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# Digoxin ameliorates autoimmune arthritis via suppression of Th17 differentiation

Jennifer Lee [a,b], Seungye Baek [a], Jaeseon Lee [a], Juhyun Lee [a], Dong-Gun Lee [a], Mi-Kyung Park [a], Mi-La Cho [a], Sung-Hwan Park [a,b], Seung-Ki Kwok [a,b,*]

[a] The Rheumatism Research Center, Catholic Research Institute of Medical Science, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 137-701, Republic of Korea
[b] Division of Rheumatology, Department of Internal Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 137-701, Republic of Korea

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ABSTRACT

Digoxin is a cardiac glycoside that is commonly used to treat heart failure. Based on its known anti-inflammatory effect, this study was undertaken to investigate the effect of digoxin on collagen-induced arthritis (CIA) and to delineate the underlying mechanism. Digoxin or vehicle was injected intraperitoneally thrice weekly in mice with CIA, from day 7 or day 35 after immunization to investigate preventive or therapeutic effect, respectively. The incidence and severity of arthritis was evaluated. Digoxin treatment suppressed the incidence of arthritis and joint inflammation in mice with CIA. The expression of IL-17 and other proinflammatory cytokines, including IL-1β, IL-6, TNF-α and IL-21, were markedly reduced in the arthritic joints of digoxin-treated CIA mice. Th17 cells and $CD4^+$ $pSTAT3^+$ cells were less frequently observed in the spleen of digoxin-treated CIA mice than controls. The mRNA expression of IL-17 and ROR γt was consistently lower in total splenocytes or draining lymph node cells obtained from digoxin-treated CIA mice. Digoxin also reduced *in vitro* Th17 differentiation and LPS-stimulated IgG production. The number of osteoclasts in the arthritic joint was lower in digoxin-treated mice, whereas digoxin treatment did not directly suppress *in vitro* osteoclastogenesis. Our findings suggest that digoxin can regulate Th17 and reciprocally promote Treg cells and suppress joint inflammation and bone erosion in CIA. Digoxin may be a therapeutic option by targeting pathogenic Th17 and immunoglobulin production, for treatment of autoimmune arthritis and other Th17-related diseases.



## 1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by hyperplasia of synovial tissue and chronic joint inflammation, which can lead to the destruction of articular cartilage and bone [1–3]. Although the exact molecular mechanism of RA pathogenesis is yet to be clearly understood, RA is currently considered as a T cell-mediated disease in which interleukin (IL)-17 producing helper T cell (Th17) plays a central pathological role [4]. Proinflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1β that promote inflammation in the arthritic joints are induced by IL-17 which is abundantly expressed in arthritic synovium [5]. Th17 also enhances the ability of B cells to produce immunoglobulin as a B cell helper [6] and up-regulates receptor activator nuclear kappa ligand (RANKL) expression, which subsequently promotes osteoclastogenesis, leading to bone erosion and consequent joint destruction in RA [7]. Collectively, Th17 is involved in multiple pathological processes of RA, and its regulation will be a good therapeutic strategy in the treatment of RA.

There are several critical pathways involved in the differentiation of naïve T cells into Th17, such as IL-6-mediated activation of Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. Indeed, we have reported preventive or therapeutic effects by targeting this pathway [8–10]. Another possible target would be retinoic acid-related orphan nuclear receptor (ROR) γt, the master transcription factor of Th17 development [11]. Digoxin drew our attention during the search for candidate molecules blocking RORγt.

Digoxin is a cardiac glycoside commonly used to treat heart failure [12]. It enhances cardiac stroke by increasing intracellular calcium concentration as a consequence of suppressed sodium/potassium pump function at the plasma membrane level [13]. Previously, Huh et al. reported that digoxin specifically inhibited the transcriptional activity of RORγt and thus could suppress Th17 differentiation [14]. The unique structure of digoxin allows it to antagonize the receptor activity of ROR γt and suppresses Th17 differentiation [15]. Later, Cascao et al. demonstrated that digoxin had a therapeutic effect in adjuvant induced arthritis albeit only in its early stages [16]. Based on these earlier observations, we investigated whether digoxin would suppress pathogenic

* Corresponding author at: Division of Rheumatology, Department of Internal Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 137-701, Republic of Korea. Tel.: +82 2 2258 6014; fax: +82 2 599 3589.
*E-mail address:* seungki73@catholic.ac.kr (S.-K. Kwok).

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Th17 and consequently ameliorate arthritis and joint damage in the CIA model, a prototype animal model of RA.

## 2. Methods

### 2.1. Mice

All experiments were performed in accordance with the Guideline for Animal Research Ethics Committee at The Catholic University of Korea. Male DBA/1J mice were purchased from Charles River Laboratories (Yokohama, Japan). The animals were maintained under specific pathogen-free conditions and were immunized at 7–10 weeks of age.

### 2.2. Induction of collagen-induced arthritis and treatment with digoxin

Type II collagen (CII) was dissolved overnight in 0.1 N acetic acid (4 mg/mL) with gentle rotation at 4 °C. Male DBA/1J mice, 7 weeks of age, were immunized intradermally at the base of the tail with 100 μg of bovine type II collagen (CII; Chondrex Inc., Redmond, WA, USA) in complete Freund's adjuvant (CFA, Chondrex Inc.). In experiments conducted to investigate therapeutic effect, mice were boosted with 100 μg of CII emulsified with incomplete Freund's adjuvant (IFA, Chondrex Inc.) via one hind footpad on day 14 after primary immunization. DBA/1J mice were injected intraperitoneally thrice weekly with digoxin (2 mg/kg or 5 mg/kg) or vehicle control (10% DMSO), beginning on day 7 (preventive effect) or day 35 (therapeutic effect) after primary immunization, and were monitored for 3 to 8 weeks to investigate the effect of digoxin on CIA.

### 2.3. Assessment of arthritis

The severity of arthritis was determined by 3 independent observers. The mice were examined thrice weekly for the development and severity of joint inflammation for up to 8 weeks after primary immunization. The severity of arthritis was assessed on a scale of 0–4 using the criteria, as described previously [17]: 0 = no evidence of erythema and swelling, 1 = erythema and mild swelling confined to the mid-foot (tarsals) or ankle joint, 2 = erythema and mild swelling extending from the ankle to the mid-foot, 3 = erythema and moderate swelling extending from the ankle to the metatarsal joints and 4 = erythema and severe swelling encompass the ankle, foot and digits. The arthritic score for each mouse was expressed as the sum of the scores for all 4 limbs. The score of one hind limb with boosted footpad has been excluded from the sum of the scores. The highest possible arthritis score for a mouse was therefore 16 for the preventive effect study or 12 for the therapeutic effect study. The mean arthritis index was used to compare the data between the control and experimental groups.

### 2.4. Histological evaluation and immunohistochemical assessment of arthritis

The mouse joint tissues were fixed in 4% paraformaldehyde, decalcified in EDTA bone decalcifier and embedded in paraffin. Seven-micrometre sections were prepared and stained with hematoxylin-eosin (H&E), safranin O and toluidine blue. The sections were dewaxed using xylene and were then dehydrated in a graded series of alcohols. The endogenous peroxidase activity was quenched with methanol and 3% $H_2O_2$.

Immunohistochemistry was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The tissues were first incubated with the primary anti-IL-1β, anti-TNFα, anti-IL-17 (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-IL-6 (Abcam Inc., Cambridge, MA), anti-IL-21 (R&D systems, Minneapolis, MN), goat IgG isotype (for TNFα and IL-21) or rabbit IgG isotype (for IL-1β, IL-6 and IL-17) Abs (all from Santa Cruz Biotechnology Inc.) overnight at 4 °C, and a biotinylated secondary antibody and a Streptavidin–peroxidase complex for 1 h. The final color product was developed using DAB chromogen (DAKO, Carpinteria, CA, USA). The sections were counterstained with hematoxylin. Images were captured using a DP71 digital camera (Olympus, Center Valley, PA) attached to an Olympus BX41 microscope at 400× magnification. For histological evaluation of CIA, sections were evaluated in a blind manner, as has been described previously. The scores were evaluated as previously described [18].

### 2.5. Measurement of the level of immunoglobulin G

Total IgG, IgG1 and IgG2a were measured using the mouse IgG1/IgG2a ELISA quantitation kit (Bethyl Lab Co., Montgomery, TX). The splenocytes were distributed in 48-well plates ($5 \times 10^5$ cells per well) and incubated with digoxin for 1 h before 100 ng/mL lipopolysaccharide (LPS) stimulation at 37 °C in a 5% $CO_2$ humidified incubator. After 4 days, the supernatant was collected and frozen at −70 °C for the assays of IgG. The absorbance values at 450 nm were determined with an ELISA microplate reader (Bio-Rad, Hercules, CA, USA).

### 2.6. Confocal microscopy

Spleen tissues were snap-frozen in liquid nitrogen and cut into sections at 7 μm and stored at −70 °C until ready for use. Following fixation in ice-cold acetone, the tissue samples were incubated with PerCP/Cy5.5 or Alexa Fluor 488-conjugated anti CD4, PE-conjugated anti-IL-17, APC-conjugated anti-CD25, FITC-conjugated anti-Foxp3 (all from eBioscience, San Diego, CA) and anti-phospho Stat3 (Y705 and S727) mAbs (BD Biosciences, San Jose, CA). The samples were analyzed with confocal microscopy (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany), after a final wash.

### 2.7. $CD4^+$ T cell purification and stimulation

$CD4^+$ T cells were purified from spleens obtained from DBA/1J mice using MACS isolation kit with an AutoMACS separator according to manufacturer's instructions. The purity of the obtained fractions was typically > 98%. $CD4^+$ T cells were activated with plated-bound anti-CD3 (0.5 μg/mL) together with anti-CD28 Abs (1 μg/mL) (all from BD PharMingen, San Diego, CA); the following cytokines and antibodies were further added to the cultures. For Th17 polarizing condition: human TGF-β (2 ng/mL), IL-6 (20 ng/mL), anti-IFN-γ (2 μg/mL), anti-IL-4 (2 μg/mL) (all from R&D Systems except TGF-β (PeproTech Rocky Hill, NJ). Pretreatment with digoxin for 2 h was followed by adding cytokines for Th17 cell differentiation.

### 2.8. Flow cytometry

For intracellular detection of IL-17 and Foxp3, cells were restimulated 25 ng/mL phorbol 12-myristate 13-acetate (PMA) plus 250 ng/mL ionomycin (Sigma-Aldrich, St Louis, MO) for 4 h in the presence of monensin-containing GolgiStop (BD Biosciences). Cells were harvested and stained with PerCP-conjugated anti-CD4 and APC-conjugated anti-CD25 mAbs (eBioscience). After fixation with fixation/permeabilization solution, cells were stained with FITC-conjugated anti-IL-17 and/or PE-conjugated anti-Foxp3 mAbs (eBioscience). The cells were examined with BD LSRIIFortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, CA).

### 2.9. Real-time RT-PCR

A LightCycler 480 II instrument (Roche Diagnostics, Basel, Switzerland) was used for PCR amplification and analysis. All reactions were performed with LightCycler480 SYBR Green I Master according to the manufacturer's instructions. The following primers were used for mouse sequences: IL-17A, 5′-CCT CAA AGC TCA GCG TGT CC-3′ (sense) and 5′-GAG CTC ACT TTT GCG CCA AG-3′ (antisense); RORγt, 5′-TGT CCT GGG CTA CCC TAC TG-3′ (sense) and 5′-GTG CAG GAG TAG GCC ACA TT-3′ (antisense); and β-actin, 5′-GTA CGA CCA GAG