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Original Paper

Intervention of Tyrosine Hydroxylase Expression Alters Joint Inflammation and Th17/Treg Imbalance in Collagen-Induced Arthritis

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Key Words
Tyrosine hydroxylase • Epinephrine • Collagen-induced arthritis • Th17 cells • Treg cells

Abstract
Background/Aims: Neuroendocrine dysregulation has been associated with rheumatoid arthritis (RA). Tyrosine hydroxylase (TH), a rate-limiting enzyme for synthesis of neuroendocrine hormones such as epinephrine, is also expressed in T lymphocytes and regulates balance between helper T (Th) 17 cells and regulatory T (Treg) cells. Herein, we aimed to show that TH expression in joints alleviates joint inflammation and Th17/Treg imbalance in collagen-induced arthritis (CIA), an animal model of RA, and these effects may be implemented by the mechanism of epinephrine action on α1-adrenoreceptor (α1-AR) in T cells. ***Methods:*** CIA was prepared by intradermal injection of collagen type II in tail base of DBA1/J mice. On the 33rd day post-immunization, lentiviral vectors encoding TH or TH shRNA were injected into ankle joints of CIA mice. Limb inflammation of the mice was assessed beginning from day 21 until day 69 post-immunization by measurement of limb swelling, erythema and rigidity. Th17 and Treg differentiation and function in ankle joints were assessed on day 69 post-immunization by test of the expression of Th17 transcriptional factor ROR-γt and the levels of proinflammatory cytokines interleukin (IL)-17 and IL-22 as well as the expression of Treg transcriptional factor Foxp3 and the levels of antiinflammatory cytokines transforming growth factor (TGF)-β1 and IL-10. T cells were obtained from the spleen of mice that had been immunized with collagen type II 41 day earlier and treated with epinephrine or α1-AR agonist phenylephrine *in vitro*. Flow cytometry was used to analyze the percentages of $CD25^{-}IL\text{-}17^{+}$ cells and $CD25^{+}Foxp3^{+}$ cells in $CD4^{+}$ T cells. ***Results:*** TH gene overexpression in ankle joints of CIA mice reduced limb inflammation and Th17-related transcription factor expression and inflammatory cytokine

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production but increased Treg-related antiinflammatory cytokine production in the joints. In contrast, TH gene silence in ankle joints of CIA mice enhanced limb inflammation and Th17 cell activity but decreased Treg cell function in the joints. Epinephrine upregulated α1-AR expression in T cells derived from CIA mice. Both epinephrine and phenylephrine reduced CIA-induced Th17 transcription factor expression and inflammatory cytokine production but enhanced Treg antiinflammatory cytokine production *in vitro*. ***Conclusion:*** Upregulating TH expression in joints alleviates joint inflammation and Th17/Treg imbalance in CIA at least partially by enhancing epinephrine action on α1-AR in T cells.

Introduction

Rheumatoid arthritis (RA) is characterized by joint inflammation, pannus formation, and immune cell invasion [1-4]. Although the clear mechanisms of RA pathogenesis still remain to be defined, it is suggested that $CD4^+$ T lymphocytes are critically implicated in perpetuation of RA [5, 6]. $CD4^+$ T cells can differentiate towards different subsets, including T helper (Th) 1, Th2, T regulatory (Treg) and Th17 cells. Previous studies showed that imbalance of $CD4^+$ T cell subtypes partaked in progression/severity of RA [7, 8]. It is considered that Th1/Th2 imbalance plays a critical role in development of RA [9-11]. Th17 cells, a recently discovered subset of Th cells, are characterized by a proinflammatory population producing proinflammatory cytokines such as interleukin (IL)-17 and IL-22 [12, 13]. Treg cells can suppress most immune activities by expressing antiinflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β [14, 15]. It is reported that Th17/Treg imbalance emphasized the crucial roles in controlling RA [16, 17]. Th17 cell level elevation and Treg function defection have been observed in patients with RA [18, 19]. Collagen type II (C II)-induced arthritis (CIA) has the pathological and histological changes similar to human RA [20], thereby we employed CIA as an animal model of RA in this study.

Epinephrine, one kind of catecholamines, has been well known to regulate cardiovascular, respiratory and digestive activities. The previous studies have reported that catecholamines regulated immune function by suppressing T-cell-mediated cellular immunity or by direct contact with lymphocytes [21-23]. Catecholamines synthesized and secreted by lymphocytes contribute to a shift of balance in Th1/Th2 toward Th2 response [24-26]. This shift may be advantageous to ameliorate proinflammatory/antiinflammatory imbalance in some autoimmune diseases. Tyrosine hydroxylase (TH) is a rate-limiting enzyme of catecholamine synthesis. Therefore, TH expression in cells represents an ability to synthesize catecholamines. It has been reported that in inflamed spleens and joints from CIA, sympathetic nerve fibers are remarkably reduced while TH-immunoreactive cells are significantly increased [27-29]. This suggests that in inflamed lymphoid tissues and joints of CIA, the lost sympathetic nerve fibers are replaced by the TH^+ catecholaminergic cells. A few studies suggest that the locally lost sympathetic nerve fibers are replaced by catecholamines, which are helpful for joint inflammatory alleviation of arthritis [11, 30]. Another kind of catecholamines, norepinephrine, has been shown to be involved in RA and CIA. A few studies show that norepinephrine has an antiinflammatory effect when its concentration is high [31, 32]. Norepinephrine inhibits Th17 cell differentiation and function during CIA [33]. Clarifying the effects and mechanisms of epinephrine on Th17/Treg balance in this study is important for us to better understand the roles of catecholamines in RA.

Epinephrine acts on target cells by binding to the receptors, α-adrenoreceptor (AR) or β-AR. Lymphocytes express α- and β-ARs [34-37]. Previous work has found that T cell function and apoptosis were suppressed by catecholamines released from lymphocytes via α1-AR [38, 39]. We previously showed that norepinephrine-induced shift of balance in Th1/Th2 towards Th2 polarization is mediated by α1-AR [26]. However, it is not known whether the action of epinephrine on Th17/Treg balance is mediated by α1-AR during CIA. Thus, we clarified the effects of TH (consequent catecholamine synthesis) on Th17/Treg balance in CIA, and then we assessed effects of epinephrine on α1-AR expression and Th17/Treg balance

in T cells in CIA. Lastly, we showed that α1-AR mediated the epinephrine effects on Th17/Treg balance in CIA condition. This investigation helps to elucidate immunomodulation by catecholamines and to provide information for RA therapy.

Materials and Methods

Mice

Male DBA1/J mice, 8-10 weeks old, were purchased from Center of Experimental Animals (Nantong University, China). Mice were housed in polystryrene cages and bred with food and water ad libitum under standard conditions with a 12-hour light/dark cycle. 80 animals were used in the study.

Statement of Ethics

All animal procedures were performed according to the National Institutes of Health (USA) guidelines and with the approval of the Animal Care and Ethics Committee of Nantong University.

Construction of lentiviral vectors expressing TH or TH-shRNA

The short hairpin RNA (shRNA) sequence targeting mouse TH (Gene Bank Accession NM 009377.1) was 5'-GCACACAGTACATCCGTCA-3'. We used a negative control for TH (TH gene overexpression) or TH-shRNA (TH gene knockdown) with just lentiviral vector or lentiviral vector expressing scrambled-shRNA (Scr-shRNA; 5'-TTCTCCGAACGTGTCACGT-3'). Genechem Co. Ltd. (Shanghai, China) generated all of the lentiviral vectors.

Induction of CIA

Mice were immunized with 100 μl of CII (1 mg/ml, Sigma-Aldrich Co., USA) on day 0. On day 21, mice were boosted with an equal volume of CII. On day 28, 20 μl LPS (1 mg/ml) injection was executed to the mice. 2×10^5 transducing units (TU) of TH lentiviral vector or TH-shRNA lentiviral vector was injected into the ankle joints of mice with CIA on day 33 post-immunization. 2×10^5 TU of lentiviral vector or Scr-shRNA lentiviral vector injection into the ankle joints was as a negative control. All observations listed below were measured until day 36 after the TH lentiviral vector or TH-shRNA lentiviral vector injection. The mice were observed for clinical score by two independently blinded examiners every 2 days from day 21 post-immunization. The clinical score was evaluated using the following scale: grade 0 = no swelling; grade 1 = slight swelling and erythema; grade 2 = pronounced swelling; and grade 3 = joint rigidity. A representative arthritis score was determined by summing the scores of all four paws. Rear paw and ankle joint thickness was measured by microcalliper before mice were killed on the 69th day after the first immunization. In a separate *in vitro* experiment, CIA mice without TH lentiviral vector or TH-shRNA lentiviral vector injection were sacrificed on day 41 post-immunization according to the previous report [40].

Purification of T cells

By using CD3 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), T cells were obtained from the mouse spleen tissues. T cells after purification were stimulated with anti-CD3 antibody (2 μg/ml, BD Pharmingen, USA) and anti-CD28 antibody (2 μg/ml, BD Pharmingen, USA) in the medium RPMI 1640 containing 10% heat-inactivated calf serum at a density of 2×10^6 cells/ml, which were cultured for 48 h at 37°C in a humidified atmosphere of 5% CO_2. Subsequently, the stimulated T cells were exposed to various treatments.

Drug treatments

The activated T cells were incubated with epinephrine (10^{-6} or 10^{-5} M, Sigma-Aldrich, USA) for 24 h. These activated T cells were also treated with a highly selective α1-AR agonist phenylephrine (10^{-6} or 10^{-5} M, Sigma-Aldrich, USA) for 72 h, or treated combined with the PLC inhibitor U73122 (10^{-5} M, Sigma-Aldrich, USA) 30 min earlier and the α1-AR agonist phenylephrine (10^{-5} M) for 72 h. Subsequent analyses as described below were performed.

Enzyme-linked immunosorbent assay (ELISA)

According to the manufacturer's protocol, anti-CII IgG antibody level in sera and cytokine (IL-17, IL-22, TGF-β1 and IL-10) level in tissue supernatants or cell cultures were analyzed by ELISA kits (eBioscience, USA).

Real-time polymerase chain reaction (PCR) analysis

RNAs were extracted with Trizol reagent (Invitrogen, USA) from *in vitro* cultured T cells or ankle joints from mice with CIA. RNA was used for reverse transcription and cDNA was obtained by reverse transcription kit (Roche, Germany). Real-time PCR was performed on the Real-Time system (Corbett Research, Australia). Primers were as follows: IL-22, 5'-GGCCAGCCTTGCAGATAACA', and 5'-GCTGATGTGACAGGAGCTGA' (NM_016971.2); IL-17, 5'-GCTCCAGAAGGCCCTCAGA', and 5'-AGCTTTCCCTCCGCATTGA-3' (NM_010552.3); IL-10, 5'-GGACAACATACTGCTAACCGAC-3', and 5'-TGGATCATTTCCGATAAGGCTTG-3' (NM_010548.2); TGF-β, 5'-GATACGCCTGAGTGGCTGTC-3', and 5'-GCTGATCCCGTTGATTTCC-3' (NM_011577.1); β-actin, 5'-CTGTCCCTGTATGCCTCTG-3', and 5'-ATGTCACGCACGATTTCC-3' (NM_007393.5). The $2^{-\Delta\Delta Ct}$ method was used to calculate each gene expression [41].

Western blot analysis

In vitro cultured T cells were harvested to extract total protein. Ankle joints were removed and pulverized. The pulverized tissue was re-suspended in lysis buffer, which was centrifuged at 4˚C at 12,000×g for 15 min. The supernatant was mixed with loading buffer, which was boiled for 10 min. The protein was separated by polyacrylamide gel and transferred onto a polyvinylidene diflfluoride membrane. The membranes were sealed with 5% BSA for 1 hour and incubated overnight at 4˚C with primary antibodies to retinoid acid receptor-related orphan receptor γt (ROR-γt) (1:500, Abcam, UK), Foxp3 (1:200, Santa Cruz Biotechnology, USA), TH (1:500, Millipore, USA), α1-AR (1:500, Abcam, UK) or β-actin (1:5,000, Sigma, USA). Membranes were incubated with secondary antibodies for 1 h at room temperature, and then analysis of the protein bands was detected by Odyssey laser scanning system (LI-COR Inc, USA).

Flow cytometric assay

Activated T cells were incubated with 2 μM monensin, 1 μM ionomycin and 50 ng/ml PMA for 4 hours. Cells were fixed and permeabilized by the Fixation/Permeabilization kit (BD Biosciences, USA) and then stained with phycoerythrin (PE)-conjugated anti-IL-17 antibodies (Clone: TC11-18H10, BD PharMingen, USA) or PE-conjugated anti-Foxp3 antibodies (Clone: R16-715, BD PharMingen, USA) at 4˚C for 30 min, followed by surface staining with peridinin chlorophyll protein-Cyanine 5.5 (PerCP-Cy5.5)-conjugated anti-CD4 antibodies (Clone: RM4-5, BD PharMingen, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-CD25 antibodie (Clone: 7D4, BD PharMingen, USA) or the appropriate isotype control antibodies. Cells were analyzed with FACS Calibur (BD Biosciences, USA).

Statistical analysis

Non-parametric test was used to analyze the data of arthritis score. The other data was assessed with one-way analysis of variance (ANOVA). *P*-values less than 0.05 were considered significant.

Results

Effects of TH gene overexpression or knockdown in ankle joints on limb inflammation of CIA mice

In ankle joints of mice with CIA, expression of TH was upregulated (Fig. 1A). TH gene overexpression in ankle joints from mice with CIA further enhanced this TH upregulation, while TH gene silence in ankle joints reduced the CIA-induced effect. In ankle joints of mice with CIA, either treatment with TH-mock or Scr-shRNA did not affect TH expression (Fig. 1A).

On day 31 post-immunization, clinical score of CIA mice began to rise with respect to that of intact mice (Fig. 1B). TH gene overexpression in ankle joints of mice with CIA significantly decreased clinical arthritis score at observed time points from the 51 th to

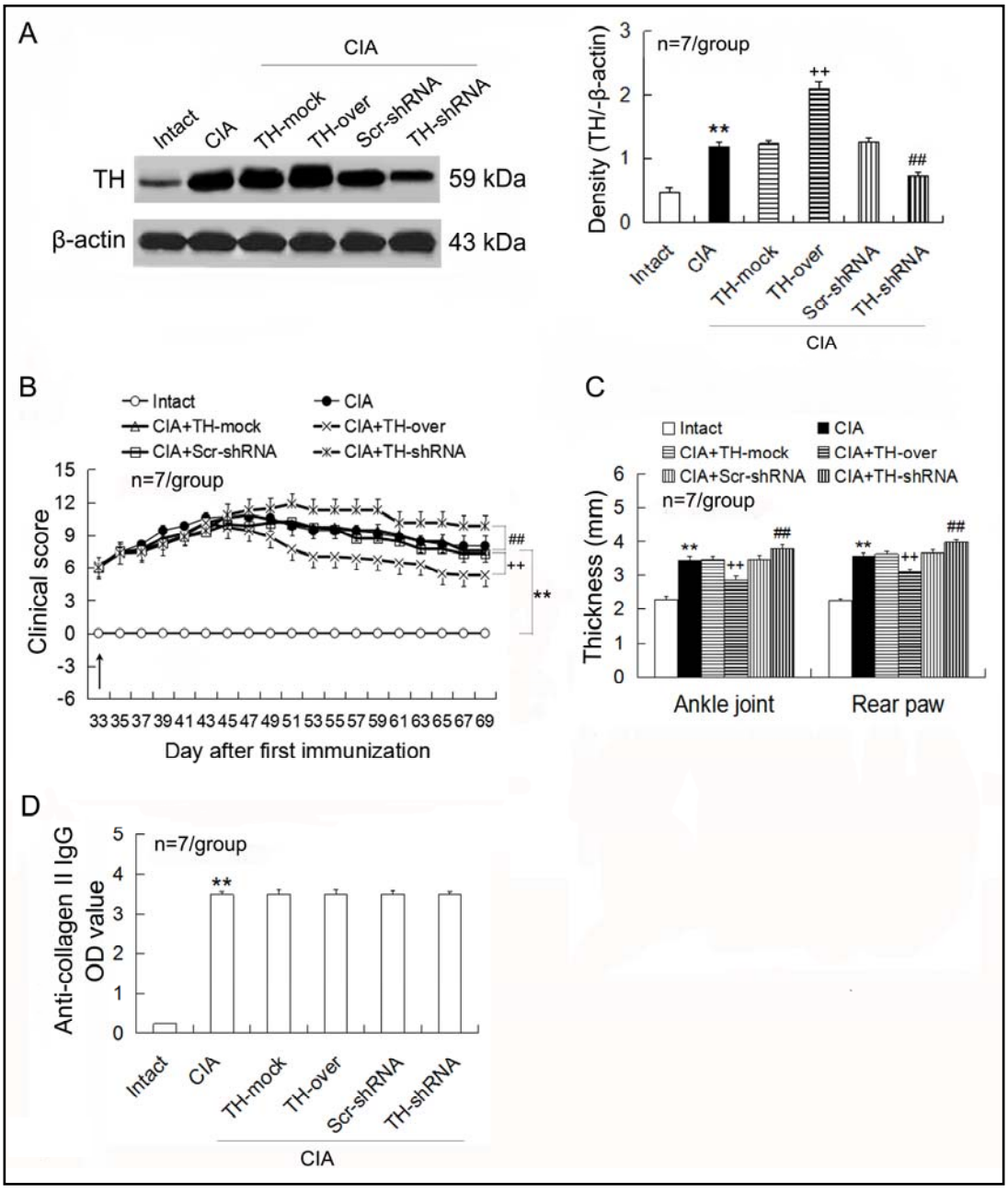

Fig. 1. Influences of TH gene overexpression or silence in ankle joints on limb inflammation in mice with CIA. 2×10^5 TU of TH lentiviral vector or TH-shRNA lentiviral vector was injected into ankle joints of CIA mice on day 33 post-immunization. Expression of TH protein in ankle joints was examined by Western blot (A). The clinical arthritis score was observed beginning from day 21 post-immunization (B). TH gene overexpression in ankle joints of mice with CIA significantly suppressed clinical arthritis score at observed time points beginning from day 51 until day 69 post immunization compared with TH-mock control. TH gene silence in ankle joints further enhanced these effects induced by CIA compared with Scr-shRNA control. Ankle joint and rear paw thickness was measured on day 69 post-immunization (C). Level of anti-CII IgG antibody was upregulated in mice with CIA, but this upregulation was not influenced by TH gene overexpression or silence in ankle joints (D). $^{**}p<0.01$, versus intact control; $^{++}p<0.01$, compared with TH mock control; $^{\#\#}p<0.01$, compared with Scr-shRNA control.

the 69 th day followed immunization compared with TH-mock control. TH gene silence in ankle joints further increased this upregulation of clinical score compared with Scr-shRNA control (Fig. 1B). Ankle joint width and rear paw thickness in CIA mice were significantly increased compared with that in intact animals (Fig. 1C). TH gene overexpression in ankle joints remarkably decreased the both ankle joint width and rear paw thickness of CIA mice. TH gene silence in ankle joints further increased these effects (Fig. 1C). Level of anti-CII IgG antibody in serum was significantly increased on day 69 post-immunization in comparison with that in intact control (Fig. 1D). Treatment with TH-overexpression or TH-shRNA did not affect the elevated level of anti-CII IgG in serum of mice with CIA (Fig. 1D).

Influences of TH gene overexpression or silence in ankle joints of mice with CIA on function of Th17 and Treg cells

CIA animals displayed a remarkable regulation of ROR-γt, IL-17 and IL-22 expression and content in ankle joints (Fig. 2A, 2B, 2C). These CIA-induced effects were reduced by TH gene overexpression and further enhanced by TH gene silence in ankle joints (Fig. 2A, 2B, 2C).

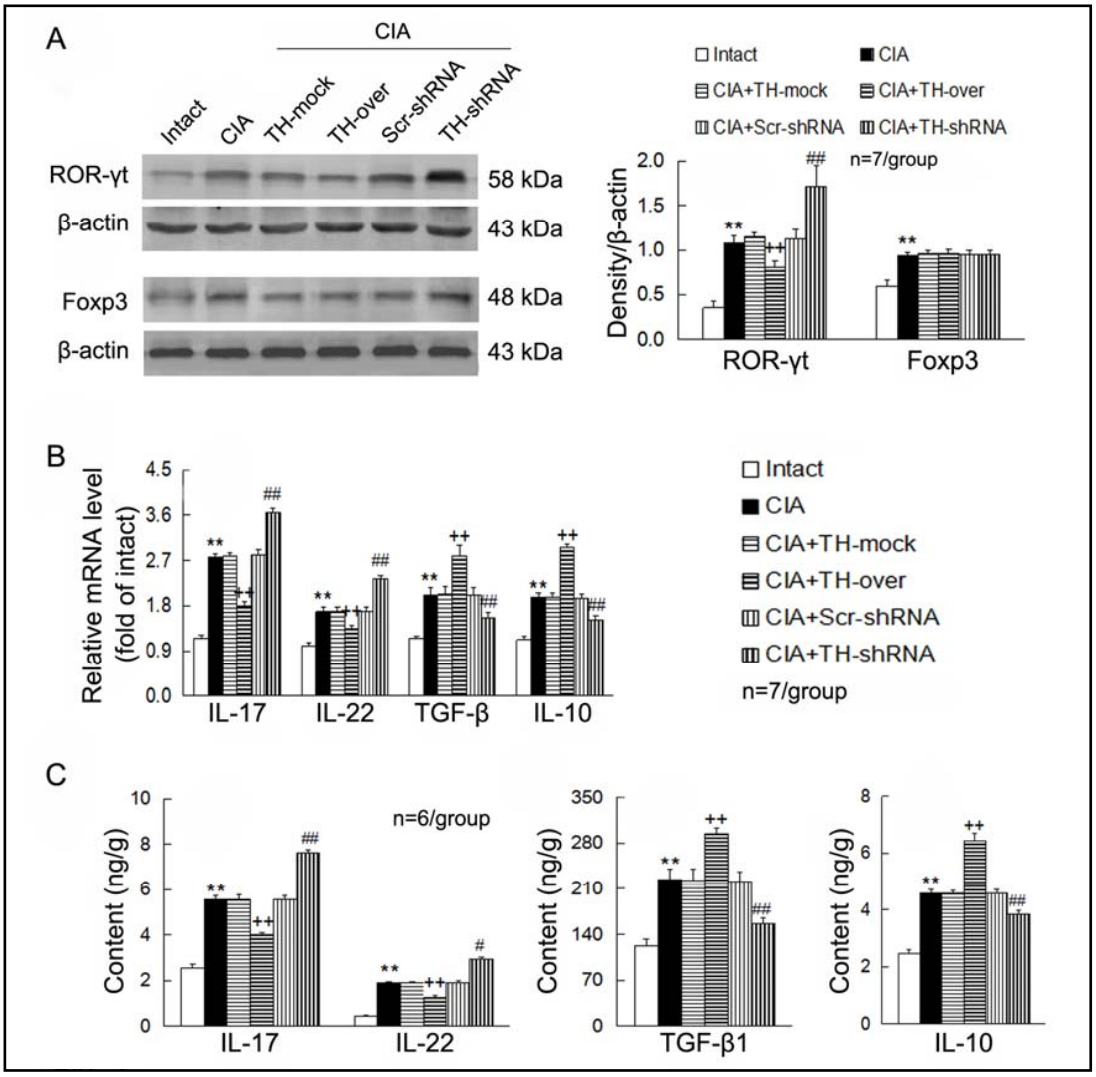

Fig. 2. Effects of TH gene overexpression or silence in ankle joints of mice with CIA on Th17 and Treg cell function. 2×10^5 TU of TH lentiviral vector or TH-shRNA lentiviral vector was injected into ankle joints of CIA mice on day 33 post-immunization. Levels of ROR-γt and Foxp3 protein expression (A) and IL-17, IL-22, IL-10 and TGF-β gene expression (B) as well as contents of IL-17, IL-22, IL-10 and TGF-β1 (C) in ankle joints were measured on day 69 post-immunization. $^{**}p<0.01$, versus intact control; $^{++}p<0.01$, compared with TH mock control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared with Scr-shRNA control.

Furthermore, CIA induced Foxp3, TGF-β and IL-10 expression and content in ankle joints (Fig. 2A, 2B, 2C). Foxp3 expression had no changes in treatment with TH-overexpression or TH-shRNA in ankle joints of mice with CIA. TGF-β and IL-10 expression and content were increased by TH gene overexpression in ankle joints of mice with CIA and these effects were decreased by TH gene silence (Fig. 2A, 2B, 2C).

Influences of epinephrine in T cells of mice with CIA on differentiation and function of Th17 and Treg cells

Percentage of $CD25^{-}IL\text{-}17^{+}$ cells in $CD4^{+}$ T cells was increased in CIA mice compared with that of intact mice (Fig. 3A). After treatment of T cells, obtained from CIA mice, with epinephrine (10^{-6} or 10^{-5} M), percentage of $CD25^{-}IL\text{-}17^{+}$ cells in $CD4^{+}$ T cells was decreased (Fig. 3A). In contrast, epinephrine treatment of T cells from CIA mice increased percentage of $CD25^{+}Foxp3^{+}$ cells in $CD4^{+}$ T cells (Fig. 3A), although CIA did not alter the percentage of $CD25^{+}Foxp3^{+}$ cells in comparison with intact mice (Fig. 3A).

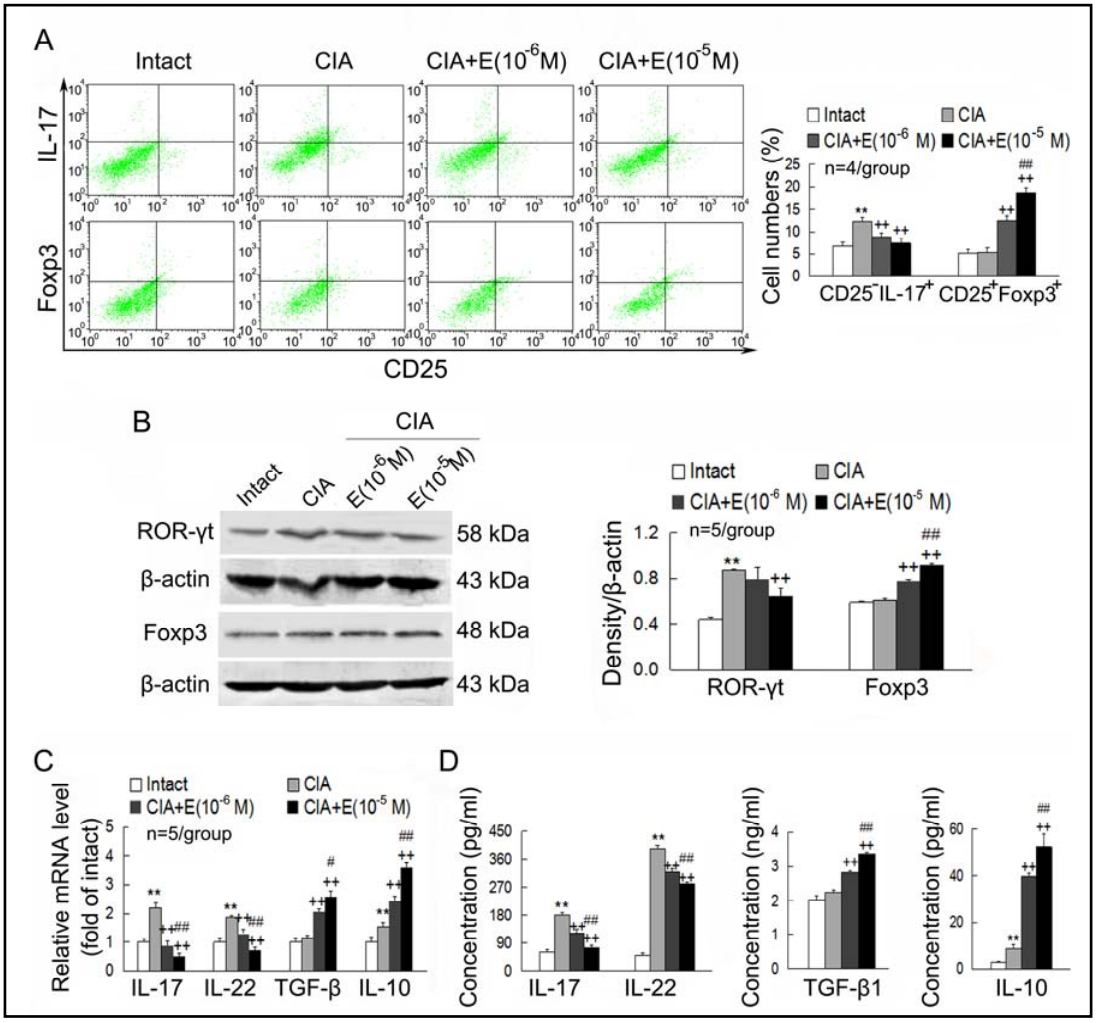

Fig. 3. Effects of epinephrine in T cells of mice with CIA on Th17 and Treg cell differentiation and function. The activated T cells were incubated with epinephrine for 24 h and labeled simultaneously with anti-CD4, anti-CD25 and anti-IL-17 or anti-Foxp3 fluorescence antibodies. The $CD4^{+}$ cells were firstly selected, and then percentage of Th17 ($CD25^{-}IL\text{-}17^{+}$) and Treg ($CD25^{+}Foxp3^{+}$) cells in $CD4^{+}$ T cells was analyzed (A). Levels of ROR-γt and Foxp3 protein expression (B) and IL-17, IL-22, TGF-β and IL-10 mRNA expression (C) in T cells as well as IL-17, IL-22, TGF-β1 and IL-10 level in the supernatants of T cell cultures (D) were assessed. E=epinephrine; $^{**}p<0.01$, versus intact control; $^{++}p<0.01$, versus CIA group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, versus CIA + E (10^{-6} M) group.

ROR-γt, IL-17 and IL-22 expression and secretion were increased in T cells from CIA mice (Fig. 3B, 3C, 3D). With respect to nothing-treated T cells of CIA mice, these effects were reduced by epinephrine (10^{-6} or 10^{-5} M) treatment in T cells of mice with CIA. In contrast, CIA did not affect Foxp3 and TGF-β expression (Fig. 3B, 3C, 3D). In addition, IL-10 expression and secretion were upregulated in T cells of mice with CIA (Fig. 3C, 3D). Nevertheless, with respect to nothing-treated T cells of CIA mice, epinephrine (10^{-6} or 10^{-5} M) treatment in T cells of mice with CIA upregulated Foxp3, TGF-β and IL-10 expression and secretion (Fig. 3B, 3C, 3D).

Epinephrine upregulates expression of α1-AR in T cells from mice with CIA

With respect to that of intact mice, α1-AR expression was downregulated in T cells of mice with CIA. Epinephrine (10^{-6} or 10^{-5} M) treatment inhibited the CIA-induced downregulation of expression of α1-AR in T cells from mice with CIA (Fig. 4).

α1-AR agonist suppresses Th17 cell differentiation and function and enhances Treg cell differentiation and function in T cells from mice with CIA and the agonist effects are blocked by PLC inhibitor

To further demonstrate the role of α1-AR expressed on T cells in CIA-induced Th17 and Treg cell differentiation and function, we applied the α1-AR agonist phenylephrine to the activated T cells derived from CIA mice. Percentage of $CD25^{-}IL\text{-}17^{+}$ cells in $CD4^{+}$ T cells was increased in CIA mice compared with that of intact animals. ROR-γt, IL-17 and IL-22 expression and secretion were increased in T cells of CIA mice compared with that of intact animals (Fig. 5A, 5B, 5C, 5D). α1-AR agonist phenylephrine (10^{-6} or 10^{-5} M) treatment of T cells from CIA mice decreased the percentage of $CD25^{-}IL\text{-}17^{+}$ cells and ROR-γt, IL-17 and IL-22 expression and secretion (Fig. 5A, 5B, 5C, 5D). Notably, the PLC inhibitor U73122 (10^{-5} M) blocked the effect of phenylephrine (10^{-5} M) inhibiting CIA-induced Th17 cell differentiation and function. These data demonstrated that activation of α1-AR impaired CIA-induced Th17 cell differentiation and function via PLC signaling.

In contrast, percentage of $CD25^{+}Foxp3^{+}$ cells in $CD4^{+}$ T cells was not influenced in CIA mice compared with that of intact mice. Foxp3 and TGF-β expression as well as TGF-β1 secretion had no changes in T cells of CIA mice relative to that of intact mice (Fig. 5A, 5B, 5C, 5D). In addition, IL-10 expression and secretion were increased in T cells of CIA mice (Fig. 5C, 5D). α1-AR agonist phenylephrine (10^{-5} M) treatment of T cells from CIA mice increased

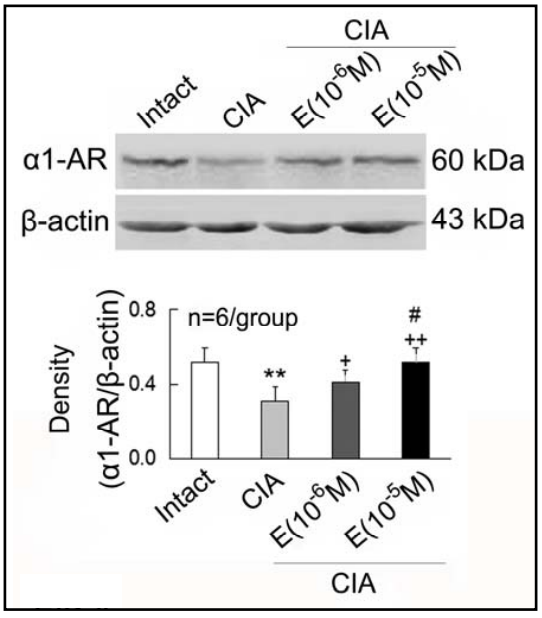

Fig. 4. Epinephrine upregulates α1-AR expression in T cells of mice with CIA. The activated T cells were incubated with epinephrine for 24 h. Protein expression of α1-AR in T cells was tested by Western blot analysis. E=epinephrine; $^{**}p<0.01$, versus intact control; $^{+}p<0.05$, $^{++}p<0.01$, versus CIA group; $^{\#}p<0.05$, versus CIA + E (10^{-6} M) group.

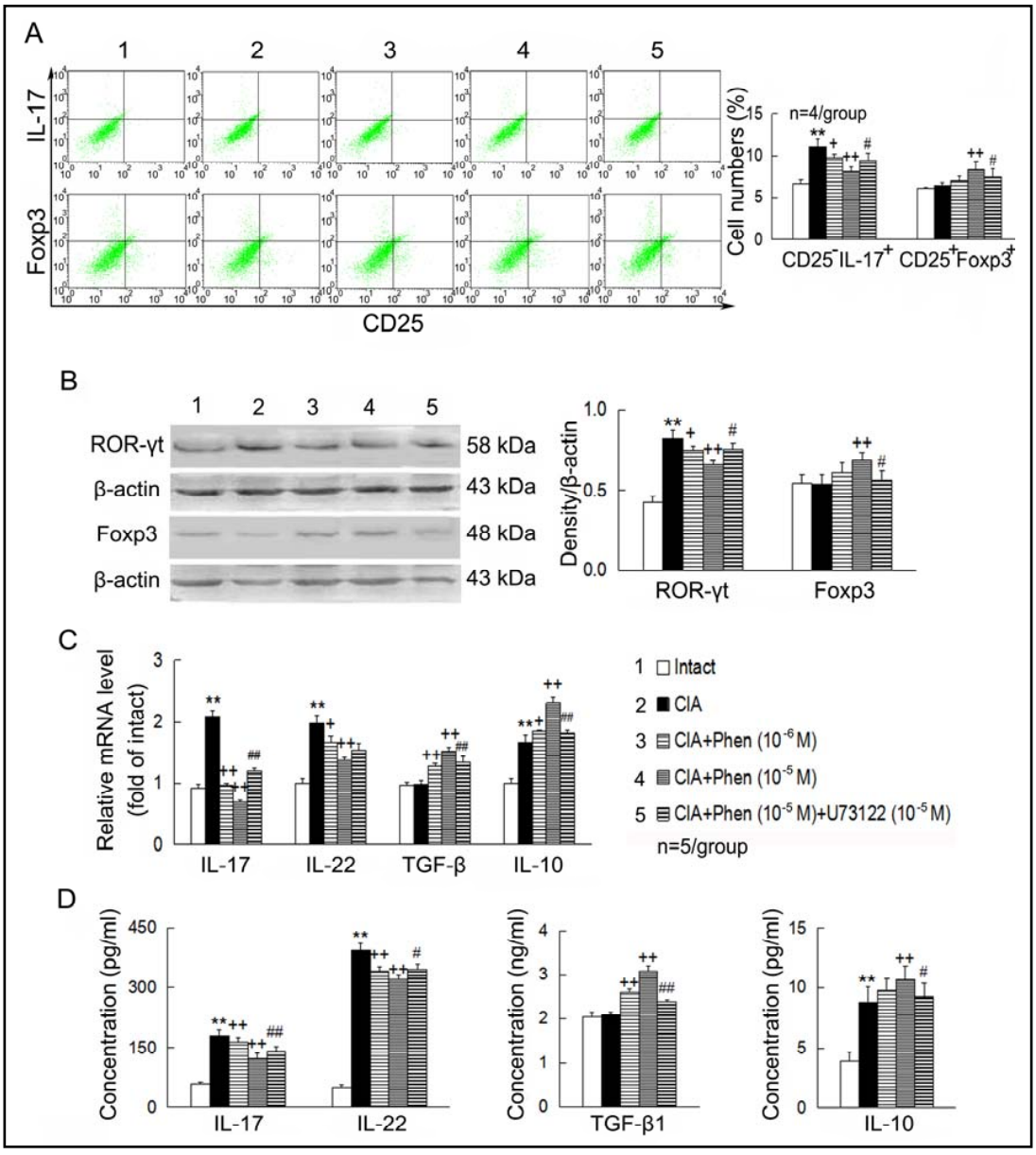

Fig. 5. α1-AR agonist suppresses Th17 cell and enhances Treg cell differentiation and function in T cells of mice with CIA and these agonist effects are abolished by PLC inhibitor. The activated T cells in the presence or absence of phenylephrine plus U73122 for 72 h were labeled with anti-CD4, anti-CD25 and anti-IL-17 or anti-Foxp3 fluorescence antibodies. The $CD4^+$ cells were firstly selected, and then percentage of Th17 ($CD25^-$ $IL\text{-}17^+$) and Treg ($CD25^+Foxp3^+$) cells in $CD4^+$ T cells was analyzed (A). Levels of ROR-γt and Foxp3 protein expression (B) and IL-17, IL-22, TGF-β and IL-10 mRNA expression (C) in T cells were tested. (D) IL-17, IL-22, TGF-β1 and IL-10 level in the supernatants of T cell cultures was determined. Phen=phenylephrine; $^{**}p<0.01$, versus intact control; $^{+}p<0.05$, $^{++}p<0.01$, versus CIA group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, versus CIA + Phen (10^{-5} M) group.

the percentage of $CD25^+Foxp3^+$ cells and Foxp3, TGF-β and IL-10 expression and secretion (Fig. 5A, 5B, 5C, 5D). The PLC inhibitor U73122 (10^{-5} M) inhibited the effect of phenylephrine (10^{-5} M) elevating Treg cell differentiation and function of CIA mice (Fig. 5A, 5B, 5C, 5D). These data demonstrated that activation of α1-AR enhanced CIA-induced Treg cell differentiation and function via PLC signaling.

Discussion

Catecholamine production in T cells is reduced by inhibition of TH, which is a rate-limited enzyme for catecholamine synthesis [24, 25, 42]. Thus, expression of TH in cells represents catecholamine synthesis ability. The present results showing the upregulation of TH expression in ankle joints from CIA mice suggest that production of catecholamines in the inflamed tissues was induced by CII injection. The findings that there is a higher TH-positive cell density in inflamed lymph tissues of CIA mice and a display of TH^+ cells only in inflamed synovial tissue of RA patients support our present results [29, 30]. In the present study, injections of TH lentiviral vector or TH-shRNA lentiviral vector into ankle joints of mice with CIA upregulated or downregulated TH protein level in ankle joints, respectively. TH gene overexpression in ankle joints inhibited CIA-induced clinical score and ankle joint and rear paw thickness, while TH gene silence enhanced these CIA-induced effects. These results show that expression of TH in ankle joints alleviates arthritic symptoms in mice with CIA. Furthermore, TH-overexpression or TH-shRNA treatment did not affect the elevated level of anti-CII IgG in serum of mice with CIA. The result suggests that local treatment with TH significantly alleviated inflammation without causing systemic effects in CIA mice.

Th17/Treg imbalance is observed in the development of RA [17, 18, 43]. It was found that TH gene overexpression in ankle joints inhibited differentiation and function of Th17 cells in CIA and TH gene silence enhanced these effects in this study. These changes suggest that TH expression in ankle joints suppresses the activity of Th17 cells. In addition, TH gene overexpression in ankle joints enhanced CIA-induced Treg cell-related cytokines, TGF-β and IL-10 production, while TH gene silence reduced these effects in mice with CIA. These results revealed that TH expression in ankle joints increased Treg cell activity in CIA mice. Collectively, TH expression in ankle joints alleviates Th17/Treg imbalance induced by CIA in the joints. Our previous work showed that changing TH activity in T cells with drugs altered catecholamine level accordingly [25, 42]. Thus, the TH expression in ankle joints in this study may promote catecholamine synthesis. Catecholamines can inhibit the *in vitro* type-1 cytokine production while favor the type-2 cytokine production [44]. In normal T lymphocytes, monoamine oxidase inhibitor pargyline increased synthesis of catecholamines and facilitated the shift of Th1/Th2 balance toward Th2 response. Decreasing catecholamines by α-MT and TH silencing promoted the shift toward Th1 response [24-26]. These changes suggest that catecholamines regulate proinflammatory/antiinflammatory response by inhibiting proinflammatory and enhancing antiinflammatory processes. Upregulation of TH expression in ankle joints in mice with CIA represents a compensatory increase in synthesis of catecholamines during CIA process. The present results suggest that the increased catecholamines alleviate CIA inflammation by inhibiting proinflammatory and enhancing antiinflammatory responses. A few *in vivo* studies support our results. In experimental arthritis, inflammation of CIA mice was markedly reduced by TH^+ neuronal cell adoptive transfer, and this effect was reversed by TH^+ cell depletion [45]. Increasing levels of cytoplasmic catecholamines by the local reserpine treatment markedly reduced inflammation of CIA mice [30].

The previous findings showed that immunomodulation of catecholamines was mediated by AR subtypes which are expressed on lymphocytes [34-37, 46]. These results suggest that catecholamine-induced lymphocyte apoptosis and shift of Th1/Th2 balance towards Th2 response are mediated by α1-AR [25, 39]. These facts support that α1-AR plays a dominant role in mediating the catecholamine immunomodulation. The findings that the catecholamine immunomodulation was mediated by receptors provide a clue for RA therapy. In this study, α1-AR expression was downregulated in T cells obtained from CIA mice and epinephrine treatment inhibited the CIA-induced downregulation of α1-AR expression in T cells *in vitro*. Epinephrine reduced CIA-induced $CD25^-IL\text{-}17^+$ cell percentage in $CD4^+$ T cells and ROR-γt, IL-17 and IL-22 expression and secretion in T cells *in vitro*. In contrast, epinephrine enhanced $CD25^+Foxp3^+$ cell percentage in $CD4^+$ T cells and Foxp3, TGF-β and IL-10 expression and secretion in T cells from CIA mice. Epinephrine is a neuroendocrine hormone belonging to catecholamines and it is synthesized also by TH catalysis. Changing TH activity also alters

epinephrine level in T cells [25, 42]. Overexpressing or silencing TH gene in $CD4^+$ T cells obtained from CIA mice alleviated or exacerbated Th17/Treg imbalance *in vitro*, respectively, in our previous study [40]. Thus, we propose that the increased or decreased expression of TH in ankle joints by the injection of TH-lentiviral vector or TH-shRNA lentiviral vector, respectively, may correspondingly alter epinephrine level in T cells and as a result, affect joint inflammation and Th17/Treg imbalance in CIA. Further investigation showed that activating α1-AR with the agonist phenylephrine attenuated CIA-induced Th17 cell activity and enhanced Treg cell activity, and PLC inhibitor reversed these effects. These findings suggest that epinephrine inhibits Th17 cell differentiation and function and enhances Treg cell differentiation and function via activating α1-AR/PLC signaling in T cells, and thereby suppresses inflammation in CIA. The present results that epinephrine inhibited the Th17/Treg imbalance suggest that autoimmune diseases can be alleviated by epinephrine. In addition, these findings that α1-AR/PLC signaling is involved in the effects of epinephrine could be applied for new treatment in some autoimmune diseases.

Conclusion

In summary, TH overexpression in ankle joints of mice with CIA inhibited Th17 proinflammatory response but enhanced Treg antiinflammatory response in the joints, and in contrast, TH knockdown produced the contrary effects. Epinephrine upregulated α1-AR expression in T cells obtained from CIA mice. Both epinephrine and the α1-AR agonist phenylephrine reduced CIA-induced Th17 response but enhanced Treg response *in vitro*. These findings suggest that upregulating TH expression in joints is involved in alleviation of joint inflammation and Th17/Treg imbalance in CIA and this effect is implemented at least partially by enhancing epinephrine action on α1-AR in T cells.

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Author Contributions

All authors participated in the analysis of the data and review of the manuscript. YPP and YHQ designed the studies and interpreted the data. XQW and TTW conducted the major experiments, and XXF and WXS contributed to prepare animal models. XQW wrote the manuscript, and YHQ edited the manuscript.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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