Expression of tyrosine hydroxylase in CD4⁺ T cells contributes to alleviation of Th17/Treg imbalance in collagen-induced arthritis

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Abstract

Tyrosine hydroxylase (TH), a rate-limiting enzyme for the synthesis of catecholamines, is expressed in T lymphocytes. However, the role of T cell-expressed TH in rheumatoid arthritis (RA) is less clear. Herein, we aimed to show the contribution of TH expression by CD4⁺ T cells to alleviation of helper T (Th)17/regulatory T (Treg) imbalance in collagen-induced arthritis (CIA), a mouse model of RA. CIA was prepared by intradermal injection of collagen type II (CII) at tail base of DBA1/J mice. Expression of TH in the spleen and the ankle joints was measured by real-time polymerase chain reaction and Western blot analysis. Percentages of TH-expressing Th17 and Treg cells in splenic CD4⁺ T cells were determined by flow cytometry. Overexpression and knockdown of TH gene in CD4⁺ T cells were taken to evaluate effects of TH on Th17 and Treg cells in CIA. TH expression was upregulated in both the inflamed tissues (spleen and ankle joints) and the CD4⁺ T cells of CIA mice. In splenic CD4⁺ T cells, the cells expressing TH were increased during CIA. These cells that expressed more TH in CIA were mainly Th17 cells rather than Treg cells. TH gene overexpression in CD4⁺ T cells from CIA mice reduced Th17 cell percentage as well as Th17-related transcription factor and cytokine expression and secretion, whereas TH gene knockdown enhanced the Th17 cell activity. In contrast, TH gene overexpression increased Treg-related cytokine expression and secretion in CD4⁺ T cells of CIA mice, while TH gene knockdown decreased the Treg cell changes. Collectively, these findings show that CIA induces TH expression in CD4⁺ T cells, particularly in Th17 cells, and suggest that the increased TH expression during CIA represents an anti-inflammatory mechanism.

Keywords: Tyrosine hydroxylase, catecholamines; type II collagen-induced arthritis, CD4⁺ T cells, Th17 cells, Treg cells

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Introduction

Tyrosine hydroxylase (TH), a rate-limiting enzyme for the synthesis of catecholamines, is expressed principally in nerve and endocrine cells. In addition to regulation of cardiovascular, respiratory and digestive activities, the catecholamines synthesized and released by nerve and endocrine cells also regulate immune function by direct contact with lymphocytes.^{1,2} In the recent decade, reports from other and our laboratories have presented that catecholamines can also be synthesized and secreted by T lymphocytes.^{3–9} The T cell-derived catecholamines modulate differentiation and function of helper T (Th) cells, with an effect of facilitating the shift of Th1/Th2 balance toward Th2 polarization.^{10–12} We hypothesized that this shift toward Th2 polarization is beneficial for amelioration of imbalance in proinflammatory/anti-inflammatory responses in some autoimmune diseases.

Rheumatoid arthritis (RA), a representative human autoimmune disease, is characterized by systemic disorder and

joint inflammation, destruction, and deformity.¹³ Although the exact pathogenesis of RA is unknown, some observations suggest that CD4⁺ T lymphocytes play a pivotal role in induction or perpetuation of this chronic inflammatory disease.¹⁴ CD4⁺ T cells, on activation and expansion, develop into different T cell subsets, including Th1, Th2, Th17, and regulatory T (Treg) cells, with different cytokine profiles and distinct effector functions. It has become clear that balances between Th1 and Th2 cells, or their cytokines, are important in induction or prevention of RA.^{15–17} Th17 cells are the newest member of the effector Th cell family and are characterized by high proinflammation via producing specific cytokines such as interleukin (IL)-17 and IL-22.^{18,19} Treg cells are characterized by the control of immune responses to self and foreign antigens and by the suppression of harmful autoimmune T cells via producing anti-inflammatory cytokines such as transforming growth factor (TGF)- β .^{20,21} A few studies show that Th17/Treg imbalance is associated

with RA process.^{22,23} Further investigation is needed to clarify the role of Th17/Treg imbalance in RA. Collagen type II (CII)-induced arthritis (CIA) is a most common and reliable animal model sharing many pathological and histological similarities with human RA.²⁴ Therefore, CIA was employed in this study as a mouse model of RA.

A reduction in density of sympathetic nerve fibers in inflamed joints and spleen of CIA has been found.^{25,26} When the sympathetic nerve fibers get lost, TH-immunoreactive cells or vesicular monoamine transporter (VMAT)-2-immunoreactive cells drastically increase in inflamed joints, spleen and lymph nodes of CIA.²⁷ This suggests a possibility that the TH⁺ catecholaminergic cells in inflamed joints and lymphoid tissues of CIA replace the lost sympathetic nerve fibers. Recently, we have shown that in inflammatory joints of CIA, TH expression in Th1 and Th2 cells is upregulated, suggesting that Th1 and Th2 cells may be a cell source of catecholamines in CIA joints.¹⁷ However, it is unclear whether Th17 and Treg cells also produce catecholamines and what role the T cell-derived catecholamines play in CIA. We hypothesized that catecholamines derived from T cells, as “sympathetic” cells, may replace the locally lost sympathetic nerve fibers and exert a compensatory inhibition of Th17/Treg imbalance in CIA.

Materials and methods

Mice

Male DBA1/J mice (8–10 weeks old, 20–25 g) were obtained from the Center of Experimental Animals, Nantong University, China. The animals were kept and bred in a specific pathogen-free environment with standard temperature and a 12-h light/dark cycle. The mice were housed in standard cages with four to five animals per cage, and food and water were provided *ad libitum*. All animal procedures were in accordance with National Institutes of Health (USA) guidelines and were approved by the Institutional Animal Care and Use Committee of Nantong University.

Induction and clinical assessment of CIA

Collagen type II (CII) (Sigma-Aldrich Co., USA) was dissolved in 0.1 M acetic acid at 2 mg/mL overnight at 4°C and emulsified with an equal volume of Freund's complete adjuvant (Chondrex, Inc., USA) in ice bath under sterile conditions. Emulsion of 0.1 mL was injected intradermally into the base of the tail of DBA1/J mice to induce arthritis on day 0. On day 21, the mice were intraperitoneally boosted with 100 µg CII emulsified with an equal volume of incomplete Freund's adjuvant (Sigma-Aldrich Co., USA). Eventually, an intraperitoneal injection of 20 µg LPS dissolved in 20 µL PBS was executed to the mice on day 28. To quantitatively evaluate the severity of the arthritis, the mice were observed for clinical symptoms and score of limbs by two independently blinded examiners every two days from day 21 post-immunization. The clinical score was assessed using the following system: grade 0, no swelling; grade 1, slight swelling and erythema; grade 2, pronounced edema; and grade 3, joint rigidity. The severity score from each paw was summed, thus giving a severity range of 0–12

per mouse. The mean arthritic score was determined using arthritic animals only. Before the mice were killed on day 35 or day 55 post-immunization, a microcaliper was used to measure the thickness of rear paws and ankle joints.

Histopathology

Ankle joints were harvested after sacrifice, fixed in 4% paraformaldehyde for three days and decalcified in 10% EDTA (pH 7.4) for two weeks. Joint sections (15 µm) were mounted on glass slides and subjected to hematoxylin and eosin staining.

Enzyme-linked immunosorbent assay for the measurement of anti-CII IgG in serum and cytokine levels in cell culture supernatants

Sera from intact and CIA mice were added into 96-well plates coated with CII (100 µg/mL), incubated overnight at 4°C, washed, and incubated for 1 h with horseradish peroxidase-labeled monoclonal antibodies against mouse IgG (Invitrogen, USA). Wells were developed using 3,3',5,5'-tetramethylbenzidine as a substrate, and the resulting color reaction was quantified using an enzyme-linked immunosorbent assay (ELISA) plate reader. Representative cytokines (IL-17, IL-22, TGF-β1, and IL-10) in cell culture supernatants involved in CIA pathogenesis and produced by CD4⁺ T cell subsets were detected using ELISA kits (eBioscience, USA), according to the manufacturer's protocol.

Purification of CD4⁺ T cells

On the 41st day following first immunization, the CD4⁺ T cells were isolated from the mouse spleen tissues and sorted using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of extraction was greater than 95% (data not shown). The CD4⁺ T cells were then resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin at the final concentration of 1.25×10^6 /mL.

Plasmids construction

The plasmid expressing full-length TH was as a TH gene overexpression, and the empty plasmid (TH mock) was used as a control of the TH gene overexpression. The sequence of short hairpin RNA (shRNA) targeting mouse TH (TH-shRNA; Gene Bank Accession NM 009377.1) was 5'-GCACACAGTACATCCGTCA-3', and the plasmid expressing scrambled-shRNA (Scr-shRNA; 5'-TTCTCCGAACGTGTACACGT-3') was used as a control of the TH-shRNA. All of the plasmids were generated by Genechem Co. Ltd. (Shanghai, China).

Transfection of plasmids used for TH overexpression or knockdown

The constructed plasmids used for TH overexpression or knockdown were transfected into CD4⁺ T cells using nucleofection technology (Amaxa Biosystems, Germany), according to the manufacturer's instructions. In the

same way, the control plasmids (empty plasmid or Scr-shRNA-expressing plasmid) were transfected. Briefly, after incubating with anti-CD3 and anti-CD28 antibodies at 37°C in an atmosphere of 5% CO₂ for 48 h, CD4⁺ T cells were resuspended in 100 µL of T cell nucleofector solution. Plasmid of 4 µg was added to 100 µL of 5 × 10⁶ CD4⁺ T cell suspension. The mixtures were subsequently transferred to an electroporation cuvette with aluminum electrodes and placed in the nucleofection device (Amaxa Biosystems, Germany). Nucleofection of these cells was accomplished using X-001 program, and the samples were immediately transferred to 12-well plates containing 2 mL pre-warmed media and incubated for 48 h. The transfection efficiency was determined by the fluorescence-positive cells under a fluorescence microscope (Leica, Germany). Number of GFP-labeled cells was approximately 50% to 70% (data not shown).

Real-time polymerase chain reaction analysis

Total RNA were extracted from spleens and ankle joints from mice 35 or 55 days following first immunization or from CD4⁺ T cells, which had been transfected and incubated for 48 h, with Trizol reagent (Invitrogen, USA), as recommended by the manufacturer. RNA was reverse-transcribed with random hexamers, and real-time quantitative polymerase chain reaction (PCR) was performed on cDNA by using the Rotor-Gene 3000 Real-time Cycler with SYBR green I as the detection system. The murine primer pairs included: TH, 5'-CGGAAGCTGATTGCA GAGAT-3' and 5'-GGGTAGCATAGAGG CCCTTC-3' (NM_009377.1); ROR-γt, 5'-AGTAGGCCACATTACACTGCT-3' and 5'-GACCCACACCTCACAAATTGA-3' (NM_011281.2); IL-17, 5'-GCTCCAGAAGGCCCTCAGA' and 5'-AGCTTTCCTCCGCATTGA-3' (NM_010552.3); IL-22, 5'-GGCCAGCCTTGACAGATAACA' and 5'-GCTGATGTGAC AGGAGCTGA' (NM_016971.2); Foxp3, 5'-TACACCCAGG AAAGACAGCAACCT-3' and 5'-TCTGCTTGCCAGTGCT TGAGAA-3' (NM_001199348.1); TGF-β, 5'-GATACGCC TGAGTGGCTGTC-3' and 5'-GCTGATCCCGTTGATTTC C-3' (NM_011577.1); IL-10, 5'-GGACAACATACTGC TAACCGAC-3' and 5'-TGGATCATTTCGATAAGGC TTG-3' (NM_010548.2); β-actin, 5'-CTGTCCCTGTATGC CTCTG-3' and 5'-ATGTCACGCACGATTTC-3' (NM_007393.5). Signals were collected using Rotor-Gene 6.0 software, and the reactions generated a melting temperature dissociation curve enabling quantification of the PCR products. Gene expression was normalized to β-actin as an endogenous reference. Data were analyzed using the comparative 2^{-ΔΔC_t} method,²⁸ and the results were expressed as relative fold change.

Western blot analysis

Total protein was extracted from spleens and ankle joints from mice 35 or 55 days following first immunization or from CD4⁺ T cells, which had been transfected with plasmids of TH overexpression or knockdown and incubated for 48 h. Tissues or cells were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 10 µl/mL protease

inhibitor cocktail, and 1 mM PMSF), and the supernatants were collected by centrifuging at 4°C at 12,000 × g for 15 min. The supernatants were mixed with loading buffer and boiled for 10 min. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Pall, USA) using a wet transfer apparatus. After blocking non-specific binding with 5% (w/v) nonfat dry milk, the membranes were probed with mouse antibodies specific for TH (1:500, Millipore, USA), Foxp3 (1:200, Santa Cruz Biotechnology, USA), or IL-10 (1:200, Santa Cruz Biotechnology, USA), or with rabbit antibodies specific for ROR-γt (1:500, Abcam, UK), TGF-β (1:500, Abcam, UK), IL-17 (1:200, Santa Cruz Biotechnology, USA) or IL-22 (1:200, Santa Cruz Biotechnology, USA) at 4°C overnight. Then, they were incubated with the IRDye 800-conjugated goat anti-mouse IgG (1:5000, Rockland Immunochemicals, USA) or with IRDye 800-conjugated goat anti-rabbit IgG (1:5000, Rockland Immunochemicals, USA) for 1 h at room temperature, followed by visualization using Odyssey laser scanning system (LI-COR Inc, USA). Blots were reprobated with monoclonal mouse anti-β-actin antibody (1:5000, Sigma, USA) and reacted with IRDye 800-conjugated goat anti-mouse IgG (1:5000, Rockland Immunochemicals, USA) to confirm equal protein loading. The molecular weight and relative quantity of the protein bands were determined by an image analysis system (Odyssey 3.0 software).

Flow cytometric assay

On the 35th and the 55th days after first immunization, the spleens were harvested from the anaesthetized mice by splenectomy. Splenic mononuclear cells were isolated using density gradient centrifugation, and washed three times with RPMI 1640 culture medium (Gibco, USA). The splenic mononuclear cells were resuspended at a concentration of 1 × 10⁷ cells/mL in 100 µL of 0.01 M PBS per sample. CD4⁺ T cell subset differentiation was evaluated by flow cytometry after staining for intracellular cytokines. Cells were cultured with 50 ng/mL PMA, 1 µM ionomycin, and 2 µM monensin for 4 h, stained for surface markers with allophycocyanin (APC)-labeled anti-CD4 or phycoerythrin (PE)-labeled anti-CD25 antibodies (BD PharMingen, USA), and further processed using a BD Fixation/Permeabilization kit (BD Biosciences, USA); cells were then incubated for 30 min at 4°C with PE-conjugated antibodies to IL-17 (BD PharMingen, USA). Afterward, 0.25 µg of anti-TH antibody (Santa Cruz Biotechnology, USA) and fluorescein isothiocyanate (FITC)-labeled secondary antibodies was added to each sample, which was incubated for 30 min and analyzed using a FACSArray flow cytometer (BD Biosciences, USA) by acquiring 10,000 cells. FACS data were analyzed using Cell Quest software (BD Biosciences, USA).

After activated with anti-CD3 and anti-CD28 antibodies and incubated with the transfection for TH overexpression or knockdown, CD4⁺ T cells were stimulated with 50 ng/mL PMA, 1 µM ionomycin and 2 µM monensin for 4 h, stained for surface markers with FITC-labeled anti-

CD25 antibodies (BD PharMingen, USA), and further processed using a BD Fixation/Permeabilization kit (BD Biosciences, USA); cells were then incubated for 30 min at 4°C with PE-labeled anti-IL-17 and APC-labeled anti-Foxp3 antibodies (BD PharMingen, USA). Analysis was performed with FACS Calibur flow cytometer equipped with an argon laser. Acquisition was analyzed with Cell Quest software (BD Biosciences).

Statistical analysis

Data were expressed as mean $\pm$ standard deviation ($M \pm SD$). Statistical analyses were performed with the Statistics Package for Social Science (SPSS, 16.0). The data were subjected to one-way analysis of variance, followed by Student-Newman-Keul's test to compare the data of all groups relative to each other. The data of clinical score were compared by independent sample T test. Differences were considered statistically significant at $p < 0.05$.

Results

CII induces limb inflammation, serum anti-CII IgG elevation, and TH expression upregulation in the spleen and ankle joints

Compared with intact mice, the mice that had received CII injection began to rise in clinical score on day 31 after the first immunization, reached a significant increase on day 35, and remained high until day 55, the last day observed in this study (Figure 1(a)). The thickness of both ankle joints and rear paws was augmented on days 35 and 55 post-immunization, and there were no significant differences between the two phases of CIA (Figure 1(b)). Anti-CII IgG antibody level was notably elevated on both day 35 and day 55 after first immunization in comparison with that of intact control, but this elevation was reduced on day 55 relative to that on day 35 (Figure 1(c)). To indicate change in synthesis of catecholamines in inflamed tissues, we determined TH expression in the spleen and ankle joints of CIA mice.

Figure 1 CII induces limb inflammation, serum anti-CII IgG elevation, and TH expression upregulation in the spleen and ankle joints. CIA mouse model was prepared by injection of the antigen CII. Clinical score was recorded from day 21 to day 55 after first immunization (a). The data show that the clinical score began to rise on day 31 post-immunization, reached a significant increase on day 35, and remained high until day 55. There was no significant difference in the clinical score between days 35 and 55 post-immunization. ** $p < 0.01$; NS: no significance. Thickness of ankle joint and rear paw indicates a significant increase on days 35 and 55 post-immunization (b). ** $p < 0.01$, versus intact. Anti-CII IgG antibody level is elevated at the two phases of CIA, but this elevation is reduced with CIA progression (c). ** $p < 0.01$, versus intact; && $p < 0.01$, versus CIA (day 35). Expression of TH gene (d1) and protein (d2) in spleen and ankle joints was examined by real-time PCR and Western blot, respectively. ** $p < 0.01$, versus intact control; && $p < 0.01$, versus CIA (day 35).

Compared with intact control, CIA mice showed a significantly upregulated expression of TH mRNA and protein in the spleen and ankle joints (Figure 1(d1) and (d2)). Between the two phases of CIA (day 35 and day 55 post-immunization), the upregulated TH expression did not have significant difference in the spleen but was weaker at the late stage than at the early stage in the ankle joints (Figure 1(d1) and (d2)).

Percentages of both CD4⁺ T cells and Th17 cells expressing TH are increased in the spleen of CIA mice

In splenic CD4⁺ T cells, percentage of IL-17-positive cells, but not CD25-labeled cells, was increased at both the early and late stages of CIA, and this increase was reduced at the late stage (Figure 2(b) and (c)). Importantly, percentages of TH-positive cells and IL-17/TH double-stained cells, but not CD25/TH double-labeled cells, were increased in splenic CD4⁺ T cells at both the stages of CIA (Figure 2(a) to (c)). Further, both the percentages of IL-17/TH double-stained cells in IL-17-positive cells and CD25/TH double-stained cells in CD25-positive cells were increased in the two phases of CIA (Figure 2(b) and (c)).

TH gene overexpression or knockdown in CD4⁺ T cells of CIA mice alters Th17 cell number but not Treg cell number

TH expression was upregulated in CD4⁺ T cells isolated from the spleen of CIA mice, compared with that of intact mice (Figure 3(a)). This upregulation was further enhanced by TH gene overexpression, but reduced by TH gene knockdown in CD4⁺ T cells of CIA mice (Figure 3(a)).

Percentage of CD25⁺IL-17⁺ cells was increased in CD4⁺ T cells of CIA mice, with respect to intact mice (Figure 3(b1)

and (b2)). TH gene overexpression in CD4⁺ T cells of CIA mice reduced the percentage of CD25⁺IL-17⁺ cells relative to TH-mock treatment, whereas TH gene knockdown increased the percentage of CD25⁺IL-17⁺ cells relative to Scr-shRNA treatment (Figure 3(b1) and (b2)). In contrast, CIA did not alter CD25⁺Foxp3⁺ cell percentage in CD4⁺ T cells, and either treatment with TH-overexpression or TH-shRNA did also not alter CD25⁺Foxp3⁺ cell percentage in CD4⁺ T cells in CIA mice (Figure 3(b1) and (b3)). As a control, either treatment with TH-mock or Scr-shRNA in CD4⁺ T cells of CIA mice affected neither percentages of CD25⁺IL-17⁺ cells and CD25⁺Foxp3⁺ cells (Figure 3(b1) to (b3)).

Effects of TH gene overexpression or knockdown in CD4⁺ T cells from CIA mice on Th17- and Treg-related transcription factors and cytokines

CIA induced the expression of both the Th17-related transcription factor ROR- γ t and the cytokines IL-17 and IL-22 as well as the secretion of IL-17 and IL-22 in CD4⁺ T cells (Figure 4(a) to (c)). TH gene overexpression in CD4⁺ T cells reduced these effects induced by CIA, while TH gene knockdown in CD4⁺ T cells further enhanced these CIA-induced effects (Figure 4(a) to (c)).

In contrast, CIA did not influence the expression of the Treg-related transcription factor Foxp3 and the cytokine TGF- β as well as the secretion of TGF- β (Figure 4(a) to (c)). Nevertheless, TH gene overexpression in CD4⁺ T cells from CIA mice increased TGF- β expression and secretion compared with TH-mock control, while TH gene knockdown decreased TGF- β expression and secretion compared with Scr-shRNA control (Figure 4(a) to (c)). In addition, IL-10, another Treg-related cytokine, had an increased expression and secretion in CD4⁺ T cells of CIA

Figure 2 Percentages of both CD4⁺ T cells and Th17 cells expressing TH are increased in the spleen of CIA mice. Splenic mononuclear cells were collected and labeled simultaneously with the three fluorescence antibodies, anti-CD4, anti-IL-17/CD25, and anti-TH antibodies. The CD4-labeled cells were firstly selected, and then percentages of the single- or double-labeled cells in the CD4-labeled cells were analyzed. The left panels are representative images of flow cytometry, and the right panels are statistical graphs which data are related to the corresponding left panels. (a) TH⁺ cell percentage in CD4⁺ cells. (b) Percentages of Th17 (IL-17⁺) cells and TH-expressing Th17 (IL-17⁺TH⁺) cells in CD4⁺ cells, as well as percentage of TH-expressing Th17 (IL-17⁺TH⁺) cells in Th17 (IL-17⁺) cells. (c) Percentages of Treg (CD25⁺) cells and TH-expressing Treg (CD25⁺TH⁺) cells in CD4⁺ cells, as well as percentage of TH-expressing Treg (CD25⁺TH⁺) cells in Treg (CD25⁺) cells. **p* < 0.05, ***p* < 0.01, versus intact; [‡]*p* < 0.05, ^{‡‡}*p* < 0.01, versus CIA (day 35). (A color version of this figure is available in the online journal.)

Figure 3 Effects of TH gene overexpression or knockdown on percentages of Th17 cells and Treg cells in CD4⁺ T cells of CIA mice. CD4⁺ T cells from the spleens of intact or CIA mice were purified and activated with anti-CD3 and anti-CD28 antibodies, and then these cells were transfected with plasmids of TH gene overexpression or knockdown. After incubating with transfection for 48 h, the CD4⁺ T cells were measured for expression of TH protein (a). Representative fluorescent dot images showing percentage of CD25⁺IL-17⁺ (Th17) cells and CD25⁺Foxp3⁺ (Treg) cells by flow cytometric analysis (b1). The statistical data for number of Th17 cells (b2) and Treg cells (b3). ***p* < 0.01, compared with the CD4⁺ T cells of intact mice; ++*p* < 0.01, compared with TH-mock control; ##*p* < 0.01, compared with Scr-shRNA control. (A color version of this figure is available in the online journal.)

mice than in the cells of intact mice (Figure 4(a) to (c)). The CIA-induced expression and secretion of IL-10 in CD4⁺ T cells were further enhanced by TH gene overexpression, but reduced by TH gene knockdown in CD4⁺ T cells (Figure 4(a) to (c)).

Discussion

In the present study, CII induced a significant increase in clinical score of four limbs on day 35 after first

immunization, suggesting that the 35th day can denote an onset of arthritis in CIA model mice. In addition, serum antibody specific to antigen CII was elevated on days 35 and 55 post-immunization, but this elevation was reduced at the late stage of CIA. The higher prevalences of CII-specific antibodies are also noted during the early phase of RA.^{29,30} Therefore, CII-specific antibodies appear to have the potential to initiate an articular inflammatory response, as evidenced by the inflammatory arthritis induced in

Figure 4 Influences of TH gene overexpression or knockdown in CD4⁺ T cells from CIA mice on Th17- and Treg-related transcription factors and cytokines. CD4⁺ T cells from the spleens of intact or CIA mice were purified and activated with anti-CD3 and anti-CD28 antibodies, and then these cells were transfected with plasmids of TH overexpression or knockdown. After incubating with transfection for 48 h, the CD4⁺ T cells were measured for gene (a) and protein (b) expression of the Th17-related transcription factor, ROR-γt and the cytokines, IL-17 and IL-22, as well as the Treg-related transcription factor, Fxp3 and the cytokines, TGF-β and IL-10. Concentrations of the cytokines were examined in the culture supernatants of CD4⁺ T cells (c). ***p* < 0.01, compared with the CD4⁺ T cells of intact mice; ++*p* < 0.01, compared with TH-mock control; #*p* < 0.05, ##*p* < 0.01, compared with Scr-shRNA control

serum transfer and collagen antibody-induced arthritis experiments.^{31–33}

An imbalance of Th17/Treg ratio is observed in peripheral blood and synovial tissue in RA patients.^{22,23,34} In the present study, CIA induced an increase in Th17 cell frequency, as determined by the increased CD25⁺IL-17⁺ cell percentage and ROR-γt expression in CD4⁺ T cells of CIA mice, but CIA did not alter Treg cell frequency, as determined by the unchanged CD25⁺Fxp3⁺ cell percentage and Fxp3 expression in CD4⁺ T cells of CIA mice. These findings confirm the imbalance of Th17/Treg ratio in CIA

and suggest that the Th17/Treg ratio imbalance principally manifests a dramatic increase in Th17 frequency. Consistent with these data, the expression and secretion of the Th17-related cytokines IL-17 and IL-22 were enhanced in CD4⁺ T cells of CIA mice, and the expression and secretion of the Treg-related cytokine TGF-β were not altered. On the other hand, CIA induced IL-10 expression and secretion by CD4⁺ T cells. Since IL-10 can be also produced by Th2 cells in addition to Treg cells, the CIA-induced IL-10 production may derive from Th2 cells and/or Treg cells. Collectively, the present results demonstrate that an enhanced Th17 cell

activity, including increased Th17 frequency and increased IL-17 and IL-22 release, is a major manifestation of Th17/Treg imbalance in CIA. As a support, the reports from other authors have presented that the increased Th17 frequency and the increased IL-17 and IL-22 release in peripheral blood have been associated with RA pathogenesis^{23,35} and that Treg immunosuppressive function is not significantly altered in RA patients.³⁶ Th17 cells can induce synovial fibroblasts in patients with RA, thus contributing to synovitis and bone destruction.³⁷

Importantly, TH expression was upregulated in the spleen and ankle joints of CIA mice. TH is a key enzyme for synthesis of catecholamines, including norepinephrine, epinephrine, and dopamine. Inhibiting TH in T cells using an inhibitor α -methyl-p-tyrosine (α -MT) or using gene interference reduces production of catecholamines including norepinephrine, epinephrine, and dopamine in the cells.^{8,10,11} Thus, the present results suggest that CIA may induce catecholamine production in the inflamed tissues. The TH⁺ cells have been found in inflamed synovial tissue of patients with RA and not in controls,^{38,39} and the TH⁺ cell density is higher in inflamed spleen and lymph nodes of CIA than in the tissues of normal mice.²⁷ These findings support our present results. Previously, we have shown that the increased TH expression in inflamed synovial tissue of CIA ankle joints is attributed at least in part to Th1 and Th2 cells.¹⁷ In this study, we found that IL-17/TH double-stained cells, but not CD25/TH double-stained cells, were increased in CD4⁺ T cells of CIA mice. The findings provide further data showing that in addition to Th1 and Th2 cells, Th17 cells are also an important cell source of catecholamines in CD4⁺ T cells of CIA mice. Immune cells in synovial tissues can produce and store catecholamines during chronic inflammatory diseases such as RA.^{39,40} Here, we provide new evidence showing that Th17 cells are one of the immune cells that produce catecholamines in CIA.

Identifying the role of T cell-derived catecholamines in RA is significant for designing a potential therapeutic strategy for RA targeting catecholamines. In the current study, CIA-induced Th17 cell activity, including increased Th17 frequency and IL-17 and IL-22 release, was inhibited by TH gene overexpression, but enhanced by TH gene knockdown in CD4⁺ T cells. These data demonstrate that TH expression (consequent catecholamine synthesis) by T cells is beneficial for alleviation of CIA by suppressing the proinflammatory Th17 cell activity. On the other hand, both TGF- β production and CIA-induced IL-10 production were enhanced by TH gene overexpression, but reduced by TH gene knockdown in CD4⁺ T cells. These changes in anti-inflammatory cytokines further show a beneficial property of TH expression or catecholamine synthesis in CD4⁺ T cells during CIA. Thus, we propose that by imbibing Th17 cells and enhancing Treg cells, T cell-derived catecholamines may alleviate CIA inflammation. In normal T lymphocytes, both α -MT and TH silencing attenuate catecholamine synthesis and consequently promote a shift of Th1/Th2 balance toward Th1 polarization, while increasing catecholamines using the monoamine oxidase inhibitor pargyline facilitates the shift toward Th2 polarization.^{10–12} These results indicate

that normally produced catecholamines by T cells can regulate Th1/Th2 balance, suggesting an effect of inhibiting proinflammatory and enhancing anti-inflammatory responses. During the inflammatory CIA, TH expression was upregulated in T cells, which represents a compensatory increase in catecholamine synthesis in the inflammatory process. The increased catecholamines inhibit proinflammatory and enhance anti-inflammatory responses and as a result ameliorate CIA manifestation. A few *in vivo* studies show the beneficial effect of catecholamines on CIA or RA. Adoptive transfer of TH⁺ neuronal cells markedly reduces CIA in mice, and 6-hydroxydopamine, which depletes TH⁺ cells, reverses this effect.⁴¹ The local treatment with reserpine, a VMAT-2 blockade that can increase cytoplasmic catecholamine levels, ameliorates the clinical score of CIA mice.³⁹ Calcagni and Elenkov⁴² have presented that catecholamines may improve autoimmune diseases, such as RA, via suppression of proinflammatory and potentiation of anti-inflammatory cytokine production. Further *in vivo* studies demonstrating the role of T cell-derived catecholamines in RA are required to better understand neural-endocrine-immune interaction in this autoimmune disease.

Conclusions

CII induces RA-like changes in mice, including joint inflammation and serum anti-CII antibody elevation. Th17/Treg imbalance principally manifesting a striking increase in Th17 frequency and IL-17 and IL-22 release occurs during CIA. CIA induces TH expression in both the inflamed tissues (spleen and ankle joints) and the CD4⁺ T cells. Between Th17 cells and Treg cells, Th17 cells are a more important cell source expressing TH during CIA. The CIA-induced TH expression in CD4⁺ T cells represents a compensatorily protective mechanism by which Th17 proinflammatory response is inhibited and Treg anti-inflammatory response is enhanced. These findings suggest that TH expression (consequent catecholamine synthesis) by T cells may alleviate CIA inflammation.

Authors' contributions: XQW performed the experiments and wrote the manuscript. YL analyzed the data. HHC performed the experiments. YPP conceived and designed the experiments and edited and revised the manuscript. YHQ conceived and designed the experiments and contributed reagents/materials/analysis tools. All authors read and approved the final manuscript.

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DECLARATION OF CONFLICTING INTEREST

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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