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# Norepinephrine Inhibits Th17 Cells via $\beta$ 2-Adrenergic Receptor ( $\beta$ 2-AR) Signaling in a Mouse Model of Rheumatoid Arthritis

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**Background:** Norepinephrine (NE), a neurotransmitter released from the sympathetic nerves, has been shown to be involved in rheumatoid arthritis (RA). However, its role in the sympathetic nervous system in RA is divergent. Herein, we demonstrate that the sympathetic neurotransmitter NE exerts an anti-inflammatory effect in collagen-induced arthritis (CIA), a mouse model of RA, by inhibiting Th17 cell differentiation and function via  $\beta$ 2-adrenergic receptor ( $\beta$ 2-AR) signaling.





**Material/Methods:** CIA was prepared by intradermal injection of collagen type II in the tail base of DBA1/J mice. On the 41<sup>st</sup> day post-immunization, the mice were used as CIA models. CD4<sup>+</sup> T cells from the spleen were purified using magnetic cell sorting and activated with anti-CD3 anti-CD28 antibodies. Th17 cells were polarized from the CD4<sup>+</sup> T cells using various antibodies and cytokines.

**Results:** Co-expression of CD4 and  $\beta$ 2-AR was observed in spleens of both intact and CIA mice. The  $\beta$ 2-AR expression in the ankle and spleen was downregulated in CIA mice. CIA induced increases in production of interleukin (IL)-17 and IL-22, CD25<sup>+</sup>IL-17<sup>+</sup> cell percentage, and ROR- $\gamma$ t expression in CD4<sup>+</sup> T cells. Importantly, NE reduced the CIA-induced CD4<sup>+</sup> T cell shift towards Th17 phenotype, and the  $\beta$ 2-AR antagonist ICI118551 blocked the NE effect. Moreover, the  $\beta$ 2-AR agonist terbutaline (Terb) inhibited CIA-induced CD4<sup>+</sup> T cell proliferation and shift towards Th17 phenotype, and the protein kinase A (PKA) inhibitor H-89 abolished the agonist effect. Terb also reduced CIA-induced Th17 enhancement, and H-89 impaired the Terb effect.

**Conclusions:** NE inhibits Th17 cell differentiation and function in CIA condition by activation of  $\beta$ 2-AR/PKA signaling.

**MeSH Keywords:** **Arthritis, Experimental • CD4-Positive T-Lymphocytes • Norepinephrine • Receptors, Adrenergic • Th17 Cells**

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## Background

Rheumatoid arthritis (RA), a representative human autoimmune disease, is characterized by joint inflammation with subsequent destruction of cartilage, pannus formation, and infiltrates of immune cells [1–3]. Although the exact pathogenesis of RA is unknown, some observations suggest that CD4<sup>+</sup> T lymphocytes play a pivotal role in induction or perpetuation of this chronic inflammatory disease [4]. CD4<sup>+</sup> T cells, on activation and expansion, develop into different T cell subsets, including helper T (Th)1, 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 [5–7]. Th17 cells are the most recently discovered members of the effector Th cell family and are characterized by high pro-inflammation via producing specific cytokines such as interleukin (IL)-17 and IL-22 [8,9]. In RA, earlier studies showed that Th1 cells are enriched in the joints of these patients [10–12]. Subsequent studies revealed similar findings for Th17 cells in the joints of RA subjects [13]. The Th17 cells are associated with immune pathology in arthritis [14]. Particularly, Th17 cells have been reported to be critical to the pathogenesis of collagen type II (CII)-induced arthritis (CIA), a murine model of autoimmune arthritis [15]. CIA shares many pathological and histological similarities with human RA [16]; therefore, it was used in this study as an animal model of RA.

Norepinephrine (NE), a neurotransmitter released from sympathetic nerve fibers in the peripheral nervous system, has been shown to be involved in RA and CIA. Synovial tissue is perfectly innervated with sympathetic nerve fibers, and the sympathetic neurotransmitter NE has anti-inflammatory effect when its concentration is high [17–19]. Unfortunately, sympathetic nerve fibers are lost in inflamed tissue of patients with RA [20] and in synovial tissue and lymph nodes of animals with CIA [21,22]. On the other hand, the effect of the sympathetic nervous system on the inflamed synovial tissue in RA is different, depending on the phase of inflammation. In the early acute phase of experimental arthritis, the sympathetic nervous system has a pro-inflammatory role [21,23,24], whereas in the late chronic phase anti-inflammatory effects have been described [21]. Therefore, clarifying the effect of the sympathetic neurotransmitter NE on Th17 cells, the crucial pro-inflammatory cells in RA pathogenetic process, is important for better understanding of the role of the sympathetic nervous system in RA.

NE acts on target cells by binding to the receptors,  $\alpha$ -adrenergic receptor ( $\alpha$ -AR) or  $\beta$ -AR. Immune cells express both  $\alpha$ -AR and  $\beta$ -AR, and T and B lymphocytes express  $\beta$ 2-AR almost exclusively [25]. Engagement of  $\beta$ 2-AR activates a cascade of signaling intermediates, including cAMP and protein kinase A (PKA),

which leads to the phosphorylation of cellular proteins [25]. We previously showed that NE promotes a shift of Th1/Th2 balance towards Th2 response by activating  $\beta$ 2-AR [26]. However, it is not known whether the  $\beta$ 2-AR-cAMP-PKA signaling pathway is involved in the action of NE on Th17 cells in CIA condition. Thus, in the present study, we firstly determined the expression of  $\beta$ 2-AR by CD4<sup>+</sup> T cells and the change of the expression in CIA condition; secondly, we assessed the effects of NE on Th17 cell differentiation and function by measurement of the percentage of Th17 cells, the expression of retinoic acid-related orphan receptor- $\gamma$ t (ROR- $\gamma$ t), a specific transcriptional factor of Th17 cells, the production of IL-17 and IL-22, and the proliferative response of CD4<sup>+</sup> T cells in CIA condition; thirdly we showed that the  $\beta$ 2-AR-cAMP-PKA signaling pathway mediated the NE effects on Th17 cells in CIA condition. This investigation thereby provides potentially useful information for treatment of RA with  $\beta$ 2-AR signaling activators or stimulators targeting Th17 cells.

## Material and Methods

### Induction of CIA mice

The induction of CIA mice was as described previously [7]. Briefly, male DBA1/J mice (8–10 weeks old, from Slack Experimental Animal Center, Shanghai, China) were intradermally injected in the tail base with 100  $\mu$ l emulsion containing 100  $\mu$ g CII (Sigma-Aldrich, USA) on day 0. On day 21, the mice were intraperitoneally boosted with 100  $\mu$ g CII emulsified with an equal volume of incomplete Freund's adjuvant (Sigma-Aldrich, USA). An intraperitoneal injection of 20  $\mu$ g lipopolysaccharide dissolved in 20  $\mu$ l PBS was executed to the mice on day 28. Mice were sacrificed on the 41<sup>st</sup> day after the first immunization with CII as CIA models for subsequent *in vivo* and *in vitro* experiments.

### Immunofluorescence staining

The spleens were fixed in 4% paraformaldehyde for 24 h. The spleen sections (25  $\mu$ m thick) were mounted on glass slides and processed for immunofluorescence staining. To block non-specific binding sites, the spleen sections were exposed to phosphate-buffered saline (PBS) containing 3% goat serum and 1% Triton X-100 for 30 min at room temperature. The sections were stained doubly with rat anti-CD4 antibody (1: 400; Serotec, UK) and rabbit anti- $\beta$ 2-AR antibody (1: 200; Abcam, UK), which were incubated with Alexa Fluor-conjugated secondary antibodies (1: 200; Molecular Probes, USA). A confocal microscope (Leica, Germany) was used to view and acquire the images.

### CD4<sup>+</sup> T cell purification and activation, and Th17 cell polarization

Naive CD4<sup>+</sup> T cells were obtained using magnetic cell sorting from the spleens of DBA1/J mice. Sorted cells were suspended in RPMI 1640 medium containing 10% heat-inactivated calf serum at the final concentration of  $5 \times 10^6$  cells/ml and stimulated with anti-CD3 antibody (2 µg/ml; BD Pharmingen, USA) and anti-CD28 antibody (2 µg/ml; BD Pharmingen, USA) for 24 h. Subsequently, the activated CD4<sup>+</sup> T cells were exposed to various treatments.

For Th17 cell polarization, as described previously [27], the purified CD4<sup>+</sup> T cells were activated with anti-CD3 and anti-CD28 antibodies and stimulated with anti-IL-4-neutralizing and anti-interferon (IFN)- $\gamma$ -neutralizing antibodies (both 10 µg/ml; BD Pharmingen, USA) plus a Th17 'cocktail' containing transforming growth factor (TGF)- $\beta$ 1 (3 ng/ml; R&D Systems, USA), IL-6 (30 ng/ml; R&D Systems, USA), tumor necrosis factor (TNF)- $\alpha$  (10 ng/ml; Peprotech, USA), IL-1 $\beta$  (10 ng/ml; Peprotech, USA), and IL-23 (20 ng/ml; Peprotech, USA) for 48 h. Subsequently, the polarized Th17 cells were exposed to various treatments.

### Drug treatments

The activated CD4<sup>+</sup> T cells were exposed to NE ( $10^{-5}$  M; Sigma-Aldrich, USA) for 24 h. To show that  $\beta$ 2-AR mediates the NE effect, a highly selective  $\beta$ 2-AR antagonist ICI118551 (ICI,  $10^{-5}$  M; Sigma-Aldrich, USA) was applied to the activated CD4<sup>+</sup> T cells for 30 min, and then NE acted on the cells for 24 h. The activated CD4<sup>+</sup> T cells were also treated with the specific  $\beta$ 2-AR agonist terbutaline (Terb,  $10^{-6}$  or  $10^{-5}$  M; Sigma-Aldrich, USA) for 24 or 72 h according to different experiments, or treated combined with the PKA inhibitor H-89 ( $10^{-5}$  or  $10^{-4}$  M; Sigma-Aldrich, USA) 30 min earlier and the  $\beta$ 2-AR agonist Terb for 72 h. Subsequent analyses as described below were performed.

In addition, the polarized Th17 cells were exposed to the  $\beta$ 2-AR agonist Terb for 24 h, or exposed combinedly to H-89 at 30 min earlier and Terb for 24 h, followed by the subsequent analyses.

### Western blot analysis

Total proteins were extracted from the spleens and ankle joints of mice or from *in vitro* cultured CD4<sup>+</sup> T cells and Th17 cells. Briefly, tissues or cells were homogenized in lysis buffer, which contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, and 10 µl/ml protease inhibitor. By centrifuging at 4°C at 12,000 rpm for 15 min, the supernatants were obtained. The proteins were separated and transferred to membranes according to our previous description [7]. After blocking nonspecific binding, the membranes were incubated with rabbit

antibodies against  $\beta$ 2-AR (1: 200; Abcam, UK), ROR- $\gamma$ t (1: 500; Abcam, UK), IL-17 (1: 200; Santa Cruz Biotechnology, USA), IL-22 (1: 200; Santa Cruz Biotechnology, USA), PKA (1: 200; Santa Cruz Biotechnology, USA), or mouse anti- $\beta$ -actin antibody (1: 5000; Sigma, USA) at 4°C overnight. Following incubation with the corresponding secondary antibodies (1: 5,000; Rockland Immunochemicals, USA), the membranes were visualized using Odyssey laser scanning system (LI-COR Inc, USA), and the protein band intensities were quantified by an image analysis system (Odyssey 3.0 software).

### Quantitative real-time PCR measurement

Total RNA was extracted from CD4<sup>+</sup> T cells or Th17 cells using Trizol reagent (Invitrogen, USA), and cDNA was subsequently generated by reverse transcription kit (Roche, Germany), following the manufacturers' instructions. The PCR reactions (94°C, 20 s; 60°C, 20 s; 72°C, 20 s) were performed on a Rotor-Gene 3000 Real-Time Cycler (Corbett Research, Australia) using Universal SYBR Green Master Mix (Roche, Germany). Species-specific mouse primers were used as follows: 5'-GCTCCAGAAGGCCCTCAGA-3' and 5'-AGCTTTCCTCCGATTGA-3' for IL-17; 5'-GGCCAGCCTTGCAGATAACA-3' and 5'-GCTGATGTGACAGGAGCTGA-3' for IL-22; 5'-ACCCACACTGTGCCATCTA-3' and 5'-GCCACAGGATTCCATACCA-3' for  $\beta$ -actin.

Relative gene expression was calculated as fold-change over control using the  $2^{-\Delta\Delta Ct}$  method after normalization to  $\beta$ -actin.

### Flow cytometric assay for CD25-IL-17<sup>+</sup> cell percentage

This method referred to our previous work [27]. Activated CD4<sup>+</sup> T cells were incubated with 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich, USA), 1 µM ionomycin (Sigma-Aldrich, USA), and 2 µM monensin (BD Pharmingen, USA) for 5 h at 37°C. After surface staining with FITC-conjugated anti-CD25 antibody (BD Pharmingen, USA), the cells were resuspended in Fixation/Permeabilization solution (Cytofix/Cytoperm kit; BD Pharmingen, USA), and then stained intracellularly with phycoerythrin-conjugated anti-IL-17 antibody (eBioscience, USA). All samples were analyzed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences, USA).

### Flow cytometric assay for CD4<sup>+</sup> T cell proliferation

Carboxy fluorescein diacetate succinimidyl ester (CFSE; Invitrogen, USA) labeling was used to measure CD4<sup>+</sup> T lymphocyte proliferation. Briefly, purified CD4<sup>+</sup> T cells were incubated with CFSE (2 µM) for 15 min at 37°C in the dark. The labeling reaction was terminated by adding an equal volume of calf serum for 1 min. The CD4<sup>+</sup> T cells were stimulated with anti-CD3 and anti-CD28 antibodies and treated with H-89 and Terb

as described above, which was incubated for 72 h. Percentage of proliferative CD4<sup>+</sup> T cells was analyzed on a flow cytometer.

### Enzyme-linked immunosorbent assay (ELISA)

The culture supernatants of either CD4<sup>+</sup> T cells or Th17 cells were harvested and analyzed for IL-17 and IL-22 concentrations with ELISA (eBioscience, USA) following the manufacturer's instructions. The lysates of cultured Th17 cells were harvested and quantified for cAMP concentration with ELISA (Biovision, USA) according to the manufacturer's instructions.

### PKA activity assay

The lysates of cultured Th17 cells were harvested and assessed for PKA activity using the assay kit (Enzo Life Sciences, USA) in accordance with the manufacturer's instructions.

### Statistical analysis

The Statistical Package for the Social Science (SPSS, 16.0) was used in the statistical analyses. The data were expressed as the mean  $\pm$  standard deviation. Comparisons between 2 groups were assessed using Student's *t* tests. Multiple comparisons among the groups were evaluated by one-way analysis of variance, followed by a post hoc analysis. The results were considered statistically significant at  $p < 0.05$ .

## Results

### **$\beta$ 2-AR is expressed by CD4<sup>+</sup> T cells and CIA downregulates this expression**

On the 41<sup>st</sup> day following the first immunization with CII, the mice reached a peak in clinical score of 4 paws and manifested 4 inflamed paws, elevated anti-CII-IgG antibody level in the serum, destruction of articular cartilages in the ankle joints, and infiltrated inflammatory cells in the synovial tissue of the ankle joints (data not shown). These results confirmed that CII induced an RA-like arthritis. Thus, the mice that were on the 41<sup>st</sup> day following the first immunization with CII were used as CIA models in subsequent experiments.

Co-expression of CD4 and  $\beta$ 2-AR was observed in the spleens of both intact and CIA mice (Figure 1A). Further experiments found that the  $\beta$ 2-AR expression was downregulated in both the ankle and spleen tissues of CIA mice compared with that of intact mice (Figure 1B). The data showed that CD4<sup>+</sup> T cells expressed  $\beta$ 2-AR, and the expression was downregulated in CIA condition.

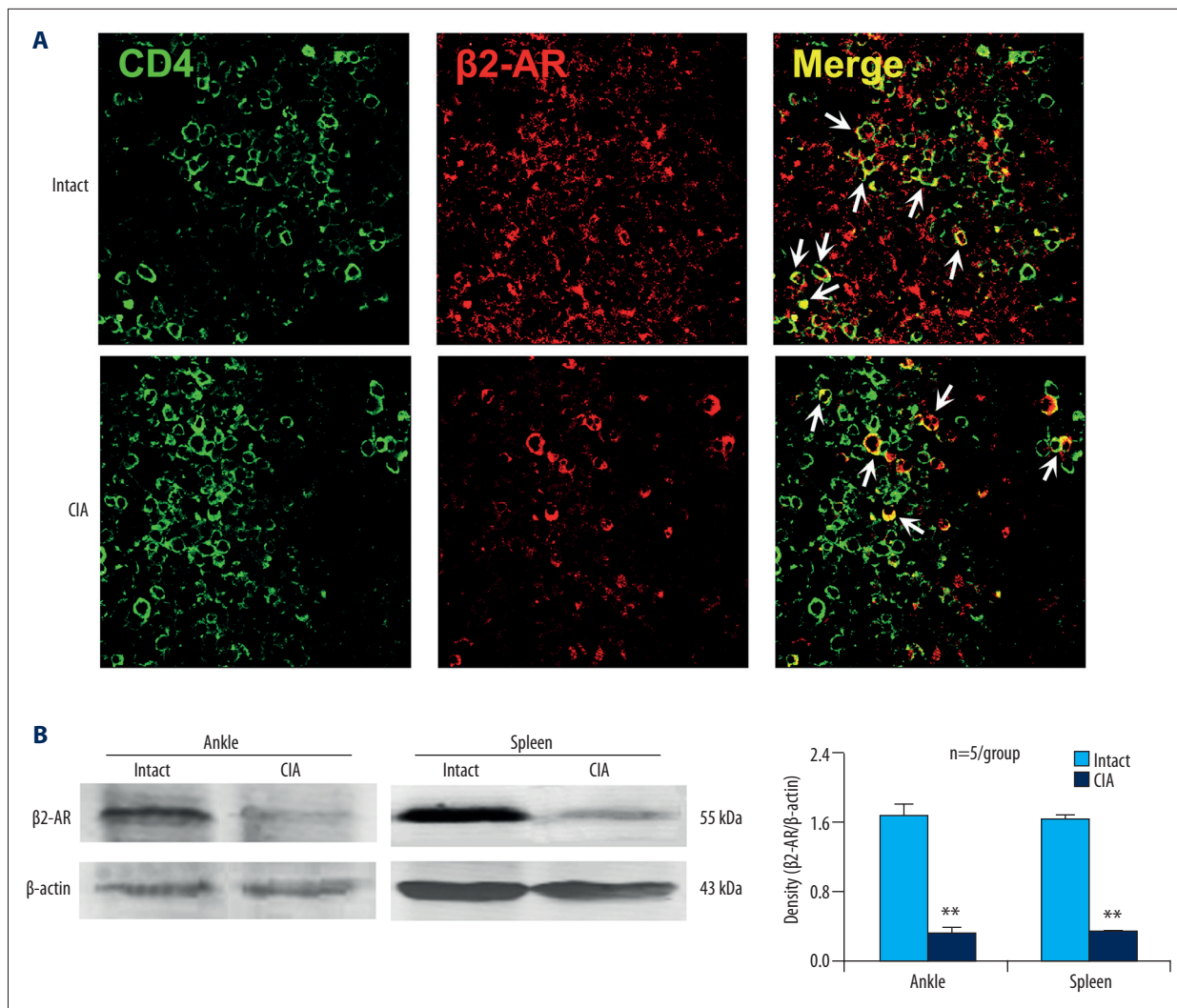
### **NE reduces CIA-induced CD4<sup>+</sup> T cell shift towards Th17 phenotype and $\beta$ 2-AR antagonist blocks the NE effect**

CD4<sup>+</sup> T cells were separated from the spleens of both CIA and intact mice by magnetic beads and activated with anti-CD3 and anti-CD28 antibodies. Percentage of CD25-IL-17<sup>+</sup> cells, which represent Th17 cells, in CD4<sup>+</sup> T cells was more in CIA condition than in intact condition (control) (Figure 2A). Simultaneously, expression of the Th17-related transcriptional factor ROR- $\gamma$ t and cytokines IL-17 and IL-22 was upregulated in CD4<sup>+</sup> T cells derived from CIA mice compared with controls (Figure 2B). Importantly, NE treatment of CD4<sup>+</sup> T cells from CIA mice reduced the percentage of CD25-IL-17<sup>+</sup> cells and the expression of ROR- $\gamma$ t, IL-17, and IL-22, with respect to NE-untreated CD4<sup>+</sup> T cells from CIA mice (Figure 2A, 2B). The results indicated that NE inhibited CIA-induced CD4<sup>+</sup> T cell shift towards Th17 phenotype.

To show that the NE effect is mediated by  $\beta$ 2-AR, we added the  $\beta$ 2-AR antagonist ICI to NE-treated CD4<sup>+</sup> T cells. The reduction of CIA-induced expression of IL-17 and IL-22 in CD4<sup>+</sup> T cells by NE was blocked by the  $\beta$ 2-AR antagonist ICI (Figure 2C). In addition, IL-17 and IL-22 levels in the supernatants of CD4<sup>+</sup> T cell cultures were higher in CIA condition than in controls (Figure 2D). Importantly, NE reduced the CIA-induced secretion of IL-17 and IL-22 from CD4<sup>+</sup> T cells (Figure 2D). More importantly, the  $\beta$ 2-AR antagonist ICI abolished the NE effect of reducing IL-17 and IL-22 secretion from CD4<sup>+</sup> T cells in CIA condition (Figure 2D). The data showed that  $\beta$ 2-AR mediated the NE effect of inhibiting CIA-induced CD4<sup>+</sup> T cell shift towards Th17 phenotype.

### **$\beta$ 2-AR agonist inhibits CIA-induced CD4<sup>+</sup> T cell shift towards Th17 phenotype and this agonist effect is abolished by PKA inhibitor**

To further demonstrate the role of  $\beta$ 2-AR expressed on CD4<sup>+</sup> T cells in CIA-induced Th17 cell differentiation and function, we applied the  $\beta$ 2-AR agonist Terb to the activated CD4<sup>+</sup> T cells derived from CIA mice. The increased percentage of CD25-IL-17<sup>+</sup> cells in CD4<sup>+</sup> T cells in CIA condition was reduced by the  $\beta$ 2-AR agonist Terb (Figure 3A). Simultaneously, the upregulated expression of ROR- $\gamma$ t, IL-17, and IL-22 in CD4<sup>+</sup> T cells in CIA condition was inhibited by the  $\beta$ 2-AR agonist Terb (Figure 3B). In addition, CIA induced CD4<sup>+</sup> T cell proliferation and this effect was also inhibited by the  $\beta$ 2-AR agonist Terb (Figure 3C). Notably, the PKA inhibitor H-89 blocked the effect of Terb inhibiting CIA-induced CD4<sup>+</sup> T cell proliferation (Figure 3C). These data demonstrated that activation of  $\beta$ 2-AR impaired CIA-induced CD4<sup>+</sup> T cell shift towards Th17 phenotype via PKA signaling.



**Figure 1.**  $\beta 2$ -AR is expressed by CD4<sup>+</sup> T cells and CIA downregulates this expression. **(A)** Immunofluorescence staining of the spleen sections. The arrows point to the typical cells of co-expressing CD4 and  $\beta 2$ -AR. **(B)**  $\beta 2$ -AR expression levels in the ankle and spleen assessed by Western blot analysis. \*\*  $p < 0.01$ , versus intact mice.

### $\beta 2$ -AR agonist reduces CIA-induced Th17 cell enhancement and the agonist effect is impaired by PKA inhibitor

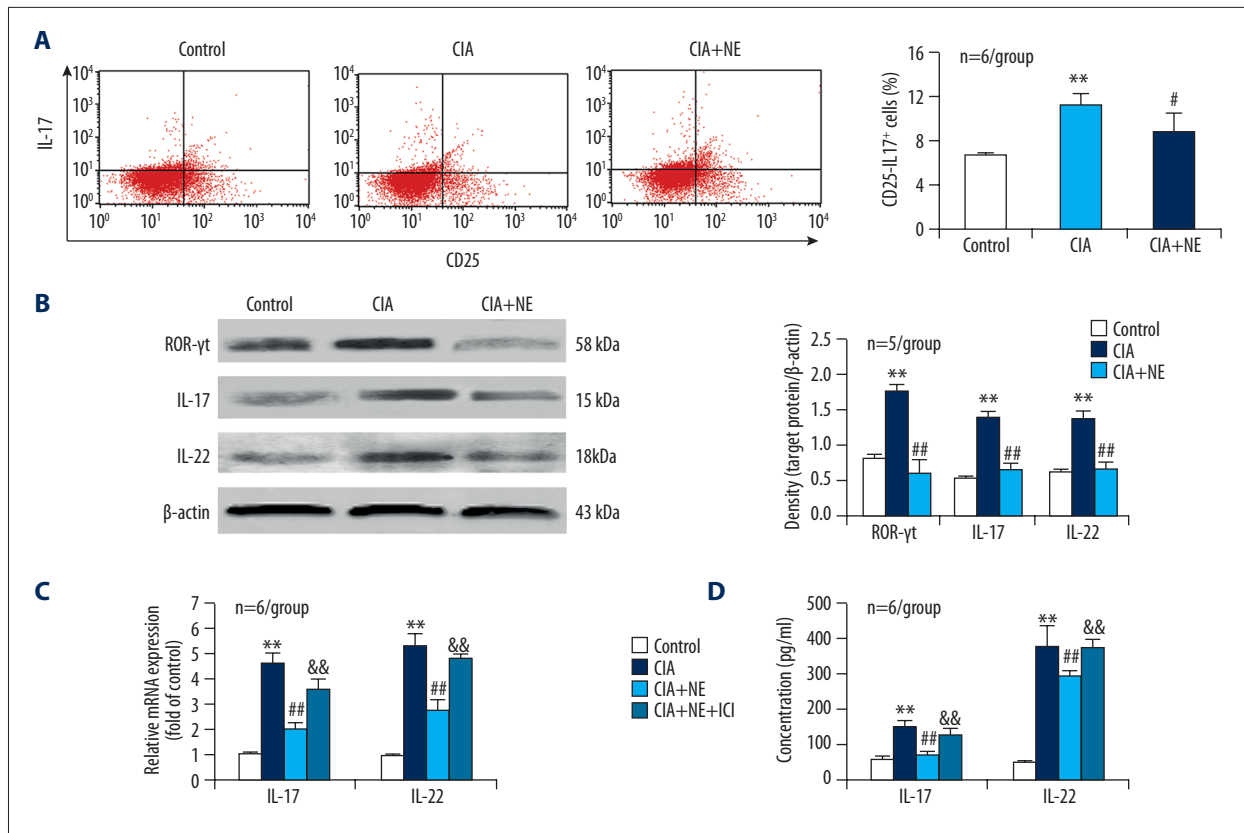
To determine whether  $\beta 2$ -AR/PKA signaling has a direct effect on Th17 cells, we applied the  $\beta 2$ -AR agonist Terb and the PKA inhibitor H-89 to Th17 cells that were activated and polarized from CD4<sup>+</sup> T cells using various antibodies and cytokines. Both the mRNA expression of IL-17 and IL-22 in Th17 cells and the concentrations of IL-17 and IL-22 in the supernatants of Th17 cell cultures were increased in CIA condition compared to controls (Figure 4A, 4B). Importantly, the  $\beta 2$ -AR agonist Terb reduced the CIA-induced IL-17 and IL-22 expression in and secretion from Th17 cells (Figure 4A, 4B). More importantly, the PKA inhibitor H-89 impaired the effects of Terb reducing CIA-induced IL-17 and IL-22 expression in and secretion from Th17 cells (Figure 4A, 4B). These findings demonstrated

that activation of  $\beta 2$ -AR attenuated CIA-induced Th17 cell enhancement via PKA signaling.

To confirm that the cAMP-PKA signaling pathway in Th17 cells is activated by the  $\beta 2$ -AR agonist Terb, we tested cAMP and PKA levels in response to Terb in Th17 cells from CIA mice. Exposure to the  $\beta 2$ -AR agonist Terb increased cAMP production (Figure 4C), PKA activity (Figure 4D) and expression (Figure 4E) in Th17 cells. These data confirmed that the cAMP-PKA signaling pathway in Th17 cells from CIA mice was activated by the  $\beta 2$ -AR agonist Terb.

### Discussion

CD4<sup>+</sup> T cells expressed  $\beta 2$ -AR and particularly Th17 cells produced a response to the  $\beta 2$ -AR agonist Terb in this study,

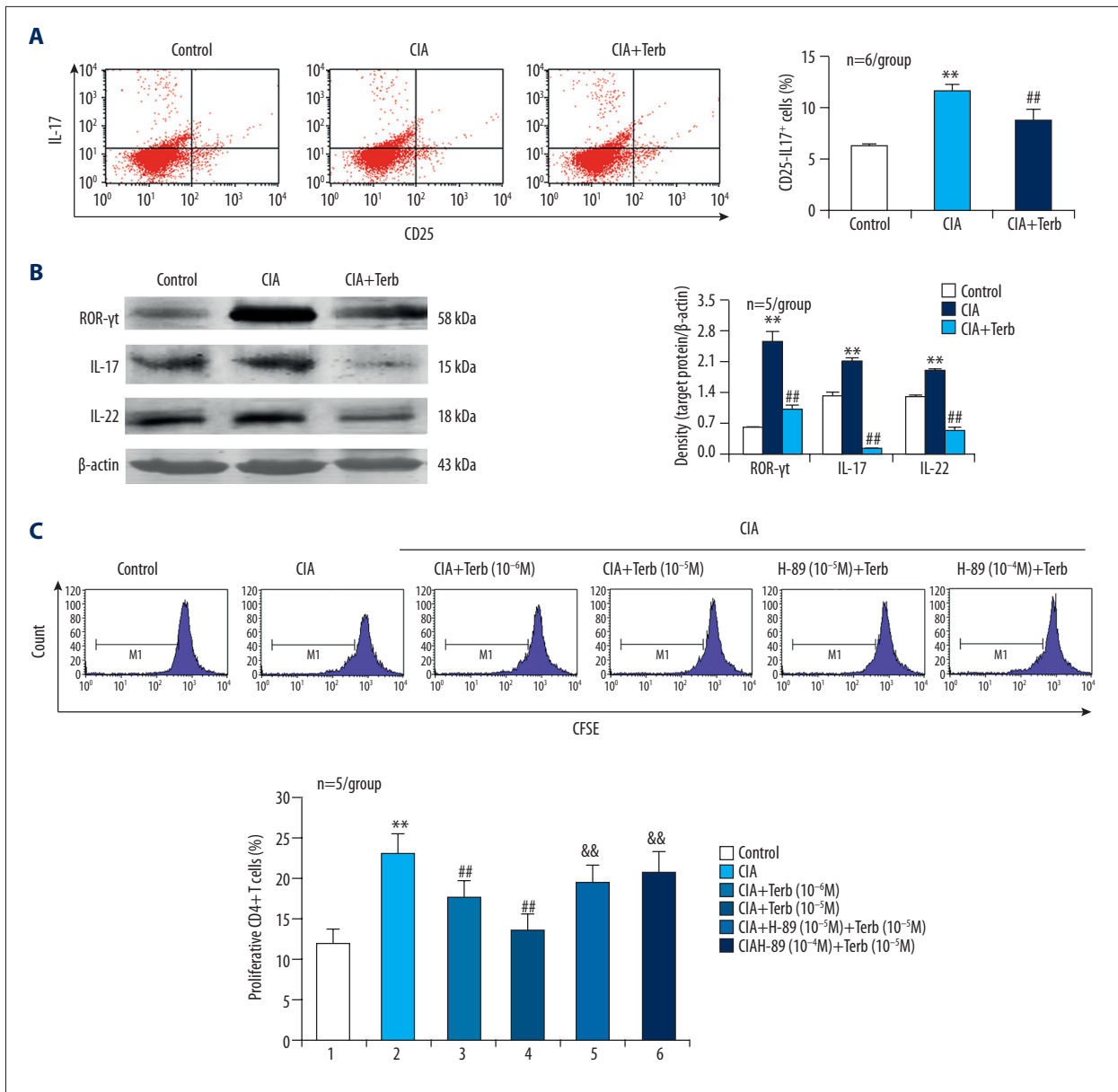


**Figure 2.** NE reduces CIA-induced CD4<sup>+</sup> T cell shift towards Th17 phenotype and this effect is blocked by the β<sub>2</sub>-AR antagonist ICI. **(A)** Flow cytometric assay for CD25<sup>+</sup>IL17<sup>+</sup> cell percentage in CD4<sup>+</sup> T cells from the spleen. **(B)** Protein expression levels of the Th17 cell specific transcriptional factor ROR-γt and the Th17 cell-related cytokines IL-17 and IL-22 in CD4<sup>+</sup> T cells assessed by Western blot analysis. **(C)** mRNA expression of IL-17 and IL-22 in CD4<sup>+</sup> T cells tested by real-time PCR. **(D)** Levels of IL-17 and IL-22 in the supernatants of CD4<sup>+</sup> T cell cultures determined by ELISA. \*\* *p*<0.01, versus control; # *p*<0.05; ## *p*<0.01, versus CIA group; && *p*<0.01, versus CIA + NE group.

suggesting that Th17 cells functionally express β<sub>2</sub>-AR. Although it is thought that β<sub>2</sub>-AR is expressed on all CD4<sup>+</sup> T cells, we demonstrated that naive CD4<sup>+</sup> T cells and effector Th1 cells express β<sub>2</sub>-AR, while effector Th2 cells do not [28–30]. Here, we provide new evidence for the expression of β<sub>2</sub>-AR on effector Th17 cells. More importantly, β<sub>2</sub>-AR expression was down-regulated in both the ankle and spleen in CIA condition in this study. Consistent with our results, in rats challenged to induce adjuvant arthritis, a model of RA, we show that during severe disease, β<sub>2</sub>-AR affinity and density decrease in the spleen and draining lymph nodes for the arthritic limbs, indicating receptor downregulation [31]. The decreased β<sub>2</sub>-AR expression in CIA suggests a disease-specific internalization and degradation of receptors, as explained by Lorton et al. [31].

Th17 cells produce IL-17 and IL-22 and express ROR-γt [32–36]. By testing IL-17, IL-22, and ROR-γt levels in CD4<sup>+</sup> T cells, we found that NE reduced CIA-induced CD4<sup>+</sup> T cell shift towards Th17 phenotype and β<sub>2</sub>-AR antagonist reversed the NE effect. Further investigation showed that activating β<sub>2</sub>-AR with the

agonist Terb attenuated CIA-induced CD4<sup>+</sup> T cell shift towards Th17 phenotype. These findings suggest that NE, via activating β<sub>2</sub>-AR on CD4<sup>+</sup> T cells, inhibits Th17 cell differentiation and function and thereby has an anti-inflammatory property in CIA. The cellular activity of naive and effector CD4<sup>+</sup> T cells is regulated primarily by cytokines and costimulatory molecules, as well as by neurotransmitters and hormones [37]. The neurotransmitter NE is stored in sympathetic nerve terminals that reside within the parenchyma of lymphoid tissue [38] and are in close association with CD4<sup>+</sup> T cells [39,40]. NE is released from these nerve terminals after antigen enters the system and binds to β<sub>2</sub>-AR expressed on immune cells [41]. Other studies support our present hypothesis that CIA stimulates sympathetic nerves to release NE, which acts on CD4<sup>+</sup> T cells by binding to β<sub>2</sub>-AR to inhibit inflammatory response. However, sympathetic nerve fibers are lost in inflamed tissue of patients with RA [20] and in synovial tissue and lymph nodes of animals with CIA [21,22]. As a compensatory mechanism for this deprivation of sympathetic neurotransmitter in the inflamed joints and lymphoid tissues, cells that are capable of producing

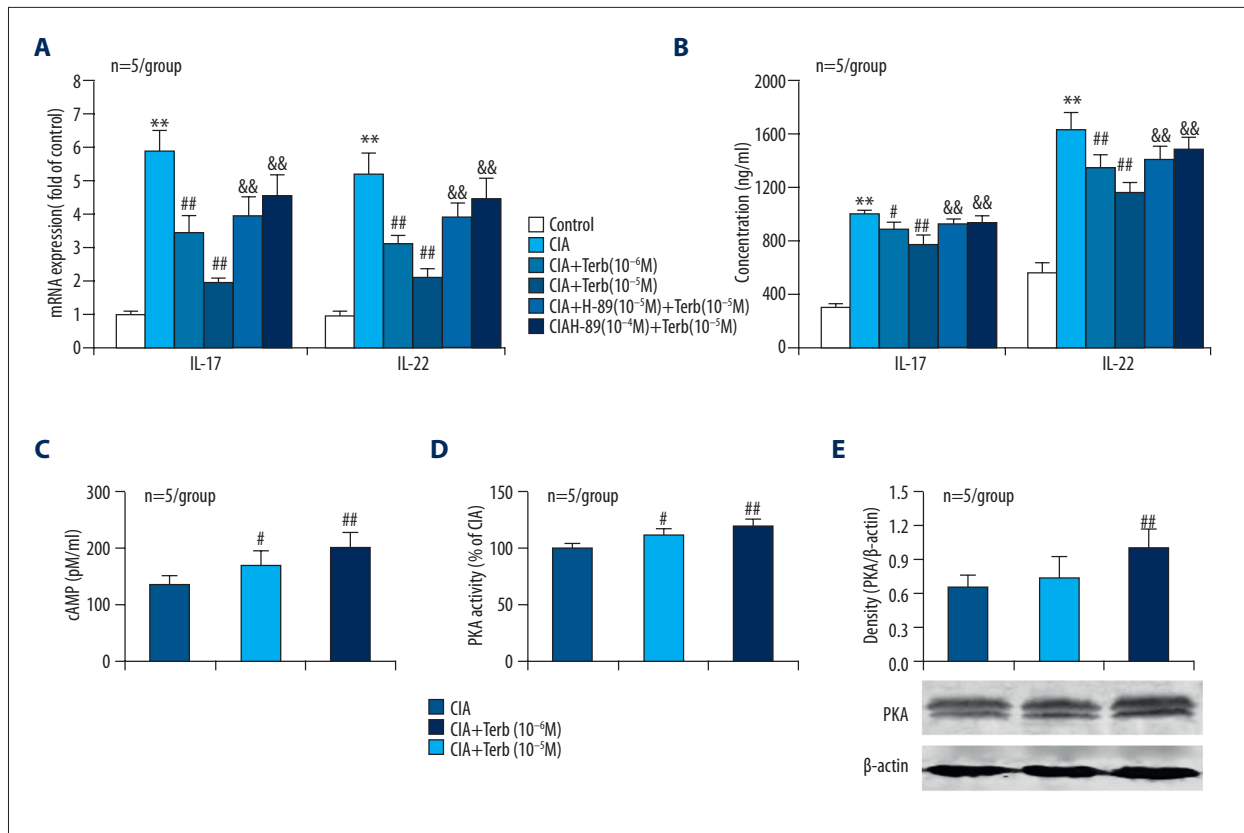


**Figure 3.** The β2-AR agonist Terb inhibits CIA-induced CD4<sup>+</sup> T cell shift towards Th17 phenotype and this effect is abolished by the PKA inhibitor H-89. **(A)** Flow cytometric assay for CD25-IL-17<sup>+</sup> cell percentage in CD4<sup>+</sup> T cells from the spleen. **(B)** Protein expression of the Th17 cell-specific transcriptional factor ROR-γt and the Th17 cell-related cytokines IL-17 and IL-22 in CD4<sup>+</sup> T cells. **(C)** Flow cytometric assay for CD4<sup>+</sup> T cell proliferative response. The cells in M1 area represent proliferative CD4<sup>+</sup> T cells. \*\* *p*<0.01, versus control; ## *p*<0.01, versus CIA group; && *p*<0.01, versus CIA + Terb group.

catecholamine neurotransmitters accumulate [7,42–44]. This tyrosine hydroxylase-positive catecholamine-producing cells have an anti-inflammatory property in human and experimental arthritis [7,43–46]. Our present findings support these reported results and provide further evidence for the anti-inflammatory property of the sympathetic neurotransmitter NE in CIA.

A direct inhibitory effect of the β2-AR agonist Terb on CIA-induced Th17 cell response was also observed in the current

study. This effect of the agonist was reduced by the PKA inhibitor H-89. These data suggest that NE can directly inhibit Th17 cell function via β2-AR/PKA signaling. Indeed, cAMP production and PKA expression and activity in Th17 cells were increased by the β2-AR agonist Terb in CIA condition in this study, confirming that in Th17 cells, β2-AR also is coupled with the downstream signaling pathway cAMP-PKA. It has been reported that cAMP-PKA signaling in CD4<sup>+</sup> T cells is important in the differentiation of Th subsets and their subsequent inflammatory



**Figure 4.** The  $\beta$ 2-AR agonist Terb reduces CIA-induced Th17 cell enhancement and this effect is impaired by the PKA inhibitor H-89. (A) mRNA expression levels of the pro-inflammatory cytokines in Th17 cells. (B) Concentrations of the pro-inflammatory cytokines in the culture supernatants of Th17 cells. (C) cAMP concentration in the lysates of cultured Th17 cells determined by ELISA. (D) PKA activity in the lysates of cultured Th17 cells. (E) PKA expression in Th17 cells. \*\*  $p < 0.01$ , versus control; #  $p < 0.05$ , ##  $p < 0.01$ , versus CIA group; &&  $p < 0.01$ , versus CIA + Terb group.

responses [47]. Here, we propose that activating  $\beta$ 2-AR-cAMP-PKA signaling in Th17 cells reduces the cell inflammatory response induced by CIA. Thus, an agonist or activator of  $\beta$ 2-AR-cAMP-PKA signaling may be helpful for alleviation of RA.

## Conclusions

$\beta$ 2-AR is expressed by CD4<sup>+</sup> T cells and this expression is down-regulated in CIA condition. NE reduces CIA-induced CD4<sup>+</sup> T cell

shift towards Th17 phenotype by activation of  $\beta$ 2-AR on the CD4<sup>+</sup> T cells. Activating  $\beta$ 2-AR-cAMP-PKA signaling in Th17 cells impairs the cell inflammatory response induced by CIA. These results suggest that the sympathetic neurotransmitter NE exerts an anti-inflammatory effect by inhibition of Th17 cell differentiation and function via  $\beta$ 2-AR/PKA signaling in CIA disease.

## Conflicts of interest

None.

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