells but Th2 cells and Tc2 cells (16). In this study, we determinated the pure peripheral blood T cells through flow cytometry, that was, Th1 type and Th2 type T cells by intracellular cytokines IFN-γ and IL-4 gating, and Th1-like cells (Th1, Tc1) and Th2-like cells (Th2, Tc2) by CRTH2 gating. As a result, $CD4^+$ T represented for Th cells, $CD8^+$ T for Tc cells, $CD4^+CRTH2^-$ T for Th1 cells, $CD4^+CRTH2^+$ T for Th2 cells, $CD8^+CRTH2^-$ T for Tc1 cells and $CD8^+$ $CRTH2^+$ T for Tc2 cells. Our studies displayed that, in ITP children, there were disequilibria between Th1-like cells and Th2-like cells, and they shifted to Th1-like superiority. Simultaneously, we noticed that in ITP children, the ratios of Th1/Th2, Tc1/Tc2 and IFN-γ^+/IL-4^+ T were all increased obviously, though the average percentages Tc (Tc1, Tc2) cells changed little, which might suggest that the ratio of Th1-like cells/Th2-like cells played an obvious role in the ITP immunopathogenesis. The high ratio of Th1-like cells/ Th2-like cells hints there might be high auto-reactive Th1 and Tc1 in ITP. Th1 cells can kill thrombocytes through secreting IL-2 and IFN-γ to induce activation of cytotoxic T cells and macrophages, and Tc1 cells, which were regarded as Tc cells commonly, can produce perforin and granzyme to damage thrombocytes especially.

Fas/Apo-1 (CD95) is a kind of type I transmembrane glycoprotein with molecular weight about 48 kD. Fas is mainly expressed on peripheral $CD4^+$ T cells, $CD8^+$ T cells, B cells, some NK cells, and myelocytes, thrombocytes and partial macrophages. Fas ligand (FasL) is a family member of tumor necrosis factor (TNF). FasL is a kind of type II transmembrane glycoprotein with molecular weight about 40 kD. FasL is mainly expressed on activated T cells and B cells, and it is an activated marker of T cells. When membrane FasL (mFasL) was bound to its receptor membrane Fas (mFas), cellular apoptosis is induced. In addition to Fas/FasL pathway, cellular apoptosis can also be induced by TNFR/ TNF pathway, p53 pathway and glucocorticoid system, and so forth. Fas/FasL pathway that induces cellular apoptosis disorder is the main factor of the disturbance between cellular immune reaction and humoral immune reaction (17). Fas and FasL participate in the regulation of lymphocyte

quantity in immune response, correlating with peripheral activated lymphocyte apoptosis. There are increasing expressions of FasL on activated lymphocytes, with the increase of expressions of Fas at the same time. After finishing their immune function, activated lymphocytes start to apoptosis immediately through Fas/FasL apoptotic pathway, so that autoimmune diseases will not happen. If there is a disorder in Fas/FasL apoptotic pathway, auto-immune diseases might take place.

Recent researches revealed that activated lymphocytosis existed in ITP patients, and thrombocytes were destructed directly by activated lymphocytes (Tc, NK) and cellular apoptosis inducing by lymphocytes (18). Yoshimura noticed that there was a close relation between Fas/FasL apoptotic pathway and ITP (19), but the expressions of Fas and FasL on T cell subsets in ITP children remained to be reported little recently. In this study, it was found that the expressions of Fas and FasL on T cell subsets were all increased, but the degree of the changes showed discordance significantly, the expressions of FasL on Th1 and Tc1 were increased sharply *vs* Fas, whereas the expressions of Fas on Th2 and Tc2 were increased obviously *vs* FasL. Our results displayed that: 1) Peripheral blood T cell apoptosis deficiency in ITP children may be caused by activated T cells apoptosis deficiency. 2) Abnormal activation existed in both Th cell subsets and Tc cell subsets, and the activated degree of Th1, Tc1 cell subsets was more obvious than that of Th2, Tc2 cell subsets. 3) High expressions of Fas and FasL may involve in Th cell subset apoptosis, but may have little effect on Tc cell subsets (20). Fas/FasL system is the main apoptotic pathway, but different kinds of T cell subsets have different sensibility to the pathway, because many positive or negative factors may affect it. Researches showed that bcl-2 was an important anti-apoptosis protein, which might have an opposite function against Fas/FasL system (21). In bcl-2 famly there were many kinds of apoptosis related genes, bcl-2 gene can inhibit cell apoptosis whereas bax gene can enhance cells apoptosis (22, 23). Zhang noticed that the expressions of Fas and FasL had little correlation with the apoptotic ratio of myelodysplastic syndrome (MDS) $CD34^+$ cells, but the expression of bcl-2 had a negative correlation with the apoptotic ratio of myelodysplastic syndrome (MDS) $CD34^+$ cells (24). In this study, we found that bcl-2 mRNA in T cells from ITP children was increased obviously and bax mRNA was decreased significantly, but the ratio of bcl-2/bax mRNA was increased sharply. Moreover, there was positive correlation between the ratio of bcl-2/bax mRNA and the ratio of Th1/Th2 (IFN-γ^+/IL-4^+ T), but in healthy children, there was no correlation between them.

In conclusion, our studies proved that abnormal expressions of Fas/FasL and bcl-2/bax mRNA might play an important role in abnormal polarization of T cell subsets, abnormal activation of T cell subsets and apoptosis of T cell subsets, which might involve in ITP immunopathogenesis. Furthermore, to study the polarization of T cell subsets and the regulation of Fas/FasL apoptotic system and bcl-2/bax gene will benefit the treatment of some immune diseases including ITP.

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