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Inactivation of Notch signaling reverses the Th17/Treg imbalance in cells from patients with immune thrombocytopenia

Shuang Yu[1], Chuanfang Liu[1], Lanhua Li[1], Tian Tian[1], Min Wang[1], Yu Hu[2], Cunzhong Yuan[3], Lei Zhang[4], Chunyan Ji[1] and Daoxin Ma[1]

T helper 17 (Th17) cells and regulatory T (Treg) cells, along with Th1 and Th2 cells, may contribute to the development of immune thrombocytopenia (ITP). The imbalance of Th17/Treg toward Th17 cells has been shown to play a pivotal role in the peripheral immune response. Notch signaling has been implicated in peripheral T-cell activation and effector cell differentiation. However, the role of Th17/Treg in the pathogenesis of ITP and the effect of Notch signaling on Th17/Treg imbalances remain largely elusive in ITP. *In vitro*, we treated peripheral blood mononuclear cells (PBMCs) from ITP and healthy controls with γ-secretase inhibitor (DAPT). Th17 cells and Treg cells were measured by flow cytometry and IL-17, IL-21, and IL-10 secretion by enzyme immunoassay technique. The mRNA expression of Ntoch1, Hes1, Hey1, RORγt, and Foxp3 was investigated by RT-PCR. Cell proliferation and apoptosis were determined by the Cell Counting Kit-8 and apoptosis detection kit. We demonstrated that DAPT was effective in inhibiting mRNA expression of Notch signaling molecules. In untreated cultured PBMCs from ITP patients, we observed elevated Th17 cell and IL-21 levels and RORγt mRNA expression, decreased Treg cells and Foxp3 mRNA expression, and an increased ratio of Th17/Treg and RORγt/Foxp3. After inactivating Notch signal by DAPT, Th17 cells and Th17/Treg ratio were dose dependently decreased and accompanied by the reduction of IL-17 in culture supernatants and RORγt mRNA expression in ITP patients. However, no significant difference was found for Treg cells and Foxp3 mRNA expression, RORγt/Foxp3 ratio, and IL-21 and IL-10 levels after DAPT treatment in ITP patients. We also present evidence that the effect of DAPT inhibition on the Th17 cell response was associated with downregulation of RORγt and IL-17 transcription using human *in vitro* polarization. In conclusion, our findings highlight the importance of Notch signaling in Th17/Treg imbalances in ITP. Inactivation of Notch signaling might be a potential immunoregulatory strategy in ITP patients.
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Immune thrombocytopenia (ITP) is an acquired immune-mediated disease characterized by autoantibody-dependent accelerated destruction and impaired production of platelets.[1,2] It is widely believed that the multi-dysfunctional immunity of T lymphocyte contributes to the development of the disease. Activated T helper (Th) cells and different Th-associated cytokines are considered to play a pivotal role in ITP pathophysiology.[3] Several studies have previously provided evidence supporting a type-1 cytokine polarization and a high Th1/Th2 ratio in the immune response in ITP.[4,5] However, the paradigm of Th1/Th2 in the pathogenesis of ITP has been challenged by the identification of Th17 cells and T regulatory (Treg) cells, two novel $CD4^+$ T subsets that are distinct from Th1 and Th2 cells.

Th17 cells are $CD4^+$ T cells that produce IL-17.[6] IL-6, along with other STAT-initiating cytokines, in concert with TGF-β, IL-21, and IL-23, contributes to the differentiation. The transcription factor retinoic acid-related orphan receptor-γt (RORγt) also appears to be required for Th17 cell differentiation in addition to the cytokines.[7] The pathogenic role of IL-17 as well as Th17 cells has been documented in numerous autoimmune diseases.[8–11] Treg cells are a group of phenotypic and functional specific T-cell subset and play a crucial role in maintenance of immune tolerance.[12] Foxp3, a member of the forkhead/winged-helix family of the transcription factor, has been identified as the best marker of Treg cells.[13] Recent studies found that missing or dysfunction of

[1]Department of Hematology, Qilu Hospital of Shandong University, Jinan, People's Republic of China; [2]Department of Oncology, Qilu Hospital of Shandong University, Jinan, People's Republic of China; [3]Department of Obstetrics and Gynecology, Qilu Hospital of Shandong University, Jinan, People's Republic of China and [4]Department of Orthopedics, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan, People's Republic of China
Correspondence: Dr D Ma, PhD, Department of Hematology, Qilu Hospital, Shandong University, 107, Wenhua Xi Road, Jinan 250012, Shandong, People's Republic of China.
E-mail: daoxinma@sdu.edu.cn

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Treg cells can lead to a variety of autoimmune diseases such as SLE and RA.[14,15] The balance of Th17/Treg controls immune response and has been reported to be a key factor in regulating Th cell function relating to the Th1/Th2 shift in autoimmune diseases and graft *vs* host disease.[16] Emerging evidence suggests that Th17/Treg imbalances contributes to the development of autoimmune diseases, such as SLE[17] and primary nephrotic syndrome.[18] We and others have previously demonstrated that Th17 cells were elevated and numbers and/or function of Treg cells were suppressed in the peripheral blood of ITP patients,[19–22] indicating a vital role of Th17/Treg imbalances in the pathogenesis of ITP. However, the molecular mechanisms that underline the Th17/Treg imbalances in ITP remain unknown.

Notch signaling is a pivotal regulator of a variety of cellular functions, including cell proliferation, differentiation, and apoptosis.[23] It is an evolutionarily conserved pathway comprising four Notch ligands (Notch 1–4) and five receptors (Delta-like 1, 3, and 4, Jagged1, and Jagged2). Upon ligand binding, Notch receptors can be split by the γ-secretase complex resulting in the release of an active intracellular domain (Notch intracellular domain (NICD)). NICD translocates to the nucleus and associates with transcription factors, modulating the gene expression of target genes and the development and growth of cells.[24] Recently, some studies have indicated the role of Notch signaling in the regulation of Th17 cells and Treg cells. Mukherjee *et al*[25] provided evidence that Notch signaling appears to regulate production of Th17 cells and inactivation of Notch inhibits Th17 cell response. Huang *et al*[26] demonstrated that Treg cells preferentially expressed Notch ligand DLL4, and blockage of DLL4–Notch signaling abrogated the activity of Treg cells, resulting in reduction or alleviation of immunity disease. We have previously shown that peripheral blood mononuclear cells (PBMCs) from patients with ITP display an increased expression profile of Notch1, Notch3, and Hes1,[27] indicating that Notch signaling could be a factor for the pathogenesis of ITP. Therefore, the imbalance of Th17/Treg may be because of the aberrant expression of Notch signaling in ITP.

In this study, we treated PBMCs from ITP patients and healthy controls with γ-secretase inhibitor to measure Th17 cells, Treg cells, Th17/Treg ratio, cytokines including IL-17, IL-21, and IL-10, transcription factors, cell apoptosis, and proliferation in ITP patients. Here, we describe the role of Notch signaling in Th17/Treg imbalances and provide novel insights into the potential therapeutic target of Notch signaling for ITP.

MATERIALS AND METHODS

Patients and Controls

A total of 38 patients with ITP were investigated in this study. They were enrolled between March 2011 and May 2012 at the Department of Hematology, Qilu Hospital, Shandong University. All the patients met the clinical diagnosis criteria of ITP reported in 2009.[28] The demographic and key clinical information of ITP patients are summarized in Table 1. In addition, 25 healthy volunteers (22 females and 3 males; age range, 22–56 years; median age, 39 years) were included simultaneously as controls. From all the subjects, 4 or 5 ml of heparinized venous peripheral blood was collected. Our study was approved by the Institutional Review Boards of Qilu Hospital, Shandong University. All blood samples were collected after informed consent from each participant before being included in the study.

Isolation and Culture of PBMCs

In individual experiments, PBMCs were separated by Ficoll-Hypaque centrifugation (Amersham Biosciences, Piscataway, NJ, USA) from heparinized venous peripheral blood of ITP patients or healthy donors. Briefly, peripheral blood was diluted 1:1 in 0.9% saline and layered over lymphoprep medium and centrifuged at 2000 *g* for 20 min. The PBMC interface was carefully collected, and was washed twice with 0.9% saline. PBMCs were suspended in RPMI-1640 medium (Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 5 ng/ml IL-2. The cells were then seeded at a density of 1×10^6 cells/well in 24-well plates and 1×10^5 cells/well in 96-well plates.

DAPT Treatment

N-[*N*-(3, 5-Difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-Butyl Ester (DAPT, CalBiochem, EMD Biosciences) used for inactivation of Notch signaling was reconstituted in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to a concentration of 10 mmol/l. PBMCs were respectively treated with DAPT at final concentrations of 2.5, 5, 10, and 20 μmol/l, whereas PBMCs treated with DMSO were used as solvent controls. In no case did addition of DMSO appear detrimental. After 72 h of incubation in the presence of DAPT or DMSO at 37 °C, 5% CO_2 incubator, PBMCs were collected

Table 1 Clinical characteristics of the patients with ITP

Patient characteristics	
No. of patients (male/female ratio)	38 (18/20)
Median age at diagnosis in years (range)	43 (13–74)
Median platelet count at diagnosis (range) ($\times$ 10^9/l)	20 (1–87)
Severe thrombocytopenia (no./case)	12/38
Treatment (no./case)	
Prednisolone	28/38
Immunosuppressive agent	7/38
Splenectomy	0/38

Abbreviation: ITP, immune thrombocytopenia.
Severe thrombocytopenia is defined as thrombocytopenia (platelet count $<10\times$ 10^9/l) at the time of collecting venous peripheral blood of ITP patients.

for intracellular staining, RNA extraction, apoptosis, and proliferation assay, whereas supernatants were harvested and frozen at $-80\,^{\circ}C$ for cytokine detection.

Human *In Vitro* Polarization

To evaluate induced Th17 cells, human *in vitro* Th17 cell polarization was performed using a modified protocol from Manel *et al*.[29] Naive $CD4^+$ T cells purified from PBMCs of three ITP patients and three healthy controls by positive selection using MACS separation according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Naive cells (1×10^6/ml) were then plated in 24-well plate and polarized under Th17 cell-polarizing cocktail consisting of IL-1 (10 ng/ml), IL-6 (10 ng/ml), and TGF-β (1 ng/ml) in addition to anti-INF-γ (10 μg/ml), anti-IL-4 (10 μg/ml), anti-CD3 (2 μg/ml), and anti-CD28 (2 μg/ml) antibodies. IL-1, IL-6, and TGF-β used in polarization were purchased from Peprotech and anti-IL-4 and anti-IFN-γ were purchased from Biolegend. To evaluate the effect of Notch inhibition on polarization of Th17 cells, naive $CD4^+$ T cells in Th17 cell-polarizing conditions for 4 days followed by treatment with DMSO or DAPT. Cells were collected after 72 h and analyzed for Th17 cell and mRNA expression of RORγt, STAT3, and IL-17.

Flow Cytometry for Analysis of Th17 Cells

For intracellular cytokine staining, treated cells suspended with 140 μl RPMI-1640 medium were incubated for 4 h at $37\,^{\circ}C$, 5% CO_2 in the presence of 25 ng/ml of phorbol myristate acetate (PMA), 1 μg/ml of ionomycin, and 1.7 μg/ml Golgiplug (monensin; all from Alexis Biochemicals, San Diego, CA, USA). PMA and ionomycin are pharmacological T cell-activating agents that mimic signals generated by the T-cell receptor (TCR) complex and have the advantage of stimulating T cells of any antigen specificity. Monensin is used to block intracellular transport mechanisms, thereby leading to an accumulation of cytokines in the cells.

After incubation, the cells were stained with PE-Cy5-conjugated anti-human CD3 and FITC-conjugated anti-human CD8 monoclonal antibodies (eBioscience, San Diego, CA, USA) at room temperature in the dark for 20 min to delimitate $CD4^+$ T cells. After surface staining, the cells were next stained with PE-conjugated anti-IL-17 monoclonal antibody after fixation and permeabilization (eBioscience). Isotype controls were given to enable correct compensation and confirm antibody specificity. Stained cells were analyzed by flow cytometric analysis using a FACS Calibur cytometer equipped with CellQuest software (BD Bioscience PharMingen, San Jose, CA, USA).

Flow Cytometry for Analysis of Treg Cells

$CD4^+CD25^+Foxp3^+$ cells as Treg cells were evaluated using human regulatory T-cell staining kit (eBioscience) according to the manufacturer's protocol. A total of 1×10^5 cells were harvested from 24-well plates after the respective treatment. Subsequently, the single-cell suspension was incubated with a cocktail of anti-CD4-FITC and anti-CD25-APC monoclonal antibodies for 30 min in the dark at $4\,^{\circ}C$ to stain the surface. After being washed with 2 ml cold flow cytometry staining buffer, the cells were incubated with 1 ml freshly prepared eBioscience Foxp3 fixation/permeabilization buffer for 30–60 min at $4\,^{\circ}C$ in the dark. The cells were washed with 2 ml freshly prepared $1\times$ permeabilization buffer twice. After that, the cells were blocked by normal rat serum for 15 min, and then stained using anti-Foxp3-PE monoclonal antibody or PE-conjugated rat IgG2a used as an isotype control for 45 min in the dark at $4\,^{\circ}C$. After being washed twice again, the frequency of $Foxp3^+$ Treg cells was expressed as a percentage of the total $CD4^+$ cells.

Detection of Cell Apoptosis

For the apoptosis analysis, cells after DAPT treatment were washed with PBS twice and stained with Alexa Fluor 488 Annexin V and PI using Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen, USA) according to the manufacturer's protocol. Annexin-positive and PI-negative cells were counted using FACS Calibur cytometer within 15 min after being stained. Data analysis was carried out using FACS Calibur cytometer equipped with CellQuest software (BD Bioscience PharMingen).

Analysis of Cell Viability Inhibition

PBMCs were harvested after cultured with DAPT for 72 h and cell proliferation was determined by the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) and measured by microplate reader scanning at 450 nm. All experiments were performed in triplicate on three separate occasions.

Cytokine Analysis

The secretion of IL-17, IL-21, and IL-10 in supernatants of cultures was analyzed with a quantitative sandwich enzyme immunoassay technique in accordance with the manufacturer's recommendations (eBioscience). The concentrations were calculated from a standard curve according to the manufacturer's protocol. The lower detection limits were as follows: IL-17, 0.5 pg/ml; IL-21, 20 pg/ml; and IL-10, 1 pg/ml.

Real-Time RT-PCR

Total RNA was isolated by Trizol (Invitrogen) according to the manufacturer's instructions. Approximately 1 μg of total RNA from each sample was subjected to first-strand cDNA synthesis using PrimeScript RT reagent Kit Perfect Real Time (Takara Bio). Reverse transcription reaction was done at $37\,^{\circ}C$ for 15 min, followed by $85\,^{\circ}C$ for 5 s. Real-time PCR was conducted using an ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. The real-time PCR contained, in a final volume of 20 μl, 10 μl of $2\times$ SYBR Green Real-time PCR Master Mix, 2 μl of cDNA, and 1.6 μl of

the forward and reverse primers. The primers are shown in Table 2. All experiments were conducted in triplicate. The PCR products were analyzed by melt curve analysis and agarose gel electrophoresis to determine product size and to confirm that no by-products were formed. The results were expressed relative to the number of β-actin transcripts used as an internal control.

Statistical Analysis

The results were expressed as median (range) or means ± s.d. Statistical significance was determined by ANOVA, and difference between two groups was determined by Newman–Keuls multiple comparison test (q-test) unless the data were not normally distributed, in which case Kruskal–Wallis test (H-test) and Nemenyi test were used. All tests were performed by SPSS 17.0 system. A P-value of <0.05 was considered statistically significant.

RESULTS

Notch Signaling Molecules in Isolated PBMCs Were Reduced by DAPT

The efficacy of DAPT for inhibition of the Notch signaling was confirmed through the analysis of Notch1, Hes1, and Hey1 mRNA expression levels by real-time PCR. As seen in Figure 1, the expression of Notch1, Hes1, or Hey1 was gradually reduced with the increasing concentrations of DAPT. In ITP patients, the reduction of Notch1 and Hes1 expression was significantly at 5, 10, or 20 μmol/l DAPT group compared with DMSO group (Figures 1a and c), but no difference in Hey1 expression was found (Figure 1e). In healthy controls, the expression of Notch1, Hes1, and Hey1 was also significantly reduced at 5, 10, or 20 μmol/l DAPT group compared with DMSO group (Figures 1b, d and f). The decreased Hes1 and Hey1 after DAPT treatment indicated that DAPT had an inhibiting effect on the Notch signaling.

DAPT Reduced the Abnormally Elevated Th17 Cells and Restored the Th17/Treg Balance in ITP Patients

To identify the role of Notch signaling in Th17/Treg imbalances in ITP patients, we explored Th17 cells and Treg cells by flow cytometry after DAPT treatment. Figures 2a–c and Figure 3a–c show the typical flow cytometric scattergrams of two T subsets in different groups. The percentages of Th17 cells, Treg cells, and Th17/Treg ratio in different groups are shown in Supplementary Table S1. In accordance with our previous results, the expression of IL-17 on $CD3^+CD8^-$ T cells (Th17 cells) was significantly increased in DMSO group of ITP patients compared with DMSO group of healthy controls (Figure 2d). After DAPT treatment, the percentage of Th17 cells showed a significant dose-dependent decrease in ITP patients (Figure 2e, left). However, DAPT had no statistical effect on Th17 cells in healthy controls (Figure 2e, right).

The percentage of Treg cells was significantly lower in DMSO group of ITP patients than DMSO group of healthy controls (Figure 3d). However, after DAPT treatment, no significant change of Treg cells was observed in ITP patients or healthy controls (Figure 3e).

As shown in Figure 4a, the ratio of Th17/Treg was significantly elevated in ITP patients compared with healthy controls before DAPT treatment. The data in Figure 4b indicated the abnormally higher Th17/Treg ratio was significantly reduced in a dose-dependent manner after DAPT treatment in ITP patients. In addition, 20 μmol/l DAPT resulted in more pronounced inhibitory effect on increased Th17 cells and Th17/Treg ratio in ITP patients.

Table 2 Primers used in the study

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Notch1	CAATGTGGATGCCGCAGTTTGTG	CAGCACCTTGGCGGTCTCGTA
Hes1	CTCTCTTCCCTCCGGACTCT	AGGCGCAATCCAATATGAAC
Hey1	GCTGGTACCCAGTGCTTTTGAA	TGCAGGATCTCGGCTTTTTCT
RORγt	TTTTCCGAGGATGAGATTGC	CTTTCCACATGCTGGCTACA
Foxp3	GTGGCCCGGATGTGTGAAG	GGAGCCCTTGTCGGATGATG
β-Actin	CCTTCCTGGGCATGGAGTCCTG	GGAGCAATGATCTTGATCTTC

DAPT Exposure Reduced IL-17 But Not IL-21 and IL-10 Secretion in Cell Culture Supernatants in ITP Patients

Similarly, we first investigated IL-17, IL-21, and IL-10 concentration in DMSO groups of ITP patients and healthy controls. Our results showed that there was a significant increase of IL-21 levels in ITP patients (median, 74.05 pg/ml (range, 54.34–132.69 pg/ml)) compared with healthy controls (median, 54.35 pg/ml (range, 46.21–127.14 pg/ml), $P<0.0001$; Figure 5b). There was an elevated trend of IL-17 concentration in ITP patients (median, 8.16 pg/ml (range, 1.35–55.58 pg/ml)) compared with healthy controls (median, 5.45 pg/ml (range, 1.23–40.64 pg/ml), $P=0.1459$), but no statistical difference was found (Figure 5a). IL-10 showed a slight decrease in ITP patients (median, 28.71 pg/ml (range, 3.19–343.02 pg/ml)) compared with healthy controls (median, 35.36 pg/ml (range, 2.50–398.56 pg/ml)), but no statistical difference was found (Figure 5c).

As shown in Figure 5d (left) and Supplementary Table S2, decreased IL-17 secretion in a dose-dependent manner was observed after DAPT treatment compared with DMSO group in ITP patients. No significant alteration of IL-17 levels was observed in healthy controls after DAPT treatment (Figure 5d, right). DAPT had no apparent influence on the secretion of IL-21 in ITP patients or controls (Figure 5e). Although IL-10 level had a slight increase after DAPT treatment in ITP patients or healthy controls, no significant difference was found (Figure 5f).

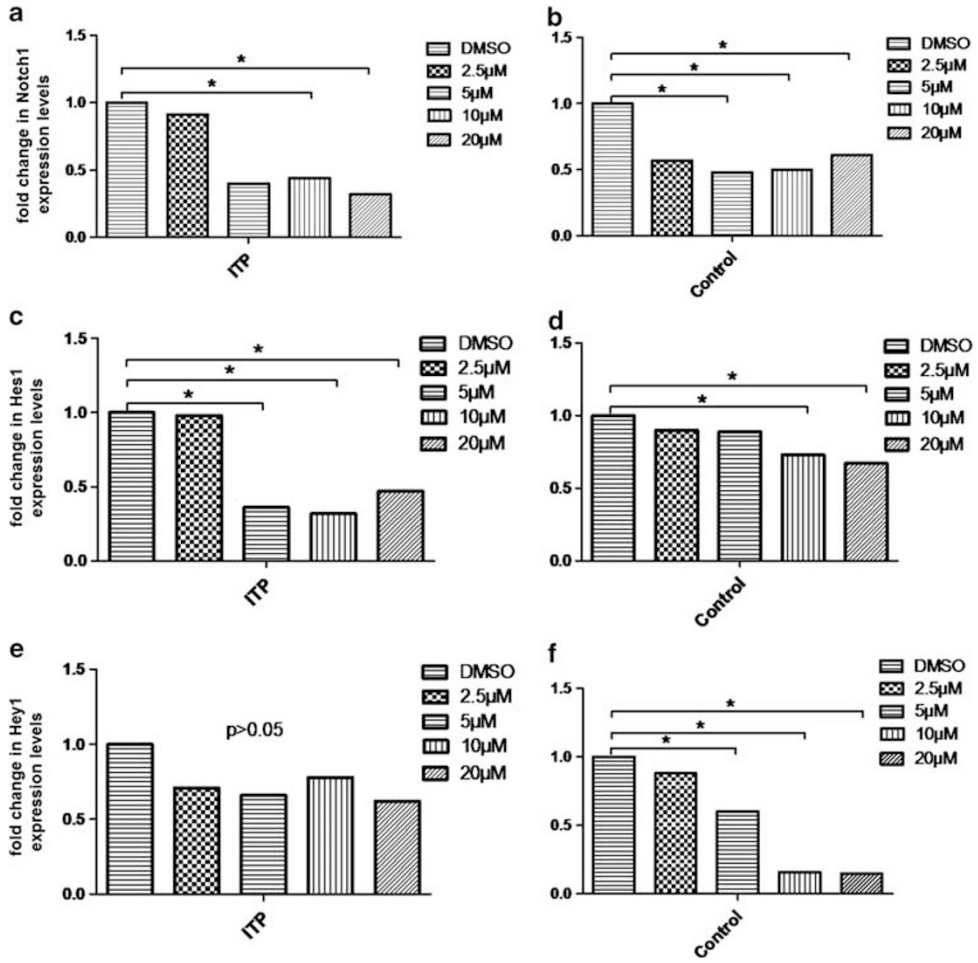

Figure 1 Inactivation of Notch signaling by DAPT. PBMCs from ITP patients and controls were treated with DAPT ranging from 2.5 to 20 μM for 72 h *in vitro*. Cells were harvested for quantitative real-time RT-PCR to analyze the change in mRNA expression of Notch1 (**a**, **b**), Hes1 (**c**, **d**), and Hey1 (**e**, **f**) in each group. Data shown were relative to DMSO group in ITP patients or healthy controls. Data represent the median (range) from three separate experiments and $*P < 0.05$.

DAPT Decreased RORγt mRNA Expression and Ratio of RORγt/Foxp3 in ITP Patients

To further determine whether the decreased Th17 cells and IL-17 level in the presence of DAPT was correlated with RORγt, we analyzed RORγt mRNA expression by RT-PCR. The results showed that RORγt expression was higher in DMSO group of ITP patients compared with healthy controls (Figure 6a and Supplementary Table S3). While PBMCs were treated with DAPT, RORγt expression was significantly reduced in a dose-dependent manner in ITP patients (Figure 6c, left). However, no significant alteration of RORγt mRNA expression was observed in healthy controls after DAPT treatment (Figure 6c, right).

Foxp3 was also determined after DAPT treatment by RT-PCR. As shown in Figure 6a and Supplementary Table S3, the mRNA expression of Foxp3 was significantly decreased in DMSO group of ITP patients compared with healthy controls. The expression of Foxp3 had no statistical alteration after DAPT treatment in ITP patients or controls (Figure 6d).

The ratio of RORγt/Foxp3 was also analyzed in ITP patients and healthy controls. As shown in Figure 6b and Supplementary Table S3, the ratio of RORγt/Foxp3 was significantly increased in ITP patients compared with healthy controls. After DAPT treatment, the ratio of RORγt/Foxp3 presented a dose-dependent decreased trend in ITP patients, but no statistical difference was found (Figure 6e, left). Also, no apparent alteration of RORγt/Foxp3 ratio was observed in healthy controls (Figure 6e, right).

DAPT Inhibited Human Th17 Cell Polarization in ITP patients by Decreasing RORγt and IL-17A Expression

To determine the effect of DAPT on Th 17 production, naive $CD4^+$ T cells were cultured in Th17 cell-polarizing conditions for 4 days followed by treatment with either DAPT or DMSO. As shown in Figure 7a and b, the percentage of Th17 cells was reduced after DAPT treatment compared with DMSO treatment in ITP patients. However, no similar changes in Th17 cell percentage in healthy controls were detected.

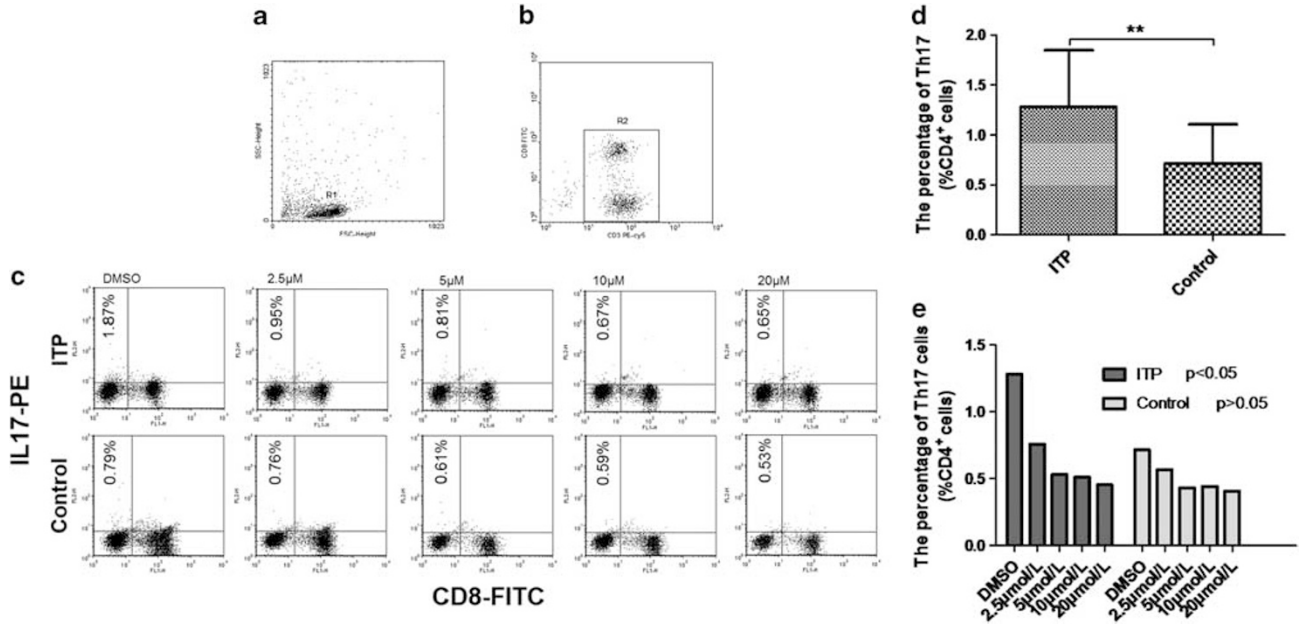

Figure 2 Inactivation of Notch signaling decreased the elevated percentage of Th17 cells in ITP patients. Cells from ITP patients and healthy controls were treated with various concentrations of DAPT ranging from 2.5 to 20 μM for 72 h, and then Th17 cells were analyzed by FACS. (**a**) Lymphocytes. (**b**) $CD3^+$ T lymphocytes. (**c**) Representative scattergrams of intracellular expression of IL-17 on $CD3^+CD8^-$ T cells in all treatments in ITP patients and healthy controls. (**d**) The comparison of Th17 cells between ITP patients and healthy controls without DAPT treatment. (**e**) The proportion of Th17 cells from ITP patients and healthy controls after DAPT treatment. Data are shown as median (range). $^{**}P<0.001$.

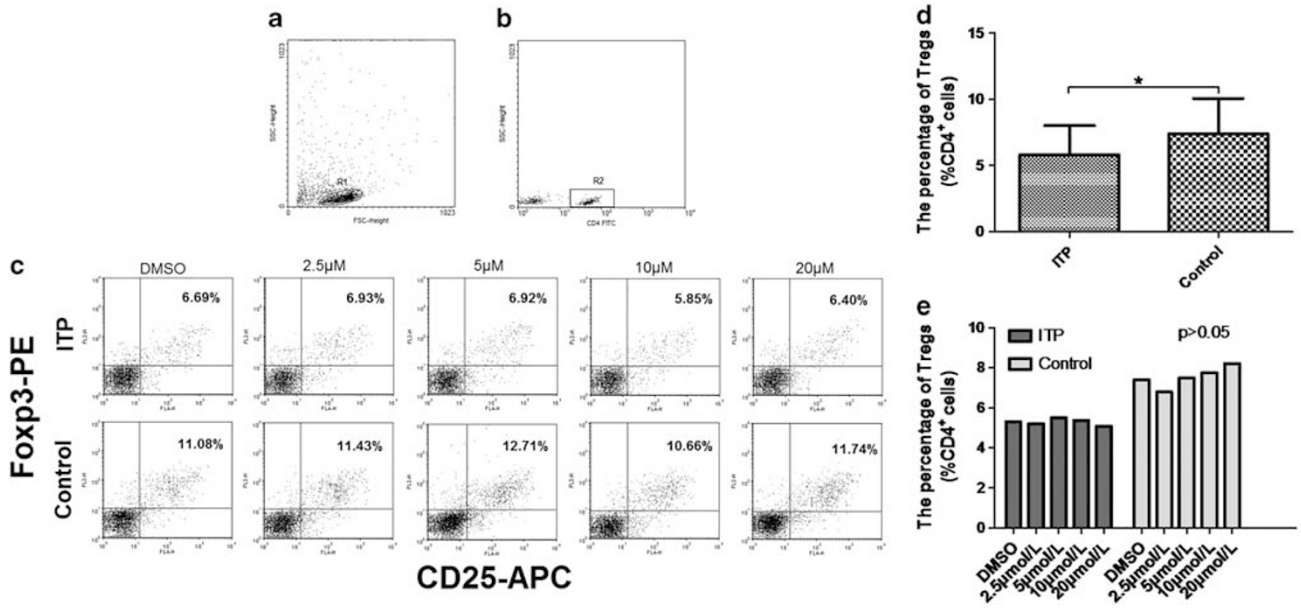

Figure 3 Effects of DAPT on Treg cells. Cells from ITP patients and healthy controls were treated with various concentrations of DAPT ranging from 2.5 to 20 μM for 72 h, and then Treg cells were analyzed by FACS. (**a**) Lymphocytes. (**b**) $CD4^+$ T lymphocytes. (**c**) Representative scattergrams of intracellular expression of Foxp3 on $CD4^+CD25^+$ T cells in all treatments in ITP patients and healthy controls. (**d**) The comparison of Treg cells between ITP patients and healthy controls without DAPT treatment. (**e**) The proportion of Treg cells from ITP patients and healthy controls after DAPT treatment. Data are shown as median (range). $^{*}P<0.05$.

To determine whether Notch inhibition influences human Th17 cell polarization by regulation of transcriptional levels of RORγt, STAT3, and IL-17, the effect of Notch inhibition on already differentiated Th17 cells was assessed. Naive $CD4^+$ T cells were cultured in Th17 cell-polarizing conditions for 4 days followed by treatment with either DAPT or

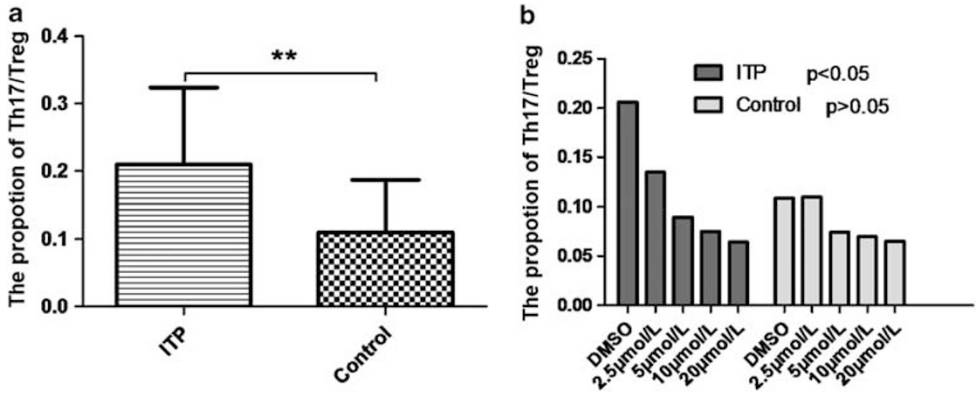

Figure 4 The alteration of Th17/Treg ratio in ITP patients and healthy controls before and after DAPT treatment. (**a**) The comparison of Treg cells between ITP patients and healthy controls without DAPT treatment. (**b**) DAPT treatment reduced the elevated Th17/Treg ratio in ITP patients. Data are shown as median (range). $^{**}P<0.001$.

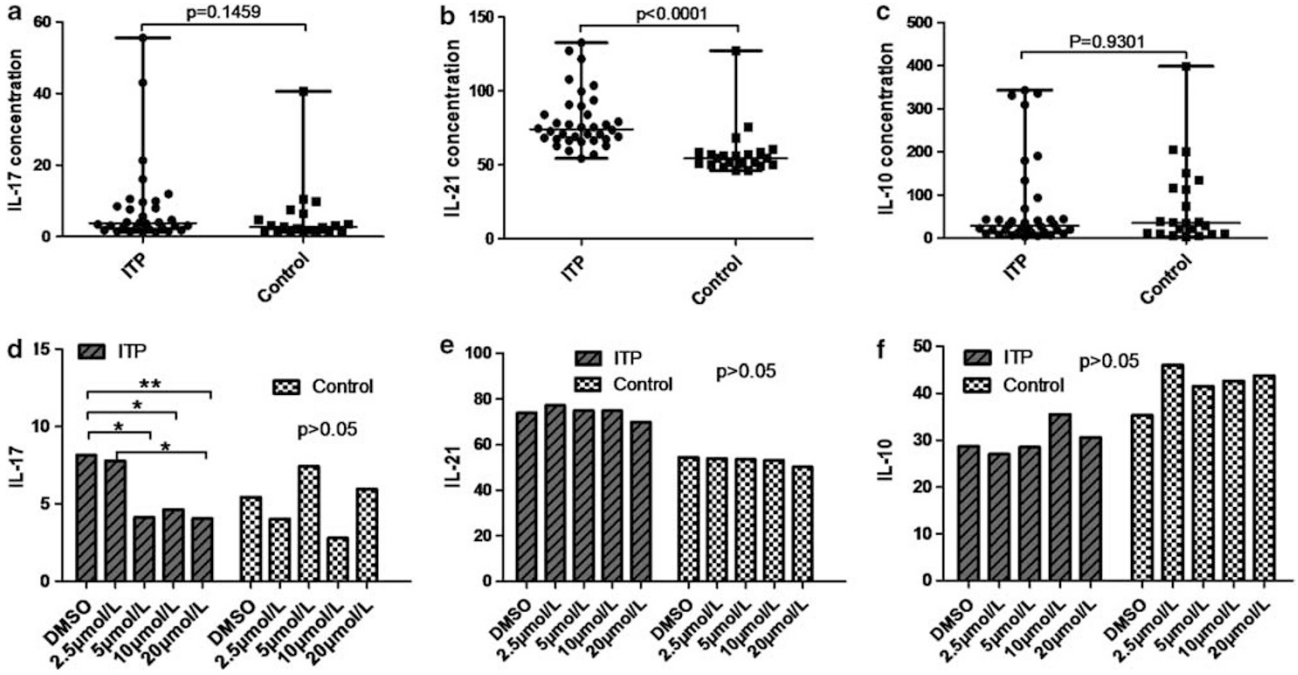

Figure 5 Inhibition of Notch signaling by DAPT significantly downregulated Th17 cell-associated cytokine IL-17 but IL-21 or Treg-associated cytokine IL-10 secretion. After 3 days of DAPT treatment, culture supernatant of each group was harvested for ELISA. The comparison of IL-17 (**a**), IL-21 (**b**), and IL-10 (**c**) was made between ITP patients and healthy controls without DAPT treatment. IL-17 (**d**), IL-21 (**e**), and IL-10 (**f**) in treated PBMCs from ITP patients and healthy controls, respectively. Data are shown as median (range). $^{*}P<0.05$, $^{**}P<0.01$.

DMSO. The mRNA expression of IL-17A and RORγt was found to be reduced in the presence of DAPT in ITP patients but healthy controls, although no significant difference was observed (Figures 7c and e). Interestingly, no significant changes in STAT3 levels were detected in ITP patients and healthy controls (Figure 7d).

DAPT Increased Apoptosis in PBMCs

The canonical scattergrams of apoptosis are shown in Figure 8a. The percentage of Annexin-positive and PI-negative cells represented the cell apoptosis rate. As shown in Figures 8b and a significant increase of apoptosis was found in 10 μmol/L DAPT groups of ITP patients. The percentage of apoptotic cells was also statistically increased in 20 μmol/l DAPT group of healthy controls. A significant increase in apoptotic cells was observed in 20 μmol/l DAPT group compared with 2.5 and 5 μmol/l DAPT groups in healthy controls.

DAPT Had No Effect on PBMC Proliferation

PBMCs were treated with DMSO or DAPT for 3 days. Cell growth was determined by the Cell Counting Kit-8 analysis.

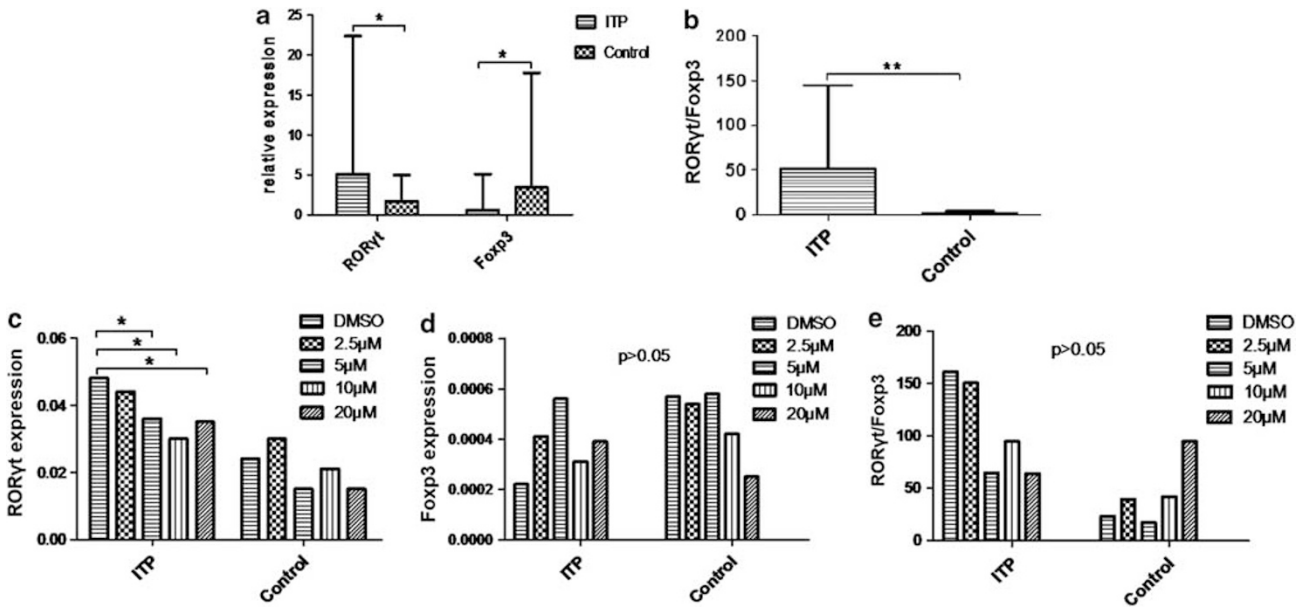

Figure 6 DAPT reduced RORγt mRNA expression and RORγt/Foxp3 ratio in ITP patients. The comparison of RORγt and Foxps (**a**) and RORγt/Foxp3 ratio (**b**) between ITP patients and healthy controls without DAPT treatment. The mRNA expression of RORγt (**c**), Foxp3 (**d**), and RORγt/Foxp3 ratio (**e**) from ITP patients and healthy controls after DAPT treatment. Data are shown as median (range). $^{*}P<0.05$, $^{**}P<0.001$.

As seen in Figures 8c and d, DAPT had no significant effect on cell proliferation from ITP patients or healthy controls.

DISCUSSION

This study demonstrates that there are Th17/Treg imbalances drifting to the direction of Th17 cells in untreated PBMCs from ITP patients, suggesting that Th17/Treg imbalances play a pivotal role in ITP. Isolated PBMCs from ITP patients could respond to DAPT, causing a reversal of Th17/Treg imbalances by blocking of Notch signaling. Notch signaling is considered as one of the main factors in regulating lymphocyte activation and differentiation. Th cell polarization and profile of cytokine production may depend on Notch ligand interacting with the Notch receptor.[30] The use of γ-secretase inhibitors to influence peripheral T-cell activation was recently documented,[31] confirming the validity of γ-secretase inhibitors as a means of Notch signal disruption. In a recent report, DAPT was employed to interrupt Notch signaling leading to a rapid reduction of Hes1 and Hes5 mRNA expression but Notch1 in retinal progenitor cells.[32] In the current study, we quantified the mRNA expression of Notch1, Hes1, and Hey1 in DAPT-cultured PBMCs. The outcome of RT-PCR demonstrated that DAPT could significantly downregulate the mRNA expression of Notch1 and Hes1.

It has recently been shown that Notch signaling may be involved in differentiation and function of Th17 cells and Treg cells. Jiao *et al*[33] reported that inhibition of Notch by DAPT and Notch3 antibody attenuated Th1- and Th17 cell-type response other than Treg cells , and other studies showed that overexpression of Notch ligand can induce regulatory cells.[34,35] It was shown that Treg-triggered Notch1 activation of the target cells by membrane-bound TGF-β and blockage of Notch1 signaling reversed the immune suppression function of Treg cells.[36] Further research also revealed that Treg cells preferentially expressed cell membrane Notch ligands and that blocking Notch signaling initiated by such ligands inhibited Treg cell suppressor function.[37] Consistent with the work of Jiao *et al*,[33] our data demonstrated that the percentage of Th17 cells was attenuated significantly by inactivation of Notch signaling with DAPT in a dose-dependent manner in ITP patients. The increased Th17/Treg ratio was also significantly reduced in ITP patients after DAPT treatment. This simultaneous reduction of Th17 cells and Th17/Treg ratio suggested a pivotal role of Notch signaling in reversing Th17/Treg imbalances in ITP.

Because Notch signaling has been implicated in cytokine secretion, the influence of Notch signaling on Th17- and Treg-associated cytokine secretion was then examined in response to alteration of Th17 cells and Treg cells induced by DAPT. Recent studies have drawn direct correlations between specific Notch receptors and IL-17 production in autoimmunity.[38] We here demonstrated that inhibition of Notch signaling by DAPT significantly decreased the secretion of IL-17 in dose-dependent manner in ITP patients. The inactivation of Notch signaling may disrupt IL-17 production, and IL-17 production might be dependent on the Notch signaling in ITP. Our results are consistent with one recent study showing that specific inhibition of Notch1 expression through the use of Notch1 siRNA abrogates IL-17A

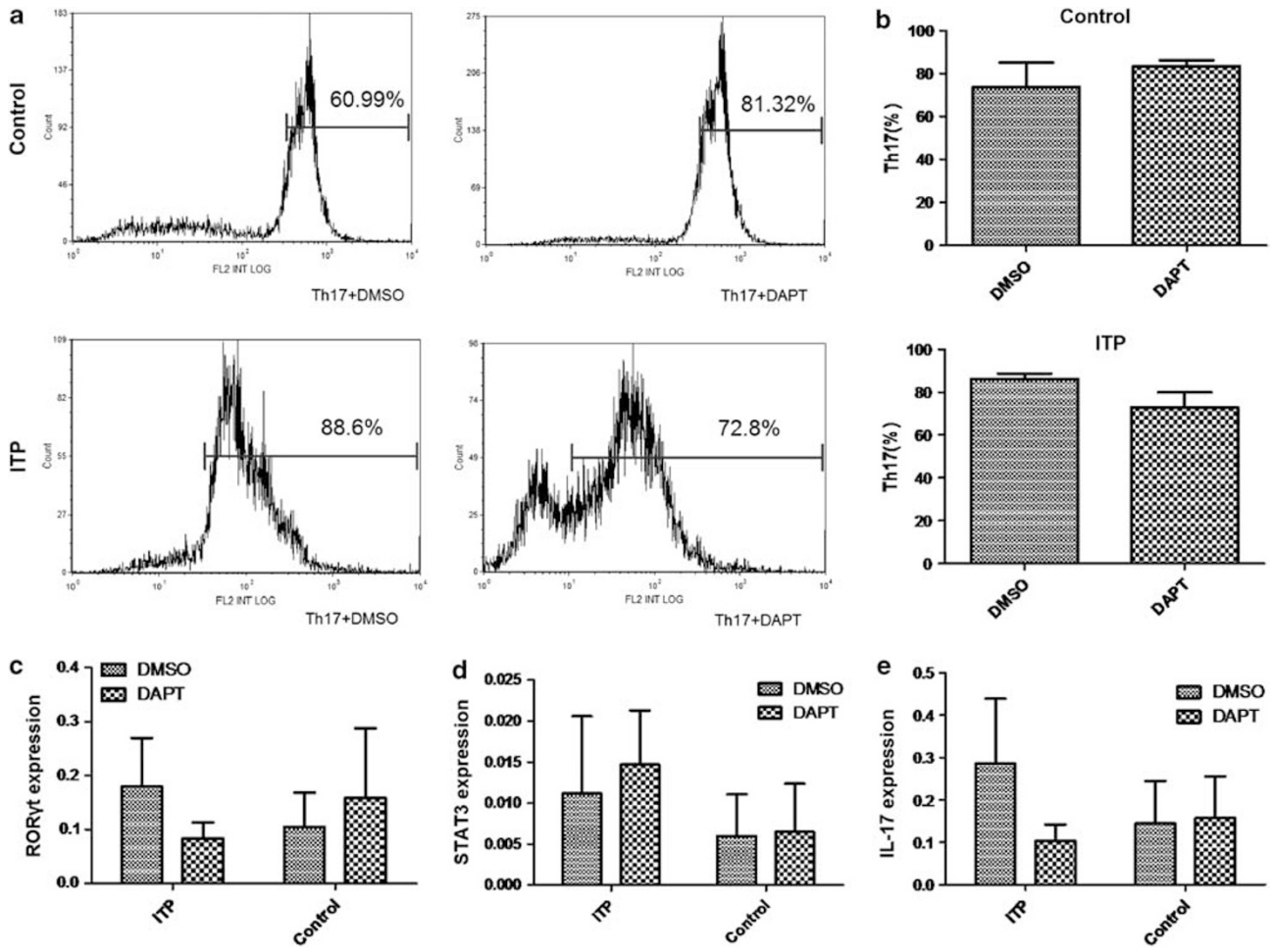

Figure 7 DAPT inhibited Th17 cell polarization by downregulation of RORγt and IL-17 transcription *in vitro* polarization assays. Naive CD4$^+$ T cells were cultured in Th17 cell-polarizing conditions for 4 days followed by treatment with DMSO or DAPT. Cells were collected after 72 h, Th17 cells were examined by flow cytometry, and mRNA expression of RORγt, STAT3, and IL-17 was examined by RT-PCR. (**a**, **b**) Alteration of Th17 cells with DAPT or DMSO treatment in ITP patients and healthy controls. The transcript levels of RORγt (**c**), STAT3 (**d**), and IL-17 (**e**). The data represent means ± s.d.

and IL-17F production in polarized human Th17 cells.[39] However, DAPT did not significantly reduce the secretion of IL-21 in ITP patients. That may be because IL-21 can be produced by multiple effector CD4$^+$ T cells and NK T cells, and IL-21 production is RORγt independent as reported before.[40] In addition, others have reported that Notch signaling was absolutely necessary for transcription of IL-10 by stimulated CD4$^+$ T cells.[41] However, we did not observe a significant decrease in IL-10 cytokine level in response to Notch inactivation, and this needs to be clarified in the future.

Th17 cytokine IL-17 or IL-21 was regulated by RORγt and cytokines associated with Treg cells such as IL-10 and TGF-β were regulated by Foxp3.[42] Previous studies have indicated that Notch signaling regulates specific transcription factor expression in T cells.[25] It has been demonstrated that Notch signaling upregulates RORγt expression by directly binding to the Rorc promoter.[42] Our findings suggested that the expression of RORγt was higher in untreated PBMCs from ITP patients compared with healthy controls, and DAPT could downregulate RORγt expression in ITP patients. The coordinated temporal downregulation of RORγt with the decreased Th17 cells and IL-17 secretion in the presence of DAPT indicates that RORγt might provide direct regulation of Th17 cells. Inhibition of the Notch signaling by DAPT may disrupt Th17 cell differentiation through downregulation of RORγt expression. The mRNA expression of Foxp3 was lower before DAPT treatment, but Foxp3 showed no significant change after DAPT treatment in ITP patients. The ratio of RORγt/Foxp3 was markedly elevated in ITP patients compared with healthy controls, and showed a reduction trend after DAPT treatment in ITP patients.

Along with defining a definitive role of Notch in Th17/Treg imbalance, our data further clarified the mechanism by which Notch regulates Th17 cell differentiation. Accumulating studies have reported that STAT3 was critically involved in Th17

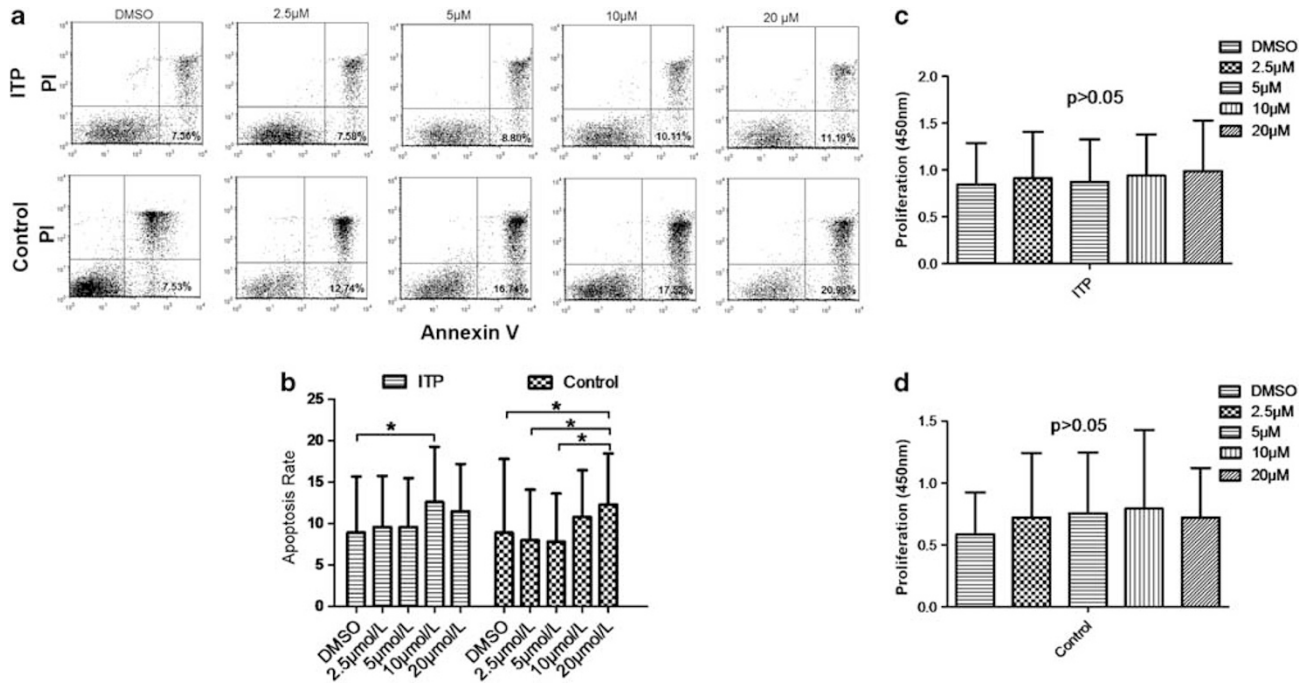

Figure 8 Results of apoptosis and cell proliferation. (**a**) The percentage of Annexin-positive and PI-negative cells on PBMCs from all treatments represented the cell apoptosis rate. (**b**) Comparison of apoptosis among 0, 2.5, 5, 10, and 20 μM DAPT treatments in ITP patients and controls. (**c**) The cell proliferation at all treatments in ITP patients. (**d**) The cell proliferation at all treatments in healthy controls. The data represent mean ± s.d. and $^*P < 0.05$.

cell lymphocyte differentiation,[43] and Notch was also found to regulate Th17 cell signature genes IL-17A and RORγt.[25] Keerthivasan *et al*[39] also present evidence that IL-17 and RORγt are direct transcriptional targets of Notch signaling in Th17 cells using promoter reporter assays, knockdown studies, as well as chromatin immunoprecipitation. To determine whether inhibition of Notch by DAPT regulates human Th17 cell polarization by influencing transcription of RORγt, STAT3, and IL-17, human $CD4^+$ T cells were differentiated *in vitro* toward the Th17 cell lineage in the presence of either DMSO or DAPT. Our results showed that transcript levels of RORγt and IL-17 were reduced in DAPT-treated Th17 cells, suggesting that Notch signaling may directly regulate Th17 cell development at least in part through regulation of RORγt and IL-17 transcription.

Notch signaling has been implicated in maintaining the balance of cell apoptosis. In addition, attenuation of Notch signaling in mature T cells has been shown to be detrimental to activation-induced proliferation.[44] Our results indicated that inhibition of Notch by DAPT can induce cell apoptosis. The enhanced apoptosis may be associated with the activation of Bcl-1 and Caspase-3 reported by Grottkau *et al.*[45] However, DAPT had no effect on PBMCs proliferation, and this is in accordance with two recent reports.[40,46]

In conclusion, our results suggested that inactivation of Notch signaling by DAPT could restore the balance of Th17/Treg in ITP patients. Notch signaling inhibitor may suppress Th17 cell response resulting from regulation of RORγt and IL-17 transcription.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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