

Clonorchis Sinensis-Derived Protein Attenuates Inflammation and New Bone Formation in Ankylosing Spondylitis

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Abstract

Background: Helminth infections and their components have been shown to have potential to modulate immunity and attenuate immune response. The objective of this study was to evaluate potential protective effects of *Clonorchis sinensis*-derived protein (CSp) on ankylosing spondylitis (AS).

Methods: Cytotoxicity of CSp at different doses was assessed by MTS and flow cytometry before performing experiments. Peripheral blood mononuclear cells (PBMCs) and Synovial fluid mononuclear cells (SFMCs) were obtained from AS patients. Inflammatory cytokine-producing cells were analyzed using flow cytometry. SKG mice were treated with CSp or vehicle. Inflammation and new bone formation were evaluated using immunohistochemistry, positron emission tomography (PET) and micro-computed tomography (CT).

Results: Treatment with CSp resulted in no reduced cell viability of PBMCs or SFMCs. In experiments culturing PBMCs and SFMCs, the frequencies of IFN- γ and IL-17A producing cells were significantly reduced after CSp treatment. In the SKG mouse model, CSp treatment significantly suppressed arthritis and enthesitis. Micro-CT analysis of hind paw revealed less new bone formation in CSp-treated mice than in vehicle-treated mice.

Conclusions: We provide the first evidence demonstrating that CSp can ameliorate clinical signs and cytokine derrangements in AS. In addition, such CSp treatment could reduce new bone formation of AS.

Background

Ankylosing spondylitis (AS) is characterized by inflammation of sacroiliac joints, axial skeleton and to a lesser degree, peripheral joints [1, 2]. The occurrence of AS is insidious and progressive, leading to spinal deformity, loss of ability to work, and disability, seriously affecting the quality of life [3]. Although the prevalence of AS is significantly different among geographic areas [4], it has been increased from 0.04% to 0.09% in USA in recent decades [5]. Several researchers have explained this increasing tendency partly by hygiene hypothesis [6], supporting an inverse relationship between worm infection and T helper type 1/17 (Th1/17)-based inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease, type 1 diabetes and multiple sclerosis. Although the exact mechanism of AS remains largely unclear, Th1/17 pro-inflammatory mediators are strongly implicated in the initiation and progression of this disease [7–9]. Treatment with Tumor necrosis factor (TNF) blocker is effective for physical function, disease activity, and health-related quality-of-life outcomes [10, 11]. However, not all AS patients respond to TNF blockers. Even the ability of TNF blockers to prevent ankylosis remains controversial [12, 13]. Thus, novel therapeutic strategy for AS is needed.

Recently, many attempts have been made to administer parasites (e.g., ingestion of eggs of the nematode *Trichuris suis*) as new modalities for treating autoimmune disorders [14, 15]. In animal models of rheumatoid arthritis, some helminth species or their proteins have been shown to be able to reduce the severity of the clinical symptoms [16–21]. The role of any trematodes-induced proteins in the

pathogenesis and disease activity of AS has not been reported yet. Thus, the aim of this study was to assess the therapeutic potential of *Clonorchis sinensis*-induced proteins (CSp) for AS. Results of this study will provide a basis for further clinical applications of *C. sinensis*.

Methods

Human samples

All patients satisfied the modified New York (NY) criteria for AS [22]. Peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs) were obtained from patients with active AS. Demographic characteristics of the patients are shown in Table 1. This study was carried out in compliance with the Helsinki Declaration. It was approved by the Ethics Committee. Written informed consent was obtained from all subjects (CNUH-2011-199).

Clonorchis sinensis crude antigen preparation

Frozen *C. sinensis* adult worms were mixed with 1 mL of homogenation buffer (5mM EDTA, 1% NP-40, 0.2mM PMSF), homogenized, vortexed for 5 min, and centrifuged at 13,000 rpm for 20 min at 4°C. After centrifugation, the supernatant was used for protein extraction using Pierce BCA Protein Assay Kit (Thermo Scientific Co., Rockford, IL, USA) according to the manufacturer's guideline. Extract protein was concentrated to be 6 µg/200 µL of working reagent.

Cell viability assay

To determine cell proliferation and cytotoxicity, cells were seeded and stimulated with CSp for indicated time durations (4 hours and 24 hours). Cell viabilities of PBMCs and SFMCs according to CSp treatment were investigated using a Cell Titer 96 AQueous One Solution Reagent (G3580, Promega, USA). Briefly, 100 µl RPMI was mixed with MTS solution (20 µl/well) and added to each well. After incubation, absorbance was recorded at wavelength of 490 nm with a 96-well microplate reader (Molecular Devices, USA). For each flow cytometry analysis, whole cells were surface stained with anti-Fixable Viability Dye-eFluor780 (65-0865-14, Invitrogen, USA).

Co-culture of human inflammatory cells with CSp

PBMCs and SFMCs were isolated and suspended in a complete medium (RPMI 1640, 2 mM L-glutamine, 100 units/ml of penicillin, and 100 µg/mL of streptomycin) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), and then seeded into 96-well plates at cell density of 1×10^6 cells/well. Cells in a 96-well culture plate were treated with CSp and then were activated with Dynabeads Human T-Activator CD3/CD28 (11163D, Invitrogen, USA) to obtain a bead to cell ratio of 1:1. Cells were then incubated in a humidified CO₂ incubator at 37°C for 24 hours. After stimulating with PMA (100 ng/mL) and ionomycin (1 µM) for 4 hours, cells were stained with Pacific Blue-conjugated anti-CD4 (300521, Biolegend, USA), and PE-conjugated anti-CD45RO (304205, biolegend, USA). Cells were washed,

fixed, permeabilised with Cytofix/Cytoperm buffer and intracellularly stained with FITC-conjugated anti-IFN- γ (552887, BD, USA), APC-conjugated anti-IL-17A (512334, BD, USA) antibodies followed by analysis with a FACS Calibur flow cytometer. Data were analyzed using FlowJo Software (BD, USA).

Experimental animal model and clinical score

SKG mice on a BALB/c background were purchased from Clea Japan (Tokyo, Japan) and bred under a specific pathogen-free facility. These mice were kept in individually ventilated cages and provided with water and standard diet *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee (CNU IACUC-H-2018-35). They were conducted in accordance with Laboratory Animals Welfare Act, Guide for the Care and Use of Laboratory Animals. Female mice were used in this study. Experiments had three groups: negative control (NC, n = 10 mice), positive control (PC, n = 10 mice), and CSp treatment group (n = 10 mice). For both PC and CSp treatment groups, a suspension of curdlan (Wako, Osaka, Japan) was intra-peritoneally (i.p.) administered at 3 mg/kg to mice aged 11 weeks. CSp treatment group received CSp (6 μ g/0.2 mL) i.p. twice before arthritis induction. The same dosage was then maintained once a week until sacrifice. Positive and negative control groups received PBS i.p. instead of CSp. At the start of therapeutic treatment, randomization was performed based on serial number generation. Clinical signs of mice were monitored twice a week and scored by two independent observers. Scores of the affected joints were summed as follows: 0 = asymptomatic, 1 = slightly swelling of the ankles or toes, 2 = ankle swelling severely, 3 = ankle severely swelling and toe swelling, and 4 = ankle and toe swelling and twisting). Sixteen points were the highest possible points.

Positron emission tomography (PET) and micro-computed tomography (micro-CT) analysis

A day before sacrifice, mice were fasted for 16 hours prior to undergoing PET/ micro CT. Briefly, mice were anaesthetised followed by an i.v. injection of 18.5 MBq ^{18}F -FDG and scanned sequentially, starting at 30 min post-injection using a small animal PET-CT (SEDECAL, SuperArgus PET/CT 4r, MARRID, SPAIN) with a detachable animal bed for maintaining animal position. Anesthesia was maintained by inhalation of approximately 1.5% isoflurane/O₂ for 1 L/min for individual scans and for 2 L/min to obtain mouse hotel scans administered via nose cone. PET images were reconstructed using OSEM3D (ordered subset expectation maximization)/MAP (maximum a posteriori) algorithm. Volume of interest (VOI) with a diameter of 6-mm was drawn at both sides of hind paws. Maximal and mean standardized uptake values (SUVx) were then measured. The following CT scan parameters were employed: energy/ intensity of 40 kV, electric current of 500 μ A, sample time of 40 msec and resolution of 768 x 972 pixels. Before CT scan, QRM-MicroCT-HA phantom (QRM GmbH; Moehrendorg, Germany) was used for calibration. For segmentation of newly formed bone and normal mature bone, segmentation thresholds values of hind paws and caudal vertebrae were used as described in a previous study [23].

Immunohistochemistry (IHC) and histologic scoring

At experimental end point, specimens of ankle were obtained from mice and fixed with 10% formalin for one week. After fixation, specimens were decalcified in 10% formic acid with shaking at 37°C for a week

and embedded in paraffin. Paraffin blocks were sectioned at a thickness of 3.5 µm and deparaffinised in neo-clear (109843, Merck, USA), hydrated with graded ethanol and stained with hematoxylin (105174, Merck, USA) and eosin (HT110216, Sigma, USA). All staining procedures followed standard protocols. Two blinded readers performed pathologic scoring. Histologic features of peripheral enthesitis were scored 1-4 as described previously [24] , where 1 = mild inflammation at tendon insertion site, 2 = mild-to-moderate inflammatory infiltrate at insertion site and along tendon, 3 = severe inflammation with bone involvement, and 4 = severe inflammation with obliteration of tendon–bone interface.

Other methods

Immunoblotting procedure and flow cytometry analysis of mice splenocytes are described in detail in the Additional file 1.

Statistical analysis

Data in all graphs are shown as mean with standard deviation. Symptom score data were assessed using two-way ANOVA with time as a dependent variable. Statistical significance of difference between means was assessed using Kruskal-Wallis test with Dunn's multiple comparisons, Wilcoxon matched-pairs signed rank test or Mann Whitney test. All statistical analyses were performed using Prism 5.0 Software (GraphPad Software, San Diego, CA, USA). For all graphs, p value less than 0.05 was considered as significant and marked as follows: *, p = 0.05–0.01; **, p = 0.01–0.001 and ***, p < 0.001.

Table 1
Clinical characteristics of ankylosing spondylitis

	Peripheral blood mononuclear cells	Synovial fluid mononuclear cells
Total number	6	8
Age, mean \pm SD (years)	24 \pm 9.5	39.1 \pm 13.7
Male, n (%)	6 (100.0)	4 (50.0)
BASDAI, mean \pm SD	5.4 \pm 4.0	7.6 \pm 2.1
AS-DAS, mean \pm SD	3.6 \pm 2.1	
HLA-B27, n (%)	6 (100.0)	6 (75.0)
CRP, mean \pm SD (range, mg/dL)	2.4 \pm 1.6	4.6 \pm 3.6
Recent treatments (last three months)		
Naive, n (%)	3 (50.0)	2 (25.0)
NSAIDs use, n (%)	3 (50.0)	5 (62.5)
Sulfasalazine use, n (%)	1 (16.6)	4 (50.0)
TNF-blocker use, n (%)	0 (0.0)	3 (37.5)

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; HLA: Human Leukocyte Antigen; CRP: C-reactive Protein; NSAIDs: Non-steroid inflammatory drugs; TNF: Tumor Necrosis Factor.

Results

Cell viability of CSp treatment

Cell proliferation and cytotoxicity of CSp treatment for 4 hours and 24 hours was assessed before performing experiments. Treatment with CSp up to 24 hours resulted in no reduced cell viability of PBMCs or SFMCs, analyzed by MTS (Fig. 1a) and flow cytometry (Fig. 1b).

CSp suppresses the pro-inflammatory cytokines *in ex vivo* experiment

To explore the anti-inflammatory properties of CSp on cytokines in a human setting, PBMCs and SFMCs obtained from patients with active AS were stimulated and cultured *ex vivo* for 24 hours in the presence or absence of CSp. Flow cytometry gating strategies were shown in the Supplementary Figure 1. Frequencies of IFN- γ and IL-17A producing cells after treatment with CSp were significantly reduced compared to those before treatment (6.46 ± 6.12 vs. 27.88 ± 9.38 , $p = 0.0313$; 0.70 ± 0.54 vs. 3.30 ± 0.95 , $p = 0.0313$, respectively) (Fig. 2a). In SFMCs, frequencies of IFN- γ and IL-17A producing cells after

treatment with CSp were significantly decreased compared to those before treatment (41.12 ± 22.02 vs. 58.12 ± 18.75 , $p = 0.0078$; 2.07 ± 1.25 vs. 3.50 ± 2.76 , $p = 0.0391$, respectively) (Fig. 2b).

To determine the role of CSp in inflammatory cell signaling, immunoblot assay was performed. We observed that the anti-inflammatory effect of CSp on immune cells was mainly due to suppression of NF- κ B phosphorylation (Supplementary Figure 2).

CSp treatment suppresses clinical symptoms in SKG mice

To investigate the effect of CSp on the development and progression of AS in curdlan-treated SKG mice, mice were treated with either CSp or vehicle (PBS) from one week before curdlan injection (Fig. 3a). The CSp treatment delayed the onset of arthritis and significantly reduced the severity of arthritis (Fig. 3b). At the end of experiment, CSp injection significantly suppressed the arthritis symptoms (12.8 ± 2.78 vs. 5.7 ± 3.68 , $p = 0.008$) (Fig. 3c). Although typical psoriatic skin lesions did not develop, PC mice developed skin redness and hair loss around the ears and nose. However, skin lesions were not obvious in the CSp treatment group (Fig. 3d).

The frequency of IL-17A producing cells from SKG splenocytes in the CSp treatment group was significantly lower than that from vehicle treated mice (1.96 ± 0.39 vs. 2.96 ± 0.77 , $p = 0.0317$). On the other hand, frequencies of IFN- γ and TNF- α producing cells were not significantly reduced in the CSp-treated group, compared to those from vehicle-treated mice (2.68 ± 0.86 vs. 4.47 ± 1.78 , $p = 0.2857$; 6.08 ± 3.02 vs. 17.59 ± 16.39 , $p = 0.2187$, respectively) (Supplementary Figure 3).

A variety of targets for inflammation imaging have been discovered and utilized. Among them ^{18}F -FDG has been successfully applied in the inflammation realm. ^{18}F -FDG-used PET features high sensitivity and specificity. PET has become one of the most frequently used molecular imaging techniques in the clinic [25]. Therefore, we used PET image for measuring inflammation on peripheral joints. Representative images are shown in Fig. 4a. Mean values of max. SUV of peripheral arthritis from the CSp-treated group were significantly lower than those from vehicle-treated mice (1.36 ± 1.03 vs. 2.35 ± 1.15 , $p = 0.0056$) (Fig. 4b). To assess the effect of CSp on local enthesal inflammation, ankle tissues also were analyzed by immunohisto-chemistry. Representative enthesal tissue stains at the end of experiment are shown in Fig. 4c. Histologic assessment showed that mice treated with CSp had less enthesitis than PC mice. (1.50 ± 0.57 vs. 3.55 ± 0.52 , $p = 0.0047$) (Fig. 4d).

CSp inhibits new bone formation in *in vivo* mice model

To evaluate whether CSp could reduce new bone formation, axial and peripheral joints were assessed by micro-CT at the end of therapeutic treatment. Representative micro-CT images for each group are shown in Fig. 5a. Analysis of hind paw revealed less low-density bone (used as a measure for new bone) in CSp-treated mice than in vehicle-treated mice (5.02 ± 0.92 vs. 7.73 ± 1.91 , $p = 0.001$) (Fig. 5b). CSp-treated mice exhibited spinal new bone formation similar to PC group (57.05 ± 9.40 vs. 65.15 ± 10.55 , $p = 0.3383$) (Fig. 5c). Quantification of normal-density bone (> 800 mg of HA/cm 3) revealed that mice with

CSp treatment had similar bone volume to other groups, suggesting no reduction in normal bone loss (data not shown).

Discussion

Helminth parasites inhabit immune-competent hosts for a long period of time and appear to develop strategies to induce strong anti-inflammatory responses in the infected host. The inverse prevalence between autoimmune diseases and helminth infection implies a potential protective role of helminth infection in autoimmune diseases. Yet, the effect of any trematode or their products on AS remains unclear. To the best of our knowledge, this is the first study that demonstrates the ameliorative effect of CSp on the clinical signs and cytokine derangements in AS. Moreover, the administration of this CSp could reduce new bone formation in an *in vivo* AS mice model.

Regarding on the helminths therapy targeting arthritis, experimental infections with *S. japonicum*, *S. mansoni*, *Ascaris suum* and *Hymenolepsis diminuta*, or administrations of their proteins have been shown to possess inhibitory effects on murine collagen-induced arthritis [17–21]. In thses studies, anti-arthritis activity is mediated by up-regulation of the Foxp3⁺ Tregs with subsequent favorable modulation of both pro- and anti-inflammatory cytokines [20]. We speculated that *C. sinensis*, a liver fluke found predominantly in Asia including Korea and China, could be an alternative candidate of *Schistosoma*. Our speculation was supported by previous studies showing an inhibitory effect of *C. sinensis* protein in mice models of asthma and colitis by inducing CD4⁺CD25⁺Foxp3⁺ T cells or IL-10 secreting macrophages [26, 27].

Up to date, only one study has reported the effect of *C. sinensis* on arthritis [28]. In that study, *C. sinensis* direct infection has a bad effect on arthritis. It induced abnormal immune response in mice with collagen-induced arthritis. Although preliminary results from human clinical trials have indicated that treating patients with inflammatory diseases using live helminth parasites has therapeutic potential, the use of helminth products as therapeutic agents might have advantages over live infection. Furthermore, it should be considered carefully regarding proper adjustment for bioavailability. Thus, we assessed the effect of CSp treatment under suitable condition excluding poor cell viability and proposed that CSp treatment could be a safe option showing minimal cytotoxicity without direct infection of *C. sinensis*. We noticed a remarkable decrease of IFN-γ and IL-17A producing cells after treatment with CSp in human PBMC and SFMCs, indicating a direct inhibitory effect of CSp on both systemic circulation and regional site. The NF-κB family of transcription factors are known to play essential roles in inflammation [29]. We found that the anti-inflammatory effect of CSp on immune cells was mainly due to suppression of NF-κB activation. This phenomenon might serve as the basis of the beneficial effect of CSp for maintaining immune-homeostasis, hence providing protection against immune-mediated diseases, although we could not demonstrate the correlation between IL-17 inhibition and IL-10 recruitment. This finding leads us to clarify the evidence of therapeutic agents against AS using a murine model.

Using SKG mice model, we found that CSp treatment group presented a markedly ameliorated disease-specific symptoms and a significantly decreased production of IL-17A in splenocytes. Levels of INF- γ and TNF- α seem to decrease in CSp-treated mice compared to PC mice. However, the decrease was not statistically significant. PET imaging and histological findings supported the inhibitory effect of CSp treatment on arthritis and enthesitis. In line with our data, similar changes in cells and cytokines such as downregulation of pro-inflammatory cytokines (INF- γ , TNF- α , and IL-17A) have been found in animals infected with *S. mansoni* [17]. Such a certain common alterations in the immune response pattern due to parasite might have contributed to the reduced severity of Th1/17-mediated immune disorders including AS. Result of this study suggest that CSp treatment is able to attenuate the symptom severity of AS via systemic and local suppression of pro-inflammatory mediators, further suggesting the potential of therapeutic agents for treating AS.

It is noteworthy that our results suggest that CSp can suppress new bone formation in an *in vivo* mice model. In addition, micro-CT analysis revealed that stat3-p Inh treatment had no significant effect on normal bone loss by preserving normal density bone. Effective and safe therapeutic approaches to AS remain a substantial clinical challenge as the suitability of TNF blockade for preventing new bone formation remains controversial. The IL-23/17 axis has been suggested to be a key player in AS pathogenesis and osteoblastogenesis directly [9, 30–32]. Although the exact mechanism remains unknown, our data highlighted that CSp could be active to inhibit new bone formation in AS which might be accompanied by attenuation of IL-17A via JAK2/STAT3 signaling [32]. The current study highlights further need for elucidating the role of CSp in osteoblastogenesis.

This study has several limitations. First, we could not clarify the exact mechanism in the inhibition of Th17 response. However, this might be mediated by upregulation of regulatory cell populations such as CD4 $^{+}$ CD25 $^{+}$ Foxp3 $^{+}$ T cells or IL-10 secreting macrophages [20, 26, 27]. The inverse correlation of IL-10 with Th17 cells has been addressed both in humans and an experimental murine model [17, 33]. All the findings of these studies support the potential of using CSp to achieve a balance between Th17 cells and Treg cells by altering IL-10 levels as a promising treatment for AS.

Conclusions

In summary, our results confirmed that CSp treatment could effectively ameliorates not only inflammation but also new bone formation in AS mainly by inhibitory manner to Th17 response. Our finding showed that CSp could suppress the pathology associated with AS without compromising the host's ability to fight disease, suggesting that therapies based on the mode of action of CSp might provide safe and novel therapeutic targets for treating AS.

Abbreviations

AS:Ankylosing spondylitis; CSp: *Clonorchis sinensis*-derived protein; i.p.: intra-peritoneally; CT: computed tomography; NC: negative control; PBMC: peripheral blood mononuclear cell; PC: positive control; PET:

Positron emission tomography; SFMC: synovial fluid mononuclear cells; TNF: Tumor necrosis factor.

Declarations

Ethics approval and consent to participate

This study was carried out in accordance with all relevant institutional guidelines. The Ethics Committee of Chonnam National University Hospital approved this study (CNUH-2011-199) and written informed consent was obtained from all subjects. All animal experiments were approved by the Institutional Animal Care and Use Committee (CNU IACUC-H-2018-35).

Consent for publication

Not applicable.

Availability of data and materials

The data and materials used in this study are available for the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yu Jeong Lee, Moon-Ju Kim, Eun Jeong Won and Tae-Jong Kim conceived the study, participated in study design, data analysis and were responsible for writing and submission of the final manuscript.

Yu Jeong Lee, Moon-Ju Kim, Sungsin Jo, So-Hee Jin, Pu-Reum Park, Jahae Kim, Ji-Young Kim carried out the experimental studies. Ho-Chun Song, Seung Cheol Shim, and Tae-Hwan Kim analyzed and interpreted data. Sung-Jong Hong offered *C. sinensis* adult worms and interpreted data. All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

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Figures

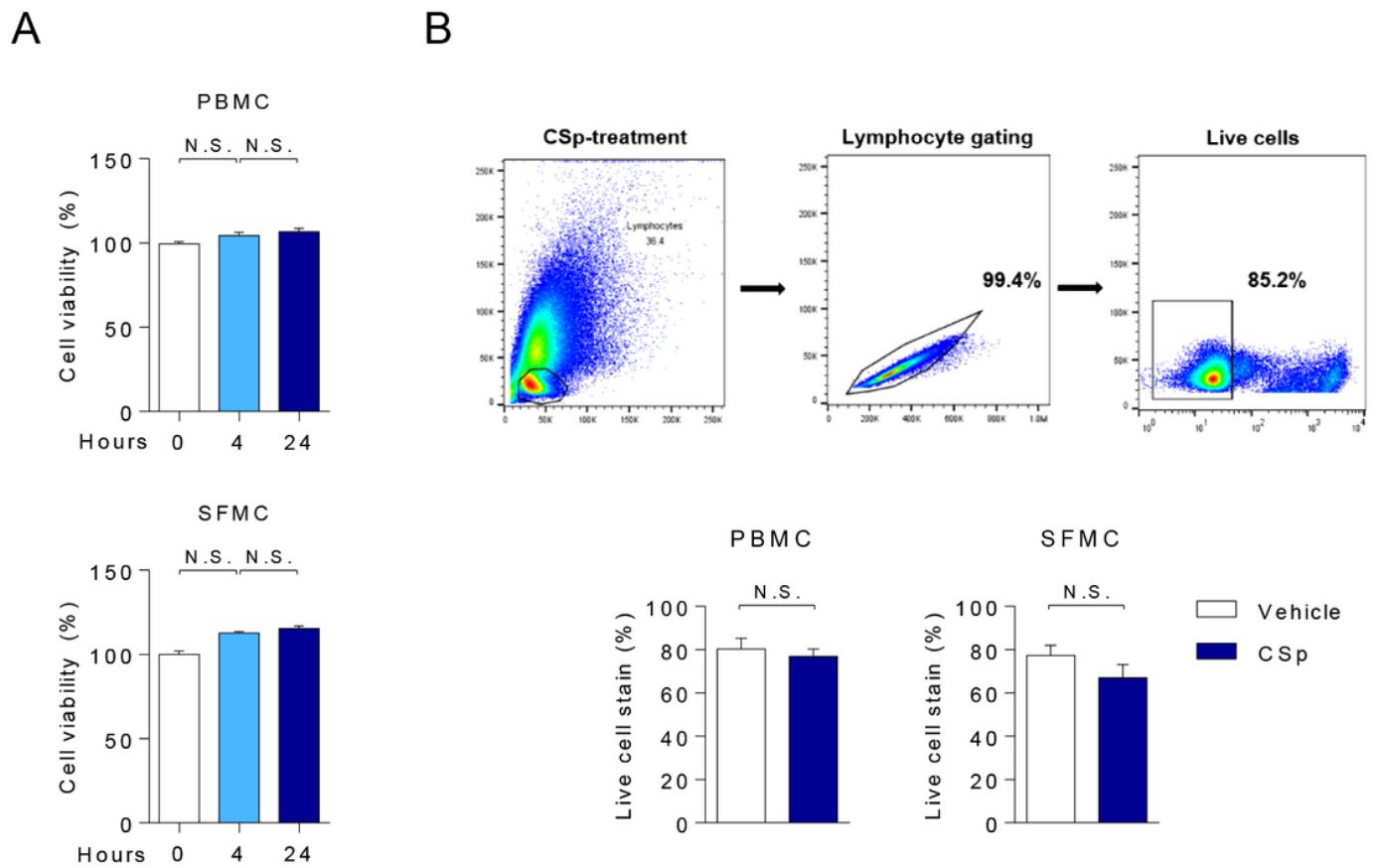
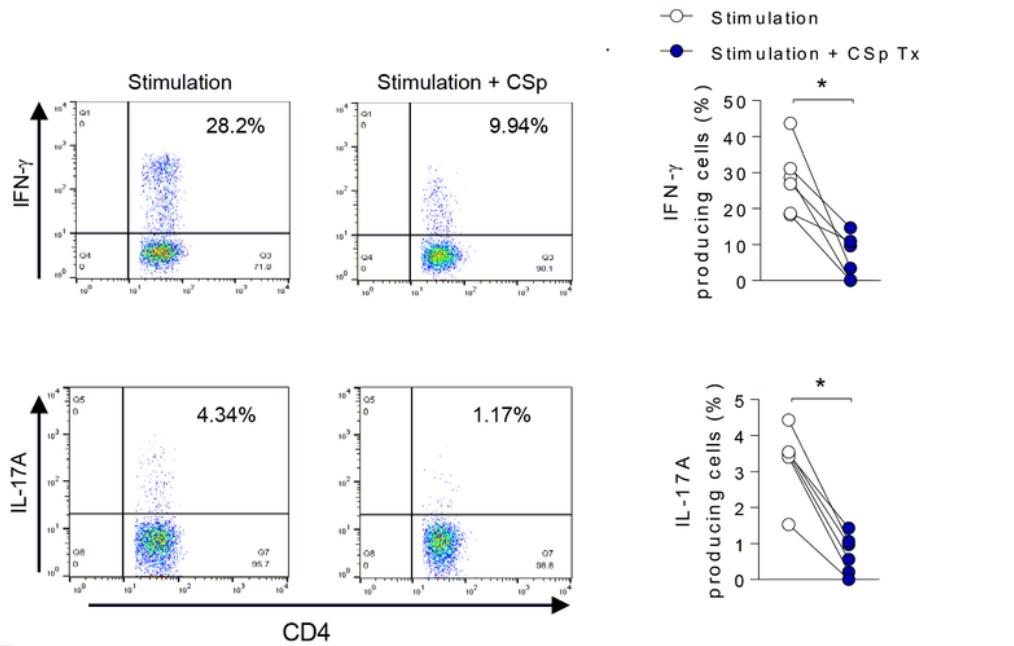


Figure 1

Figure 1

Assessment of cell viability with CSp. (a) Cell viability of PBMCs and SFMCs were analyzed by MTS assay. Kruskal-Wallis test with Dunn's multiple comparisons was performed to determine statistical significance. Values are the mean \pm SEM. (b) Anti-Fixable Viability Dye of PBMCs and SFMCs were stained and measured by flow cytometry. Mann-Whitney U test was performed to determine statistical significance. Values are the mean \pm SEM. N.S.: not significant. CSp: Clonorchis sinensis-derived protein; PBMCs: peripheral blood mononuclear cell; SFMCs: synovial fluid mononuclear cells.

A



B

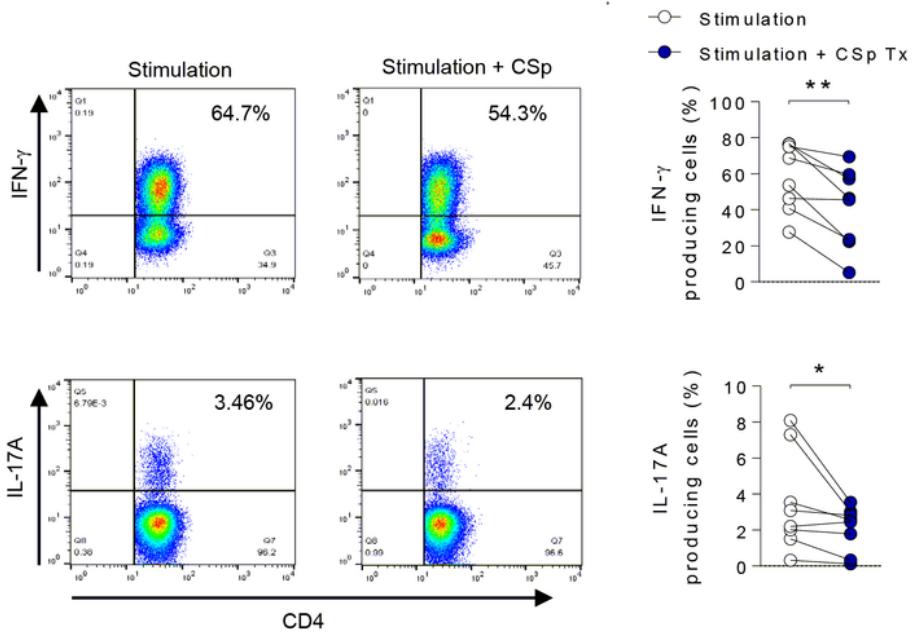


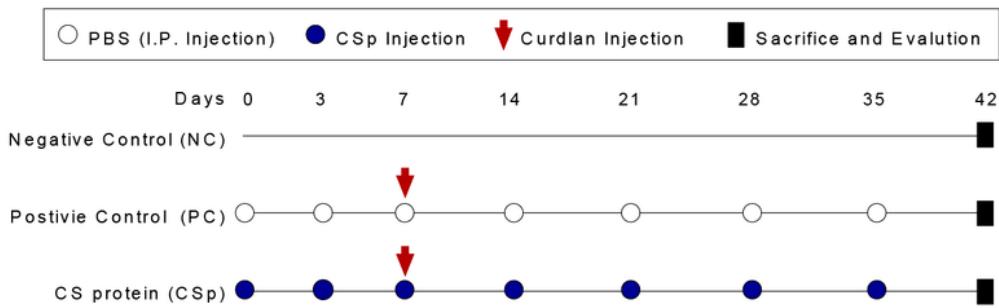
Figure 2

Figure 2

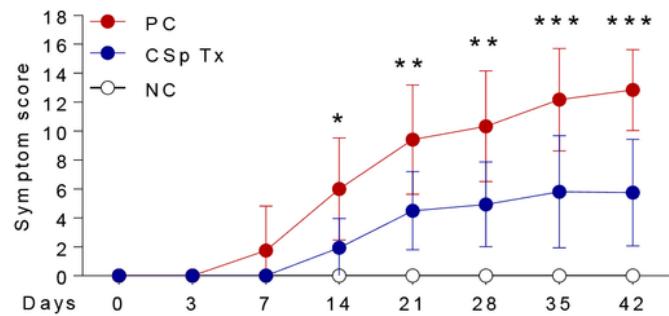
CSp decreases production of inflammatory cytokines from PBMCs and SFMCs in patients with ankylosing spondylitis (AS). Cells were activated with Dynabeads Human CD3/CD28 in the presence or absence of CSp for 24 hours. Then cells were stimulated with PMA and ionomycin for 4 hour. Percentages of INF- γ and IL-17A positive cells from PBMCs (a) and SFMC (b) were analyzed. Symbols represent individual sample. * = $P < 0.05$, ** = $P < 0.01$, by Wilcoxon matched-pairs signed rank test. CSp:

Clonorchis sinensis-derived protein; PBMCs: peripheral blood mononuclear cell; SFMCs: synovial fluid mononuclear cells.

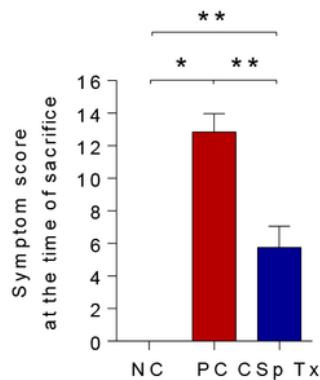
A



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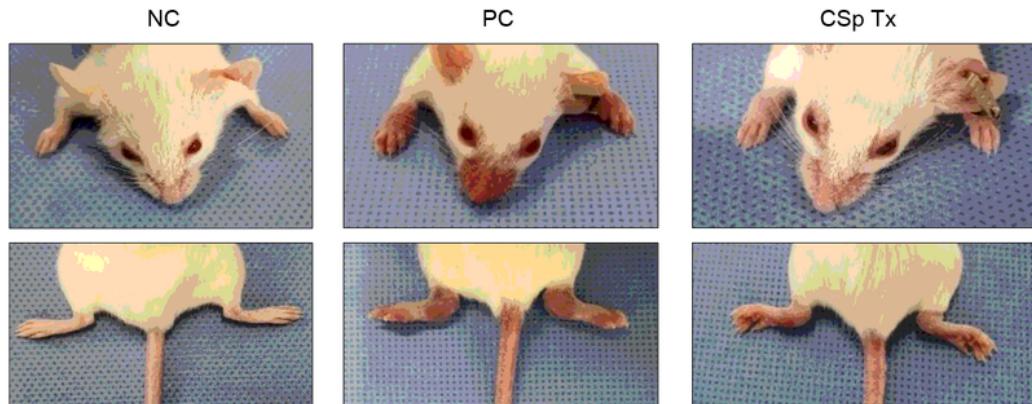


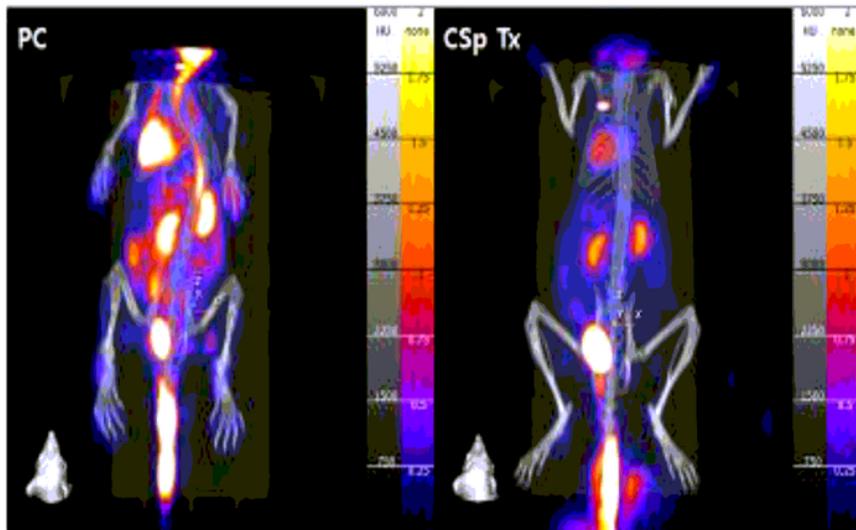
Figure 3

Figure 3

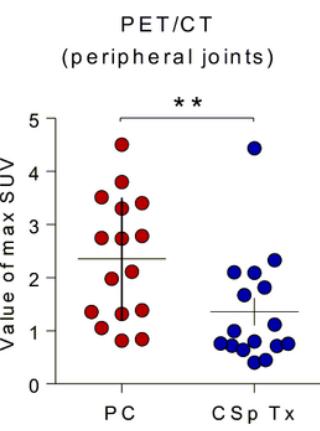
CSp treatment reduces clinical symptoms in SKG mice. (a) SKG mice were treated with CSp or vehicle i.p. for 6 weeks. (b) The arthritis scores were determined based on clinical arthritis severity in each group ($n = 10$ mice for each group). Values of left panel are the mean \pm SEM. * = $P < 0.05$, ** = $P < 0.01$, *** = $P <$

0.001, by two-way analysis of variance (ANOVA). (c) Symptom scores at the end of experiment. Values in right panel are the mean \pm SEM. * = $P < 0.05$, ** = $P < 0.01$, by Kruskal-Wallis test with Dunn's multiple comparisons. (d) Representative mouse from each group at the end of experiment. CSp: Clonorchis sinensis-induced protein; NC: negative control; PC: positive control.

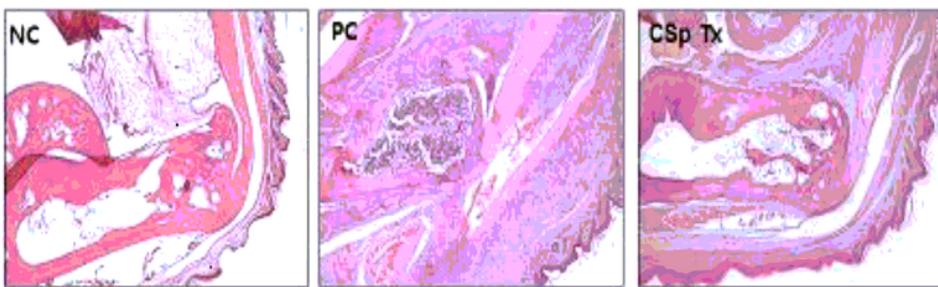
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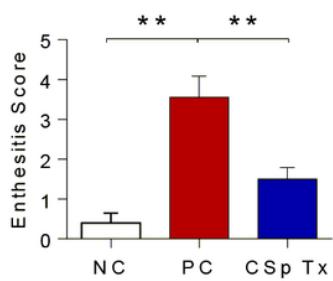


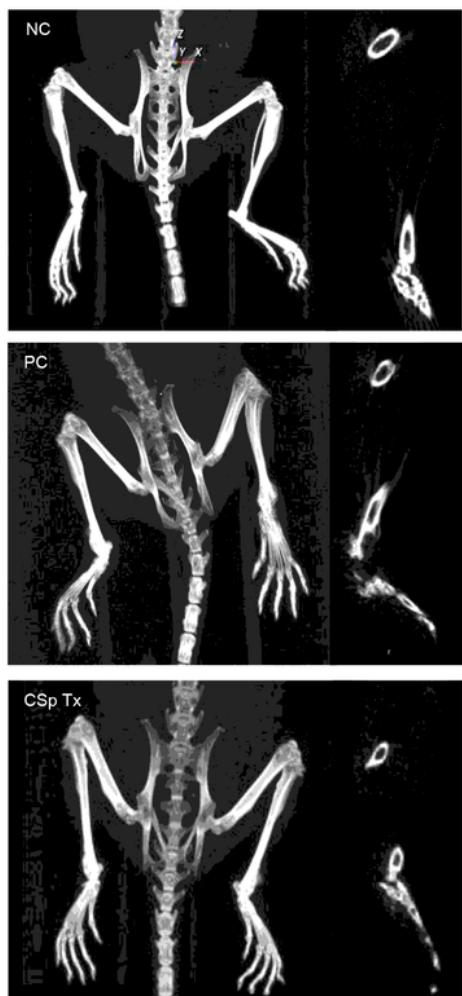
Figure 4

Figure 4

CSp treatment reduces arthritis and enthesitis in SKG mice. Mice were scanned using 18F-FDG PET at the end of experiment. (a) Representative PET image are shown in each group. (b) The mean values of max. SUV of peripheral joint were measured. Symbols represent individual ankle joint ($n = 8$ mice for each group). ** = $P < 0.01$, was determined by Mann Whitney test. (c) Representative heel tissue stains at the end of experiment are shown. (d) Analysis of histological scores for enthesitis were shown in bar graphs.

Values are the mean \pm SEM. ** = $P < 0.01$, by Kruskal-Wallis test with Dunn's multiple comparisons. CSp: Clonorchis sinensis-induced protein; NC: negative control; PC: positive control.

A



B

C

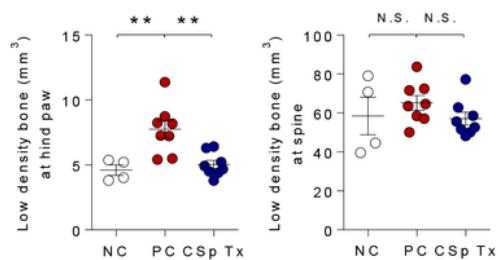


Figure 5

Figure 5

Reduction of new bone formation by CSp in SKG mice model. (a) Representative radiographic images are shown for each group. Quantification of low-density bone (used as a measure for new bone) in hind paws (b) and spine (c) from each group. Symbols represent individual mouse ($n = 4$ mice for NC, $n = 8$ mice for

PC and CSp group). N.S., not significant, ** = P < 0.01, by Kruskal-Wallis test with Dunn's multiple comparisons. CSp: Clonorchis sinensis-induced protein; NC: negative control; PC: positive control.

Supplementary Files

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