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ORIGINAL PAPER

Schistosoma japonicum cystatin attenuates murine collagen-induced arthritis

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Abstract Recombinant SjCystatin (rSjCystatin), a recombinant protein of *Schistosoma japonicum* cystatin, has been reported to have an effect on immunoregulation mediated by IL-10 induction. Rheumatoid arthritis (RA) is a common autoimmune inflammatory arthropathy, and recombinant immune-modulating drugs for RA treatment are under development. We aimed to study the putative immune regulation of rSjCystatin and its prophylactic/therapeutic effects on murine collagen-induced arthritis (CIA). CIA was induced in DBA/1 mice by inoculation with bovine collagen II (CII). rSjCystatin was administered prior or post development of CIA. The severity of CIA was assessed using established clinical and histopathological scoring systems. The incidence was also determined. The CII-specific antibodies in sera and cytokines in splenocyte culture supernatants were measured by ELISA. Th1/Th2/Th17 cells and Tregs development in splenocytes were monitored by flow cytometry. The inflammatory mediators in the diseased joint were semiquantitated by qPCR. Prophylactic injection of rSjCystatin attenuated paw clinical scores, incidence, and histopathology scores of joints in CIA mice. The arthritis-alleviative effects were closely associated with the augmentation of IL-4, IL-10, and collagen-specific IgG1, and with the distinct reduction of IFN-γ, collagen-specific IgG2a, and the marked decrease of proinflammatory cytokines IL-6, IL-17, and TNF-α and RANKL. The data indicate that rSjCystatin may prevent cartilage destruction and inflammation of joints in CIA mice. The effects are related to the inhibitory modulation of Th1 and Th17 and upregulation of Tregs and Th2 via a shift of cytokines profiling from Th1 to Th2 response.

Keywords Collagen-induced arthritis · *Schistosoma japonicum* · Cystatin · Immune modulation

Fang Liu and Weisheng Cheng contributed equally to this work.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory and systemic destructive autoimmune disorder that affects approximately 0.5–1 % of the world's population (Silman and Pearson 2002). Ideal therapies should combine immunomodulatory, anti-inflammatory, and antibone resorptive properties in view of the autoimmune and bone/cartilage degenerative pathology of RA (Schepetkin et al. 2015). In recent years, some novel biologics have been proposed to substitute conventional disease-

modifying antirheumatic drugs (DMARDs) (Breedveld et al. 2006; Klareskog et al. 2004). Technological advances have promoted the introduction of the tailor-made cytokine-targeted therapies (anti-TNF, anti-IL-1, and anti-IL-6) into clinical practice. Cytokine antagonists that inhibit TNF-α, IL-6, or IL-1β establish a preventive effects (Brzustewicz and Bryl 2015; Luo et al. 2015; Smolen et al. 2007). Yet despite that, various biologic agents, as well as DMARDs, are not applicable to all the RA patients. Thus, the development of innovative prophylactic or therapeutic strategy is obligatory. Development of CIA is related to T and B cells (Holmdahl et al. 1995). T helper (Th) cells are clearly central to RA pathogenesis as they are involved in the initiation and sustainment of the disease (Cope 2008). Abnormal proportions of Th1/Th17/Tregs lymphocytes occur in the peripheral blood of RA patients. RA is generally considered to be mediated by the Th1 response, which aggravates joint inflammation by way of Th1 cells releasing IFN-γ, TNF-α and lymphotoxin β in synovial tissue (Germann et al. 1995; Yamada et al. 2008). The IL-17-producing $CD4^+$ T cell subpopulation, termed Th17 cells, represents a different proinflammatory Th-cell lineage that has been confirmed to play a critical role in the generation of several types of autoimmune arthritis such as collagen-induced (CIA) and glucose-6-phosphate isomerase (GPI)-induced arthritis (Furuzawa-Carballeda et al. 2007; Iwanami et al. 2008; Nakae et al. 2003). Regulatory T cells (Tregs) maintain immune system homeostasis and tolerance to self-antigens. Defects of Tregs are important in the pathogenesis of autoimmune diseases. Adoptive transfer of activated Tregs was shown to suppress osteoclastogenesis and CIA (Kelchtermans et al. 2009). Additionally, induction of Tregs by immunomodulatory agents could significantly reduce the incidence and severity of CIA and suppress the autoreactive response to restore immune tolerance (Gonzalez-Rey et al. 2007).

Helminth parasites have co-evolved with modern humans for hundreds of thousands of years and are widely prevalent in tropical and subtropical areas (Weinstock 2012). Many helminth species colonize the human host with a range of medical consequences and constitute a public health risk (Fleming and Weinstock 2015). However, some chronic infections are typical of Th2-dominant response, which would be addition to overall already down-regulated immune system (Dunne and Cooke 2005). It has been proposed that intentional infection of humans with helminth parasites may be therapeutic in autoimmune/immune-mediated diseases (Dunne and Cooke 2005; Fleming and Weinstock 2015). *Schistosoma japonicum* infection has shown to be protective in a murine model of CIA (Song et al. 2011). Nevertheless, intensity and chronicity of infection are important factors associated with the amelioration of allergic inflammation, whereas acute infections may procure aggravation of allergic responses (Smits et al. 2007). Hence, unique worm proteins are considered as candidates for drugs of autoimmune diseases.

Recently, some cysteine protease inhibitors (cystatins) have been identified in parasites, most of which belong to the type II family. Researchers have investigated the influence of these proteins on some inflammatory or allergic diseases such as asthma and inflammatory bowel diseases (IBD), with promising results (Schnoeller et al. 2008b). Cysteine protease is extensively expressed in organisms and participates in many vital physiological processes, and the papain-like cysteine proteases in the inflamed joint contribute to the tissue damage (Gabrijelcic et al. 1990; Lenarcic et al. 1988; Trabandt et al. 1990). It is also crucially involved in bone remodeling, including osteogenesis and reabsorption (Brage et al. 2004). Therefore, cysteine proteases have been considered as potential drug targets to treat degenerative tissue and inflammatory conditions (Yasuda et al. 2005). *S. japonicum* cystatin is a member of cystatin and the recombinant SjCystatin (rSjCystatin) possesses enzymatic activity by inhibiting the proteolytic activity of papain. However, it possesses negative immune regulatory property by inhibiting antigen presentation processes in vitro experiment (Zhou et al. 2014). The precise mechanism of the potential immunomodulatory or therapeutical effects mediated by rSjCystatin in rheumatoid inflammation remains to be determined. We investigated the potential effects of rSjCystatin on CIA mice.

Materials and methods

Animals

Forty DBA/1 J healthy male mice, 7 to 8 weeks old, were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). The mice were fed ad libitum in specific pathogen-free (SPF) level animal facility of the Laboratory Animal Centre of Anhui Medical University. This study was carried out in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The institutional review board of the Anhui Medical University Institute of Biomedicine approved all of the animal procedures performed (approval No. AMU26-08061).

Expression and purification of recombinant *S. japonicum* cystatin

The cDNA of SjCystatin was cloned and expressed in *Escherichia coli* as described previously (Zhou et al. 2014). After induction, the bacteria cells were harvested, ultrasonicated, and centrifuged. The recombinant proteins were expressed efficiently as soluble and purified by a His-Bind® Purification Kit (Merck Millipore, Billerica, MA, USA) according to the manufacturer's instruction, then dialyzed against PBS/0.05 % Triton X-100. To remove endotoxin

contaminations, we used Affinity Pak™ Detoxi-Gel™ Endotoxin Removing Gel (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instruction. Protein concentrations were determined using Pierce bicinchoninic protein assay, BCA (Thermo Scientific, Waltham, MA, USA).

Administration of rSjCystatin injection and arthritis induction in mice

The 40 mice were randomly divided into 4 treatment groups each made up of 10 mice. They included four groups which constituted the normal control (group A), CIA model control (group B), prophylactic (group C), and therapeutic (group D) mice, respectively. Group A mice were untreated with rSjCystatin or phosphate-buffered saline (PBS). Also, they were not exposed to arthritis induction. Mice in group B received 100-μl PBS whereas those in group C were treated with rSjCystatin (100 μg) dissolved in 100 μl PBS once weekly for 4 weeks (28 days) through intraperitoneal injection. This was followed by arthritis induction. Bovine type II collagen (CII; Chondrex, Redmond, WA, USA; 2 mg/ml in 0.05 M acetic acid) was emulsified in Freund's complete adjuvant (CFA) (Chondrex, 2 mg/ml of *Mycobacterium tuberculosis*) (1:1, *v*/*v*). On day 0, each mouse in groups B and C received 100-μl emulsion through intradermal injection at the base of the tail. On day 21, the mice in both groups were boosted intraperitoneally with 100-μg CII in PBS. Almost all the mice showed clinical signs and symptoms of CIA consistently by day 25. The clinical signs and symptoms of CIA in the mice were evaluated from days 21 to 40. To investigate the therapeutic efficacy of rSjCystatin, arthritis was induced in each mouse in group D using the same induction regimen described for groups B and C mice. Treatment began at day 25 postinduction. Treatment was continued on days 25, 28, 31, 34, 37, and 40. All the mice in groups B, C, and D were sacrificed on day 42 postinduction for further investigations. Group A mice were also sacrificed at the same time. Experimental groups are shown in Table 1.

Clinical scoring and incidence of CIA

The severity of arthritis was assessed using an established scoring system for evaluation: score 0 = No evidence of erythema and swelling; 1 = erythema and mild swelling confined to the tarsals or ankle joint; 2 = erythema and mild swelling extending from the ankle to the tarsals; 3 = erythema and moderate swelling extending from the ankle to metatarsal joints; and 4 = erythema and severe swelling encompassing the ankle, foot and digits, or ankylosis of the limb (Brand et al. 2007). The cumulative scores for all four paws of each mouse (maximum possible score 16) were used to represent overall disease severity and progression. For the evaluation of incidence, mice were considered to have arthritis if the clinical arthritis score increased by at least two points compared with the score at the start of treatment.

Histopathology scoring

At day 42 after first CII immunization, all mice were sacrificed under euthanasia. Mouse hind knee joints ($n = 7$) were collected, fixed in 10 % paraformaldehyde solution for 48 h, decalcified in 1 M ethylene diamine tetra-acetic acid (EDTA) solution for 2 to 3 weeks. The femur-tibial tissues were then embedded in paraffin, cut into 7-μm sagittal sections, and stained with hematoxylin and eosin (H&E). Histopathological scores for each joint were determined according to the following criteria: score 0 = normal; 1 = synovial hyperplasia and mild inflammatory infiltration; 2 = pannus formation with cartilage degeneration; and 3 = heavy inflammatory infiltration, chondrocyte, and cartilage matrix loss (Camps et al. 2005).

Lymphocyte isolation

Mice spleens were collected aseptically. Total spleen cell suspension was isolated by a 200-mesh strainer. Then, splenocytes were isolated by density gradient centrifugation using mouse lymphocyte separation medium (TBD sciences, Beijing, China), following the manufacturer's instructions.

Flow cytometric analysis

For regulatory T cell analysis, splenocytes were extracellularly stained with FITC-conjugated anti-CD4 and APC-conjugated anti-CD25 (BD Pharmingen™, Erembodegen, Belgium), and then intracellularly stained with PE-conjugated anti-Foxp3

Table 1 Groups of experiments for rSjCystatin efficacy in CIA mice

Group	Agent liquor	Animals, *n*	Dose per time	Day 0	Day 21	Injection project	Day 42
A (normal)	–	10	–	–	–	–	Sacrifice
B (control CIA)	PBS	10	100 μl	CII immunization	CII boost	as following procedure	Sacrifice
C (prophylactic)	rSjCystatin	10	100 μg	CII immunization	CII boost	day:−28,−21,−14,−7, i.p.	Sacrifice
D (therapeutic)	rSjCystatin	10	100 μg	CII immunization	CII boost	day: 25,28,31.34,37,40, i.p.	Sacrifice

CIA collagen-induced arthritis, *CII* bovine type II collagen, *i.p.* intraperitoneal injection, *group A* no manipulation

(BD). For detection of intracellular accumulations of cytokines including IFN-γ, IL-4, and IL-17, a leukocyte activation cocktail (BD Pharmingen™, Erembodegen, Belgium), with BD GolgiPlug™ mixture (BD Pharmingen™, Erembodegen, Belgium), was utilized to elicit a response from T cells and stimulate cytokine production. A mouse Th1/Th2/Th17 phenotyping kit (BD Pharmingen™, Erembodegen, Belgium) was used following the general protocol. Splenocytes were resuspended with 10^6 cells/ml in minimum essential medium alpha modification (α-MEM; HyClone, Logan, UT, USA), containing 3 % fetal bovine serum (FBS; HyClone, Logan, UT, USA) and cultured according to the manufacturer's guidance in 12 well plates at 37 °C and 5 % CO_2. Data were acquired on a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Measurements of spleen size and T cell expansion

Macroscopic measurement of spleen diameter was performed for determination of spleen size. The number of total splenocytes and $CD4^+$ cells was determined by cell counting and flow cytometry in CIA mice for assessment of the putative inhibitory effect of rSjCystatin on T lymphocytes and cell proliferation

Detection of serum collagen-specific IgG

Sera were collected on day 42 from mice under anesthesia. Samples of diluted sera for detection of anticollagen total IgG were incubated overnight at 4 °C in Nunc 96-well microtiter plates (Thermo Scientific, Waltham, MA, USA) coated with 2 μg/ml CII in PBS. Bound total IgG was detected by incubation with HRP-conjugated anti-mouse CII IgG (abcam, Cambridge, UK) at 37 °C for 1 h. The plates were washed with PBS-0.5 % Tween 20, developed with TMB substrate and terminated with 4.5 N H_2SO_4. Optical density (OD) values were measured at 450 nm using the BLx808 Automatic Microplate Reader (BIO-TEK, Winooski, Vermont, USA). Because the titer of IgG was so high that it was beyond the linear range even at dilution ratio of 1:10,000, we evaluated the variation of total IgG by OD values using five titers of dilution (1:1000, 1:2500, 1:5000, 1:10,000, 1:15,000). Collagen-specific antibody subtypes IgG1 and IgG2a were measured by standard sandwich ELISA kits (Chondrex, Redmond, WA, USA), following the manufacturer's instructions.

Cytokine profiles assessment

The supernatants were collected after the splenocytes (5×10^6 cells/ml) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (HyClone, Logan, UT, USA) containing 10 % FBS (HyClone, Logan, UT, USA) with ConA (5 μg/ml) for 48 h. The levels of cytokines, including TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-17A were measured by ELISA Kits (Ray biotech, Norcross, GA, USA), following the manufacturer's instructions.

RNA extraction and real-time PCR analysis

Total RNA of joint synovium tissues were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed with Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Primers for qRT-PCR were designed using Primer 5.0. The sequences of PCR primers for mouse are shown in Supplementary file 1: Table S1. The levels of mRNA expression were estimated using real-time quantitative PCR with LightCycler® 480 SYBR Green I Master (Roche, Basel, Switzerland) according to the manufacturer's descriptions, and all reactions were performed on a Cobas Z480 system (Roche, Basel, Switzerland). To quantitate gene relative expression levels, the comparative C_T method previously described by (Schmittgen and Livak 2008) was adopted.

Statistical analysis

Statistical analysis was completed using Student's *t* test, log-rank test of Kaplan–Meier survival curves, or Mann–Whitney *U* test. Significant differences were considered at $p < 0.05$ or < 0.01.

Results

Prophylactic effects of rSjCystatin on the development of CIA

Clinical signs of mice were evaluated for the preventive effect of rSjCystatin on the development of arthritis, once every 2 days from day 21 to 40 (Fig. 1). The clinical parameters including incidence and clinical scores of arthritis were distinctly suppressed at a dose of 100 μg rSjCystatin compared with the PBS control. Histopathology demonstrated that rSjCystatin markedly reduced the inflammatory cell infiltration at the joint, synovial hyperplasia, and bone destruction in prior rSjCystatin-injected CIA mice compared with the PBS control mice.

Therapeutic effects of rSjCystatin on established CIA

Similarly, we evaluated the therapeutic efficacy of rSjCystatin on the established CIA (Fig. 1). There was no significant difference in the clinical parameter incidence and histological examination scores between rSjCystatin-treated mice and PBS control mice.

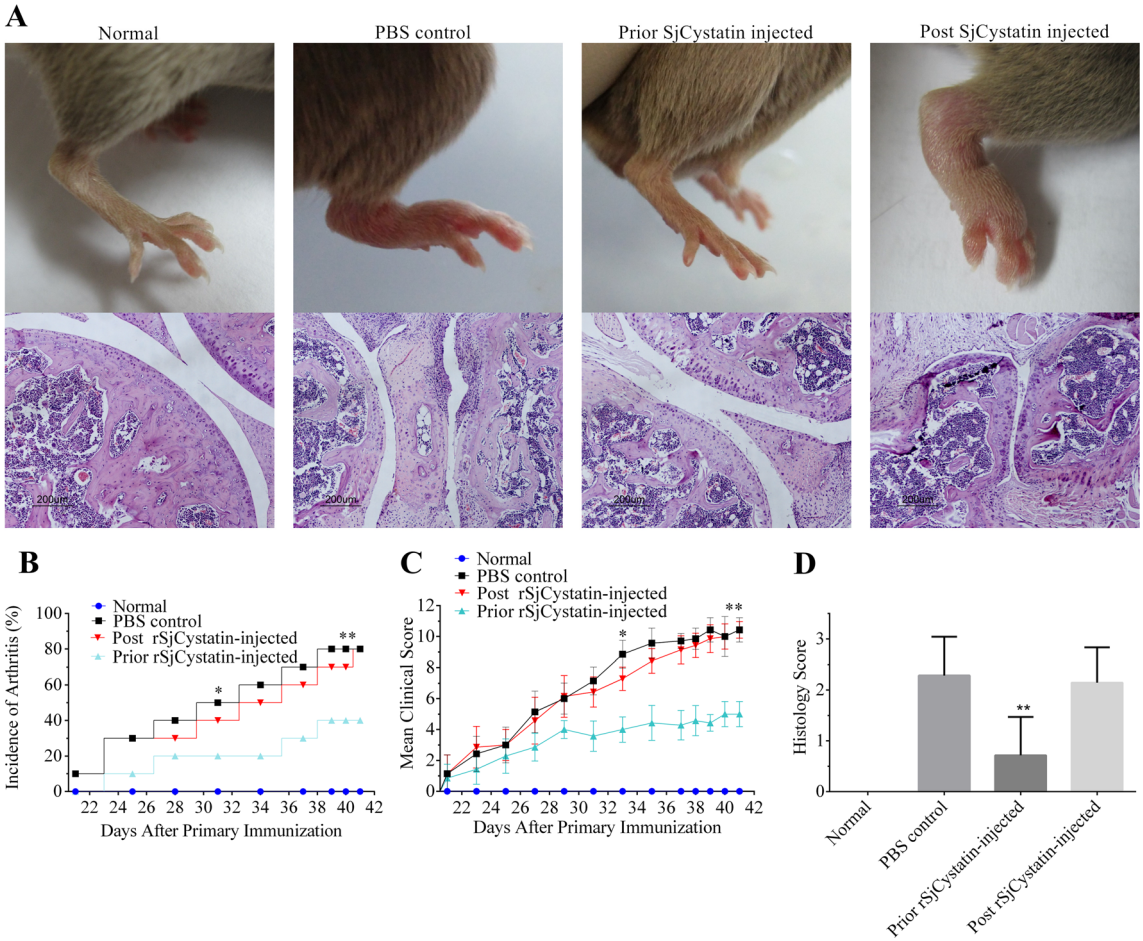

Fig. 1 Preventive effects of rSjCystatin on collagen-induced arthritis (CIA) in DBA/1 mice. Collagen-induced arthritis was induced in male DBA/1 mice. Attenuated clinical parameters of arthritis were seen in prophylactic rSjCystatin-injected mice. The incidence and severity of arthritis were evaluated as previously described in the "Materials and methods" section. The survival curve and clinical score only differ significantly between prophylactic rSjCystatin-injected CIA mice and PBS control mice. **a** The hind paw pictures and the knee-joint sections stained with H&E ($n = 7$/group). **b** The incidence of arthritis in different groups ($n = 10$/group). **c** Clinical scores over time evaluated from days 21 to 41 of the different treatment groups ($n = 10$). **d** Histology scores determined ($n = 7$). Data are mean ± SD (*$p < 0.05$ and **$p < 0.01$ vs control group by log-rank test of Kaplan–Meier survival curves or by Mann–Whitney *U* test)

Prophylactic intervention of rSjCystatin suppresses spleen size and T cell expansion in vivo

We estimated the size of fresh spleens isolated from the mice of prior injection. The size of spleens was definitely reduced compared to the PBS control mice (Fig. 2). Prior rSjCystatin injection decreased the numbers of total splenocytes and $CD4^+$ lymphocytes in the spleen of the CIA mice, while the post-rSjCystatin administration did not result in distinct changes when compared with the cells from PBS control mice.

Prophylactic administration of rSjCystatin regulates the proportion of helper T subsets in CIA mice

The modulation of rSjCystatin injection in Th1, Th2, and Th17 cells was investigated in CIA mice. As shown in Fig. 3a, the counts of Th1 and Th17 cells were distinctly elevated in PBS control mice. Prophylactic administration of rSjCystatin enhanced the ratio of Th2 cells; meanwhile, the proportions of the Th1 cells ($CD4^+IFN\text{-}\gamma^+$) and Th17 cells ($CD4^+IL\text{-}17A^+$) were reduced notably. The results demonstrated that rSjCystatin can effectively ameliorate arthritis by inducing Th2 cell response to suppress Th1 and Th17 cell expansion in CIA mice.

Intervention of rSjCystatin increased the Foxp3-expressing Tregs in CIA mice

We investigated the population of regulatory T cells (Tregs) in CIA mice. As shown in Fig. 3b, a significant increase of percentage of $CD4^+CD25^+Foxp3^+$ Tregs rather than $CD4^+CD25^-Foxp3^+$ cells was seen in the preventive intervention group when compared with PBS control mice. Also, post-injection treatment of rSjCystatin had a slightly enhanced proportion of Tregs.

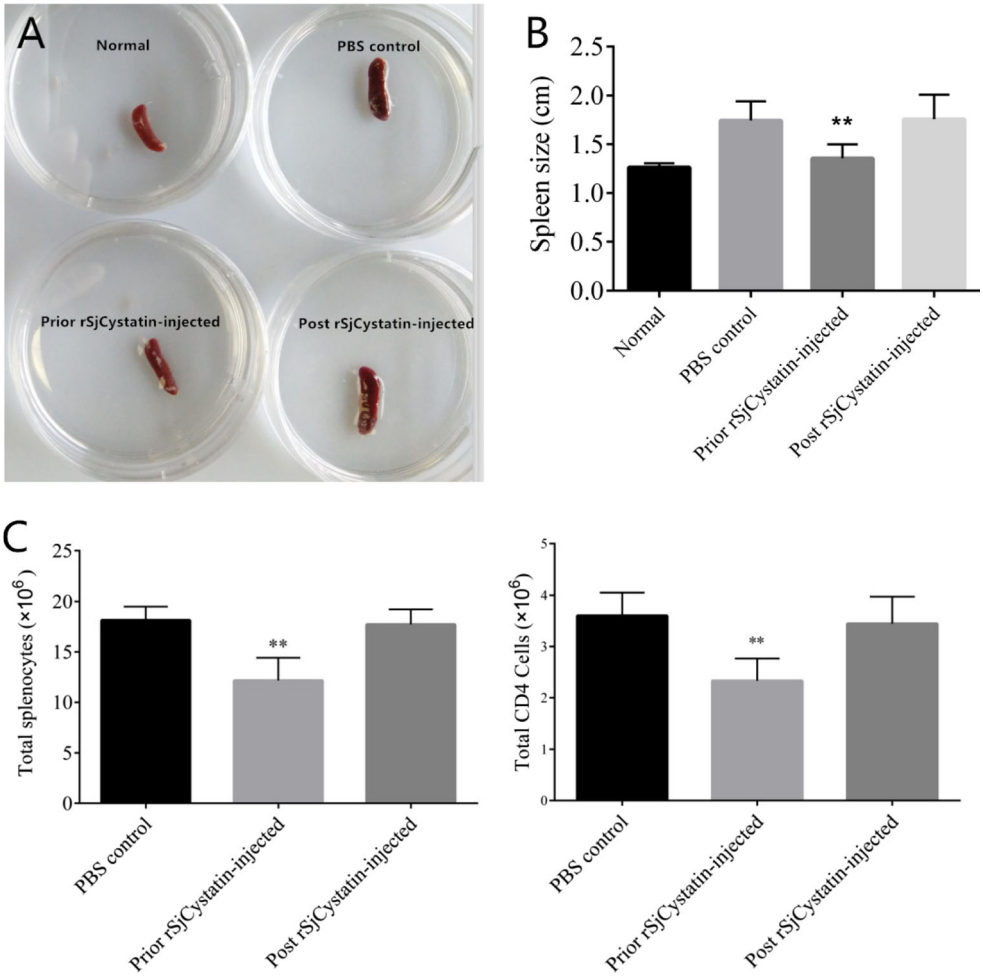

Fig. 2 Prophylactic intervention of rSjCystatin suppressed the spleen size and T-cell expansion in vivo. CIA mice were injected with rSjcystatin prior or post CII immunization (Table 1) and spleens were collected and analyzed. **a** Photograph of freshly isolated spleens. **b** Spleen size in CIA mice with prior rSjcystatin injection. **c** The number of total splenocytes and CD4 cells determined by cell counting and flow cytometry. Data are means ± SD (*$p < 0.05$ and **$p < 0.01$ vs control group by Student's t test ($n = 7$)

Prophylactic injection of rSjCystatin alters the antibody response in CIA mice

Antibodies against CII collagen were determined to assess the influence of rSjCystatin on the humoral response in vivo. The total CII-specific IgG and its subtypes IgG1 and IgG2a were measured in the sera of the mice. The titer of IgG_1, but not IgG_{2a}, in the prior rSjCystatin-injected group was conspicuously elevated (Fig. 4). The rSjCystatin injection, however, had no noticeable influence on the level of total IgG, suggesting that instead of a general B-cell-depletion/suppression, rSjCystatin selectively controlled the CII-specific B-cell response induced by Th cell cytokines in the CIA mice.

rSjCystatin promotes a shift in cytokine production from proinflammatory to anti-inflammatory profile in vitro

The supernatants of splenocytes were harvested after culturing with Con A. The mice with prior rSjCystatin injection presented a marked expression of IL-4 and IL-10 assessed by ELISA, while the splenocytes of the PBS control mice produced considerably high levels of IFN-γ, TNF-α, IL-6, and IL-17 (Fig. 5). In contrast, rSjCystatin used as treatment had no significant influence on the levels of IL-1β and IL-2. The preventive rSjCystatin treatment had no significant influence on the levels of IL-1β and IL-2. Additionally, therapeutic rSjCystatin injection could only inhibit TNF-α production and slightly raise IL-10 in established CIA mice.

Prophylactic administration of rSjCystatin attenuates the augmentation of inflammatory mediators in the inflamed joints

To examine the efficacy of rSjCystatin in the inflamed joints, we determined the mRNA levels of the receptor activator of NF-kB ligand (RANKL) in inflamed joints. As shown in Fig. 6, the levels of TNF-α, IL-17 and IL-6 mRNA expression were notably attenuated, while IL-10 was increased in the mice with prior rSjCystatin injection. In vivo examination of the specific transcription factors of Th cell subpopulations demonstrated that the mRNA levels of T-bet and retinoic

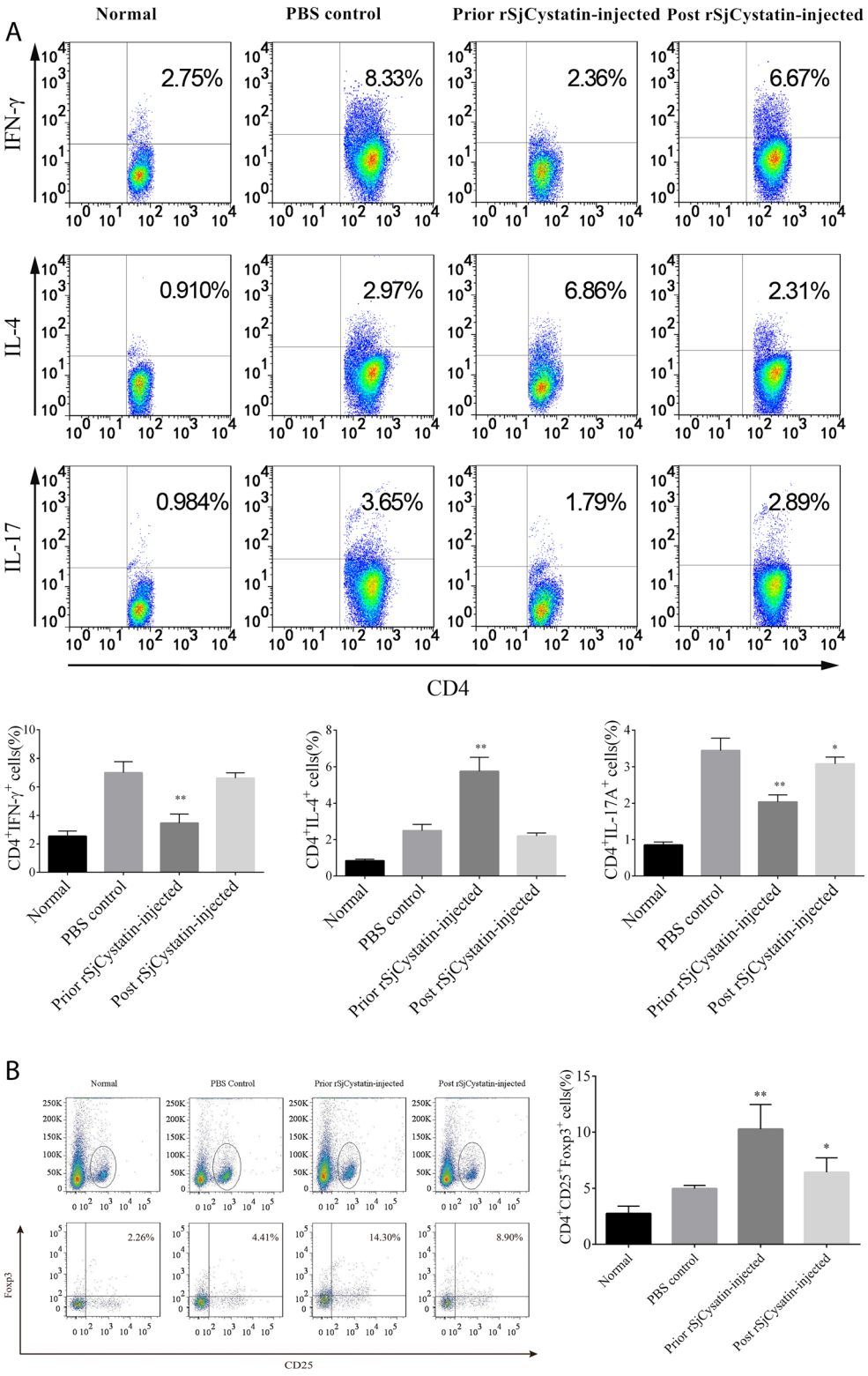

Fig. 3 rSjCystatin regulated the proportion of Th1 or Th2, Th17 cells, and Tregs in CIA mice. Splenocytes were activated in vitro with phorbol myristate acetate and ionomycine. **a** To assess T-helper (Th) cells, splenocytes were stained with anti-CD4-PerCP-cy5.5 and anti-interferon (IFN) γ-FITC, or anti-interleukin (IL) 4-APC, or anti-IL-17A-PE. After prophylactic administration of rSjcystatin, the proportion of $CD4^+IL\text{-}4^+$ Th2 cells was upregulated, and the percentage of $CD4^+IFN\text{-}\gamma^+$ Th1 cells and $CD4^+IL\text{-}17^+$ Th17 cells were suppressed. In addition, the splenocytes were analyzed by flow cytometry (see the "Materials and methods" section). **b** For evaluation of regulatory T cells (Tregs), splenocytes were stained with anti-CD4-FITC, anti-CD25-APC, and Foxp3-PE. After prophylactic administration of rSjcystatin, the proportion of $Foxp3^+CD25^+$ Tregs in the spleen was upregulated. The rSjcystatin treatment also slightly enhanced $Foxp3^+CD25^+$ Tregs in CIA mice. Data are mean ± SD (*$p < 0.05$ and **$p < 0.01$ vs CIA control group by Student's t test ($n = 7$/group)

orphan receptor γt (RORγt) were obviously down-regulated, while Foxp3 were decidedly up-regulated by prior rSjCystatin injection. Simultaneously, a substantial reduction in RANKL, an essential activator for osteoclast development, was noted in prior rSjCystatin-injected CIA mice.

Discussion

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disorder that causes inflammation and destruction of the joints and other parts of the body. However, no clinically useful

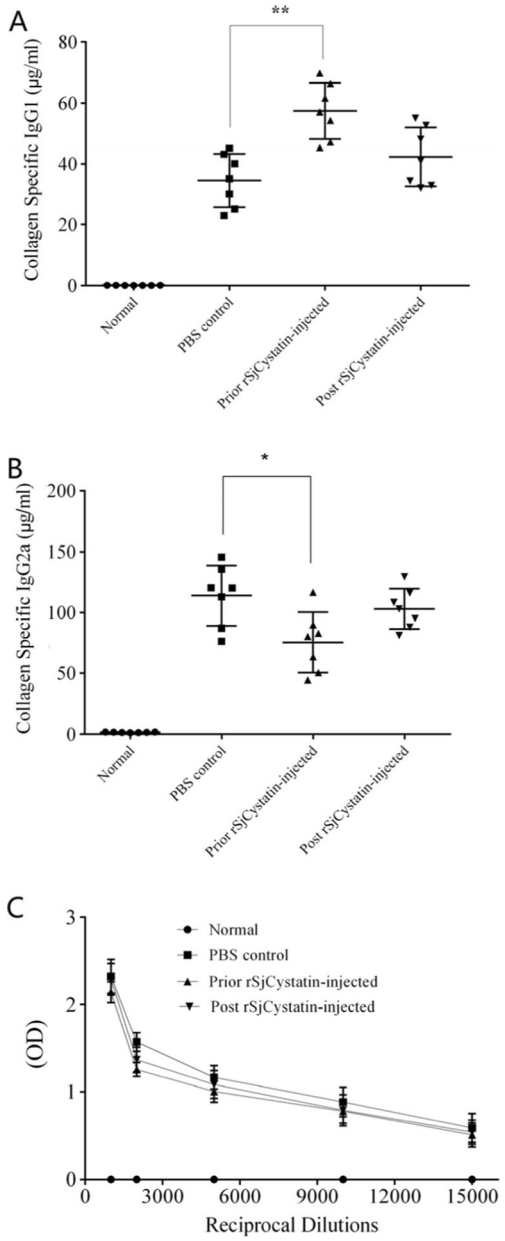

Fig. 4 Prophylactic intervention of rSjcystatin selectively inhibited production of antibodies against type II collagen in CIA mice. Prior rSjcystatin injection altered the levels of autoantibodies. The concentration of anti-CII IgG1 and anti-CII IgG2a in sera were determined by ELISA. The levels of total anti-CII IgG were measured by optical density (OD) values. Prior injection of rSjCystatin increased anti-CII IgG1 and decreased anti-CII IgG2a production in CIA mice, while it had no influence on total anti-CII IgG production. No difference was observed between rSjcystatin-treated and PBS control CIA mice. Data are mean ± SD (*$p < 0.05$ and **$p < 0.01$ vs control group by Student's t test ($n = 7$/group)

biomarkers have been identified for use in individually tailored treatment of RA (Cuppen et al. 2016), and patients usually require long-term therapy, in which side effects are often inevitable.

Classically, Th cells are divided into Th1 ($CD4^+$ IFN-γ^+), Th2 ($CD4^+$IL-4^+), and Th17 ($CD4^+$IL-17^+) subsets, which perform an essential role in chronic autoimmune inflammation by producing distinct cytokines (Kondo et al. 2015). In the synovium of RA patients, numerous inflammatory chemokines upregulate the trafficking of Th1 and Th17 cells into the tissue, with Th1 cells being the most prevalent (Snir et al. 2012). It has been widely accepted that the Th1 response (mainly IFN-γ and IL-2) dominates in the collagen-induced arthritis (Manoury-Schwartz et al. 1997). Th1 cells are more frequently observed than are Th2 cells (mainly IL-4) in arthritis synovium, and the Th17 population (essentially IL-17) should be considered as factors in RA pathogenesis (Hirota et al. 2007). The inflammatory cytokines lead to the overgrowth of synovial cells by upregulating synoviolin expression in mouse synovial fibroblasts (Gao et al. 2006). Additionally, it has been reported that a variety of cytokines positively or negatively regulate osteoclast differentiation by modulating expression of RANKL on synovial fibroblasts (Komatsu and Takayanagi 2015). RANKL is an essential differential and activating factor expressed by osteoclastogenesis-supporting cells like activated T cells, osteoblasts, and synoviocytes, and it also induces osteoclast-mediated bone destruction (Lorenzo et al. 2008).

It has been reported that cystatins of filaria, tissue-dwelling nematodes, inhibit class II MHC-restricted antigen processing, induce the polarization of immunosuppressive cells, and restrain immune cell activation and migration and are responsible for degrading the immune components of the host (Manoury et al. 2001; Schnoeller et al. 2008a). Similarly, recombinant SjCystatin (rSjCystatin) could inhibit the antigen presentation process, reduce monocyte activation, enhance IL-10 generation, and decrease proinflammatory factor production involving TNF-α and IL-6 (Zhou et al. 2014). Previous studies also indicated that the mouse will develop arthritis at a high incidence when it lacks cystatin C (Backlund et al. 2011), and cysteine protease inhibitor has been suggested as a potential drug to treat arthritis (Connor et al. 2009; Hayami et al. 2012; McDougall et al. 2010).

We previously demonstrated the prophylactic effects of schistosome infection on alleviation of the arthritis in CIA mice at the histopathological level. We also provided direct evidence that the Th2 response evoked by prior schistosome infection suppresses the Th1 response and proinflammatory mediators, which are closely associated with upregulation of the Treg response and downregulation of the Th17 response (Song et al. 2011). In this study, we had an insight into the suppressive immunomodulatory influence of *S. japonicum*-derived recombinant rSjCystatin on CIA mice. We found that prior administration of rSjCystatin before CIA modeling significantly attenuated the structural pathology of the joints of CIA mice. This alleviative effect is linked to the augmentation of Th2 cytokines of IL-4 and IL-10, collagen-specific IgG1 (mostly induced by IL-4), and to the reduction of IFN-γ, collagen-specific IgG2a (mostly induced by IFN-γ), and pro-inflammatory cytokines IL-6, IL-17, and TNF-α.

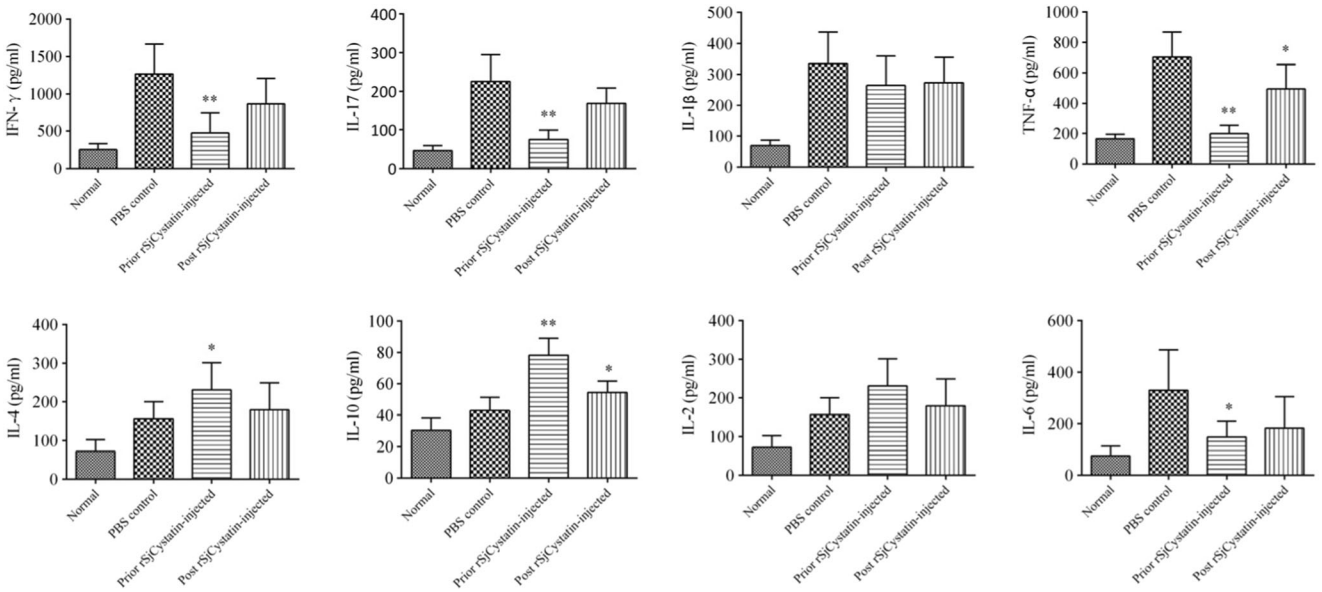

Fig. 5 Injection of rSjcystatin suppressed proinflammatory cytokine production and increased anti-inflammatory cytokine production in vitro. CIA mice were injected with rSjcystatin prior or postcollagen immunization (Table 1), and splenocytes were cultured and analyzed. Cultural supernatants (RPMI 1640, 10 % FCS, 5 mg/ml ConA) were determined by ELISA as described in the "Materials and methods" section. In prior-injection mice, the cytokine levels of IFN-γ, TNF-α, IL-6, and IL-17 were significantly decreased, while IL-4 and IL-10 were notably enhanced. In postinjection mice, the cytokine level of TNF-α was slightly lowered and IL-10 was mildly increased. Data are mean ± SD (*$p < 0.05$ and **$p < 0.01$ vs control group by Student's t test ($n = 7$/group)

Additionally, the percentage of Th17 cells was downregulated. The supernatant of splenocyte culture in the prior rSjCystatin-injected mice contained low concentrations of IFN-γ, TNF-α, IL-6, and IL-17 but had high levels of IL-4 and IL-10 compared with the PBS control group. The imbalance of cytokine production by Th1/Th2/Th17 lymphocytes doubtlessly contributes to RA pathogenesis. Taken together, it is strongly suggested that restraining of the Th1 dominant response induced by rSjCystatin might contribute to the prophylactic effect on amelioration of the pathology of CIA mice.

Tregs are vital in suppressing immune responses. Mice deficient in $Foxp3^+$ Tregs would develop autoimmune diseases (Sakaguchi 2004; Wan and Flavell 2007). $Foxp3^+$ Tregs are made up of $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-Foxp3^+$ populations (Komatsu et al. 2009; Miyao et al. 2012). We noted that following the prophylactic application of rSjCystatin, the percentage of $CD4^+CD25^+$ $Foxp3^+$ cells was enhanced. Simultaneously, we observed the ascending numbers of $CD4^+CD25^-$ $Foxp3^+$cells in the spleen of PBS control CIA mice, which might be responsible for the increased number of Th17, since $CD4^+CD25^-$ $Foxp3^+$ cells could pathologically convert into Th17 cells in autoimmune arthritis (Komatsu et al. 2014). Thus, we infer that the rSjCystatin could be an effective stimulus for the upregulation of Treg/Th17 ratio in murine experimental autoimmune arthritis, and the efficacy of prior rSjCystatin injection may be, at least partly, due to the improved Treg/Th17 balance.

Furthermore, post-rSjCystatin injection slightly enhanced the expression of IL-10 and Foxp3 and decrease TNF-α concentration in the splenocyte culture supernatant, but it does not reduce the RANKL level in synovitis of CIA mice (Fig. 6). It seems that injection of rSjCystatin in preexisting arthritis does not dampen the cartilage degradation. Because the protein treatment began after the onset of chronic arthritis, rSjCystatin did not affect the earliest inflammatory events involving differentiation of CII-specific T cells, infiltration of inflammatory cells, and the synovial hyperplasia. Following the development of CIA, increasing levels of anti-CII antibodies bind to the articular cartilage collagen, activate the complement system, and initiate the tissue damage; the therapeutic protocol of rSjCystatin might be insufficient to alter cartilage pathology and alleviate the loss of mobility and joint function in established CIA. Thus, we propose that postinjection of rSjCystatin does not prevent the rapid outbreak of inflammatory in CIA mice, including synovitis and cartilage degradation. Additionally, rSjCystatin might function primarily through its immunoregulatory ability more than inhibiting enzymatic activity, since blockage of synovial cathepsin activity by overexpression of cystatin C in the synovium failed to alleviate synovitis or cartilage degradation in established osteoarthritis (Kyostio-Moore et al. 2015). However, a novel approach is needed to better understand the specific mechanisms affected by rSjCystatin on complex autoimmune diseases.

Conclusion

We have demonstrated for the first time that rSjCystatin, a recombinant *S. japonicum* cysteine protease inhibitor, can

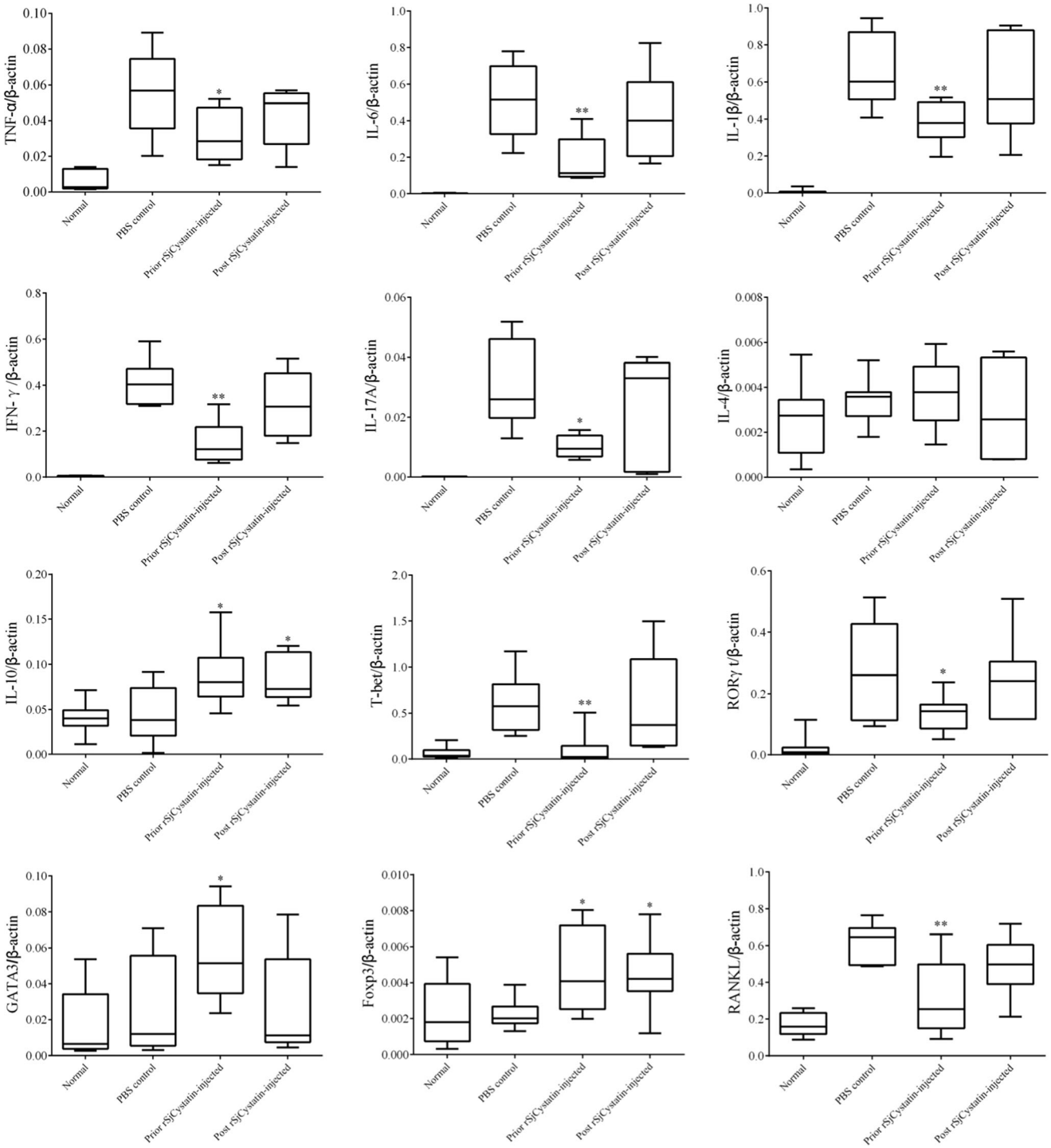

Fig. 6 Prior rSjcystatin injection attenuated the augmentation of inflammatory mediators in the inflamed joint. The mRNA expression was quantified by real-time PCR. Prior rSjcystatin injection modulated mRNA expression of proinflammatory and anti-inflammatory cytokines and reduced the mRNA expression of RANKL in the inflammatory joints. Values along the vertical axis represent relative expression levels normalized to β-actin. Data are mean ± SEM (*$p < 0.05$ and **$p < 0.01$ vs CIA control group, $n = 7$). Data are representative of three repeated experiments

effectively alleviate the pathology of murine adjuvant arthritis by prophylactic intervention. The amelioration of clinical parameters, decline of histopathological scores, and improvement of the detection indexes appear to be attributed to the downregulated role of rSjCystatin in pathogenesis of arthritis in mice. Thus, further study of rSjCystatin is indicated in order to gain new insights on its prophylactic and complementary use in the therapeutic strategy of rheumatoid arthritis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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