

A Notch signaling antagonist (DAPT) ameliorates autoimmune arthritis in mice by suppressing Th1 and Th17 immune responses

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Abstract: Background: Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease for which targeted therapies remain limited. The Notch signaling pathway has been implicated in T cell differentiation and autoimmune inflammation. **Objectives:** This study aimed to investigate the therapeutic effects of the Notch signaling antagonist DAPT on autoimmune arthritis in mice and to explore its underlying immunological mechanisms. **Methods:** A collagen-induced arthritis (CIA) mouse model was established. DAPT (100 ng/kg) or PBS was administered intraperitoneally every other day from day 0 to day 36. Notch pathway activation in CD4⁺ T cells and synovial tissues was assessed by Western blot and immunohistochemistry. Arthritis severity was evaluated by clinical scoring, radiological examination and histopathology. Th1, Th17 and Treg cell frequencies and absolute numbers in spleen and lymph nodes (LNs) were analyzed by flow cytometry. Plasma cytokine levels were measured by multiplex assay. *In-vitro* Th17 differentiation assays were performed with DAPT or a Notch agonist. **Results:** Compared with normal mice, CIA mice showed significant upregulation of NICD expression in CD4⁺ T cells and synovial tissues. DAPT treatment significantly reduced clinical arthritis scores, joint erosion and cartilage destruction. DAPT also decreased the frequencies and absolute numbers of Th1 and Th17 cells in the spleen and LNs, along with reduced plasma levels of IFN- γ and IL-17. *In-vitro*, DAPT suppressed Th17 differentiation, while Notch agonist enhanced it. Treg cells were not significantly altered by DAPT. **Conclusion:** The Notch signaling antagonist DAPT ameliorates autoimmune arthritis in mice by suppressing Th1 and Th17 immune responses, highlighting Notch signaling as a potential therapeutic target for RA.

Keywords: Autoimmune arthritis; DAPT; Notch signal; Th17; Th1

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INTRODUCTION

Rheumatoid arthritis (RA) is the most common chronic inflammatory arthritis, particularly affecting women, smokers and patients with a family history of the disease (Israel *et al.*, 2026). In China, approximately 6 million adults are affected by RA, with 41 newly diagnosed cases per 100,000 people annually (Wei *et al.*, 2025). Studies have shown that within the first decade following initial diagnosis, approximately 35–45% of RA patients become disabled as a result of the disease (Ward, 2022). Moreover, because of its association with the increased morbidity and mortality, RA was considered to be one of the major causes of disability globally. Although this disease has a significant impact on individuals and society, there was evidence that RA might be reversed as long as the disease can be diagnosed and treated within the first few months after the onset of symptoms (Mai *et al.*, 2024). Notch signaling is a conserved evolutionary pathway essential for systemic homeostasis and metazoan development. Its receptors, as well as its typical ligands belonging to the Serrate (Ser)/Jagged (JAG) and Delta (DL)/DLL families, have a transmembrane protein structure and are composed of multiple epidermal growth factor-like (EGF) repeats within their extracellular domain (Wang *et al.*, 2025; Vullings *et al.*, 2025). The interaction of Notch receptors with their ligands on

neighboring cells triggers the Notch signal transduction or transactivation. Previous studies have shown that the γ -secretase inhibitor DAPT effectively blocks Notch receptor signaling by preventing the release and transcriptional activation of the intracellular domain (ICD) (Wang *et al.*, 2022). In addition, studies have indicated that Notch signaling plays an essential role in the differentiation and function of various immune cells, which are involved in driving the chronic and destructive nature of autoimmune arthritis (Schneider *et al.*, 2023). *In-vitro* experiments have exhibited (Yang *et al.*, 2023) that the activation of Notch signal and its ligand δ -like 1 can foster the Th17/Th1 response. Recent research (Chen *et al.*, 2024) has revealed that Th17 cells act as a principal element in the disease occurrence of CIA and RA. In patients in the early course of RA, Th17 cells, rather than Th1 cells, can function as effective inducers of matrix metalloproteinases and pro-inflammatory cytokines upon interacting with fibroblasts.

Recent studies have further enriched the mechanistic understanding of Notch signaling in autoimmune arthritis. A 2024 study (Jiang *et al.*, 2024) found that selective inhibition of NOTCH1 suppresses pathogenic Th17 cells without affecting the function of protective Treg cells. In addition, γ -secretase inhibitors have shown therapeutic potential in various inflammatory diseases (Li *et al.*, 2026; Essayan-Perez and Sudhof., 2023). These advances

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support the exploration of Notch signaling as a therapeutic target for autoimmune arthritis. Therefore, the present study aimed to investigate the *in-vivo* effects of Notch signal blockade on Th1/Th17-related immune responses and the progression of autoimmune arthritis.

MATERIALS AND METHODS

Experimental animals

Forty male DBA/1J mice (8–10 weeks old) were purchased from Fenok Biotech (Shanghai) Co., Ltd (China) and housed in an SPF facility at Wenzhou Central Hospital.

CIA model induction

Following the method described in the literature (Sahu *et al.*, 2024), 100 µg of bovine type II collagen (Maokang Biotechnology Co., Ltd., China) was emulsified in Freund's complete adjuvant (Sartorius Bioreagent, Germany) and injected subcutaneously at the tail base of DBA/1J mice. 21 days later, another 100µg of the same type II bovine collagen, emulsified in Freund's incomplete adjuvant (Nantong Egens Biotechnology Co., Ltd., China), was used for secondary immunization, while the control mice received PBS instead of type II collagen. The severity of arthritis in mice was assessed utilizing a five-point Likert scale (Brand *et al.*, 2007).

DAPT treatment and animal grouping

The Notch signaling antagonist DAPT (γ -secretase inhibitor, Selleck, USA) was dissolved in dimethyl sulfoxide (DMSO). One day prior to the first immunization, mice received an intraperitoneal injection of DAPT (100 ng/kg) or an equal volume of DMSO. A total of 40 mice were randomly divided into the following four groups, with 10 mice per group; Normal control group (Normal): non-immunized, injected with PBS; CIA control group (CIA): CIA model + DMSO; DAPT treatment group (CIA + DAPT): CIA model + DAPT (100 ng/kg); Vehicle control group (CIA + DMSO): CIA model + DMSO (to verify that DMSO had no effect). The dosage was selected based on a previous study (Ma *et al.*, 2025), which demonstrated that 100 ng/kg DAPT effectively inhibits Notch signaling and ameliorates autoimmune arthritis.

Each group was further divided into two independent experimental batches, with 5 mice per batch, for subsequent different assays (e.g., flow cytometry, Western blot, IHC, etc.). The results showed no significant difference between the DMSO group and the CIA control group; therefore, the DMSO group data are not presented separately.

The treatment was administered every other day until day 36. On day 36, the mice were euthanized and ankle joints, spleens, inguinal lymph nodes and peripheral blood were

collected. Since the aim of this study was to verify the therapeutic effect of Notch signaling blockade, multiple dose gradients were not included.

Western blot

CD4⁺ T cells were lysed in Laemmli sample buffer (Maokang Biotechnology Co., Ltd., China) and then centrifuged at 12 000 × g for 10 minutes. The supernatant was processed for Western blotting (WB) analysis. After the protein separation utilizing 10% SDS-PAGE, the whole protein samples were transferred to nitrocellulose membrane (Chengdu Bio-Atom Biotechnology Co., Ltd., China). Afterward, the samples were incubated overnight with the antibody targeting lysed NICD (Notch Intracellular Domain) (1:500, Chengdu Bio-Atom Biotechnology Co., Ltd., China) at 4°C. Following the co-incubation with the secondary antibody labeled with horseradish peroxidase, the labeled samples were processed to visualization utilizing the Pierce enhanced chemiluminescence detection system (1:500, Chengdu Bio-Atom Biotechnology Co., Ltd., China). Eventually, quantification was performed utilizing a fluorescence and chemiluminescence imaging system (Beijing Shengxian Creation Science Co., Ltd., Beijing, China) with anti- β -actin (Lihan Biotechnology Co., Ltd., China) as a loading control.

Immunohistochemical staining

The ankle joints from hind feet were fixed for 72 hours using 10% phosphate-buffered formalin. Then the samples were processed for 48 hours of decalcification using 10% nitric acid before paraffin embedding. Afterward, they were sliced (in 4µm-thickness) for H and E staining. After the exposure of surface antigens, the paraffin-embedded tissue sections of murine ankle joints were probed with blocking with 10% rat serum, followed by the combined incubation with the anti-lysed NICD primary antibody (Val 1744; Maokang Biotechnology Co., Ltd., China) and the horseradish peroxidase-labeled anti-rabbit IgG secondary antibody (Maokang Biotechnology Co., Ltd., China). After washing with TBST, the samples were subjected to color development using diaminobenzidine (DAB) substrate (Nantong Egens Biotechnology Co., Ltd., China).

Radiological assessment

X-ray imaging of the mouse hind limbs was performed using an X-ray radiography system (Biotests Biotechnology Co., Ltd., China) under the following parameters: 22 kVp, 6 mAs. The degree of joint destruction was independently assessed by three radiologists blinded to the experimental groups, using the following scoring system (per ankle joint) (Jia *et al.*, 2025): 0- normal; 1- mild soft tissue swelling; 2- mild bone erosion; 3- marked bone erosion with joint space narrowing; 4- complete joint destruction or ankylosis. The scores were averaged.

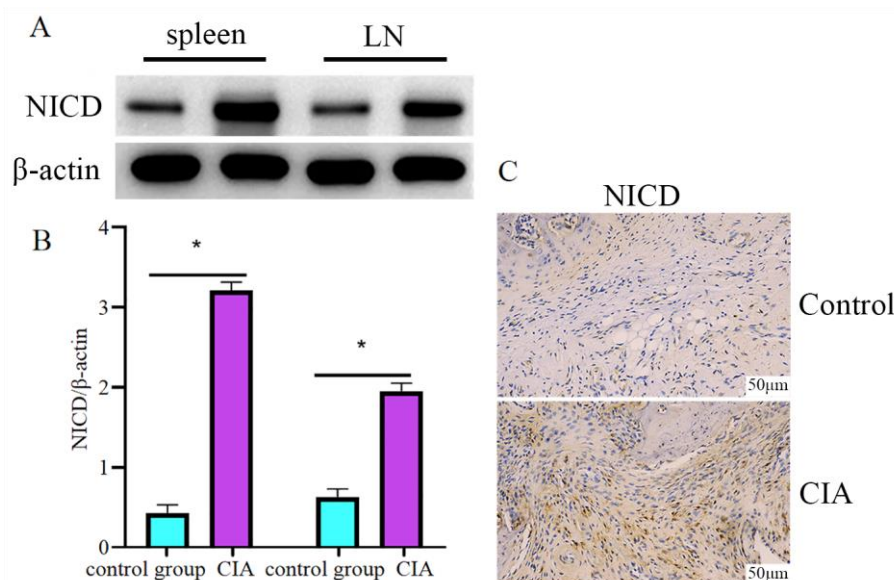


Fig. 1: Notch signal was activated in CD4⁺ T cells and synovial tissues of CIA mice. (A) Expression of NICD in CD4⁺ T cells and synovial tissues; (B) Quantitative evaluation of NICD expression; (C) Graphs of NICD expression in murine synovium via IHC analysis (original $\times 400$). All data were denoted as mean $\pm$ SD. * $P < 0.05$.

Flow cytometry analysis

Cells were incubated for 5 hours at 37°C in 5% CO₂ with 5 μ g/mL brefeldin A, 50 ng/mL phorbol myristate acetate (PMA) and 1 μ g/mL ionomycin (all from Biotests Biotechnology Co., Ltd., China). Cell-surface staining was performed using anti-CD4-FITC antibody. Subsequently, cells were treated with the IntraPrep fixation/permeabilization reagent kit for intracellular staining with anti-IFN- γ -PE (for Th1 detection), anti-IL-17A-PE (for Th17 detection) and anti-Foxp3-PE (for Treg detection). Treg cells were identified using the CD4⁺Foxp3⁺ double-positive strategy, with isotype controls and fluorescence minus one (FMO) controls. Flow cytometry was performed on a FACSCalibur instrument and data were analyzed using Cell Quest 3.2 software. The gating strategy was as follows: lymphocytes were first gated based on FSC/SSC, followed by gating on CD4⁺ cells and then the percentages of IFN- γ ⁺, IL-17A⁺, or Foxp3⁺ cells were analyzed. For absolute cell number calculation, single-cell suspensions from the spleen or lymph nodes were prepared, total cell counts were obtained and then the number of a specific cell subset was calculated by multiplying the total cell count by the percentage of that subset detected by flow cytometry.

Enzyme-linked immunosorbent assay

The concentrations of cytokines (IL-17, IFN- γ , IL-10, IL-4, IL-22, etc.) in plasma were measured using a 13-plex FlowCytomix multiplex assay kit (BioAtom Biotechnology Co., Ltd., Chengdu, China). The procedure was performed according to the manufacturer's instructions and data were acquired and analyzed using a FACSCalibur flow cytometer.

In-vitro expansion of Th17 cells

Referring to the method described in the literature (Hu *et al.*, 2023), CD4⁺ T cells were purified from the spleens of naïve mice. The obtained cells were then cultured with anti-CD3/CD28 antibodies in the presence of IL-6, TGF- β , IL-23 and IL-1 β to induce the expansion of Th17 immune cells *in-vitro*. Meanwhile, 1 μ mol/L of the Notch receptor agonist valproic acid (VPA) was added. After 48 hours, the cells were collected to detect intracellular IL-17A expression and IL-17 levels in the culture supernatant. This concentration was selected based on preliminary experiments and the reported EC50 value in the literature (Cheng *et al.*, 2023).

Statistical analysis

Statistical analysis was performed using SPSS 26.0 software. Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA), followed by Tukey's HSD test for homogeneous variances or Games-Howell test for heterogeneous variances as the post hoc test. Comparisons between two groups were performed using an independent two-tailed Student's t-test. All data are presented as mean $\pm$ standard deviation (SD) and a P value < 0.05 was considered statistically significant.

RESULTS

Enhanced Notch signal presented in the synovial tissues as well as the CD4⁺ T cells from CIA mice

Compared with control mice, CIA mice showed significantly increased NICD expression in CD4⁺ T cells from the lymph nodes and spleen (Figs. 1A and 1B) and in synovial tissues (Fig. 1C).

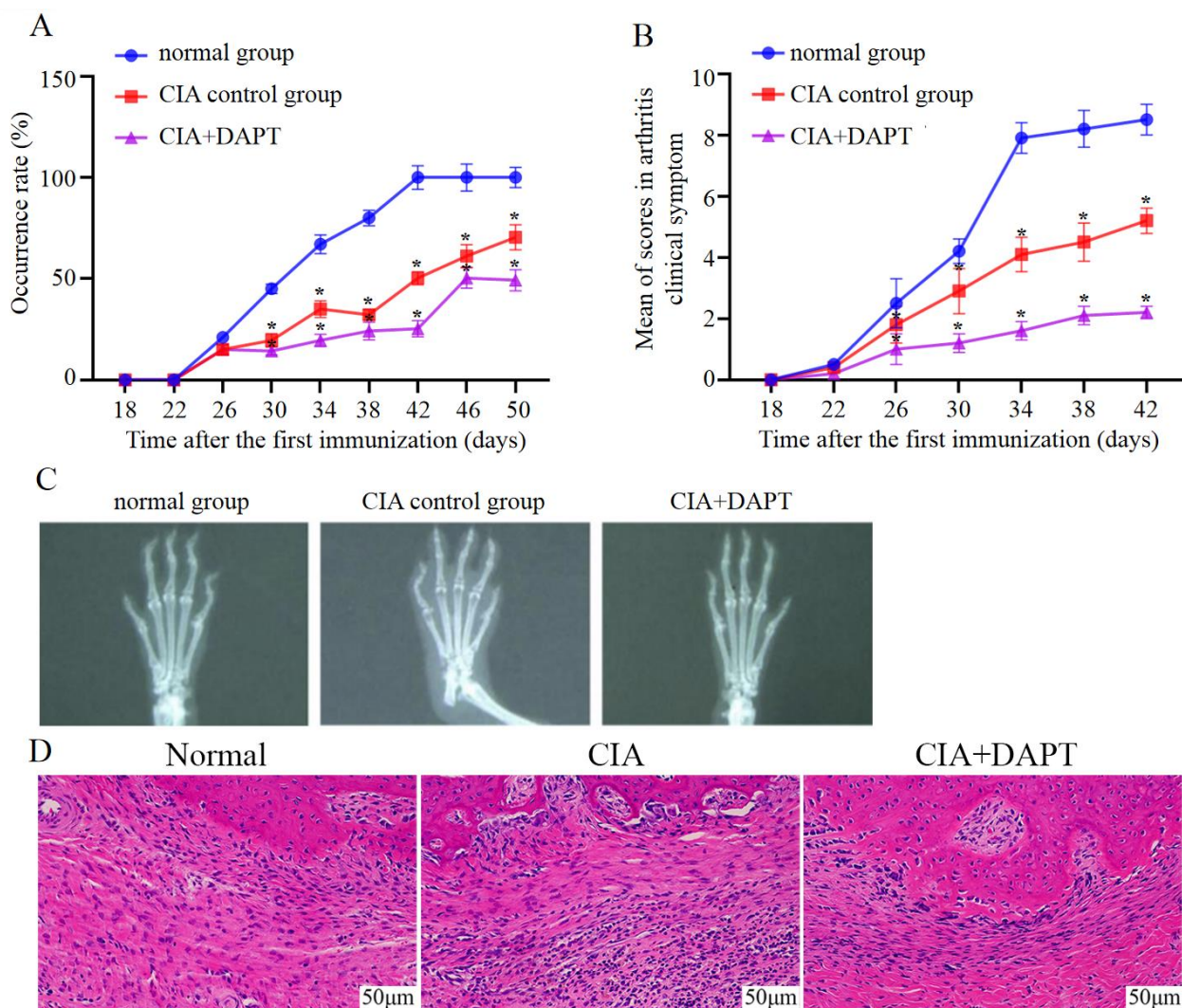


Fig. 2: The blockage of Notch signal ameliorated the arthritis condition in CIA mice. (A) The occurrence rate of arthritis progression in mice; (B) The clinical scoring of murine arthritis; (C) Representative photos of murine ankle joints in the radiological examination; (D) Representative images of murine ankle joints after H and E staining (original $\times 100$). All data were denoted as Mean $\pm$ SD, * $P < 0.05$ with control mice as the internal reference.

Inhibition of Notch signaling in CIA mice ameliorated arthritis development.

Compared with the CIA control group, the severity of arthritis manifestations in the DAPT (100 ng/kg) treatment group mice was significantly reduced (Figs. 2A and 2B). The radiological examination disclosed that DAPT-treated CIA mice manifested significantly reduced erosion of the articular bones (Fig. 2C), accompanied by significantly diminished cartilage destruction, pannus formation and synovial hyperplasia (Fig. 2D).

Inhibition of the Notch signaling pathway impeded the Th1/Th17 response in mice with CIA.

In contrast to the CIA control group, the NICD quantity declined significantly in CD4⁺ T cells, which were enriched from the LNs and spleen of mice with CIA after receiving DAPT treatment (Figs. 3A and 3B). In contrast

to the normal control group, CIA mice exhibited significantly elevated populations of Th17 and Th1 cells in the LNs and spleen. In contrast to the CIA control mice, CIA mice that received DAPT treatment exhibited a significantly lower proportion of Th17 and Th1 cells in the LNs and spleen (Figs. 3C and 3D; Figs. 4A and 4B). In addition, compared with normal control mice, CIA mice showed a significantly enlarged population of Treg lymphocytes within the LNs and spleen, accompanied by a significant reduction in the absolute numbers of Th1 and Th17 cell subsets. Although DAPT treatment had no significant effect on Treg population elevation, it still significantly impeded the reduction in Th1 and Th17 cell numbers (Figs. 5A and 5B). These outcomes revealed that the blockade of Notch signal transduction *in-vivo* can retard the production of Th17 as well as Th1 cells in mice with CIA.

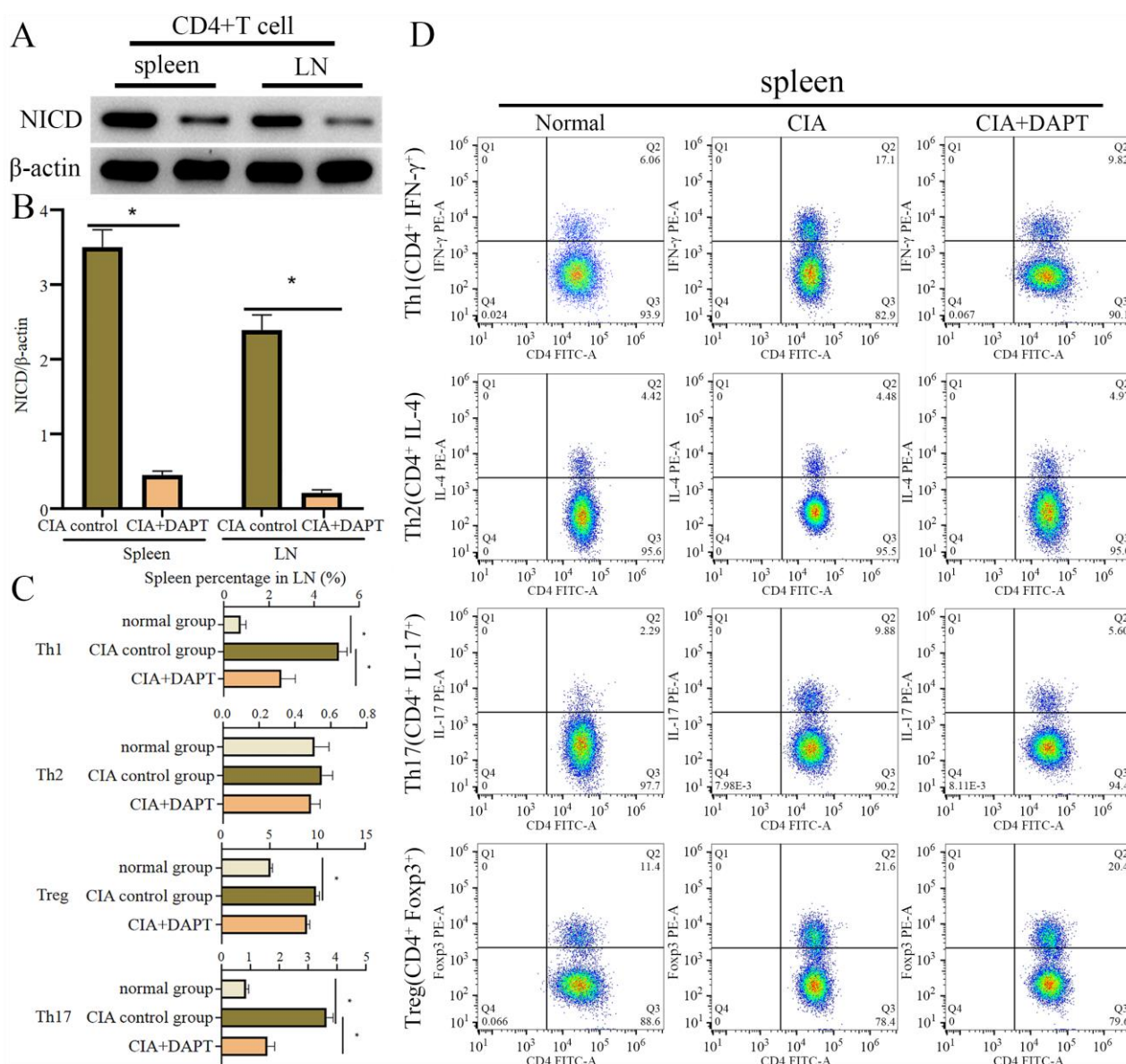


Fig. 3: Blockade of Notch signaling reduces the frequency of Th1 and Th17 cells in the spleens of CIA mice. (A) The expression of NICD in CD4⁺ T cells from the spleens of mice in different groups; (B): Quantitative evaluation of NICD expression in each group; (C) The frequency determination of Th1/Th2, Treg and Th17 cells in the spleen via flow cytometry; (D) Representative plots of T cell subpopulations among CD4⁺ T cells in the spleen from flow cytometry. All data were denoted as mean $\pm$ SD. * $P < 0.05$.

Suppression of the Notch signal can downregulate IL-17 and IFN- γ levels in the peripheral blood of mice with CIA.

Compared to normal mice, the CIA control mice exhibited elevated IL-17 and IFN- γ levels in plasma (Fig. 5C), whereas DAPT treatment significantly lowered IFN- γ and IL-17 levels. No significant alterations of IL-10, IL-4 or IL-22 levels were observed.

Notch signaling modulated the differentiation process of Th17 cells in-vitro.

In comparison with the control cells, those cells that were

administered with DAPT exhibited a significantly reduced percentage of Th17 cells (Fig. 6A), which was also accompanied by a lower total amount of Th17 immunocytes as well as a diminished amount of IL-17 in the supernatant (Figs. 6B and 6C), suggesting that Notch signaling played a necessary part in the cellular differentiation procedure of Th17 immunocytes *in-vitro*. Additionally, after being treated with Notch agonist, these cells displayed significantly elevated percentage and absolute amount of Th17 immunocytes, along with a significantly strengthened IL-17 level in the supernatant (Figs. 6D-6F).

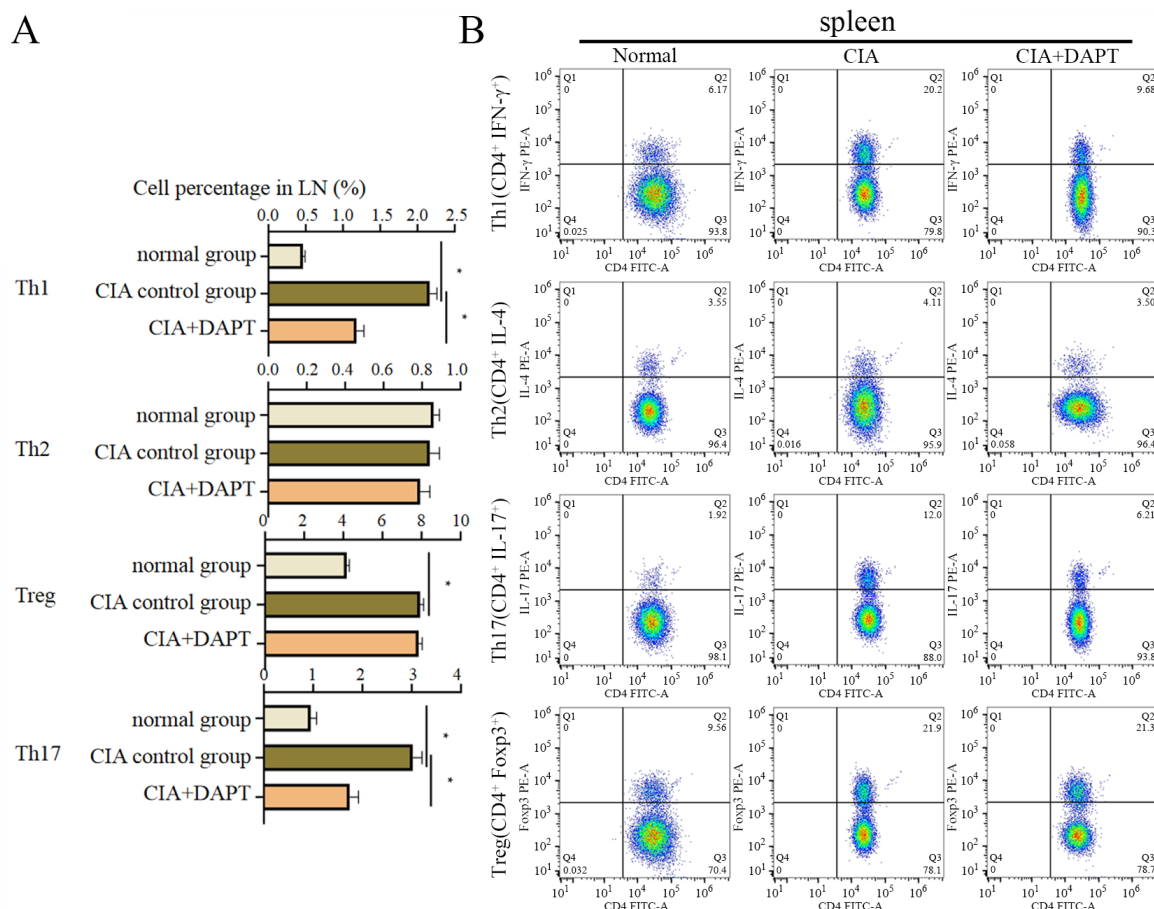


Fig. 4: The blockage of Notch signal transduction weakened the frequency of Th1 and Th17 cells in the LNs of CIA mice. (A) The frequency determination of Th1/Th2, Treg and Th17 cells in the LNs via flow cytometry; (B) Representative plots of T cell subpopulations among CD4⁺ T cells in the LNs from flow cytometry. All data were denoted as mean $\pm$ SD. * $P < 0.05$.

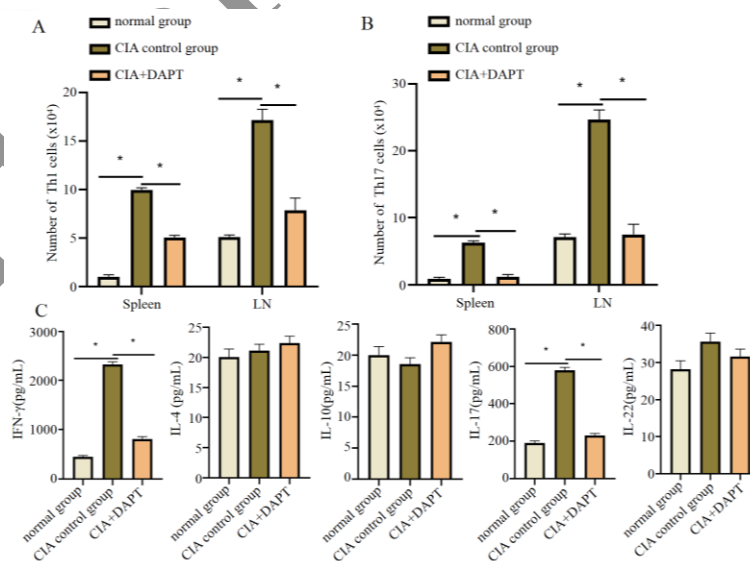


Fig. 5: The blockage of Notch signal transduction reduced the number of Th1 and Th17 cells in the spleen and LNs as well as lowered the cytokine levels in the plasma of CIA mice. (A) The absolute number of Th1 cells in the spleens and LNs; (B) The absolute number of Th17 cells in the spleens and LNs; (C) The cytokine levels in the plasma. All data were denoted as mean $\pm$ SD. * $P < 0.05$.

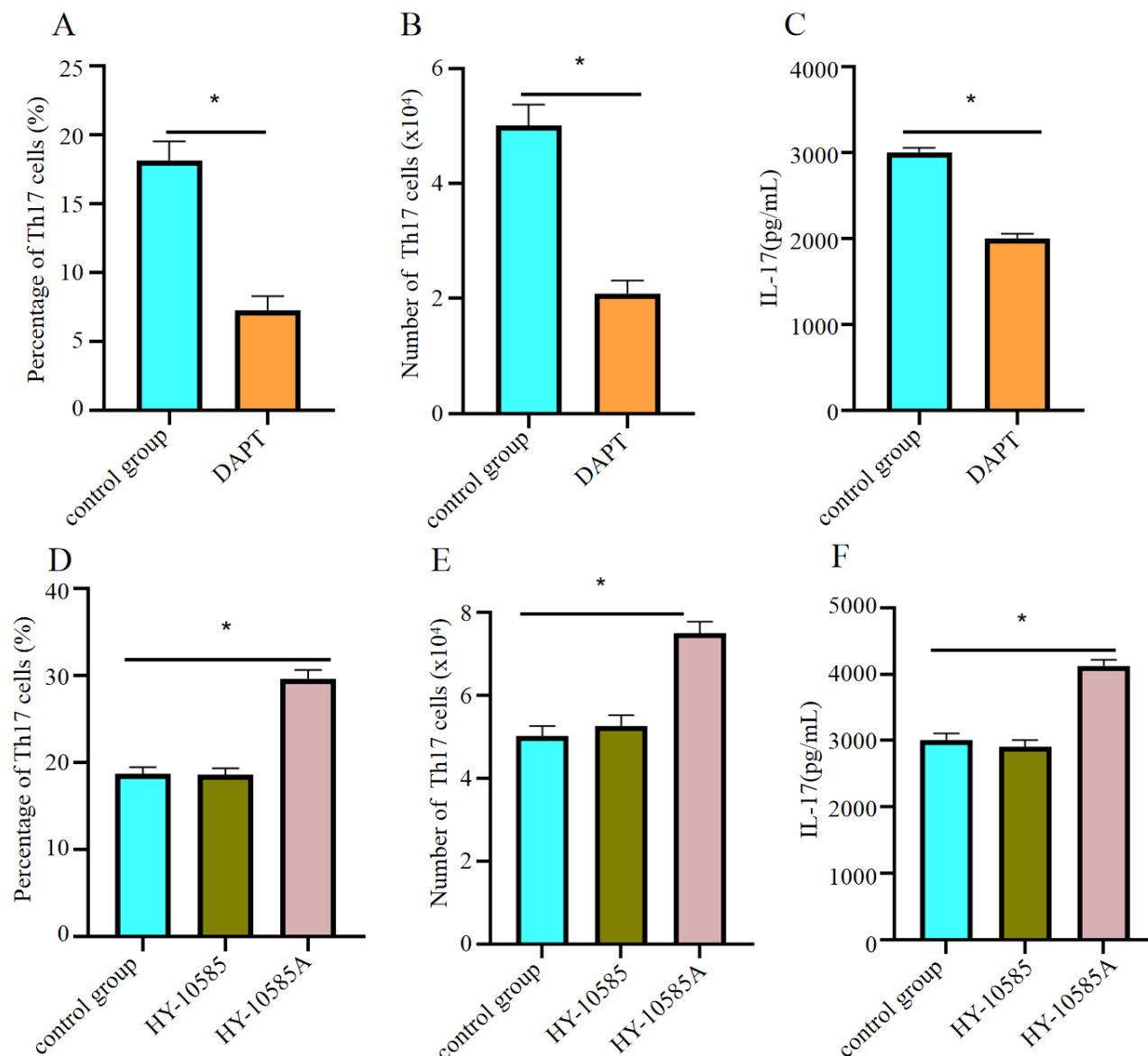


Fig. 6: Notch signal modulated the *in vitro* expansion of Th17 cells. (A) The frequency of Th17 cells after DAPT administration; (B) The absolute number of Th17 cells after DAPT administration; (C) The IL-17 quantity in the supernatant; (D) The frequency of Th17 cells after treatment with Notch agonist; (E) The absolute number of Th17 cells after treatment with Notch agonist; (F) The IL-17 quantity in the supernatant after treatment with Notch agonist. All data were denoted as mean $\pm$ SD. * P < 0.05.

DISCUSSION

Studies have reported significant changes in T helper cell populations in the peripheral blood of patients diagnosed with RA and have also noted abnormal activation of multiple intracellular signaling pathways (Niu *et al.*, 2024). In contrast to healthy controls, patients would exhibit enhanced Notch receptor function, together with intensified activation of their associated downstream signaling. The results of the current study revealed enhanced Notch signaling in CD4⁺ T cells within the LNs and spleen, with this abnormal activation also observed in

the synovial tissues of CIA mice.

Previous literature has shown that the Notch signaling antagonist GSI exerts beneficial therapeutic effects in several inflammatory autoimmune diseases (Mora and Chapouly., 2023; Giambra *et al.*, 2024). Observational records have shown that Notch signaling is important for regulating the differentiation of neural stem cells into neurons, astrocytes and oligodendrocytes, revealing its multiple functions in neuroinflammation, neurodegeneration and myelin formation (Askari *et al.*, 2024). DAPT's antagonistic effect on Notch signaling can

restrain lung fibrosis and the overactivation of the autoimmune response in mice (Lian *et al.*, 2024). In addition, the literature reports that antagonists targeting Notch signaling can effectively inhibit Th1 differentiation, thereby altering the disease course of cough variant asthma (Xu *et al.*, 2025). The results of the present study showed that DAPT can efficiently block Notch signaling in the CIA mouse model, thereby alleviating disease severity and joint destruction. Th17 cells were considered a principal group of inflammation-enhancing helper T cells in the disease course of CIA. They possessed the ability to secrete cytokines, including IL-17F and IL-17A, and to express RORC (a lineage-specific transcription factor), all of which enabled them to play a principal role in the pathogenesis of human RA (Chen *et al.*, 2024). Some studies have revealed that, in addition to the function of Th17 immunocytes and their effector cytokines in RA, they are also closely related to the occurrence of various autoimmunity- and inflammation-associated diseases (Itano *et al.*, 2023; Wu *et al.*, 2026). It was demonstrated in the present study that DAPT administration led to a significant decline in NICD levels in CD4⁺ T cells isolated from the lymph nodes and spleen of CIA mice, accompanied by effective inhibition of Notch signaling. Moreover, DAPT can result in a dramatic reduction in Th17 and Th1 immunocytes in the LNs and spleen of mice with CIA, in both percentage and total cell numbers, thereby lowering IFN- γ and IL-17 levels in the plasma. Recent studies have reported that blocking Notch signaling *in-vivo* can ameliorate the inflammatory condition and Th17 differentiation observed in experimental autoimmune encephalomyelitis, accompanied by reduced proliferation of collagen-specific T cells and a weakened Th1-Th17 immune response (Han *et al.*, 2025). Consistent with the results of this study, previous studies have shown that inhibiting γ -secretase activity in the experimental autoimmune thyroiditis model can reduce IL-17-producing CD4⁺ T cells and improve thyroid symptoms (Liu *et al.*, 2023). Furthermore, another study suggests that Notch signaling may interact with the AhR pathway to jointly regulate the Th17 cell polarization process in immune thrombocytopenia (Cheng *et al.*, 2023). These studies support the significant role of Notch signaling in autoimmune arthritis from various perspectives. The readouts of this study revealed that, under Th17-differentiation triggered by specific stimulation, DAPT administration notably reduced both the proportion and total number of Th17 immunocytes, along with reduced production of Interleukin-17. While the supplementation of Notch ligand $\Delta 3$ can remarkably boost both the proportion and total amount of Th17 immunocytes, along with intensified IL-17 levels in the supernatant under a Th17-polarized culture situation.

There are still certain limitations in this study. First, DAPT is a non-selective γ -secretase inhibitor and not a specific Notch antagonist. In addition to Notch receptors,

γ -secretase is involved in the cleavage and activation of various substrate proteins. Therefore, the inhibition of Th1/Th17 responses and alleviation of arthritis symptoms observed in this study may involve signaling pathways other than NOTCH. Future studies should employ genetic approaches or selective blocking antibodies to validate the specific contributions of different Notch receptor subtypes. Second, the NICD antibody used in this study primarily recognizes the activated form of Notch1 and fails to comprehensively assess the changes in NOTCH 2, NOTCH 3 and NOTCH 4 in the CIA model. Third, this study examined only a single DAPT dose and did not conduct dose-response experiments, so the dose-dependent effect of DAPT cannot be confirmed. Moreover, although this study observed that DAPT treatment reduced the proportions of Th17 and Th1 cells and ameliorated arthritis symptoms, it did not provide molecular biological evidence for the direct regulation of Th17/Th1 differentiation by Notch signaling. Specifically, this study did not examine the expression levels of the lineage-specific transcription factors ROR γ t and T-bet in Th17 and Th1 cells, nor did it analyze the transcriptional activity of the classical Notch target genes Hes1 and Hey1. Therefore, the direct molecular mechanism by which Notch signaling regulates Th17/Th1 differentiation remains unclear.

CONCLUSION

In summary, this study demonstrates that Notch signaling (primarily NOTCH1) is activated in CD4⁺ T cells in a mouse model of autoimmune arthritis. Pharmacological inhibition of Notch signaling using DAPT, a γ -secretase inhibitor, alleviates disease severity and joint destruction in CIA mice, accompanied by reduced Th1 and Th17 responses. However, given the off-target effects of DAPT and the lack of genetic validation, the causal role of Notch signaling in regulating Th1/Th17 differentiation needs further confirmation using more specific approaches, such as Notch receptor-specific gene knockout or blocking antibodies. This study provides preliminary experimental evidence for Notch signaling as a potential therapeutic target in autoimmune arthritis, but the clinical relevance and specificity of this pathway require further investigation.

Acknowledgments

None.

Authors' contributions

Guangjie Pan; Conceived and designed the study; Pingshan Yang; Performed the animal experiments, Western blot and immunohistochemistry; Hui Chen; Conducted flow cytometry analysis and ELISA, performed the radiological assessment and statistical analysis; Jun Xie and Guangjie Pan; Wrote the original draft; Hui Chen; Reviewed and edited the manuscript. All

authors approved the final version of the manuscript.

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Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval

The research methods and procedures were approved by the Animal Ethics Committee and the Animal Use Committee of Wenzhou Central Hospital (Approval No.: IA20251008). All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest

The authors declare no conflict of interest.

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