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Activation of the Notch signaling pathway disturbs the $CD4^+/CD8^+$, Th17/Treg balance in rats with experimental autoimmune uveitis

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Abstract

Objective and design The present study aimed to investigate the relationship between the disturbed balance of $CD4^+/CD8^+$, Th17/Treg and the activation of the Notch signaling pathway in experimental autoimmune uveitis (EAU).

Methods An EAU rat model was induced in Lewis rats, and pathology analysis was performed by hematoxylin and eosin (H&E) staining. $CD4^+$, $CD8^+$, Th17, and Treg levels in spleen, lymph nodes and eye tissues were determined by flow cytometry. Meanwhile, the expression of Notch1, DLL4, IL-10, and IL-17 was determined by quantitative polymerase chain reaction (Q-PCR) and enzyme-linked immunosorbent assay (ELISA). In addition, the inhibitory effect of *N*-(*N*-(3,5-difluorophenacetyl-L-alanyl))-*S*-phenylglycine t-butyl ester (DAPT) on Th17 differentiation by Notch signaling in vitro was further investigated using T lymphocytes from EAU rats on day 12 post-immunization by flow cytometry.

Results The pathological results showed that inflammatory cell infiltration occurred in ocular tissues in EAU rats. The $CD4^+/CD8^+$ and Th17/Treg ratios in EAU rats were apparently higher than those in normal control individuals. Q-PCR and ELISA analyses indicated the expression of Notch1, DLL4, IL-10, and IL-17 in EAU rats gradually increased on day 6 after immunization, peaked on day 12, and then gradually decreased. The dynamic trends in Notch1 and DLL4 expression in EAU rats were identical to those of $CD4^+/CD8^+$ and Th17/Treg levels. DAPT can significantly inhibit the activation of Notch signaling, decrease Th17 cell differentiation, and attenuate the level of the Th17 cell lineage, contributing to the balance of the Th17/Treg ratio.

Conclusion The activation of the Notch signaling pathway can regulate Th17 and Treg cell differentiation, disrupt the $CD4^+/CD8^+$ and Th17/Treg balance, and aggravate the severity of EAU; inactivation of the Notch signaling pathway contributes to the $CD4^+/CD8^+$ and Th17/Treg balance in EAU rats. Our findings highlighted that the dynamic change in the $CD4^+/CD8^+$ and Th17/Treg ratio was consistent with the expression trend of Notch signaling in EAU rats, suggesting that Notch signaling may be a potentially important therapeutic target in clinical practice.

Keywords Experimental autoimmune uveitis · Notch signaling pathway · T helper 17 cell · Regulatory T cell

Abbreviations

BD	Behcet's disease
CFA	Complete Freund's adjuvant

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DAPT	*N*-(*N*-(3,5-Difluorophenacetyl-L-alanyl))-*S*-phenylglycine t-butyl ester
DMSO	Dimethyl sulfoxide
EAU	Experimental autoimmune uveitis
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
H&E	Hematoxylin and eosin
IFN-γ	Interferon-γ
IL-17	Interleukin-17
IRBP	Interphotoreceptor retinoid-binding protein
MCP-1	Monocyte chemoattractant protein-1
NC	Normal control
PBS	Phosphate buffer saline
Q-PCR	Quantitative polymerase chain reaction
TB	Tuberculin
TGF	Transforming growth factor
Th	T helper
TNF	Tumor necrosis factor

Introduction

Uveitis is an important cause of legal blindness in the developed world, characterized by an inflammatory ocular disease in clinical practice, and mainly includes Behcet's disease (BD), sarcoid-related uveitis, and Vogt–Koyanagi–Harada disease. The etiology of uveitis is complex and can cause irreversible damage to eye tissues. At present, acute attacks of uveitis are usually controlled by steroids, but in the long term, patients cannot continue to receive therapeutic doses of steroids due to serious side effects. Therefore, the creation of controllable animal models, exploration of the pathogenesis of uveitis and the identification of new therapeutic targets are of great significance for clinical treatment. T helper (Th) cells play an important role in regulating the immune response. Traditionally, uveitis has been recognized as a Th1-mediated inflammatory disease. In recent years, it has been confirmed that the absence of regulatory control of Th1 and Th17 cells is responsible for many human autoimmune diseases [1]. Th17 cells can regulate the pathological process of inflammation by producing different cytokines, including interleukin (IL)-17, IL-21, and IL-22. These cytokines can also influence the activation of signaling pathways and thus regulate naïve $CD4^+$ T cell differentiation. It is known that both Th17 and Treg cells share a common development pathway [2], and the expression of downstream cytokines and effector cells from the Th17 and Th1 subsets are sequentially activated, contributing to the development of diseases [3].

T cells that express the surface marker CD4 are known as Th cells and play critical roles in the pathogenesis of uveitis [4]. Aktas et al. found that compared with healthy subjects, Th1, Th22 and IL-17-producing Th17 cells are increased significantly, whereas the regulatory T (Treg) cell percentage is decreased significantly in BD patients, suggesting that the incidence of BD may be associated with a decreased Treg level and increased Th1, Th22 and Th17 cell levels [5]. Moreover, the $CD4^+/CD8^+$ and Th17/Treg balance is of great importance for everyone, and unbalanced $CD4^+/CD8^+$ and Th17/Treg ratios can result in the occurrence and development of diseases including uveitis. Studies have shown that Th17 cells and gamma delta T lymphocytes and their secreted IL-17 play an essential role in the pathogenesis of autoimmune diseases, especially autoimmune uveitis [6, 7]. It has been confirmed that Th17/Treg imbalance is closely related to the development of experimental autoimmune uveitis (EAU) in rats. Th17 and Treg are interrelated in terms of their differentiation and antagonism functions [8]. Moreover, Ahmadi et al. observed a disturbed Th17/Treg balance in peripheral blood patients with BD [9]. Dave et al. also found that the ratio of $CD4^+/CD8^+$ T lymphocytes in the aqueous humor of patients with granulomatous uveitis was elevated [10]. Thus, a disturbed $CD4^+/CD8^+$, Th17/Treg balance is associated with the pathogenesis of uveitis, and the coordinated expression of Th17/Treg and related cytokines can achieve immune balance, contributing to the recovery of uveitis [11].

In recent years, both IL-10 and IL-17 have attracted wide attention because of their very important roles in the development of autoimmune diseases. Treg cells can induce phenotypic and functional changes in monocytes, thereby maintaining peripheral immune tolerance by regulating several T cell lineages. Monocytes cocultured with expanded Tregs could upregulate IL-10 and reduce the production of expanding IL-17-producing T cells [12]. Diefenhardt et al. found that IL-10 receptor (IL-10R) signaling in Treg cells was related to the downregulation of Th17 cells. The deficient IL-10Ra signaling transduction in Treg cells could increase Th17-induced cytokine production [13]. Molins et al. showed that the level of IL-10 in peripheral blood mononuclear cells of patients with uveitis was significantly lower than that of normal subjects [14]. Therefore, both IL-10 and IL-17 are key cytokines that are involved in the development of uveitis, and IL-17 is closely associated with uveitis, whereas IL-10 is negatively correlated with uveitis [15].

The Notch signaling pathway is an important signaling pathway that is responsible for cell fate by inhibiting cell differentiation and regulating the inflammatory response to maintain immune balance. It can affect the proportional balance of Th cells by regulating the differentiation of naïve $CD4^+$ T cells into Th17 and Treg cells [16]. Clinical evidence has shown that Notch signaling-related molecules were highly activated in patients with active uveitis, accompanied by a high level of Th17 response. Notch blockers preferentially inhibit the Th17 response in patients with active uveitis, suggesting that activation

of the Notch signaling pathway is associated with a high Th17 response [17]. Rong et al. found that infiltrating Treg cells in uveitic eyes of mice expressed Notch1, Notch2, JAG1 and DLL1. Transplantation of Treg cells with Notch1 deficiency markedly reduced the production of inflammatory cytokines and inflammatory cell infiltration in anterior uveitis. Therefore, Notch signaling negatively regulates the immunosuppressive function of infiltrating Treg cells in EAU mice [18].

Activation of the Notch signaling pathway may play a fundamental role in the pathogenesis of EAU. However, the dynamic changes at the level of the gene and protein expression of Notch signaling-related molecules in uveitis and the underlying mechanism involved in the pathogenesis of uveitis remain unclear. In the present study, we induced EAU in rats by injecting an interphotoreceptor retinoid-binding protein (IRBP) emulsion supplemented with complete Freund's adjuvant (CFA) and tuberculin (TB) and determined the levels of Notch1, DLL4, IL-10, and IL-17 in spleen, lymph nodes and eye tissues. Moreover, the inhibitory effect of the γ-secretase inhibitor DAPT on Th17 differentiation by Notch signaling in vitro was further investigated by flow cytometry using T lymphocytes from EAU rats on day 12 post-immunization. Our findings will expound the role of the activation of the Notch signaling pathway in the development of uveitis, providing new insights into the pathogenesis of uveitis and paving a way forward in the treatment of uveitis by targeting the Notch signaling pathway in clinical practice.

Materials and methods

Animals

The principles for the care and use of laboratory animals in this study strictly abided by the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health of China and the ARVO Statement on the Application of Animals in Ophthalmological and Visual Research. All experiments were approved by the ethics committee of the Eye Institute of Shandong University of Traditional Chinese Medicine (2015-XK-013). All animals were designed to reduce animal discomfort and stress. In the present study, female Lewis rats (aged 6–8 weeks, 160–180 g) were purchased from Beijing Vital River Laboratory Animal Co., Ltd. (Beijing, China). The animals were exposed to light and dark for 12 and 12 h, respectively, at room temperature (25 ± 1 °C) with a relative humidity of 50 ± 10%. Prior to the experiment, animals were first acclimated to their housing for 1 week.

Reagents

FITC-CD4, PE-CD8, PE-IL-17, APC-CD25, PE-Foxp3 (eBioscience, USA); Fixation/Permeabilization Kit with BD Golgistop™ (BD Biosciences, USA); cell activation cocktail (Biolegend Inc., USA); beta-mercaptoethanol, glutamine (Ameresco, USA); fetal bovine serum (FBS), RPMI 1640 medium (HyClone, USA); lymphocyte separating solution (Solarbio, Beijing, China); IRBP peptide (residues 1177-1191, sequence: ADGSSWEGVGVVPDV), phosphate buffered saline (PBS, pH 7.2) (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China); Mycobacterium tuberculin H37RA (TB) (Difco, USA); CFA (Sigma, USA); RNA Tissue/Cell Rapid Extraction Kit (Vazyme Biotech Co., Ltd., Nanjing, China); DNA Reverse Transcription Kit, 2*SYBR Green I Kit (QIAGEN, Germany); Rat Notch1, DLL4 ELISA Kits (Wuhan Colorful Gene Biological Technology Co., Ltd., Wuhan, China); IL-10, IL-17 ELISA kits (Dakewe Biotech Co. Ltd., Beijing, China); DAPT (MedChemExpress, USA); dimethyl sulfoxide (DMSO) (Sigma, USA); anti-rat CD3, anti-rat CD28 (BD Biosciences, USA); rIL-1β, rIL-23, rIL-6, TGF-β (Sino Biological, Beijing, China); FITC rat IgG2b κ Isotype Control, PE rat IgG2b κ Isotype Control (BD Biosciences, USA); Rat IgG2b κ Isotype Control APC (eBioscience, USA).

Induction of EAU

Healthy Lewis rats were randomly divided into a normal control (NC) group ($n = 15$) and an EAU group ($n = 63$). To induce EAU, IRBP emulsion was prepared with 100 μg of IRBP peptide dissolved in sterilized PBS supplemented with 100 μg of TB and 150 μL of CFA to a total volume of 300 μL. Every rat in the EAU group was immunized subcutaneously with a total of 300 μL of IRBP emulsion, while rats in the NC group received the identical volume containing only 150 μL of CFA plus 100 μg of TB.

Clinical evaluation of EAU

After immunization, the severity of inflammation in EAU rat eyes was observed with a Genesis-D camera (Kowa Company Ltd., Japan) on days 6, 9, 12, 15 and 18. The severity of inflammation was scored according to criteria [19, 20] and ranged from 0 (no disease) to 4 (the most serious disease): 0 = no disease; 1 = the iris is slightly dilated and the pupillary structure is abnormal; 2 = the iris is hyperemic, the pupil dilatation is limited, and the anterior chamber is slightly cloudy; 3 = the iris is heavily congested, the anterior chamber is moderately cloudy, and pupils are still visible;

and 4 = the iris is seriously congested, the anterior chamber is seriously cloudy and empyemic, and the pupil is closed.

Histopathological examination

On day 12 after immunization, rats in both the NC group and EAU group ($n = 3$ for each group) were randomly selected to perform histopathological examination. After euthanasia, rats were killed, and the eyes were isolated and fixed in eyeball fixative solution for 24 h. After dehydration, embedding, and sectioning, hematoxylin–eosin (H&E) staining was performed. The histopathological examination of the retina and ciliary body was carried out by using a microscope (Ti; Nikon Corporation, Tokyo, Japan).

Determination of $CD4^+$, $CD8^+$, Th17, and Treg cell levels

The spleen, lymph nodes and eye tissues of rats in the NC group and EAU group were isolated at different time points. After grinding, cells were first filtered through a 200-mesh cell sieve. The cell suspension was filtered through a nylon wool column. Then, approximately 1×10^6 T lymphocytes were collected from each tissue with rat lymphocyte separation solution. Next, 6 μL of cell activation cocktail, 4 μL of protein transport inhibitor, and 1 mL of RPMI 1640 medium (containing 100 μL of FBS and 2 μL of beta-mercaptoethanol) were gently blended and added into the collected cells and maintained in a CO_2 incubator at 37 °C for 5 h. At the indicated time point, the cells were rinsed with PBS, centrifuged at 500 g for 6 min, and stained with relevant fluorescence antibodies. The cells were stained with FITC-CD4, PE-CD8, and APC-CD25 for surface staining and PE-IL-17 and PE-Foxp3 for intracellular staining. The levels of $CD4^+$, $CD8^+$, Th17, and Treg cells in spleen, lymph nodes and eye tissues of rats were detected by a flow cytometer (BD FACSVerse™, USA). Meanwhile, an isotype control assay was performed to eliminate unwanted background cell staining.

Expression of Notch1, DLL4, IL-10, and IL-17 mRNA determined by quantitative-PCR (Q-PCR)

Rats ($n = 6$ for each group) were randomly selected from either the NC group or EAU group at 6, 9, 12, 15 and 18 days after immunization. After euthanasia, the spleen, lymph nodes and eye tissues of rats were separated in an aseptic environment, and portions of the tissues were stored at − 80 °C for enzyme-linked immunosorbent assay (ELISA). Additionally, total RNA was extracted from the remaining samples using an RNA Tissue/Cell Rapid Extraction Kit (Sparkjade Science Co., Ltd., China) according to the manufacturer's protocols. After the collection of RNA samples, the purity and concentration of total RNA were detected by agarose gel electrophoresis and a K5600 spectrophotometer (Beijing Kaiao Technology Development Co., Ltd., Beijing, China), respectively. Subsequently, cDNA synthesis was performed using a DNA Reverse Transcription Kit, and the levels of Notch1, DLL4, IL-10 and IL-17 mRNA were detected by Q-PCR. GAPDH was used as the internal reference for the Notch1, DLL4, IL-10, and IL-17 genes. The primer sequences are listed in Table 1. In the present study, Q-PCR was performed on a LightCycler® 480 II instrument (Roche Diagnostics, Mannheim, Germany). The reaction conditions for Q-PCR were set as follows: 94 °C for 5 min for 1 cycle, and 94 °C for 5 s, 57 °C for 15 s, and 72 °C for 10 s for 45 cycles. After Q-PCR detection, the levels of the Notch1, DLL4, IL-10 and IL-17 genes were calculated according to the $2^{-\Delta\Delta Ct}$ method.

Measurements of Notch1, DLL4, IL-10, and IL-17 protein levels by ELISA

The spleen, lymph nodes and eye tissues of the two groups were ground under liquid nitrogen and dissolved in 350 μL of RIPA lysate. Subsequently, samples were sonicated on ice for 20 min and centrifuged at 8000 g at 4 °C for 20 min. After centrifugation, the supernatants were collected and quantified using a K5600 spectrophotometer (Beijing Kaiao Technology Development Co., Ltd., Beijing, China). Additionally, the levels of Notch1, DLL4, IL-10, and IL-17 proteins at different time points were detected by an ELISA technique using a multifunctional microplate reader (Envision, Perkin-Elmer, USA); the detection limits for Notch1, DLL4, IL-10, and IL-17 were 2 pg/mL, 33 pg/mL, 1.5 pg/mL, and 3.3 pg/mL, respectively.

Determinations of $CD4^+$, $CD8^+$, Th17 and Treg levels after silencing notch signaling in vitro

On day 12 post-immunization, the spleen, lymph nodes and eye tissues of EAU rats were isolated. After grinding, cell

Table 1 Primer sequences for differentially expressed mRNAs

Gene	Primer sequences
GAPDH	Forward: 5′-GACCACAGTCCATGACATCACT-3′
	Reverse: 5′-TCCACCACCCTGTTGCTGTAG-3′
Notch1	Forward: 5′-ATGGCCCCACCTGCAGACAAGATG-3′
	Reverse: 5′-GGCACGGCAGGCACAGCGATAG-3′
DLL4	Forward: 5′-CAAGAATAGCGGCAGTGGTCGTAA-3′
	Reverse: 5′-GTAGCGCAGTCTTGTGAGGGTGTT-3′
IL-10	Forward: 5′-TTCCATCCGGGGTGACAATAA-3′
	Reverse: 5′-TTCTGGGCCATGGTTCTCTGC-3′
IL-17	Forward: 5′-TTGCTGCTACTGAACCTGGAG-3′
	Reverse: 5′-GCATGGCGGACAATAGAG-3′

suspensions were collected using a 200-mesh cell sieve, followed by filtration through a nylon wool column to collect T lymphocytes. Subsequently, T lymphocytes collected from each tissue were purified using Ficoll–Paque™ PLUS density gradient centrifugation medium (GE Healthcare Life Sciences, Piscataway, NJ) for the following experiment.

To evaluate the effect of DAPT, a classical Notch signaling (γ-secretase) inhibitor, on the activation and polarization of Th17 cells, T lymphocytes were divided into an EAU model group, DAPT treatment group, and DMSO control group. First, DAPT was dissolved in DMSO to be used to treat T lymphocytes, and then T lymphocytes from each tissue collection were seeded in 24-well flat plates (1.0×10^6/mL/well) and polarized under Th17 cell-polarizing cocktail consisting of anti-rat CD3 (5 μg/mL), anti-rat CD28 (2 μg/mL), rIL-1β (20 ng/mL), rIL-6 (60 ng/mL), rIL-23(30 ng/mL), and TGF-β (2 ng/mL). Cells in the DAPT treatment group were treated with DAPT solution (final concentration: 40 μmol/L), while cells in the DMSO control group were treated solely with DMSO at an identical concentration to that in the DAPT treatment group. All cells were cultured at 37 °C for 48 h in a humidified incubator with 5% CO_2. After culturing for 48 h, cells were collected to detect the levels of $CD4^+$, $CD8^+$, Th17, and Treg cells using a flow cytometer (BD FACSVerse™, USA). Meanwhile, an isotype control assay was also performed to eliminate unwanted background cell staining.

Statistical analysis

All experiments were repeated three times. Data were expressed as the mean ± SD (standard deviation) and analyzed by SPSS statistical software (SPSS for Windows, version 22.0, IBM-SPSS, Chicago, IL, USA). The Levene test was used to test the variance, and the LSD test was used to compare two groups. $P<0.05$ was statistically significant.

Results

Ocular inflammation

Genesis-D animal eye camera observation showed that there was no iris or conjunctival vasodilation or congestion in normal rats. On day 6 after immunization, slight iris vasodilation and congestion occurred in EAU rats and then increased on day 9. On day 12 after immunization, the most serious ocular inflammation was found in EAU rats, including severe iris dilation and congestion, substantial fibrin exudation in the anterior chamber, pupil obliteration and empyema. Afterwards, the severity of inflammation in EAU rat eyes was gradually alleviated. On day 15 after immunization, the iris vascular congestion symptoms were apparently alleviated, and on day 18 after immunization, the severity of inflammation in EAU rat eyes was significantly mitigated (data not shown).

Ocular histopathological examination

Histopathological examination revealed that a large number of inflammatory cells infiltrated in the anterior chamber, iris, ciliary body and retina in EAU rats. It was noted that severe iris adhesion, swelling of the iris and ciliary body, disorder of the eye tissue structure and destruction of the retina occurred in EAU rats on day 12 after immunization; this result indicates that we successfully established an EAU rat model (data not shown).

Alteration of the levels of $CD4^+$, $CD8^+$, Th17, and Treg cells in EAU rats

The results of flow cytometry determination showed that compared with those in control rats, the levels of $CD4^+$, Th17, and Treg cells in EAU rats increased significantly after immunization. It was noted that the elevated ratios of both $CD4^+/CD8^+$ and Th17/Treg peaked on day 12 after immunization, indicating the disturbance of the immune microenvironment in EAU individuals (Figs. 1, 2, 3).

Changes in the expression of Notch1, DLL4, IL-10, and IL-17 genes in EAU rats

The Q-PCR results indicated that the expression of Notch1, DLL4, IL-10, and IL-17 in spleen, lymph nodes and eye tissues of EAU rats changed compared with that in normal control rats, indicating that these alterations were more closely correlated with the development of uveitis. It was found that the levels of the Notch1, DLL4 and IL-17 genes in spleen, lymph nodes and eye tissues of EAU rats were in agreement with the development of uveitis, and the expression of Notch1, DLL4 and IL-17 in spleen, lymph nodes and eye tissues of EAU rats increased on day 6, peaked on day 12 after immunization, and then gradually decreased. Nevertheless, they were still higher than those in the control group on days 15 and 18 ($P<0.05$) (Fig. 4a, b, d). Moreover, the levels of IL-10 in spleen, lymph nodes and eye tissues of EAU rats were also higher than those of the NC group on day 6 (all $P<0.05$). IL-10 levels reached their peak on day 12 in lymph nodes and eye tissues of EAU rats but peaked on day 15 in spleen ($P<0.05$), followed by a gradual decrease on days 15 and 18 (both $P<0.05$) (Fig. 4c).

Alterations of Notch1, DLL4, IL-10, and IL-17 protein levels in EAU rats

After immunization, the protein levels of Notch1, DLL4, IL-10, and IL-17 in spleen, lymph nodes and eye tissues of

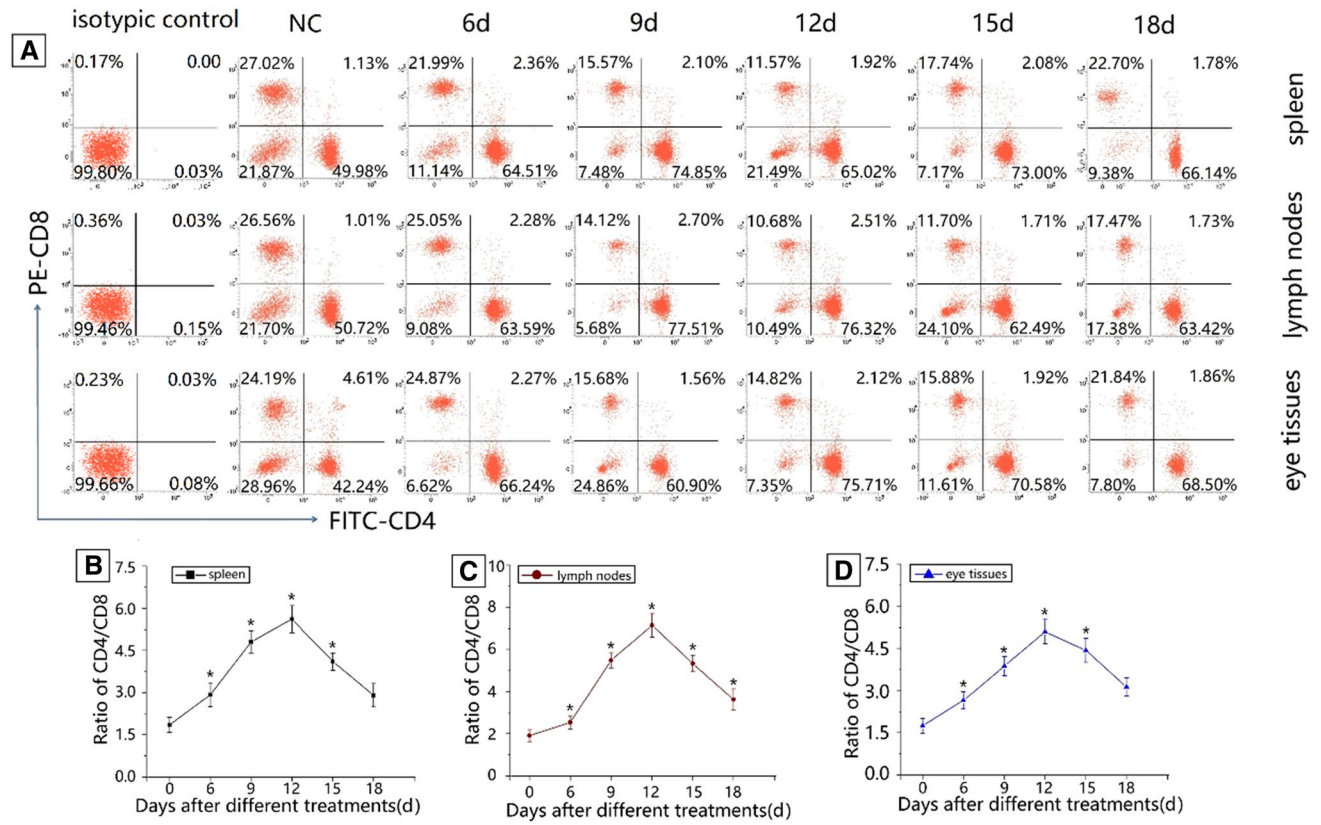

Fig. 1 Dynamic changes of $CD4^+$, $CD8^+$ levels in EAU rats. The levels of $CD4^+$ and $CD8^+$ at 6, 9, 12, 15 and 18 days after immunization in spleen, lymph nodes, and eye tissues in NC and EAU rats were detected by flow cytometry (**a**). The changes in the $CD4^+/CD8^+$ ratio in spleen (**b**), lymph nodes (**c**), and eye tissues (**d**) in NC and EAU rats were further analyzed at different time points. $^*P<0.05$ compared with those of NC individuals

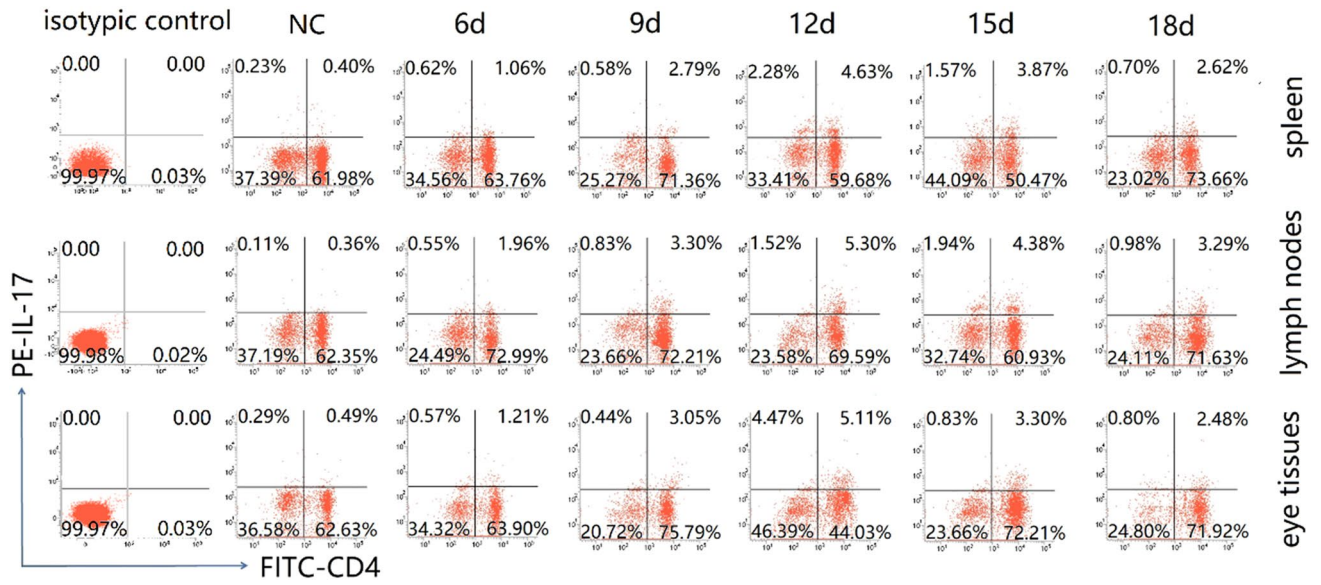

Fig. 2 Changes of Th17 levels in spleen, lymph nodes, and eye tissues after immunization at different time points. The Th17 levels in spleen, lymph nodes, and eye tissues at 6, 9, 12, 15 and 18 days after immunization were separately detected by flow cytometry. It was noted that Th17 levels were elevated after immunization compared to those in relevant NC subjects

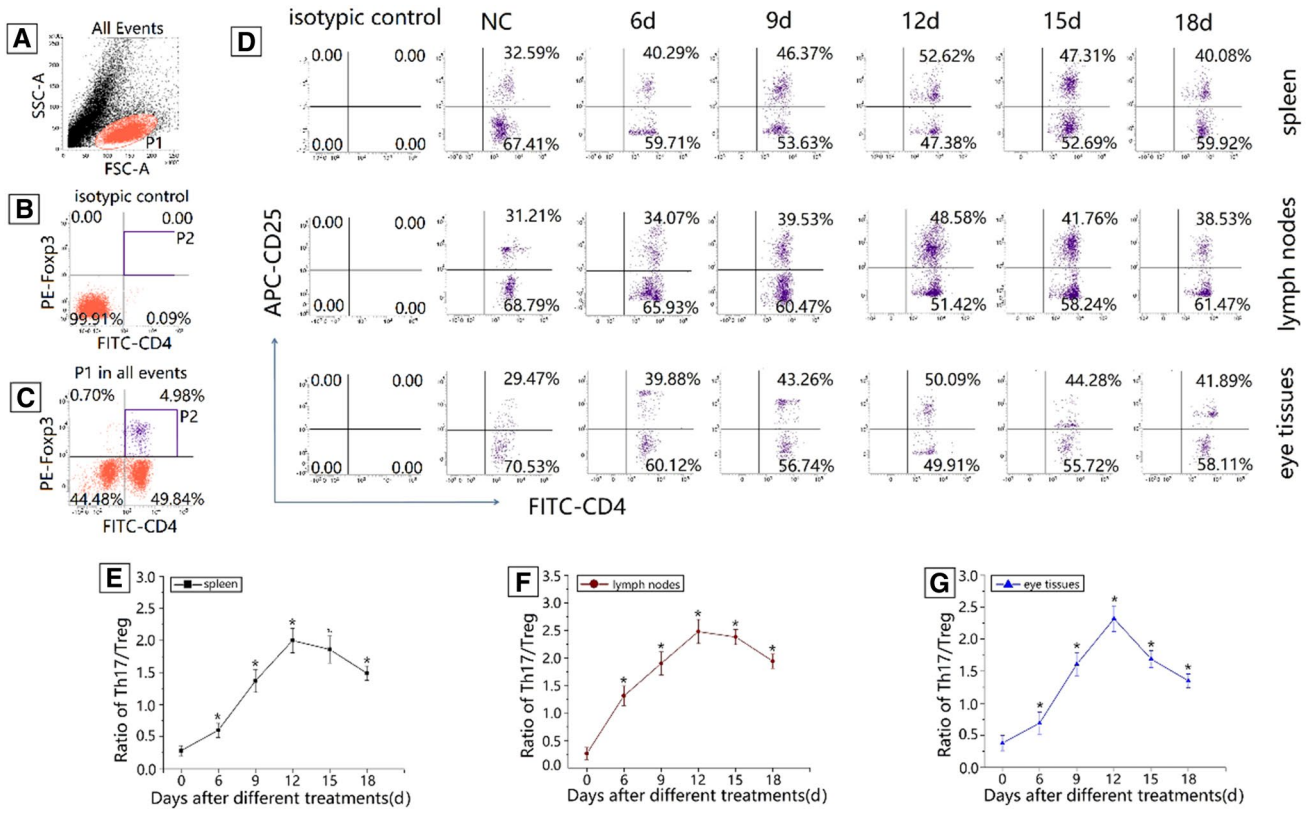

Fig. 3 Dynamic changes of Treg levels in spleen, lymph nodes, and eye tissues after immunization at different time points. T lymphocytes were gated in P1 of **a**. **b** and **C** were from P1, $CD4^+$ $Foxp3^+$ T cells were gated in P2 of **b** and **c**. **d** was from P2 of **b** and **c**, and $CD4^+CD25^+Foxp3^+$ T cells (Treg cells) are shown in the right-upper quadrant of **d**. The changes in the Th17/Treg ratio in spleen **e**, lymph nodes **f**, and eye tissues **g** at different time points in NC and EAU rats were further analyzed. $^*P<0.05$ compared with normal control individuals

EAU rats showed the same trends as those of gene expression. Compared with the NC group, the expression of the Notch1, DLL4, IL-10, and IL-17 proteins in spleen, lymph nodes and eye tissues of EAU rats gradually increased on day 6, peaked on day 12, and then gradually decreased until day 18 (Fig. 5). Nevertheless, the levels were still higher than those of the NC group (all $P=0.000$).

Measurement of $CD4^+$, $CD8^+$, Th17, and Treg cell levels after silencing Notch signaling in vitro

After cells were cocultured with 40 μmol/L DAPT for 48 h, flow cytometry determination revealed that the levels of $CD4^+$ and Th17 in the DAPT treatment group were apparently decreased compared with those of the EAU model group; meanwhile, $CD4^+/CD8^+$ and Th17/Treg were also notably reduced (both $P<0.01$). However, there was no significant difference between the EAU model and DMSO control groups (both $P>0.05$). This result indicated that DAPT treatment can successfully inhibit Notch signaling activation and Th17 cell differentiation and thus reduce the proportions of $CD4^+/CD8^+$ and Th17/Treg (Figs. 6, 7, 8).

Discussion

Uveitis is a serious eye disease with intraocular inflammation that often presents at in much younger adults and consequently causes years of visual loss. Previously, Th1 and Th2 lymphocytes were recognized as important pathogenic factors of uveitis [21]. Studies confirmed that interferon (IFN)-γ secreted by Th1 cells plays an important role in the pathogenesis of EAU [22]. Therefore, the therapeutic effect of EAU in mice was considered to be related to the inhibition of Th1 and Th2 cell functions [23]. The changes in cytokines secreted by Th cells, especially Th17 and Treg cells, are closely correlated with the pathogenesis of uveitis [24, 25]. IL-10 is a crucial cytokine that is mainly produced by Th2 and Treg cells, exhibiting an anti-inflammatory effect. IL-10 can retard the development of EAU by inhibiting the aggregation and function of inflammatory cells [26]. In this study, we evaluated the changes in $CD4^+$, $CD8^+$, Th17, and Treg cell turnover and related inflammatory factors, including IL-10 and IL-17, in an EAU rat model. After receiving antigen stimulation signal, naïve $CD4^+$ T lymphocytes are activated and differentiate into different effector T cell

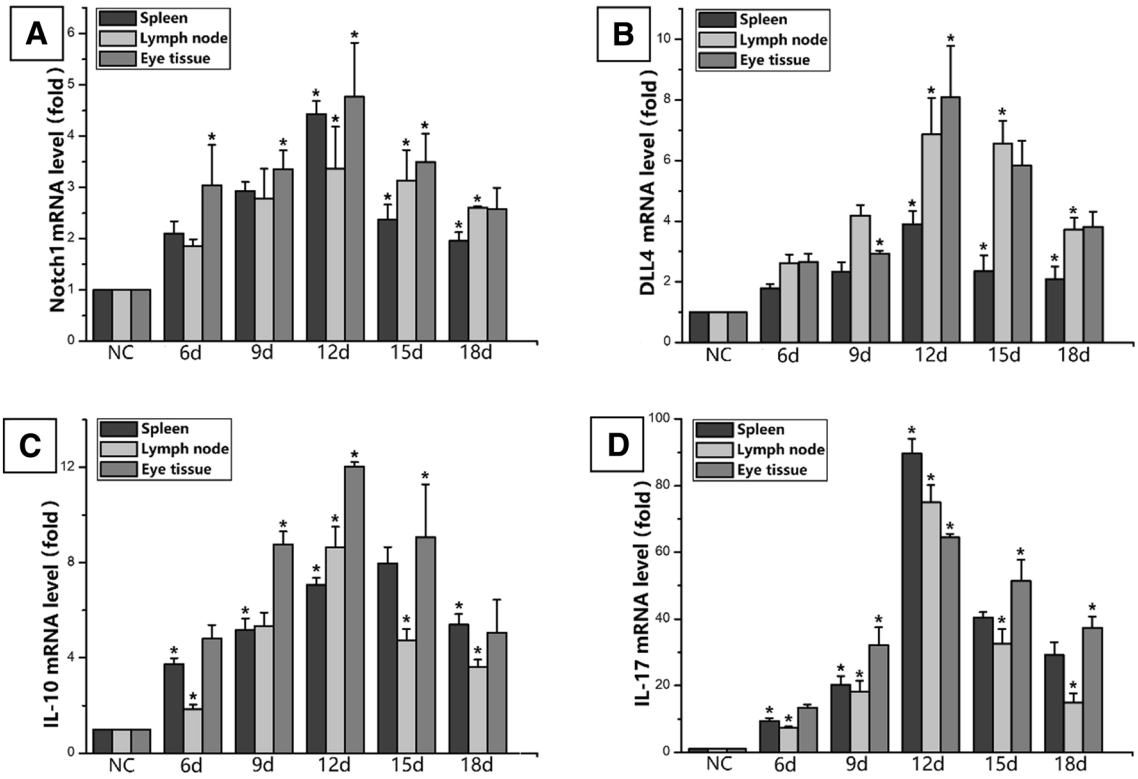

Fig. 4 Dynamic expression of Notch1, DLL4, IL-10, and IL-17 mRNA levels in spleen, lymph nodes, and eye tissues in EAU rats. The expression of Notch1 (**a**), DLL4 (**b**), IL-10 (**c**), and IL-17 (**d**) mRNA levels at 6, 9, 12, 15 and 18 days in spleen, lymph nodes, and eye tissues in NC and EAU rats were determined by quantitative-PCR. $^{*}P < 0.05$ compared with the corresponding NC subjects

lineages under different conditions. Tang et al. noted that after inhibiting the differentiation of naïve $CD4^+$ T cells, the expression of IFN-γ, IL-17 and TNF-α decreased, whereas the production of IL-10 was elevated, contributing to the alleviation of the symptoms of EAU [27]. Kaabachi et al. found that $CD4^+$ T cells and monocytes stimulated by IL-26 promoted the production of Th17-related cytokines (IL-17A, IL-23) and inhibited Treg-related cytokines (IL-10, TGF-β) [28]. The increased level of CD11c in $CD8^+$ T cells in patients with BD suggests that the upregulation of CD11c in $CD8^+$ T cells may be involved in the pathogenesis of BD [29]. STAT3 can regulate the survival and differentiation of $CD4^+$ T cells. The increased activation of STAT3 induced by IL-6 during inflammation can also inhibit the expansion of CD8-Treg cells, thereby impeding recovery from uveitis [30]. IL-6 and TGF-β are key cytokines that determine the differentiation of naïve $CD4^+$ T lymphocytes into Th17 or Treg cells [31], while Th17 and Treg cells are interrelated in terms of their differentiation and antagonism functions [32]. It was found that detection of the $CD4^+/CD8^+$ ratio in vitreous fluid has a high diagnostic value for granulomatous uveitis [33, 34]. In our study, we also found that the expression of IL-10 in spleen, lymph nodes and eye tissues of EAU rats was gradually elevated and peaked on days 12/15, followed by alleviation of the severity of inflammation, suggesting that IL-10 can exert an inhibitory effect on inflammation. Li et al. found that by inhibiting the levels of monocyte chemoattractant protein (MCP)-1, IL-17, and IFN-γ and increasing the level of IL-10 in aqueous humor, the local cytokine environment of EAU rats could be improved [35]. Compared with the control group, the frequency of peripheral Treg cells was significantly lower in patients with tuberculous uveitis, and the levels of IFN-γ, IL-17A, and IL-10 in the eyes also increased [36]. All of these findings indicate that disturbance of the immune environment is involved in the differentiation of naïve $CD4^+$ T cells into Th1 and Treg cells and accompanied by alterations in IL-17 and IL-10 levels.

IL-17-producing Th17 cells, which are related to the pathophysiology of inflammatory diseases, are thought to be developmentally related to Treg cells. Thus, imbalance of the Th17/Treg ratio plays an important role in the pathogenesis of EAU [37]. IL-17 has been recognized in the pathogenesis and development of many autoimmune/inflammatory diseases. Recent genome-wide association studies highlighted the association between the IL-23/IL-17 pathway and IL-10. Immunological studies also support the role of IL-17 in the pathogenesis of diseases [38]. Under stimulated conditions, Th17 cells can secrete IL-17 and

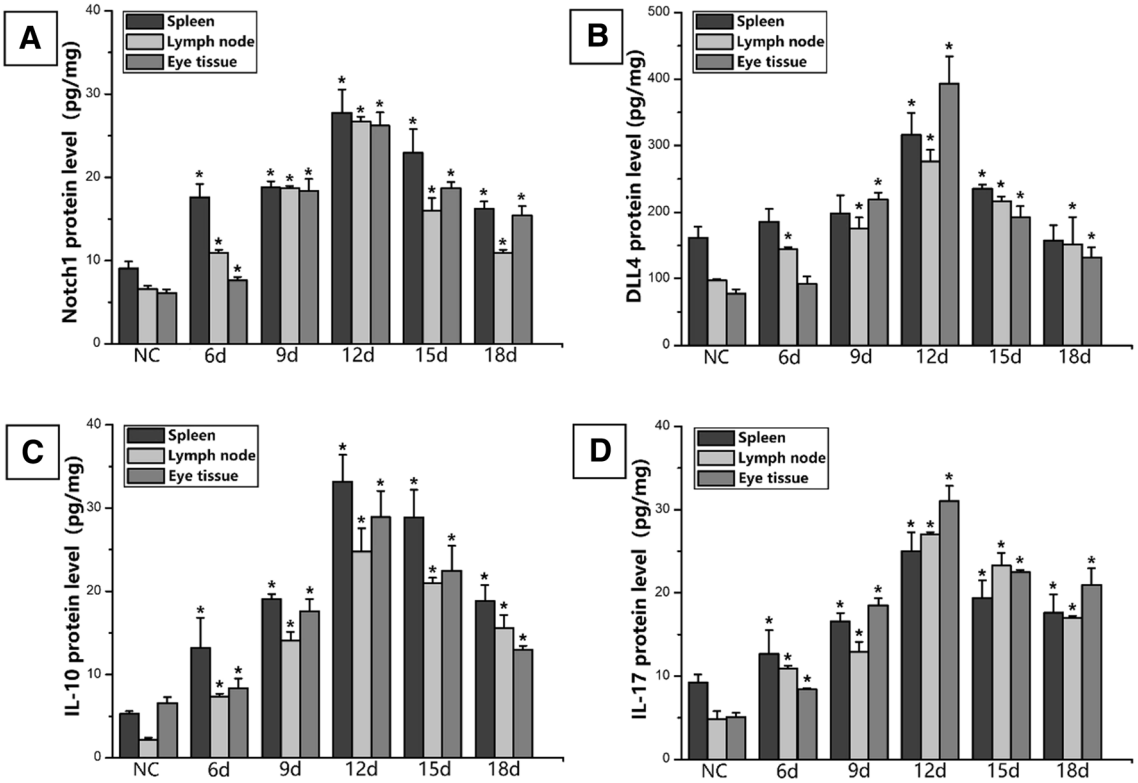

Fig. 5 Dynamic expression of Notch1, DLL4, IL-10, and IL-17 protein levels in spleen, lymph nodes, and eye tissues in EAU rats. The expression of Notch1 (**a**), DLL4 (**b**), IL-10 (**c**), and IL-17 (**d**) protein levels at 6, 9, 12, 15, and 18 days in spleen, lymph nodes, and eye tissues in EAU rats was determined by ELISA. $^*P<0.05$ compared with the corresponding NC subjects

IFN-γ simultaneously in BD patients, resulting in activation of Th17 and Th1 reactions in BD in vitro [39]. Th17 cells exert their effects by secreting cytokines such as IL-17 and IL-21. As an autocrine regulator of Th17 cells, IL-21 can induce Th17 differentiation and inhibit the functions of Th1 and Treg cells [40]. Th17 cells have been shown to play an important role in the development of uveitis [41]. Treg cells are a subset of T cells that control the autoimmune response in vivo. They can achieve immune tolerance and prevent autoimmune diseases by inhibiting the activation and proliferation of effector T cells [42]. As a broadly acting and potent anti-inflammatory population of $CD4^+$ T cells, Treg cells are essential for maintaining immune homeostasis and preventing autoimmunity. Therefore, research on Treg cells is not only necessary for understanding the mechanism of immune homeostasis, but also very useful for developing treatments of various immune diseases. It is reported that clinical remission for patients with uveitis is closely associated with a significant increase in serum levels of TGF-β and IL-10, exhibiting a positive correlation with Treg cells. Compared to those in patients with active uveitis, serum levels of IFN-γ, IL-17A and IL-22 were significantly decreased [43]. Moreover, clinical studies have also revealed that an abnormal Th17/Treg ratio plays a critical role in the pathogenesis of uveitis. Th17 and Treg cells are involved in the pathogenesis of many autoimmune diseases and are a new $CD4^+$ T helper cell lineage. Zhuang et al. found that Th17 cells increased and Treg cells decreased significantly in HLA-B27-related acute anterior uveitis patients [37]. Decreased or dysfunctional Treg cells and Th17/Treg cell imbalance can lead to uveitis [44]. Therefore, the proportion of Th17/Treg cells can affect the body's immune response and regulate the development of uveitis. In the present study, we found that the percentage of $CD4^+$ T cells and the level of Th17 cells in spleen, lymph nodes and eye tissues began to increase on day 6 after immunization, and the levels of $CD4^+/CD8^+$ and Th17/Treg peaked on day 12, accompanied by the most serious ocular inflammation in EAU rats. Subsequently, the ratios of $CD4^+/CD8^+$ and Th17/Treg gradually decreased, ultimately showing balance. In this study, we observed that there was a Th17/Treg imbalance derived from the shifting of Th17 cells in untreated T lymphocytes from EAU rats, suggesting that imbalanced Th17/Treg levels play a pivotal role in the development of uveitis.

The Notch signaling pathway plays an important role in regulating Th17/Treg cell balance. Researchers confirm that after blocking the Notch signaling pathway with the specific γ-secretase inhibitor DAPT, JAG1 signaling molecules in the

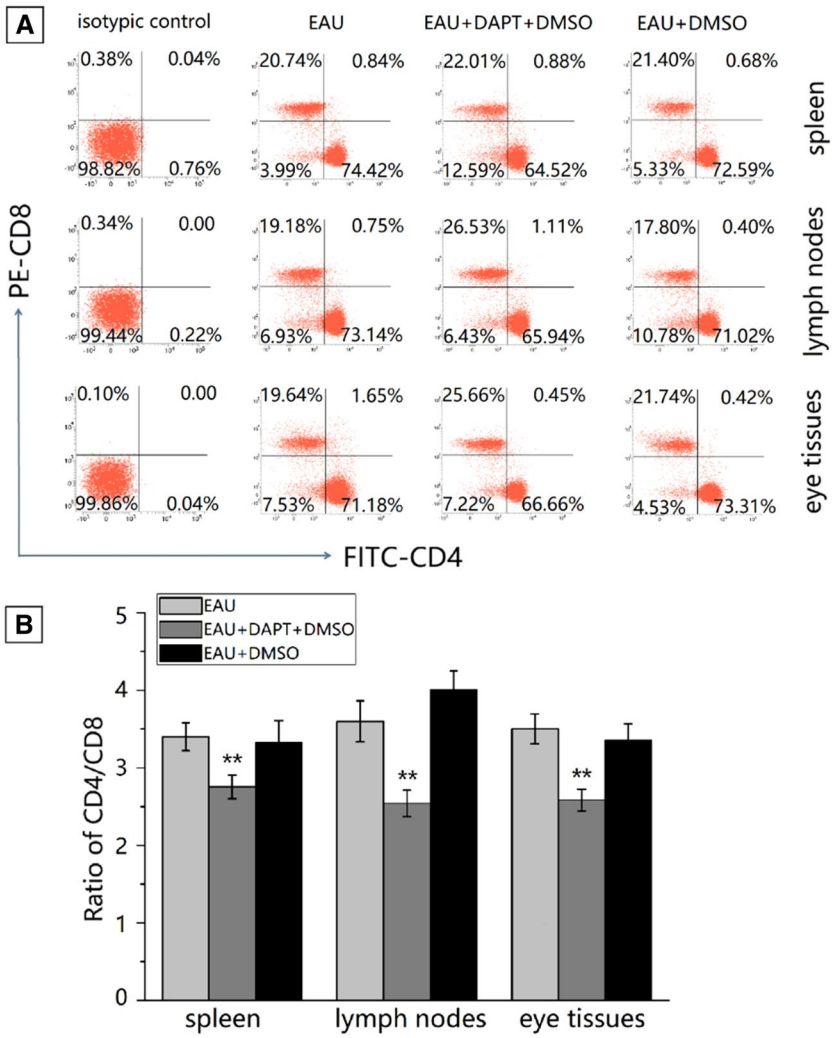

Fig. 6 Dynamic changes of $CD4^+$, $CD8^+$ levels of T lymphocytes in spleen, lymph nodes, and eye tissue in the EAU model, DAPT treatment and DMSO control groups in vitro after culture for 48 h. **a** $CD4^+$, $CD8^+$ levels in the EAU, DAPT treatment and DMSO control groups were determined by flow cytometry. **b** Histogram analysis of the $CD4^+/CD8^+$ ratio. $^{**}P<0.01$ compared with the EAU model group

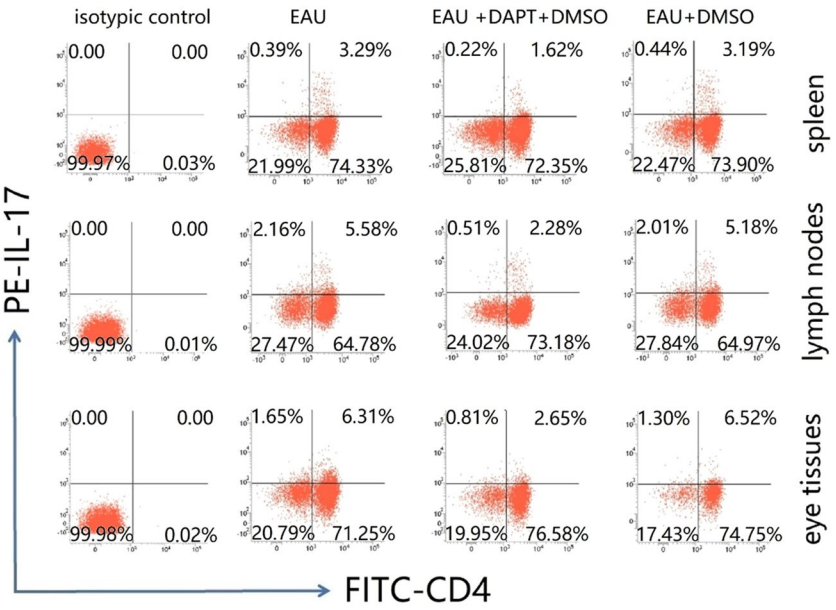

Fig. 7 Changes of Th17 levels in spleen, lymph nodes, and eye tissues in the EAU model, DAPT treatment and DMSO control groups in vitro after culture for 48 h

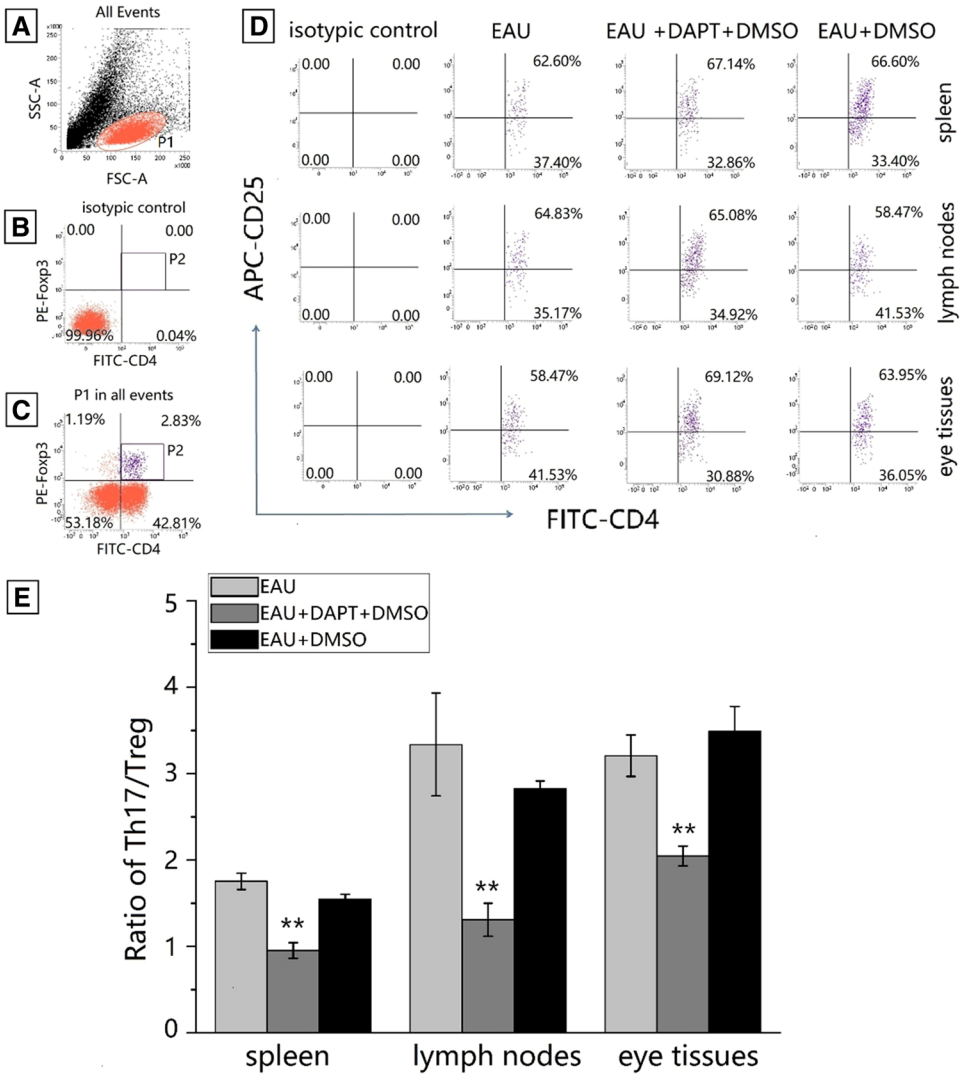

Fig. 8 Dynamic changes of Th17 and Treg levels after culture for 48 h in the EAU, DAPT treatment and DMSO control groups in vitro. T lymphocytes were gated in P1 of **a**, **b** and **c** were from P1, and $CD4^+$ $Foxp3^+$ T cells were gated in P2 of b and **c**. **d** was from P2, and $CD4^+CD25^+Foxp3^+$ T cells (Treg cells) were shown in the right-upper quadrant of **d**. Changes of the Th17/Treg ratio in the EAU, DAPT treatment and DMSO control groups were showed in **e**. $^{**}P<0.01$ compared with the EAU model group

pathway can significantly reduce the expression of ROR-γt in $CD4^+$ T cells and downregulate the levels of Th17-related inflammatory cytokines such as IL-17A, IL-17F, IL-23a and IL-12rb1, thereby inhibiting the differentiation of $CD4^+$ T cells into Th17 cells [45]. Moreover, injection of an anti-DLL4 antibody can also significantly reduce the differentiation level of Th17 cells [46], suggesting that the Notch signaling pathway plays an important role in regulating the differentiation of Th17 cells and in maintaining the balance of Th17/Treg. Yang et al. noted that the activation of Notch signaling was related to the aggravation of ocular inflammation. Notch1, Notch4, DLL4, and NICD were upregulated in EAU mice. Intraperitoneal injection of the Notch signaling pathway blocker DAPT can reduce the expression of NICD, Hes-1 and pro-inflammatory cytokines and markedly reduce the intraocular inflammation in EAU mice [47]. It was found that Notch signaling pathway blockers could inhibit the development of uveitis in C57BL/6 uveitis mice at different times [48]. Jiao et al. found that DAPT, an inhibitor of Notch1 signaling, could notably reduce the differentiation of naïve T cells into Th17 cells, inhibit the response of Th1 and Th17 cells in spleen and lymph nodes, and reduce the levels of IFN-γ and IL-17 in serum [49]. After blocking the Notch signaling pathway with DAPT, the proportion of Th17 cells and Th17/Treg in the peripheral blood of patients with immune thrombocytopenia decreased significantly, accompanied by decreased IL-17 and ROR-γt mRNA expression [50]. Geri et al. found that IL-21 plays a key role in the pathogenesis of BD patients by promoting Th17 and inhibiting Treg cell functions [51]. A study also showed that Notch signaling molecules play a critical role in regulating the balance of Th17/Treg cells by targeting the differentiation of naïve $CD4^+$ T cells into Th17 and Treg cells [52]. The synergistic gene expression of Th17- and Treg-related molecules is related to the regression of monophasic EAU. By means of the synergistic expression of Th17- and Treg-related factors, EAU tends to restore the immune balance [53]. In our experiment, we found that the expression levels of Notch1, DLL4,

IL-10 and IL-17 in spleen, lymph nodes and eye tissues of EAU rats increased on day 6 after immunization and peaked on day 12, followed by a decrease. Further ELISA confirmed the above viewpoint. Increased levels of Notch1, DLL4 and the activation of the Notch signaling pathway can regulate the differentiation of naïve $CD4^+$ T lymphocytes into Th17 and Treg cells and influence the Th17/Treg balance, thus affecting the development and recovery of uveitis. We have demonstrated that blocking Notch signaling by DAPT can notably inhibit Th17 differentiation and reduce the level of the Th17 cell lineage, and thus decreasing the secretion of IL-17, indicating that the inactivation of Notch signaling may attenuate IL-17 generation. Hence, IL-17 production may be dependent on the activation of Notch signaling in uveitis. This finding suggests that the pathogenesis of EAU is closely related to the activation of the Notch signaling pathway. Importantly, the dynamic changes in the $CD4^+$/$CD8^+$ and Th17/Treg ratio were consistent with the expression trend of Notch signaling in EAU rats.

Moreover, the activation of Notch signaling can lead to a disturbed $CD4^+$/$CD8^+$, Th17/Treg balance and aggravate EAU. By contrast, the isolated T lymphocytes from EAU rats can respond to DAPT, leading to a reversal of the Th17/Treg imbalance by blocking the Notch signaling pathway. The Notch signaling pathway is considered one of the main pathways regulating lymphocyte activation and differentiation. Th cell polarization and the cytokine production profile may depend on Notch ligand interacting with the Notch receptor [54]. Application of γ-secretase inhibitor can influence T lymphocyte activation and differentiation [50], indicating the effectiveness of γ-secretase inhibitor as a means of disrupting Notch signaling. In the present study, we quantified the mRNA levels of Notch1 and DLL4. The results indicated that both Notch1 and DLL4 mRNA levels were significantly elevated in spleen, lymph nodes and eye tissues in EAU rats, and the Th17 cell lineage level was increased. However, DAPT treatment can markedly reduce Notch1 and DLL4 mRNA expression (data not shown) and attenuate the level of Th17 cells. Meanwhile, the elevated Th17/Treg ratio in T lymphocytes in EAU rats was also markedly decreased after DAPT treatment; that is to say, DAPT treatment can efficiently reverse imbalance of the Th17/Treg ratio. The simultaneous reduction of Th17 cells and the Th17/Treg ratio demonstrated the fundamental role of Notch signaling activation in the disturbed $CD4^+$/$CD8^+$, Th17/Treg balance in EAU rats. The inhibition of Notch signaling contributes to the amelioration of uveitis.

In conclusion, the expression of Notch1, DLL4, IL-10, and IL-17 in spleen, lymph nodes and eye tissues was increased at the mRNA and proteins levels in EAU rats. Moreover, the $CD4^+$/$CD8^+$ and Th17/Treg ratios also increased significantly compared to those of normal control individuals. DAPT can efficiently inhibit the activation of Notch signaling, leading to decreased Th17 cell differentiation and activation. Taken together, our findings demonstrate that the activation of the Notch signaling pathway contributes to the disturbed $CD4^+$/$CD8^+$, Th17/Treg imbalance in rats with experimental autoimmune uveitis; inactivation of the Notch signaling pathway restores the $CD4^+$/$CD8^+$, Th17/Treg balance, suggesting that the changes in Notch1, DLL4, IL-10 and IL-17 in EAU rats are closely correlated with the pathogenesis of uveitis. Our findings provide new insight into the treatment of uveitis by targeting Notch signaling in clinical practice.

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Author contributions DG conceived and designed the study; XY, BL, HW, SW, FX, and LG performed the experiments; XY and TL analyzed the data; HB and DG contributed reagents/materials/analysis tools; XY and DG wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicting financial interests.

References

1. Chen X, Vodanovic-Jankovic S, Johnson B, Keller M, Komorowski R, Drobyski WR. Absence of regulatory T cell control of TH1 and TH17 cells is responsible for the autoimmune-mediated pathology in chronic graft-versus-host disease. Blood. 2007;110(10):3804–13.
2. Hamzaoui K. Th17 cells in Behcet's disease: a new immunoregulatory axis. Clin Exp Rheumatol. 2011;29(4 Suppl 67):S71–6.
3. Hsu YR, Chang SW, Lin YC, Yang CH. Expression of MicroRNAs in the eyes of lewis rats with experimental autoimmune anterior uveitis. Mediat Inflamm. 2015;2015:457835.
4. Weinstein JE, Pepple KL. Cytokines in uveitis. Curr Opin Ophthalmol. 2018;29(3):267–74.
5. Aktas Cetin E, Cosan F, Cefle A, Deniz G. IL-22-secreting Th22 and IFN-γ-secreting Th17 cells in Behcet's disease. Mod Rheumatol. 2014;24(5):802–7.
6. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C. A distinct lineage of $CD4^+$ T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol. 2005;6:1133–41.
7. Cui Y, Shao H, Lan C, Nian H, O'Brien RL, Born WK, Kaplan HJ, Sun D. Major role of gamma delta T cells in the generation of IL-17 + uveitogenic T cells. J Immunol. 2009;183:560–7.
8. Forrester JV, Klaska IP, Yu T, Kuffova L. Uveitis in mouse and man. Int Rev Immunol. 2013;32(1):76–96.
9. Ahmadi M, Yousefi M, Abbaspour-Aghdam S, Dolati S, Aghebati-Maleki L, Eghbal-Fard S, Khabbazi A, Rostamzadeh D, Alipour S, Shabani M, Nouri M, Babaloo Z. Disturbed Th17/

Treg balance, cytokines, and miRNAs in peripheral blood of patients with Behcet's disease. J Cell Physiol. 2018. https://doi.org/10.1002/jcp.27207.
10. Dave N, Chevour P, Mahendradas P, Venkatesh A, Kawali A, Shetty R, Ghosh A, Sethu S. Increased aqueous humor $CD4^+$/$CD8^+$ lymphocyte ratio in sarcoid uveitis. Ocul Immunol Inflamm. 2018;8:1–8.
11. Jia X, Hu M, Wang C, Wang C, Zhang F, Han Q, Zhao R, Huang Q, Xu H, Yuan H, Ren H. Coordinated gene expression of Th17- and Treg-associated molecules correlated with resolution of the monophasic experimental autoimmune uveitis. Mol Vis. 2011;17:1493–507.
12. Romano M, Fanelli G, Tan N, Nova-Lamperti E, McGregor R, Lechler RI, Lombardi G, Scottà C. Expanded regulatory T cells induce alternatively activated monocytes with a reduced capacity to expand T helper-17 cells. Front Immunol. 2018;9:1625.
13. Diefenhardt P, Nosko A, Kluger MA, Richter JV, Wegscheid C, Kobayashi Y, Tiegs G, Huber S, Flavell RA, Stahl RAK, Steinmetz OM. IL-10 receptor signaling empowers regulatory T cells to control Th17 responses and protect from GN. J Am Soc Nephrol. 2018;29(7):1825–37.
14. Molins B, Mesquida M, Lee RW, Llorenç V, Pelegrín L, Adán A. Regulatory T cell levels and cytokine production in active non-infectious uveitis: in vitro effects of pharmacological treatment. Clin Exp Immunol. 2015;179(3):529–38.
15. Zhou ZY, Chen SL, Shen N, Lu Y. Cytokines and Behcet's disease. Autoimmun Rev. 2012;11(10):699–704.
16. Tindemans I, Peeters MJW, Hendriks RW. Notch signaling in T helper cell subsets: instructor or unbiased amplifier? Front Immunol. 2017;18(8):419.
17. Qi J, Yang Y, Hou S, Qiao Y, Wang Q, Yu H, Zhang Q, Cai T, Kijlstra A, Yang P. Increased Notch pathway activation in Behcet's disease. Rheumatology. 2014;53(5):810–20.
18. Rong H, Shen H, Xu Y. Yang Notch signalling suppresses regulatory T cell function in murine experimental autoimmune uveitis. Immunology. 2016;149(4):447–59.
19. Yoshimura T, Sonoda KH, Miyazaki Y, Iwakura Y, Ishibashi T, Yoshimura A, Yoshida H. Differential roles for IFN-gamma and IL-17 in experimental autoimmune uveoretinitis. Int Immunol. 2008;20:209–14.
20. Jha P, Manickam B, Matta B, Bora PS, Bora NS. Proteolytic cleavage of type I collagen generates an autoantigen in autoimmune uveitis. J Biol Chem. 2009;284(45):31401–11.
21. Crane IJ, Forrester JV. Th1 and Th2 lymphocytes in autoimmune disease. Crit Rev Immunol. 2005;25(2):75–102.
22. Caspi RR. Th1 and Th2 responses in pathogenesis and regulation of experimental autoimmune uveoretinitis. Int Rev Immunol. 2002;1:197–208.
23. Huang XT, Wang B, Zhang WH, Peng MQ. Lin D Total glucosides of paeony suppresses experimental autoimmune uveitis in association with inhibition of Th1 and Th2 cell function in mice. Int J Immunopathol Pharmacol. 2018. https://doi.org/10.1177/0394632017751547.
24. Hamzaoui K. Th17 cells in Behçet's disease: a new immunoregulatory axis. Clin Exp Rheumatol. 2011;29(4 Suppl 67):S71–6.
25. Zhuang Z, Wang Y, Zhu G, Gu Y, Mao L, Hong M, Li Y, Zheng M. Imbalance of Th17/Treg cells in pathogenesis of patients with human leukocyte antigen B27 associated acute anterior uveitis. Sci Rep. 2017;7:40414.
26. Agarwal RK, Horai R, Viley AM, Silver PB, Grajewski RS, Su SB, Yazdani AT, Zhu W, Kronenberg M, Murray PJ, Rutschman RL, Chan CC, Caspi RR. Abrogation of anti-retinal autoimmunity in IL-10 transgenic mice due to reduced T cell priming and inhibition of disease effector mechanisms. J Immunol. 2008;180:5423–9.
27. Tang K, Guo D, Zhang L, Guo J, Zheng F, Si J, Bi H. Immunomodulatory effects of Long dan Xie gan Tang on CD4+/CD8+T cells and associated inflammatory cytokines in rats with experimental autoimmune uveitis. Mol Med Rep. 2016;14(3):2746–54.
28. Kaabachi W, Bouali E, Berraïes A, Dhifallh IB, Hamdi B, Hamzaoui K, Hamzaoui A. Interleukin-26 is overexpressed in Behcet's disease and enhances Th17 related -cytokines. Immunol Lett. 2017;190:177–84.
29. Park YJ, Park MJ, Park S, Lee ES. CD11c is upregulated in CD8 + T cells of patients with Behcet's disease. Clin Exp Rheumatol. 2016;34(6 Suppl 102):S86–91.
30. Yu CR, Dambuza IM, Lee YJ, Frank GM, Egwuagu CE. STAT3 regulates proliferation and survival of CD8 + T cells: enhances effector responses to HSV-1 infection, and inhibits IL-10 + regulatory CD8 + T cells in autoimmune uveitis. Mediat Inflamm. 2013;2013:359674.
31. Ueno A, Jeffery L, Kobayashi T, Hibi T, Ghosh S, Jijon H. Th17 plasticity and its relevance to inflammatory bowel disease. J Autoimmun. 2018;87:38–49.
32. Ivanova EA, Orekhov AN. T helper lymphocyte subsets and plasticity in autoimmunity and cancer: an overview. Biomed Res Int. 2015;2015:327470. https://doi.org/10.1155/2015/327470.
33. Kojima K, Maruyama K, Inaba T, Nagata K, Yasuhara T, Yoneda K, Sugita S, Mochizuki M, Kinoshita S. The CD4/CD8 ratio in vitreous fluid is of high diagnostic value in sarcoidosis. Ophthalmology. 2012;119:2386–92.
34. Sanz-Marco E, Garces M, Sempere A, Diaz-Llopis M. CD4/CD8 ratio in aqueous humor in Uveitis. Ocul Immunol Inflamm. 2013;21:408–9.
35. Li J, Qic C, Zhang Z, Yuan W, Ge z, Tan B, Yang P, Liu J, Zhu X, Qiu C, Lai D, Guo L, Yu L. Subretinal transplantation of human amniotic epithelial cells in the treatment of autoimmune uveitis in rats. Cell Transpl. 2018;27(10):1504–14.
36. Sharma RK, Gupta A, Kamal S, Bansal R, Singh N, Sharma K, Virk S, Sachdeva N. Role of regulatory T cells in tubercular uveitis. Ocul Immunol Inflamm. 2018;26(1):27–36.
37. Zhuang Z, Wang Y, Zhu G, Gu Y, Mao L, Hong M, Li Y, Zheng M. Imbalance of Th17/Treg cells in pathogenesis of patients with human leukocyte antigen B27associated acute anterior uveitis. Sci Rep. 2017;7:40414.
38. Ozen S, Eroglu FK. Pediatric-onset Behcet's disease. Curr Opin Rheumatol. 2013;25(5):636–42.
39. Deniz R, Tulunay-Virlan A, Ture Ozdemir F, Unal AU, Ozen G, Alibaz-Oner F, Aydin-Tatli I, Mumcu G, Ergun T, Direskeneli H. Th17-inducing conditions lead to in vitro activation of both Th17 and Th1 responses in Behcet's disease. Immunol Invest. 2017;46(5):518–25.
40. Fasching P, Stradner M, Graninger W, Dejaco C, Fessler J. Therapeutic potential of targeting the Th17/Treg axis in autoimmune disorders. Molecules. 2017;22(1):134.
41. Guedes MC, Borrego LM, Proença RD. Roles of interleukin-17 in uveitis. Indian J Ophthalmol. 2016;64(9):628–34.
42. Miyara M, Ito Y, Sakaguchi S. TREG-cell therapies for autoimmune rheumatic diseases. Nat Rev Rheumatol. 2014;10(9):543–51.
43. Gilbert RM, Zhang X, Sampson RD, Ehrenstein MR, Nguyen DX, Chaudhry M, Mein C, Mahmud N, Galatowicz G, Tomkins-Netzer O, Calder VL, Lightman S. Clinical remission of sight-threatening non-infectious uveitis is characterized by an upregulation of peripheral T regulatory cell polarized towards T bet and TIGIT. Front Immunol. 2018;9:907.
44. Zou W, Wu Z, Xiang X, Sun S, Zhang J. The expression and significance of T helper cell subsets and regulatory T cells CD4 + CD25 + in peripheral blood of patients with human leukocyte antigen B27-positive acute anterior uveitis. Graefes Arch Clin Exp Ophthalmol. 2014;252(4):665–72.

45. Wang Y, Xing F, Ye S, Xiao J, Di J, Zeng S, Liu J. Jagged-1 signaling suppresses the IL-6 and TGF-β treatment-induced Th17 cell differentiation via the reduction of ROR-γt/IL-17A/IL-17F/IL-23a/IL-12rb1. Sci Rep. 2015;5:8234.
46. Weng C, Chong L, Jia X, Zheng R, Huang Y, Zhu T, Li C, Zhang W. Anti-Dll4 antibody inhibits the differentiation of Th17 cells in asthmatic mice. Inflammation. 2017;40(6):1975–82.
47. Yang H, Zheng S, Mao Y, Chen Z, Zheng C, Li H, Sumners C, Li Q, Yang P, Lei B. Modulating of ocular inflammation with macrophage migration inhibitory factor is associated with notch signalling in experimental autoimmune uveitis. Clin Exp Immunol. 2016;183(2):280–93.
48. Bhuyan ZA, Asanoma M, Iwata A, Ishifune C, Maekawa Y, Shimada M, Yasutomo K. Abrogation of Rbpj attenuates experimental autoimmune uveoretinitis by inhibiting IL-22-producing CD4 + T cells. PLoS One. 2014;9(2):e89266.
49. Jiao Z, Wang W, Hua S, Liu M, Wang H, Wang X, Chen Y, Xu H, Lu L. Blockade of Notch signaling ameliorates murine collagen-induced arthritis via suppressing Th1 and Th17 cell responses. Am J Pathol. 2014;184(4):1085–93.
50. Yu S, Liu C, Li L, Tian T, Wang M, Hu Y, Yuan C, Zhang L, Ji C, Ma D. Inactivation of Notch signaling reverses the Th17/Treg imbalance in cells from patients with immune thrombocytopenia. Lab Invest. 2015;95(2):157–67.
51. Geri G, Terrier B, Rosenzwajg M, Wechsler B, Touzot M, Seilhean D, Tran TA, Bodaghi B, Musset L, Soumelis V, Klatzmann D, Cacoub P, Saadoun D. Critical role of IL-21 in modulating TH17 and regulatory T cells in Behçet disease. J Allergy Clin Immunol. 2011;128(3):655–64.
52. Qin L, Zhou YC, Wu HJ, Zhuo Y, Wang YP, Si CY, Qin YM. Notch signaling modulates the balance of regulatory T cells and T Helper 17 cells in Patients with chronic hepatitis C. DNA Cell Biol. 2017;36(4):311–20.
53. Jia X, Hu M, Wang C, Wang C, Zhang F, Han Q, Zhao R, Huang Q, Xu H, Yuan H, Ren H. Coordinated gene expression of Th17- and Treg-associated molecules correlated with resolution of the monophasic experimental autoimmune uveitis. Mol Vis. 2011;17:1493–507.
54. Kostianovsky AM, Maier LM, Baecher-Allan C, Anderson AC, Anderson DE. Up-regulation of gene related to anergy in lymphocytes is associated with Notch-mediated human T cell suppression. J Immunol. 2007;178(10):6158–63.