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Therapeutic effect of Cryptotanshinone on experimental rheumatoid arthritis through downregulating p300 mediated-STAT3 acetylation

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ABSTRACT

Background and purpose: The balance between T helper 17 (Th17) cells and regulatory T (Treg) cells, plays a critical role in rheumatoid arthritis (RA). The differentiation of Th17 cells requires the activation of STAT3, which determines the balance of Th17/Treg. Here, we investigated the therapeutic effect of Cryptotanshinone (CTS) on collagen induced mouse arthritis and explored the underlying mechanisms.
Experimental approach: Arthritis was induced in DBA/1 mice with bovine collagen type II and complete Freund's adjuvant. CTS was given at 20 mg kg^{-1} d^{-1} or 60 mg kg^{-1} d^{-1} by gavage for 6 weeks. The immuno-inflammation and joint destruction were evaluated and the balance of Th17/Treg was determined. STAT3 acetylation and phosphorylation were detected by western blotting, and the involvement of p300 was investigated by siRNA and plasmid overexpression.
Key results: CTS at a dose of 60 mg kg^{-1} d^{-1} ameliorated the inflammation and joint destruction in CIA mice. It improved Th17/Treg imbalance, and inhibited both acetylation and phosphorylation of STAT3. CTS reduced p300 expression and its binding to STAT3, but increased phosphorylated AMPK. Knockdown of p300 mimicked the inhibitory effect of CTS on STAT3 acetylation and phosphorylation, which could be partially rescued by overexpression of p300-WT, but not p300-dominant negative (DN) construct.
Conclusion and implications: Our study suggested that the anti-arthritis effects of CTS were attained through suppression of p300-mediated STAT3 acetylation. Our data suggest that CTS might be a potential immune modulator for RA treatment.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by hyperplasia and inflammation of synovial tissue, leading to the progressive destruction of joints. Currently, biological disease-modifying anti-rheumatic drugs (DMARDs), targeting either tumor necrosis factor (TNF), interleukin 1 (IL-1) receptor, IL-6 receptor or T-cell co-stimulation [1,2], have been considered as promising treatments to prevent joint destruction. However, according to American College of Rheumatology (ACR), there are still approximately 30% RA patients who failed to achieve obvious improvements. Besides, long-term biological therapy increased the risks of serious infection and malignancy. The unsatisfactory situation in RA drug development prompts us to search for alternative therapy with immunomodulatory effects.

$CD4^+$ T helper (Th) cells are essential regulators in autoimmune diseases including RA. Among $CD4^+$ Th cells, IL-17 producing Th17 cells are the major players in RA [3]. In contrast, $CD4^+$ $CD25^+$

Abbreviations: RA, rheumatoid arthritis; CTS, Cryptotanshinone; DMARDs, disease-modifying anti-rheumatic drugs; FLS, fibroblast-like synoviocytes; CIA, type-II collagen-induced arthritis; STAT, Signal transducer and activator of transcription; IGU, iguratimod; Treg, regulatory T cell; Th17, T helper 17.

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Foxp3$^+$ regulatory T (Treg) cells inhibit the function of Th17 cells. Briefly, Treg cells express cytotoxic T-lymphocyte associated protein 4 to compete for T cell costimulation and secret anti-inflammatory cytokines including IL-10 and TGF-β [4]. The pathogenesis of RA is closely correlated with the imbalance of Th17/Treg and characterized by inflammatory microenvironment supporting continuous differentiation of Th17 cells [5]. Considering the differentiation of Th17 and Treg cells is determined by IL-6/STAT3 activation [6], STAT3 might be developed as a promising target for RA therapy.

IL-6 is a pleiotropic cytokine with immunomodulatory activities. IL-6 induces the differentiation of naïve T cells towards Th17 cells by inhibiting TGF-β-induced Treg generation [7–9]. IL-6 activation requires a complex consisting of IL-6 receptor (IL-6R) and glycoprotein 130 (gp130). Complexes of IL-6, IL-6R and gp130 then phosphorylate Janus Kinase (JAK) to activate signal transducer and activator of transcription 3 (STAT3) [10–12]. Despite the classical activation of p-STAT3 by JAK2, STAT3 acetylation by p300/CBP histone acetyltransferase (HAT) is also essential for STAT3 to form stable dimers and to activate STAT3 target gene transcription [13]. Acetylated STAT3 forms stable dimer to translocate into nucleus and binds strongly to the DNA to enhance the transcription of STAT3 target genes. Thus, targeting STAT3 acetylation would be an alternative approach to avoid side effects caused by directly inhibiting STAT3 upstream signaling.

Cryptotanshinone (CTS) is a quinoid diterpene compound isolated from the root of *Salvia Miltiorrhiza* bunge. Recent study showed that CTS could rapidly inhibit the phosphorylation of STAT3 on Tyr705 site in a JAK-2 independent manner in tumor cells [14]. Besides, our previous studies on rat collagen-induced arthritis (CIA) showed that CTS ameliorated CIA without causing such adverse effects on immunity as Leflunomide [15]. However, what still confused us is the precise underlying mechanism. In order to illuminate it, we studied the effects of CTS on DBA/1 mice with CIA as well as the in vitro cell models. Our study showed that CTS suppressed CIA by regulating Th17/Treg balance, which might be achieved by inhibiting p300-mediated STAT3 activation. Our results suggested that CTS might be a potential immune modulator for RA treatment.

2. Materials and methods

2.1. Materials

CTS used in vivo study was kindly provided by Professor Peiqing Liu in the Laboratory of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-sen University (Guangzhou, Guangdong, China). The chemical purity was >98%. The inclusion complex of CTS with hydroxypropyl-β-cyclodextrin containing 19.5% CTS was prepared to improve the bioavailability in vivo [16]. For in vitro studies, CTS, AG490 and β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iguratimod (IGU) tablets were purchased from Simcere Pharmaceutical Group (Nanjing, Jiangsu, China). Bovine type II collagen, Freund's Adjuvant and Mouse anti-type II collagen IgG2α subtype assay kit were purchased from Chondrex (Redmond, WA, USA). For animal experiments, either CTS or IGU tablets were dissolved and ground using sterilized 0.5% carboxymethylcellulose sodium (CMC-Na). For in vitro studies, CTS or AG490 were dissolved in DMSO and diluted with DMEM containing 0.1% FBS. Recombinant mouse IL-6, human IL-6 and human sIL-6 receptor α (sIL-6R) were purchased from Peprotech (Rocky Hill, NJ, USA). One step staining mouse Treg flow kit was purchased from BioLegend (San Diego, CA, USA). TGF-β1, IL-6, IL-10 and IL-17α ELISA kits were purchased from Dakewe Biotech Company (Shenzhen, Guangdong, China). Antibodies against p-STAT3, STAT3, Ace-STAT3, p-JAK2, p-AMPK, AMPK and Acetyl-NF-κB p65 were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibody against p300 was purchased from Millipore (Billerica, MA, USA). Antibody against GAPDH was purchased from Beyotime Biotechnology (Guangzhou, Guangdong, China). Nuclear Extract Kit was purchased from Active Motif (Carlsbad, CA, USA). *E. coli* DH5α competent cells were purchased from Takara (Tyoto, Japan). HiSpeed Plasmid Midi Kit was purchased from Qiagen (Hilden, Germany). Lipofectamine 3000 was purchased from Thermo Fisher scientific (Waltham, MA, USA). p300 siRNA was produced by RiboBio Co., Ltd (Guangzhou, Guangdong, China). Constructs of pcDNA3.1-p300 WT and pcDNA3.1-p300 DN (HAT$^-$) were supplied by Addgene (Cambridge, MA, USA).

2.2. Animals

All animal experiments were conducted according to the guidance of China Animal Welfare Legislation and the Ethics Committee on the Care and Use of Laboratory Animals of Sun Yat-sen University. For in vivo studies, six to seven-week old male DBA/1 mice (18–22 g) for CIA induction were purchased from Shanghai Slac Laboratory Animal Co. Ltd one week before the first immunization. They were housed under specific pathogen-free (SPF) conditions (temperature: 22 ± 2 °C; humidity: 40–60%), kept under a 12 h dark/light cycle and fed standard lab chow and water *ad libitum*. For in vitro studies, female Wistar rats (90–110 g) were supplied by Shanghai Slac Laboratory Animal Co. Ltd and immunized to induce CIA [15]. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [17,18].

2.3. CIA induction and administration

Fifty DBA/1 mice were divided into 5 groups in a randomized double blind method: normal (NOR) group, collagen induced arthritis (CIA) group, 10 mg kg^{-1} d^{-1} Iguratimod treated (IGU) group, 20 mg kg^{-1} d^{-1} CTS treated (CTSL) group and 60 mg kg^{-1} d^{-1} CTS (CTSH) group (n = 10 for each group). Despite NOR group, CIA was induced in mice by subcutaneous injection at the base of tail with 100 μL of emulsion of equal volumes of bovine collagen type II and complete Freund's adjuvant [19] denoted at day 1. The mice were then given booster injection in the same way on day 21. NOR mice were injected with saline instead. Drug treatment began from the day of onset to appear by gavage. NOR and CIA groups were treated with 0.5% CMC-Na. After six weeks of treatment, all mice were sacrificed following Pentobarbital anesthesia. Paws and spleens were isolated for micro-CT, histopathological examination or flow cytometry.

2.4. Arthritis assessment

Clinical symptoms of arthritis were graded by two observers unware of the experiment on a scale of 0–4 every 2 days for each paw: 0 = no erythema or swelling, 1 = slight erythema and/ or swelling of one digit, 2 = erythema or mild swelling of the limb or swelling more than two digits, 3 = moderate erythema and swelling of the limb, 4 = severe erythema and swelling of the limb or even ankylosis, with a slight modification of previously described protocol [20]. The final score for each mouse was the sum of the scores of four paws, which might reach the maximum of 16.

2.5. Micro-computed tomography (micro-CT)

Paws of mice in each group were imaged by two observers unware of the experiment using Inveon PET/CT imaging system provided by SYSU Center for Animal Molecular Imaging [21]. Joint