

Schistosoma japonicum cystatin suppresses osteoclastogenesis via manipulating the NF- κ B signaling pathway

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Abstract. Abnormal osteoclastic activation and secretion of cysteine proteinases result in excessive bone resorption, which is one of the primary factors in the development of bone metabolic disorders, such as rheumatoid arthritis and osteoporosis. Mammalian cystatins have been demonstrated to restrain osteoclastic bone resorption and to alleviate severe osteolytic destruction via blocking the activity of cysteine proteinases. However, the specific effects of parasite cystatins on the formation and function of osteoclasts remain unclear. The purpose of the current study was to explore the effects of cystatins from *Schistosoma japonicum* (Sj-Cys) on macrophage colony-stimulating factor (M-CSF) and receptor activator of NF- κ B ligand (RANKL)-induced osteoclast differentiation, as well as the underlying molecular mechanisms. Recombinant Sj-Cys (rSj-Cys) dose-dependently restrained osteoclast formation, with a half-maximal inhibitory concentration (IC₅₀) value of 0.3 μ M, and suppressed osteoclastic bone resorptive capability *in vitro*. The findings were based on tartrate resistant acid phosphatase (TRAP) staining and bone resorption assays, respectively. However, the cell viability assay showed that the repression of rSj-Cys on osteoclast formation did not depend on effects on cell viability or apoptosis. Based on the results of reverse transcription-quantitative PCR and western blot analysis, it was found that rSj-Cys downregulated the

expression levels of osteoclastogenesis-related genes and proteins, by interfering with M-CSF and RANKL-induced NF- κ B signaling and downstream transcription factors during early-phase osteoclastogenesis. Overall, the results of the present study revealed that rSj-Cys exerted an inhibitory role in osteoclast differentiation and could be a prospective biotherapeutic candidate for the treatment and prevention of bone metabolic disorders.

Introduction

Skeletal remodeling and integrity, as well as bone homeostasis, are strictly controlled by the coordinated interactions of osteoblast-mediated ossification and osteoclast-regulated bone resorption (1). The abnormal osteoclastic activation and/or defective osteoblastic function may contribute to the decline in bone quality and lead to internal microstructure damage to bone, ultimately leading to the development of bone metabolic disorders, such as rheumatoid arthritis and osteoporosis. Osteoclasts are hematopoietic stem cell-derived, multinucleated giant cells that have a unique role in bone-mineralized matrix degradation (2). Osteoclast formation is a sophisticated multiple-stage process that comprises proliferation, differentiation, and the fusion of monocyte/macrophage precursors (3). There are two cytokines that are essential for the regulation of osteoclastogenesis-related cell behavior. Specifically, osteoclast precursors rely on macrophage colony-stimulating factor (M-CSF) for survival and proliferation and receptor activator of NF- κ B ligand (RANKL) binding for osteoclast differentiation (4-6). Upon RANK/RANKL interaction, the adaptor protein tumor necrosis factor receptor-associated factor 6 (TRAF6) is recruited to the cytoplasmic tail of RANK for activation of several signal transduction pathways. TRAF6-induced downstream events include initially the activation of distinct signaling pathways that are mediated by NF- κ B and MAPK, and subsequently the synthesis of transcription factors, such as nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) and Fos proto-oncogene AP-1 transcription factor subunit (c-Fos). These transcription factors serve as definitive regulators and modulate the expression of multiple specific genes that regulate

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terminal osteoclastogenesis, including tartrate resistant acid phosphatase (TRAP), osteoclast-associated receptor (OSCAR), integrin $\beta 3$ (ITGB3) and cathepsin K (CTSK), thus, driving osteoclast differentiation and fusion (2,7,8).

Notably, osteoclastic bone resorption is dependent not only on the number of osteoclasts formed but also on the extent of osteoclast activation. Activated mature osteoclasts closely adhere to the bone surface, followed by a ruffled border, and thus form a sealing chamber from the extracellular environment. The process of bone resorption takes place in Howship's lacunae. Inside the segregated chamber, calcium hydroxy-apatite crystals are dissolved in an acidic environment due to the acidification by hydrogen ion pumps on the cell surface, resulting in exposure of the organic bone matrix (9,10). Additionally, different proteolytic enzymes, particularly cysteine proteinases and matrix metalloproteinases, are also activated at low pH and are involved in digestion of the organic bone matrix. Although the exact cysteine proteinases that are in charge of osteoclast-mediated bone degradation are not fully understood, substantial evidence has shown that, as one member of the papain-cysteine proteinase family, cathepsin K is abundantly expressed in mammalian osteoclasts and serves a dominant role in efficient cleavage of collagenous bone matrix (11,12). Single-point mutations in the human cathepsin K gene have been associated with a rare autosomal recessive lysosomal storage disorder termed as pycnodysostosis, a specific osteopetrotic-like phenotype, characterized by dwarfism and facial deformity, as well as a predisposition to fragility fractures (13). Similarly, the phenotype of pycnodysostosis was later reproduced in cathepsin K-deficient mice, which display moderate osteopetrosis with hypermineralization, as well as many undigested collagen fragments in the subosteoclastic area, mainly in their long bones (14). Consequently, these observations support the hypothesis that cathepsin K may be a potential antiresorptive target for pharmacologic intervention in bone metabolic disorders.

Cystatins, endogenous, reversible, and tight-binding inhibitors of cysteine proteinases, have been successfully extracted, cloned, and characterized in different taxonomic groups, from viruses to vertebrates (15). Based on amino acid sequence similarity and crystal structure variation, the cystatin family is categorized into three distinct subgroups. Type I cystatins (stefins) are primarily intracellular but have been seldomly reported in body fluids. Stefins are unglycosylated proteins consisting of ~100 amino acids without disulfide bonds or carbohydrate chains. Type II cystatins, which are secretory proteins with nearly 120 amino acids and possess 2 conserved disulfide bridges toward the carboxyl terminus, are mostly extracellular and contain cystatins C, D, E/M, F, G, S, SA, and SN. Type III cystatins (kininogens), multi-domain glycoproteins composed of ~350 amino acids, are synthesized in the liver and circulate predominantly in blood plasma (16,17). To date, cystatins have been widely researched and confirmed to participate in various physiopathological processes. Mammalian cystatins serve vital roles in immunomodulation, tumorigenesis, and bone remodeling, whereas parasite cystatins significantly contribute to immunosuppression in humans and may be biotherapeutic candidates for autoimmune or allergic diseases, such as asthma and inflammatory colitis (15,18-20).

Schistosoma japonicum is a digenetic blood fluke with a widespread distribution in mainland China. Cystatins from *S. japonicum* (Sj-Cys) are similar to other parasite cystatins and have also been demonstrated in recent years to serve a vital role in modulation of the host immune response. Yang *et al* (21) reported that Sj-Cys could restrain the production of tumor necrosis factor (TNF)- α and interleukin (IL)-6 in lipopolysaccharide-induced RAW264.7 cells *in vitro*. Furthermore, our previous study demonstrated that Sj-Cys alleviated severe inflammation and ameliorated sepsis-induced multiple organ failure, by reducing pro-inflammatory cytokines (22). Although widespread reports of Sj-Cys have focused on the immunoregulation of inflammation and inhibitory effects on cysteine proteinase, little is known regarding the functional diversification. Given that both Sj-Cys and stefin B (cystatin B) belong to the homologous type I cystatins class, they may have common features to some extent (23). Stefins B, a single-chain protein, is ubiquitously expressed by multiple cell types, including osteoclasts. Stefins B administration restrains osteoclastic bone resorption via blocking the activity of cathepsin K (24-26). However, the specific effect of Sj-Cys on regulation of the formation and activation of osteoclasts remains unclear. The current study investigated whether Sj-Cys could affect M-CSF and RANKL-induced osteoclastogenesis and bone resorption *in vitro*, and explored the underlying molecular mechanisms.

Materials and methods

Production and purification of the recombinant Sj-Cys (rSj-Cys) protein. The expression and purification of rSj-Cys has been described previously (27). Briefly, DNA encoding Sj-Cys was cloned into the pET-28a vector (Promega Corporation). The sequence was confirmed to be inserted successfully before transformation of the restructured plasmid into *Escherichia coli* (BL 21). Next, the transformants were induced for large-scale protein expression using 1 mM isopropyl- β -D-1-thiogalactopyranoside (Sigma-Aldrich; Merck KGaA) at 37°C for 5 h. After purification with the HisPur™ Ni-NTA Spin Column (Thermo Fisher Scientific, Inc.), endotoxin contamination was mostly eliminated using the ToxOut™ High-Capacity Endotoxin Removal kit (BioVision, Inc.), and residual endotoxin detection was performed using the ToxinSensor™ Chromogenic Limulus Amebocyte Lysate Endotoxin Assay kit (GenScript), in accordance with the manufacturer's protocols. Finally, a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology) was used to determine the concentration of rSj-Cys.

Cell culture and in vitro osteoclastogenesis assay. RAW264.7 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotic-antimycotics (Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. Prior to induction into osteoclasts, the cells were seeded in 96-well plates at a density of 3x10³ cells/well with α -minimal essential medium (α -MEM; Gibco; Thermo Fisher Scientific, Inc.) and incubated overnight. The culture medium was then

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
Bax	TGAAGACAGGGGCCTTTTGT	AATTCGCCGGAGACACTCG
Bcl-2	GCTACCGTCGTGACTTCGC	CCCCACCGAACTCAAAGAAGG
CTSK	GAAGAAGACTCACCAGAAGCAG	TCCAGGTTATGGGCAGAGATT
TRAP	TGTCATCTGTGAAAAGGTGGTC	ACTGGAGCAGCGGTGTTATG
ITGB3	GGCGTTGTTGTTGGAGAGTC	CTTCAGGTTACATCGGGGTGA
RANK	CCAGGAGAGGCATTATGAGCA	ACTGTCGGAGGTAGGAGTGC
OSCAR	CCGTGCTGACTTCACACCAA	GGGGTGACAAGGCCACTTTT
TREM-2	CTGGAACCGTCACCATCACTC	CGAAACTCGATGACTCCTCGG
NFATc1	GGAGAGTCCGAGAATCGAGAT	TTGCAGCTAGGAAGTACGTCT
c-Fos	CGGGTTTCAACGCCGACTA	TTGGCACTAGAGACGGACAGA
IκBα	TGAAGGACGAGGAGTACGAGC	TTCGTGGATGATTGCCAAGTG
p65	AGGCTTCTGGGCCTTATGTG	TGCTTCTCTCGCCAGGAATAC
β-actin	AGAGGGAAATCGTGCGTGAC	CCAAGAAGGAAGGCTGGAAA

CTSK, cathepsin K; TRAP, tartrate resistant acid phosphatase; ITGB3, integrin β 3; RANK, receptor activator of NF- κ B; OSCAR, osteoclast-associated receptor; TREM-2, triggering receptor expressed on myeloid cells 2; NFATc1, nuclear factor of activated T-cells cytoplasmic 1; c-Fos, Fos proto-oncogene AP-1 transcription factor subunit; I κ B α , NF- κ B inhibitor α ; p65, RELA proto-oncogene NF- κ B subunit.

replaced with complete α -MEM containing either M-CSF alone (25 ng/ml; R&D Systems, Inc.) or M-CSF (25 ng/ml) and RANKL (30 ng/ml; R&D Systems, Inc.) in the presence of rSj-Cys (0, 0.01, 0.1, 0.3, 1 or 10 μ M), and cells were cultured for 5 days. The medium was replaced with fresh medium every 2 days.

TRAP staining. After induction for 5 days as aforementioned, osteoclast formation was analyzed using a commercial TRAP staining kit (Sigma-Aldrich; Merck KGaA), following the manufacturer's instructions. In brief, the medium was removed and the cells were fixed with 4% polyformaldehyde for 10 min at room temperature. After the removal of stationary liquid and rinsing thoroughly with distilled water, the cells were incubated for 1 h at 37°C with TRAP staining solution. The number of TRAP-positive multinucleated cells with three or more nuclei per well was visualized and counted under an inverted fluorescence microscope (Olympus Corporation). Additionally, the estimated half-maximal inhibitory concentration (IC₅₀) value of rSj-Cys was determined and used for subsequent experiments.

Bone resorption assay. Bone resorptive ability was evaluated using the Corning Osteo Assay Surface (COAS; Corning Inc.). RAW264.7 cells were plated onto the COAS at a density of 3×10^3 cells/well and cultured in complete α -MEM containing M-CSF (25 ng/ml) and RANKL (30 ng/ml) with or without rSj-Cys (IC₅₀, 0.3 μ M) for osteoclastogenesis. The medium was replaced with fresh medium every 2 days. Following culture for 5 days, the medium was discarded, and the cells were lysed with 10% sodium hypochlorite for 10 min before the wells were air-dried. The surface images of pits were randomly captured using an inverted fluorescence microscope, and the number and area of pit formation were quantified using the ImageJ software (version 1.38X; National Institutes of Health).

Cell viability assay. Cell viability in response to rSj-Cys was assessed using the MTT colorimetric assay (Beijing Solarbio Science & Technology Co., Ltd.). In short, the RAW264.7 cells were seeded in 96-well plates at a density of 3×10^3 cells/well and cultured in complete DMEM. After 4 h, the cells were treated with M-CSF (25 ng/ml) and rSj-Cys (concentration, 0 or IC₅₀), followed by further cultivation for 24, 48 or 72 h. The medium was removed and MTT reagent was added to each well for an additional 4 h. The optical density was measured at a wavelength of 570 nm using a microplate reader.

Reverse transcription-quantitative PCR (RT-qPCR). For the analysis of mRNA expression levels of osteoclast-related genes, RAW264.7 cells were seeded in 6-well plates at a density of 5×10^4 cells/well and co-stimulated with M-CSF (25 ng/ml) and RANKL (30 ng/ml). The cells were then treated with or without rSj-Cys (concentration, IC₅₀) and cultured until the indicated time points (4, 24, 48 or 72 h). Total RNA was isolated from cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse-transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. Amplification of the cDNA template was conducted using the Q6 Flex Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the SYBR® Premix Ex Taq II kit (Takara Bio, Inc.) with specific oligonucleotide primers, as listed in Table I (Sangon Biotech Co., Ltd.). The thermocycling conditions used for qPCR were as follows: Denaturation at 95°C for 30 sec; followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The β -actin housekeeping gene was used as an internal control. Relative fold changes in mRNA expression were calculated using the formula $2^{-\Delta\Delta C_q}$ (28).

Western blot analysis. The culture conditions and interventions for osteoclast differentiation were as aforementioned. After

24 or 72 h, total protein was collected using the RIPA lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration was calculated using the BCA protein assay kit (Beyotime Institute of Biotechnology). Aliquots (containing 30 μ g protein) were separated by 10% SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride (Millipore) membranes. The membranes were blocked with 5% skimmed milk for 1 h at room temperature prior to incubation overnight at 4°C with primary antibodies against CTSK (1:1,000; cat. no. DF6614), TRAP (1:1,000; cat. no. DF6989), ITGB3 (1:1,000; cat. no. AF6086), RANK (1:1,000; cat. no. DF12532), triggering receptor expressed on myeloid cells 2 (TREM-2; 1:1,000; cat. no. DF12529), NFATc1 (1:1,000; cat. no. DF6446), c-Fos (1:1,000; cat. no. AF0132), NF- κ B inhibitor α (I κ B α ; 1:1,000; cat. no. AF5002), RELA proto-oncogene NF- κ B subunit (p65; 1:1,000; cat. no. AF5006), β -actin (1:2,000; cat. no. AF7018) (all from Affinity Biosciences) and OSCAR (1:1,000; cat. no. AF1633; R&D Systems, Inc.). Horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000; cat. no. S0001; Affinity Biosciences) was then added for 90 min at room temperature; then, the immunoreactive proteins were visualized using chemiluminescent HRP substrate (EMD Millipore). Band intensity was semi-quantified using ImageJ software and normalized against β -actin levels.

Statistical analysis. All experiments were conducted independently in triplicates, and numerical data were presented as means \pm standard deviation. SPSS Statistics version 20.0 (IBM Corp.) and GraphPad Prism 6.0 (GraphPad Software, Inc.) were used for statistical analysis. Two-group comparisons were assessed using the Student's t-test, whereas multiple comparisons were performed using one-way analysis of variance followed by the Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

rSj-Cys suppresses M-CSF and RANKL-induced osteoclastogenesis in vitro. After the RAW264.7 cells were incubated with either M-CSF or M-CSF and RANKL in the absence or presence of rSj-Cys for 5 days, mature osteoclasts were fixed and subjected to TRAP staining. The results demonstrated that the stimulation of RAW264.7 cells with M-CSF alone failed to induce the formation of TRAP-positive multinucleated cells, whereas these cells were frequently found upon treatment with both M-CSF and RANKL (Fig. 1A). However, the number of TRAP-positive cells was gradually reduced in a dose-dependent manner following exposure to rSj-Cys (Fig. 1A and B). Osteoclast formation was restrained by $\sim 30\%$ with 0.1 μ M rSj-Cys, and this was almost completely inhibited with 10 μ M rSj-Cys treatment (Fig. 1A and B). Based on the morphological size and the number of TRAP-positive cells, the IC_{50} value of rSj-Cys was found to be 0.3 μ M. Therefore, rSj-Cys exerted an inhibitory effect on M-CSF and RANKL-induced osteoclastogenesis in RAW264.7 cells.

rSj-Cys decreases the bone resorptive capability of osteoclasts. Since osteoclast formation is a necessary presupposition for osteoclastic bone resorption, it was hypothesized that the bone resorptive ability of osteoclasts would also be

markedly restricted by rSj-Cys. Thus, a bone resorption assay was conducted to investigate the effects of rSj-Cys on M-CSF and RANKL-induced bone resorption in RAW264.7 cells. As anticipated, the addition of rSj-Cys significantly decreased the area of resorption pits, even though the osteoclasts induced by M-CSF and RANKL generated large resorption pits (Fig. 2A). Quantitative analysis of the pit-formation area confirmed these results (Fig. 2B) and suggested that rSj-Cys treatment attenuated the bone resorptive capability of osteoclasts.

Effects of rSj-Cys on the survival of RAW264.7 cells. The survival of osteoclast precursors is indispensable for osteoclast differentiation and formation. To investigate the survival of the osteoclast precursor cells used in the present study, the potential cytotoxic effects of rSj-Cys on the viability of RAW264.7 cells in the presence of M-CSF were analyzed by performing an MTT colorimetric assay. The results demonstrated that M-CSF-stimulated cell proliferation was not evidently influenced by rSj-Cys at IC_{50} concentrations, even after 72 h of exposure (Fig. 3A). Simultaneously, RT-qPCR was performed to assess expression levels of the proapoptotic gene Bax and the antiapoptotic gene Bcl-2 to evaluate the effects of rSj-Cys on M-CSF and RANKL-induced apoptosis. As shown in Fig. 3B, the mRNA expression levels of Bax were decreasing in a time-dependent manner following stimulation with M-CSF and RANKL, and there was no appreciable effect of rSj-Cys. By contrast, the mRNA expression levels of Bcl-2 exhibited a reverse trend and were significantly elevated following the addition of rSj-Cys (Fig. 3B). Collectively, the possibility that rSj-Cys adversely affected the survival of RAW264.7 cells and consequently inhibited osteoclast formation by promoting apoptosis or by decreasing cell viability was excluded.

rSj-Cys inhibits the expression of osteoclastogenesis-related genes and proteins. To further elucidate the effects of rSj-Cys on osteoclastogenesis, the expression levels of genes and proteins associated with the osteoclast phenotype (CTSK, TRAP and ITGB3) and the cell surface receptors of osteoclast precursors (RANK, OSCAR and TREM-2) were explored by RT-qPCR and western blot analysis, respectively. The results demonstrated that treatment with M-CSF and RANKL exerted a stimulative effect on the mRNA expression levels of CTSK, TRAP, ITGB3, RANK and OSCAR in a time-dependent manner (Fig. 4A). By contrast, these enhancements were dramatically repressed following rSj-Cys intervention (Fig. 4A). These observations were identical to the findings from western blot analysis at 72 h (Fig. 4B). Notably, there were no evident variations in both mRNA and protein expression levels of TREM-2 during the entire induction period (Fig. 4A and B). The present results indicated that rSj-Cys inhibited osteoclast differentiation by regulating the expression of osteoclast phenotype markers and cell surface receptors of osteoclast precursors, with the exception of TREM-2.

rSj-Cys represses the M-CSF and RANKL-induced NF- κ B signaling pathway during early-phase osteoclastogenesis. The NF- κ B signaling pathway serves a critical role in the early phase of osteoclastogenesis and is responsible for the activation of crucial downstream transcription factors, namely NFATc1 and c-Fos, which are master regulators of

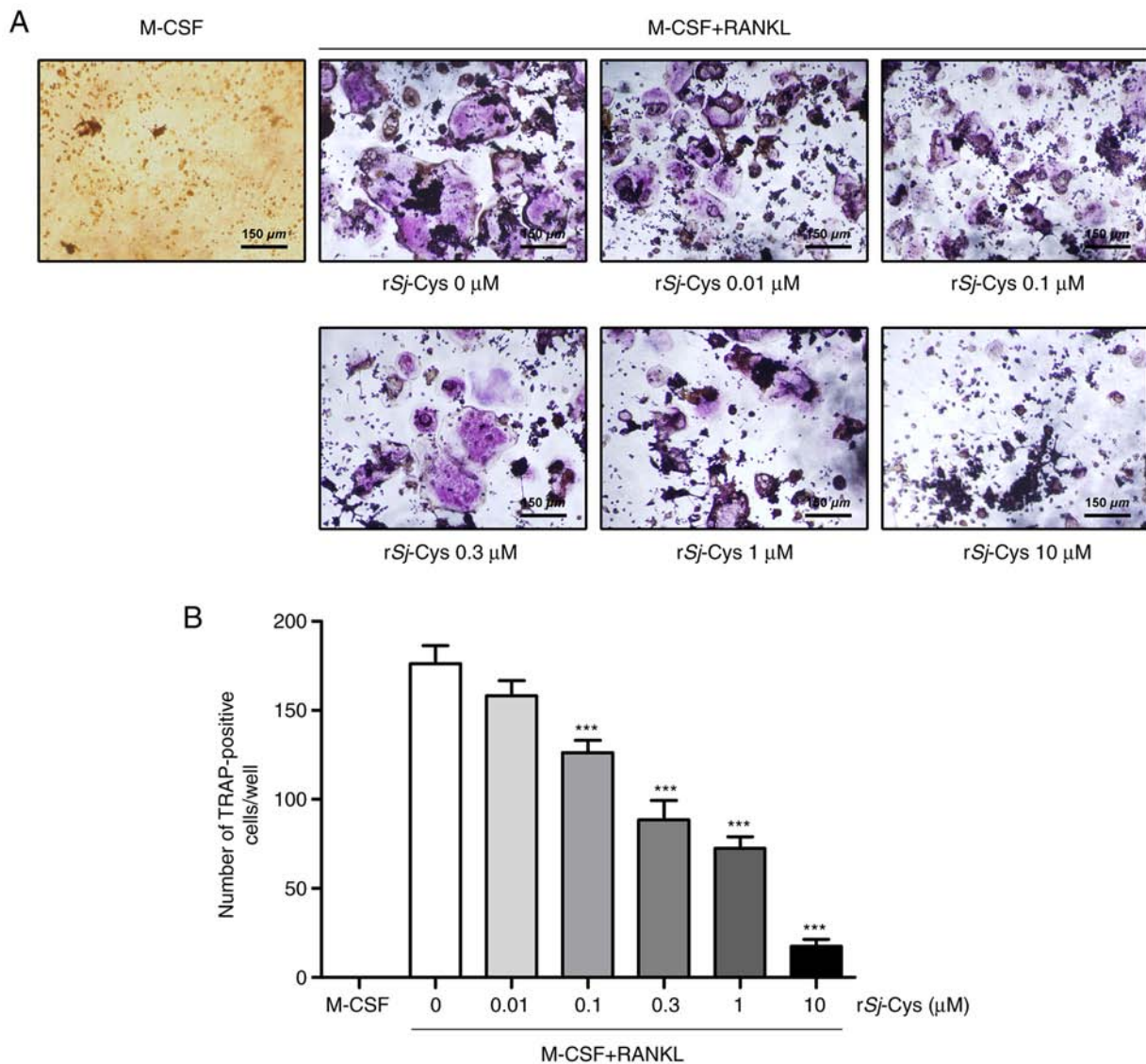


Figure 1. rSj-Cys suppresses M-CSF and RANKL-induced osteoclastogenesis in a dose-dependent manner *in vitro*. (A) RAW264.7 cells were incubated with either M-CSF (25 ng/ml) alone, or with M-CSF and RANKL (30 ng/ml) in the absence or presence of rSj-Cys (0, 0.01, 0.1, 0.3, 1 or 10 μ M) for 5 days, and then TRAP staining was performed. TRAP-positive multinucleated cells were visualized under an inverted microscope. Original magnification, $\times 100$. (B) The numbers of TRAP-positive multinucleated cells were counted and the IC_{50} of rSj-Cys was found to be 0.3 μ M. Data are presented as mean \pm SD of three independent experiments. *** $P < 0.001$ vs. M-CSF + RANKL group. rSj-Cys, recombinant *Schistosoma japonicum* cystatins; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF- κ B ligand; TRAP, tartrate resistant acid phosphatase; IC_{50} , half-maximal inhibition concentration.

osteoclast differentiation. Hence, the mRNA and protein expression levels of NF- κ B-associated signaling molecules (I κ B α and p65) and of NFATc1 and c-Fos were assessed by RT-qPCR and western blot analysis, respectively, in order to explore the underlying molecular mechanisms of the antiosteoclastogenic effects of rSj-Cys. As presented in Fig. 5A, the mRNA expression levels of I κ B α , p65, NFATc1 and c-Fos were significantly upregulated upon exposure to M-CSF and RANKL over time, whereas these increases were significantly inhibited by rSj-Cys intervention. These results suggested that rSj-Cys might suppress the NF- κ B signaling pathway by downregulating the mRNA expression levels of I κ B α and p65 and might further impact levels of NFATc1 and c-Fos. This was consistent with the findings from western blot analysis at 24 h (Fig. 5B). The present results indicated that rSj-Cys may neutralize early-phase osteoclastogenesis primarily by regulating the NF- κ B signaling pathway.

Discussion

Excessive bone resorption due to the abnormal osteoclastic activation and massive secretion of cysteine proteinases is one of the characteristics of bone metabolic disorders; and as such, reducing the number of osteoclasts and/or constraining osteoclast function may be promising approaches for treatment and prevention of osteolytic destruction. It is generally known that cysteine proteinases, especially cathepsin K, act a predominant role in osteoclastic bone matrix degradation (29). To this end, cystatins, as natural antagonists of these enzymes, have garnered extensive attention. Although multiple studies have indicated that several mammalian cystatins, such as cystatin B, C and D, effectively inhibit osteoclast differentiation and formation, the effects of parasite cystatins on osteoclastogenesis have not been reported to date (26,30). The present study explored the potential role of rSj-Cys and

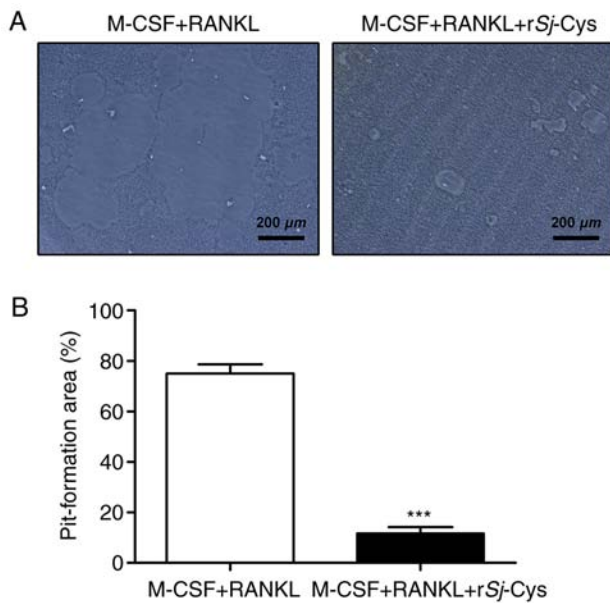


Figure 2. rSj-Cys decreases the bone resorptive capability of osteoclasts. (A) RAW264.7 cells were plated onto the COAS and cultured with M-CSF (25 ng/ml) and RANKL (30 ng/ml) in absence or presence of rSj-Cys (0.3 μM) for 5 days. The area of pit formation generated by osteoclasts was observed on the COAS bottom under an inverted microscope. Original magnification, x100. (B) Quantitative analysis of pit-formation area by ImageJ software. Data are presented as the mean ± SD of three independent experiments. ***P<0.001 vs. M-CSF + RANKL group. rSj-Cys, recombinant *Schistosoma japonicum* cystatins; COAS, Corning Osteo Assay Surface; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand.

its regulatory mechanism on M-CSF and RANKL-induced osteoclast differentiation of RAW264.7 cells.

Currently, cystatins have been discovered in various types of parasite species and mostly belong to the type II class (31). Despite the numerous studies on cystatins in a broad range of organisms, there have been few studies on these molecules from *S. japonicum*, especially type I class. Sj-Cys has an inhibitory effect on the proteolytic activity of papain, primarily owing to the similarity between their conserved domains and type I cystatins (23). However, the precise effect of Sj-Cys on the formation and activation of osteoclasts remains unknown, which motivated our team to conduct the presents study. Based on previous research and data, it was hypothesized that Sj-Cys might inhibit osteoclastogenesis and block the activity of cysteine proteinases to protect against excessive bone resorption.

TRAP is generally utilized as an osteoclast phenotypic marker because of its specific expression in osteoclasts (32). The present study established an osteoclast model using RAW264.7 cells that were induced by M-CSF and RANKL stimulation, and then evaluated osteoclast formation by TRAP staining, which is a standard method to identify mature osteoclasts (TRAP-positive cells). Functionally, osteoclasts possess the unique role of bone resorption; hence, a bone resorption assay was conducted to measure pit-formation ability. The results of these assays demonstrated that rSj-Cys dose-dependently decreased the number of TRAP-positive cells, with an IC₅₀ value of 0.3 μM. In terms of the effect of rSj-Cys on bone resorption, the results showed that rSj-Cys

could significantly reduce the area of resorption pits formed by osteoclasts. Altogether, the present study suggested that rSj-Cys inhibited osteoclast formation and impaired the bone resorptive capability of osteoclasts.

During osteoclastogenesis, M-CSF is crucial for the proliferation and survival of osteoclast precursors and exhibits an antiapoptotic function by regulating the expression of Bcl-2 (33,34). In the present study, results of the MTT colorimetric assay and RT-qPCR both demonstrated that rSj-Cys did not show cytotoxicity accompanied by a decrease in cell viability or the promotion of apoptosis in RAW264.7 cells.

Expression of RANK in osteoclast precursors is induced by M-CSF, and the RANK/RANKL system is critical for osteoclast differentiation and activation (6). The binding between RANK and RANKL triggers the recruitment of c-Fos, and subsequent stimulation directly activates the expression of NFATc1 (3). Previous studies have indicated that NFATc1 and c-Fos are master transcription factors that regulate osteoclast differentiation by initiating the transcription of downstream targets of osteoclastogenesis-related genes (5). Osteoclast differentiation also requires costimulatory signals induced by immunoglobulin-like receptors, including TREM-2 and OSCAR. These cell surface receptors that are expressed by osteoclast precursors are associated with adaptor proteins, such as DNAX-activating protein 12 or Fc receptor common γ-chain, and their activation leads to the amplification and translocation of NFATc1 (10). In the present study, the results demonstrated that rSj-Cys dramatically repressed the expression of osteoclast phenotype markers (CTSK, TRAP, and ITGB3) and cell surface receptors of osteoclast precursor (RANK and OSCAR) at both the mRNA and protein levels. These findings suggested that the inhibition of osteoclastogenesis caused by rSj-Cys may be relevant to the downregulation of osteoclastogenic genes and proteins.

Furthermore, the activation of c-Fos and NFATc1 is a downstream event in the NF-κB signaling pathway, which acts an essential role in the early phase of osteoclast development (35). Following stimulation with RANKL, phosphorylated IκBα is degraded and releases p65, which is translocated into the nucleus and initiates the transcription of related genes (36). In the present study, the mRNA and protein expression levels of NF-κB-associated signaling molecules (IκBα and p65), and of downstream targets (c-Fos and NFATc1), were down-regulated by rSj-Cys. These findings suggested that rSj-Cys not only suppresses the NF-κB pathway, but also impacts the expression of its downstream transcription factors c-Fos and NFATc1 during early-phase osteoclastogenesis.

Taken together, the present study is the first to shed light on the inhibitory effects of rSj-Cys on M-CSF and RANKL-induced osteoclast differentiation using RAW264.7 cells. rSj-Cys downregulated the expression of osteoclastogenesis-related genes and proteins, thereby restraining osteoclast formation and impairing the bone resorptive capability of osteoclasts. Furthermore, the repression of rSj-Cys did not rely on effects on cell viability or apoptosis, but rather functioned by interfering with the M-CSF and RANKL-induced NF-κB signaling during early-phase osteoclastogenesis. Nevertheless, there are limitations of the present study. The main limitation is that the inhibitory effects and regulatory mechanisms of rSj-Cys in osteoclastogenesis

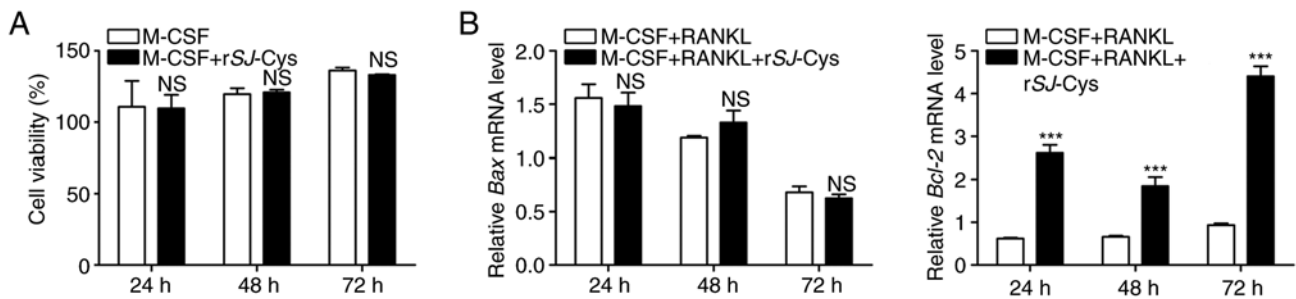


Figure 3. Effects of rSj-Cys on the survival of RAW264.7 cells. (A) RAW264.7 cells were treated with M-CSF (25 ng/ml) in the absence or presence of rSj-Cys (0.3 μ M), and MTT colorimetric assays were conducted to measure cell viability at 24, 48 or 72 h. (B) RAW264.7 cells were co-stimulated with M-CSF (25 ng/ml) and RANKL (30 ng/ml) and then treated with or without rSj-Cys (0.3 μ M) for the indicated amounts of time. The mRNA expression levels of the proapoptotic gene Bax and the antiapoptotic gene Bcl-2 were assessed by reverse transcription-quantitative PCR. The β -Actin housekeeping gene was utilized as an internal control. Data are presented as the mean \pm SD of three independent experiments. *** P <0.01 vs. M-CSF + RANKL group. rSj-Cys, recombinant *Schistosoma japonicum* cystatins; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF- κ B ligand; NS, no significance.

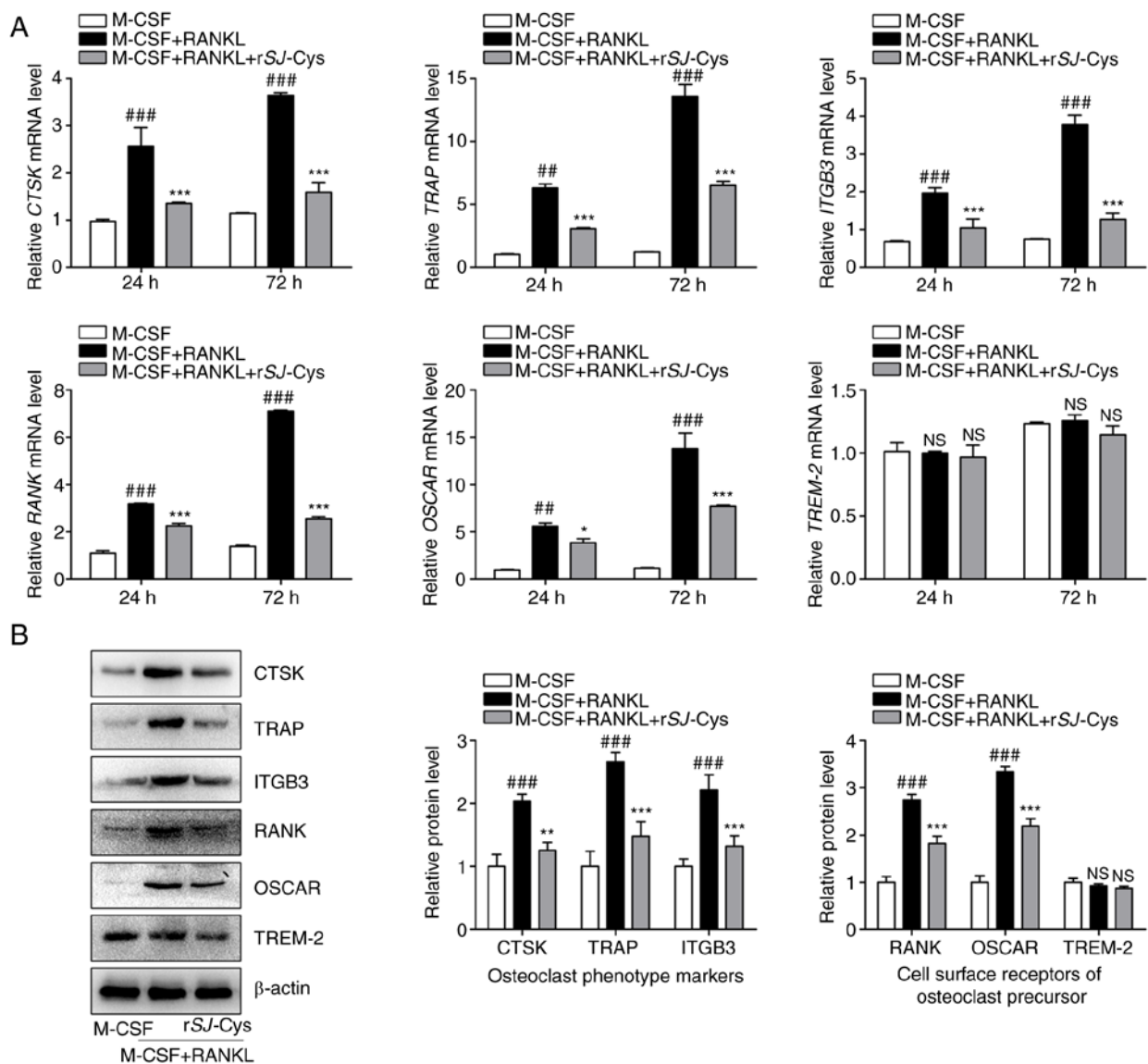


Figure 4. rSj-Cys inhibits the expression of osteoclastogenesis-related genes and proteins. RAW264.7 cells were co-stimulated with M-CSF (25 ng/ml) and RANKL (30 ng/ml) and then treated with or without rSj-Cys (0.3 μ M) for different time periods (24, 48 or 72 h). The expression levels of genes and proteins associated with osteoclast phenotype markers (CTSK, TRAP, ITGB3) and cell surface receptors of osteoclast precursors (RANK, OSCAR, TREM-2) were determined by (A) reverse transcription-quantitative PCR and (B) western blot analysis. Band intensity was semi-quantified with ImageJ software and normalized against β -actin levels. Data are presented as the mean \pm SD of three independent experiments. ** P <0.01, *** P <0.001 vs. M-CSF alone group; * P <0.05, ** P <0.01, *** P <0.001 vs. M-CSF + RANKL group. rSj-Cys, recombinant *Schistosoma japonicum* cystatins; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF- κ B ligand; CTSK, cathepsin K; TRAP, tartrate resistant acid phosphatase; ITGB3, integrin β 3; OSCAR, osteoclast-associated receptor; TREM-2, triggering receptor expressed on myeloid cells 2; NS, no significance.

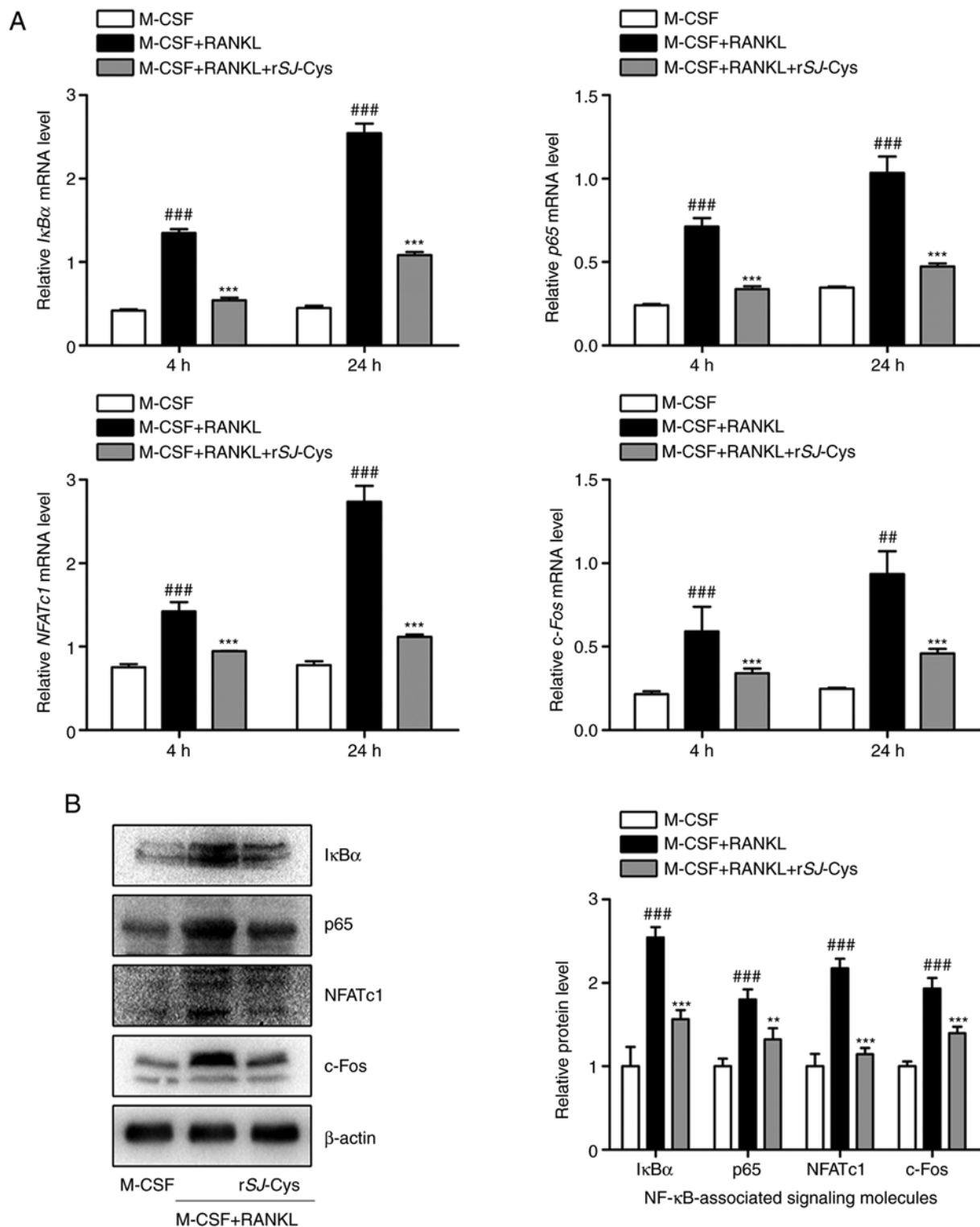


Figure 5. rSj-Cys represses M-CSF and RANKL-induced NF-κB signaling during early-phase osteoclastogenesis. RAW264.7 cells were co-stimulated with M-CSF (25 ng/ml) and RANKL (30 ng/ml) and then treated with or without rSj-Cys (0.3 μM) for different time periods (4 or 24 h). The mRNA and protein expression levels of NF-κB-associated signaling molecules *IkBα* and *p65*, and of downstream targets *NFATc1* and *c-Fos*, were assessed by (A) reverse transcription-quantitative PCR and (B) western blot analysis. Band intensity was semi-quantified using the ImageJ software and normalized against β-actin levels. Data are presented as mean ± SD of three independent experiments. ##*P*<0.01, ###*P*<0.001 vs. M-CSF alone group; **P*<0.01, ****P*<0.001 vs. M-CSF + RANKL group. rSj-Cys, recombinant *Schistosoma japonicum* cystatins; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand; *IkBα*, NF-κB inhibitor α; *p65*, RELA proto-oncogene NF-κB subunit; *NFATc1*, nuclear factor of activated T-cells cytoplasmic 1; *c-Fos*, Fos proto-oncogene AP-1 transcription factor subunit.

were examined in only one cell line *in vitro*; thus, additional *in vivo* investigations will be required to fully understand its specific functions. Despite this, the present findings suggested

that rSj-Cys, as an inhibitor of osteoclast differentiation, may serve as a prospective biotherapeutic candidate for the treatment and prevention of bone metabolic disorders.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

YC, BW, PX, HT, LY and YW performed the experiments, contributed to literature search, analyzed the data and wrote the manuscript. XY, YF and YM contributed to the conception and design of the study and reviewed the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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