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Activation of β2-adrenergic Receptor Alleviates Collagen-induced Arthritis by Ameliorating Th17/Treg Imbalance

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ABSTRACT

Background: Recent research in our laboratory shows that $CD4^+$ T cells express the β2 adrenergic receptor (β2-AR), and the sympathetic neurotransmitter norepinephrine regulates the function of T cells via β2-AR signaling. However, the immunoregulatory effect of β2-AR and its related mechanisms on rheumatoid arthritis is unknown.

Objective: To explore the effects of β2-AR in collagen-induced arthritis (CIA) on the imbalance of T helper (Th) 17/ regulatory T (Treg) cells.

Methods: In DBA1/J mice, collagen type II was injected intradermally at the tail base to prepare the CIA model. The specific β2-AR agonist, terbutaline (TBL), was administered intraperitoneally beginning on day 31 and continuing until day 47 after primary vaccination, twice a day. Magnetic beads were used to sort $CD3^+$ T cells subsets from spleen tissues.

Results: *In vivo*, β2-AR agonist TBL alleviated arthritis symptoms in the CIA mice including histopathology of the ankle joints, four limbs' arthritis score, the thickness of ankle joints, and rear paws. After TBL treatment, in the ankle joints, the levels of proinflammatory factors (IL-17/22) notably decreased and the levels of immunosuppressive factors (IL-10/TGF-β) significantly increased. *In vitro*, ROR-γt protein expression, Th17 cell number, mRNA expression and the releasing of IL-17/22 from $CD3^+$ T cells reduced following TBL administration. Moreover, TBL enhanced the anti-inflammatory responses of Treg cells.

Conclusion: These results suggest that β2-AR activation exerts anti-inflammatory effects through the amelioration of Th17/Treg imbalance in the CIA disease.

Keywords: β2-adrenergic receptor, Inflammation, Terbutaline, Th17 cells, Treg cells, Type II collagen-induced Arthritis

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INTRODUCTION

Sympathetic nerves innervate lymphocytes and regulate their function by releasing the neurotransmitter norepinephrine (NE). Similarly, epinephrine, transported to target cells by the adrenal gland in the bloodstream, regulates lymphocytes. NE and epinephrine prime target cells by activating their receptors, including the α- and β-adrenergic receptors (α-AR and β-AR). T lymphocytes express α-AR and β-AR. Recently, studies have shown that β2-AR is expressed in $CD3^+$ T cells [1]. In different immune stimulations or states, $CD4^+$ T cells differentiate into helper T (Th) 1/2/17, or regulatory T (Treg) cells [2]. Four subsets of $CD4^+$ T cells exert different immune activities by releasing their predominant cytokines. Th17 cells secrete interleukin (IL)-17/22 or other pro-inflammatory cytokines to be involved in many inflammatory and autoimmune diseases [2, 3]. In contrast, Treg cells possess the properties of suppressing immune responses and maintaining immune tolerance. IL-10/transforming growth factor (TGF)-β are anti-inflammatory cytokines produced by Treg cells [2, 4]. We have shown Th1/Th2 balance is shifted by NE to Th2 response via the activation of β2-AR [5]. The activated β2-AR triggers an intracellular signaling cascade including cAMP and protein kinase A (PKA) to regulate lymphocyte proliferation, differentiation, maturation, and function [6]. However, it is poorly known whether β2-AR activation regulates Th17 and Treg cell responses, particularly in autoimmune diseases.

Rheumatoid arthritis (RA), a human autoimmune disease, is distinguished by articular cartilage destruction, chronic inflammation, and deformity of joints [7, 8]. Collagen type II-induced arthritis (CIA) mimics many properties of RA, and therefore is a commonly used animal model of the human autoimmune disease. Sympathetic nerves innervate joint synovial tissues, but sympathetic nerve density is reduced in inflamed synovial tissues in RA patients or mice with the CIA [9-11]. This suggests that sympathetic nerves are involved in autoimmune diseases. A balance between Th1 and Th2 cells is crucial in inducing or preventing RA [12, 13]. Th17 cell was identified in 2005, challenging the classic paradigm [14, 15]. Pro-inflammatory cytokines produced by Th17 cells have been implicated in RA development [16, 17]. Recently, we have shown that β2-AR/PKA signaling is activated by NE in the CIA, inhibiting the differentiation and function of Th17 cells [18]. On the other hand, both RA therapeutic strategies and pathogenesis have been linked to Treg cells. In addition, Th17/Treg imbalance is involved in the pathogenesis of RA [19, 20]. Nevertheless, it needs clarification whether the activation of the β2-AR/PKA pathway in $CD3^+$ T cells mitigates Th17/Treg imbalance in the CIA and promotes recovery of the CIA symptoms.

In the present study, the CIA mice were intraperitoneally injected with terbutaline (TBL), a specific β2-AR agonist. $CD3^+$ T cells from the CIA mice were exposed to TBL *in vitro*. Both joint inflammation and joint pathological injury were assessed. Simultaneously, the frequency and activity of Th17/Treg cells were measured after treatments.

MATERIALS AND METHODS

Mice

Chinese Experimental Animals' Center at Nantong University provided male DBA1/J mice at the age of 8-10 weeks, weighing 20-25 g. Standard cages were used to house the animals with ad libitum access to water and food. The Nantong University Institutional Animal Care and Use Committee approved all animal procedures performed following the National Institute of Health guidelines.

Induction and Evaluation of the CIA

Small modifications were made to the CIA mice' induction as previously described [21, 22].

Collagen type II (CII, 2 mg/ml) was diluted with 0.1 M acetic acids, followed by emulsion with complete Freund's adjuvant. On day 0, as a primary immunization, 0.1 ml emulsion was intradermally injected into the mice's tail base. On day 21, 100 μg CII emulsions diluted with incomplete Freund's adjuvant were injected intraperitoneally. On day 28, 20 μg lipopolysaccharide was boosted intraperitoneally into mice. Each limb was scored on a scale of 0-3: 0 indicates no turgidity; 1 indicates mild turgidity and erythema; 2 indicates remarkable turgidity, and 3 indicates joint rigidity. Using a cumulative score for all paws, each animal's score ranged from 0-12. The mice were sacrificed on day 47, and the thickness of the ankle joints and rear paws were measured by a microcalliper.

Treatment of the CIA Mice

A total of 4 groups of DBA1/J mice were examined: intact, the CIA, the CIA+vehicle, and the CIA+TBL500. Specific β2-AR agonist, TBL (Sigma-Aldrich, USA) diluted with normal saline, was administered (500 μg/kg/d) intraperitoneally beginning from day 31 to day 47, twice a day. The CIA+vehicle mice were injected with an equal volume of normal saline.

Histopathology

On day 47, the ankle joints of mice were harvested. 4% paraformaldehyde was used to fix them for 72 hrs., followed by 2 weeks of decalcification in 10% EDTA. Hematoxylin and eosin-staining was performed on the joint sections with 15 μm in thickness.

Purification of $CD3^+$ T Cells and Drug Treatments

Magnetic beads were used to sort $CD3^+$ T cells from spleen tissues in DBA1/J mice. PRMI 1640 complete medium was used to dilute the sorted cells to a final concentration (5×10^6 cells/ml). 2 μg/ml anti-CD3 antibody and 2 μg/ml anti-CD28 antibody stimulated them for 48 hrs. Activated $CD3^+$ T cells were divided into the 3 groups: the control, the CIA, and the CIA+TBL (10^{-5} M). TBL (10^{-5} M) was administered to cells for 72 hrs.

Quantitative Real-time PCR Analysis

Ankle joints or $CD3^+$ T cells were extracted for the total RNA using a total RNA isolation kit. After skin, muscle, and connective tissue removal, the ankle joints were pulverized with liquid nitrogen and then transferred to 1.5 ml EP by adding 1 ml TRIzol. M-MuLV reverse transcriptase was used to transcribe the total RNA reversely. Using SYBR Green I, the temperature was initially elevated to 95 °C for 5 min, then decreased to 94 °C for 15 s, 60 °C for 1 min, and 40 cycles. As PCR products accumulated in real time, Rotor-Gene software was used to analyze the results. They were displayed as $2^{-\Delta\Delta Ct}$ [23].

Enzyme-linked Immunosorbent Assay

Anti-CII serum IgG antibody level and cytokine contents in the ankle joints and cell culture supernatants were determined by Enzyme-linked immunosorbent assay (ELISA). On day 47 post-immunization, the mice (25-30 g) were deeply anesthetized with isoflurane inhalation. 0.2-0.3 ml blood was obtained from the orbital sinus, and then the mice were sacrificed [24]. After blood coagulation, the samples were centrifuged at room temperature, and the supernatants were obtained. An optical density (OD) value was acquired at 450 nm with a multimode reader. The ankle joints were pulverized with liquid nitrogen, added with lysis buffer, and then centrifuged. The supernatants were collected. Cytokines (IL-10, -17, -22, and TGF-β1) in the ankle joints and cell culture supernatants were detected using ELISA kits (cat. no. BMS6001, BMS6022, BMS608/4, BMS614/2; eBioscience, USA).

Western Blot Analysis

Cultured $CD3^+$ T lymphocyte lysates were extracted with ultrasound and then centrifuged to collect supernatants. An equal amount of proteins was electrophoresed.

Polyvinylidene difluoride membranes (Pall, USA) were used to transfer the proteins. With 5% skim milk blocking nonspecific binding, membranes were incubated with rabbit ROR-γt specific antibody (1:1000), mouse-specific Foxp3 antibody (1:500), or with mouse-specific β-actin antibody (1:3000). Then, the membranes were incubated with 1:5000 IRDye 700-conjugated affinity-purified goat anti-mouse IgG or with 1:5000 IRDye 800-conjugated affinity-purified goat anti-rabbit IgG, followed by visualization with a double infrared laser scanner. Software Odyssey 3.0 was used to quantify the protein band intensity.

Flow Cytometric Assay

Through intracellular cytokine staining, $CD4^+$ T cell differentiation was observed using the flow cytometry. $CD3^+$ T cells were stimulated with monensin (2 μmol/l), ionomycin (1 μmol/l), and phorbol 12-myristate 13-acetate (50 ng/ml) at 37 °C for 4 hrs. Anti-CD4 and anti-CD25 antibodies were used to stain the cells for surface markers before fixing and permeabilizing them (BD Pharmingen, USA). Finally, anti-IL-17 antibody or anti-Foxp3 antibody was used to incubate cells for 30 min at 4 °C.

Statistical Analysis

SPSS 21.0 software was used in this study to determine the mean and standard deviation of the data. For comparisons between the groups, a one-way ANOVA followed by Student-Newman-Keul's test were applied. The difference was considered statistically significant when the p-value was less than 0.05.

RESULTS

β2-AR Agonist Terbutaline Alleviates Arthritic Symptoms in the CIA Mice

A histopathological examination of the ankle joints revealed inflammatory cell infiltration, synovial hyperplasia, arthrostenosis, and cartilage destruction in the CIA mice. After TBL treatment, these histopathological changes were alleviated (Figure 1A). Compared with the intact group, on day 31, arthritis scores of the CIA and the CIA+vehicle groups started to rise significantly, reached their highest level on

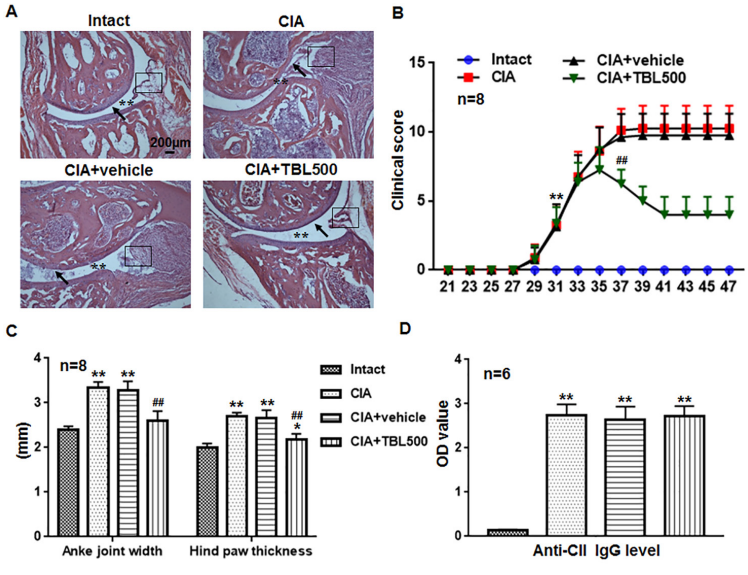

Figure 1. β2-AR agonist terbutaline alleviates arthritic symptoms. The specific β2-AR agonist, TBL, was administered intraperitoneally beginning on day 31 and continuing until day 47 after primary vaccination, twice a day. (A) Histopathology of the ankle joints was observed by H&E staining. Asterisks present articular cavity, arrows indicate articular cartilage, and frames denote the synovium. (B) Arthritis scores of mouse limbs were recorded from 0-12. (C) The thickness of the ankle joints and rear paws were measured on day 47. (D) Anti-CII IgG serum antibody level was determined by ELISA. Scale bar=200 μm. *P<0.05, **P<0.01 vs. intact; ##P<0.01 vs. the CIA+vehicle. CIA: Collagen type II-induced arthritis; TBL: Terbutaline

day 37, and remained at this high level until day 47. The CIA and CIA+vehicle groups did not differ significantly. The clinical score of the CIA mice injected with β2-AR agonist decreased on day 37, and the difference was statistically significant compared with the vehicle-treated CIA mice (Figure 1B). Additionally, the CIA and the CIA+vehicle mice showed an increase in the thickness of the ankle joints and rear paws compared with the intact mice on day 47. TBL injection notably reduced the thickness of ankle joints and rear paws compared with the CIA+vehicle mice (Figure 1C). As well as arthritis inflammatory manifestations, anti-CII serum IgG levels in the remaining three groups notably elevated compared with the intact control, but the difference between the three groups was not significant (Figure 1D).

β2-AR Agonist Terbutaline Inhibits the Function of Th17 Cells in the CIA Mice

IL-17/22 mRNA expression levels in the ankle joints from the CIA and the CIA+vehicle groups were greater than in the intact control. The difference between the CIA and the CIA+vehicle groups was not significant. Compared with the CIA+vehicle group, IL-17/22 mRNA levels in the agonist group decreased, but the expression levels were still higher than the normal (Figure 2A). IL-17/22 contents in the ankle joints from the CIA and the CIA+vehicle groups also increased higher than those in the intact control. In comparison with the CIA+vehicle group, IL-17/22 contents in the CIA+TBL500 group notably reduced (Figures 2B, C).

β2-AR Agonist Terbutaline Enhances the Function of Treg Cells in the CIA Mice

TGF-β/IL-10 mRNA levels in the ankle joints from the CIA and the CIA+vehicle groups upregulated compared with the intact control, and the difference between the CIA and the CIA+vehicle groups was not significant. After being injected with β2-AR agonist TBL, TGF-β/IL-10 mRNA levels dramatically increased compared with the CIA+vehicle group (Figure 3A). Similarly, TGF-β1/IL-10 contents in the ankle joints from the CIA and the CIA+vehicle groups significantly enhanced than those in the intact group. TGF-β1 and IL-10 contents in the CIA+TBL500 group significantly increased compared with the CIA+vehicle group, (Figures 3B, C).

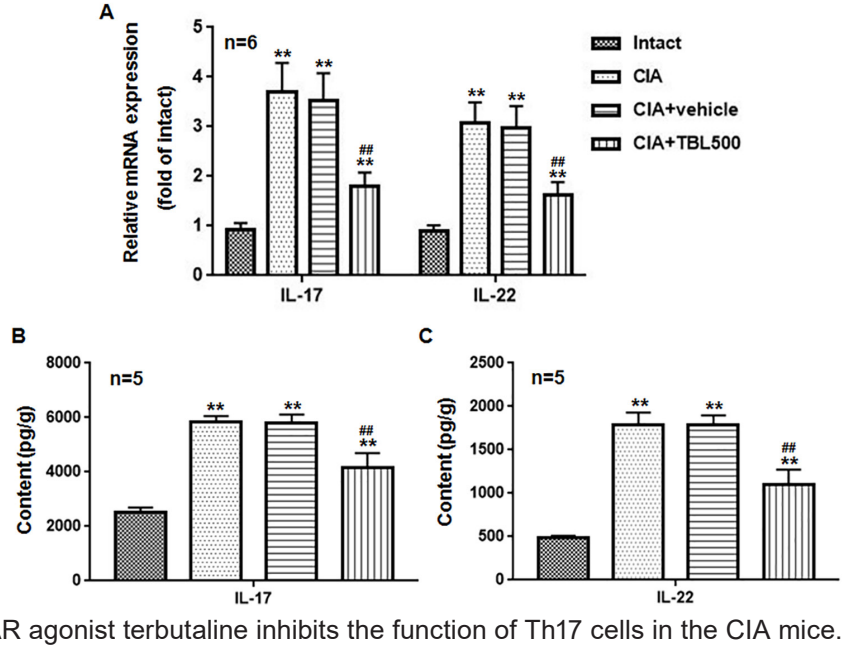

Figure 2. β2-AR agonist terbutaline inhibits the function of Th17 cells in the CIA mice. (A) The mRNA expressions of IL-17/22 in the ankle joints were examined by real time-PCR. (B, C) Contents of cytokines (IL-17/IL-22) were measured by ELISA in the ankle joints of mice. **P<0.01 vs. intact; ##P<0.01 vs. the CIA+vehicle. CIA: Collagen type II-induced arthritis; TBL: Terbutaline

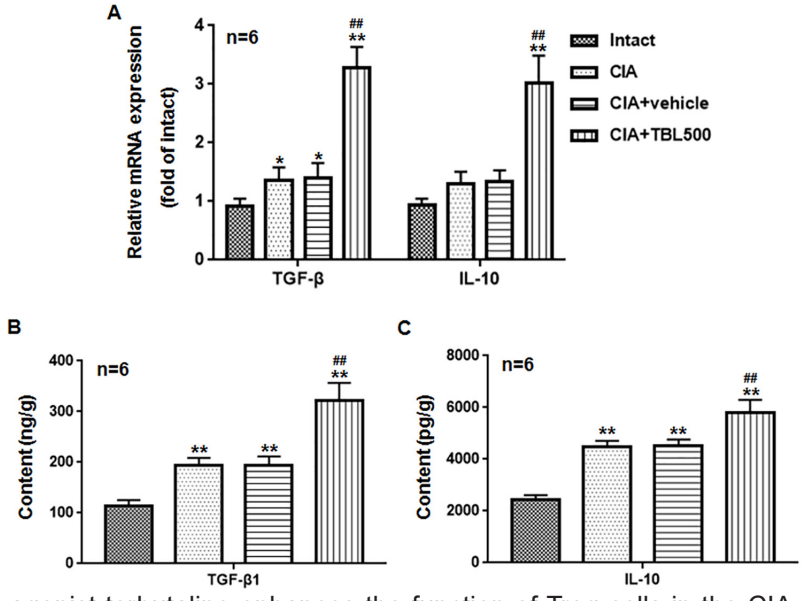

Figure 3. β2-AR agonist terbutaline enhances the function of Treg cells in the CIA mice. (A) TGF-β/IL-10 gene expressions in the ankle joints were tested by real time-PCR. (B, C) TGF-β1/IL-10 contents were assessed by ELISA in the ankle joints of mice. **P<0.01 vs. intact; ##P<0.01 vs. the CIA+vehicle. CIA: Collagen type II-induced arthritis; TBL: Terbutaline

β2-AR Agonist Terbutaline Attenuates Th17 Cell Differentiation and Function in Vitro

Protein expression of ROR-γt (Th17 cell-specific transcription factor) detected by the Western blot analysis and Th17 cell number detected by flow cytometry were explored for Th17 cell differentiation. In contrast to the control group, ROR-**γt** protein expression and Th17 cell number in the CIA group significantly increased. After the addition of TBL, they decreased (Figures 4A, B). In addition, IL-17/22 mRNA expressions

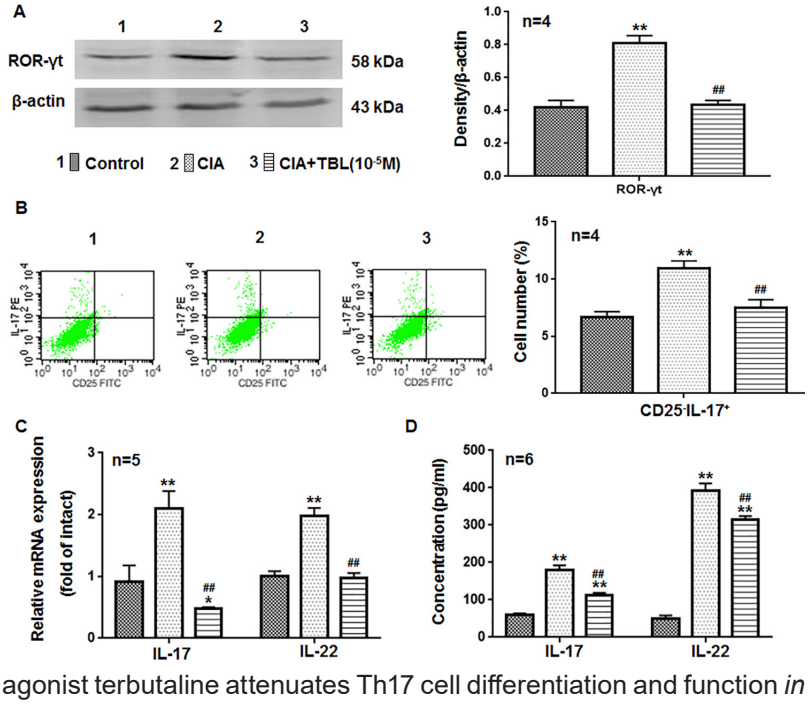

Figure 4. β2-AR agonist terbutaline attenuates Th17 cell differentiation and function *in vitro*. (A) ROR-γt protein expression was detected by the Western blot for Th17 cell's differentiation. (B) $CD25^{-}IL17^{+}$ cell number in $CD4^{+}$ T cells was explored by the flow cytometric assay for Th17 cell's differentiation. (C) The mRNA expressions of IL-17/22 were detected by real time-PCR for Th17 cell's function. (D) Concentrations of IL-17/IL-22 in cell supernatants were determined by ELISA for Th17 cell's function. *P<0.05, **P<0.01 vs. the control; ##P<0.01 vs. the CIA. CIA: Collagen type II-induced arthritis; TBL: Terbutaline

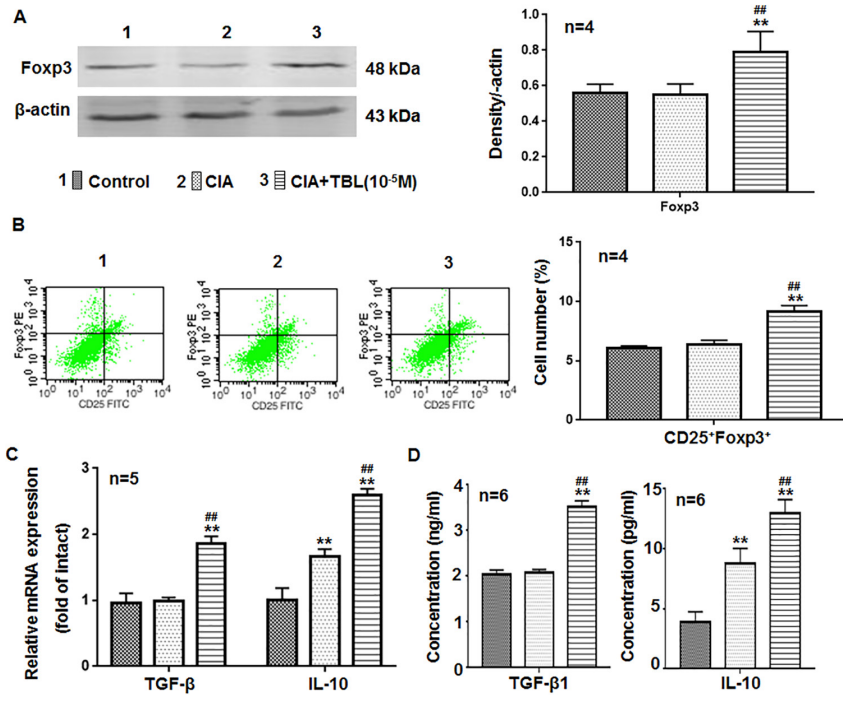

Figure 5. β2-AR agonist terbutaline enhances Treg cell differentiation and function *in vitro*. (A) Foxp3 protein expression was assessed by the Western blot for Treg cell's differentiation. (B) $CD25^{+}Foxp3^{+}$ cell number in $CD4^{+}$ T cells was tested by the flow cytometric assay for Treg cell's differentiation. (C) The gene expressions of TGF-β/IL-10 were detected by real time-PCR for Treg cell's function. (D) The concentrations of TGF-β1/IL-10 were performed by ELISA for Treg cell's function. **P<0.01 vs. the control; ##P<0.01 vs. the CIA. CIA: Collagen type II-induced arthritis; TBL: Terbutaline

detected by real time-PCR and IL-17/22 concentrations in supernatants from cell culture detected by ELISA were performed for Th17 cell's function. In contrast to the control group, IL-17/22 gene expressions in the CIA group notably increased. After the addition of TBL, they dramatically decreased (Figure 4C). IL-17/IL-22 concentration changes were consistent with their changes of mRNA expressions (Figure 4D).

β2-AR Agonist Terbutaline Enhances Treg Cell Differentiation and Function in Vitro

Protein expression of Foxp3 (Treg cell-specific transcription factor) detected by the Western blot analysis and Treg cell number detected by the flow cytometry were explored for Treg cell differentiation. The difference between the control and the CIA groups was not significant. After TBL addition, Foxp3 protein expression and Treg cell number dramatically increased (Figures 5A, B). In addition, TGF-β/IL-10 mRNA expressions detected by real time-PCR and TGF-β1/IL-10 concentrations in supernatants from cell culture detected by ELISA were performed for Treg cell's function. In contrast to the control, the IL-10 gene level in the CIA group increased. After TBL addition, TGF-β/IL-10 mRNA expressions dramatically increased (Figure 5C). TGF-β1/IL-10 concentration changes were consistent with their changes in the mRNA expressions (Figure 5D).

DISCUSSION

In this study, the CIA mice manifested joint inflammation, cartilage destruction, synovial hyperplasia, and serum anti-CII IgG antibody elevation. Importantly, the TBL treatment alleviated the joint inflammation and joint pathological injury, showing that β2-AR activation ameliorates the CIA symptoms. The β2-AR is activated in the body by both the NE released from the sympathetic

nerves and the epinephrine secreted from the adrenal gland. Joint tissues and lymphocytes have sympathetic innervation and β2-AR expression [1, 9-11]. The TBL treatment *in vivo* mimicked an enhanced NE and epinephrine release and as a result, β2-AR was activated. Accordingly, our present data suggest that the NE or epinephrine may promote the CIA recovery by activating β2-AR.

Th17/Treg imbalance has been linked to RA and the CIA pathogenesis [19, 20, 25]. In the current study, in the CIA mice, IL-17/22 production increased in the ankle joints, demonstrating that Th17 cell functional activity is enhanced in the CIA. Interestingly, IL-10/TGF-β production also increased in the ankle joints, although the increased degree was not as high as that of IL-17 and IL-22. The unexpected increase may be explained by a compensatory or protective mechanism of Treg cell functional activity in the CIA [20]. Since the enhancement of Treg cells did not reach the level of Th17 cells, Th17/Treg are imbalanced at a high level according to the CIA. More importantly, in the CIA mice, TBL treatment reduced IL-17/22 production but elevated TGF-β/IL-10 levels in the ankle joints. The data reveal that Th17 pro-inflammatory response is inhibited by the activation of β2-AR, while Treg anti-inflammatory activity is enhanced, and therefore ameliorates Th17/Treg imbalance. These effects are beneficial for the slowdown of the CIA's progress.

To further demonstrate that β2-AR activation mitigates Th17/Treg imbalance in the CIA, we assessed *in vitro* TBL effects on $CD3^+$ T cell differentiation and function from the CIA mice. $CD3^+$ T cells of the CIA highly expressed ROR-γt, a transcriptional factor specifically expressed by Th17 cells and had an enhancive proportion of $CD4^+CD25^-$ $IL\text{-}17^+$ cells, Th17 cell's phenotype. This shows that the CIA induces an increase in Th17 cell frequency. Consistently, IL-17/22 production by $CD3^+$ T cells from the CIA mice elevated, indicating that the CIA induces enhancement of function or activity of Th17 cells. These results demonstrate that the CIA promotes the polarization of $CD3^+$ T cells toward Th17 cells. Notably, TBL attenuated the CIA-induced polarization of $CD3^+$ T cells toward Th17 cells. The findings imply that β2-AR activation on $CD3^+$ T cells of the CIA inhibits differentiation of the cells toward pro-inflammatory Th17 cells. Unlike Th17 cells, the Treg cell level that is represented by specific transcriptional factor Foxp3 expression and $CD4^+CD25^+Foxp3^+$ cell percentage of $CD3^+$ T cells did not alter in the CIA. Moreover, TGF-β production did not change either in $CD3^+$ T cells of the CIA, although the IL-10 level increased by the CIA. Since the IL-10 is produced not only by the Treg cells but also by Th2 cells, an increase in this protein may explain a Th2 response in the CIA [12, 13]. Accordingly, we propose that the CIA does not attenuate $CD3^+$ T cell differentiation toward Treg cells. The findings *in vitro* are consistent with the results *in vivo* and other reports [26, 27]. Importantly, TBL raised the Treg cell's frequency and function in the CIA. These findings suggest that β2-AR activation of the CIA $CD3^+$ T cells promotes cell differentiation and function toward Treg cells. Collectively, the activation of β2-AR on $CD3^+$ T cells of the CIA shifts the bias of differentiation and function from pro-inflammatory Th17 cells to anti-inflammatory Treg cells. These effects alleviate Th17/Treg imbalance and therefore ameliorate the CIA symptoms. Thus, promoting sympathetic and adrenal activities or giving an agonist of β2-AR may become a therapeutic strategy for the CIA.

In summary, the intraperitoneal injection of β2-AR agonist TBL improved the CIA symptoms including the joint inflammation and joint pathological injury. Simultaneously, in the CIA, TBL impaired the Th17 cell's activity and enhanced the Treg cell's function. In addition, the exposure of $CD3^+$ T cells of the CIA to TBL attenuated Th17 cell frequency and function but raised Treg cell frequency and activity. The results indicate that the activation of β2-AR in $CD3^+$ T cells

promotes cell differentiation and function from predominant Th17 cells toward Treg cells in the CIA. This represents pro-inflammatory activity reduction and anti-inflammatory activity enhancement, i.e., mitigation of Th17/Treg imbalance. Thus, the activation of β2-AR in T lymphocytes may become a novel therapeutic strategy for the CIA and RA.

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Conflict of Interest: None declared.

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