Ursolic acid inhibits Th17 cell differentiation via STAT3/RORγt pathway and suppresses Schwann cell-mediated Th17 cell migration by reducing CXCL9/10 expression

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Abstract
Th17 cells represent important immune cells. Ursolic acid (UA) can regulate immune cell activities. This study was aimed to explore the effects of UA on Th17 cell differentiation and Schwann cell (SCs)-mediated migration and the potential mechanism. Naïve CD4⁺ T cells were isolated from rat peripheral blood, induced for Th17 cell differentiation, and treated with UA. The proportion of Th17 cells was detected by flow cytometry assay. SCs were co-cultured with Th17 cells. Th17 cell migration was detected by Transwell assay. The molecule expression was determined by Western blot and qRT-PCR. UA inhibited the Th17 cell differentiation and suppressed the STAT3/RORγt pathway. STAT3 overexpression upregulated p-STAT3 and RORγt expression and promoted Th17 cell differentiation under the UA treatment. In LPS- and IFN-γ-stimulated-SCs, UA suppressed the expression of chemokines CXCL9/10, but had no significant effect of CCL20 expression. The expression CXCL9/10 receptor CXCR3 was higher in Th17 cells than that in Treg cells, while the expression CCL20 receptor CCR6 was lower in Th17 cells than that in Treg cells. UA, anti-CXCR3, and anti-CCR6 treatment inhibited SCs-mediated Th17 cell migration, and anti-CXCR3 exerted stronger inhibitory effect than Anti-CCR6. UA inhibited Th17 cell differentiation through STAT3/RORγt pathway and suppressed Th17 cell migration through down-regulating CXCL9/10 expression in SCs.

Keywords
Ursolic acid, Th17 cell differentiation, Th17 cell migration, Schwann cells

Introduction
Th17 cells, one of the subsets of effector CD4⁺ T helper cells, are important immune cells in the adaptive immune system. Such cells play important roles in diverse immune-related diseases, including experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease, psoriasis, and periodontal disease. During the pathological process of those diseases, Th17 cells are differentiated from naïve CD4⁺ T cells in the presence of certain cytokines such as IL-6, TGF-β1, and IL-23, and then the chemokine receptors functionally expressed by Th17 cells are recognized by chemokines, such as CXCL9, CXCL10 and CCL20, thereby mediating Th17 cell migration. After migration, Th17 cells produce and release IL-17A, IL-17F, IL-22, IL-9, and chemokines e.g. granulocyte macrophage colony-stimulating factor (GM-CSF) and other proteins.

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e.g. apolipoprotein D (APOD), cathepsin L (CTSL), and thus leading to the damage of tissues or cells.6–13 Thus, inhibiting Th17 cells activities has important implications for treating immune-related diseases.

Ursolic acid (UA) is a pentacyclic triterpenoid compound that is naturally produced by kinds of plants, and it is often used as a Chinese herbal medicine.14 It is widely recognized for its various pharmacological functions, such as anti-tumor, anti-angiogenesis, hepatic protection, and lipid-lowering effects.15,16 It has been proved that UA can also exert functions in altering activities of immune cells. For example, UA can inhibit the cytokine production from Th1 cells to relieve adjuvant-induced arthritis.17 UA can also suppress the activation, proliferation and cytokine secretion of T cells, B cells and macrophages in the graft-versus-host disease.18 Yang et al., observed that UA can inhibit the proliferation of T lymphoma cells.19 In addition, the impacts of UA on Th17 cells have also been confirmed. Previous studies have demonstrated that UA can inhibit Th17 cell differentiation by regulating retinoic orphan receptor yt (RORyt), a nuclear receptor required for g CD4+ Th17 T cells.20–22 Nevertheless, the specific signaling pathways of UA inhibiting Th17 cell differentiation and the effect of key factors on Th17 cell migration have not been fully elucidated.

In the present study, we aimed to investigate the effects of UA on the differentiation and Schwann cells (SCs)-mediated migration of Th17 cells and the potential mechanism. Our results showed that UA inhibited Th17 cell differentiation by repressing STAT3/RORyt pathway. As well, our data showed that UA suppressed SCs-mediated Th17 cell migration via decreasing the expression of CXCL9 and CXCL10.

Materials and methods

Isolation of naïve Cd4+ T cells

The male rats (6-wk-old, 176–200 g mass, Beijing Vital River Laboratory Animal Technology Co. Ltd) were anesthetized by intraperitoneal injection of 1% pentobarbital sodium, then the abdominal cavity of the rats was cut open, and the main venous blood was extracted with vacuum blood collection needle. The naïve CD4+ T cells were isolated from the PBMCs using the magnetic bead cell sorting (MACS) method. Briefly, the PBMCs were used to select positive cells by the anti-CD4 magnetic beads on MS-positive selection columns (Milenyi Biotech, Germany) according to its manufacture instructions. Then the combined CD4+ T cells were stained with a cocktail of biotin-conjugated CD25 Ab (eBioscience, USA), CD44 Ab (eBioscience), DC69 Ab (eBioscience), and CD-45RO Ab (eBioscience). After that, cells were subjected to select negative cells, i.e., naïve CD4+ T cells, by the biotin coupled beads on magnetic columns (Milenyi Biotech). The naïve CD4+ T cells were stained with CD3, CD4, CD25, CD69, CD45RA, and CD45RO and then performed flow cytometry analysis. More than 96% of purified cells were naïve CD4+ T cells identified by flow cytometry. The isolated naïve CD4+ T cells were cultured in the RPMI medium containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 50 μm 2-ME and incubated at 37°C, 5% CO2.

Induction of Th17 cell differentiation

The isolated naïve CD4+ T cells were treated with anti-CD3 (0.5 μg/ml), anti-CD28 (1 μg/ml), anti-IFN-γ (2 μg/ml), and anti-IL-4 (2 μg/ml) Abs, recombinant TGF-β (2 ng/ml), and recombinant IL-6 (20 ng/ml) for Th17 differentiation induction for 72 h.

UA treatment of naïve Cd4+ T cells and Sc cells

UA with a purity of 97% was purchased from Nanjing Zeling Medical Technology Co. Ltd (Nanjing, China), and 228 mg UA was dissolved in 25 ml DMSO. The prepared 100 mM UA solution was then diluted with medium. The naïve CD4+ T cells were treated with UA at indicated concentrations (1 μM, 4 μM) for 72 h. Then the cells were collected for flow detection, quantitative real-time PCR and Western blot detection.

Flow cytometry

Flow cytometry assay was performed to assess the proportion of Th17 cells and Treg cells. In brief, induced naïve CD4+ T cells were first incubated with PE-conjugated CD4 Ab (12-0040-82, eBioscience). After being fixed and permeabilized, cells were incubated with the following Abs: FITC-conjugated Foxp3 Ab (0.2 mg/ml, 11-5773-82, eBioscience), FITC-conjugated IL-17A Ab (0.2 mg/ml, 11-7177-81, eBioscience), CCR6 PE-conjugated Ab (0.2 mg/ml, Cat. No. FAB8320P, R&D Systems), or CXCR3 PE-conjugated Ab (0.2 mg/ml, Cat. No. FAB8109P, R&D Systems). Then cells were sorted on a flow cytometer.

ELISA

The concentrations of IL-17A were measured by ELISA system kits (Cat. No. DY8410-05, R&D Systems) according to its manufacture instructions.

Total RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from induced naïve CD4+ T cells by Trizol reagent (Invitrogen, USA) according to the manufacture’s standard procedures. The reverse transcription of
total RNA for cDNA synthesis was performed with a cDNA Reverse Transcription Kit (Cat. No. 4368814, Applied Biosystems, USA). The qRT-PCR was conducted on an ABI 7900HT system (Applied Biosystems, USA) with the SYBR Green Master Mix (Applied Biosystems, USA). Primers were synthesized by Sangon Biotech (Shanghai, China). β-Actin served as internal control. The 2^−ΔΔCt method was used to evaluate the relative expression level. The primer sequences are listed in Table 1.

Western blot

Western blot was performed to analyze protein expression levels. The induced CD4⁺ T cells were treated with lysis buffer (Beyotime Biotechnology, China) containing protease inhibitor. Protein concentration was measured with a Bio-Rad protein assay system (Bio-Rad, USA), and then separated by SDS-PAGE with an electrophoresis system (Bio-Rad, USA). Proteins were transferred to the polyvinylidene difluoride (PVDF) membrane (Invitrogen, USA), and blocked in TBST containing 5% skimmed milk at room temperature for 1 h. The membrane was incubated with primary Abs (Abcam, UK) against RORγt (ab207082, 1/2000), p-STAT3 (ab32143, 1/1000), or β-actin (ab8226, 1 µg/ml) at 4°C overnight, and then the membrane was incubated with HRP-bounded Abs at room temperature for 2 h. The target proteins were visualized using ECL Plus Western Blotting Substrate (Thermo Scientific, USA). For protein level quantification, β-actin was used as the control.

### Table 1. Primer sequences for reverse transcription-quantitative PCR.

| Gene       | Primer sequence (5′→3′)          |
|------------|----------------------------------|
| RORγt      | F: ACAGAGAACCACCCGGAAACTC        |
|            | R: ATGCCAGATGACTTTGTCGCC        |
| STAT3      | F: AAGCCTCCGATGGAAACCTG         |
| TNF-α      | F: CCAACAAAGGAGAAGTCTCC         |
|            | R: CCTGTCTGGCTTTTGGTAC          |
| iNOS       | F: CTTTGTCAGCTACGCCCTC          |
|            | R: AAGGCCAATACCCGATACC          |
| IL-6       | F: GCAAGAGAATCCGGCCACTG         |
| CCL20      | F: GGTAATGCTGGTTTTACCT          |
|            | R: TCAGCCATCTGTTGTTGGA          |
| CXCL9      | F: ACCTCAGATTCCTGCCC           |
|            | R: CGGGAGTTGGGTTTTTGA          |
| CXCL10     | F: TGGCTGACTGCTAGGGACT         |
|            | R: GCAGCTGAGATGACTGCA          |
| β-Actin    | F: GCACCTAGCCCATCTCTTC         |
|            | R: AGCCCTAAGGAGCATCACC         |

F, forward; R, reverse.

### Cell transfection

The induced CD4⁺ T cells (2 × 10⁶ cells) were re-suspended in 100 µl of complete RPMI medium. In each transfection, 100 µl cell suspension was mixed with 1 µM lentivirus vector (Lenti-STAT3 or Lenti-NC), transferred into a cuvette, and transfected with a Nucleofector device using program X-001. Immediately after Nucleofection, cells were transferred into 500 µl of pre-warmed media.

### Scs isolation, culture, and treatment

SCs were isolated from rat sciatic nerves. Briefly, the bilateral sciatic nerves and brachial plexuses of rats were harvested and then washed with PBS and digested with 0.05% collagenase. The cell suspension was collected by repeatedly pipetting the digested mixture and cultured in DMEM-F12 supplemented with 10% FBS in an incubator containing 5% CO₂ at 37°C. Then fibroblasts and other types of cells were removed by the mitotic inhibitors, cytosine arabinoside and G-418 was used to remove fibroblast. The bFGF was used to promote the growth of SCs. After the cells were confluent, SCs were collected with TrypLE (ThermoFisher Scientific) for qRT-PCR and Western blot detection.

### Transwell assay

The induced CD4⁺ T cells in serum-free medium were put into the upper chamber of the Transwell inserts with pre-coated Matrigel matrix (BD Bioscience, USA), with anti-CXCR3 or anti-CCR6 added. UA-treated-SCs were cultured with the serum-free medium supplemented with LPS (100 ng/ml) and IFN-γ (100 U/ml) in the lower chamber. After 48 h of incubation at 37°C, cells in the lower chamber were sorted on a flow cytometer using FITC-conjugated IL-17A Ab, and FITC-conjugated Foxp3 Ab. The migration rate of Th17 cells (%) = the count of CD4⁺IL17⁺ T cells migrated to the lower chamber/ the count of CD4⁺ T seeded in the upper chamber. The migration rate of Treg cells (%) = the count of CD4⁺Foxp3⁺ T cells migrated to the lower chamber/ the count of CD4⁺ T seeded in the upper chamber.

### Statistical analysis

The SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Differences among different groups were tested by one-factor analysis of variance.
(ANOVA), followed by Least Significant Difference (LSD) test as a post-hoc test. Results were presented as mean ± SD and a level of $P < 0.05$ was considered significant.

**Results**

**UA inhibited Th17 cell differentiation and suppressed STAT3/RORγt pathway**

The naïve CD4$^+$ T cells were induced for Th17 differentiation and treated with UA at different concentrations (1 μM or 4 μM) to assess the impact of UA on Th17 cell differentiation. The results of flow cytometry assay showed that the proportion of Th17 cells was reduced by UA, and the higher dosage of UA exhibited stronger effects, suggesting that UA inhibited the Th17 cell differentiation (Figure 1A). Nevertheless, the percentage of Treg cells was elevated with UA treatment, and the higher dosage of UA, the bigger percentage of Treg cells (Figure 1B).

It has been proved that STAT3/RORγt pathway plays vital roles in differentiation of Th17 cells. Thus, we then investigated the effect of UA on the STAT3/RORγt pathway. The results showed that UA significantly decreased the mRNA expression of RORγt and the concentration of IL-17A (Figure 1C,D). UA also evidently down-regulated the protein levels of p-STAT3.

![Figure 1](image_url)

*Figure 1.* The effects of UA on Th17 cell differentiation and the STAT3/RORγt pathway. The naïve CD4$^+$ T cells were induced for Th17 differentiation and treated with UA at different concentrations (1 μM or 4 μM). (A) The proportion of Th17 cells was evaluated by flow cytometry assay. (B) The proportion of Treg cells was evaluated with flow cytometry assay. (C) The mRNA expression of RORγt was quantified with qRT-PCR. (D) The concentration of IL-17A in the cell supernatant was detected by ELISA assay. (E) The protein levels of p-STAT3 and RORγt were analyzed with Western blot. *$P < 0.05$, **$P < 0.01$. 
and RORγt (Figure 1E). Those findings showed that UA inhibits Th17 cell differentiation and represses STAT3/RORγt pathway.

**STAT3 overexpression reduced the inhibitory effect of UA on Th17 cell differentiation**

In view of the suppression of STAT3/RORγt pathway in Figure 1, we next examined whether STAT3/RORγt pathway is critical for anti-Th17 cell differentiation of UA. Lentivirus vector encoding STAT3 (Lenti-STAT3) was transfected into naïve CD4+ T cells before Th17 differentiation induction and UA treatment. This transfection up-regulated p-STAT3 and RORγt protein and mRNA expressions (Figure 2A,B), increased the percentage of Th17 cells, and decreased the percentage of Treg cells (Figure 2C,D). In addition, Lenti-STAT3 transfection increased the IL-17A concentration (Figure 2E). These results demonstrated that STAT3/RORγt pathway is important for UA inhibiting the Th17 cell differentiation.

**UA suppressed the expression of CXCL9 and CXCL10 in LPS and IFN-γ induced-SCs**

A previous study reported that SCs can mediate T cells migration through producing chemokines. Hence, we explored the impacts of UA on the expression of inflammatory factors (TNF-α, iNOS, and IL-6) and chemokines (CXCL9, CXCL10, and CCL20) in LPS and IFN-γ-stimulated SCs. As shown in Figure 3A–C, LPS and IFN-γ induced the expression of TNF-α, iNOS, and IL-6, while UA suppressed their expressions in a dose-dependent manner. Moreover, LPS and IFN-γ promoted the expression of CXCL9, CXCL10, and CCL20, while 4 µM UA down-regulated the expression of CXCL9 and CXCL10 but had no significant effect of CXCL20 expression.

![Figure 2](image)

**Figure 2.** The effects of STAT3 overexpression on Th17 cell differentiation and the STAT3/RORγt pathway. Naïve CD4+ T cells were transfected with Lenti-STAT3 or Lenti-NC and then induced for Th17 differentiation and treated with 4 µM UA. (A) The protein levels of p-STAT3 and RORγt were analyzed by Western blot. (B) The mRNA levels of STAT3 and RORγt were detected by qRT-PCR. (C) The percentage of Th17 cells was evaluated by flow cytometry assay. (D) The percentage of Treg cells was evaluated by flow cytometry assay. (E) IL-17A concentration was detected by ELISA assay. Lenti-NC: Negative control of Lenti-STAT3. *P < 0.05, **P < 0.01.
UA inhibited SCs-mediated Th17 cell migration by suppressing CXCL9/10 expression

As Th17 cells can functionally express chemokine receptors, such as CXCR3 and CCR6, we detected the expression of CXCR3 and CCR6 in Th17 cells. The flow cytometry results showed that the expression of CXCR3 (the receptor of CXCL9 and CXCL10) was abundant in Th17 cells, while CCR6 (the receptor of CCL20) expression was enriched in Treg cells (Figure 4A).

In consideration of the inhibitory effect of UA on the expression of CXCL9/10 and the abundant expression of CXCR3 in Th17 cells, we further investigated whether UA could affect SCs-mediated Th17 cell migration. Induced CD4+ T were co-cultured with SCs and then the ratio of migrated Th17 or Treg cells was detected by the Transwell assay. As shown in Figure 4B, 4 µM UA, anti-CXCR3, or anti-CCR6 treatment of LPS and IFN-γ-stimulated SCs resulted in decreases of migrated Th17 cells proportion. In addition, 4 µM UA, anti-CXCR3, or anti-CCR6 treatment of LPS and IFN-γ-stimulated SCs caused reductions of migrated Treg cells proportion (Figure 4C). These results suggested that UA inhibited SCs-mediated Th17 cell migration, which was associated with its inhibitory effect on the expression of CXCL9/10 in SCs.

Discussion

In this study, we demonstrated the effects of UA on the Th17 cell differentiation and SCs-mediated migration and elucidated the potential mechanisms. Our results revealed that UA inhibited the Th17 cell differentiation and suppressed the STAT3/RORγt pathway. Overexpression of STAT3 up-regulated the expressions of p-STAT3 and RORγt and promoted Th17 cell differentiation, indicating the significant role of STAT3/RORγt pathway in UA inhibiting Th17 cell differentiation. UA also suppressed the expression of CXCL9 and CXCL10 in LPS- and IFN-γ-stimulated SCs, which related to its inhibitory effects on SCs-mediated migration of Th17 cells.
The effects of UA on immune cells activities have been widely studied. As reported, UA can inhibit the proliferation, activation, and cytokine production and secretion of immune cells, such as T cells, B cells, macrophages or lymphocytes. In addition, studies also indicated that UA could influence Th17 cell differentiation. Baek et al. reported that UA blocked Th17 cell differentiation in autoimmune arthritis. Xu and his team demonstrated that UA...
inhibited the differentiation of Th17 cells in EAE.20 Consistent with results mentioned above, our present study also suggested that UA suppressed Th17 cell differentiation in a dose-dependent manner. In consideration of the important roles of Th17 cell migration in the pathological process of many immune-related diseases, we also investigated the effects of UA on Th17 cell migration in the current study. The results showed that UA inhibited the SCs-mediated Th17 cell migration. Taken together, the present results demonstrated the inhibitory effects of UA on the differentiation and SCs-mediated migration of Th17 cells. In addition, previous studies used T cells derived from humans or mice for experiments, while T cells derived from rats were used in this study. The results of T cells from the three sources were consistent, indicating that UA is widely applied across species.

STAT3 phosphorylation activation is a crucial step progress in Th17 cell differentiation.26 Phosphorylated STAT3 (p-STAT3) form a dimer and enter into nucleus to regulate the expression of relative genes such as RORγt, and thereby controlling Th17 cell differentiation.27–29 The inhibition of Th17 cell differentiation was closely associated with the decreased number of RORγt-expressing CD4+ cells and down-regulated p-STAT3,30 indicating the key regulation role of STAT3/RORγt pathway in Th17 cell differentiation. A previous study showed that UA can selectively hinder the function of RORγt in EAE.30 Another study revealed that UA suppresses the phosphorylation of STAT3 and down-regulates RORγt expression in autoimmune arthritis.21 In this study, we found that UA suppressed the STAT3/RORγt pathway in a dose-dependent manner, implying that the STAT3/RORγt pathway might be involved in UA inhibiting Th17 cell differentiation. To verify this speculation, we preformed STAT3 overexpression experiments. Our results indicated overexpression of STAT3 up-regulated p-STAT3 and RORγt expression as well as facilitated Th17 cell differentiation under UA treatment. Therefore, our findings indicated that UA inhibited Th17 cell differentiation by suppressing STAT3/RORγt pathway.

SCs are a type of glia cells in the peripheral nervous system.31 Such cells derive from myelin and Remak cells and exert important neuroprotection function and nerve regeneration.25 Under the stimulation of high Glc, inflammatory cytokines, and other adverse factors, SCs produce chemokines and then chemokines recognize and bind to their receptors on T cells, and thus mediating the T cell migration.25 In the present study, we found that LPS and IFN-γ stimulation induced the expression of chemokines CXCL9/10 and CCR20 in SCs, and UA treatment inhibited CXCL9/10 expression but not CCL20. Previous studies reported that Th17 cells can functionally express trafficking receptors CXCR3 and CCR6, which can be specifically recognized by CXCL9/10 and CCL20, respectively.7,33,34 In the current study, we found that the expression of CXCR3 was abundant in Th17 cells. Anti-CXCR3 and anti-CCR6 both suppressed SCs-mediated Th17 cell migration, and anti-CXCR3 showed stronger inhibitory effect. Therefore, our results demonstrated that SCs mainly mediate Th17 cell migration through CXCL9/10, and UA inhibited SCs-mediated Th17 cell migration by suppressing CXCL9/10 expression.

In conclusion, we further explored the mechanism of UA inhibiting Th17 differentiation and suggested that UA inhibited Th17 cell differentiation through the STAT3/RORγt pathway and suppressed the SCs-mediated Th17 cell migration by repressing the expression of CXCL9/10. These findings might reveal another mechanism by which UA plays a role and provided new insight for the therapy of Th17 cell-related immune diseases.

Ethics approval and consent to participate
All animal experimental procedures were in accordance with the Animals Ethics Guidelines of Shandong First Medical University (2015019). The study was carried out in compliance with the ARRIVE guidelines.

Consent for publication
The study was undertaken with the patient’s consent.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental material
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