



Research Article

SR-A neutralizing antibody: potential drug candidate for ameliorating osteoclastogenesis in rheumatoid arthritis

Yang Xie¹ , Xiang Jiang^{1,2}, Ping Wang¹, Xi Zheng^{1,2}, Jing Song^{1,2}, Mingxin Bai¹ , Yundi Tang¹, Xiangyu Fang¹, Yuan Jia¹, Zhanguo Li^{1,2,3} and Fanlei Hu^{1,3,4,*}

¹Department of Rheumatology and Immunology, Peking University People's Hospital & Beijing Key Laboratory for Rheumatism Mechanism and Immune Diagnosis (BZ0135), Beijing, China

²Peking-Tsinghua Center for Life Sciences, Peking University, Beijing, China

³State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing, China

⁴Department of Integration of Chinese and Western Medicine, School of Basic Medical Sciences, Peking University, Beijing, China

*Correspondence: Fanlei Hu, Department of Rheumatology and Immunology, Peking University People's Hospital, 11 Xizhimen South Street, Beijing 100044, China. Email: fanleihu@bjmu.edu.cn

Abstract

Rheumatoid arthritis (RA) is an autoimmune disease characterized by proliferative synovitis with deterioration of cartilage and bone. Osteoclasts (OCs) are the active participants in the bone destruction of RA. Although with great advances, most current therapeutic strategies for RA have limited effects on bone destruction. Macrophage scavenger receptor A (SR-A) is a class of pattern recognition receptors (PRRs) involved in bone metabolism and OC differentiation. More recently, our study revealed the critical role of SR-A in RA diagnosis and pathogenesis. Here, we further demonstrated that serum SR-A levels were positively correlated with bone destruction in patients with RA. Anti-SR-A neutralizing antibodies significantly inhibited OC differentiation and bone absorption *in vitro* in patients with RA, but not in healthy individuals, dampening the expression of OC-specific genes such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK), and matrix metalloproteinase-9 (MMP-9). Similar results were also seen in collagen-induced arthritis (CIA) mice *in vitro*. Moreover, the anti-SR-A neutralizing antibody could further ameliorate osteoclastogenesis *in vivo* and *ex vivo* in CIA mice, accompanied by decreased serum levels of C-terminal telopeptide and IL-6, exhibiting potential protective effects. These results suggest that blockade of SR-A using anti-SR-A neutralizing antibodies might provide a promising therapeutic strategy for bone destruction in the RA.

Keywords: scavenger receptor A, osteoclastogenesis, rheumatoid arthritis

Abbreviations: ACPA: anti-citrullinated protein antibodies; BMMs: bone marrow-derived monocytes; CFA: complete Freund adjuvant; CIA: collagen-induced arthritis; CTSK: cathepsin K; IFA: incomplete Freund adjuvant; JSN: joint space narrowing; MMP-9: matrix metalloproteinase-9; NSAIDs: nonsteroidal anti-inflammatory drugs; OCs: osteoclasts; PB: peripheral blood; PBMCs: peripheral blood mononuclear cells; PRRs: pattern recognition receptors; RA: rheumatoid arthritis; RF: rheumatoid factor; SHS: Sharp/van der Heijde score; SMLs: small-molecule inhibitors; SR-A: scavenger receptor A; TRAP: tartrate-resistant acid phosphatase

Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease with the typical characteristic of periarticular bone erosion [1]. The inflammation can lead to synovium proliferation, bone destruction, deformity, and function decline [2]. In 1971, Sharp *et al.* proposed a system including the observation and scoring of hands and wrists in patients with RA to assess structural change [3]. Van Der Heijde further modified the Sharp score for a better description of bone destruction, including bone erosion score and joint space narrowing (JSN) score [4]. Bone erosion is a critical outcome indicator in RA, predicting a more severe course of disease with a higher degree of disability [5].

Osteoclasts (OCs) are the main active participants in RA bone destruction [6]. They are multinucleated cells derived from hematopoietic precursors of the myeloid lineage with

the potential of bone resorption. In RA, the generation, differentiation, and activation of OCs are increased as induced by proinflammatory cytokines such as TNF- α and IL-6 [7], and autoantibodies such as anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF) [8], resulting in bone resorption and destruction. Therefore, inhibiting the formation and functions of OCs are ideal and effective approaches for RA targeted therapy. Although several inhibitors have been developed, including bisphosphonates [9], denosumab [10], and natural compounds such as xylitol [11], till now there are few therapeutic drugs targeting OCs available. Current drugs for RA treatment mainly consist of nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids (GC), and disease-modifying antirheumatic drugs (DMARDs) including conventional DMARDs (cDMARDs), biological DMARDs (bDMARDs), and targeted synthetic DMARDs

(tsDMARDs). Although with great advances, their effects on inhibiting OCs and preventing joint and bone destruction are insufficient with limitations.

Macrophage scavenger receptor A (SR-A, CD204, MSR-1, SCARA1) is a kind of classical pattern recognition receptors (PRRs) primarily expressed on macrophages [12]. Recent studies have further indicated its critical involvement in T- and B-cell immune responses [13, 14]. Besides cancer, cardiovascular disease, and Alzheimer's disease, the role of SR-A in autoimmune diseases, such as systemic lupus erythematosus, autoimmune hepatitis, and inflammatory bowel diseases have also been recognized [15]. Moreover, compared to WT mice, SR-A^{-/-} mice revealed increased bone mass, bone thickness, bone density, and trabeculae number, which suggested the role of SR-A in bone metabolism and OC differentiation [16]. SR-A was then demonstrated to be able to directly promote OC differentiation [17]. SR-A may also activate the ERK and JNK signaling pathways and increase IL-6 production to further stimulate the formation of OCs [18]. In our previous large-scale, multicenter study, we unveiled a significant elevation of soluble SR-A (sSR-A) in the patient serum with RA, serving as a potential diagnostic marker [19]. Moreover, our group revealed that SR-A^{-/-} mice were resistant to collagen-induced arthritis (CIA). Administration of SR-A recombinant protein exacerbated the incidence and progression of CIA, while SR-A neutralizing antibody ameliorated the severity of arthritis [19]. However, the therapeutic potential of SR-A blockade against bone destruction remains to be elucidated.

In this study, we aimed to characterize the correlation between sSR-A and bone destruction in RA and reveal the capacity of SR-A neutralizing antibody in inhibiting OC differentiation, trying to provide a potential approach for RA bone destruction therapy.

Materials and methods

Patients and samples

Serum samples were obtained from 80 patients with RA as well as 60 age- and sex-matched healthy volunteers, and peripheral blood (PB) samples were obtained from six patients with RA. All patients fulfilled the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification diagnostic criteria for RA. The detailed characteristics of patients with RA are provided in Table 1. The study was approved by Research Ethics Committee at Peking University People's Hospital, Beijing, China. All patients and healthy donors provided informed consent.

Radiological damage of patients with RA was evaluated using Sharp-van der Heijde (SHS) total score as reported previously [4].

Mice

Six to eight-week-old male DBA/1 mice were purchased from Huafukang Bioscience Company (Beijing, China). All mice were housed in a specific pathogen-free environment under controlled conditions (22 °C ambient temperature, 40% humidity). All animal procedures complied with relevant ethical regulations for animal research and were approved by the Institutional Animal Care and Use Committee (IACUC) of Peking University People's Hospital.

Table 1. Clinical and demographic characteristics of patients with RA

Characteristics	Patients with RA and with radiographic images (n = 80)
Age, mean (range), yrs	59 (26–84)
Gender, female/male	66/14
Duration, median (range), yrs	10 (0.05–40)
ESR, median (range), mm/h	48 (6–129)
CRP, median (range), mg/l	16.2 (1–243)
ACPA, no. positive/no. negative/no. nd	67/12/1
RF, median (range), IU/ml	139.5 (20–7330)
Tender joint count of 28 joints, median (range)	6 (0–26)
Swollen joint count of 28 joints, median (range)	4 (0–26)
DAS28, mean (range)	5.24 (2.39–8.21)
SHARP score, median (range)	82 (2–280)
Bone erosion score, median (range)	44 (0–160)
Joint space narrowing score, median (range)	44 (1–120)
Medication, no. (%)	
Steroids	19 (23.75)
NSAIDs	31 (38.75)
Methotrexate	24 (30)
Other DMARDs	54 (67.5)
Biologics	4 (5)
No treatment	8 (10)

Abbreviations: ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; nd: not detected; DAS28: disease activity score 28.

ELISA analyses

The serum levels of sSR-A in patients with RA and healthy individuals were detected by ELISA as reported previously [19], using a commercially available human SR-A ELISA kit (Sino Biological Inc., Beijing, China). The levels of IL-6 in CIA mouse serum and the cell culture supernatant of patients with RA were detected by ELISA kit from Neobioscience (Beijing, China), while the levels of C-terminal telopeptide in CIA mouse serum were detected by ELISA kit from Chondrex (Redmond, WA). The results were obtained on a Synergy™ 4 Multi-Mode Microplate Reader with software GEN5CH 2.0 (BioTek, Winooski, VT).

CIA induction and treatment

Collagen-induced arthritis (CIA) models were established in DBA/1 mice by immunization on Day 1 with 200 µg bovine type II collagen (Chondrex Inc., Redmond, WA) emulsified in complete Freund adjuvant (CFA), and on Day 21 with 100 µg bovine type II collagen emulsified in incomplete Freund adjuvant (IFA) as a booster. The severity of arthritis was scored based on the level of inflammation as described previously [19].

After the arthritis initiation, the mice with similar scores of 2–3 were randomly divided into two groups. As reported in previous studies concerning blocking antibodies, the experimental group mice were intravenously administered with anti-mouse SR-A neutralizing antibodies (AF1797, R&D Systems, Minneapolis, MN; 2 µg/mice in 200 µl PBS), while the control group mice were intravenously administered

with isotype control IgG every other day for a total of five times. The mice were sacrificed on Day 12 after the first treatment for further analysis.

OC differentiation

For human OC differentiation *in vitro*, freshly isolated PB mononuclear cells (PBMCs) from patients with RA or healthy individuals were suspended in complete α -MEM containing 10% FBS and planted in a 96-well plate at a density of 1×10^5 cells/well. After culturing for 4 h, the non-adherent cells were removed, remaining the adherent OC precursors. Then the cells were co-cultured with 2 μ g/ml SR-A neutralizing antibody or isotype antibody along with RANKL (100 ng/ml) and M-CSF (50 ng/ml). Sometimes, the cells were co-cultured with RA serum pre-incubated with SR-A neutralizing antibody or isotype antibody in the presence of RANKL and M-CSF. The medium was changed every 3 days, and on Day 14, OC formation was measured through TRAP staining with leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, MO). TRAP-positive multinucleated cells (MNCs) containing three or more nuclei in the entire single well of 96-well plate were observed and counted under an inverted fluorescence microscope (Olympus IX71-141, Tokyo, Japan).

For mouse OC differentiation *in vitro*, primary bone marrow cells (BMMs) from CIA or naïve mice were cultured in a 96-well plate at a density of 1×10^5 cells/well for 3 days in complete α -MEM with 10% FBS and 25 ng/ml M-CSF. Then non-adherent cells were discarded and the adherent cells were further cultured in the presence of 50 ng/ml RANKL, 25 ng/ml M-CSF, 2 μ g/ml SR-A neutralizing antibody or isotype antibody. Six days later, tartrate-resistant acid phosphatase (TRAP) staining was performed for OC detection.

For mouse OC differentiation *ex vivo*, BMMs from CIA mice or naïve mice treated with SR-A neutralizing antibody or isotype antibody as described above were collected for OC formation accordingly.

Bone resorption assay

peripheral blood mononuclear cells (1×10^5 cells/well) from patients with RA were performed for OC differentiation as described above in 96-well plates coated with bovine cortical bone slices (6 mm in diameter and approx. 200 μ d thick, IDS, Boldon, UK). On Day 14, the bone slices containing the OCs were collected and washed with distilled water. Then, the resorption pits were visualized by staining with 1% toluidine blue and detected under a polarized light microscope (Olympus BX51-P, Tokyo, Japan). The results were expressed as the area of resorption lacunae in the total plate area.

Mouse paw histopathology and micro-CT analyses

Mice were sacrificed and the hind paws were fixed in 4% buffered formaldehyde. The tissues were collected, fixed, paraffin-embedded, sectioned, stained with TRAP, and analyzed with NDP.view2 (Hamamatsu Photonics K.K., Japan).

Micro-CT images of the mouse paws were acquired on the Tri-Modality FLEX Triumph™ Pre-Clinical Imaging System (Gamma Medica-Ideas, Northridge, CA). CT image set

acquisitions lasted 10 min and utilized beam parameters of 130 μ A and 80 kVP. Analyze 10.0 (AnalyzeDirect, Overland Park, KS) was used to perform the image analysis.

RT-PCR and qPCR

RNA extraction, reverse transcription, and qPCR analyses were performed as described previously [20, 21]. Briefly, after the formation of OCs as described above, total RNA was extracted by RNAsimple Total RNA kit (Tiangen, Beijing, China), and reverse transcription was performed with the reverse transcriptase kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Then, qPCR was performed to analyze the OC-specific gene expression using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The primers used were as follows:

Human GAPDH sense primer: 5'-AAGGTGAAGGTCGGAGTCAA-3', antisense primer: 5'-AATGAAGGGTTCATTGATGG-3';

Human TRAP sense primer: 5'-GATCCTGGGTGCAGACTTCA-3', antisense primer: 5'-GCGCTTGGAGATCTTAGAGT-3';

Human CTSK sense primer: 5'-ACCGGGGTATTGACTCTGAA-3', antisense primer: 5'-GAGGTCAGGCTTGCATCAAT-3';

Human MMP-9 sense primer: 5'-CGCTACCACCTCGAACTTTG-3', antisense primer: 5'-GCCATTCACGTCGTCCCTAT-3'.

Mice GAPDH sense primer: 5'-GGTGAAGGTCGGTGTGAACG-3', antisense primer: 5'-CTCGCTCCTGGAAGATGGTG-3';

Mice TRAP sense primer: 5'-CGACCATTGTTAGCCACATACG-3', antisense primer: 5'-TCGTCCTGAAGATACTGCAAGTT-3';

Mice CTSK sense primer: 5'-GCTTGGCATCTTTCCAGTTTAC-3', antisense primer: 5'-TATCCAGTGCTTGCTTCCCCTCT-3';

Mice MMP-9 sense primer: 5'-CGTGTCTGGAGATTCCGACTTGA-3', antisense primer: 5'-TTGGAAACTCACAGCCAGA-3'.

Statistical analysis

GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA) was used for statistical analysis. Differences between various groups were evaluated by the Spearman's rank correlation test, Mann-Whitney *U* test, Wilcoxon matched-paired signed-rank test, paired Student's *t*-test, and one-way ANOVA test. All data are expressed as mean \pm SEM. A confidence level above 95% ($P < 0.05$) was considered statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant).

Results

Elevated serum sSR-A correlates with bone destruction in patients with RA

A previous study by our group revealed the diagnostic value of sSR-A and its pathogenic activity in RA. In the present study, we further analyzed the correlation between sSR-A and bone destruction in patients with RA. Serum samples from patients with RA with radiographic images ($n = 80$)

and healthy controls (HC, $n = 60$), were collected for detection of the levels of sSR-A. The result showed that sSR-A in patients with RA was significantly higher than that in HC (median 2.906 ng/ml, mean 6.006 ng/ml, SD 8.441 ng/ml, $***P < 0.001$, Fig. 1A). Sharp/van der Heijde score (SHS) was then performed and simple linear regression showed that the level of sSR-A positively correlated with the SHARP score (Fig. 1B). Further analysis showed that the serum sSR-A level in patients with RA correlated positively with both erosion score and JSN score (Fig. 1C, D).

SR-A neutralizing antibody inhibits OC differentiation and bone destruction in patients with RA

We first tried to reveal the potential involvement of sSR-A in RA bone destruction. As shown in Fig. 2, treatment of healthy individual monocytes with serum of the patient with RA fundamentally stimulated OC formation *in vitro*. However, the stimulatory effects of serum of the patients with RA were aborted when pre-incubated with SR-A neutralizing antibody, which indicated the role of sSR-A in osteoclastogenesis.

We then examined the effects of blockade of SR-A on osteoclastogenesis in patients with RA. Monocytes from patients with RA and healthy individual peripheral blood were cultured with SR-A neutralizing antibody or isotype antibody for OC differentiation. Tartrate-resistant acid phosphatase (TRAP) staining showed that compared with isotype antibody, SR-A neutralizing antibody could substantially inhibit the OC formation and decrease the number of OCs in patients with RA (Fig. 3A). Nevertheless, only a minimal inhibitory effect of SR-A neutralizing antibody on OC differentiation was seen in healthy individuals (Fig. 3B), indicating the disease-specific pathologic role of SR-A. Bone resorption assay further showed that SR-A neutralizing antibody could functionally dampen the bone erosion and destruction in patients with RA (Fig. 3C). Moreover, the expression of TRAP, cathepsin K (CTSK), and matrix metalloproteinase-9 (MMP-9), marker genes for OCs, were significantly down-regulated after SR-A neutralizing antibody treatment in RA (Fig. 3D). The levels of IL-6 in the cell culture supernatants were also fundamentally decreased after the treatment (Fig. 3E).

Although SR-A neutralizing antibody blocks both soluble and membrane-bound SR-A, our preliminary results revealed that the expression of membrane-bound SR-A on monocytes

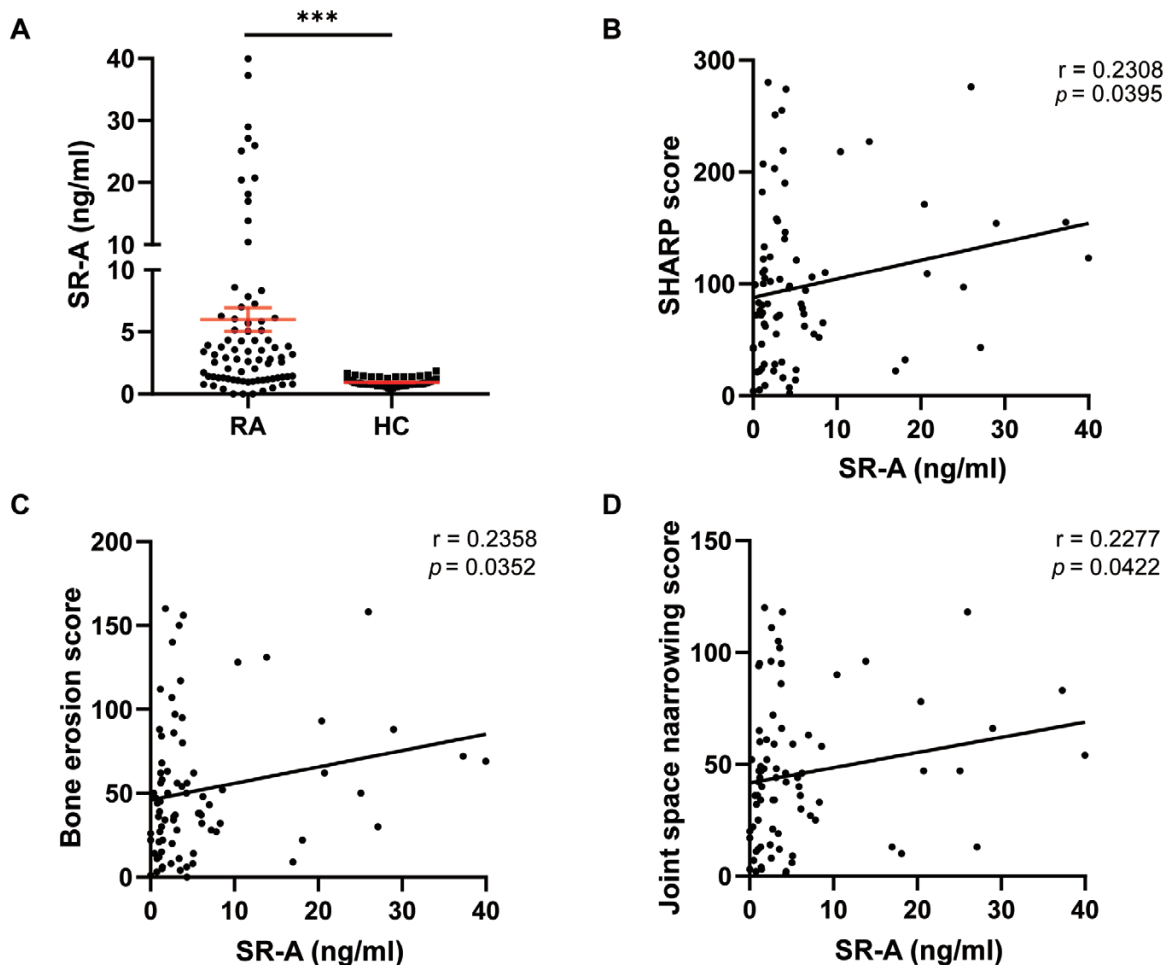


Figure 1. Correlation analysis of the level of sSR-A with bone destruction in patients with RA. (A) The serum level of sSR-A was higher in patients with RA than that in healthy individuals (RA, $n = 80$; HC, $n = 60$; $***P < 0.001$). (B–D) The level of sSR-A in patients with RA was positively correlated with the SHARP score (B; $r = 0.2308$, $*P = 0.0395$), the erosion score (C; $r = 0.2358$, $*P = 0.0352$), and the joint space narrowing score (D; $r = 0.2277$, $*P = 0.0422$). The results are presented as mean \pm SEM (two-tailed Mann–Whitney U test (A) and two-tailed Spearman's rank correlation test (B–D)).

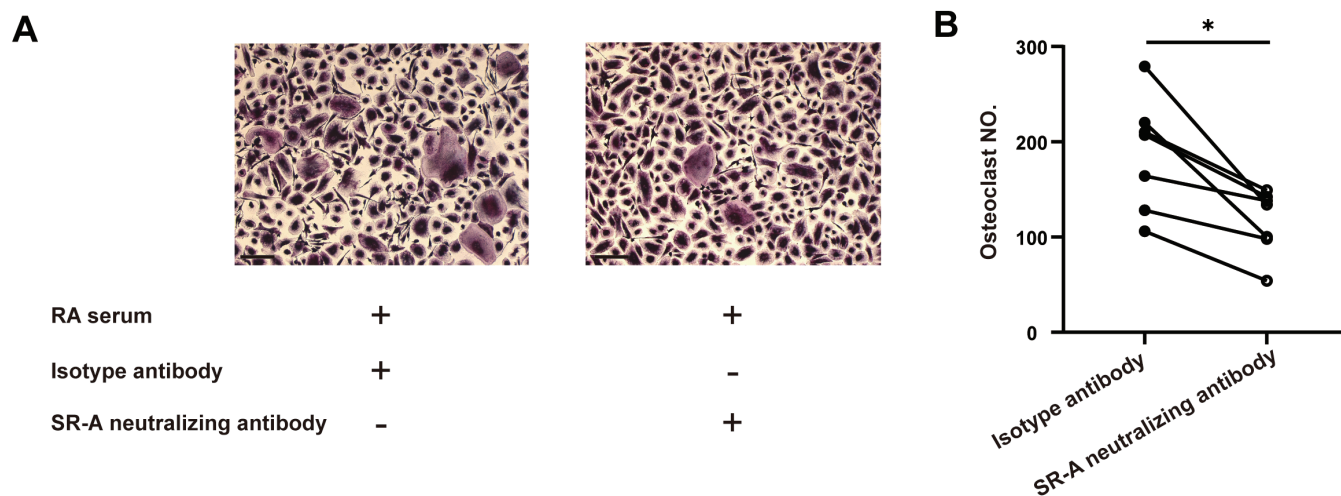


Figure 2. The potential involvement of sSR-A in RA bone destruction. Monocytes from healthy individuals ($n = 6$) were co-cultured with the serum of the patient with RA pre-incubated with 2 $\mu\text{g/ml}$ anti-human SR-A neutralizing antibody or isotype antibody, 100 ng/ml RANKL, and 50 ng/ml M-CSF. After 14 days, the cells were fixed and set for TRAP staining, and multinucleated cells (more than three nuclei) with TRAP positivity were counted. The representative figures (A) and the statistical result (B) were shown, respectively (two-tailed Wilcoxon matched-paired signed-rank test, $*P < 0.05$). Scale bar = 50 μm .

of the patient with RA were significantly decreased as compared with healthy individuals, while the levels of SR-A mRNA as well as soluble SR-A in the serum were significantly elevated (data not shown). Therefore, we speculated that SR-A neutralizing antibodies inhibited osteoclastogenesis in patients with RA mainly through blocking soluble SR-A. Nevertheless, the detailed functions and underlying mechanisms need to be further studied.

SR-A neutralizing antibody dampens OC differentiation in arthritis mice *in vitro*

We next detected the effects of SR-A neutralizing antibody on OC differentiation in collagen-induced arthritis (CIA) mice *in vitro*. Bone marrow-derived monocytes (BMMs) from CIA mice and naïve mice were cultured with SR-A neutralizing antibody or isotype antibody for 9 days in the presence of M-CSF and RANKL. As shown in Fig. 4A, TRAP staining revealed that SR-A neutralizing antibody could markedly alleviate the OC formation in CIA mice. However, only a faintest effect was detected in naïve mice (Fig. 4B), similar to that in healthy individuals. These results further demonstrated the function of SR-A neutralizing antibodies in compromising OC differentiation in RA.

SR-A neutralizing antibody ameliorates osteoclastogenesis in arthritis mice *in vivo*

We further revealed whether blockade of SR-A by SR-A neutralizing antibodies could affect OC formation and bone destruction in CIA mice *in vivo*. Established CIA mice with similar scores of 2–3 was injected with SR-A neutralizing antibody or isotype antibody intravenously once every 2 days for five injections before sacrifice. As shown in Fig. 5A, TRAP staining revealed that SR-A neutralizing antibodies could decrease the number of OCs in CIA mice. Micro-CT imaging further demonstrated that compared with isotype antibody, SR-A neutralizing antibody also significantly alleviated bone destruction (Fig. 5B). Unfortunately, we failed to provide the

quantification result because the equipment we used could not provide parameters for quantification of bone erosions and destruction. Moreover, the serum levels of IL-6 that promote osteoclastogenesis, as well as the serum levels of C-terminal telopeptide, a biomarker for bone remodeling, were significantly decreased in CIA mice receiving SR-A neutralizing antibody treatment (Fig. 5C, D). All these results indicated the function of SR-A neutralizing antibody in ameliorating arthritis severity and osteoclastogenesis *in vivo*.

SR-A neutralizing antibody attenuates OC differentiation in arthritis mice *ex vivo*

To confirm the direct effects of SR-A neutralizing antibody on osteoclastogenesis in CIA mice *in vivo*, BMMs from CIA mice receiving SR-A neutralizing antibody or isotype antibody treatment as described above were harvested for OC differentiation *ex vivo*. TRAP staining showed that the number of OCs in CIA mice treated with SR-A neutralizing antibody was significantly lower than that in CIA mice treated with isotype antibody (Fig. 6A). Moreover, the expression of OC-specific-marker genes, CTSK, TRAP, MMP-9, was also down-regulated after SR-A neutralizing antibody treatment (Fig. 6B).

Taken together, these results revealed the direct function of SR-A neutralizing antibodies in ameliorating arthritis severity and osteoclastogenesis *in vivo*.

Discussion

Our previous study has revealed the diagnostic value and pathogenic activity of SR-A in patients with RA and mice with experimental arthritis [19]. In this study, we further described the positive correlation between sSR-A and bone destruction in patients with RA. Moreover, we revealed the therapeutic potential of blocking SR-A by neutralizing antibodies in ameliorating OCs differentiation and bone destruction both in patients with RA and CIA mice. In addition, SR-A neutralizing antibody down-regulated the expression of OC-specific genes

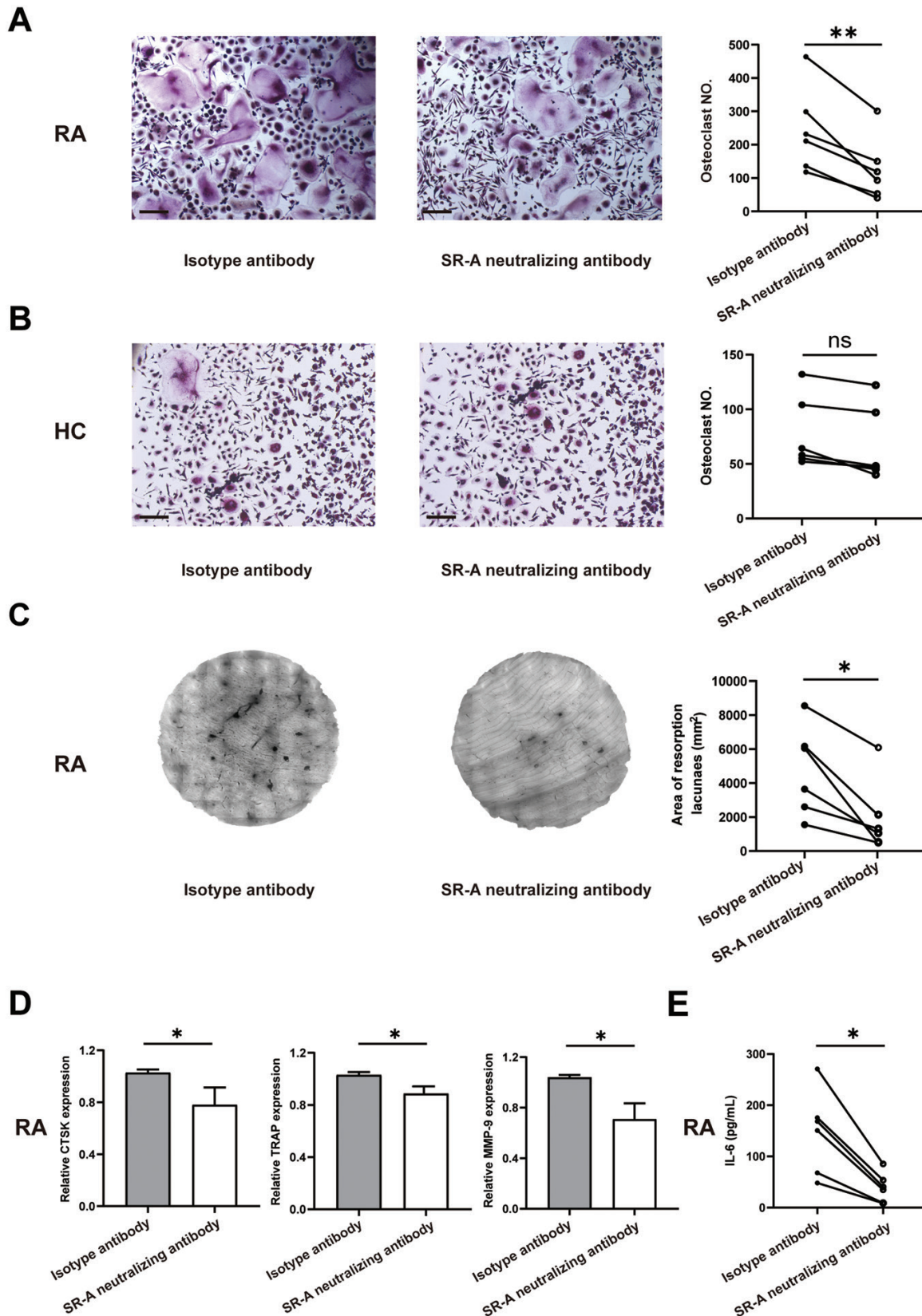


Figure 3. SR-A neutralizing antibody inhibits OC differentiation and bone destruction in patients with RA. (A) Monocytes from patients with RA ($n = 6$) were co-cultured with isotype antibody or anti-human SR-A neutralizing antibody at $2 \mu\text{g/ml}$ for 14 days in the presence of 100 ng/ml RANKL and 50 ng/ml M-CSF. Then the cells were fixed and set for TRAP staining, and multinucleated cells (more than three nuclei) with TRAP positivity were counted. The representative figures and the statistical result were shown, respectively. Scale bar = $50 \mu\text{m}$. (B) SR-A neutralizing antibody showed minimal effects on OC differentiation in healthy individual monocytes. OC differentiation was performed as described above ($n = 6$), and the representative figures and statistical results were shown, respectively. Scale bar = $50 \mu\text{m}$. (C) SR-A neutralizing antibody inhibited bone destruction in patients with RA. OC

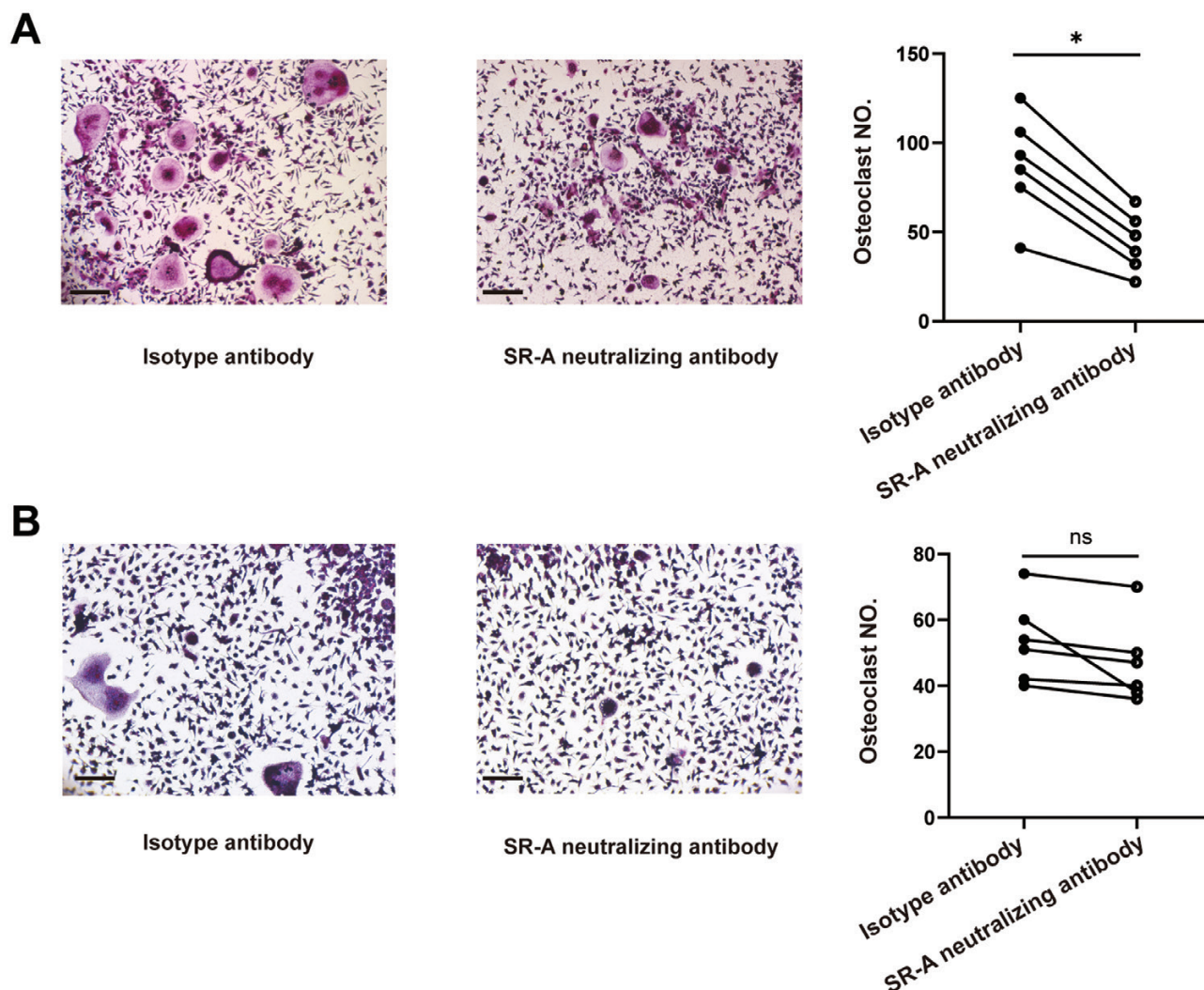


Figure 4. SR-A neutralizing antibody dampens OC differentiation in collagen-induced arthritis (CIA) mice *in vitro*. Bone marrow-derived monocytes (BMMs) from CIA mice (A, $n = 6$) and naïve mice (B, $n = 6$) were co-cultured with isotype antibody or anti-mouse SR-A neutralizing antibody at $2 \mu\text{g/ml}$ in the presence of 100 ng/ml RANKL and 50 ng/ml M-CSF. After 9 days, the cells were fixed for tartrate-resistant acid phosphatase (TRAP) staining. The typical figures and the statistical results were shown, respectively (two-tailed paired Student's t test, $*P < 0.05$, ns, not significant). Scale bar = $50 \mu\text{m}$.

such as *TRAP*, *CTSK*, and *MMP-9*, and attenuated the production of inflammation cytokine IL-6 and bone remodeling biomarker C-terminal telopeptide. Therefore, SR-A neutralizing antibodies may provide a promising strategy for RA therapy especially in dampening bone destruction.

Ideal treatment in RA aims for both reductions of chronic inflammation and structural protection of the impaired bones and joints. However, current therapeutic regimens hardly achieve this perfect goal. Clinical observations indicated that compared with biologic agents, cDMARDs such as methotrexate (MTX), sulfasalazine (SSZ), leflunomide (LEF), and hydroxychloroquine (HCQ) display minimal protective effect on bone destruction in RA [22]. As a bDMARDs, tumor necrosis factor (TNF)-blocking reagents, such as infliximab, could virtually arrest the progression of bone destruction in RA. Emerging studies have provided evidence that TNF- α may

potentiate RANKL-induced OC differentiation, and impair the function of Treg cells which are potential suppressors of OC formation [23]. Moreover, Abatacept, which can mimic the effect of cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), could directly target cell surface receptor CD80/CD86 on precursor cells of OCs [24]. tsDMARDs such as Janus-associated kinase (JAK) inhibitor were also reported to directly affect osteoclastogenesis [25]. But these existing b/tsDMARDs are far from perfect due to the potential adverse effects, such as the higher risk of reactivation of latent tuberculosis and other serious opportunistic infections, malignancy, and the inadequate response in a proportion of patients [26, 27].

In this study, we demonstrated that SR-A neutralizing antibodies could significantly inhibit OC differentiation and bone destruction in patients with RA and CIA mice *in vivo* and *in vitro*. Given the anti-inflammatory effects of blocking SR-A

differentiation was performed as describe above with the bone slices at the bottom of the 96-well plates ($n = 6$). Then the resorption area was stained and analyzed. (D) The differentiated OCs from patients with RA as described in (A) were set for qPCR analysis of the expression of the OC maker genes, including *CTSK*, *TRAP*, and *MMP-9*. (E) The level of IL-6 in the cell culture supernatants of patients with RA was also detected by ELISA. The statistical results are presented as mean \pm SEM (two-tailed Wilcoxon matched-paired signed-rank test, $*P < 0.05$, $**P < 0.01$, ns, not significant).

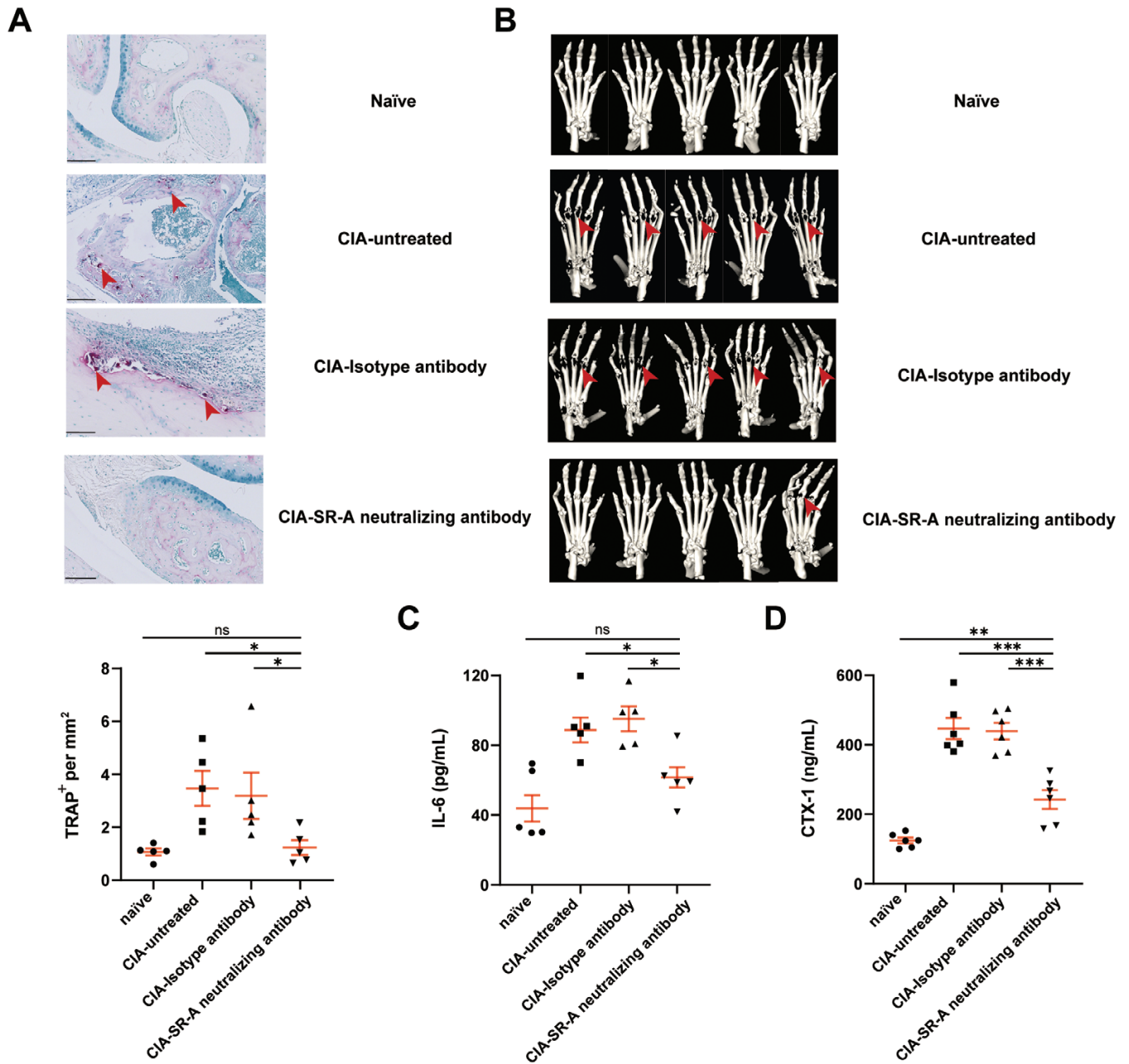


Figure 5. SR-A neutralizing antibody ameliorates osteoclastogenesis in CIA mice *in vivo*. After the initiation of disease, CIA mice with similar scores of 2–3 were injected with anti-mouse SR-A neutralizing antibody or isotype antibody (2 μg/mouse) intravenously every 2 days for a total of five injections (*n* = 5 per group). (A) After sacrifice, the paws from SR-A neutralizing antibody-, or isotype antibody-treated CIA mice, or untreated CIA mice, as well as naïve mice were fixed, paraffin-embedded, sectioned, stained with TRAP, and analyzed by NDP:View2. Arrows indicate osteoclastogenesis. Scale bar = 100 μm. (B) Micro-CT imaging of paws from different group mice as in (A) was also performed. Arrows indicate the bone erosion and destruction. (C–D) The serum levels of IL-6 (C) and C-terminal telopeptide (D) in different group mice were detected by ELISA. The statistical results are presented as mean ± SEM (One-way ANOVA test followed by Dunnett’s posttest for multiple comparisons, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, ns, not significant).

in CIA mice as proved in our previous study [19], SR-A neutralizing antibodies may provide a latent and supplementary strategy for RA therapy with dual protective functions. This also partially explained the fast effect of SR-A neutralizing antibodies administration against osteoclastogenesis observed in the study.

Nevertheless, currently, few SR-A neutralizing antibodies are available. In this study, we used two SR-A neutralizing antibodies from R&D systems, anti-mouse SR-A neutralizing polyclonal antibody (pAb) and anti-human monoclonal antibody (mAb). However, pAbs are not the ideal reagents

for therapeutic application in the clinic. Novel anti-mouse SR-A mAbs for neutralization need to be further designed for therapeutics. Moreover, anti-human SR-A neutralizing antibodies targeting the predominant epitopes, especially the pathogenic domain of soluble SR-A, need to be further developed, since our results indicate that soluble SR-A and membrane-bound SR-A might demonstrate different functions [19]. In addition, further modification of SR-A neutralizing antibodies, such as galactosylation and sialylation that enhances the anti-inflammatory activity, as well as partially or fully humanization that minimizes the immunogenicity,

ex vivo osteoclast differentiation

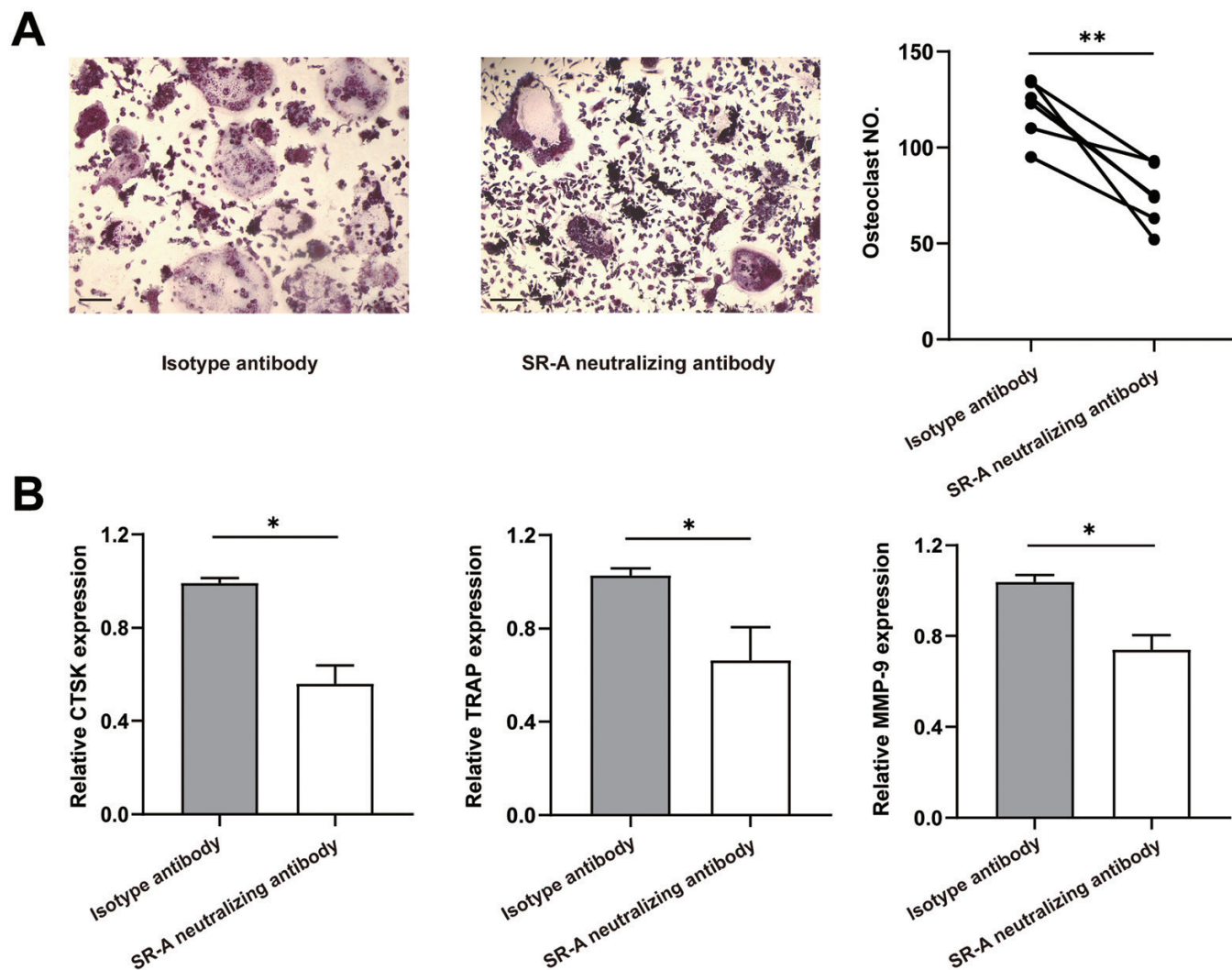


Figure 6. SR-A neutralizing antibody attenuates osteoclastogenesis in CIA mice *ex vivo*. BMMs from CIA mice treated with SR-A neutralizing antibody or isotype antibody as described in Figure 5 were harvested for osteoclast differentiation *ex vivo*. After 9 days, the cells were fixed and set for TRAP staining, and multinucleated cells (more than three nuclei) with TRAP positivity were counted. The typical figures and the statistical result were shown, respectively (A). Scale bar = 50 μm . The relative expression of CTSK, TRAP, and MMP-9 was also evaluated by qPCR (B). The statistical results are presented as mean \pm SEM (two-tailed paired Student's *t* test, * $P < 0.05$, ** $P < 0.01$).

would further facilitate their potential clinical application [28, 29].

Besides SR-A neutralizing antibodies, several other reagents targeting SR-A, including small-molecule inhibitors (SMIs) of SR-A and inhibitory peptides, could also be developed for RA bone destruction treatment. Currently, tannic acid and rhein were identified as SMIs of SR-A [30–32]. However, only tannic acid has been revealed the ability to ameliorate inflammation and joint damage in arthritis mice [30]. Moreover, through phage-displayed peptide library, a novel peptide antagonist of SR-A, PP1, was selected to target the carriers to atherosclerotic aortic artery lesions [33]. Nevertheless, its effect against RA bone destruction remains unclear. Due to their unique mechanism of action and the simultaneous effect on multiple mediators, screening for more specific and competent SMIs and inhibitory peptides may offer promising and supplementary strategies for the treatment of RA inflammation and bone destruction.

In conclusion, here we revealed that the SR-A neutralizing antibodies could inhibit OCs differentiation both in patients with RA and CIA mice, protecting against bone destruction. Developing more specific and optimal SR-A monoclonal antibodies and designing SMIs or inhibitory peptides will provide novel therapeutic strategies for treating bone destruction in RA.

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Conflict of interests

The authors declare that they have no competing interests.

Authors' contributions

Y.X., X.J., P.W., X.Z. performed the experiments and analyzed the data. J.S., M.X.B., Y.D.T., X.Y.F. provided reagents, materials and analysis tools. Y.X. wrote the original draft of the manuscript. Y.J., Z.G.L. and F.L.H. revised the manuscript. F.L.H. designed the study and defined the final version of the manuscript.

Ethical approval

The study protocol was approved by the ethics committees of Peking University People's Hospital.

Data availability

The data underlying this article are available in the article.

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