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# Tangeretin promotes regulatory T cell differentiation by inhibiting Notch1/Jagged1 signaling in allergic rhinitis

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ABSTRACT

*Background and objective:* Tangeretin demonstrates broad anti-inflammatory effects. The present study aimed to assess whether tangeretin functions in regulating T-regulatory cells (Tregs) and alleviating allergic rhinitis (AR).
*Methods:* An ovalbumin (OVA)-induced AR animal model was constructed to monitor the changes in the allergic symptom score, OVA-specific IgE titers, histopathological characteristics and T-helper cell (Th1, Th2, and Th17)-related cytokine levels under tangeretin or dexamethasone (DXM) administration. The expression levels of Notch1/Jagged1 and FOXP3, and the proportion of Tregs in the spleens of these animals, were also detected. Furthermore, purified naive CD4 + T cells were utilized to assess the effects of tangeretin on Notch1 expression and their differentiation in vitro.
*Results:* Both tangeretin and DXM administration alleviated airway inflammation, decreased the production of serum OVA-induced IgE, but only tangeretin administration restored the balance of cytokine profiles compared with those in the AR group. The abundance of splenic CD4 + CD25 + FOXP3 + Treg cells and the transcription factor FOXP3 were significantly increased under tangeretin treatment, either in AR mice or in naïve CD4 + T-cell differentiation, followed by a concomitant reduction in Notch1/Jagged1 expression. However, as a positive control, the treatment of allergic rhinitis with dexamethasone was not related to the expression of Notch1/Jagged1 or the differentiation of Treg cells.
*Conclusion:* Tangeretin could promote regulatory T cell responses by inhibiting Notch1/Jagged1 expression, followed by promoting FOXP3/Treg cell differentiation and thus could serve as a novel curative therapeutic for AR.

## 1. Introduction

Allergic rhinitis (AR) comprises an immunoglobulin E (IgE)-mediated type I hypersensitivity reaction that occurs when atopic individuals react to an inciting inhaled allergen, characterized by symptoms of nasal obstruction, rhinorrhea sneezing, and nasal itching, and up to 40% of the global population is affected by AR [1]. AR is also associated with other atopic disorders, such as, food allergy, asthma, and atopic dermatitis [2]. To restore the balance between regulatory and effector cells, various methods have been tested, including glucocorticoid treatment, allergen-specific immunotherapy [3,4] and biologicals targeting cytokines [5]; but each technique has limitations [6–8]. Thus, there is an important need to identify effective therapies to manage airway inflammation in AR.

Tangeretin (chemical name: 4′,5,6,7,8-pentamethoxyflavone), is a polymethoxylated flavonoid that is abundant in citrus fruit peel [9]. Tangeretin has many beneficial bioactivities, such as anti-oxidant, anti-inflammatory, and anti-cancer [10–12]. Recently, the potent anti-inflammatory and anti-asthmatic effects of tangeretin have addressed using neonatal asthmatic mouse model, which exhibited an extreme reversal of the T-helper cell (Th1, Th2, and Th17) imbalance via the Notch and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) sig-

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naling pathway [13]. Allergic diseases share some same immune signatures [14–16]; therefore, those findings highlighted the potential efficacy of tangeretin to treat a range of atopic disorders, including allergic rhinitis.

In our previous study, we found that Notch1/Jagged1 is abnormally activated in patients with AR, and Notch signaling could promote AR development by inhibiting T-regulatory cell (Treg) differentiation and forkhead box P3 (FOXP3) expression [17]. Tangeretin can suppress Notch1 expression, and that of its ligand Jagged1, in the radiation-induced epithelial-mesenchyme transition (EMT) of gastric cancer cells [18]. Notch signaling is suggested to be involved in AR; however, whether tangeretin could inhibit the progression of inflammatory rhinitis and its mechanism remain unclear. In the present study, we determined that tangeretin-mediated inhibition of Notch1 signaling could ameliorate the AR process either in vivo or in vitro by promoting the differentiation of regulatory T cells.

## 2. Methods

### 2.1. Animals

Twenty-four female specific pathogen free (SPF) C57BL/6 mice (18 to 22 g, aged 4 to 6 weeks), were purchased from Beijing Weitonglihua Experimental Animal Technology Co., Ltd. (License No.: SCXK (J) 2016-0006) and maintained in the Animal Experiment Center of the Renmin Hospital of Wuhan University (License No.: SYXK (E) 2015-0027) under pathogen-free conditions. The mice were housed at approximately 18–22 °C, with moderate humidity (approximately 50–60%). The mice were reared in microisolator cages and received free access to food and water. The mice were acclimatized for a minimum of one week before experimentation. The protocols for animal care and handing were approved by the Institutional Animal Care and Use Committee of Renmin Hospital of Wuhan University (License No.: WDRM-20170310).

### 2.2. Animal model

The mice were grouped randomly ($n$ = 6/group): Normal group, AR group, AR + Tan (tangeretin) group, and AR + DXM (dexamethasone) group. To sensitize the mice they received seven intraperitoneal (i.p.) injections of 100 μg of endotoxin-free ovalbumin (OVA) (Sigma, St. Louis, MO, USA) in 2 mg of alum (Pierce Chemical, Rockford, IL, USA) at 0, 2, 4, 6, 8, 10, and 12 days. On days 14 to 28, mice were challenged intranasally (i.n.) with 10 μL per nasal cavity of 10% OVA in a saline suspension. Finally, the mice were sacrificed on day 30 (AR group). To examine the effects of tangeretin, mice were fed tangeretin (purity > 95%; Aladdin, Shanghai, China) orally (50 mg/kg) regularly 1 h before each OVA administration (AR + Tan group), as described previously [19]. Furthermore, DXM (1 mg/kg) was injected 1 h before OVA administration as a positive control (AR + DXM group). These administration processes are shown in Fig. 1A.

### 2.3. Allergic rhinitis test

Mice ($n$ = 6/group) were monitored for allergic symptom scores 30 min after the last nasal challenge; rubbing and sneezing frequencies were recorded, and the scores were calculated as follows [20]: 1, several slight wipes of the nose or less than three sneeze; 2, wipes the nose repeatedly or more than three but less than ten sneezes; 3, continuous rubbing from nose to face or > 11 sneezes.

### 2.4. OVA-specific serum immunoglobulin E (IgE) assay

At the time of death, blood samples were collected from anesthetized mice from the orbital venous plexus. The sera were separated from the whole blood samples using centrifugation at 600$g$ for 10 min. Sera were stored at −80 °C for further experimentation. The titers of OVA-specific IgE were measured in the sera using enzyme-linked immunosorbent assay (ELISA) kits (Cayman chemical, Ann Arbor, MI. USA) according to the manufacturer's protocols. Data are presented of three independent experiments, each group with $n$ = 6.

### 2.5. Morphological observations of nasal mucosa

Paraformaldehyde (10%) was used to immobilize the nasal mucosa, which was then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for eosinophils and periodic acid-Schiff stain (PAS) for goblet cells. The final cell counts were derived from the mean of the results from five randomly high-power fields (magnification 400×), as described in our previous work [17]. Data are presented of three independent experiments, each group with $n$ = 6.

### 2.6. Western blotting of spleen samples

Protein samples from spleen tissues were prepared using homogenization. Total protein (40 μg) was electrophoresed through a 10% SDS-PAGE gel. The proteins were transferred electrically to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked using 5% skim milk in TBST (20 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween-20, pH 7.5) for 1 h at room temperature. Thereafter, the membranes were incubated with primary antibodies recognizing Notch1 (1: 500; Cell Signaling Technology, Danvers, MA, USA), Notch2 (1: 500; Cell Signaling Technology), Jagged1 (1:1000; Cell Signaling Technology), FOXP3 (1:1000; Cell Signaling Technology) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1: 2000 dilution; Servicebio, Wuhan, China) overnight at 4 °C. The membranes were then incubated with anti-rabbit lgG (1:20000) for 1 h at room temperature. The immunoreactive bands were visualized using an infrared laser imaging system (LI-COR, USA) and quantified using Odyssey software. The results are expressed as the ratio of the mean band density of the experimental groups compared with that of the control group after normalization to the GAPDH value. Data are presented of three independent experiments, each group with $n$ = 6.

### 2.7. Detection of CD4 + CD25 + FOXP3 + Tregs

For the in vivo assays, spleen samples from each mouse group were harvested and prepared as single-cell suspensions by grinding. ACK lysis buffer was used to remove erythrocytes and the remaining cells were resuspended in PBS, half of which were used for Treg cell detection. Data are presented of three independent experiments, each group

with $n = 6$. For the in vitro assays, CD4 + CD25 + FOXP3 + Tregs were analyzed among magnetic bead–purified (Miltenyi Biotech, Bergisch Gladbach, Germany), tangeretin, or dimethyl sulfoxide (DMSO)-treated inducible Tregs (iTregs).

For cell surface staining, the single-cell suspensions were incubated with antibody cocktails (anti-CD4-fluorescein isothiocyanate (FITC), anti-CD25-allophycocyanin (APC); BD Pharmingen, San Jose, CA, USA) at 4 °C for 30 min. Fixation/permeabilization staining (eBioscience, San Diego, CA, USA) was performed according to the manufacturer's instructions. Anti-FOXP3-phycoerythrin (PE) antibodies (BD Pharmingen) were used to stain the cells at 4 °C for 30 min. The cells were washed twice and then the proportion of CD4 + CD25 + FOXP3 + Treg cells was expressed as a percentage of the total CD4 + cells.

### 2.8. Determination of cytokine levels

For the in vivo assays, the remaining half of the lymphocyte single-cell suspension was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS), with 1 μL/mL of lipopolysaccharide stimulation. Multiplex analyses of cytokines, including interleukin (IL)-2, IL-4, IL-6, interferon gamma (IFN-γ), tumor necrosis factor (TNF), IL-17A and IL-10, were then performed after 12 h of incubation using a cytokine bead array kit (BD Pharmingen) following the manufacturer's protocol. Data are presented of three independent experiments, each group with $n = 6$.

### 2.9. Naïve CD4 T-cell isolation and determination of optimum culture conditions for iTreg polarization

Spleens of 8 to 12-week-old WT C57BL/6 mice were used as sources of CD4 + CD25–T cells, isolated using MACS columns. To achieve efficient iTreg polarization in vitro, several stimulus concentrations (TGF-β; anti-CD28; anti-CD3; and IL-2) in culture medium were investigated. Data are presented of three independent experiments.

### 2.10. In vitro iTreg generation

For in vitro iTreg generation, magnetic bead–purified CD4 + CD25–T cells were seeded at $1 \times 10^6$ per well in 24-well plates and stimulated with 10 μg/mL of plate-bound anti-CD3 antibodies (BD Pharmingen), 1 μg/mL anti-CD28 antibodies (BD Pharmingen), 10 ng/mL IL-2 (R&D Systems, Minneapolis, MN, USA), and 8 ng/mL TGF-β (R&D Systems) in complete medium (RPMI1640 containing 10% heat-inactivated FBS [vol/vol], 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 μM L-glutamine) at 37 °C in 5% $CO_2$. Tangeretin (4, 10, or 18 μM) or DMSO was added once on day 0, and then the numbers of CD4 + CD25 + FOXP3 + iTreg were determined using flow cytometry. Data are presented of three independent experiments. *Notch1* and *Foxp3* mRNA expression levels were analyzed using quantitative real-time reverse transcription PCR (qRT-PCR) on day 3.

### 2.11. Quantitative real-time reverse transcription PCR assay

Total RNA from cell lysates was extracted using Trizol (Invitrogen, Waltham, MA, USA). cDNA was reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time quantitative was performed using FastStart Universal SYBR Green Master Mix (Rox; Roche, Branchburg, NJ, USA). The primers used are listed as follows (Table 1).

Samples were run in triplicate and the relative expression levels of *Notch1* and *Foxp3* were normalized to that of *Actb* (encoding β-actin) using the $2^{-\Delta\Delta Ct}$ method [21]. Data are presented of three independent experiments.

### 2.12. Statistical analysis

We used SPSS software V.19.0 (IBM Corp., Armonk, NY, US) to perform the statistical analysis. The results are presented as the mean ± standard deviation (SD). To test whether the data were normally distributed, the Shapiro–Wilk method was used. To test the homogeneity of variance, the Levene method was used. Multigroup comparisons that met the normal distribution and homogeneity of variance were operated by one-way analysis of variance test with post hoc contrasts by Turkey test. To assess the correlation between *Foxp3* and *Notch1* expression levels, the Pearson correlation coefficient (r) was calculated. $P < 0.05$ was defined as statistically significant.

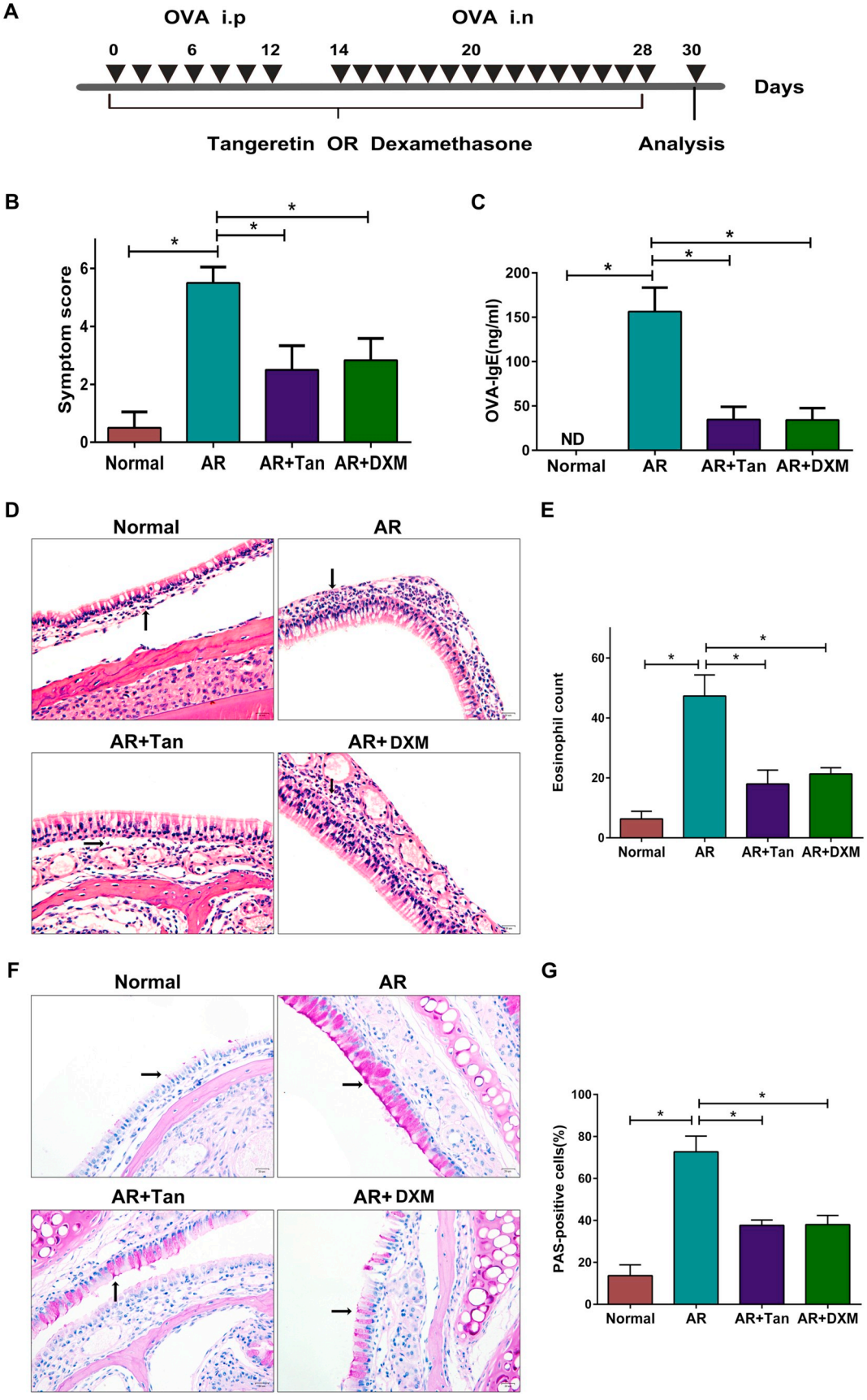
## 3. Results

### 3.1. Tangeretin treatment alleviates the inflammatory responses in an AR model

To investigate whether tangeretin could ameliorate inflammatory responses generated in AR, mice were sensitized and challenged using OVA, and then treated with a regular gavage of tangeretin or injection of DXM (Fig. 1A). Compared with the normal group, the AR model demonstrated increased allergic symptom scores (Fig. 1B; $p < 0.05$), OVA-specific serum IgE titers (Fig. 1C; $p < 0.05$), eosinophil infiltrates, and goblet cells hyperplasia (Fig. 1D–G; $p < 0.05$) indicated that the model was reliably constructed. Notably, mice treated with either tangeretin or DXM displayed markedly reduced allergic symptom scores (Fig. 1B; $p < 0.05$) and serum levels of OVA-specific IgE production (Fig. 1C; $p < 0.05$) compared with those of the AR group, as well as significant reductions in nasal eosinophil infiltration and mucus hypersecretion (Fig. 1D–G; $p < 0.05$). The results indicated the high efficiency of tangeretin in suppressing OVA-induced allergic inflammation.

**Table 1**
List of primers used in quantitative RT-PCR.

| Group | Primer sequences | | Annealing temperature (°C) | PCR product size (bp) |
|---|---|---|---|---|
| | Forward | Reverse | | |
| Notch1 | ATGCTGCTGTTGTGCTCCTGAAG | CGGCAATCGGTCCATGTGATCC | 60 | 167 |
| Foxp3 | AAGAATGCCATCCGCCACAACC | GGCGTTGGCTCCTCTTCTTGC | 60 | 118 |
| β-actin | GTGACGTTGACATCCGTAAAGA | GCCGGACTCATCGTACTCC | 60 | 245 |




*(caption on next page)*

**Fig. 1.** Tangeretin treatment alleviates the inflammatory responses in an AR model. (A). OVA in alum was injected intraperitoneal seven times into mice to sensitize them. The mice were then treated with 20 μL of 10% OVA nasal drops via intranasal administration from day 14 for 15 days. Tangeretin was given orally or DEX was injected 1 h before each OVA administration. At 24 h after the last intranasal administration, analyses were performed. (B) Allergic symptom scores were calculated in each group. Values represent the mean ± SD. *$P < 0.05$. (C) OVA-specific serum IgE levels were evaluated using an enzyme-linked immunosorbent assay (ELISA). Values represent the mean ± SD. *$P < 0.05$. (D) Hematoxylin and eosin (H&E) were used to stain nasal mucosa sections. Original magnification, 400×. Scale bar = 20 μm. (E) Quantification of eosinophil infiltration. Values represent the mean ± SD. *P < 0.05. (F) Nasal mucosa sections were stained with periodic acid–Schiff reagent (PAS). Original magnification, 400×. Scale bar = 20 μm. (G) Quantification of goblet cell hyperplasia. Values represent the mean ± SD. *P < 0.05. Data are presented of one (B) or three independent experiments (C/E/G), each group with $n = 6$. AR, allergic rhinitis; DXM, dexamethasone; OVA, ovalbumin; Tan, tangeretin.

### 3.2. Tangeretin treatment induced a reversal of the Th1/Th2/Th17 imbalance in the AR model

Next, we investigate the effect of tangeretin on the Th1/Th2/Th17 imbalance in AR mice. The quantities of both Th2 cytokines IL-6 and IL-10, and proinflammatory cytokine IL-17A, were significantly increased following OVA challenge, whereas Th1 cytokines IFN-γ and IL-2 decreased (Fig. 2A, B; $p < 0.05$), demonstrating typical Th2 polarization features. Therapeutic administration of tangeretin during OVA challenge induced remarkable protection against the Th1/Th2/Th17 imbalance, with increased IFN-γ, and decreased IL-17A, IL-10, and IL-6 levels (Fig. 2A, B; p < 0.05). Among all groups, TNF levels remain unaltered. In addition, the content of IL-4 was extremely low; therefore its bar graph is not displayed in this manuscript. Additionally, there was no significant change of cytokine profiles under DXM treatment (Fig. 2A, B; $p > 0.05$), which indicated that this treatment had no effect on Th1/Th2/Th17 cytokines.

(A) Bead-based cytokine analysis (IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A, and IL-10) were evaluated by flow cytometry. (B) Statistical analysis of the levels of IL-2, IL-6, TNF-α, IL-10, IL-17A, and TNF in splenocytes. Values represent the mean ± SD. *$P < 0.05$. Data are presented of three independent experiments, each group with $n = 6$. AR, allergic rhinitis; DXM, dexamethasone; Tan, tangeretin.

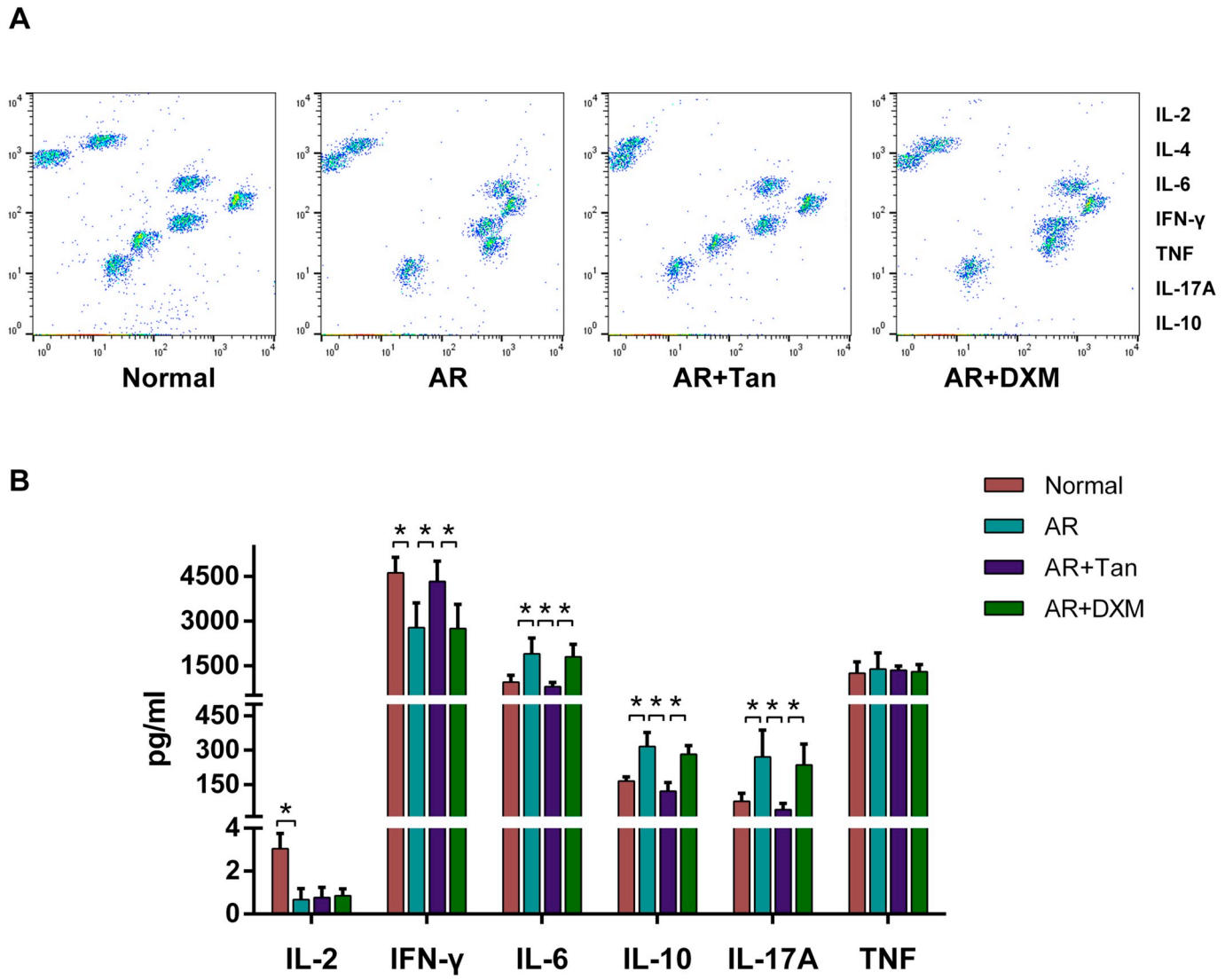



**Fig. 2.** Tangeretin treatment induced a reversal of Th1/Th2/Th17 imbalance in AR model.

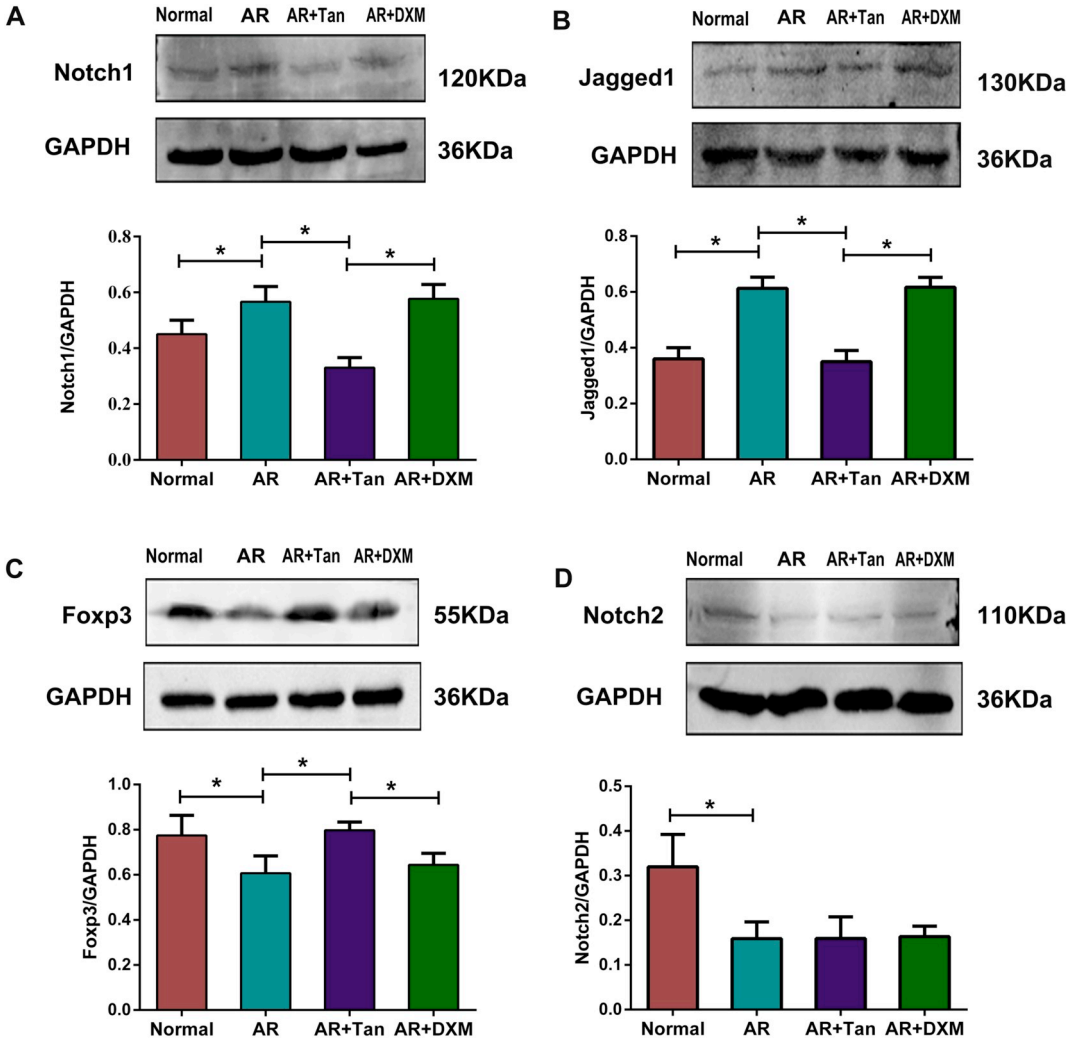



**Fig. 3.** Tangeretin treatment could specially inhibit the expression of Notch1/Jagged 1 and increase the expression of FOXP3 in an AR model. (A) Relative expressions of Notch1/GAPDH proteins as assessed using western blotting. Values represent the mean ± SD. *$P < 0.05$. (B) Relative expression levels of Jagged1/GAPDH proteins as assessed using western blotting. Values represent the mean ± SD. *P < 0.05. (C) Relative expressions of Foxp3/GAPDH proteins as assessed using western blotting. Values represent the mean ± SD. *P < 0.05. (D) Relative expressions of Notch2/GAPDH proteins as assessed using western blotting. Values represent the mean ± SD. *P < 0.05. Data are presented of three independent experiments, each group with $n = 6$. AR, allergic rhinitis; DXM, dexamethasone; Tan, tangeretin.

### *3.3. Tangeretin treatment could specially inhibit the expression of Notch1 and Jagged1, and increased the expression of FOXP3 in the AR model*

To further investigate the mechanisms of tangeretin's anti-inflammatory effects, we aimed to identify the specific molecules targeted by the treatment when administered systemically. Notably, compared with the Normal group, the AR group showed an increase in Notch1 and Jagged1 production, and decreased FOXP3 expression. Interestingly, markedly decreased Notch1 and Jagged 1 production, as well as significantly increased FOXP3 expression, were observed under tangeretin treatment compared with those in the AR group (Fig. 3A–C; $p < 0.05$). These results highlighted the remarkably restrictive effect of tangeretin on Notch1/Jagged1 signaling, and the inverse correlation between Notch1/Jagged1 and FOXP3 protein levels. We also found that in this AR model, the expression of Notch2 was not significantly affected by tangeretin (Fig. 3D; $p > 0.05$). Moreover, the expression levels of Foxp3, Notch1, Notch2, and Jagged1 remained unaltered under DXM administration (Fig. 3; p > 0.05).

### *3.4. Tangeretin treatment expanded CD4 + CD25 + FOXP3 + Tregs in the AR model*

Next, we explored the contribution of Notch1 and Jagged1 inhibition by tangeretin to the fundamental Treg biology in the tangeretin-treated murine model. Compared with those in the Normal group, the proportion of CD4 + CD25 + FOXP3 + Treg cells and the expression level of the transcription factor FOXP3 in the AR group were significantly decreased, whereas tangeretin-treated mice showed higher FOXP3 expression levels compared with mice in the AR model group (Fig. 3C; $p < 0.05$), followed by an increased proportion of Tregs (Fig. 4A, B; p < 0.05). Additionally, mice treated with DXM displayed no significant changes in FOXP3 expression, as well as the proportion of splenic Treg cells, when compared with those in the AR group (Figs. 3C and 4A, B; $p > 0.05$). These data demonstrated that tangeretin treatment effectually expands splenic CD4 + CD25 + FOXP3 + Tregs in the AR model.

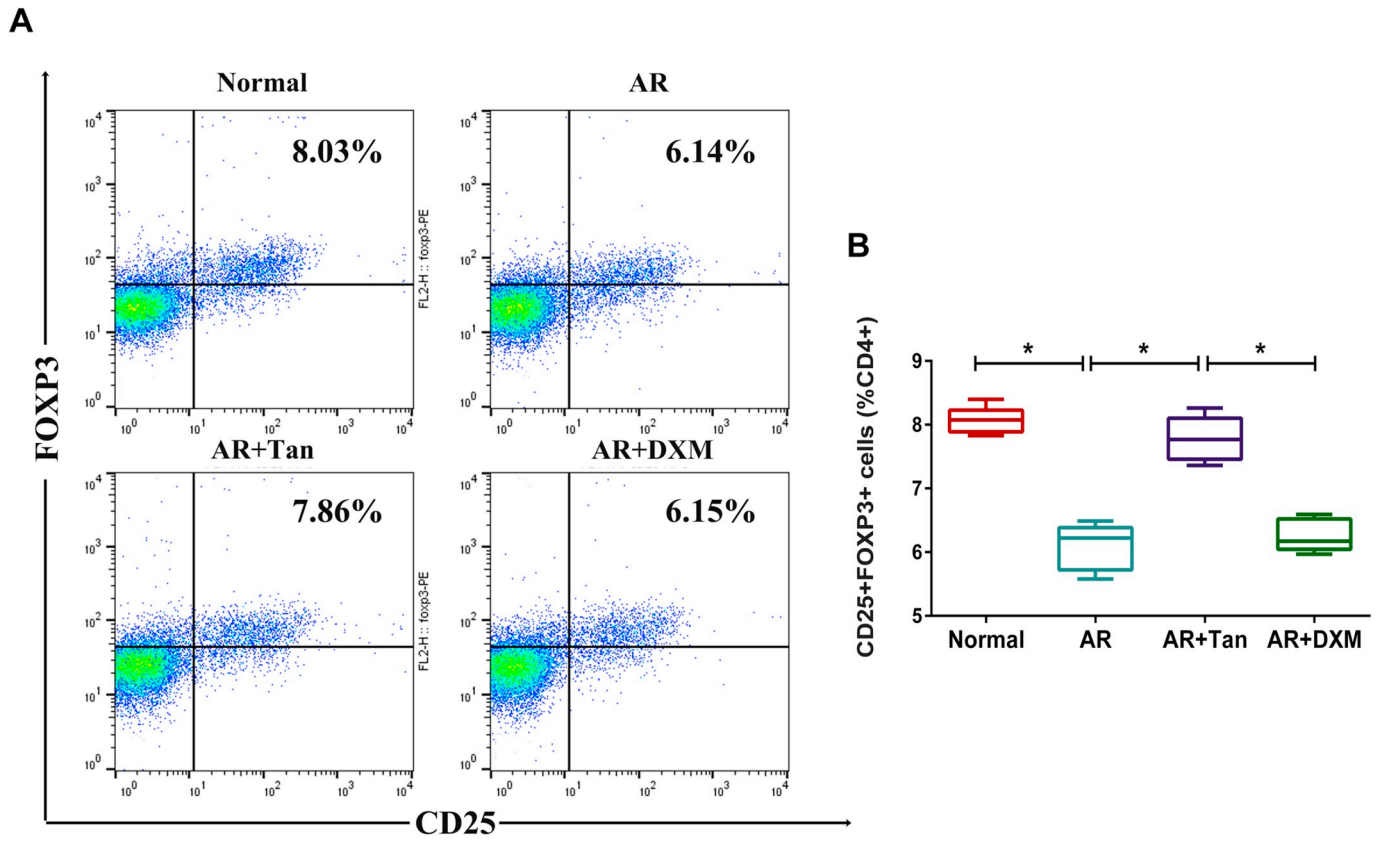



**Fig. 4.** Tangeretin treatment expanded CD4 + CD25 + FOXP3 + Tregs in an AR model. (A) Representative cytofluorimetric plot of the gating strategy for CD25 + FOXP3 + cells evaluation from CD4 + T cells. (B) Quantitative analyses of the flow cytometry data for CD25 + Foxp3 + cells among CD4 + T cells. Values represent the mean ± SD. *$P < 0.05$. Data are presented of three independent experiments, each group with $n = 6$. AR, allergic rhinitis; DXM, dexamethasone; Tan, tangeretin.

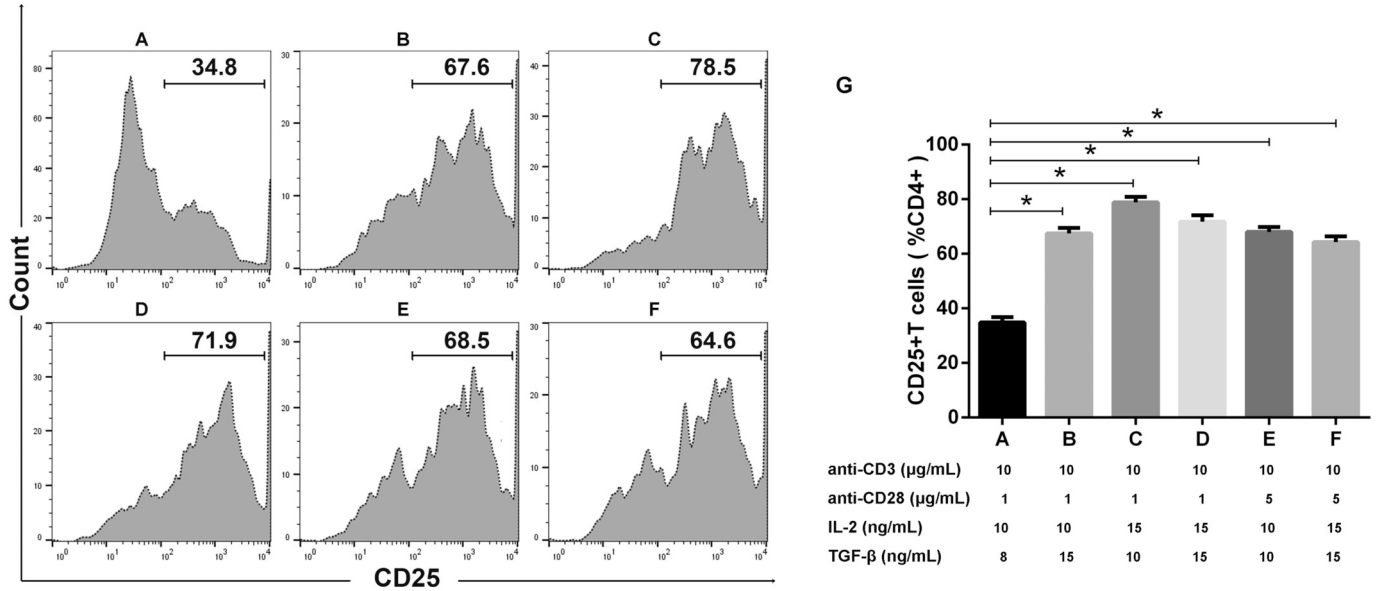



**Fig. 5.** Determination of optimum culture conditions for iTreg polarization. Proportion of CD25 + cells among CD4 + T cells in each culture condition: (A) 10 μg/mL anti-CD3, 1 μg/mL anti-CD28, 10 ng/mL IL-2 and 8 ng/mL TGF-β. (B) 10 μg/mL anti-CD3, 1 μg/mL anti-CD28, 10 ng/mL IL-2 and 15 ng/mL TGF-β. (C) 10 μg/mL anti-CD3, 1 μg/mL anti-CD28, 15 ng/mL IL-2 and 10 ng/mL TGF-β. (D) 10 μg/mL anti-CD3, 1 μg/mL anti-CD28, 15 ng/mL IL-2 and 15 ng/mL TGF-β. (E) 10 μg/mL anti-CD3, 5 μg/mL anti-CD28, 10 ng/mL IL-2 and 10 ng/mL TGF-β. (F) 10 μg/mL anti-CD3, 5 μg/mL anti-CD28, 15 ng/mL IL-2 and 15 ng/mL TGF-β. (G) Summary graph. *$P < 0.05$. Data are presented of three independent experiments. iTREG, induced T regulatory cells.

### 3.5. Tangeretin treatment promotes CD4 + CD25 + FOXP3 + Tregs differentiation through inhibiting Notch1 pathway in vitro

In this study, we first explored the optimum culture conditions for iTreg polarization (Fig. 5). The results showed that the optimal conditions were CD4 + CD25–T cells stimulated with 10 μg/mL of plate-bound anti-CD3 antibodies, 1 μg/mL of anti-CD28 antibodies, 15 ng/mL of IL-2, and 10 ng/mL of TGF-β (Fig. 5C), which provided good culture conditions for Treg-related in vitro experiments. However, lower stimulant concentrations (Fig. 5A) were chosen subsequently so that we could verify the vital role of tangeretin in the promotion of Tregs generation markedly.

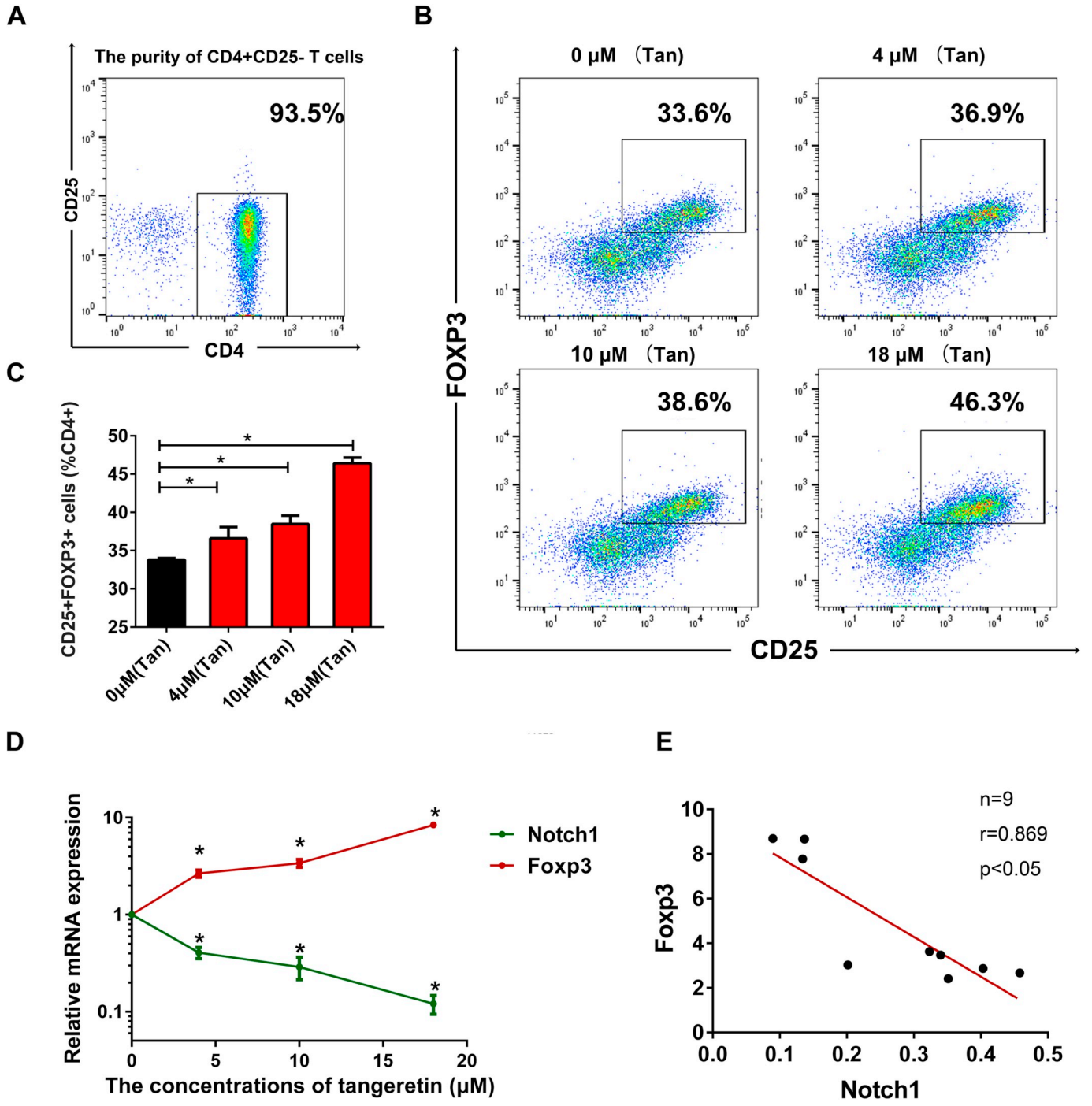



**Fig. 6.** Tangeretin treatment exactly promote CD4 + CD25 + FOXP3 + Tregs differentiation through inhibiting Notch1 pathway in vitro. (A) CD4 + CD25-T cell purity. (B) Representative cytofluorimetric plot of the gating strategy for CD25 + FOXP3 + cells evaluation from CD4 + T cells. (C) Proportion of CD25 + Foxp3 + cells among CD4 + T cells in polarized iTregs. Values represent the mean ± SD. *$P < 0.05$. (D) qRT-PCR analysis of FOXP3/Notch1 expression in polarized iTregs. Values represent the mean ± SD. *P < 0.05. (E) Pearson correlation analysis between FOXP3 and Notch1 expression. *P < 0.05. Data are presented of three independent experiments. iTREG, induced T regulatory cells; Tan, tangeretin.

The purity of the CD4 + CD25-T cells used in iTreg generation was over than 90% (Fig. 6A). Next, we sorted naive CD4 + T cells into iTregs in the presence of tangeretin (4, 10, or 18 μM) or DMSO control. As indicated, iTreg conversion from naïve CD4+ precursors under Treg polarization conditions was variably increased by all concentrations of tangeretin compared with that under DMSO, in a dose-dependent manner, and 18 μM tangeretin appeared to exert the maximum iTreg differentiation (Fig. 6B, C; $p < 0.05$). These data were consistent with the increased *Foxp3* expression, as determined by qRT-PCR (Fig. 6D; $p < 0.05$), which suggested that tangeretin could be administered as a potential therapy to treat Treg-related immune diseases.

We then evaluated *Notch1* mRNA expression during Treg polarization. Indeed, Notch1 mRNA levels exerted a remarkable dose-dependent suppressive effect after tangeretin treatment (Fig. 6D; $p < 0.05$). Moreover, Pearson correlation analysis revealed that the levels of Notch1 negatively correlated with the expression of *Foxp3* (Fig. 6E; $p < 0.05$).

Taking these results together, we found a previously unknown function for tangeretin in promoting the generation of FOXP3+ iTregs by controlling of Notch1 expression in vitro.

## 4. Discussion

Allergic rhinitis reflects the failure to invoke tolerance toward a specific allergen, producing an allergen-specific Th2 cell response, eosinophilia, airway goblet cell hyperplasia, and generation of allergen-specific IgE [22,23]. In the present study, we showed that both tangeretin and DXM could decrease allergic symptom scores, suppress eosinophil infiltration into the airways, reduce mucus hypersecretion, and restrain the production of OVA-specific serum IgE titers. In addition, treatment with tangeretin also restored the Th1/Th2/Th17 balance, which is typically altered during allergic inflammation. Succinctly, the levels of both Th2 cytokines (IL-10 and IL-6) and proinflammatory cytokine IL-17A were significantly decreased upon tangeretin administration, whereas Th1 cytokines (IFN-γ) increased, thus providing definite evidence for the crucial role of tangeretin in alleviating OVA-induced allergic inflammation.

Treg cells are crucial to prevent the differentiation and migration of other effector cells (such as Th1, Th2, and Th17) during infection, inflammation, and autoimmunity [24–27]. Hence, we focused the related mechanisms of tangeretin on regulatory T (Treg) lymphocytes, and found it promoted efficient FOXP3 expression and consequent generation of CD4 + CD25 + FOXP3 + Tregs that inhibit effector T cell functions. Additionally, in vitro differentiation of Tregs showed a higher ratio of iTreg/CD4 + T cells ratio in cells treated with tangeretin when compared with those treated with DMSO, in a dose-dependent manner, documenting the pivotal role for tangeretin in creating a sufficiently protolerogenic environment to induce iTregs. These data conclusively established the anti-inflammation effect of tangeretin in AR via promoting Foxp3/Treg cell differentiation. There has been no previous report of effects of tangeretin on the polarization of CD4 + T cells, our in vitro and in vivo data provided a novel candidate for the treatment of Treg-related immune diseases.

The Notch signaling pathway comprises four different Notch transmembrane receptors (Notch1–4) and five types of transmembrane ligands (Delta1,3,4; Jagged1–2) in mammals [28]. In our previous work, we have found that in patients with AR, Notch1 and Jagged1 were significantly highly expressed, which also closely associated with allergy severity [17]. As several studies have shown the potent Notch-1 inhibiting capacity of tangeretin [13,18], we explored whether tangeretin alleviates allergic rhinitis via its specific inhibition of Notch1 signaling. In this study, the AR murine model confirmed that after tangeretin gavage, the Notch1 and Jagged1 expression levels in the spleens of mice were significantly decreased, while the expression of Notch2 showed no significant change. After treatment with tangeretin, we also observed a significant dose-dependent decrease in the expression of Notch1 in the naïve CD4+ precursors isolated from the spleen. The results demonstrated that tangeretin could specifically inhibit the expression of Notch1 and Jagged1 in CD4 + T cells.

Of the four Notch receptors, Notch1 was most highly expressed in Treg cells, and.

blockade of Notch1 signaling in Treg cells, by means of lineage-specific targeted gene inactivation, resulted in increased Treg cell frequency [29]. Additionally, Notch1 have been proved that it could regulated the Th17/Treg immune imbalance in allergic asthma [30]. In some Th2-dominant immune diseases, such as rheumatoid arthritis, pharmacological and genetic inhibition of Notch1 signaling could promote the population and function of Treg cells [28]. In our previous study, we found in the animal model of AR that CD4 + FOXP3 + Treg cells were significantly increased after the Notch signal inhibitor GSI was applied to inhibit the expression of Notch1/Jagged1. We confirmed Notch1/Jagged1suppressed the differentiation of Treg cells and promoted the development of AR, highlighting the negative regulation role of Notch signaling on Treg cells differentiation in allergic rhinitis [17]. Together, we concluded here that tangeretin promotes FOXP3/Treg cell differentiation by inhibiting Notch1/Jagged1 signaling in allergic rhinitis.

Importantly, the experimental mice showed no significant weight loss or changes to major organs, indicating that high dose gavage of tangeretin was not toxic [31], represents a promising treatment for allergic diseases. The results of the present and previous studies [13,18] demonstrated that tangeretin could specifically inhibit Notch1 and Jagged1 rather than Notch2 signaling; therefore, tangeretin might represent a less toxic alternative than a broad-spectrum blockade of the Notch pathway (i.e., gamma secretase inhibitors) [32–35]. Interestingly, we found that Notch2 signaling, in contrast to Notch1 signaling, promotes the differentiation of Treg cells and ameliorates allergic diseases, which will be discussed in another paper. Thus, the targeted inhibition on the Notch1-Jagged1 axis will lead to fewer side effects and increase its application prospects.

Steroid hormones, especially those of local spray, are the most commonly used drugs in clinical treatment of AR [36]. To investigate the role of tangeretin in the treatment of AR, DXM was selected as the positive control. We found that tangeretin had similar effects to DXM in treating AR, but no changes in TH1/TH2/TH17 cytokines, no changes in the expression of Notch1/Jaggd1 signal, and no changes in the differentiation of Treg cells in the DXM treatment group. So, what is the mechanism of DXM in treating AR?

As we have showed, dexamethasone (1 mg/kg) treatment of AR had no relationship with Foxp3/Treg cell differentiation and Th1/Th2/Th17 cytokines, in light with the research that Foxp3 mRNA and Th2 cytokine mRNA (IL-6, IL-10) remained constant after in vitro exposure of PBMC to corticosteroid for 11 days [37]. Treg cells typically serve to regulate Th1/Th2/Th17 balance, the unaltered Treg cells may result in unaltered cytokine expression. Additionally, the Th17 cell response has been shown to be resistant to hormones [38]. However, it is noticed that steroid treatment can alleviate allergic reactions by inhibiting IgE receptor (FcεRI) expression on mast cells and basophils [39], reducing chemokines produced by mast cells [40], downregulating histamine synthesis in nasal mucosa [41] and preventing vasoconstriction [42]. Thus, we speculate here that DXM has multiple ways to alleviate AR development, rather than influencing the cytokines we have measured.

In summary, we demonstrated that tangeretin can alleviate AR development by inhibiting the expression of Notch1 and Jagged1, and promoting FOXP3/Treg cells differentiation in vivo and in vitro. Further research into the bioavailability, efficacy, dose, and safety are needed to support the use of tangeretin to treat AR and related human allergic disorders.

*Abbreviations*

| | |
|---|---|
| AR | allergic rhinitis |
| DXM | dexamethasone |
| i.n. | intranasal |
| i.p. | intraperitoneal |
| OVA | ovalbumin |
| sIgE | specific immunoglobulin E |
| Treg | regulatory T cell |
| Tan | tangeretin |

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## Author contributions

S.M.C., Y.G.K., Z.Z.T., conceived and designed the experiments of the current study. S.X., W.E.J., performed the experiments and wrote the manuscript. R.Y.,Y.R.Q., and Y.X., were responsible for data acquisition, analysis and interpretation. All the authors read and approved the final version of the manuscript.

## Competing interests

The authors have declared clearly that no competing interest exists.

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