Association of systemic and intra-articular osteoclastogenic potential, pro-inflammatory mediators and disease activity with the form of inflammatory arthritis

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Abstract

Purpose We aimed to assess osteoclastogenic potential of peripheral blood mononuclear cells (PBMC) and synovial fluid-derived mononuclear cells (SFMC) in different forms of arthritis and to correlate it with inflammatory mediators within intra-articular and circulatory compartments.

Methods Paired PBMC and SFMC samples of patients with rheumatoid arthritis (RA; $n=10$) and psoriatic arthritis (PsA; $n=10$), and PBMC of healthy controls were cultured to assess osteoclastogenic potential by the number of tartrate-resistant

acid phosphatase (TRAP)-positive osteoclasts (OCs) and expression of OC-related genes (receptor activator of nuclear factor- κ B (*RANK*), *cFMS*, and *TRAP*). Osteoclastogenesis was correlated with the arthritis-related inflammatory indicators in serum and synovial fluid (SF).

Results Number of OCs differentiated from PBMC was significantly higher in RA and PsA compared with control, with RA having more OCs compared with PsA. There was no difference in SFMC OC number between arthritic patients, but *RANK* expression in OCs differentiated from SFMC was higher in PsA compared with RA. SF of PsA patients more potently induced OC differentiation from control CD3⁺CD19⁺CD56⁺CD11b⁺CD115⁺ PBMC compared with RA, paralleled with higher *RANK-ligand* expression in PsA SFMC. Positive correlations of OC number with erythrocyte sedimentation rate, serum level of CCL2, and PBMC gene expression of *interleukin-18* and *Fas-ligand* were observed.

Conclusion Osteoclastogenic potential is systemically enhanced in patients with RA, paralleled by disordered systemic and local expression of proinflammatory mediators, whereas PsA involves specific deregulation in RANKL/RANK axis. Our study reveals arthritis-specific mediators associated with the form of arthritis, indicating clinical relevance for diagnosis and treatment.

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Introduction

Inflammatory arthritides comprise a heterogeneous group of joint disorders that are characterized by chronic inflammation, and intra-articular and generalized bone loss due to deregulated

bone homeostasis: bone resorption by osteoclasts (OCs) and bone formation by osteoblasts [1]. Erosions of periarticular bone are the central feature of rheumatoid arthritis (RA) and also occur in spondyloarthritis (SpA) and the erosive form of osteoarthritis [2].

Development of bone erosions is critically dependent on OCs, capable of resorbing the mineralized matrix. OCs are multinuclear cells derived from mononuclear osteoclast progenitors (OCPs) of the monocyte-macrophage lineage, which can be matured in vitro in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL) [1, 3]. OCPs are found in the peripheral blood (PB) and synovial tissue of patients with RA and SpA [4], mediating bone loss locally, in the form of bone erosions and joint osteolysis, and systemically, with the loss of bone mineral density (BMD) [5]. Under pathological conditions associated with arthritis, the process of osteoclastogenesis is markedly enhanced by various osteoclastogenic factors, including proinflammatory cytokines (interleukin (IL)-1, IL-6, IL-17, IL-18, tumor necrosis factor α (TNF- α)), chemokines (such as CC-chemokine ligand 2 (CCL2), CCL3, CCL4), and apoptotic mediators (Fas-ligand (FasL), TNF-related apoptosis-inducing ligand, herpesvirus entry mediator ligand) [6, 7]. It has been shown that even a small rise in the level of systemic inflammation can precipitate osteodestruction, leading to fractures and disabilities related to arthritis [2, 8].

Although the enhanced osteoclastogenesis and osteoresorption have been confirmed in several forms of arthritis, the relative contribution of systemic versus local changes to OC differentiation and activity has not been fully revealed. Moreover, discrimination criteria between forms of arthritis, regarding the mechanisms of osteoclastogenic response and key osteoclastogenic factors, have not been fully defined. The major aim of our study was to correlate the osteoclastogenic potential of peripheral blood mononuclear cells (PBMC) and synovial fluid-derived mononuclear cells (SFMC) with the parameters of systemic inflammation as well as circulating and intra-articular levels of proinflammatory/immunoregulatory mediators. In addition, we aimed to identify disease-specific factors that discriminate between control and arthritic subjects, and are best associated with osteoclastogenic potential, form of arthritis and disease activity.

Patients and methods

Patients

A total of 20 patients with the following diseases: RA ($n=10$) and psoriatic arthritis (PsA) ($n=10$), admitted to the Rheumatology Department of the Clinical Hospital Center “Sisters of Mercy”, were included in the study after obtaining approval from the Ethics Committee and informed consent from patients

(Table 1). The study was conducted in accordance with the Declaration of Helsinki. RA was diagnosed according to the revised American College of Rheumatology (ACR) criteria and PsA according to the Moll and Wright criteria. Paired synovial fluid (SF) and PB samples were obtained from RA and PsA groups, age and sex matched, and were further evaluated using the standard clinical variables (listed in Supplementary table 1) and laboratory parameters (C-reactive protein [CRP; mg/L], measured by standard nephelometric assay and erythrocyte sedimentation rate [ESR; mm/h], determined according to the Westergren method) (Table 1). PB samples of the age and sex matched control subjects (Ctrl) admitted due to non-inflammatory conditions, with normal values for inflammatory indicators (ESR and CRP; Table 1) and without history of autoimmune or joint diseases, were used for enzyme-linked immunosorbent assay (ELISA) ($n=14$) and OC cultures ($n=8$). Patients with RA and PsA significantly differ from controls in the systemic indicators of inflammation (ESR and CRP; Table 1).

Osteoclast differentiation

SF of arthritic patients was aspirated during therapeutic puncture of the affected knee. PB of arthritic patients and control subjects was obtained as part of a routine clinical assessment. PBMC and SFMC were separated using Histopaque (Sigma-Aldrich, St. Louis, MO, USA).

Isolated PBMC and SFMC were plated in α -minimal essential medium (α -MEM) supplemented with 10 % fetal bovine serum (FBS). After 24 hours non-adherent cells were harvested and plated (3.5×10^5 cells/well in 96-well plate) over glass coverslips in the presence of 40 ng/mL recombinant human (rh)M-CSF and 60 ng/mL rhRANKL for an additional 8 days [4]. OCs were identified as tartrate-resistant acid phosphatase (TRAP)-positive cells with ≥ 2 nuclei/cell. OC differentiation was additionally confirmed by the expression of OC-specific genes: receptor activator of nuclear factor- κ B (*RANK*), macrophage colony-stimulating factor receptor (*cFMS*, CD115) and *TRAP* using quantitative (q)PCR.

Gene expression analysis by qPCR

Total RNA was extracted from PBMC/SFMC and OC cultures using TRIzol (Invitrogen by Life Technologies (LT), Grand Island, NY, USA), converted to complementary DNA and amplified in duplicates by qPCR in an ABI Prism 7500 Sequence Detection System (Applied Biosystems by LT). Gene expression of OC-specific genes (*RANK*, *cFMS*, *TRAP*) and proinflammatory/immunoregulatory mediators (*TNF- α* , *IL-17*, *IL-18*, *FasL*, *RANKL*) were assessed using TaqMan Assays (Applied Biosystems by LT) (Supplementary Table 2) and presented as RNA relative quantity as previously described [7].

Table 1 Selected demographic and clinical characteristics of control subjects and patients with rheumatoid arthritis (RA) and psoriatic arthritis (PsA)

Characteristic	Control	RA	PsA
Number of subjects	14	10	10
Male/female	5/9	3/7	3/7
Age, years	59.7±14.7	55.8±9.1	48.3±11.5
Disease duration, years	NA	12.1±12.6	11.2±10.2
ESR, mm/h	9.0±4.0	51.4±37.8	32.7±20.5
CRP, mg/L	2.7±0.8	57.1±23.9	38.3±21.2
Swollen joint count (28-joint score)	NA	6.4±4.1	4.8±4.2
Tender joint count (28-joint score)	NA	13.3±7.5	9.9±7.4
DAS28 (0–10)	NA	6.0±0.9	5.1±1.3
BASDAI (0–10)	NA	NA	3.3±3.0
MASES (0–13)	NA	NA	4.2±4.6
Larsen score for RA (0–4); Radiological score of sacroileitis for PsA (0–4)	NA	2.7±0.5	2.8±0.4
NSAIDs	NA	7/10	6/10
DMARDs	NA	5/10	5/10
Corticosteroids	NA	1/10	2/10

ESR erythrocyte sedimentation rate, CRP C-reactive protein, DAS28 Disease Activity Score including a 28-joint count, BASDAI Bath Ankylosing Spondylitis Disease Activity Index, MASES Maastricht Ankylosing Spondylitis Enthesitis Score, NSAIDs non-steroid anti-inflammatory drugs, DMARDs disease-modifying antirheumatic drugs, NA not applicable

Values are presented as mean ± standard deviation. Sera of 14 control patients were analyzed by ELISA, whereas eight PBMC control samples were subjected to osteoclastogenic assay. Patients with RA and PsA were not treated with biological agents

ELISA

The serum and SF concentrations of TNF- α (Quantikine High Sensitivity Immunoassay, R&D systems, Minneapolis, MN, USA), CCL2, IL-17, FasL (Quantikine Immunoassay, R&D systems), and IL-18 (Quantikine Immunoassay, Medical & Biological Laboratories Co., LTD, Naka-ku, Nagoya, Japan) were determined in accordance with the manufacturer's instructions. Optical density was determined within 15 minutes on the microplate reader (Bio-Rad, Hercules, CA, USA) set to excitation wavelength at 450 nm (for CCL2, IL-17, IL-18, and FasL) or 490 nm (for TNF- α).

Cell sorting and co-culture

PBMC of healthy volunteers ($n=5$) were pooled and labeled using the lymphoid (CD3/CD19/CD56) and myeloid markers (CD11b and CD14). OCPs were defined as CD3⁺CD19⁺CD56⁺CD11b⁺CD14⁺ cells and sorted using BD FACSaria I (BD Biosciences, Franklin Lakes, NJ, USA), with sorting

purity >98 %. Sorted cells (50,000/cm²) were cultured in α -MEM/10 % FBS supplemented with rhRANKL and rhM-CSF (20 ng/mL for both). Additionally, SF of individual RA and PsA patients was added in 40 % volume proportion to assess its osteoclastogenic potential.

Statistical analysis

Clinical data were presented as mean ± standard deviation (SD). OC number and gene expression values were expressed as median with the interquartile range (IQR) and compared using non-parametric Kruskal-Wallis test followed by Mann–Whitney test with Bonferroni's correction for multiple testing. Correlations were assessed by Spearman's coefficient rho (ρ) with its 95 % confidence interval (CI). Receiver-operating characteristic (ROC) curve analysis was presented as area under the curve (AUC) with its 95 % CI, and used to discriminate between arthritic and control groups. Statistical analysis was performed using the MedCalc software package (Mariakerke, Belgium). For all experiments, α -level was set at 0.05.

Results

Enhanced osteoclastogenic potential in arthritic patients

Number of TRAP-positive OCs differentiated from PBMC of patients with RA and PsA was significantly higher compared with control (RA vs ctrl $p=0.008$, PsA vs ctrl $p<0.001$; Fig. 1a and b), with the most prominent osteoclastogenic potential in RA (RA vs PsA $p=0.043$; Fig. 1b). In parallel, expression of RANK and TRAP was higher in freshly isolated PBMC (day 0) from RA and PsA patients, but reached the statistical significance only for RANK in PsA compared with control ($p=0.003$; Fig. 1c).

In contrast, there was no significant difference in the number of TRAP-positive OCs differentiated from SFMC (Fig. 1a and b). Nevertheless, SFMC derived OCs from PsA patients were morphologically different (larger with more nuclei/cell; Fig. 1a). Gene expression for RANK and TRAP in freshly isolated SFMC (day 0) of PsA patients was higher compared with RA, but did not reach statistical significance (Fig. 1c).

OC differentiation was confirmed by the increase in the expression of OC-specific genes with the duration of osteoclastogenic cultures from arthritic patients (comparison of day zero [white bars] and day ten [grey bars]; Fig. 1c), particularly for RANK and TRAP. We found more than threefold higher RANK expression at the OC-culture end-point for PBMC (day ten) of RA compared with PsA ($p=0.004$; Fig. 1c), and more than fivefold higher RANK expression for SFMC (day ten) of PsA compared with RA ($p=0.048$; Fig. 1c).

Fig. 1 Osteoclastogenic cultures of peripheral blood mononuclear cells (PBMC) and synovial fluid-derived mononuclear cells (SFMC) from arthritic patients. **a** Osteoclasts (OCs) were identified as TRAP-positive cells with ≥ 2 nuclei/cell (representative cultures). **b** Number of TRAP-positive OCs/well differentiated from control subjects (PBMC), and arthritic patients with RA and PsA (PBMC and SFMC), presented as median (IQR). **c** Expression of OC-specific genes in PBMC (PB) and SFMC (SF) at culture days 0 and 10, presented as RNA relative quantity (median [IQR]). Group-to-group comparisons were performed using Mann–Whitney test, p values ≤ 0.05 are shown. *RANK* receptor activator of nuclear factor- κ B, *cFMS* macrophage colony-stimulating factor receptor, *TRAP* tartrate-resistant acid phosphatase

Association of systemic and intra-articular osteoclastogenic potential in arthritis

To determine the profile of soluble systemic and local osteoclastogenic factors, we selected TNF- α , CCL2, IL-17 and IL-18, and assessed them in PB and SF at both protein and gene expression levels (Fig. 2a and b). Overall, concentrations of selected inflammatory mediators were higher in SF than in serum, with RA SF having the peak values for CCL2, TNF- α and IL-17 (Fig. 2a).

Serum concentration of CCL2 was significantly higher in RA patients compared with control ($p=0.016$; Fig. 2a) and positively correlated with the number of OCs derived from PBMC ($p=0.036$; Fig. 3). For TNF- α , we could not find a statistically significant difference at the protein or gene expression level between groups, although the values were highest for SF of RA patients (Fig. 2a). Serum levels of IL-17 in patients with PsA were elevated compared with controls (PsA vs ctrl, $p=0.013$) as well as in SF of RA patients compared with PsA

($p=0.054$) (Fig. 2a). Gene expression of *IL-18* in PBMC was significantly higher in both forms of arthritis compared with controls (RA vs ctrl, $p<0.001$; PsA vs ctrl, $p<0.001$; Fig. 2b). At the protein level, IL-18 was the most prominent in both serum and SF of RA patients, but the difference was not statistically significant (Fig. 2a). Furthermore, statistically significant positive correlation was observed between the *IL-18* PBMC gene expression and PBMC OC number ($p=0.049$; Fig. 3).

Fig. 2 Pro-inflammatory/immunoregulatory mediators in circulatory and intra-articular compartments of arthritic patients. **a** Mediators were analyzed in serum and synovial fluid (SF) at the protein level by ELISA, and in peripheral blood mononuclear cells (PBMC) and synovial fluid-derived mononuclear cells (SFMC) at the gene expression level by qPCR (**b**). Values are presented as protein concentration or RNA relative quantity (median [IQR]) for each group. Group-to-group comparisons were performed using Mann–Whitney test; p values ≤ 0.05 are shown. CCL2 CC-chemokine ligand 2, TNF- α tumor necrosis factor α , FasL Fas-ligand, IL interleukin

Fig. 3 Correlation between osteoclastogenic potential and inflammation indicators. Significant correlations were found for the number of osteoclasts (OCs) differentiated from peripheral blood mononuclear cells (PBMC) with erythrocyte sedimentation rate (ESR), as an indicator of systemic inflammation, and osteoclastogenic mediators: serum

concentration of CC-chemokine ligand 2 (CCL2), PBMC gene expression of interleukin-18 (*IL-18*) and Fas-ligand (*FasL*). Values were correlated using rank correlation and Spearman's coefficient ρ (95 % confidence interval [CI] for ρ); p values ≤ 0.05 are shown. TRAP tartrate-resistant acid phosphatase

In addition, we found increased gene expression of *FasL* in PBMC of patients with PsA compared with control ($p=0.003$; Fig. 2b) as well as positive correlation between both soluble FasL concentration in sera ($\rho=0.412$, 95 % CI 0.067–0.669, $p=0.024$; not shown) and *FasL* PBMC gene expression ($p=0.017$; Fig. 3) with the number of PBMC derived OCs.

Finally, we functionally tested the osteoclastogenic potential of SF by co-culture of sorted OCPs (defined as $CD3^+CD19^+CD56^+CD11b^+CD14^+$ population) in osteoclastogenic media containing 40 % volume proportion of the individual SF from RA or PsA patients (Fig. 4a). In that model we confirmed significantly more efficient osteoclastogenesis in co-cultures treated with PsA SF compared with RA SF ($p=0.040$; Fig. 4b). Moreover, PsA patients had significantly higher *RANKL* expression in SFMC ($p=0.042$; Fig. 4c) but similar in PBMC compared with RA patients (Fig. 4c).

Correlation between osteoclastogenic potential and indicators of inflammation

ESR, an indicator of chronic systemic inflammation, was in positive correlation with the number of differentiated OCs from PBMC ($p=0.023$; Fig. 3). However, we could not

confirm significant correlations with osteoclastogenic potential for other clinical variables, probably due to the small group size. Furthermore, we subdivided the patients regarding the sulphasalazine (SSZ) treatment, used as a disease-modifying antirheumatic drug, and found that they had significantly reduced levels of FasL ($p=0.013$, Mann–Whitney test) in SF ($n=10$) compared with patients not receiving SSZ ($n=10$).

ROC curve analysis revealed that the control and patient groups could be distinguished based on the number of ex vivo matured OCs (AUC=0.921, 95 % CI 0.750–0.987, $p<0.001$), serum level of CCL2 (AUC=0.697, 95 % CI 0.499–0.852, $p=0.045$), and PBMC gene expression of *IL-18* (AUC=1.000, 95 % CI 0.871–1.000, $p<0.001$) and *FasL* (AUC=0.793, 95 % CI 0.594–0.923, $p=0.001$) (Fig. 5).

In contrast, there was no significant correlation between the SF osteoclastogenic mediators or circulatory indicators of inflammation and the number of differentiated OCs from SFMC, suggesting that SF represents the specific compartment that is locally regulated by infiltrating immune cells. Nevertheless, we found the significant positive correlation between *CCL2* gene expression in SFMC osteoclastogenic culture end-point (day ten) and number of differentiated OCs

Fig. 4 Comparison of synovial fluid (SF) osteoclastogenic effect from patients with rheumatoid arthritis (RA) and psoriatic arthritis (PsA). **a** Osteoclastogenic effect of SF in a co-culture model of sorted osteoclast (OC) progenitors (CD3⁻CD19⁻CD56⁻CD11b⁺CD14⁺ cells from control peripheral blood mononuclear cell [PBMC] samples, $n=5$), differentiated (50,000 cells/cm²) with the receptor activator of nuclear factor- κ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), and individual SFs from RA or PsA patients (in 40 % volume proportion). **b**

($\rho=0.546$, 95 % CI 0.070–0.820, $p=0.034$), indicating that intra-articular OCs may contribute to the enhanced osteoclastogenic potential within the affected joints.

Discussion

Our study confirmed systemic enhancement of osteoclastogenesis in arthritis, particularly RA. Increased osteoclastogenic potential of PBMC positively correlated with proinflammatory mediators in circulation and inflammation intensity, confirming that inflammation is a trigger for enhanced OC activity. OCPs are contained among both PBMC and SFMC, but not with the same frequency in RA and PsA, and even within the PBMC and SFMC of the same patient. We concluded that circulatory and intra-articular compartments

possess distinct profiles of osteoclastogenic factors and OC-inducing capacity. Higher number of differentiated OCs formed in cultures from PBMC of RA and PsA patients compared with controls. Moreover, more differentiated OCs and induced *RANK* expression were found in RA compared with PsA patients, reflecting high osteoclastogenic potential of circulatory compartment. Human OCPs are present at low frequency in normal PB within the monocyte population [1], and increase in number and osteoclastogenic potential in arthritis [9, 10], but this is the first study to include control subjects and patients suffering from different forms of arthritis, using paired PBMC and SFMC arthritic samples for osteoclastogenic assay.

In addition to the systemic changes, inflamed joints are marked with severe lymphocyte infiltration especially in RA, with the predominance of T lymphocytes within the SF [11].

Fig. 5 Discriminatory ability of osteoclast (OC) differentiation potential and proinflammatory mediators between arthritic and control samples. Receiver operating characteristic (ROC) curves for the number of OCs differentiated from peripheral blood mononuclear cells (PBMC), serum concentration of CC-chemokine ligand 2 (CCL2), and PBMC gene expression of interleukin-18 (*IL-18*) and Fas-ligand (*FasL*). Diagnostic efficacy for those values was assessed using the sensitivity and specificity at a cut-off point. ROC curve analyses with p values (for area under curve = 0.5) ≤ 0.05 are shown

Therefore, by plating the same number of SFMC for osteoclastogenic cultures as for PBMC, we included lower number of OCPs, giving us the overall lower number of ex vivo matured OCs and no difference between RA and PsA. Bone loss in RA and PsA occurs to a similar extent, either locally in the peripheral joints, or systemically with loss of BMD [2, 12]. However, in the functional assay SF of PsA patients showed more potent OC-inducing activity compared with RA, paralleled by higher *RANKL* and *RANK* expression, corroborating that PsA involves specific changes in the *RANKL*/*RANK* axis [5]. Ritchlin et al. demonstrated intense *RANKL* expression throughout the synovial lining layer, implicating enhanced *RANKL*-mediated osteoclastogenesis in PsA [10].

A proinflammatory profile in our patients included the highest level of CCL2 in RA patients, and positive correlation with the number of PBMC derived OCs. Also, CCL2 was highly induced with OC maturation, with the positive correlation between OC *CCL2* expression and the number of OCs differentiated from SFMC. It has been shown, using CCL2-

deficient mice and human PBMC, that CCL2 regulates OC differentiation in an autocrine/paracrine manner and that OCPs express CCL2 receptors, CCR2 and CCR4 [13, 14]. Kim et al. proposed that *RANKL*-stimulated OC-lineage cells send CCL2 chemokine signal to attract OCPs to the site of *RANKL* signaling, resulting in cell fusion to multinuclear OCs [15].

Association of proinflammatory factors with osteoclastogenic potential confirmed that they play a role not only in creating an inflammatory environment but also in inducing osteoresorption. IL-17 and IL-18 were both elevated systemically and, together with TNF- α , the most prominent in SF of RA patients. TNF- α was not increased in serum, but overall concentrations in all groups were very low. TNF- α and IL-17 are able to induce *RANK* expression on human OCPs and OC differentiation [16, 17], and are substantial factors in inflammation-mediated bone destruction [18, 19]. Results for IL-18 effects on osteoclastogenesis are still controversial, suggesting both anti-osteoclastogenic [20] and pro-osteoclastogenic effects, via induction of TNF and *RANKL* in synovial T lymphocytes and fibroblast-like synoviocytes [21, 22]. Intra-

articular osteoclastogenic milieu, observed in our study, may support OCPs not only in SF but also on bone surfaces, causing periarticular bone erosions [10]. Moreover, we believe that lack of correlation between proinflammatory mediators and number of OCs differentiated from SFMC could be explained by the fact that those mediators act on the pannus-bone interface and enhance OCPs attachment to the resorption lacunas where they are not available for harvesting by SF aspiration.

Besides inflammatory mediators, we found increased expression of *FasL* in PBMC of PsA patients and a positive correlation with the number of PBMC-derived OCs. A disease-modifying drug SSZ was able to reduce *FasL* in SF of treated patients. Although the *Fas/Fas* ligand system is involved in the regulation of bone homeostasis, the role in arthritis is still controversial [7, 23, 24]. Resident synoviocytes and infiltrating immune cells are resistant to *FasL*-mediated apoptosis, contributing to joint lesions [24]. Increased expression could represent a compensatory mechanism to induce apoptosis of autoreactive T lymphocytes in autoimmune conditions.

Conclusions

In conclusion, our study revealed disease-specific changes despite high heterogeneity of SF milieu between patients, reflecting the chronic nature of the disease and great variability in SF content [11]. In particular, RA patients have the highest values of inflammatory mediators in both serum and SF, implicating alternate osteoclastogenic pathways independent of RANKL and TNF- α [25]. On the other hand, PsA involves a specific increase in *RANKL/RANK* expression in the synovial compartment, creating a potent osteoclastogenic environment in the affected joints. Our findings indicate arthritis-specific mediators associated to the form of arthritis, which may be clinically relevant for the development of diagnostic or therapeutic strategies targeting proinflammatory and osteoclastogenic effects of arthritis.

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Conflict of interest The authors declare that they have no conflict of interest.

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