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The potential therapeutic benefit of resveratrol on Th17/Treg imbalance in immune thrombocytopenic purpura

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ABSTRACT

Background: Immune thrombocytopenic purpura (ITP) is an autoimmune disease characterized by the restrained production of new platelets and the persistent reduction of existing platelets. An imbalance between Th17 and Treg cells is associated with a decrease in platelets. However, few therapeutic strategies aim to modulate this imbalance between Th17 and Treg cells in ITP.

Methods: ITP patients and healthy controls were enrolled in this study. Quantitative real-time PCR (qRT-PCR) and Western blotting were performed to measure the expression of the aryl hydrocarbon receptor (AhR), cytochrome P450 family 1 member A1 (CYP1A1), RAR-related orphan receptor gamma t (ROR-γt) and forkhead-box P3 (Foxp3). ELISA was employed to measure the secretion of IL-17A, IL-22 and IL-10. Flow cytometry was used to assess the proportion of Th17 and Treg cells. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to measure cell viability.

Results: The proportion of Th17 cells and the secretion of the pro-inflammatory cytokines IL-17A and IL-22 were both elevated, whereas the proportion of Treg cells and the production of the anti-inflammatory cytokine IL-10 were both reduced in ITP patients compared to healthy controls. The ratio of Th17/Treg cells and the expression of IL-17A and IL-22 displayed a positive correlation with the severity of ITP. Low and moderate concentrations of resveratrol did not affect the viability of $CD4^+$ T cells from ITP patients but repressed Th17 differentiation and promoted Treg differentiation. Moreover, resveratrol could markedly downregulate the production of IL-17A and IL-22 and upregulate the secretion of IL-10 in $CD4^+$ T cells in a time- and concentration-dependent manner. Mechanistic studies revealed that resveratrol exerted its beneficial function mainly through suppressing the AhR pathway, which led to the impaired expression of ROR-γt and reduced secretion of IL-17A and IL-22, as well as enhanced expression of Foxp3 and augmented secretion of IL-10. The induction of AhR by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in $CD4^+$ T cells led to a Th17/Treg imbalance and the upregulation of IL-17A and IL-22, an effect that could be reversed by resveratrol treatment.

Conclusion: This study revealed that resveratrol reversed the Th17/Treg imbalance by a mechanism involving the suppression of the AhR pathway. Since ITP is characterized by a Th17/Treg imbalance, resveratrol might be beneficial for the treatment of this condition.

1. Introduction

Immune thrombocytopenic purpura (ITP) is an autoimmune disease characterized by enhanced platelet destruction and impaired platelet production, which usually results in a low quantity of platelets and mucocutaneous bleeding, such as petechiae and purpura [1]. Generally, ITP patients will develop thrombocytopenia if the platelet count is lower than 150×10^9/L [2]. The estimated incidence of ITP for adults is 33 cases per 1 million persons every year, while for children, the incidence is between 19 and 64 per 1 million every year [3]. The pathogenesis and clinical features of ITP are heterogeneous in different patients, as is their response to various treatments. Currently, the first-line therapies for ITP comprise corticosteroids, intermittent intravenous IgG injection [4] and anti-RhD immunoglobulin (anti-D) treatment [5]. However, the effect of these drugs is restrained due to the relatively short-term responses and the common side effects. The second-line

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therapies include splenectomy and immunomodulatory drugs such as azathioprine, cyclosporine, mycophenolate, cyclophosphamide, danazol and dapsone [6]. In addition, two second-line agents, eltrombopag and romiplostim, have been approved by the FDA for ITP treatment in adults. Both of these drugs are thrombopoietin (TPO) receptor agonists, which can stimulate megakaryopoiesis to produce more platelets [7]. Nevertheless, the overall response rate varies among patients, and the side effects are quite obvious. Splenectomy is a traditional treatment that shows an early response rate up to 90% and a long-term response rate closer to 70% [8]. However, relapse still occurs in some patients. Moreover, the risk of thrombosis and severe infection after splenectomy limits its application for ITP. Thus, developing new drugs for ITP is required to improve the therapeutic efficacy.

Previous studies elucidated that the low density of platelets in ITP was closely associated with the differentiation and proportion of Th17 cells [9]. Separate from Th1, Th2 and Treg lineages, Th17 cells can secrete the pro-inflammatory cytokine IL-17 and contribute to the development of various autoimmune diseases [10]. RAR-related orphan receptor gamma t (ROR-γt) is a Th17-specific nuclear transcription factor that plays critical roles in the differentiation and development of the Th17 subset [11]. Previous reports revealed that both the proportion of Th17 cells and the production of IL-17A were increased in ITP patients, and the increase was positively correlated with the incidence of ITP [12]. In addition, accumulated evidence showed that the proportion of Treg cells was downregulated and that the function of Treg cells was impaired in ITP patients [13]. However, these symptoms could be mitigated by rituximab administration [14]. Therefore, the balance between Th17 and Treg cells is crucial for maintaining the homeostasis of the immune system and improving the therapeutic efficacy of ITP.

Aryl hydrocarbon receptor (AhR) is a highly conserved and ligand-activated transcription factor that is involved in the regulation of cellular responses to planar aromatic (aryl) hydrocarbons. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a well-studied AhR ligand that can bind AhR to mediate its toxicity and tumour-promoting function [15]. Previous studies showed that AhR could regulate the differentiation and cytokine release of Th17 cells [16]. Meanwhile, some reports demonstrated that TCDD administration enhanced the proliferation of Treg cells and thus suppressed the progression of autoimmune diseases in mouse models [17], or improved the graft survival ratio [18]. Therefore, AhR signalling may influence the balance between Th17 and Treg cells [19]. Upon ligand engagement, AhR can translocate to the nucleus and interact with the corresponding xenobiotic response elements to activate the transcription of cytochrome P450 family 1 member A1 (CYP1A1). CYP1A1 is one of the main cytochrome P450 enzymes that can catalyse the bioactivation of procarcinogens and pro-teratogens [20]. Therefore, we speculated that the regulation of the imbalance between Th17 and Treg cells via the AhR pathway should be a reliable method to treat ITP.

As a known AhR antagonist, resveratrol has been studied in the prevention and treatment of cardiovascular diseases, metabolic diseases, neurodegenerative diseases, ageing, inflammation and cancers [21]. Moreover, early studies confirmed that resveratrol could ameliorate the severity of autoimmune diseases [22], which were associated with elevated Th17 differentiation and impaired Treg function. In one recent report, Yao et al. found that resveratrol treatment could decrease the Th17 proportion while increasing the Treg population in an ulcerative colitis mouse model [23]. Thus, we hypothesized that treating ITP using resveratrol to modulate Th17/Treg imbalance would be an appealing and promising approach.

In general, our study focused on the potential therapeutic benefit of resveratrol on the Th17/Treg imbalance in ITP patients. In this study, we first found that the Th17 proportion and Th17-related cytokine production were both enhanced, while the Treg proportion and IL-10 secretion were downregulated in ITP patients. A mechanistic study revealed that resveratrol treatment did not affect cell viability but significantly inhibited Th17 differentiation while promoting Treg differentiation. Moreover, resveratrol administration reversed the imbalance between Th17 and Treg subsets, as well as the cytokine profiles, by suppressing the AhR-CYP1A1 pathway. Herein, our study provides more detailed information on the pathogenesis and treatment of ITP, which might aid in the design of new drugs for ITP in the future.

2. Methods

2.1. Reagents

RPMI1640 medium, foetal bovine serum (FBS), TRIzol Plus RNA Purification Kit, SuperScript IV reverse transcriptase, Pierce BCA Protein Assay Kit, MTT Assay Kit, Pierce ECL Western blotting substrate, mouse anti-human AhR (RPT1) antibody, rabbit anti-human CYP1A1 (PA1-340) antibody, rat anti-human ROR-γt (AFKJS-9) antibody, goat anti-mouse IgG-HRP, goat anti-rat IgG-HRP and goat anti-rabbit IgG-HRP antibodies were obtained from Thermo Fisher Scientific. Rabbit anti-human Foxp3 (D25D4) and rabbit anti-human GAPDH (14C10) antibodies were obtained from Cell Signalling Technology. FITC-labelled anti-human CD4 antibody, PE-labelled anti-human CD25 antibody, Alexa 647-labelled anti-human Foxp3 antibody, APC/Cy7-labelled anti-human IL-17A antibody and Foxp3 fixation/permeabilization buffer were obtained from Biolegend. All of the qRT-PCR primers were synthesized by Invitrogen. SYBR Green Master Mix was purchased from Bio-Rad. Human IL-17A, IL-22 and IL-10 cytokine ELISA kits, human IL-1β, IL-2, IL-6, IL-23 and TGF-β1, anti-IL-4 antibody and anti-IFN-γ antibody were all from R&D systems. Resveratrol, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and DMSO were obtained from Sigma-Aldrich. Ficoll-Paque PREMIUM was from GE Healthcare. An EasySep Human $CD4^+$ T cell Enrichment Kit was obtained from Stem Cell Technologies.

2.2. Patient recruitment

A total of 30 inpatients with ITP from the Second Affiliated Hospital of Nanchang University (Nanchang, Jiangxi, China) were enrolled in this study. Active cases were defined as patients with platelet counts $< 50 \times 10^9$/L and typical clinical features of bleeding; relapsed and newly diagnosed ITP patients were also included. Stable cases were generally identified by stable platelet count $> 50 \times 10^9$/L and $< 150 \times 10^9$/L without large fluctuations or with no bleeding having occurred over the prior six months. Remission cases were defined as patients who maintained a normal platelet count for at least six months without clinical manifestation of bleeding [24]. Meanwhile, 30 healthy donors who were age- and gender-matched with the ITP patients were enrolled in this study from health checks at our hospital. Prior to collecting the clinical data and blood samples, written informed consent forms were signed by the patients and healthy donors. This research was approved by the Medical Ethics Committee at our hospital. The clinical features of these patients are shown in Table 1.

Table 1
Basic information about 30 patients with ITP.

	Age (median, range)	Sex (male/female)	Course of disease (months)	Platelet count (median, range) $\times 10^9$/L
Active (n = 10)	42 (18–57)	4/6	18 (12–45)	25 (8–50)
Stable (n = 9)	47 (19–62)	5/4	32 (12–90)	94 (56–158)
Remission (n = 11)	33 (19–64)	8/3	36 (18–75)	217 (180–323)

ITP, primary immune thrombocytopenia; date was expressed as median (range).

2.3. The isolation of peripheral blood mononuclear cells (PBMC) and $CD4^+$ T cells from blood

Peripheral blood specimens were collected from the ITP patients and healthy donors, and PBMCs were isolated by density gradient centrifugation using Ficoll-Paque PREMIUM. Subsequently, human $CD4^+$ T cells were isolated from PBMCs using the EasySep Human $CD4^+$ T Cell Enrichment Kit according to the manufacturer's instructions.

2.4. The in vitro induction and treatment of Th17 and Treg cells

Human $CD4^+$ T cells were cultured in 24-well culture plates precoated with anti-CD3 antibody (5 μg/mL) together with soluble anti-CD28 antibody (1 μg/mL) in RPMI1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 50 μg/mL streptomycin. To induce Th17 differentiation, the medium was supplemented with IL-6 (10 ng/mL), IL-1β (10 ng/mL), TGF-β1 (10 ng/mL), IL-23 (10 ng/mL) and the neutralizing antibodies anti-IL-4 (10 μg/mL) and anti-IFN-γ (10 μg/mL). To induce Treg differentiation, the medium was supplemented with IL-2 (300 U/mL), TGF-β1 (10 ng/mL), anti-IL-4 (10 μg/mL) and anti-IFN-γ (10 μg/mL). The cells were cultured for 96 h at 37 °C and then harvested for analysis.

To test the effect of resveratrol on Th17 and Treg differentiation, vehicle (DMSO) or 10, 30, or 50 μM resveratrol was added to the culture medium 24 h before the end of induction, or 30 μM resveratrol was added to the culture medium at 12 h, 24 h, and 48 h before the end of induction.

2.5. Th17 and Treg analysis by flow cytometry

Human $CD4^+$ T cells (1×10^6/mL) were stimulated with 10 ng/mL TPA and 500 ng/mL ionomycin in complete medium for 3 h, then 10 μg/mL brefeldin A was added to block secretion. After an additional 3 h, human $CD4^+$ T cells were harvested and washed with PBS. To analyse the proportion of Th17 cells, $CD4^+$ T cells were first stained with FITC-conjugated anti-human CD4 antibody at 4 °C for 30 min. Then, they were fixed and permeabilized with fixation/permeabilization buffer and were intracellularly stained with APC/Cy7-conjugated anti-human IL-17A antibody at room temperature in the dark for 30 min. To analyse the proportion of Treg cells, $CD4^+$ T cells were simultaneously stained with FITC-conjugated anti-human CD4 antibody and PE-conjugated anti-human CD25 antibody, then fixed and permeabilized and intracellularly stained with Alexa 647-labelled anti-human Foxp3 antibody at room temperature in the dark for 30 min. Isotype-matched control antibodies were used in all staining processes. Flow cytometric analysis was performed on an LSRII flow cytometer (Becton Dickinson, USA), and the data were further analysed by FlowJo v7.6.5 software (TreeStar, USA).

2.6. Cell viability measurement by MTT assay

$CD4^+$ T cells were cultured in 96-well plates at a density of 10^4 cells per well in 100 μL complete medium. For each concentration of resveratrol, five repeated wells were prepared, and a blank control group with culture medium only was also set. An equal volume of DMSO was added to the vehicle group. Cell viability was measured at the indicated time points by using the MTT Assay Kit following the manufacturer's instructions. Briefly, 10 μL MTT solution was gently loaded into each well and incubated at 37 °C in a humidified incubator with 5% CO_2 for 4 h. Next, 75 μL of the medium was removed from each well, and then 50 μL of DMSO was added and thoroughly mixed with the pipette. Then, the 96-well plate was incubated at 37 °C for 10 min. The optical density (OD) was measured at 540 nm by a plate reader (SpectraMax® M5e, Molecular Devices). All OD data were corrected by subtracting the OD value of the culture medium only group.

2.7. Western blot analysis

A total of 5×10^5 $CD4^+$ T cells were directly lysed in 50 μL of RIPA buffer supplemented with protease and phosphatase inhibitors. After centrifugation, the supernatant was collected for subsequent protein quantification with a BCA kit. Then, the supernatant from each group was mixed with loading buffer, and the solution was boiled for 10 min. Equal amounts of proteins were separated by SDS-PAGE, and the separated proteins in the gel were transferred to PVDF membranes, which were subsequently blocked with BSA. Next, the PVDF membranes were incubated with the indicated primary antibodies. After washing, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies. Following extensive washing, the PVDF membranes were incubated with ECL substrate and subjected to X-ray film exposure in a dark room. The proteins were quantified using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.8. RNA extraction and quantitative real-time PCR

Total RNA was extracted by TRIzol Plus RNA Purification Kit according to the manufacturer's instructions. The concentration of total RNA was determined by measuring UV absorbance at 260 nm and 280 nm using a spectrophotometer. The first-strand cDNA, which was used for subsequent quantitative real-time PCR, was obtained with SuperScript™ IV reverse transcriptase and $Oligo(dT)_{15}$ primers by reverse transcription PCR with SYBR Green Master Mix. The relative expression levels of AhR, CYP1A1, ROR-γt and Foxp3 mRNA were normalized to GAPDH mRNA. Relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. The specific primers used in real-time PCR were as follows: *Ahr* forward: 5′-CAAATCCTTCCAAGCGGCATA-3′; *Ahr* reverse: 5′-CGCTGAGCCTAAGAACTGAAAG-3′; *Cyp1a1* forward: 5′-TCGGCCACGGAGTTTCTTC-3′; *Cyp1a1* reverse: 5′-GGTCAGCATGTGCCCAATCA-3′; *Ror-γt* forward: 5′-GTGGGGACAAGTCGTCTGG-3′; *Ror-γt* reverse: 5′-AGTGCTGGCATCGGTTTCG-3′; *Foxp3* forward: 5′-GTGGCCCGGATGTGAGAAG-3′; *Foxp3* reverse: 5′-GGAGCCCTTGTCGGATGATG-3′; *Gapdh* forward: 5′-AGCCACATCGCTCAGACAC-3′; *Gapdh* reverse: 5′-GCCCAATACGACCAAATCC-3′. The quantitative real-time PCR experiment was performed on a QuantStudio 3 Real-Time PCR System (Applied Biosystems).

2.9. Cytokine secretion by enzyme-linked immunosorbent assay (ELISA)

$CD4^+$ T cells were cultured in Th17 or Treg induction medium together with vehicle (DMSO) or 10 μM, 30 μM, or 50 μM resveratrol for 24 h or with 30 μM resveratrol for 0, 12 h, 24 h, and 48 h. Subsequently, the culture supernatant was collected by centrifugation, and the concentrations of IL-17A, IL-22 and IL-10 in the supernatant were measured using the corresponding human ELISA Kits (R&D systems) according to the manufacturer's instructions. The cytokine levels in human serum from ITP patients and healthy donors in Fig. 1 were directly quantified with the above human ELISA Kits. All experiments were performed in triplicate, and the plates were analysed with a microplate reader (Synergy NEO, BioTek).

2.10. Data analysis

All experiments were performed at least three times, and the data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed by GraphPad Prism 6 (GraphPad Software, Inc.). The significance of the difference was determined as indicated in the figure legends. $*P < 0.05$ was considered significant. An unpaired two-tailed *t*-test was used for comparison between two groups. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for multiple comparisons.

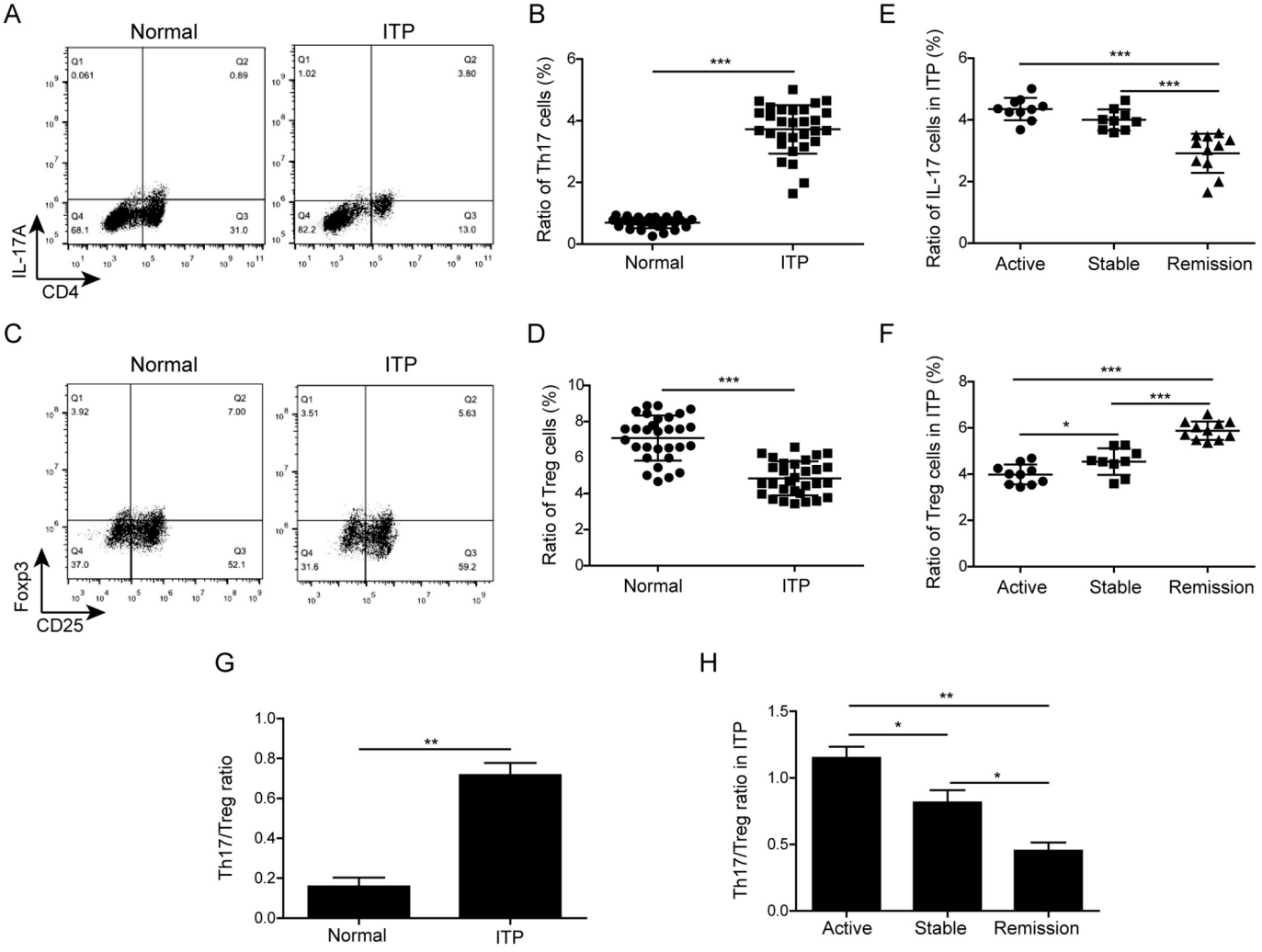

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Fig. 1. Inflammatory cytokine dysregulation and imbalance between Th17 and Treg cells in ITP patients.
Representative data showing the proportion of Th17 cells ($CD4^+IL\text{-}17A^+$) (A) or Treg cells ($CD4^+CD25^+Foxp3^+$) (C) in $CD4^+$ T cells isolated from the peripheral blood of ITP patients or healthy donors. Mean data are also shown (B, D). Also shown is the proportion of Th17 cells (E) or Treg cells (F) in $CD4^+$ T cells from ITP patients in active, stable and remission stages and the ratio of Th17/Treg cells in ITP patients or healthy controls (G), or in active, stable and remission stages of ITP patients (H). IL-17A, IL-22 and IL-10 levels in the serum of ITP patients and healthy donors (I), and in active, stable and remission stages of ITP patients (J) were monitored. Data are representative of three independent experiments using the same batch of ITP patients. Data represent mean ± SD. *p* values were determined by unpaired two-tailed *t*-test (B, D, G, I) or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (E, F, H, J). $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$.

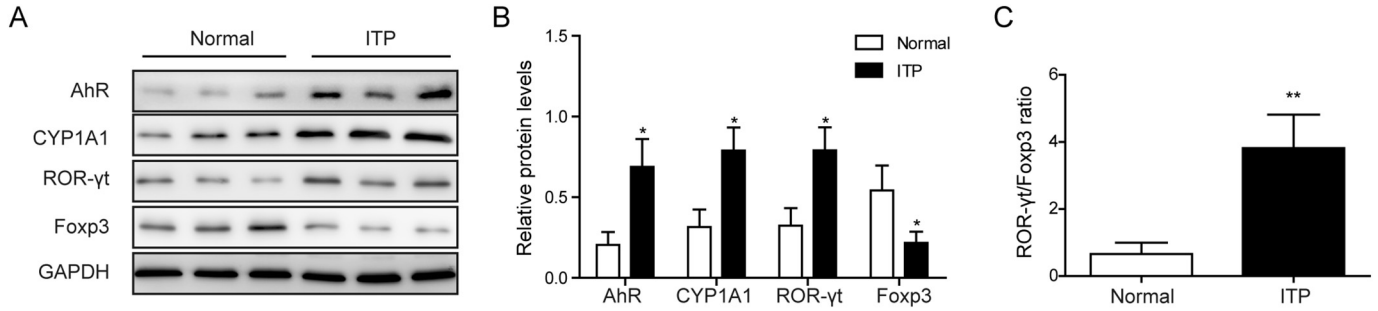

Fig. 2. The expression levels of AhR, CYP1A1 and ROR-γt were up-regulated while Foxp3 was down-regulated in ITP patients.
(A) The expression levels of AhR, CYP1A1, ROR-γt and Foxp3 in $CD4^+$ T cells sorted from ITP patients or healthy controls were assessed by Western blotting. GAPDH was used as the loading control. Results are representative of three independent experiments using the same batch of ITP patients. (B) Data show the relative expression of AhR, ROR-γt, CYP1A1 and Foxp3 after normalization to GAPDH. (C) The ratio of ROR-γt/Foxp3 is also shown. Data represent mean ± SD. *p* values were determined by unpaired two-tailed *t*-test. $^{**}p < 0.01$, $^{*}p < 0.05$.

3. Results

3.1. The clinical features of patients with ITP

In this study, we enrolled 30 patients with ITP and 30 healthy donors with matched age and gender. Among these ITP patients, there were approximately 33% of patients in the active stage, 30% in the stable stage, and the remaining 37% were in the remission stage (Table 1). The youngest patient was 18 years old, the oldest patient was 64 years old, and most patients were between 30 and 50 years old. There were 17 males and 13 females in this group of patients; in particular, only three female patients were in the remission group, which implied that the male patients might be more susceptible to ITP. The course of disease extended from the active stage to the remission stage, and the platelet quantity increased significantly during this process, from 25×10^9/L in the active stage to 217×10^9/L in the remission stage. Moreover, the median age of ITP patients in the remission group was 33, which was lower than in the active group (42) or stable group (47), suggesting that it was easier for young patients to reach the remission stage of ITP.

3.2. The imbalance between Th17 and Treg cells in ITP patients

Previous reports have shown that Th17/Treg imbalance might play crucial roles in the pathogenesis of autoimmune diseases [25]. To analyse the phenotype of Th17 and Treg cells in ITP, we first sorted $CD4^+$ T cells from the peripheral blood of ITP patients and healthy donors and then performed surface or intracellular staining for Th17- or Treg-specific markers. Flow cytometric analysis clearly showed that the proportion of Th17 cells was increased in ITP patients, whereas the proportion of Treg cells was decreased when compared with healthy donors (Fig. 1A–D). The Th17/Treg ratio further confirmed the elevated number of Th17 cells and reduced number of Treg cells in ITP patients (Fig. 1G). Moreover, patients in different stages of ITP displayed distinct proportions of Th17 or Treg cells. For example, patients in the active stage had the highest ratio of Th17 cells and the lowest ratio of Treg cells, while patients in the remission stage had the reverse phenotype (Fig. 1E–F). The Th17/Treg ratio in various stages of ITP was positively correlated with the severity of ITP (Fig. 1H). In addition, we measured the concentrations of IL-17A, IL-22 and IL-10 in the serum of ITP patients and healthy donors by ELISA. Consistently, the Th17-derived pro-inflammatory cytokines IL-17A and IL-22 were both upregulated, whereas the Treg-derived anti-inflammatory cytokine IL-10 was downregulated in ITP patients (Fig. 1I). Specifically, we analysed the secretion of IL-17A, IL-22 and IL-10 in various stages of ITP, and the results showed that patients in the active stage had the highest levels of IL-17A and IL-22 and the lowest level of IL-10, which was contrary to patients in the remission stage (Fig. 1J). In summary, the experiments here suggested that ITP patients had an increased proportion of Th17 cells and increased production of IL-17A and IL-22 with a diminished Treg cell proportion as well as reduced IL-10 secretion. Furthermore, the Th17/Treg ratio and the expression of IL-17A and IL-22 displayed a positive correlation with the severity of ITP.

3.3. AhR activation was positively correlated with the imbalance between Th17 and Treg cells in ITP patients

To explore the mechanism behind the dysregulation of Th17 and Treg cells in ITP, we performed Western blotting experiments to assess the expression of ROR-γt and Foxp3, which are the specific nuclear transcription factors in Th17 and Treg cells, respectively. Consistently, $CD4^+$ T cells from ITP patients showed higher expression of ROR-γt and lower expression of Foxp3 when compared with $CD4^+$ T cells from healthy donors (Fig. 2A–B). In addition, evidence indicates that the AhR pathway plays a crucial role in the regulation of Th17 and Treg cells. Therefore, we examined the expression of AhR and its downstream protein CYP1A1 by Western blotting, and the results showed that both AhR and CYP1A1 were significantly upregulated in $CD4^+$ T cells from ITP patients (Fig. 2A–B). Moreover, the elevated ratio of ROR-γt/Foxp3 further demonstrated that the differentiation of Th17 cells was augmented while Treg differentiation was suppressed in ITP (Fig. 2C). Herein, we revealed that AhR activation was closely related to the dysregulation of Th17 and Treg cells in ITP patients.

3.4. Low and moderate doses of resveratrol did not affect the viability of $CD4^+$ T cells

Resveratrol has been reported to be an antagonist of the AhR

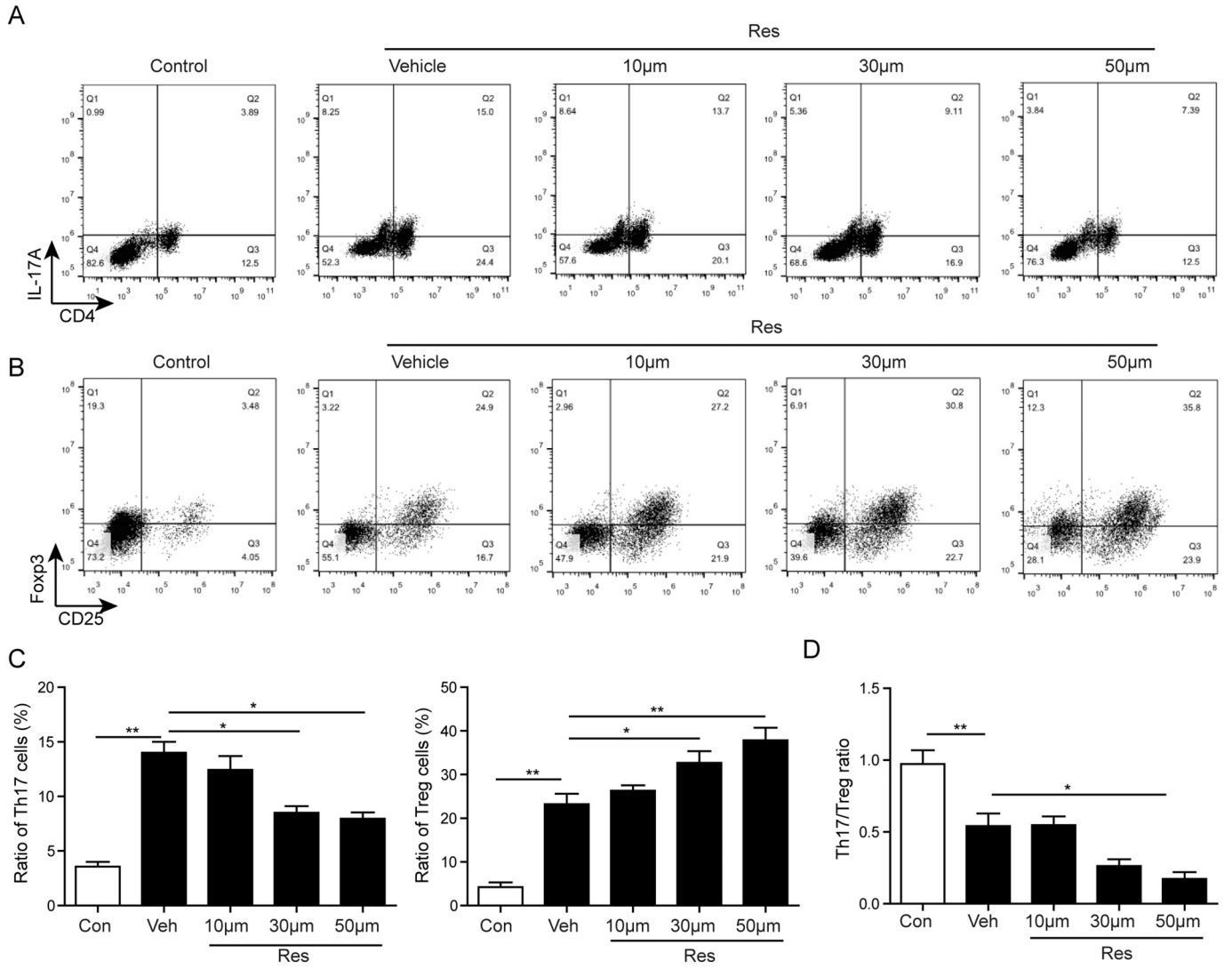

Fig. 3. Resveratrol inhibited Th17 differentiation yet promoted Treg differentiation in a concentration-dependent manner.
$CD4^+$ T cells isolated from ITP patients were cultured in either (A, C) Th17- or (B, C) Treg-inducing conditions, then they were treated with increasing concentrations of resveratrol for 24 h or not. DMSO was added to the vehicle group. The proportion of Th17 cells (A, C) and Treg cells (B, C) in the $CD4^+$ T cell population was analysed by flow cytometry. (D) The ratio of Th17/Treg following treatment with increasing concentrations of resveratrol is shown. For (A, B) data are representative of three independent experiments using newly differentiated Th17 or Treg cells on each occasion. Data represent mean ± SD. *p* values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $^{**}p < 0.01$, $^{*}p < 0.05$.

pathway that can ameliorate some autoimmune diseases by modulating Th17 and Treg cells. To explore the potential benefits of resveratrol for ITP, we first examined its effect on cell viability by culturing $CD4^+$ T cells from ITP patients with varying concentrations of resveratrol for 12 h, 24 h, 48 h and 96 h. Then, the cell viability was determined by MTT assay. The results showed that low doses of resveratrol (5 μM, 10 μM, 30 μM) could slightly enhance the viability of $CD4^+$ T cells. However, high doses of resveratrol (70 μM, 90 μM, 110 μM) showed some cytotoxicity in $CD4^+$ T cells (Supplementary Fig. 1). Moreover, the repression of cell viability by resveratrol was aggravated as time extended; for example, 96 h treatment with 110 μM resveratrol led to an almost 60% reduction in cell viability (Supplementary Fig. 1). Here, our results indicated that low and moderate concentrations of resveratrol had no influence on cell viability, and high concentrations of resveratrol could inhibit cell viability in a concentration- and time-dependent manner. Therefore, we mainly used low and moderate doses of resveratrol (10 μM, 30 μM, 50 μM) to treat $CD4^+$ T cells for < 48 h in the subsequent experiments.

3.5. Resveratrol attenuated Th17 cell differentiation while facilitating Treg cell differentiation

In previous studies, Bakheet, Saleh A. et al. reported that resveratrol could mitigate the dysregulation of Th1, Th2, Th17 cells, and Treg-related transcription factors in a mouse model of Autism [26]. To study the effect of resveratrol on Th17 and Treg differentiation, we collected naive $CD4^+$ T cells from the peripheral blood of ITP patients and cultured them under Th17- or Treg-inducing conditions in the absence or presence of resveratrol. The proportions of differentiated Th17 or Treg cells were assessed by flow cytometry. Compared with the vehicle, resveratrol potently suppressed Th17 differentiation (Figs. 3A, 4A), whereas it promoted Treg differentiation (Figs. 3B, 4B). Meanwhile, the effect of resveratrol on Th17 or Treg cells was dose- and time-dependent (Figs. 3C, 4C). The ratios of Th17/Treg cells in response to different resveratrol treatments also showed that resveratrol could modulate the balance between Th17 and Treg cells (Figs. 3D, 4D). Based on these data, we concluded that resveratrol could attenuate Th17 differentiation while promoting Treg differentiation in a concentration- and

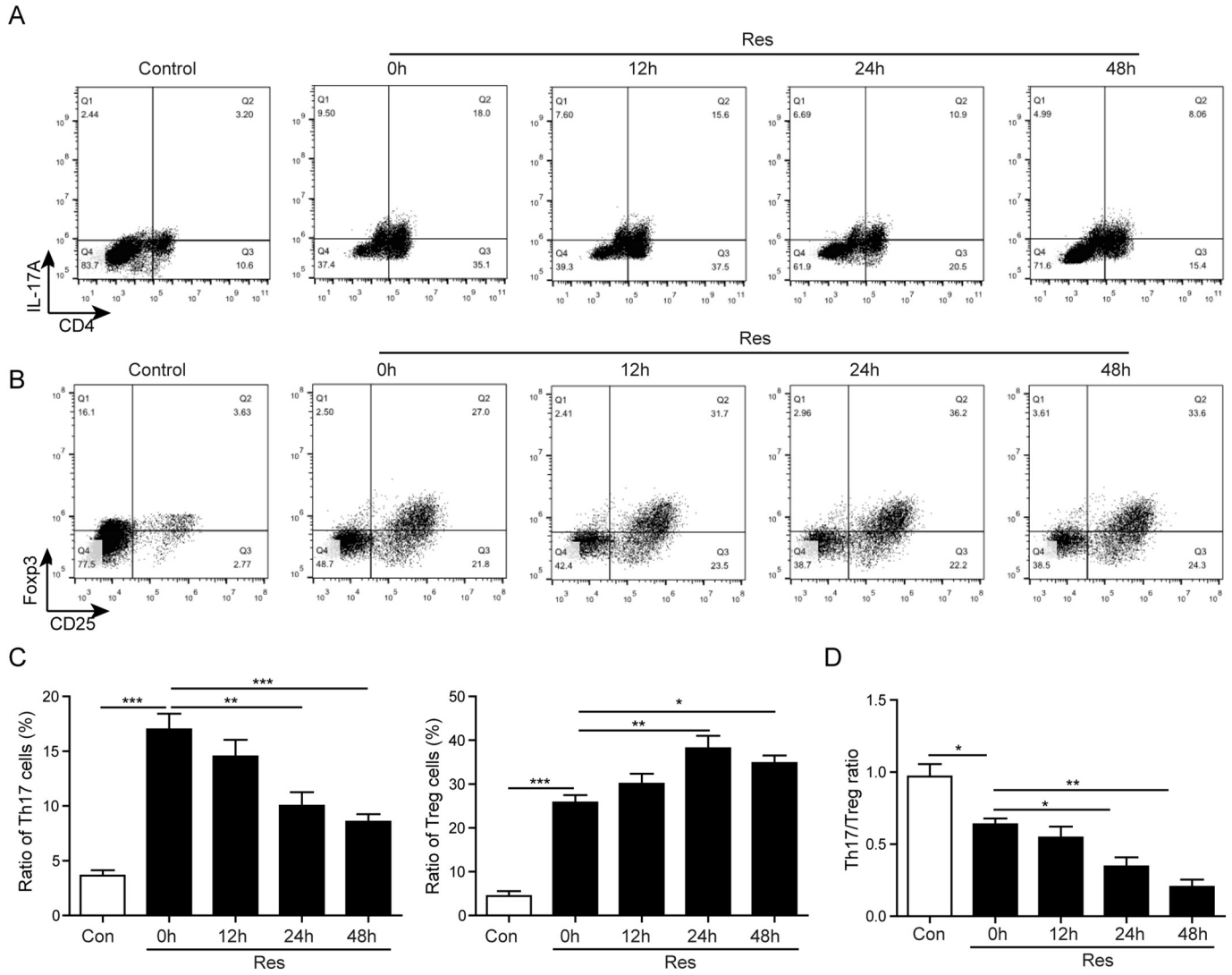

Fig. 4. Resveratrol suppressed Th17 differentiation yet facilitated Treg differentiation in a time-dependent manner.
$CD4^+$ T cells isolated from ITP patients were cultured in either (A, C) Th17- or (B, C) Treg-inducing conditions, after which they were treated with 30 μM resveratrol for varying periods of time. DMSO equivalent was added to the vehicle group. The proportion of Th17 cells (A, C) and Treg cells (B, C) in the $CD4^+$ T cell population was analysed by flow cytometry. (D) The ratio of Th17/Treg following treatment with resveratrol (30 μM) for different time periods is shown. For (A, B) data are representative of three independent experiments using newly differentiated Th17 or Treg cells on each occasion. Data represent mean ± SD. *p* values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

time-dependent manner.

3.6. The effect of resveratrol on the secretion of IL-17A, IL-22 and IL-10

To determine the effect of resveratrol on the cytokine secretion of Th17 cells, we collected $CD4^+$ T cells from the peripheral blood of ITP patients and then induced the differentiation of Th17 cells in the absence or presence of resveratrol. The production of IL-17A, IL-22 and IL-10 was determined by ELISA. Resveratrol treatment significantly diminished the production of the pro-inflammatory cytokines IL-17A and IL-22, whereas it enhanced the secretion of the anti-inflammatory cytokine IL-10 (Fig. 5A–B). Furthermore, the regulation of cytokine production by resveratrol also showed a concentration- and time-dependent feature (Fig. 5A–B). Taken together, the results demonstrated that resveratrol not only regulated the differentiation of Th17 and Treg cells but also modulated cytokine production, both in a concentration- and time-dependent manner.

3.7. Resveratrol regulated the Th17/Treg balance via the AhR pathway

To uncover the molecular mechanism for the effect of resveratrol on Th17 and Treg cells, we first checked the expression of ROR-γt and Foxp3 in the presence of resveratrol during the induction of Th17 subsets by Western blotting. As expected, resveratrol treatment led to impairment of ROR-γt in a dose- and time-dependent manner. However, Foxp3 expression was significantly elevated, which was the opposite of the ROR-γt pattern (Fig. 6A, C). The ratio of ROR-γt/Foxp3 from different doses or different treatment lengths further confirmed that the attenuation of ROR-γt and the augmentation of Foxp3 caused by resveratrol were concentration- and time-dependent (Fig. 6B, D). Because it is a pivotal regulator of Th17 differentiation, we also assessed the expression of AhR. Notably, resveratrol could significantly repress the expression of AhR as well as its downstream protein CYP1A1 in a concentration- and time-dependent manner (Fig. 6A, C). Moreover, the mRNA levels of AhR and CYP1A1 upon resveratrol treatment were consistent with the Western blot results (Fig. 6E–F). Although ROR-γt mRNA was downregulated after resveratrol treatment, it did not show

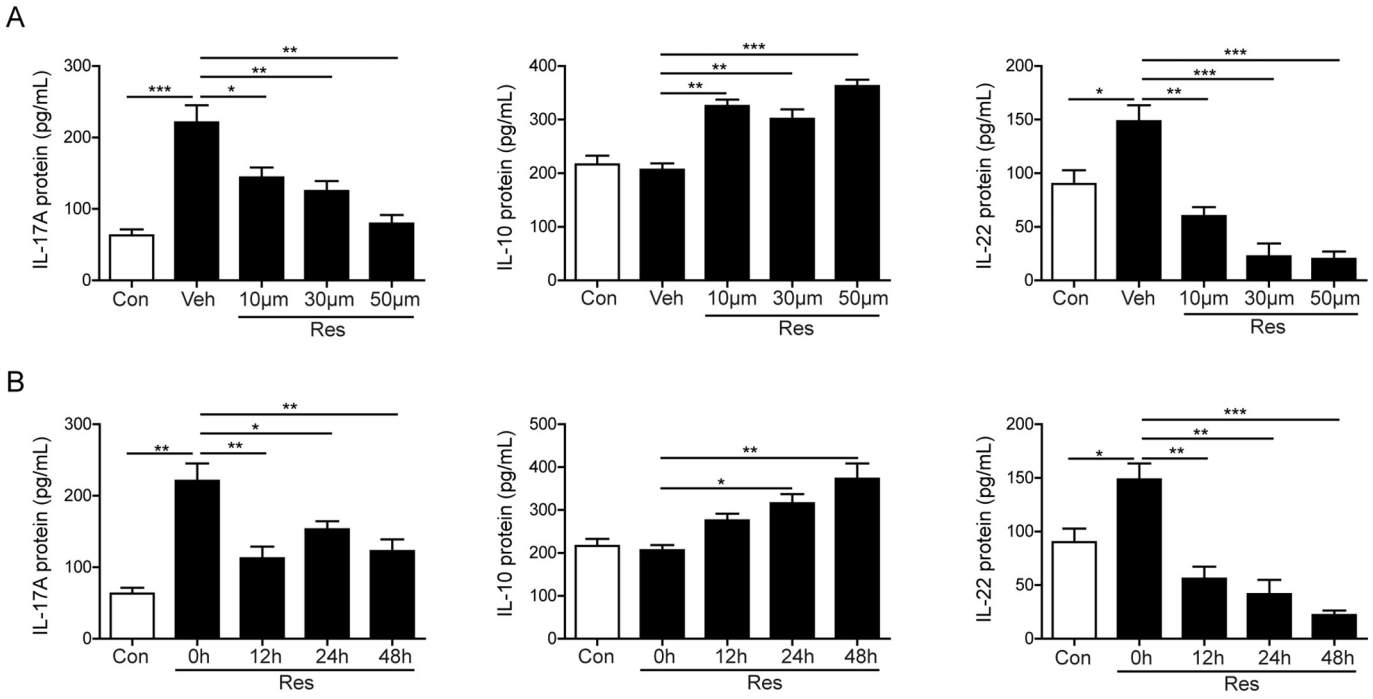

Fig. 5. Resveratrol impaired IL-17, IL-22 production yet enhanced IL-10 secretion in a concentration- and time-dependent manner.
$CD4^+$ T cells isolated from ITP patients were cultured in Th17-inducing condition, after which the cells were treated with either (A) varying concentrations of resveratrol for 24 h or (B) with a single concentration (30 μM) of resveratrol for varying time periods (0 to 48 h). DMSO equivalent was added to the vehicle group. IL-17A, IL-22 and IL-10 levels were measured in the supernatants by ELISA. The data are representative of three separate experiments using newly differentiated Th17 cells on each occasion. Data represent mean ± SD, p values were determined by the one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

obvious concentration- and time-dependent features. In contrast, Foxp3 mRNA did not display significant enhancement after resveratrol treatment. This indicated that other regulatory mechanisms might participate in the regulation of Foxp3 expression upon resveratrol administration (Fig. 6E–F). In conclusion, we found that resveratrol could regulate the Th17/Treg balance by modulating the AhR pathway.

3.8. The AhR pathway is critical for Th17/Treg balance regulated by resveratrol

To ascertain the association between the suppression of the AhR pathway and the remission of the Th17/Treg imbalance during resveratrol treatment, we used the AhR agonist TCDD to induce the activation of AhR. Western blotting results showed that TCDD treatment could robustly increase the expression of AhR and CYP1A1 compared with vehicle treatment, which showed an opposite effect on the AhR pathway from resveratrol. Furthermore, ROR-γt expression was upregulated, while Foxp3 expression was slightly downregulated upon TCDD administration (Fig. 7A). It was noted that TCDD plus resveratrol treatment resulted in the significant suppression of AhR, CYP1A1 and ROR-γt but obvious augmentation of Foxp3 compared with TCDD treatment (Fig. 7A). The ROR-γt/Foxp3 ratio in different groups further confirmed that AhR pathway suppression was crucial for the reversal of Th17/Treg imbalance (Fig. 7B). Moreover, the relative mRNA levels of AhR, CYP1A1, ROR-γt and Foxp3 upon various treatments displayed a similar pattern as that in the Western blotting experiment, which indicated that the downregulated AhR pathway was closely related to the impairment of ROR-γt and the enhancement of Foxp3 (Fig. 7C). During the differentiation of Th17 cells, TCDD administration significantly increased the proportion of Th17 cells, whereas the combined resveratrol and TCDD treatment completely diminished the effect of TCDD on Th17 differentiation (Fig. 7D–E). In contrast, the Treg population was reduced upon TCDD treatment but was reinstated after treatment with resveratrol plus TCDD (Fig. 7D–E). Additionally, the alteration of the Th17/Treg ratio upon various treatments further demonstrated that AhR pathway inhibition was closely associated with the reinstatement of the Th17/Treg balance (Fig. 7E). Finally, the secretion of IL-17A, IL-10 and IL-22 in Th17 or Treg cells was measured by ELISA. The data revealed that IL-17A and IL-22 were significantly upregulated while IL-10 was severely impaired by TCDD, while additional resveratrol treatment almost abolished the effect of TCDD on cytokine secretion (Fig. 7F). Taken together, these data demonstrated that AhR pathway inhibition was crucial for Th17/Treg balance regulated by resveratrol.

4. Discussion

Extensive studies have demonstrated that ITP is an acquired autoimmune disease related to a dysregulated immune system, in which T cells play a critical role [27]. As two distinct subsets of $CD4^+$ T cells, both Th17 and Treg cells participate in the pathogenesis of ITP. Emerging evidence has shown that the Th17 proportion is upregulated, while Treg cell numbers are obviously reduced in ITP patients. Furthermore, the Th17/Treg ratio is also closely correlated with the disease stage of ITP [28]. Moreover, Th17/Treg imbalance is associated with the severity of other autoimmune diseases, including systemic lupus erythematosus and arthritis [29]. Therefore, we decided to investigate the Th17/Treg imbalance in ITP patients and to reveal the underlying mechanism and the association between the Th17/Treg imbalance and the clinical features of ITP. In addition, we aimed to restore the Th17/Treg balance with resveratrol. This study of immune dysfunction in ITP may help researchers understand the pathogenesis of ITP and improve the efficacy of ITP therapies.

In the present study, we first surveyed the clinical features of ITP patients at different stages of the disease. As shown in Table 1, patients in later stages generally had higher platelet counts in their peripheral blood, which meant that the platelet generation had gradually

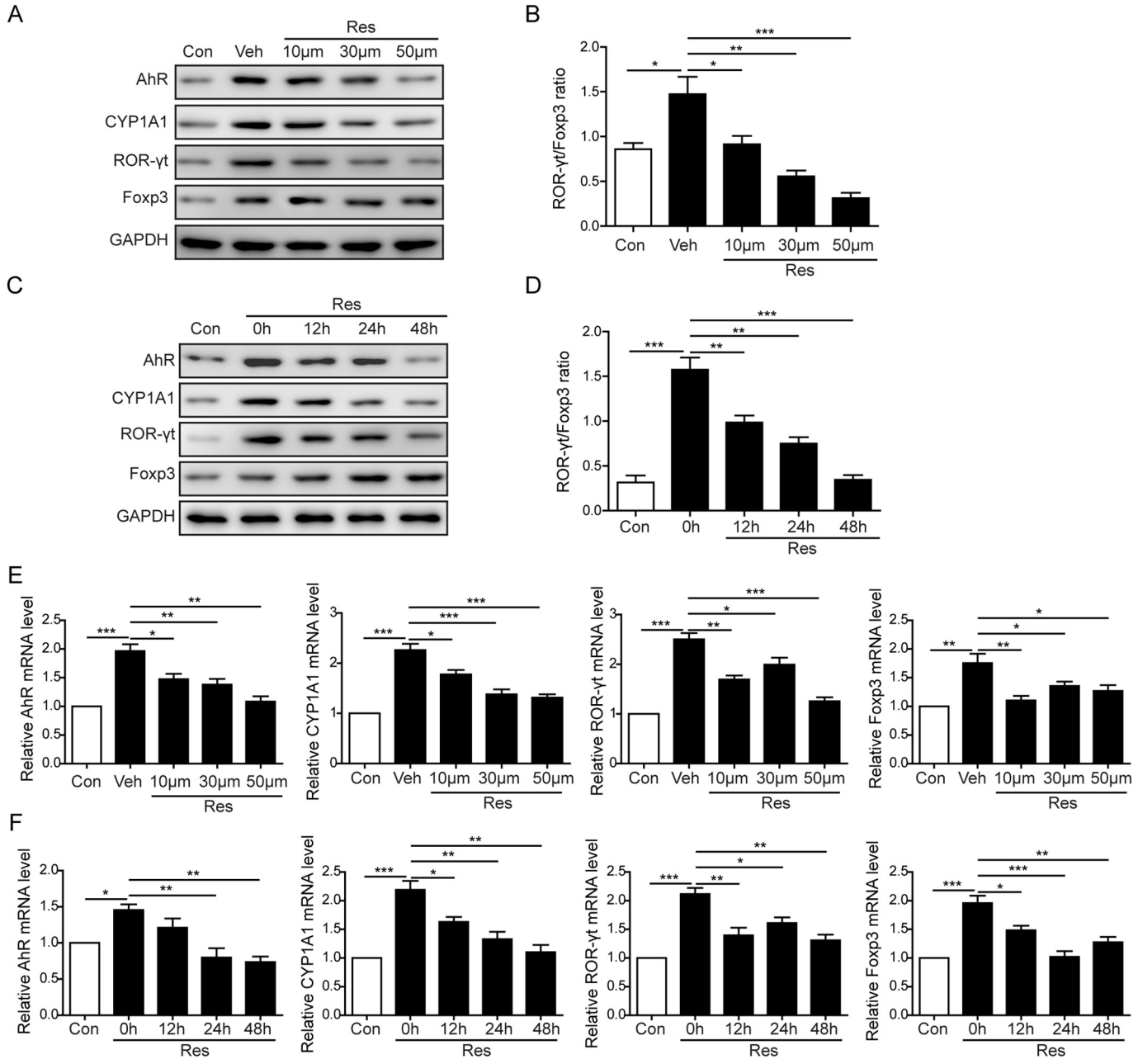

Fig. 6. Resveratrol could reinstate the balance between Th17 and Treg cells via suppressing the expression of AhR, ROR-γt, CYP1A1 and enhancing Foxp3. $CD4^+$ T cells isolated from ITP patients were cultured in Th17-inducing condition, after which the cells were treated with either (A, B, E) varying concentrations of resveratrol for 24 h or (C, D, F) with a single concentration (30 μM) of resveratrol for varying time periods (0 to 48 h). DMSO equivalent was added to the vehicle group. (A, C) The expression levels of AhR, ROR-γt, CYP1A1 and Foxp3 in $CD4^+$ T cells were assessed by Western blotting. GAPDH was used as the loading control. (B, D) The ratio of ROR-γt/Foxp3 was assessed by densitometry. (E, F) The relative mRNA levels of AhR, ROR-γt, CYP1A1 in $CD4^+$ T cells were determined. (A, C, E, F) Results are representative of three separate experiments using newly differentiated Th17 cells on each occasion. Data represent mean ± SD. *p* values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

recovered and the destruction of platelets by the immune system was attenuated after long-term treatment. Consistently, a previous report found that the anti-platelet antibody in serum was decreased in ITP patients in the remission stage [30], which further supports evidence that the adaptive immune system plays a pivotal role in the recovery of ITP. To ascertain the roles of Th17 and Treg cells in the progression of ITP, we analysed the primary $CD4^+$ T cells from the peripheral blood of ITP patients by FACS. The results clearly showed that the proportion of Th17 cells was elevated while Treg cells decreased in ITP. This result is consistent with previous reports that Th17 cells were upregulated in ITP patients [31]. Moreover, in-depth data analysis revealed that the Th17 proportion was negatively correlated with the Treg proportion, and the Th17/Treg ratio was positively associated with disease progression from active to stable and remission stages. In addition to Th17 and Treg cells, a new subset of T helper cells, Th9, was also shown to be increased in ITP patients and might contribute to the pathogenesis of ITP by having a synergetic effect with Th17 cells [32]. As a potent suppressor of Th17 cells, Treg cells have been shown to exert critical functions in preventing the anti-platelet autoimmune response. The dysregulation of Treg cells was greatly improved in ITP patients after treatment with dexamethasone, rituximab, or thrombopoietin receptor agonists (A-TPOs) [13]. At the same time, A-TPO administration

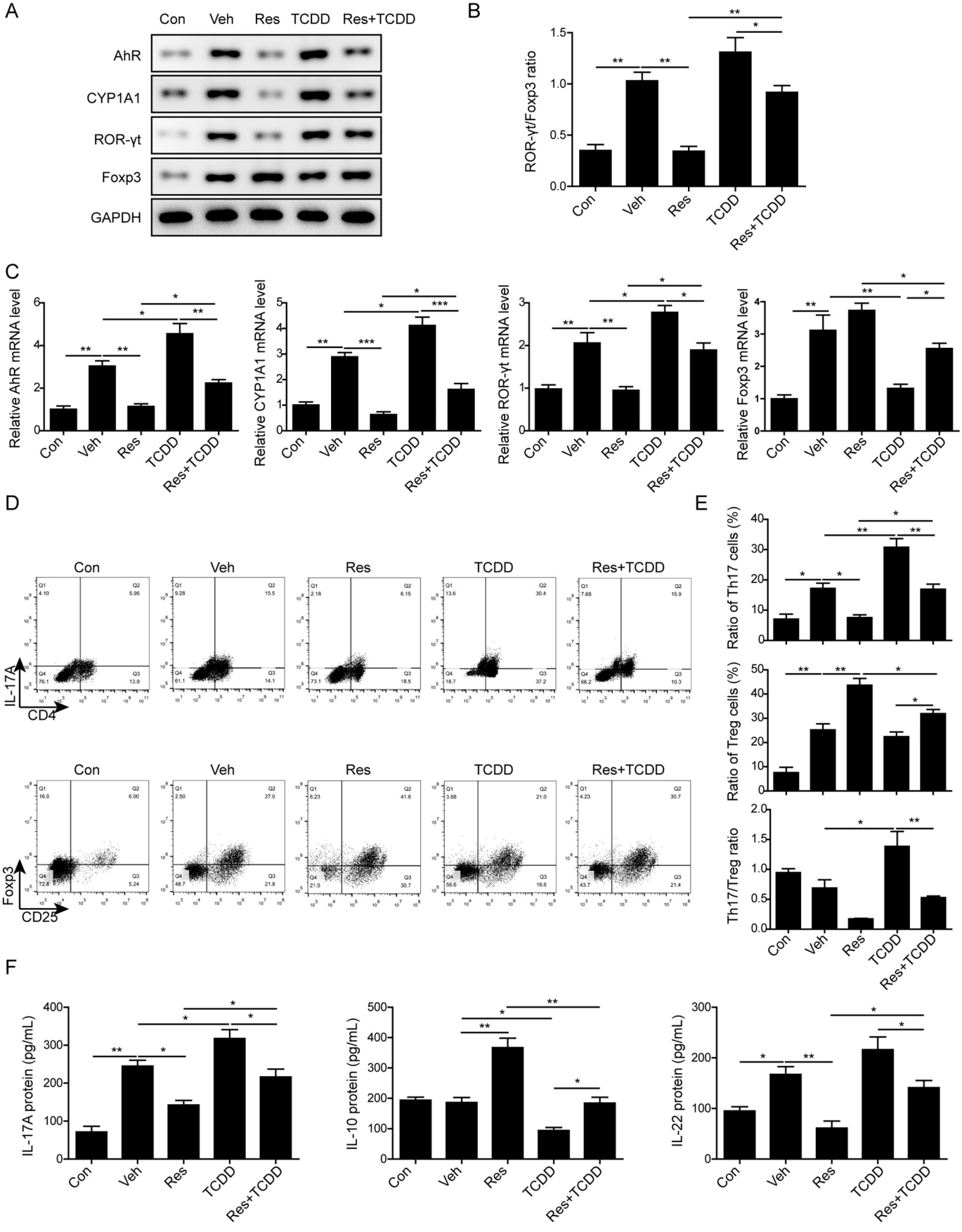

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Fig. 7. AhR pathway was critical for the regulation of Th17/Treg balance by resveratrol.
$CD4^+$ T cells isolated from ITP patients were cultured in either (A, C, D, F) Th17- or (D) Treg-inducing conditions, after which the cells were treated with 50 μM resveratrol, AhR agonist TCDD, 50 μM resveratrol plus TCDD for 48 h, respectively. DMSO equivalent was added to the vehicle group. (A) The expression levels of AhR, CYP1A1, ROR-γt and Foxp3 were assessed by Western blotting. GAPDH was used as the loading control. (B) The ratio of ROR-γt/Foxp3 was assessed by densitometry. (C) The relative mRNA levels of AhR, CYP1A1, ROR-γt and Foxp3 were determined by qRT-PCR. (D) The proportion of Th17 cells (upper panel) or Treg cells (lower panel) in the $CD4^+$ T cell population was analysed by flow cytometry. (E) The ratio of Th17/Treg following indicated treatment is shown. (F) IL-17A, IL-22 and IL-10 levels were measured in the supernatants by ELISA. (A, C, D, F) Data are representative of three separate experiments using newly differentiated Th17 or Treg cells on each occasion. Data represent mean ± SD. p values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$.

resulted in the elevation of regulatory B cells (Bregs), which could significantly enhance the secretion of IL-10 and lead to complete remission in some cases [33]. In addition, natural killer (NK) cells were also reduced in ITP patients [34], which might facilitate the production of auto-antibodies due to the suppressive function of NK cells on B cells. Furthermore, ELISA experiments demonstrated that IL-17A and IL-22 were upregulated, whereas IL-10 was downregulated in serum from ITP patients. In agreement with previous reports [12], the levels of IL-17A and IL-22 in patients also indicated the Th17-dominated cytokine profile in ITP. In addition, other pro-inflammatory cytokines, such as IL-6, IL-12, IL-23, IL-27 and IFN-γ, were also upregulated in the serum of ITP patients [34], while the anti-inflammatory cytokines IL-4 and IL-35 were downregulated in ITP [33]. The abnormal expression of cytokines facilitated the development of ITP into a chronic disease, and our data further support that the expression of IL-17A and IL-22 is positively associated with the progression of ITP, while IL-10 expression is negatively associated with disease progression. The alteration of cytokine production was consistent with the changes in the Th17/Treg ratio. Moreover, we found that the enhanced expression of ROR-γt and impaired expression of Foxp3 were the underlying molecular mechanism for the above phenotype. Similarly, additional research demonstrated that the mRNA level of ROR-γt was higher in ITP patients than in healthy donors before and after A-TPOs treatments [35]. Moreover, the AhR pathway, which serves as a potent regulator of Th17 and Treg cells, was found to be upregulated in $CD4^+$ T cells of ITP patients in our study, which implies that AhR might be involved in the pathogenesis of ITP. In previous reports, several studies investigated the roles of the AhR pathway in the pathogenesis of other autoimmune diseases using mouse models, and they found that the activation of AhR could ameliorate colitis [36]. However, AhR deficiency in T cells suppressed collagen-induced arthritis [36]. The functional diversity of AhR might depend on disease types and stages and the distinct microenvironment of autoimmune diseases.

Based on the above findings, we further explored the effect of resveratrol on the Th17/Treg imbalance and cytokine production. As expected, low and median doses of resveratrol did not change the cell viability but potently attenuated the secretion of IL-17A and IL-22 while enhancing the production of IL-10 in a concentration- and time-dependent manner. In previous reports, Fuggetta et al. also found that resveratrol treatment could downregulate the expression of IL-17 at both the mRNA and protein levels in HTLV-1-infected T cells [37]. Thomas M. Petro demonstrated that oral administration of resveratrol led to increased expression of IL-10 in T cells during EAE progression [38]. Our results are consistent with these reports and confirm the anti-inflammatory effect of resveratrol. Moreover, the reversal of the cytokine profile indicated that blocking AhR signalling could shift the balance towards the Treg subset, which was further verified by the proportional alterations of Th17 and Treg cells. Notably, the downregulation of Th17 cells and upregulation of Treg cells could significantly mitigate ITP in mice [39]. Unfortunately, no study has been carried out to investigate the protective effect of resveratrol on platelets in an ITP mouse model. However, this mouse model research will be performed in our lab. Finally, we demonstrated that the key transcription factors of Th17 and Treg cells, ROR-γt and Foxp3, were downregulated and upregulated, respectively, upon resveratrol treatment. Meanwhile, the expression of AhR and CYP1A1 was also suppressed by resveratrol, which is consistent with a previous report about the effect of resveratrol on the AhR pathway [40].

To ascertain the relationship between the AhR pathway and ROR-γt or Foxp3 as well as the Th17/Treg balance, we modulated the activation of the AhR pathway with the AhR agonist TCDD. As expected, TCDD administration significantly increased the expression of AhR, CYP1A1, and ROR-γt but suppressed Foxp3 expression. TCDD also elevated the proportion of Th-17 cells and secretion of IL-17A and IL-22 while it impaired the proportion of Treg cells and secretion of IL-10. However, resveratrol plus TCDD treatment completely abrogated the effects induced by TCDD. These data revealed that AhR pathway inhibition was required for the Th17/Treg balance regulated by resveratrol, and are consistent with one previous report about the association between the AhR pathway and the Th17/Treg balance [41].

In summary, our study revealed that the Th17/Treg imbalance in ITP patients might be restored by resveratrol via suppression of the AhR pathway. These results reveal the significance of the AhR pathway in ITP research from two aspects. On the one hand, some agonists that exist in the environment or in the body can activate the AhR pathway in T cells, which will result in the hyperactivation of the Th17 subset and the impairment of immune tolerance. Subsequently, some auto-reactive B cells will produce substantial amounts of auto-antibodies, such as anti-platelet antibodies, which is one of the predominant reasons for the pathogenesis of ITP. On the other hand, targeting the AhR pathway with potent inhibitors, such as resveratrol, can repress the proliferation and function of Th17 cells and promote the function of Treg cells. AhR inhibition may help to mitigate the symptoms of ITP and enable new ITP drug discoveries in the future.

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Conflict of interest

The authors declare that there is no conflict of financial interest.

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