International Immunopharmacology 77 (2019) 105914

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Therapeutic treatment of a novel selective JAK3/JAK1/TBK1 inhibitor, CS12192, in rat and mouse models of rheumatoid arthritis

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A B S T R A C T

Rheumatoid arthritis (RA) is a representative autoimmune disease characterized by chronic inflammation and joint destruction. Although biological inhibitors such as TNF-α and IL-6 antibodies have achieved success in clinical therapy, small molecule inhibitors against the Janus kinases (JAKs) involved in the signaling pathways of various cytokine receptors have gained more attraction as safe and efficacious options. In this study, we identified CS12192 as a novel selective JAK3/JAK1/TBK1 inhibitor and investigated its pharmacological effects on the experimental arthritis models in rat and mouse. We found that CS12192 showed a more selective inhibitory activity on JAK3, and to a less extent on JAK1 and TBK1, that were verified by decreased activation of p-STATs and p-IRF3 as well as down-regulation of IFN gene expression in the cultured cells with relevant stimuli. Furthermore, oral treatment with CS12192 dose-dependently ameliorated the disease severity, hind paw swelling, body weight loss, and bone destruction in rat models of adjuvant-induced arthritis (AIA) and collagen-induced arthritis (CIA). In a mouse CIA model, CS12192 also attenuated the disease severity, which was correlated with the suppressed $CD4^+$ T cell activation and Th17 function, as well as the reduced cytokine levels in sera and pro-inflammatory cytokine and chemokine gene expression in joint tissue. Corroboratively, RANKL-induced osteoclast formation was inhibited by CS12192. Thus, these results suggest that CS12192 as a novel selective JAK inhibitor has therapeutic potential for the treatment of RA and may provide a new strategy for the control of autoimmune diseases.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by chronic synovitis leading to the progressive destruction of joints accompanied by systemic inflammation and the production of autoantibodies [1]. Although the etiology of RA is still unknown, it has been well recognized that activated immune cells such as T cells, B cells, macrophages, and even fibroblast-like synoviocytes play critical roles in the pathogenesis of RA. While the productions of rheumatoid factors (RF) and/or anti-citrullinated protein antibodies (ACPAs) by auto-reactive B cells may trigger RA-specific immunity and facilitate the onset of RA [2], a variety of pro-inflammatory cytokines and chemokines secreted by tissue-infiltrating effector immune cells and/or resident cells result in perpetuating joint inflammation and aggravating tissue destruction at the chronic disease stage [3,4]. Therefore, understanding the immunological mechanisms involved in RA pathogenesis has led in large part to the clinical development of therapeutic drugs targeting specific cells and molecules [5].

Among numerous cytokines implicated in RA pathogenesis, tumor necrosis factor-α (TNF-α) and IL-6 are the two key players that have been extensively studied over the past two decades, and biological inhibitors targeting these two cytokines have been successfully developed as the pioneer molecular targeting drugs for the treatment of RA [6,7]. Although treatment with TNF-α or IL-6 blockers have achieved a fundamental improvement in RA care, some critical issues related to cytokine antagonist therapy including the increased risk of infection, primary non-responders, secondary loss of efficacy, and antidrug antibody formation still remain unresolved [8]. In addition, since cytokines often interact with each other and act in synergy to increase and maintain the inflammation, targeting a single cytokine signaling pathway could not be sufficient for complete disease remission [9].

The Janus kinases (JAKs) are a small family of receptor-associated tyrosine kinases that provide intracellular transmission signals from type I and type II cytokine, interferon, and many hormone receptors. There are four JAKs–JAK1, JAK2, JAK3, and TYK2 (tyrosine kinase 2)–each associates with different cytokine receptors [10]. Unlike other JAKs, JAK3 is more restrictedly expressed in hematopoietic cells and mediates signals through the common γ chain shared by many immune-related cytokine receptors [11]. Upon stimulations by cytokines, the paired JAKs become phorsphorylated by active receptors and

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https://doi.org/10.1016/j.intimp.2019.105914
Received 9 April 2019; Received in revised form 6 September 2019; Accepted 12 September 2019
Available online 18 October 2019
1567-5769/

phorsphorylate signal transducer and activator of transcription (STATs). Activated STATs then translocate into the nucleus, bind to DNA and induce the transcription of targeted genes [10]. Considering that JAKs are essential signaling mediators downstream of many pro-inflammatory cytokine receptors, small molecule inhibitors of JAKs have gained attraction as safe and efficacious options for the treatment of inflammation-driven pathologies such as RA, psoriasis, and inflammatory bowel disease [12]. As one of the first generation JAK inhibitors, tofacitinib, a JAK3/JAK1/JAK2 inhibitor, has been approved by FDA for the treatment of RA patients unresponsive to or intolerant of methotrexate in 2012 [13]. Another JAK1/JAK2 inhibitor, baricitinib, was approved for the treatment of moderate-to-severe RA patients who are inadequate to anti-TNF therapy in 2018. Nonetheless, several treatment-related severe side effects, such as thrombocytopenia, anemia, and neutropenia, are possibly caused by the inhibition of JAK2 [14]. Thus, more next generation of selective JAK inhibitors with improved safety are currently under investigation [12].

Recently, a type I IFN signature has been found to be associated with the early diagnosis and clinical outcome prediction in RA patients [15,16]. As TANK-binding kinase 1 (TBK1) is an IKK-related serine/threonine kinase best known for the induction of type I interferon and interferon-stimulated genes [17], the possibility of targeting TBK1 for therapeutic inhibition of type I interferon induction has received attention as a treatment strategy in autoimmune diseases [18].

In this study, we have identified a novel selective JAK inhibitor, CS12192, with a potent inhibition on JAK3, and to a less extent on JAK1 and TBK1. The potential therapeutic effects and immunological mechanisms of CS12192 were investigated in both rat and mouse models of experimental arthritis, including adjuvant-induced arthritis (AIA) and collagen-induced arthritis (CIA).

2. Materials and methods

2.1. Animals

Both female Lewis rats at 6–8 weeks and female DBA/1 at 8–10 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were acclimated for 7 days prior to experimental procedures. The experimental arthritis models in rats were conducted in PharmaLegacy Laboratories (Shanghai, China). The mouse arthritis model was conducted in Shenzhen Chipscreen Biosciences (Shenzhen, Guangdong, China). All procedures were preapproved by the Institutional Animal Care and Use Committee.

2.2. Materials

CS12192 was designed and synthesized in Shenzhen Chipscreen Biosciences. The purity of CS12192 was over 99%. CP690550 and BX795 were purchased from Selleck Chemicals (Houston, TX, USA). All compounds were dissolved in sterile DMSO for *in vitro* experiments. For *in vivo* administration, compounds were suspended in pure water for oral gavage. Human natural killer cell line (KHYG-1) was purchased from the Human Science Research Resources Bank (Tokyo, Japan). Human erythroleukemia cell line (UT-7) was purchased from Cell Resource Center of Peking Union Medical College (Beijing, China). Human monocytic cell line (THP-1) and mouse macrophage cell line (RAW264.7) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cytokines including IL-2, IL-4, EPO, RANKL, and M-CSF were purchased from PeproTech (Rocky Hill, NJ, USA). Poly (I:C) was purchased from InvivoGen (San Diego, CA, USA). LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary rabbit antibodies used for immunoblotting were purchased from either Cell Signaling Technology (Beverly, MA, USA) or Santa Cruz Biotechnology (San Diego, CA, USA). The HRP-conjugated secondary goat anti-rabbit antibody was purchased from Sigma. All primers for human and mouse genes were synthesized by Genewiz (Suzhou, Jiangsu, China). Chicken type II collagen emulsified in complete or incomplete Freund's adjuvant (CFA or IFA) were purchased from Hooke Laboratories (Lawrence, MA, USA). Phorbol 12-myristate 13-acetate (PMA) and Ionomycin were purchased from Sigma. The fluorochrome-conjugated anti-mouse monoclonal antibodies for cell surface markers (CD4, CD8, CD44 and CD62L) and intracellular cytokines (IL-17 and IFN-γ) were purchased from eBiosciences (San Diego, CA, USA). Intracellular fixation and permeabilization Buffer set was also purchased from eBiosciences.

2.3. Enzymatic activity analysis

The biochemical activity of CS12192 for human kinases was assayed by Eurofins Scientific (UK). The *in vitro* inhibition rate for a panel of 75 human kinases by CS12192 at 1 μM were also tested.

2.4. Immunoblotting

To determine the inhibition of cytokine-stimulated phosphorylation of STATs by compounds, KHYG-1, THP-1 and UT-7 (3×10^6) were incubated in 6-well plates with FBS-free medium overnight for synchronization. After pre-treatment with CS12192 or CP690550 at indicated concentrations for 1 h, cytokines including IL-2 (200 IU/ml), IL-4 (20 ng/ml) or EPO (10 ng/ml) were added for stimulation, respectively. 15 min later, the whole cell lysate proteins were collected and analyzed by western blotting with the corresponding primary rabbit antibodies specific for phospho-STAT1 (Tyr701) (Cell Signaling, #7649) and total STAT1 (Cell Signaling, #9172), phospho-STAT3 (Tyr705) (Cell Signaling, #9145) and total STAT3 (Cell Signaling, #4904), phospho-STAT5 (Tyr694) (Cell Signaling, #4322) and total STAT5 (Abcam, #ab194898), phospho-STAT6 (Tyr641) (Cell Signaling, #9361) and total STAT6 (Cell Signaling, #5397), as well as β-actin (Santa Cruz, #8432) used as the internal control. The phosphorylation of STATs in the splenocyte lysate from CIA mice were measured using the above primary antibodies due to their cross reactivity with mice.

For determination of the phosphorylation of TBK1 and IRF3 induced by poly (I:C), THP-1 (1×10^7) were seeded in 9 cm-culture dishes and cultured in FBS-free RPMI-1640 overnight for synchronization. After pre-treatment with CS12192 (3 μM), BX795 (0.5 μM) or CP690550 (3 μM) for 1 h, the cells were transfected with poly (I:C) at 1 μg/ml for 2 h. Then the whole cell lysate proteins were collected and analyzed by western blotting with the corresponding primary rabbit antibodies specific for phospho-TBK1 (Ser172) (Cell Signaling, #5483) and total TBK1 (Cell Signaling, #3504), Phospho-IRF3 (Ser396) (Cell Signaling, #29047) and total IRF3 (Cell Signaling, #4302), as well as β-actin used as the internal control.

2.5. Rat AIA and CIA induction and assessment

The rat AIA model was induced by an intradermal injection of 0.5 ml CFA at the base of the tail and the lower back on day 0. After the onset of the disease on day 7, animals were divided randomly into 5 groups (n = 10 per group) with 3 naïve rats as a normal control. CS12192 (20, 40, 80 mg/kg) and CP690550 (3 mg/kg) were intragastrically administered, twice a day, respectively, until day 28. The rat CIA model was induced by an intradermal injection of 0.5 ml bovine type II collagen emulsified in IFA at the base of the tail and the lower back on day 0. Booster injections were administered on day 7 with 0.5 ml emulsion at the same injection site. At the onset of the disease on day 11, animals were divided randomly into 5 groups (n = 10 per group). CS12192 and CP690550 at the indicated doses were intragastrically administered, twice a day, respectively, until day 28. The disease severity of rat arthritis were evaluated every 2–3 days with the cumulative scores of all four paws on a total scale of 0–16, where each paw was scored as follows: 0 = no arthritis; 1 = swelling and/or

redness of one to two interphalangeal joints; 2 = involvement of three to four interphalangeal joints or one larger joint; 3 = more than four joints red/swollen; 4 = severe arthritis of an entire paw. The volume of two hind paws were measured with a plethysometer (Ugo Basile, Italy), and animal body weights were also monitored every 2–3 days.

2.6. Radiological evaluation of rat arthritis

Upon the termination of the above experiments, X-ray radiographs of one hind paw per rat were taken by Faxitron MX-20-DC5 (Faxitron X-ray Corporation, Wheeling, IL, USA). The severity of bone erosion was ranked by a blinded radiologist using a modified version of the Sharp scoring method: 0 = no radiographic changes; 1 = equivocal findings; 2 = beginning erosions and joint space narrowing; 3 = advanced erosions and joint space narrowing; 4 = severe erosions and loss of joint space.

2.7. Mouse CIA induction and assessment

CIA was induced in female DBA/1 mice by intradermal immunization with chicken type II collagen (50 μg) emulsified in CFA purchased from Hooke Laboratories at the base of the tail on day 0, followed by a booster immunization with chicken type II collagen (50 μg) emulsified in IFA at day 21. After the onset of the disease on day 25, the mice were divided randomly into two groups (n = 8–9 per group) with 4 naive mice as normal control. CS12192 were intragastrically administered at 80 mg/kg, twice a day (BID), for 22 days. The disease severity of CIA was assessed every 2–3 days with the cumulative scores of all four paws on a total scale of 0–16, where each paw was scored as follows: 0 = normal paw; 1 = one toe inflamed and swollen; 2 = more than one toe, but not entire paw inflamed and swollen, or mild swelling of entire paw; 3 = entire paw inflamed and swollen; 4 = very inflamed and swollen or ankylosed paw.

2.8. Cellular staining and flow cytometry

The single cell suspension were isolated from the spleen of naïve, vehicle- or CS12192-treated CIA mice. The cells were stained with cell surface markers of CD4, CD8, CD44, and CD62L for the determination of T cell activation. For intracellular staining, the splenocytes (1×10^6) were stimulated with PMA (100 ng/ml) and Ionomycin (1000 ng/ml) for 6 h. After cell surface staining, the cells were cytofixed and permeabilized, and then stained with the permeabilization buffer containing anti-IL-17 and anti-IFNγ antibodies. Samples were acquired on a BD FACScanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and the results were analyzed by Flowjo software (TreeStar, Ashland, OR, USA).

2.9. Cytokines in sera

The serum levels of several pro-inflammatory cytokines and Th17 cell differentiation-related cytokines including IL-1β, IL-6, TNF-α, IL-2, IL-15, IL-21, and IL-22 from naïve, vehicle- and CS12192-treated CIA mice were evaluated by ProcartaPlex assay (Thermo Fisher Scientific, Waltham, MA, USA).

2.10. RNA isolation and quantitative RT-PCR

After pre-treatment with CS12192 (3 μM), BX795 (0.5 μM) or CP690550 (3 μM) for 1 h, THP-1 or RAW264.7 cells were either transfected with poly (I:C) at 1 μg/ml or stimulated with LPS at 100 ng/ml for 3 h, respectively. The total RNA from the cultured cells or joint tissues of CIA mice were isolated by TRIzol reagent according to the manufacturer's instructions (Ambion, Austin, TX, USA). 5 μg of extracted RNA was reverse transcribed into cDNA first-strand using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Synthesized cDNA was diluted 50 times with nuclease-free water prior to the quantitative real-time polymerase chain reaction (PCR) analyses. Quantitative PCR was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master (ROX) dye (Roche Diagnostics), and threshold cycle numbers were obtained using ABI Prism 7000 SDS software version 1.0. The amplification condition consisted of a pre-incubation at 94 °C for 3 min followed by 40 cycles of 94 °C for 10 s, 55 °C for 10 s, and 72 °C for 10 s, then 1 cycle of 72 °C for 10 min. All reactions were followed by melting curve analysis and performed in triplicate. The primer sequences used in this study are listed in Table 1. The relative expression of cytokine/chemokine genes were normalized to the internal control of β-actin gene and analyzed using the 2-ΔΔCq method [19].

Table 1
Primer sequences for tested human and mouse genes.

Gene name (species)	Sequence
IFNβ (human)-Forward	GGCACAACAGGTAGTAGGCG
IFNβ (human)-Reverse	GTGGAGAAGCACAACAGGAGA
CXCL10 (human)-Forward	CCTGCAAGCCAATTTTGTCCA
CXCL10 (human)-Reverse	TGTGGTCCATCCTTGGAAGC
β-actin (human)-Forward	GAGCACAGAGCCTCGCCTTT
β-actin (human)-Reverse	TCATCATCCATGGTGAGCTGGC
IFN-β (mouse)-Forward	CAACAGCTACGCCTGGATGG
IFN-β (mouse)-Reverse	CCTGCAACCACCACTCATTC
IL-1β (mouse)-Forward	AAGCTCTCCACCTCAATGGAC
IL-1β (mouse)-Reverse	AGGCCACAGGTATTTTGTCGT
IL-6 (mouse)-Forward	GGAGCCCACCAAGAACGATAG
IL-6 (mouse)-Reverse	GTGAAGTAGGGAAGGCCGTG
IL-17A (mouse)-Forward	ACTACCTCAACCGTTCCACG
IL-17A (mouse)-Reverse	TTCCCTCCGCATTGACACAG
CXCL2 (mouse)-Forward	TCCAGAGCTTGAGTGTGACG
CXCL2 (mouse)-Reverse	AGGTACGATCCAGGCTTCCC
CCL2 (mouse)-Forward	CCTGCTGCTACTCATTCACCA
CCL2 (mouse)-Reverse	ATTCCTTCTTGGGGTCAGCA
CCL5 (mouse)-Forward	GTGCCCACGTCAAGGAGTAT
CCL5 (mouse)-Reverse	TTCTCTGGGTTGGCACACAC
IFNα (mouse)-Forward	ATTTCCCCTGACCCAGGAAGATG
IFNα (mouse)-Reverse	CCCAGCACATTGGCAGAGG
CXCL10 (mouse)-Forward	AGTGCTGCCGTCATTTTCTG
CXCL10 (mouse)-Reverse	TCCCTATGGCCCTCATTCTCA
β-actin (mouse)-Forward	GTCGAGTCGCGTCCACC
β-actin (mouse)-Reverse	ACGATGGAGGGGAATACAGC

2.11. Osteoclast formation and TRAP assay

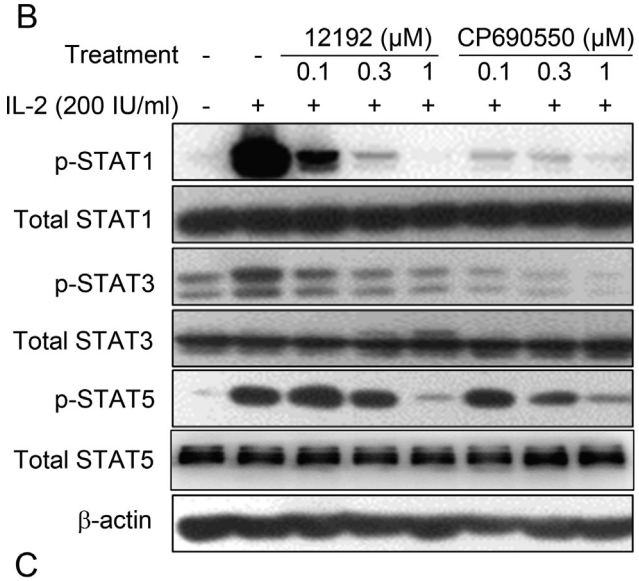
For the induction of osteoclastogenesis, mouse bone marrow-derived macrophages (BMMs) were seeded at a density of 1×10^5 cells/well in 24-well plates in the presence of RANKL (50 ng/mL) and M-CSF (50 ng/ml) with or without CS12192 or CP690550 at 3 μM for 7 days. The culture medium was replaced by half every other day. Osteoclasts were identified by Tartrate-resistant acid phosphatase (TRAP) staining kit according to the manufacturer's instructions (Sigma). The multinucleated TRAP-positive cells per low power microscopic field (10×) were counted and averaged as mean ± SD from 5 random fields. Three repeated wells per treatment were assessed in three independent experiments.

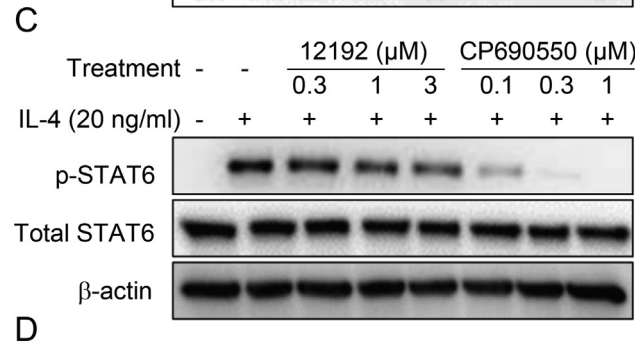
2.12. Statistical analysis

All data were analyzed and represented as the mean ± standard deviation (SD), using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). The Student's *t*-test for comparison of mean values between two groups and the One-Way ANOVA for comparison among three or more groups were used for statistical differences. A value of $p < 0.05$ was considered statistically significant.

A

Human kinases	Biochemical inhibition, IC_{50} (nM)
JAK3	11
JAK1	105
JAK2	1,404
Flt4	107
TBK1	162





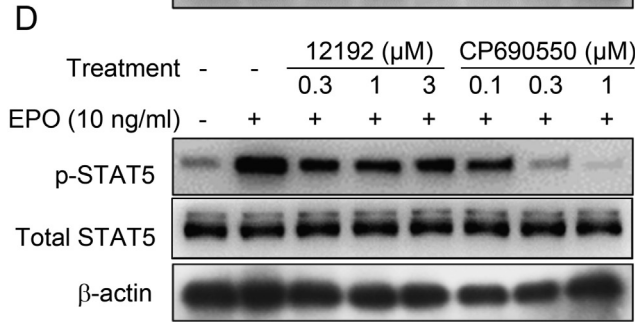


Fig. 1. Identification of CS12192 as a novel JAK inhibitor. (A) Enzymatic inhibition of CS12192 in human JAK and other kinases. Human natural killer cell line (KHYG-1) (B), human monocytic cell line (THP-1) (C), or human erythroleukemia cell line (UT-7) (D) were pre-treated with CS12192 or CP690550 at indicated concentrations for 1 h, and then stimulated with recombinant IL-2 (200 IU/ml), IL-4 (20 ng/ml), or EPO (10 ng/ml) for 15 min, respectively. The levels of phosphorylation of STATs and the corresponding total STATs were analyzed by immunoblotting. The shown data are representative of three independent experiments.

3. Results

3.1. Characterization of CS12192 as a novel selective JAK3/JAK1/TBK1 inhibitor

In order to find a JAK inhibitor with better selectivity, we designed, synthesized, and screened a serials of small molecular compounds by a cell-free enzymatic activity assay. CS12192 showed a more selective JAK3 inhibition with an IC_{50} of 11 nM, and to a less extent of JAK1 inhibition with an IC_{50} of 105 nM (Fig. 1A). In contrast, CS12192 inhibits JAK2 with an IC_{50} of 1404 nM, indicating a > 120 fold selectivity for JAK3 over JAK2. Interestingly, CS12192 inhibited TBK1 and Flt4 with the IC_{50} of 162 nM and 107 nM, respectively (Fig. 1A). The preliminary results also indicated that CS12192 at 1 μM showed slightly over 50% of inhibitory activity only to a few other kinases (such as BMX, FLT3, KDR, but not TYK2) in an activity screening assay for a panel of 75 human kinases (data not shown).

To verify the inhibitory effects of CS12192 on JAK/STAT signaling pathways at cellular level, the activation of STATs in different cell lines stimulated by relevant cytokines were tested. In a human natural killer cell line (KHYG-1), IL-2 stimulation induced a clear phosphorylation of STAT1, 3, and 5, which were inhibited by CS12192 in a dose-dependent manner (Fig. 1B). As a positive control, CP690550 showed a stronger inhibition on these STATs probably due to its relatively low nanomolar inhibitory potency against all JAK enzymes [20]. Confirmatively, the dose-dependent inhibition of phosphorylation of STAT5 upon IL-2 stimulation by CS12192 were also observed in other human and mouse T cell lines (Fig. S1). In another human monocytic cell line (THP-1), phosphorylation of STAT6 induced by IL-4 stimulation was dose-dependently inhibited by CS12192 although at a relatively high concentration (Fig. 1C). On the contrary, CS12192 did not affect the phosphorylation of STAT5 in a human erythroleukemia cell line (UT-7) under the stimulation of erythropoietin (EPO) via JAK2 activation, whereas CP690550 inhibited p-STAT5 dose-dependently (Fig. 1D). Therefore, these results indicate that CS12192 is a selective inhibitor against JAK3/JAK1, but not JAK2.

To further evaluate the effect of CS12192 on TBK1-mediated IFN response, both the activation of TBK1/IRF3 and the expression of IFN genes were tested in poly (I:C)-stimulated THP-1 cells. Poly (I:C) transfection activated TBK1/IRF3 as indicated by the phosphorylation of TBK1 and IRF3 (Fig. 2A), and induced the robust gene expression of both IFNβ and CXCL10 (Fig. 2B). Interestingly, although CS12192 caused a compensatory increase of p-TBK1 probably due to a feedback reactivation response, p-IRF3 as the substrate of p-TBK1 and the two IFN gene expression were sharply inhibited by CS12192 and the TBK inhibitor, BX795 (Fig. 2A and B). In contrast, the JAK inhibitor C9690550 had no effect on TBK1 signaling pathway. Similarly, CS12192 but not CP690550 inhibited IFN gene expression LPS-stimulated mouse macrophage cell line (RAW264.7) (Fig. 2C). Thus, CS12192 is also a novel dual JAK/TBK1 inhibitor.

In addition, the cellular growth/proliferation of 17 hematopoietic or non-hematopoietic cell lines treated with CS12192 were screened to exclude any off-target or cytotoxic effect of this compound. While the 50% of growth inhibition (GI_{50}) of CS12192 for two IL-2-dependent cell lines (CTLL-2 and KHYG-1) were less than 0.2 μM as expected, the proliferation of the majority of tested cell lines were not affected by CS12192 (GI_{50} > 20 μM) (Fig. S2). The partial growth inhibition of CS12192 for certain leukemia cell lines, K562, Jurkat, and HL-60 was likely due to the inhibition of TBK1, which was previously reported to participate in mitosis [21].

3.2. CS12192 ameliorates disease severity of both AIA and CIA in rats

To investigate the *in vivo* pharmacological effect of CS12192, an experimental arthritis model in rat induced by adjuvant (AIA) was initially used. Immediately after the onset of the disease, CS12192 were

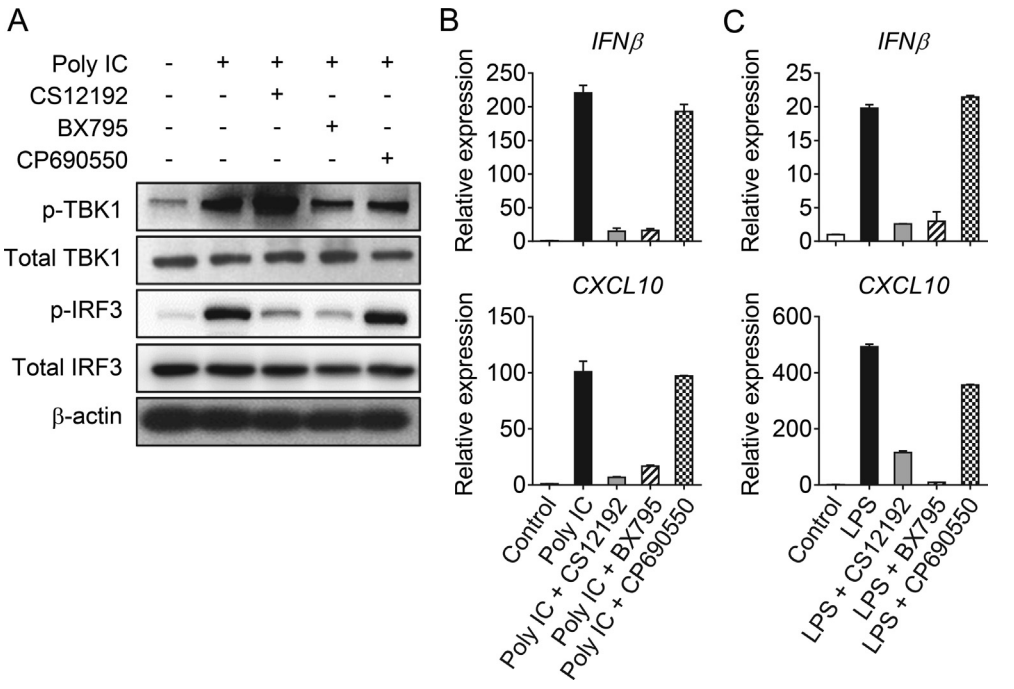


Fig. 2. CS12192 suppresses IFN gene expression through the inhibition of TBK1 signaling pathway. (A) THP-1 cells were pre-treated with CS12192 (3 μM), BX795 (0.5 μM), or CP690550 (3 μM) for 1 h, and then transfected with poly (I:C) at 1 μg/ml for 2 h. The levels of phosphorylation of TBK/IRF3 and the corresponding total TBK/IRF3 were analyzed by immunoblotting. (B) The relative gene expression of human *IFNβ* and *CXCL10* in THP-1 cells after transfection for 3 h were measured by quantitative RT-PCR. (C) Mouse RAW264.7 cells were pre-treated with CS12192 (3 μM), BX795 (0.5 μM), or CP690550 (3 μM) for 1 h, and then stimulated with LPS at 100 ng/ml for 3 h. The relative gene expression of mouse *IFNβ* and *CXCL10* were measured by quantitative RT-PCR. The shown data are representative of three independent experiments.

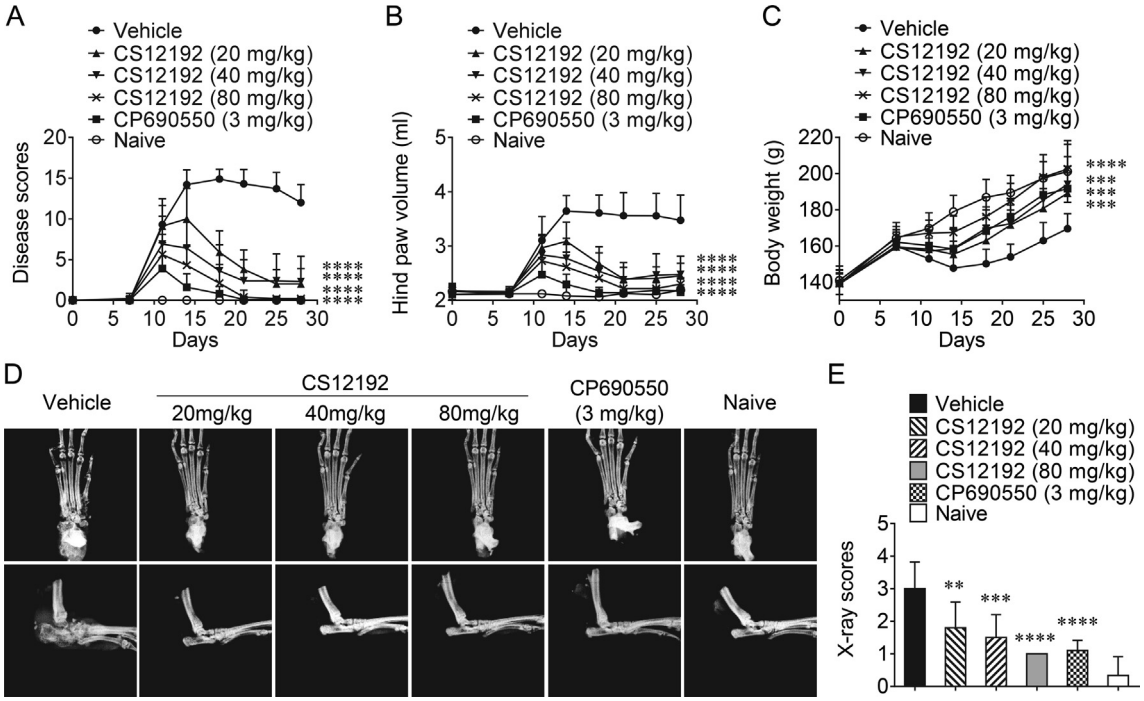


Fig. 3. CS12192 ameliorates disease severity of AIA in rats. The rat AIA model was induced by an intradermal injection of CFA at the base of the tail and the lower back on day 0. On day 7, animals were randomly grouped and intragastrically administered with the indicated doses of CS12192 or CP690550, twice a day, until day 28. The disease scores (A), hind paw volume (B), and body weights (C) were monitored every 2–3 days. The representative X-ray results (D) and X-ray scores (E) of rat hind paws from each group were evaluated on day 28. The significance of the differences between vehicle- and compound-treated groups were determined by One-Way ANOVA test. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

intragastrically administered into animals at the indicated dosages, twice a day. Compared to vehicle-treated AIA rats, treatment with CS12192 attenuated very significantly disease score and hind paw swelling in a dose-dependent manner (Fig. 3A and B). Besides the efficacy of CS12192 at 80 mg/kg that was comparable to CP690550 at 3 mg/kg, body weight gains in CS12192-treated AIA rats at this dose were even better than those in CP690550-treated group (Fig. 3C). The radiographic evaluation of rat joints revealed that severe bone erosion and joint space narrowing shown in vehicle-treated group were significantly reduced by treatment with CS12192 in a dose-dependent manner (Fig. 3D and E). Confirmatively, histopathological evaluation demonstrated that joints in vehicle-treated AIA rats showed severe inflammatory infiltration as well as joint tissue destruction with synovial hyperplasia and cartilage degradation, whereas the cartilage and the synovial tissue structure from both CS12192- and CP690550-treated AIA rats displayed much less damage (Fig. S3A).

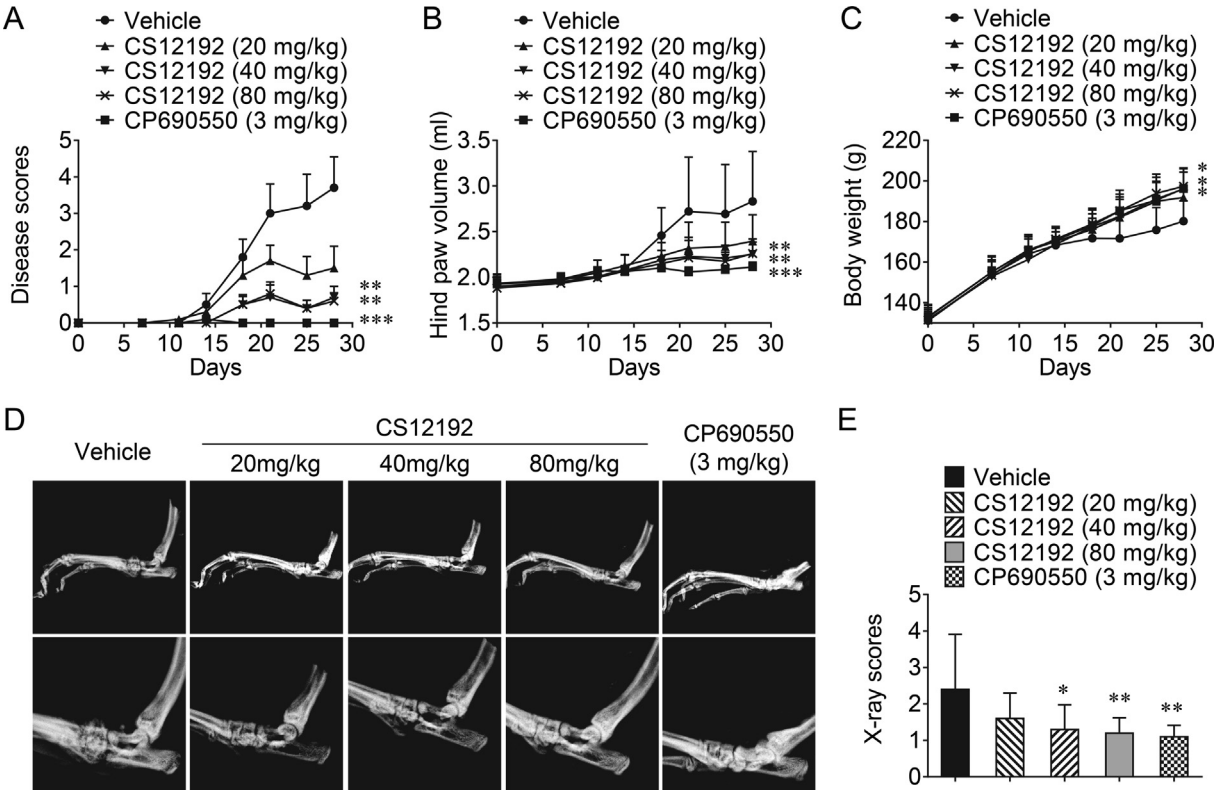


Fig. 4. CS12192 ameliorates disease severity of CIA in rats. The rat CIA model was induced by an intradermal injection of bovine type II collagen emulsified in IFA at the base of the tail and the lower back on day 0, and boosted with the same injection on day 7. On day 11, animals were randomly grouped and intragastrically administered with the indicated doses of CS12192 or CP690550, twice a day, until day 28. The disease scores (A), hind paw volume (B), and body weights (C) were monitored every 2–3 days. The representative X-ray results (D) and X-ray scores (E) of rat hind paws from each group were evaluated on day 28. The significance of the differences between vehicle- and compound-treated groups were determined by One-Way ANOVA test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The therapeutic effect of CS12192 was further verified in the antigen-specific arthritis model of CIA in rat. CS12192 significantly alleviated disease scores, hind paw swelling and body weight loss of rat CIA model in a dose-dependent manner (Fig. 4A–C). Similarly, the results from both X-ray and histopathology in joint tissues showed that treatment with CS12192 dose-dependently impeded the bone erosion and joint tissue inflammation with synovial hyperplasia and cartilage degradation compared with vehicle-treated control (Fig. 4D, E and Fig. S3B). Taken together, these results indicate that CS12192 ameliorates disease severity in both rat AIA and CIA models.

3.3. CS12192 attenuated disease severity of mouse CIA through inhibiting Th17 differentiation and suppressing pro-inflammatory cytokine gene expression in joint tissue

Next to explore the possible immune alterations induced by CS12192, the activation and cytokine production of $CD4^+$ T cells in a mouse CIA model were examined. At the beginning of the disease, CIA mice were orally administered with CS12192 at the dose of 80 mg/kg, twice a day, for 21 days. CS12192 significantly attenuated disease progression of mouse CIA (Fig. 5A). At the termination of the experiment, treatment with CS12192 not only significantly down-regulated the frequency of $CD44^+$ $CD62L^-$ $CD4^+$ T cells, but also decreased the percentage of IL-17-producing $CD4^+$ T cells (Th17) in the spleen compared to vehicle-treated control (Fig. 5B). Consistently, treatment with CS12192 significantly inhibited the serum levels of both proinflammatory cytokines (IL-1β, IL-6, TNF-α) and Th17 proliferation and/or differentiation-related cytokines (IL-2, IL-15, IL-21, IL-22) from CIA mice (Fig. 5C). Interestingly, although both CS12192 and CP690550 inhibited *in vitro* Treg differentiation/proliferation in a dose-dependent manner likely through the suppression of IL-2/STAT5 pathway, treatment with CS12192 has little effect on the frequency of splenic Tregs from CIA mice (Fig. S4). Probably, the maintanence of Tregs under chronic inflammatory conditions is IL-2-independent [22]. The phosphorylation of STAT1 and STAT3 in the spleen from CS12192-treated mice were also suppressed (Fig. 5D), confirming the *in vivo* pharmaceutical effects of CS12192 on JAK-STAT signaling pathway. These results suggested that both $CD4^+$ T cell activation and Th17 differentiation which are critical for RA disease development were substantially impaired by CS12192.

Furthermore, while the relative gene expression of pro-inflammatory cytokines (*IL-1β*, *IL-6*, and *IL-17*) and chemokines (*CCL2*, *CCL5*, and *CXCL2*) as well as type I IFN genes (*IFNα* and *CXCL10*) in joint tissues from vehicle-treated CIA mice were sharply increased compared to naïve mice, treatment with CS12192 significantly reduced the expression of these above genes (Fig. 5E). Therefore, inhibition of T cell activation and Th17 differentiation as well as suppression of tissue inflammation by CS12192 may contribute to the alleviation of mouse CIA.

3.4. CS12192 suppressed RANKL-induced osteoclast formation

To determine whether reduced bone erosion was mediated through a direct effect on osteoclastogenesis, the potential of CS12192 to impact osteoclast differentiation in an *in vitro* model were evaluated. As reported previously, bone marrow-derived macrophages in the presence of RANKL and M-CSF proliferate and differentiate into osteoclast with TRAP positive staining through activation of TBK1 signaling pathway [23]. In contrast, CS12192 significantly inhibited osteoclast formation with reduced quantification of TRAP staining, whereas CP690550 showed little effect (Fig. 6). Thus, CS12192 may have a direct effect on osteoclastogenesis through TBK1 inhibition.

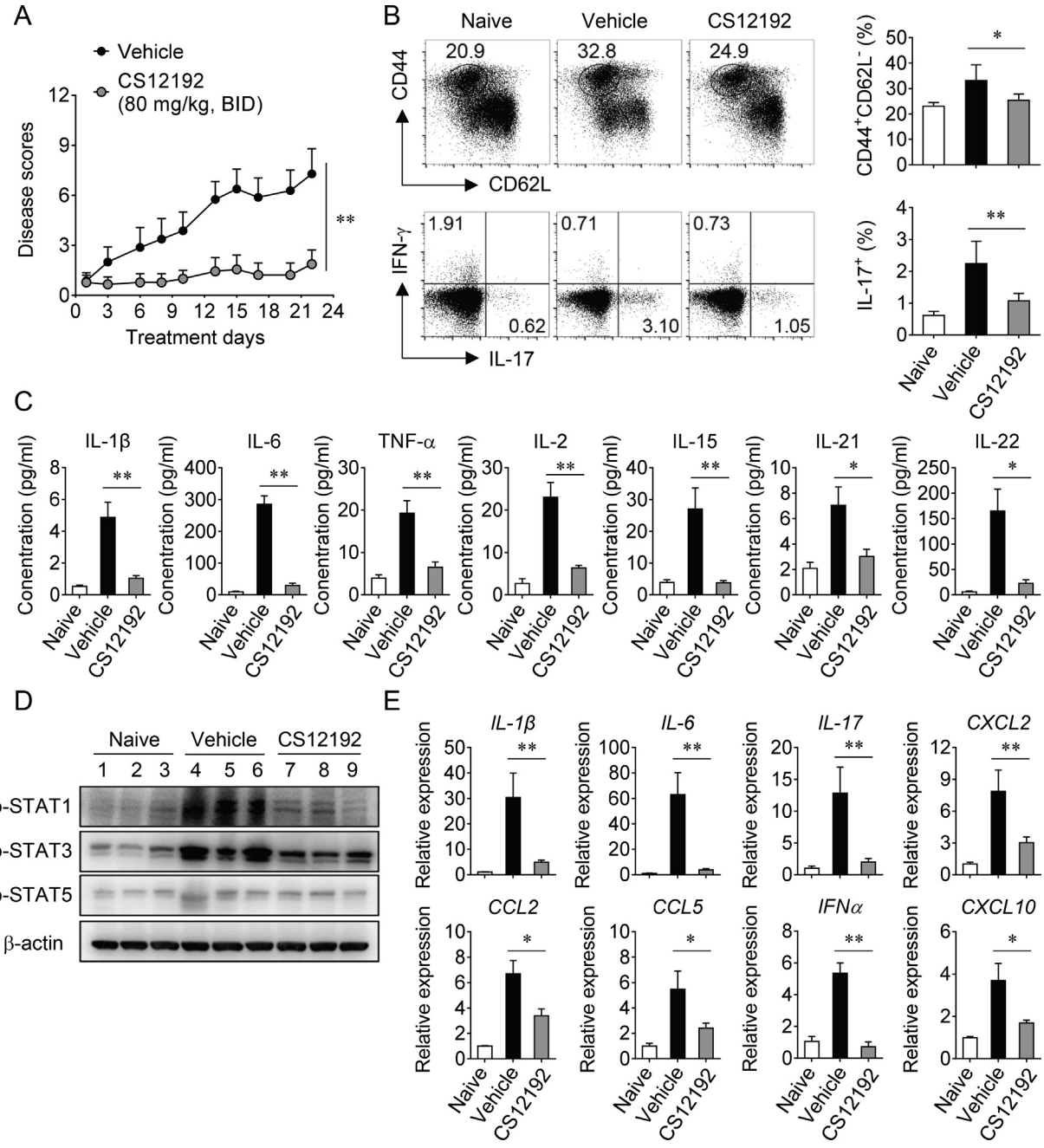


Fig. 5. CS12192 attenuates mouse CIA through inhibition of Th17 and suppression of pro-inflammatory gene expression. The mouse CIA model was induced in female DBA/1 mice by intradermal immunization with chicken type II collagen emulsified in CFA at the base of the tail on day 0, followed by a booster immunization with chicken type II collagen emulsified in IFA at day 21. On day 25, animals were randomly grouped and intragastrically administered with vehicle or CS12192 (80 mg/kg), twice a day (BID), for 22 days. (A) The disease scores were assessed every 2–3 days. (B) The activation of $CD4^+$ T cells shown as $CD44^+$ $CD62L^-$ and IL-17-producing $CD4^+$ T cells (Th17) in the spleen were determined by flow cytometry. The representative plot data and the cumulative frequencies of $CD44^+$ $CD62L^-$ and $IL\text{-}17^+$ in $CD4^+$ T cells from naïve, vehicle- and CS12192-treated mice were shown. (C) The serum levels of pro-inflammatory cytokines and Th17 differentiation-related cytokines from CIA mice were evaluated by ProcartaPlex assay. (D) The levels of p-STAT1, p-STAT3, and p-STAT5 in the spleen from 3 individual animal of each groups were evaluated by immunoblotting. (E) The relative gene expression of pro-inflammatory cytokines and chemokines in the joint tissues from each group were evaluated by RT-PCR. The significance of the differences between vehicle- and compound-treated groups were determined by *t*-test. *, $P < 0.05$; **, $P < 0.01$.

4. Discussion

In the present study, we have characterized CS12192 as a novel small-molecule JAK inhibitor selective for JAK3/JAK1/TBK1 using *in vitro* enzymatic and cellular assays, and demonstrated its therapeutic effectiveness in both rat and mouse models of experimental arthritis, including CIA and AIA models. The mechanistic studies revealed that CS12192 treatment ameliorated disease severity of mouse CIA through inhibiting effector $CD4^+$ T cell activation and Th17 cell differentiation together with the suppression of pro-inflammatory cytokine and chemokine gene expression in joint tissue. Furthermore, the suppression of type I interferon production and osteoclast formation by CS12192 through TBK1 inhibition may contribute to the prevention of tissue inflammation and bone destruction.

The importance of $CD4^+$ T cells and its differentiated subpopulations such as Th1, Th17 and T follicular helper (Tfh) cells in the

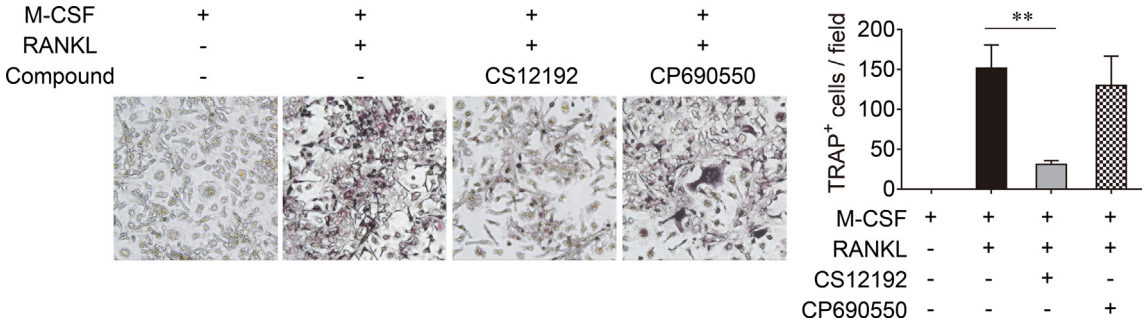


Fig. 6. Inhibition of RANKL-induced osteoclast formation by CS12192. BMMs were treated with or without CS12192 or CP690550 at 3 μM in the presence of RANKL (50 ng/ml) and M-CSF (50 ng/ml) for 7 days. Cells were then fixed and stained for TRAP assay. The TRAP-positive multinucleated cells as osteoclasts per field (10×) were counted and averaged as mean ± SD from five random fields. The shown data are representative of three independent experiments.

pathogenesis of RA have been well illustrated [24,25]. In fact, the common cytokine-receptor γ-chain family cytokines including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 are not only essential for lymphoid homeostasis [26], but also play important roles in effector T cell expansion and Th cell differentiation [27]. For example, while IL-21 are fundamental for both Tfh cell generation and Th17 cell proliferation [28], IL-7 and IL-15 are critical for maintaining pathogenic memory Th17 cells [29]. Since JAK3 and JAK1 kinases are the key signal mediators for the common γ-chain cytokine receptors [30,31], targeting JAK3/JAK1 could efficiently abolish the signals from common γ-chain cytokine receptors and prevent Th cell activation and differentiation, which has been verified by the first generation of pan JAK inhibitor tofacitinib [32]. Our results showed that CS12192 selectively inhibited IL-2-dependent T cell line proliferation via JAK3/JAK1 rather than affecting EPO-dependent leukemia cell line growth via JAK2. In addition, the inhibited $CD4^+$ T effector cell activation and Th17 cell function with reduced Th17 differentiation-related cytokines in sera were well correlated to the ameliorated CIA disease by CS12192 treatment. Therefore, CS12192 as a selective JAK3/JAK1 inhibitor demonstrated its therapeutic effects on experimental arthritis probably through the signaling inhibition of common γ-chain cytokine receptors.

Although the generation and/or differentiation of Th17 cells is induced from naïve T precursor cells in the context of a milieu of inflammatory cytokines, IL-6 and particularly STAT3 are the master regulators for Th17 development [33,34]. Consistently, our results showed the decreased serum level and joint tissue gene expression of IL-6 and suppressed STAT3 phosphorylation in the splenocytes from CS12192-treated CIA mice. Since IL-6 mainly activates STAT3 through its receptor gp130-associated JAK1 [35], and a selective JAK1/2 inhibitor efficiently inhibits IL-6-stimulated phosphorylation of STAT3 [36], it is very likely that CS12192 targeted on JAK1 to inhibit IL-6/STAT3 signaling pathway, leading to Th17 suppression. The signaling from other cytokines, like IL-21, which induces Th17 differentiation in a STAT3-dependent manner, could also be the targets of CS12192 [37].

With the emerging evidence that the activated type I IFN signaling is implicated in the development of autoimmune diseases including systemic lupus erythematosus (SLE) and RA [38,39], one of the key kinases required for type I IFN induction, TBK1, has been considered as a potential therapeutic target in various disease models [17,18]. For example, TBK1 deficiency reduces the gene and protein expression of type I IFN and CXCL10 in fibroblast-like synoviocytes [40]. Also, small molecule inhibitors of TBK1 have demonstrated the therapeutic potentials in animal models of SLE [41] and RA [42]. CS12192 inhibits both TBK1 and JAK1 so that the induction of type I IFNs and the activation of type I IFN signaling could be simultaneously blocked. This may be correlated with the decreased gene expression of IFN-α and CXCL10 in joint tissue from CS12192-treated mice. Interestingly, TBK1 also controls the migration of autoreactive T cells during neuroinflammation [43], suggesting another regulatory mechanism of CS12192 on T cell infiltration into the inflammed tissues through TBK1 inhibition.

Inflammed joints in RA are often associated with periarticular bone loss which precedes focal bone erosion [44]. Previous study has shown that the pan-JAK inhibitor tofacitinib suppressed structural joint damage in a rat model of AIA. However, tofacitinib had no direct impact on RANKL-dependent osteoclast differentiation but rather suppressed osteoclast-mediated bone resorption indirectly through decreasing RANKL production from T cells via JAK inhibition [45]. Similar results were also reported in the study of another JAK1/2 inhibitor, baricitinib [46]. Recently, TBK1 was found to play a role in RANKL-induced NF-κB activation, and inhibition of TBK1 suppressed RANKL-mediated osteoclastogenesis and prevented ovariectomy-induced bone loss [23,47]. Therefore, CS12192 may not only down-regulate RNAKL expression through JAK inhibition, but also directly block RANKL signaling through TBK1 inhibition, indicating a superior feature to other JAK inhibitors on the prevention of bone loss-associated osteoclastogenesis.

In conclusion, we have demonstrated that a novel selective JAK3/JAK1/TBK1 inhibitor, CS12192, is efficacious in both rat and mouse models of experimental arthritis. Its mechanistic effects on the suppression of immune response and tissue inflammation together with bone damage protection may provide a new strategy for the control of autoimmune diseases.

Author contributions

S Shan, Y Zhou, L Xin and X Lu conceived, designed and directed the study; Y Zhou, Y Wang, L Li, J Zhu and Y Zhang performed the experiments; J Yu, Q Yang, and Z Li contributed to the compound design and synthesis; S Shan, Y Zhou, D Pan and L Xin collected and analyzed data; L Xin wrote the paper; S Shan, Y Zhou, D Pan, S Huang, Z Ning and X Lu contributed to the revision of the paper.

Declaration of Competing Interest

The authors have declared that no competing interests exist.

Acknowledgements

This study was supported by the Major Science and Technology Projects of Guangdong Province (2013A022100013), and Shenzhen Technology Innovation Project (JSGG20170412153957140).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105914.

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