# A method of experimental rheumatoid arthritis induction using collagen type II isolated from chicken sternal cartilage

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Abstract. At present, collagen-induced arthritis (CIA) is the best known and most extensively used model for the immunological and pathological characteristics of human rheumatoid arthritis (RA). This model is useful not only in aiding our understanding of the pathogenesis of this disease, but also in the development of new therapies. Bovine, porcine and human collagen has been used to induce CIA; however, response has been identified to vary between strains and injection conditions, and false positive results and reduced potency are common as a result of minor contaminants or deglycosylated protein. Therefore, in the present study, type II collagen (CII) was isolated and purified from chicken sternal cartilage and was found to successfully induce the RA model. Furthermore, T helper 17 (Th17) cells were observed to infiltrate the joint on day 45 following induction by CII. In vitro, expression of toll-like receptor 2 (TLR2) increased in peritoneal macrophages stimulated by CII. In addition, blockage of TLR2 was identified to markedly decrease levels of TGF-B and IL-6 in the cell culture supernatant. The results indicate that CII isolated from chicken sternal cartilage may be recognized by TLR2 on macrophages, leading to TGF- $\beta$  and IL-6 production and subsequent activation of Th17 cells which mediates CIA development.

## Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis which affects all synovial-lined diarthrodial joints; however, the disease has a predilection for the wrist and

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small joints of the hand. RA occurs in ~1% of adults and is ~2.5 times more prevalent in females than males. A number of genetic and environmental factors are known to affect this disease (1,2). RA leads to progressive articular damage, joint deformities and disability and is considered to be an autoimmune disease. A previous study in the joints of patients with RA revealed the presence of T cells in synovium (3). In an additional study, cluster of differentiation (CD) 4<sup>+</sup> cells isolated from the joints of patients with RA and other inflammatory joint diseases were reported to be less responsive to mitogenic stimulation in the presence of IL-2 compared with control patients. By contrast, no difference was found in the CD8<sup>+</sup> cell response, indicating that joint-resident CD4<sup>+</sup> T cells are potential effectors in RA (4). However, at present, limited knowledge of target antigens relevant to the disease process remains a major limitation for understanding of the role of the adaptive immune response to RA (5).

Animal models have been used extensively in studies of RA pathogenesis. Despite numerous inherent limitations, several models have significantly contributed to the understanding of fundamental pathological RA mechanisms and to major advances in therapy. These models include collagen-induced arthritis (CIA) (6), collagen antibody-induced arthritis (7), zymosan-induced arthritis (8), methylated BSA and genetically manipulated or spontaneous arthritis, including TNF- $\alpha$ -transgenic, K/BxN and SKG mice (9). CIA is the best known and most extensively used model for the analysis of immunological and pathological similarities with human RA.

CIA is an autoimmune disease of joints, characterized by T and B cell response to autologous type II collagen (CII). This model is reproducible in genetically susceptible mouse strains, including DBA/1 and B10.Q by immunization with heterologous type II collagen in complete Freund's adjuvant (CFA). Bovine, porcine and human collagen has been used to reproduce this model. However, variations in response have been identified between various strains and injection conditions, and false positive results and reduced potency are common as a result of minor contaminants or deglycosylated protein (10,11). In the current study, CII was isolated and purified from chicken sternal cartilage and observed to successfully induce RA. The model was characterized by T helper 17 (Th17) cell infiltration.

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#### Materials and methods

CII isolation and purification. Chicken sternal cartilage was selected as raw material and CII was isolated according to the following protocol. Briefly, fresh sternal cartilage was removed from the periosteum and the calcified portion, cut into slices and conserved at -20°C. Proteoglycans were removed using guanidine hydrochloride. The precipitation was washed by Tris-HCl (0.05 mol/l) and acetic acid (0.5 mol/l), then digested by 5 times the volume of pepsin (1 g/l, pH 7.5), collecting the supernatant following centrifugal, repeated two times; all the supernatant was collected. The supernatant was keep pH 7.5 and chlorine sodium (NaCl) (1 mol/l), salting out overnight at 4°C, collecting the precipitation following centrifugal. The precipitation was dissolved with 0.1 mol/l acetic acid and dialysis equilibrium by 0.05 mol/l Tris-HCl-0.2 mol/l NaCl (pH 7.5), namely primary collagen. And then the CII was purified by DE22 cellulose or DEAE-agarose.

The molecular weight of CII was identified by SDS-PAGE and the amino acid composition was analyzed by the State Key Laboratory of Medical Biotechnology, Nanjing University (Nanjing, China). The absorption spectrometry was identified by spectrophotometer (Shimadzu, Kyoto, Japan).

*Mice*. DBA/1 mice were purchased from Shanghai Laboratory Animal Center, CAS (Shanghai, China) and maintained in the Animal Center of Jiangsu University (Zhenjiang, China) in compliance with the Guide for the Care and Use of Laboratory Animals (no. 85-23, revised 1996). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Jiangsu University.

CIA model induction. Male DBA/1 mice (6-9 weeks) were immunized intradermally at the base of the tail with 100  $\mu$ g chicken sternal hyaline CII dissolved in 100  $\mu$ l acetic acid (0.05 mol/l) and mixed with an equal volume of CFA (Difco Laboratories, Detroit, MI, USA). After 3 weeks, animals were reimmunized with 100  $\mu$ g CII emulsified in incomplete Freund's adjuvant (Difco). Mice were observed 3 times/week. After 45 days, mice were anesthetized with pentobarbital sodium (30 mg/g body weight, i.p.) and sacrificed by cervical dislocation. They then underwent rapid joint excision.

Mice were inspected for the development of CIA and inflammation of the four paws was graded between 0 and 4: 0, paws with no swelling; 1, paws with swelling of finger joints or focal redness; 2, paws with mild swelling of wrist or ankle joints; 3, paws with severe swelling of the entire paw; and 4, paws with deformity or ankylosis. Each paw was graded and the four scores were totaled (12).

*Histological analysis.* Knee joints from the mice were removed and fixed in 10% formalin. After 4 days, joints were placed in 5% formic acid for decalcification. Tissue sections were stained with hematoxylin and eosin.

*Immunofluorescence*. Immunofluorescence staining of paraffin-embedded mouse joints was performed as described in a previous study (13). Following deparaffinization, rehydration and antigen unmasking, samples were immersed in blocking buffer for 60 min. Then, primary antibodies against CD4 and

interleukin (IL)-17 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were applied for 2 h at room temperature. Following washing, fluorescein isothiocyanate and phycoerythrin labeled secondary antibodies were added for 1 h. Sections were viewed under a fluorescence microscope (Olympus, Tokyo, Japan) and then analyzed using Image J software.

*Macrophages cells isolates and treatment*. Macrophages isolated from DBA/1 mice were cultured at  $1x10^6$  cells/well in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum and 1% streptomycin/penicillin and then stimulated with 0.5 µg/ml CII for 30 min, 1 h, 90 min and 2 h for mRNA analysis or treated for 1, 2 and 3 days for cytokine analysis.

For blockade of toll-like receptor 2 (TLR2), 10  $\mu$ g/ml anti-TLR2 antibody (Santa Cruz Biotechnology) was added prior to 1 h treatment and then stimulated with 0.5  $\mu$ g/ml CII for 3 days. The supernatant was collected and used for IL-1 $\beta$ , transforming growth factor (TGF)- $\beta$  and IL-6 assays.

Cytokine measurement. Mouse serum and cell culture supernatant were collected and stored at -80°C until use. Serum levels of TGF- $\beta$  and IL-6 and -17 were detected using ELISA kits (Bender MedSystems GmbH, Vienna, Austria), according to the manufacturer's instructions.

Real-time quantitative polymerase chain reaction (RT-qPCR). TLR2, 4, 5, 7, 8 and 9 and Dectin 1 and 2 mRNA levels were assessed by RT-qPCR as described previously (14). Briefly, total RNA was isolated from cells using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions and reverse transcribed into first-strand cDNA using the Moloney murine leukemia virus reverse transcriptase system. Following cDNA synthesis, real-time PCR was performed with iQ SYBR-Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), using a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) with  $\beta$ -actin as an internal control. Primer sequences are presented in Table I. Quantification of gene expression was calculated relative to  $\beta$ -actin.

## Results

Characteristics of CII isolated and purified from chicken sternal cartilage. CII was successfully isolated and purified. SDS-PAGE analysis demonstrated that CII was constituted of  $\alpha$ ,  $\beta$  and  $\gamma$  isoforms.  $\beta$  forms a dimer with  $\alpha$  and  $\gamma$  forms a trimer with  $\alpha$ . The relative molecular weight of the  $\alpha$  monomer was 12 kDa (Fig. 1A). High performance liquid chromatography (HPLC) was performed to analyze the amino acid constitution of the  $\alpha$  monomer and identified that the  $\alpha$  monomer is rich in glycine, proline and alanine, particularly glycine (1/3 of the structure). Aromatic amino acid content was low, phenylalanine was 1.53% and tyrosine was not detected. Analysis of absorption spectrometry was in accordance with HPLC results (Fig. 1B).

*Isolated CII induced CIA, a Th17 cell-related disease.* To determine whether isolated CII induced CIA in mice, models were induced as described previously (15). On day 34, the

Table I. Primer sequences used for RT-qPCR.

Gene	Sequence(5'-3')	Amplicon length	
TLR2	CATGGGCCCCAGGTCCTT	269	
	CAACTCTCTCAACGGCCA		
TLR4	CTGAGCAGCCGCTCTGGC	307	
	AGCCCCAGGTGAGCTGTA		
TLR7	GGGTGTGTGATGGCCGCT	279	
	AAAGGGCCCGAACCAG		
TLR8	TGGCTTCTTCTCCGAAGC	366	
	AGGTGGTGAACCAGAGCA		
TLR9	ACGCAGCGCCCAAACTCC	301	
	GCGGTCTTCCAACAGGCG		
Dectin1	TGGGTTAGTGAGCCTCAT	278	
	CACCCAGCACTGCAGCAA		
Dectin2	TGTGGGGGATGCTTGCCCA	285	
	TGGGTTCATGGGGGGTGCC		
IFN-γ	TATTCGGTAACTGACTTG	378	
	AATCACATAGCCTTGC		
IL-17	TCTCCAAAGGAAGCCTGA	231	
	CAAGACTGAACACCGACT		
β-actin	CACGAAACTACCTTCAACT 265 CATACTCCTGCTTGCTGA		

TLR, toll-like receptor; IFN, interferon; IL, interleukin; RT-qPCR, real time quantitative polymerase chain reaction.

Table II. Concentration of IL-6 and TGF- $\beta$  in cell culture supernatant.

Protein (pg/ml)	Collagen II	Collagen II + blockade TLR2	P-value
TGF-β	258.12±32.13	38.32±17.34	<0.005
IL-6	435.79±13.34	123.56±21.13	<0.005

TGF, transforming growth factor; IL, interleukin.

joints of experimental mice were swollen. Control mouse joints were unchanged (Fig. 2A and B). On day 45, histological examination revealed marked infiltration of inflammatory cells in the experimental group and characteristics typical of RA. All model mice severity scores were >1 (Fig. 2C and D).

Immunofluorescence analysis demonstrated that Th17 cell infiltration in the CIA group was significantly increased compared with the control (P<0.05; Fig. 2E). Serum IL-17 levels were 321.43±19.32 and 34.76±10.23 pg/ml in the experimental and control groups, respectively (P<0.05).

Th17 cell induction by a TLR2-dependent pathway. Next, the mechanism by which CII induces Th17 cell infiltration was



Figure 1. Characteristics of CII isolated from chicken sternal cartilage. (A) SDS-PAGE analysis. Lanes A, marker; B, CII from Sigma-Aldrich; and C, CII isolated from chicken sternal cartilage. (B) Analysis of absorption spectrometry. CII, type II collagen.

determined. Peritoneal macrophages were isolated and treated with 0.5  $\mu$ g/ml CII in vitro. The pattern recognization receptors, TLR2, 4, 5, 7, 8 and 9 and Dectin 1 and 2 were detected. Results revealed that TLR2 significantly increased at 90 min and peaked at 120 min (P<0.05), indicating that CII may be recognized by TLR2 expressed on macrophages and associated with induction of the Th17 cell response.

Following this, an anti-TLR2 antibody was utilized to block TLR2 expressed on the peritoneal macrophages and the cells were stimulated using CII. After 3 days, TGF- $\beta$  and IL-6 levels were determined in the cell culture supernatant. The results were as follows: TGF- $\beta$ , 258.12±32.13 pg/ml (stimulated group) vs. 38.32±17.34 pg/ml (stimulated + blockade group); IL-6, 435.79±13.34 pg/ml (stimulated group) vs. 123.56±21.13 pg/ml (stimulated + blockade group), respectively. Following TLR2 blockade, TGF- $\beta$  and IL-6 levels were significantly decreased (P<0.05; Table II).

### Discussion

Animal models of autoimmune arthritis have proven to be valuable research tools for the analysis of the pathogenesis of diseases as well as exploring new therapeutic targets. More recently, transgenic and knockout mice have also enabled important advances and led to a greater understanding of the role of a number of cytokines, particularly IFN- $\gamma$ , in disease pathogenesis. In animal models, one mechanism by which IFN- $\gamma$  functions is by suppressing IL-17 production, which is emerging as a key pathogenic cytokine of lymphoid tissue inducer-like Th17 cells (16-19).

Animal models have also proved useful for understanding the complexity of the immune system, particularly various



Figure 2. Characteristics of CIA induced by CII isolated from chicken sternal cartilage. DBA/1 mice were analyzed. At day 35, mouse RA began to develop and peaked at day 45. Images of mouse joints in (A) control and (B) CIA groups. (B) Joint swelling was marked following development of CIA. Histological analysis by hematoxylin and eosin staining in the (C) control and (D) CIA groups (magnification, x200). (D) Mice with CIA were identified with higher numbers of lymphocytes infiltrated in the knee joint cavity. Th17 cell infiltration into the joint in (E) control and (F) CIA groups. On day 45, Th17 cells infiltrated the joint. (G) The number of infiltrated Th17 cells was quantified using Image J software. CIA, collagen-induced arthritis; CII, type II collagen; RA, rheumatoid arthritis.



Figure 3. PRR mRNA expression in peritoneal macrophages. Values are expressed as PRRs compared with  $\beta$ -actin. Data are presented as the mean  $\pm$  SD from three independent experiments. PRR, pattern recognization receptor; TLR, toll-like receptor.

subgroups of CD4<sup>+</sup> T cells in disease pathogenesis. It is well known that specific CD4<sup>+</sup> T cells and their associated cytokines are able to promote or negate the development of RA in a number of models (20). For example, IFN- $\gamma$  and IL-4 are able to inhibit Th17 infiltration in the adjuvant-induced arthritis, SKG, CIA and proteoglycan-induced arthritis models (21-23). In addition, human and mouse CD4<sup>+</sup> T cells are known to possess a certain degree of plasticity (24-26), for example Th17 cells are able to convert into Th1 cells. Therefore, crosstalk between CD4<sup>+</sup> T cells may be necessary to fine-tune T cell lineage commitment to control diseases.

In mice with CIA, CD4<sup>+</sup> T cells are important in disease induction and Th1 cells are considered as the major mediator. However, the hypothesis that CIA is a Th1-mediated disorder has been challenged (27-30). In the present study, Th17 cells were demonstrated to represent the dominant effector T helper cell subset in the CIA model. Results are consistent with previous studies in IL-17-deficient mice or mice treated with anti-IL-17 antibody demonstrating the importance of IL-17 in the pathology of CIA (31,32). Following this, the mechanism by which Th17 cells mediate induction of the immune response was investigated as well as the hypothesis that macrophages induce Th17 cells.

Macrophages are important antigen presenting cells as well as an important link between the innate and adaptive immune response. Stimulation of macrophages by antigen presentation induces secretion of proinflammatory molecules which induce CD4<sup>+</sup> T cell differentiation and determine the type of immune response. In addition, macrophages are also responsible for numerous immunological and inflammatory processes (33). A number of previous studies have demonstrated that macrophages perform important roles in RA development (34-36). Therefore, in the present study, peritoneal macrophages were isolated and treated with CII. After 2 h, TLR2 mRNA levels were identified to be significantly increased, indicating that TLR2 is involved in recognition of CII. In mice, it is well known that Th17 differentiation from naïve T-cells requires TGF-ß and IL-6, molecules which are critical in CIA (16,24). To confirm the observation that CII binds TLR2 to activate macrophages and induce Th17 cells, anti-TLR2 was used to block TLR2 on macrophages. Following blockage, levels of TGF-B and IL-6 were identified to be significantly decreased, consistent with the hypothesis.

In the current study, CII was isolated from chicken sternal cartilage and successfully induced RA via a TLR2-dependent pathway. In addition, the RA model was found to represent a Th17 cell-related disease.

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