

# Beta-endorphin prevents collagen induced arthritis by neuroimmuno-regulation pathway\*

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## Abstract

**OBJECTIVE:** To observe the effects and mechanisms of beta-endorphin ( $\beta$ -END) preventing collagen induced arthritis (CIA) by neuroimmuno-regulating pathway. **METHODS:** Female wistar (Ws) rats were used in this study. CIA was induced by Native bovine type II collagen emulsified with complete Freund's adjuvant (CFA).  $\beta$ -END was administered i.p. to CIA rats every other day from the 14th day (secondary immunization) to the 35th day after primary immunization. Clinical assessments were performed by two independent, blinded examiners every other day. Pathological and radiological observations were taken on the 35th day after the primary immunization. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), regulated upon activation, normal T-cell expressed and secreted (RANTES), inducible NO synthases (iNOS), matrix metalloproteinase-2 (MMP-2) and MMP-9 mRNA expression of synovium tissues of CIA rats was estimated by quantitative RT-PCR. The frequency of spleen Th1 and Th2 cells were assessed by fluorescence activated cell sorter (FACS) assay. **RESULTS:** Clinical manifestation of rats with CIA were significantly abrogated or ameliorated by treatment with  $\beta$ -END.  $\beta$ -END treatment in vivo could down-regulate mRNA expression of several pro-inflammatory cytokines, chemokines and MMPs in CIA synovial, and polarize Th1/Th2 balance to Th2. **CONCLUSION:**  $\beta$ -END alleviates CIA through both depressing Th1 responses and down-regulating proinflammatory and other rheumatic factors, suggesting  $\beta$ -END is a promising anti-inflammatory and anti-rheumatic agent in treating CIA.

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## Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that leads to inflammation in the joints and subsequent destruction of the cartilage and bone. Collagen induced arthritis (CIA) is a classical experimental model of RA induced by type collagen (C) [1]. The pivotal pathological change of RA and CIA is synovitis with a massive infiltration of immune cells releasing an excessive amount of chemokines and pro-inflammatory cytokines, consequently excessive synovial cells proliferating, at last leading to tissue destruction. It has also been discovered a polarization of Th1/Th2 response to Th1 in RA and CIA. All above suggests an important unbalance of the T response and excessive synovial cells function could be important in the etiology and development of RA and CIA [2,3].

Clinical features and experimental observations suggest a role of neuroimmune mechanisms in the pathogenesis of RA and CIA. Recently it is reported that the opioid peptides modulate excessive RA synovial cell functions in vitro, implying that these peptides not only act in an antinociceptive manner but also ameliorating synovitis in RA [4]. As an opioid neuropeptide,  $\beta$ -Endorphin ( $\beta$ -END) contributes to control of pain transmission by interacting with opioid receptors within the nervous system, exerting multiple important functions in the nerves and immune system [5]. In this study, we demonstrated  $\beta$ -END prevented CIA through modulating multiple inflammatory components, suggesting  $\beta$ -END is a potent ameliorating agent in CIA. Research on the role of  $\beta$ -END in RA is promising.

## Materials and methods

### Animals

Female wistar (Ws) rats between 6 and 8 weeks old were purchased from and housed in a special pathogen-free animal facility of the Peking Union Medical College Hospital (PUMCH) Animal Center. Animal care and handling conformed to institutional guidelines.

### Reagents

Native bovine type collagen (C), complete Freund's adjuvant (CFA),  $\beta$ -endorphin Rat ( $\beta$ -END,  $\beta$ -lipotropin 61-91, C157H254N42O44S, FW 3466) were purchased from Sigma (St. Louis, MO, U.S.A.). Reagents were initially diluted in phosphate buffered saline (PBS) to 10mmol/L, filter sterilized, and stored at -20 until use. Calipers were obtained from Peking Measuring Tools Co. Ltd. (Peking, China). Fluorescein isothiocyanate (FITC) labeled rat CD4 antibody and phycoerythrin (PE) labeled rat IL-4, IFN- $\gamma$  antibodies for flow cytometer (FCM) as well as goat anti-rat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Restriction endonucleases were purchased from Biolab Co. Ltd. (U.S.A.), and M-MULV was purchased from GIBCO-BRL (U.S.A.). Primers were designed using Primer premier 5.0 software and synthesized by Shanghai Sangon Co. Ltd (Shanghai, China).

### Inducement of CIA

Native bovine type II collagen was dissolved in 0.05mol/L acetic acid at 4°C overnight, then emulsified with CFA. 96 female wistar rats were injected s.c. at the base of the tail with 0.1ml emulsion containing 200 $\mu$ g of C for the primary immunization. 200 $\mu$ g C with PBS was i.p. injected on the 14th day later to reinforce immunization [1, 6-9].

### Assessment of CIA

For the analysis of CIA rats, signs of arthritis were monitored using representative parameters including paw swelling and clinical score by two independent, blinded examiners every other day from the 1st to the 35th day after the primary immunization. The level of paw swelling was measured by the thickness of the affected hind paws with 0 to 10-mm calipers. Arthritis symptoms were assessed by using a scoring system (grade 0, no swelling; grade 1, slight swelling and erythema; grade 2, middle edema; grade 3, pronounced edema; grade 4, joint rigidity and ankylosis). On the 35th day after the primary immunization, X-ray was performed on the hind paws and knees of the rats. Then rats were sacrificed by cervical dislocation, and paws were fixed with 10% paraformaldehyde, decalcified in 5% formic acid, embedded in paraffin. Sections (5 $\mu$ m) were stained with hematoxylin-eosin (HE) for histological observation [1,6-9]. At the same time spleens and synovial tissue were removed, Th/Th2 cell frequency was analyzed by fluorescence activated cell sorter (FACS) and mRNA level of several cytokines in synovial tissue was detected by quantitative RT-PCR.

### Treatment protocols

In each experiment, CIA rats were randomly divided into 4 groups, 1 control and 3 different concentration of  $\beta$ -END. Three separate experiments (8 CIA rats /group per experiment) were performed, two groups of experimenters assessed CIA in double-blinded manner. Therapy began after the secondary immunization (day 14 after the primary immunization), and rats were administered i.p. at the specified doses (0.1nmol, 1nmol, 5nmol) respectively every other day until day 35 after the primary immunization. The control rats were injected with the same amount of PBS.

### mRNA analysis (reverse transcription-polymerase chain reaction, RT-PCR)

The mRNA expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, RANTES, MMP-2, MMP-9 and iNOS in CIA synovial cells were estimated by quantitative RT-PCR. Briefly, rats were sacrificed by cervical dislocation on the 35th day after the primary immunization, synovial tissues of rat knees were quickly isolated and removed, then divided into 2-mm segments. These segments were immediately frozen in liquid nitrogen and stored at -70. Total RNA was isolated by a previously using TRIZOL (Promega, U.S.A.) described method [10]. RNA quality was checked by 1% denaturing formaldehyde agarose gels, exhibiting clear 28S and 18S bands (not shown). Aliquots of 2.5-5 $\mu$ g total RNA were used for first-

strand cDNA synthesis in a 20 µl final reaction with oligo dT primers (Promega, U.S.A.) and AMV reverse transcriptase (Promega, U.S.A.). Complementary DNA (2.5µl per test) was amplified by PCR with 2.5 Units of Taq buffer (Promega, U.S.A.), 0.2mM deoxyribonucleotide triphosphate (dNTP) mix and 0.25µmol/L of specific sense and antisense primers together with the same amount of housekeeping gene ( $\beta$ -actin/GAPDH) primers. For all reactions, temperature cycling was follows: step 1, 94 for 1min; step 2, Tm for 2min; and step 3, 72 for 1min. Step 1 through step 3 were repeated 35 times, followed by 72 for 10min. After amplification, 10µl of PCR products were resolved on 1.5% agarose in TBE (90mmol/L Tris-borate, 2mmol/L EDTA, at PH 8.0). The specific bands were scanned and analyzed by FluroChem 8800 Scan System (Alpha innotech, U.S.A.). The reaction primers and Tm used are shown in Table 1. We adapted RT-PCR analysis using limiting dilutions of cDNA to accurately compare the relative amounts of mRNA expressed in different samples, as reported [11].

#### Study on spleen T cell response

CIA rats (modeled successfully) were sacrificed on day 35 after primary immunization, and the spleens were removed, then were washed in RPMI 1640 (modified with 5mmol/L HEPES, 5 mmol/L glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml, all from Gibco, Life Technologies, Inc.) and teased apart to a single-cell suspension. Erythrocytes were lysed by treatment with 0.83% NH<sub>4</sub>Cl (pH 7.2). The remaining cells were washed twice in serum-free RPMI 1640, and the number of viable lymphocytes was assessed by trypan blue exclusion. The cells were then resuspended in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS) (Hyclone). Adherent

cells were depleted by nylon wool columns. Nylon wool-nonadherent (T-enriched) cells were eluted from the columns, counted, and resuspended in fresh RPMI containing 10% FCS [12]. FACS assay was used to assess the frequency of Th1 and Th2 cells as described before and according to the assay kit. CD4+ T cells that produced IFN- $\gamma$  were regarded as Th1 cells, and CD4+ T cells that produced IL-4 were regarded as Th2 cells. Briefly, cells were harvested and stimulated with PMA (50ng/ml final concentration; Sigma) and calcium ionomycin (250ng/ml final concentration; Sigma) in the presence of monensin (2µmol/L final concentration; Sigma) for 6h in 37°C. Then cells were stained with FITC-labeled anti-CD4 mAb, fixed, permeabilized, and subsequently stained with 0.25µg of PE-labeled anti-rat IFN- $\gamma$  antibody or IL-4 antibody, following PharMingen's staining protocol. The quadrant markers for the bivariate dot pots were set based on autofluorescence controls, and verified using the unlabelled antibody blocking control.

#### Data Analysis

All data presented in this report were of mean $\pm$ SD of nine replicates from three separate experiments. Statistical differences were evaluated using ANOVA to parametric data, Mann-U test on all clinical results, considering significance at the p<0.01 levels. Figures were obtained from at least three independent experiments with similar patterns.

## Results

#### Reduced severity of arthritis after $\beta$ -END treatment

75% (72/96) rats were established successfully as CIA model according to clinical parameter, radiology test. Histology analysis (HE) was used to confirm the estab-

**Table 1.** Primers, Tm and amplified products size (bp) used in this study

Primer	Tm (°)	Amplified Products Size (bp)	Sequence
$\beta$ -actin	58.3	320	sense: 5' -GCA TGG AGT CCT GTG GCA T-3' antisense: 5' -CTA GAA GCA TTT GCG GTG G -3'
TNF- $\alpha$	60	688	sense: 5' - ACT CCC AGA AAA GCA AGC AA -3' antisense: 5' - TGG AAG ACT CCT CCC AGG TA -3'
IL-1 $\beta$	60	506	sense: 5' - GTG GCA GCT ACC TAT GTC TT -3' antisense: 5' - GAG AGG TGC TGA TGT ACC AG -3'
IL-6	59.9	495	sense: 5' - CTT CCA GCC AGT TGC CTT-3' antisense: 5' - AGT TTG GTG TCG CGG CGG AGC AC -3'
RANTES	61.1	279	sense: 5' - CGT GAA GGA GTA TTT TTA CAC CAG C-3' antisense: 5' - CTT GAA CCC ACT TCT TCT CTG GG -3'
iNOS	57.8	552	sense: 5' - ACC TAC TTC CTG GAC ATC AC-3' antisense: 5' -ACC CAA ACA CCA AGG TCA TG -3'
MMP-2	57.8	661	sense: 5' - GAG TTG GCA GTG CAA TAC CT-3' antisense: 5' -GCC GTC CTT CTC AAA GTT GT -3'
MMP-9	61.9	737	sense: 5' - CTT CCA GCC AGT TGC CTT-3' antisense: 5' - AGT TTG GTG TCG CGG CGG AGC AC -3'

lishment of the model (data not shown). Several doses of  $\beta$ -END were given i.p. on alternate days for 3wks after the onset of the disease. The clinical symptoms of CIA were significantly improved by administration of  $\beta$ -END in a dose-dependent manner ( $p < 0.01$ ) (Fig.1). Radiological and histopathological analysis of joints shows that CIA-characterized chronic inflammation of synovial tissue (synoviocyte proliferation and leukocyte infiltration), pannus formation, cartilage destruction, and bone erosion were significantly abrogated or ameliorated by treatment with  $\beta$ -END (Fig.2,3).

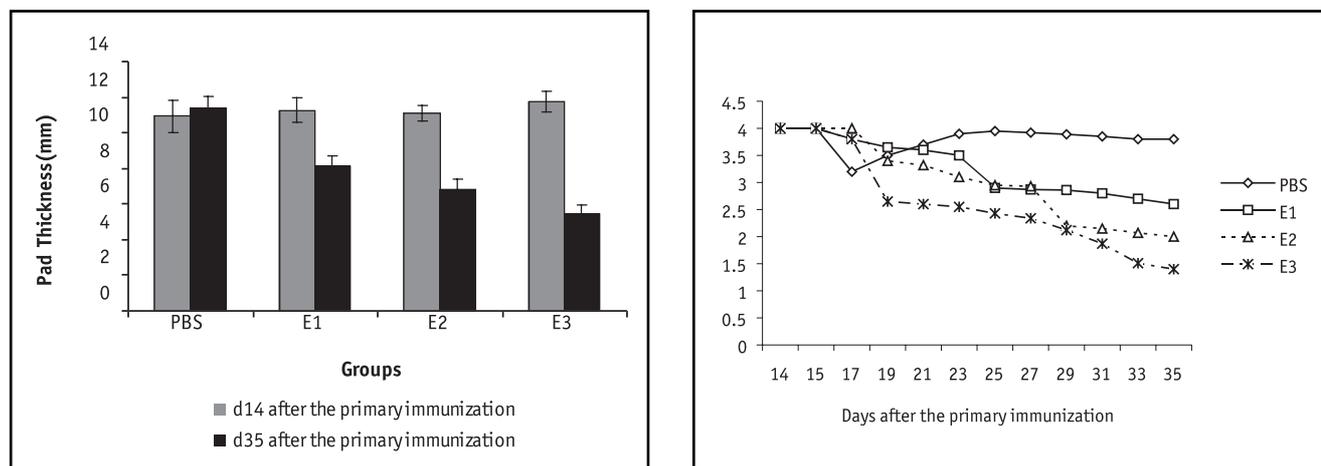
*$\beta$ -END modulates Th1/Th2 response in CIA*

There is an evidence that CIA is an autoimmune disorder mediated by Th1 cytokines, whereas the supply of Th2 cytokines has great benefit in the development of severe arthritis. Because  $\beta$ -END has been noticed to polarize the Th response to Th2 [4], we next studied the effect of  $\beta$ -END on T cytokine production in CIA rats. As shown in Fig.4, CIA resulted in the development of spleen CD4+T cells, producing high levels of

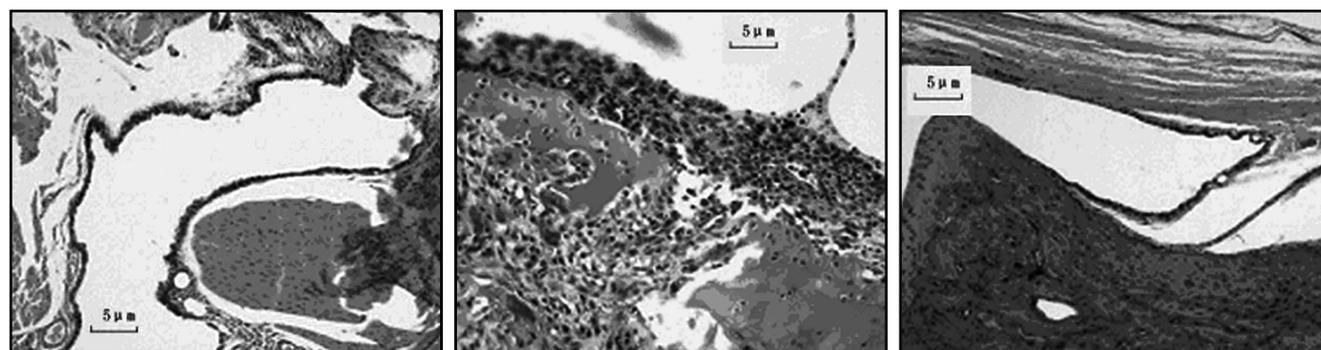
IFN- $\gamma$  and low levels of IL-4, showing a Th1 secretion pattern, which is one of the characteristics of the disease. Inversely, spleen CD4+T cells isolated from  $\beta$ -END treated rats produced large amount of IL-4 and low levels of IFN- $\gamma$  ( $p < 0.01$ ) (Fig.4), suggesting  $\beta$ -END lead the T cell response to Th2 response in CIA rats. No significant cytokines production change by T cells was detectable in the presence of PBS.

*Effect of  $\beta$ -END treatment on cytokines production of synovium tissue of CIA rats*

mRNA expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, RANTES, iNOS, MMP-2, and MMP-9 in synovial tissues of CIA rats were down-regulated by the treatment of  $\beta$ -END ( $p < 0.01$ ), suggesting that  $\beta$ -END significantly inhibit inflammatory cytokines mRNA expression in vivo (Fig. 5).



**Figure 1.** Remission in the clinical symptoms was observed in 3wks during  $\beta$ -END administration. a: Mean of behind paw thickness accessed on the d14 after the primary immunization ( $\beta$ -END administration started) and d35 after primary immunization ( $\beta$ -END administration finished). The specified doses of  $\beta$ -END (E1: 0.1nmol; E2: 1nmol; E3: 5nmol) were administered i.p. every other day. A control group of rats injected with PBS was used in each experiment. b: Continuous observation of the arthritis index (AI) of CIA from d14 to d35 after the primary immunization. Results are expressed as the mean  $\pm$ SD from three separate experiments (8 rats / group per experiment);  $p < 0.01$  vs control.



**Figure 2.** Section of rat knee joints taken on 35d after the primary immunization. a: normal rat; b: CIA model rat; Synovial proliferation and intense infiltration by mononuclear cells is producing cartilage and bone erosion; c: CIA rat received  $\beta$ -END 5nmol qod  $\times$ 3wks, very slightly manifestation of synovitis. HE $\times$ 40.

## Discussion

RA is a chronic and debilitating autoimmune disease that leads to chronic, progressive, and symmetrical inflammation in the joints and subsequent erosive destruction of the cartilage and bone. CIA is a well-demonstrated model of RA with several common characters in clinical and pathology. We established CIA model using female wistar rats and 75% were established successfully. The results were close to prior reports. Both clinical and pathologic characters indicate that CIA model is a chronic relapsing arthritis with a clinical pattern that more closely resembles human disease, suggesting it may be a good animal model for the investigation of various immunogenetic and drug therapeutic traits in RA [1,6-9].

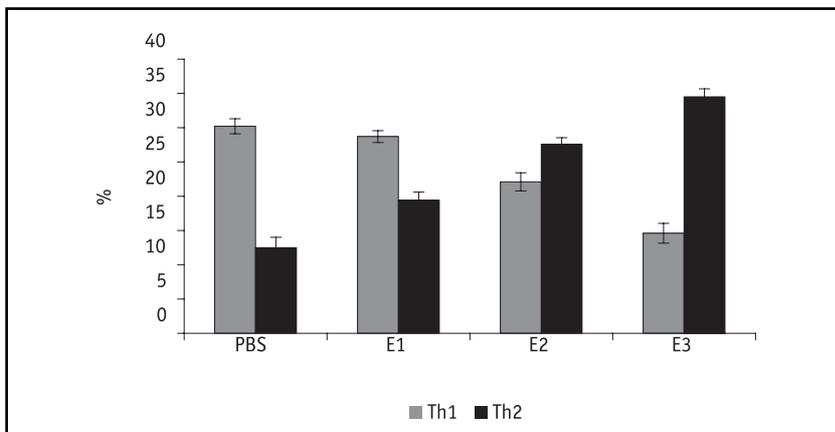
Currently drugs used in RA treatment such as disease-modifying reagents seem to be only effective on the underlying process and show harmful side effects.

The blockade of TNF- $\alpha$  and IL-1 with monoclonal antibodies (mAbs) seems to be efficient to ameliorate the synovial inflammation in RA, but a long term treatment with the Abs may result in an increased incidence of other autoimmune diseases. Because a specific causative agent of antigen (Ag) has not been identified yet, bypassing all the potential Ag and targeting the cytokine unbalance could represent a solid way to control RA and CIA.

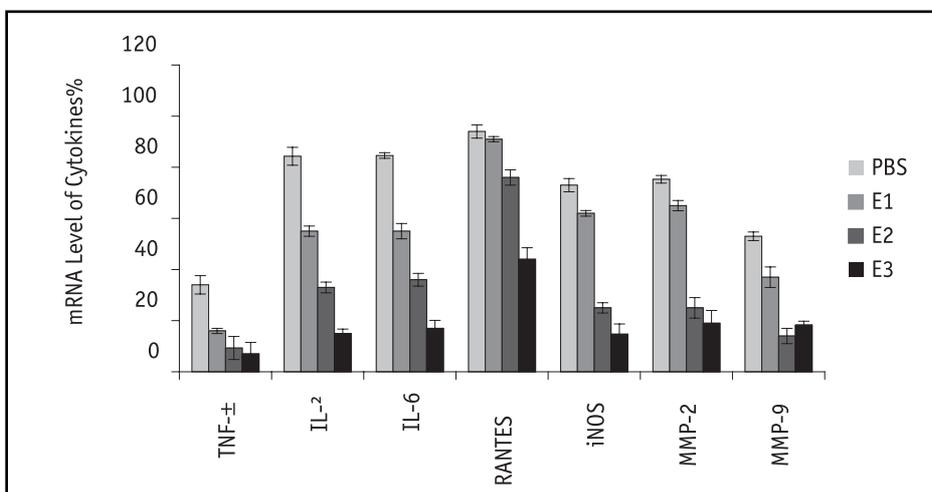
Clinical features and experimental observations suggest  $\beta$ -END as an opioid peptide, plays an important role in pathophysiology and neuro-immunoregulation of RA and CIA. Several studies have reported that circulating opioid peptide levels are reduced in patient with RA. It is confirmed that proopiomelanocortin (POMC), preproenkephalin and END are produced by synovium infiltrating lymphocytes and RA synovial cells, whereas absent in noninflamed tissue. Opioid peptides and their receptors expressed on various cell



**Figure 3.** X-ray of rat hind foot that taken approximately 35d after primary immunization. **a:** untreated CIA rat; Joints were swollen, cartilage and bone were erosive; **b:** CIA rat received  $\beta$ -END 5nmol qod for 3wks, slight inflammatory changes of joints.



**Figure 4.**  $\beta$ -END treatment in vivo polarized Th1/Th2 balance to Th2 in rats with CIA. **Th1:** rats spleen CD4+ IFN- $\gamma$ + T cells; **Th2:** rats spleen CD4+ IL-4+ T cells. E means the specified doses of  $\beta$ -END; **E1:** 0.1nmol; **E2:** 1nmol; **E3:** 5nmol.



**Figure 5.** Effects of  $\beta$ -END on mRNA levels of different inflammatory cytokines in CIA synovial tissue. TNF- $\alpha$ , IL-1 $\beta$ , IL-6, RANTES, iNOS, MMP-2 and MMP-9 mRNA expression were all down-regulated by the treatment of  $\beta$ -END. E means the specified doses of  $\beta$ -END; **E1:** 0.1nmol; **E2:** 1nmol; **E3:** 5nmol.

types within synovial tissue may constitute a regulatory circuit to control inflammatory reactions in RA [4,5].  $\beta$ -END is also demonstrated to directly suppress inflammatory mediator production by altering Th1/Th2 balance [13]. Therefore it is possible that  $\beta$ -END acts as upstream modulation of inflammation that bypassing several potential Ag and targeting the cytokine unbalance, suggesting a potential pathway in RA and CIA.

TNF- $\alpha$ , IL-1 $\beta$  and IL-6 had been considered to have critical roles in RA and CIA in that they promotes the production of most other proinflammatory mediators. iNOS and RANTES are two important representative proinflammatory and chemokines of RA and CIA. Our data showed  $\beta$ -END could directly reduce the mRNA levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, iNOS, RANTES, suggesting  $\beta$ -END plays a role in anti-inflammatory function of CIA rats. We also demonstrated  $\beta$ -END has antirheumatic effects because it reduces the levels of soluble mediator like MMPs (MMP-2 and MMP-9), which contributes to destruction of the joints structure by attacking the conjunctive support.

There is a strong evidence that the majority of the T cells in the inflamed tissues and blood in RA and CIA show a Th1 cytokine pattern [2,3], in which the synthesis of IFN- $\gamma$  and IL-4 is an important marker of this disease. In fact, certain factors that promote a Th2 response instead of a Th1 response are beneficial for RA and CIA with these disorders. The data of this study demonstrates the Th2-inducing response of  $\beta$ -END, possibly being one of the factors that contribute to the remission of the disease. Therefore the anti-inflammatory effect of  $\beta$ -END is associated with both in situ modulation on multiple inflammatory factors and systemic effect on T response.

It has been reported that the effects of  $\beta$ -END on immune function are biophasic [14], with a reduction of responses at the higher concentration and an increasing at lower concentration. Our study showed that nearly physiological dose of  $\beta$ -END prevented CIA through modulating multiple inflammatory factors, suggesting  $\beta$ -END is a potent ameliorating agent in CIA. All these facts should be taken into account in future study on the role of  $\beta$ -END in the treatment of RA.

The mechanisms of the anti-inflammatory and anti-rheumatic functions of  $\beta$ -END remain unknown, but it is reported that  $\beta$ -END exerts its anti-inflammatory action through various molecular mechanisms including binding to the receptors of immune cells and activating cAMP pathway such as MAPK or PKA pathways [15]. Further researches on signal transduction of anti-inflammatory mechanisms of  $\beta$ -END on CIA and RA are necessary.

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