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PRINCIPAL INVESTIGATOR: Xiang-Yang Wang, PhD

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| <b>14. ABSTRACT</b><br>Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes irreversible joint damage and significant disability. The pathogenesis of RA is often associated with activation of immune cells and osteoclasts that cascade into a vicious cycle of inflammation and bone erosion. However, the fundamental mechanisms underlying how excessive inflammation and bone erosion develop and are sustained chronically in RA remain largely unknown. Using clinically relevant mouse models, we revealed that genetic ablation of scavenger receptor A (SRA, CD204), an innate pattern recognition receptor primarily expressed in innate myeloid cells, rendered mice fully protected from induction of collagen-induced arthritis. Lack of SRA abolished cartilage erosion of articular surface, and synovial hyperplasia in the articular cavity. Monocytic MDSCs (M-MDSCs) can be important precursors of OCs given their inherent capability to differentiate into OCs. Additionally, SRA was shown to function as a critical molecular factor governing OC differentiation from M-MDSCs, which may be its previously unrecognized activity in modulating the RANKL signaling. These data indicate that SRA is involved in osteoclastogenic processes of autoimmune arthritis and provide a scientific rationale for targeting SRA to potentially mitigate the symptoms of RA. |  |   |   |   |   |
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## 1. INTRODUCTION

Rheumatoid arthritis (RA) is a common autoimmune disease that is characterized by chronic inflammation, progressive joint destruction, progressive disability and systemic complications. RA is the most common type of inflammatory arthritis and affects 1% of the US population overall, rising to 2% in those 60 years of age and older. It causes both significant morbidity and increased mortality that is associated with socioeconomic costs. Although the management of RA has advanced significantly, there is still no cure for RA. Treatment for RA is unsatisfactory because some patients do not respond to the existing therapies and certain drugs cause serious side effects. Therefore, it remains an unmet need for more efficient and safe treatment options.

Scavenger receptor A (SRA), also called CD204, is an innate pattern recognition molecule primarily expressed on the cells of myeloid origin. Using a mouse model that mimics human RA, we showed that genetic ablation of SRA renders mice highly resistant to collagen-induced arthritis (CIA). In addition to a reduced inflammatory response (e.g., Th17 response) in the inflamed tissues, lack of SRA abolished cartilage erosion of articular surface, and synovial hyperplasia in the articular cavity. On a cellular level, SRA absence disrupts the cross-talk of MDSCs and Th17 cells, resulting in impaired Th17 polarization as well as OC differentiation. During this funding period, we used CIA model together with the SKG transgenic mice that spontaneously develop symmetric inflammatory arthritis to examine the SRA impact on osteoclastogenesis and bone erosion, both of which are the key pathologic features of inflammatory arthritis. We showed that monocytic MDSCs (M-MDSCs) can be important precursors of OCs given their inherent capability to differentiate into OCs as well as their significant expansion during the progression of RA. Additionally, SRA was shown to function as a critical molecular factor governing OC differentiation from M-MDSCs, which may be its previously unrecognized activity in modulating the RANKL signaling. These data together with our previous findings indicate that SRA is involved in both pro-inflammatory and osteoclastogenic processes of autoimmune arthritis and provide a scientific rationale for targeting SRA to potentially mitigate the symptoms of RA.

## **2. KEYWORDS**

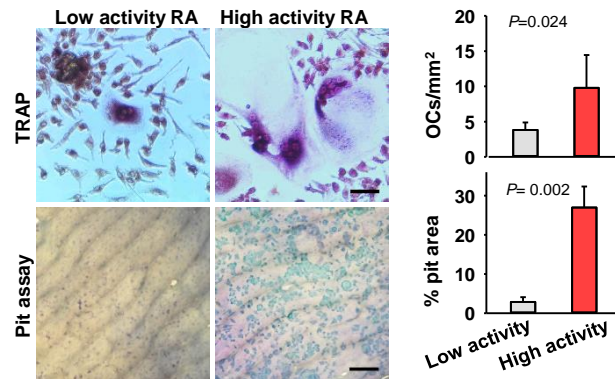
Rheumatoid arthritis, autoimmune inflammation, scavenger receptor A, myeloid derived suppressor cells, bone erosion, osteoclasts.

### 3. ACCOMPLISHMENTS

The major goals of the project for the period from Sep. 30<sup>th</sup>, 2020 through Sep. 29<sup>th</sup>, 2021 is to define the molecular and cellular basis of SRA-facilitated osteoclast differentiation and bone erosion during the progression of inflammatory arthritis.

#### Accomplishments during this funding period:

During this funding period, we have investigated the pathogenic activity of SRA in promoting osteoclastogenesis and the progression of rheumatoid arthritis (RA). We made new findings that human MDSCs, particularly monocytic MDSCs (M-MDSCs), exhibit inherent OC-differentiating capacity and positively correlate with clinical bone erosion in RA patients. More importantly, MDSCs from Wild-type (WT) mice with collagen-induced arthritis (CIA) or SKG transgenic mice are more efficient than that those from SRA<sup>-/-</sup> counterparts in differentiation into OCs. There is enhanced bone destruction associated with abnormal formation/activation of OCs in WT mice following CIA as compared to those in SRA<sup>-/-</sup> mice.



**Figure 1. Human M-MDSCs from RA patients promote osteolysis.** A. M-MDSCs from peripheral blood of RA patients with low disease activity (28-joint disease activity score, DAS28 $\leq$ 3.2) or high disease activity (DAS28 $>$ 5.1) were cultured in the presence of M-CSF and RANKL *in vitro* for 14 days. TRAP staining and pit assay were performed to assess differentiation and biological activity of OCs. Scale bar in the images of TRAP and pit assay represent 50  $\mu$ m and 200  $\mu$ m, respectively.

#### Task 1. Determine the differential capabilities of SRA expressing M-MDSCs in differentiation into osteoclasts (OCs).

Our previous study reported that human MDSCs were significantly elevated in RA patients and correlated with the level of inflammatory cytokine IL-17A.<sup>1</sup> Given that IL-17-producing Th17 cells can promote RANK-L signaling dependent OC differentiation.<sup>2,3</sup> We analyzed the frequency of MDSCs and joint destruction in RA patients to determine a potential association between these

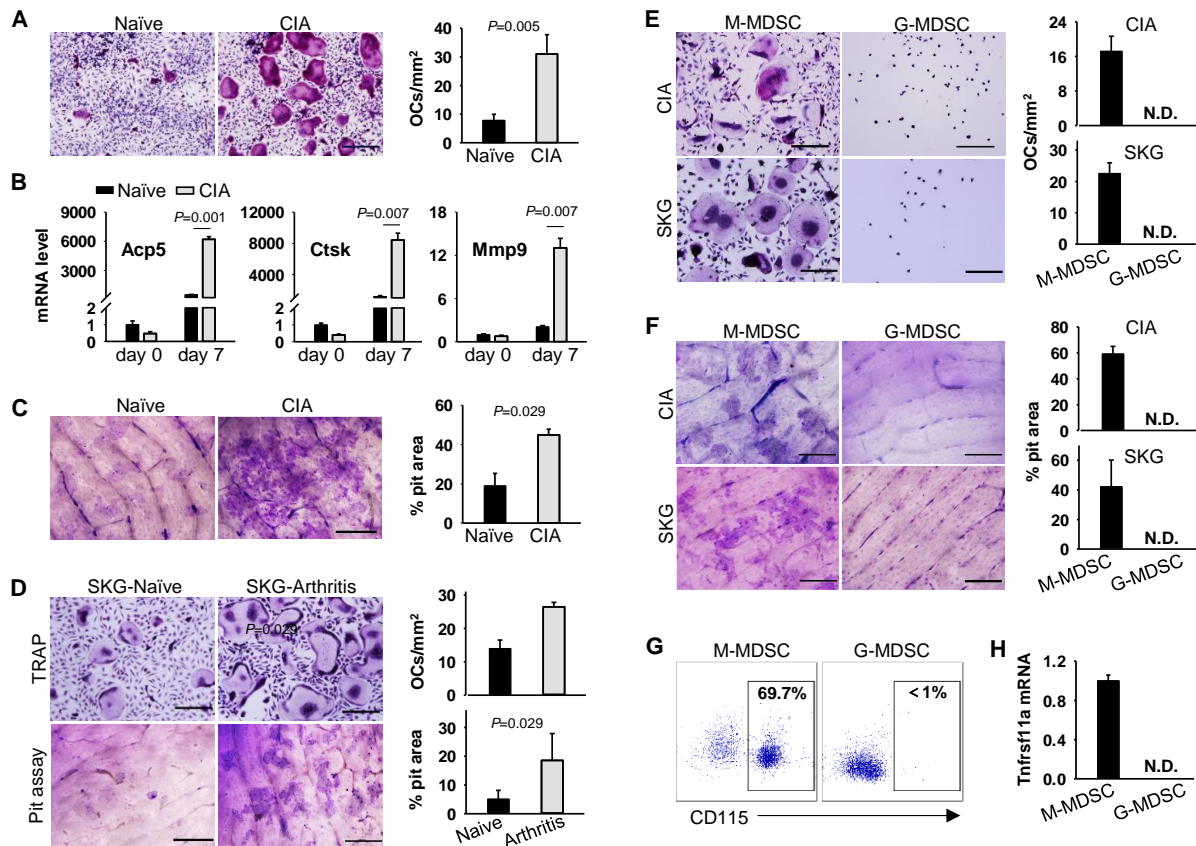
**Table 1. Correlation between frequency of MDSC subsets and bone destruction in patients with RA.**

| Correlation analysis | Bone destruction scores |        | TRAP5b (U/L)         |       | $\beta$ -CTX (ng/ml)  |       |
|----------------------|-------------------------|--------|----------------------|-------|-----------------------|-------|
|                      | r (95% CI)              | P      | r (95% CI)           | P     | r (95% CI)            | P     |
| MDSC                 | 0.494 (0.222-0.694)     | <0.001 | 0.336 (0.099-0.537)  | 0.005 | 0.331 (0.094-0.533)   | 0.006 |
| M-MDSC               | 0.521 (0.257-0.713)     | <0.001 | 0.388 (0.158-0.578)  | 0.001 | 0.329 (0.092-0.532)   | 0.006 |
| G-MDSC               | 0.067 (-0.243-0.364)    | 0.667  | 0.090 (-0.328-0.159) | 0.467 | -0.106 (-0.342-0.143) | 0.389 |

Note: Bone destruction scores in patients with RA ( $n=44$ ) were assessed by joint ultrasound. Levels of TRAP5b and  $\beta$ -CTX in the serum of RA patients ( $n=68$ ) were examined by ELISA. Frequencies of MDSCs as well as their subsets in peripheral blood mononuclear cell (PBMC) ( $n=69$ ) were determined by Flow cytometry. Spearman rank test was used for analyzing the correlation between frequency of MDSC subsets and bone destruction scores and level of TRAP5b and  $\beta$ -CTX.

cells and bone erosion. We showed that the frequency of MDSCs or M-MDSCs, not G-MDSCs, positively correlated with bone erosion scores as assessed by joint ultrasound in RA patients

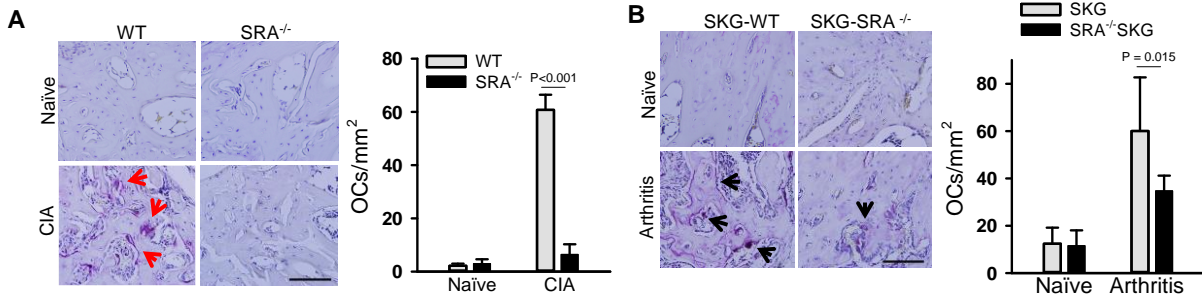
(Table 1). This correlation was also supported by significantly higher levels of TRAP5b and  $\beta$ -CTX, two bone destruction markers,<sup>4,5</sup> in RA patients with more circulating M-MDSCs (Table 1). We further showed that CD11b<sup>+</sup>CD33<sup>+</sup>HLA-DR<sup>-low</sup>CD14<sup>+</sup>CD15<sup>-</sup> M-MDSCs, isolated from peripheral blood of RA patients, were able to differentiate into TRAP<sup>+</sup> OCs that can induce bone



**Figure 2. MDSCs from arthritic mice display enhanced capacity in differentiation into functional OCs.** **A.** MDSCs from naïve or CIA mice were cultured in the presence of M-CSF and RANK-L for OCs differentiation. Representative images of TRAP staining and OCs numbers are shown. **B.** Relative mRNA levels of *Acp5*, *Ctsk*, and *Mmp9* genes were assessed using qRT-PCR. **C.** MDSCs were seeded on bone slices in the presence of M-CSF and RANK-L. Bone resorption pit was examined using pit assays. **D.** MDSCs sorted from naïve or arthritic SKG mice were differentiated into OCs as described above. **E** and **F.** OC-differentiating potential of M-MDSCs or G-MDSCs and bone resorption activity were assessed. **G.** Expression of CD115 on MDSCs subsets was assayed by flow cytometry. **H.** Transcription of *Tnfrsf11a* (*RANK*) was determined by qRT-PCR. Scale bar, 200  $\mu$ m. Results shown are representative of three independent experiments. N.D., not detected.

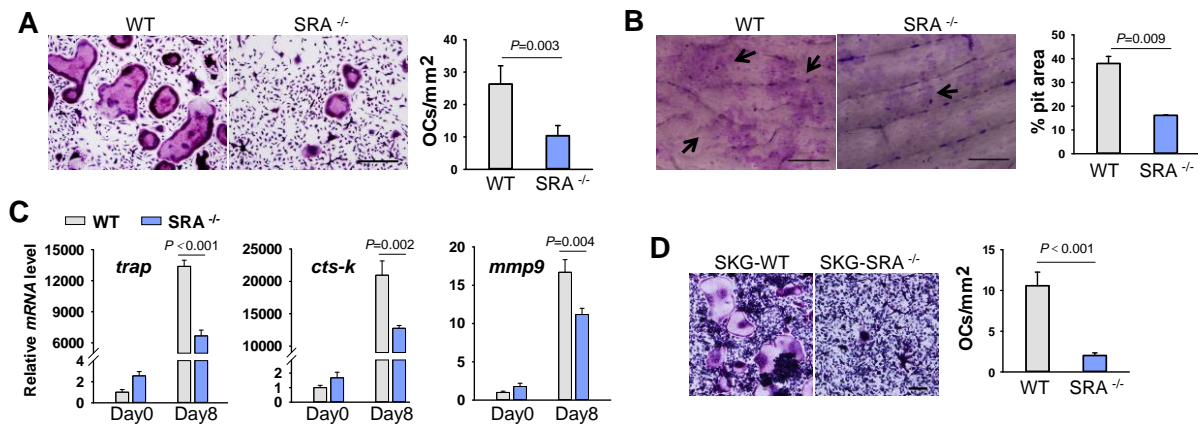
resorption (Fig. 1). Intriguingly, M-MDSCs from RA patients with high disease activity were more readily to differentiate into OCs as compared to those from patients with low disease activity (Fig. 1), suggesting that the intrinsic OC-differentiating potential of M-MDSCs may be determined by the disease status.

Given that human MDSCs from patients with RA can differentiate into fully functional OCs, we also compared the OC-differentiating capacity of MDSCs from naïve or CIA mice in the presence of M-CSF and RANK-L. CIA-associated MDSCs displayed a marked increase in its



**Fig. 3. Absence of SRA impairs OC differentiation in the inflamed joints of arthritic mice.** CIA was induced in WT and SRA<sup>-/-</sup> mice by immunization with collagen emulsified in CFA. (A) Osteoclasts in the inflamed joints were quantified by TRAP staining. Arthritis was induced in SKG mice or SRA<sup>-/-</sup> SKG mice by i.p. injection with mannan (20mg). Quantification of osteoclasts was performed by TRAP staining (B). Bar = 100  $\mu$ m.

compacity for differentiation into OCs, indicated by elevated numbers of OCs (Fig. 2A), heightened transcription of *Acp5* (tartrate-resistant acid phosphatase), *Ctsk* (cathepsin k), or *Mmp9* (matrix metalloproteinase 9) genes (Fig. 2B), as well as enhanced activity in bone degradation (Fig. 2C). SKG mice, a genetic model of experimental autoimmune arthritis,<sup>6</sup> were also used to examine MDSC-initiated osteoclastogenesis and bone absorption. Similarly, MDSCs from arthritic SKG mice were more readily to differentiate into TRAP-positive OCs and to induce bone resorption (Fig. 2D). Considering that M-MDSCs and G-MDSCs not only are phenotypically and morphologically distinct, but also have different functional characteristics,<sup>7</sup> we further examined the OC-differentiating capacity of these two MDSC subsets. TRAP staining and pit assays showed that M-MDSCs derived from CIA mice or arthritic SKG mice, not G-MDSCs, were able to differentiate into OCs (Fig. 2E and F). Consistent with their differential OC-differentiating capacities, G-MDSCs displayed significantly lower levels of the M-CSF receptor CD115 (Fig. 2G) and the RANK-L receptor RANK (Fig. 2H), as compared to M-

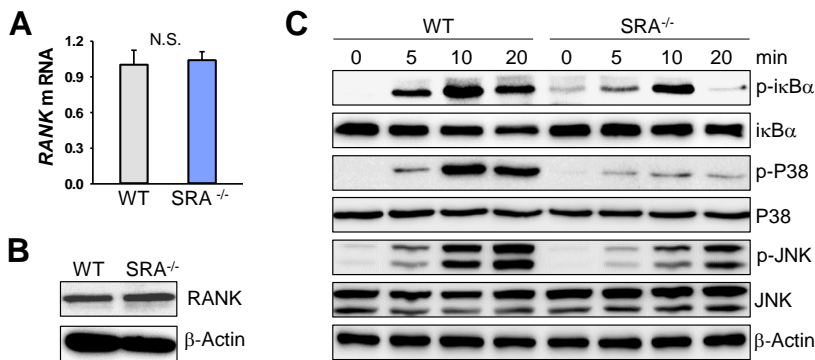


**Fig. 4. SRA ablation impairs differentiation of monocytic MDSC into osteoclasts.** MDSCs sorted from WT or SRA<sup>-/-</sup> arthritic mice were cultured in the presence of M-CSF and RANK-L for OCs differentiation. Representative images of TRAP staining and OCs numbers are shown (A). MDSCs were seeded on bone slices in the presence of M-CSF and RANKL. Bone resorption pit was examined using pit assays (B). (C) Relative mRNA levels of *Trap*, *Ctsk*, and *Mmp9* genes were assessed using real time PCR (C). MDSCs were sorted from SKG mice or SRA<sup>-/-</sup> SKG mice that have been established with arthritis by i.p. injection with mannan. Representative images of TRAP staining and OCs numbers are shown (D). Bar = 100  $\mu$ m.

MDSCs. These suggest that only arthritis-associated M-MDSCs exhibit a unique potential of differentiating into functionally active OCs.

Bone erosion seen in RA pathogenesis has been attributed to abnormal activation of OCs, which is a central feature of RA. Our previous data showed that absence of SRA abolished cartilage erosion of articular surface in mice following induction of CIA. In support of this observation, we found that the number of OCs was substantially reduced in the joints of both SRA<sup>-/-</sup> CIA mice (**Fig. 3A**) and SRA<sup>-/-</sup> SKG mice with inflammatory arthritis (**Fig.3B**), suggesting that SRA is involved in the osteoclastogenesis in arthritic mice.

Considering that SRA is primarily expressed on M-MDSCs and M-MDSCs (not G-MDSCs) can act as the precursors of OCs in arthritic mice, we next examined the hypothesis that SRA enhances the intrinsic capability of M-MDSCs to differentiate into OCs. To this end, SRA effect on OC differentiation from M-MDSCs were evaluated by culturing WT or SRA<sup>-/-</sup> mice-derived M-MDSCs in the presence of M-CSF and RANKL *in vitro*. SRA deficiency dramatically impaired



**Fig. 5. SRA enhances RANKL signaling in myeloid cells.** Bone marrow cells from WT and SRA<sup>-/-</sup> mice were cultured in the presence of M-CSF for 3 days. RANK expression was analyzed by real time PCR or western blotting (**A** and **B**). Phosphorylation of IκBα, p38 and JNK was assayed by western blotting after stimulation with RANKL for the indicated time points (**C**).

the capability of M-MDSCs to differentiate into functional OCs, indicated by the reduced number of OCs (**Fig. 4A**). When compared to those differentiated from SRA<sup>-/-</sup> M-MDSCs, OCs differentiated from WT precursors showed enhanced capability in inducing osteolysis as evidenced by a significant increase in absorbed areas on bone slices (**Fig. 4B**). There was also decreased transcription of *Acp5*, *Ctsk*, or *Mmp9* genes in OCs differentiated from SRA<sup>-/-</sup> precursors (**Fig. 4C**). Moreover, M-MDSCs from WT SKG mice were also more efficient than that those from SRA<sup>-/-</sup>SKG mice in differentiation into OCs (**Fig. 4D**). Together, these data suggest that SRA is a critical determinant factor in osteoclastogenesis driven by MDSCs and possibly other SRA-expressing myeloid cells.

## Task 2: Define the molecular mechanisms of SRA-expressing or -deficient M-MDSCs in differentiation into OCs.

The differentiation program of OC precursors is primarily regulated by the interaction of RANKL with its receptor RANK, whose expression can be induced by M-CSF.<sup>8</sup> We next examined whether SRA functionally modulates the RANKL-RANK signaling that is critical for the differentiation of M-MDSCs into OCs. Although SRA did not appear to affect the M-CSF-induced RANK expression on these myeloid cells (**Fig. 5A** and **B**), upon stimulation with RANKL, lack of SRA downregulated the signaling events downstream of the RANKL-RANK interaction, which include phosphorylation of IκBα, degradation of IκBα, phosphorylation of P38 and c-JNK (**Fig. 5C**). Therefore, our data indicate for the first time that SRA regulates the molecular signaling

involved in the differentiation program of OCs, which represents a novel molecular mechanism responsible for SRA-facilitated osteoclastogenesis and bone erosion in inflammatory arthritis.

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### **Opportunities for training and professional development has the project provided:**

This funding provided a Post-doc training opportunity for Dr. Zheng Liu.

### **The results disseminated to communities of interest:**

Part of research findings has been published in *Rheumatology* 2021,14;60(5): 2409-2420.

### **Plan to do during the next reporting period:**

We will continue to examine the SRA effect on M-MDSC-enhanced Th17 response and the regulation of RANKL production from Th17 cells by SRA-expressing M-MDSCs. We will focus on testing the feasibility of targeting SRA in the inflamed joints using SRA monoclonal antibodies to reduce Th17 response and bone erosion.

#### **4. IMPACT**

We have demonstrated a novel pro-osteoclastogenic activity of MDSCs, particularly M-MDSCs, in RA, highlighting their functional plasticity and versatility in disease pathogenesis. These results will advance the current understanding of the functional distinctions of MDSCs in RA-associated osteoclastogenesis. We also provide a compelling evidence that SRA is required for the pathogenic activity of MDSCs in this disease, which contribute to abnormal OC activation and bone erosion in inflammatory arthritis. Strategically targeting SRA to reduce inflammation and bone destruction may provide a novel approach to the treatment of RA.

#### **The impact on the development of the principal discipline(s) of the project:**

Nothing to Report

#### **The impact on other disciplines:**

Nothing to Report

#### **The impact on technology transfer:**

Nothing to Report

#### **The impact on society beyond science and technology:**

Nothing to Report

## **5. CHANGES/PROBLEMS**

Nothing to Report

## **6. PRODUCTS:**

### **PUBLICATIONS:**

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### **ABSTRACTS AND PRESENTATIONS:**

Nothing to Report

### **INVENTIONS, PATENT, AND LICENSES**

Nothing to Report

### **OTHER PRODUCTS**

Nothing to Report

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

|                             |   |  |   |  |   |
|-----------------------------|---|--|---|--|---|
| Name                        | Xiang-Yang Wang   | Chunqing Guo   | Christopher Wise  | Zheng Liu  | Luiza Bretas  |
| Project Role                | Principal Investigator  | Co-Investigator  | Co-Investigator   | Post-doc   | Nurse Coordinator   |
| Nearest person month worked | 3   | 2.2  | 0.6   | 12   | 1   |
| Contribution to Project     | Oversaw the overall project and executed the research plan. Supervised Dr. Liu to carry out proposed studies. | Work with Dr. Wang to oversee the progress of the project and supervise Dr. Liu to perform the proposed studies. | Work with Dr. Wang for research progress of the project and provide clinical expertise in RA. | Performed work in arthritis induction, histology and pathology analysis of arthritic mice, evaluate autoimmune inflammation in arthritic mice. | Assist Dr. Wise with clinical research involving specimens from patients with RA. |
| Funding Support             | NIH/DOD   | NIH/DOD  | DOD   | DOD  | DOD   |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report

**What other organizations were involved as partners?**

Nothing to Report



## **8. SPECIAL REPORTING REQUIREMENTS**

N/A

## 9. Appendices

## Original article

## Monocytic MDSCs skew Th17 cells toward a pro-osteoclastogenic phenotype and potentiate bone erosion in rheumatoid arthritis

Shixian Chen<sup>1,2,†</sup>, Chunqing Guo<sup>3,4,†</sup>, Ran Wang<sup>1,2,†</sup>, Zhitao Feng<sup>1,a</sup>, Zheng Liu<sup>3,4</sup>, Lisheng Wu<sup>1</sup>, Di Zhao<sup>1</sup>, Songyuan Zheng<sup>1</sup>, Feilong Chen<sup>1</sup>, Dingding Zhang<sup>1</sup>, Juan Xu<sup>1</sup>, Junqing Zhu<sup>1</sup> , Xiaoguang Chen<sup>5</sup>, Zhanguo Li<sup>6</sup>, Christopher M. Wise<sup>7</sup>, Juan Li<sup>1,2,\*</sup> and Xiang-Yang Wang<sup>1</sup> <sup>3,4,8,\*</sup>

## Abstract

**Objectives.** While myeloid-derived suppressor cells (MDSCs) were previously shown to promote a proinflammatory T helper (Th) 17 response in autoimmune conditions, a potential impact of the MDSC-Th17 immune axis on abnormal bone destruction in RA remains largely unknown.

**Methods.** We investigated the correlation between the frequency of MDSCs or its subsets and joint destruction in RA patients. The reciprocal actions of patient-derived MDSCs and Th17 cells were studied using osteoclast (OC) differentiation and bone resorption assays *in vitro*, which were further validated using mouse models of RA. Contribution of MDSCs to osteoclastogenesis and bone erosion *in vivo* was determined by depletion or transfer of MDSCs.

**Results.** Human MDSCs, particularly monocytic MDSCs (M-MDSCs), exhibit inherent OC-differentiating capacity and positively correlate with clinical bone erosion in RA patients. Strikingly, patient-derived M-MDSCs can program Th17 cells towards a pro-osteoclastogenic phenotype, which in return potentiates OC differentiation via the receptor activator of nuclear factor  $\kappa$ B ligand (RANK-L)-RANK signalling. This enhanced osteolysis driven by the reciprocal actions of M-MDSCs and Th17 cells is further confirmed using mouse models of RA. Selective depletion of M-MDSCs significantly ameliorates osteoclastogenesis and disease severity in arthritic mice, whereas transfer of M-MDSCs aggravates bone erosion associated with increased OCs in recipient mice.

**Conclusion.** Our findings highlight the functional plasticity of MDSCs and identify a novel pro-osteoclastogenic pathway governed by interplay between myeloid cells and T lymphocytes in autoimmune RA.

<sup>1</sup>Department of Traditional Chinese Internal Medicine, School of Traditional Chinese Medicine, <sup>2</sup>Department of Rheumatic & TCM Medical Center, Nanfang Hospital, Southern Medical University, Guangzhou, China, <sup>3</sup>Department of Human & Molecular Genetics, <sup>4</sup>Institute of Molecular Medicine, Virginia Commonwealth University School of Medicine, Richmond, VA, USA, <sup>5</sup>Guangdong Provincial Key Laboratory of Tropical Disease Research, Department of Pathogen Biology, Southern Medical University School of Public Health, Guangzhou, <sup>6</sup>Department of Rheumatology and Immunology, Peking University People's Hospital & Beijing Key Laboratory for Rheumatism Mechanism and Immune Diagnosis, Beijing, China, <sup>7</sup>Department of Internal Medicine, Virginia Commonwealth University Medical Center and <sup>8</sup>McGuire VA Medical Center, Richmond, VA, USA

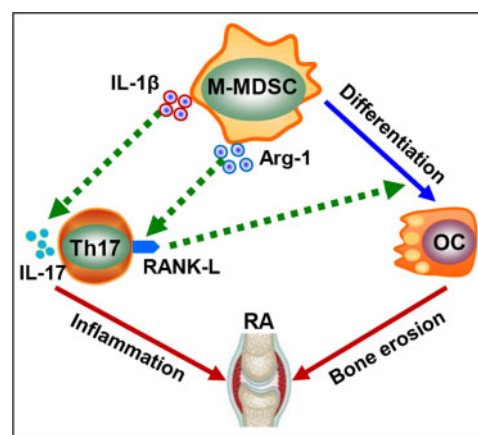
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Correspondence to: Juan Li, Department of Traditional Chinese Internal Medicine, School of Traditional Chinese Medicine, Southern Medical University, Guangzhou, China. E-mail: lijuan@smu.edu.cn

<sup>a</sup>Present address: Third-Grade Pharmacological Laboratory on Chinese Medicine Approved by State Administration of Traditional Chinese Medicine, Medical College of China Three Gorges University, Yichang, China

\*Juan Li and Xiang-Yang Wang share senior authorship.

†Shixian Chen, Chunqing Guo and Ran Wang contributed equally to this work.



**Key words:** rheumatoid arthritis, bone erosion, osteoclast, myeloid-derived suppressor cell, Th17

### Rheumatology key messages

- Monocytic MDSCs from patients with rheumatoid arthritis can differentiate into osteoclasts with bone resorbing activity.
- MDSC-Th17 interaction upregulates the pro-osteoclastogenic signal RANK-L on Th17 cells.
- MDSC-‘conditioned’ Th17 cells reprogram MDSCs into functional osteoclasts.

## Introduction

RA is a chronic autoimmune disorder that is characterized by exacerbated synovial inflammation, progressive joint destruction and physical disability [1]. It has been well documented that uncontrolled differentiation and activation of osteoclasts (OCs), the primary somatic cell type capable of resorbing bone matrices, contribute to bone erosion in inflammatory arthritis [2, 3]. *In vitro* and *in vivo* studies have shown that the receptor activator of NF- $\kappa$ B ligand (RANK-L) is indispensable for the differentiation and activation of OCs [4–6]. While an important role of the RANK-L-induced OC formation and bone destruction in RA has been recently established, a better understanding of the underlying cellular and molecular mechanisms will provide new therapeutic targets for effective disease intervention.

Myeloid-derived suppressor cells (MDSCs) are a population of immune cells that have emerged as a critical regulator of many pathologic conditions, including autoimmune disorders [7, 8]. MDSCs in human are typically defined as CD11b<sup>+</sup>CD33<sup>+</sup>HLA-DR<sup>low/-</sup> cells and divided into CD14<sup>+</sup> monocytic and CD15<sup>+</sup> granulocytic populations. MDSCs in mice, characterized by the expression of myeloid cells markers CD11b and Gr-1 (Ly6G/Ly6C), consist of two major subsets: CD11b<sup>+</sup>Ly6G<sup>-</sup>Ly6C<sup>high</sup> monocytic MDSCs (M-MDSCs) and CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>low</sup> granulocytic MDSCs (G-MDSCs) [9]. Although the immunosuppressive function of MDSCs are well established in cancer, the emerging evidence reveals previously under-appreciated features of these myeloid cells in aggravation of inflammation in cancer [10, 11] and autoimmune disorders [12–15]. Recently, preclinical and clinical studies from our and other groups showed that MDSCs, substantially expanded under autoimmune pathological conditions (e.g. RA), can promote T helper (Th) 17 polarization and IL-17 production, thereby driving excessive inflammation, tissue injury, and disease pathogenesis [12–15]. However, the potential involvement of this MDSC-Th17 immune axis in RA-associated osteoclastogenesis and bone metabolism remains to be elucidated.

In this study, we investigate the functional interactions of MDSCs and its subsets with Th17 cells in the context of osteoclastogenesis using specimen from patients with RA and two mouse models of autoimmune arthritis.

For the first time, we report that human MDSCs, particularly M-MDSCs, exhibit inherent capability to differentiate into OCs inducing bone resorption, further underscoring the functional plasticity and diversity of these myeloid cells in RA. More importantly, our findings uncover a novel immuno-osteoclastogenic pathway involving MDSC-Th17 interplay and the pro-osteoclastogenic factor RANK-L.

## Methods

### Patient samples

Whole blood samples were collected from 69 patients fulfilling the 1987 ACR revised criteria for the classification of RA or 2010 ACR/EULAR classification criteria for RA from Nanfang Hospital, Southern Medical University. Clinical and laboratory data were collected for evaluation of disease activity score in 28 joints (DAS28). RA disease activity were defined based on DAS28-ESR:  $\leq 3.2$ , remission or low disease activity; 3.2 ~ 5.1, moderate disease activity;  $> 5.1$ , high disease activity [16].

Bone erosion scores were quantified based on finger joints (total 20 joints, including proximal interphalangeal joints and metacarpophalangeal joints) ultrasonography [17, 18]. Each joint was scored according to a semi-quantitative scoring system (grades 0–3) [18]: 0, regular bone surface; 1, irregularity of the bone surface without formation of a defect; 2, formation of a defect in the surface of the bone; 3, bone defect creating extensive bone destruction. Bone erosion score was the sum of the grades for each joint. All participants were given written informed consent, and all procedures in this study were approved by the ethics committee of Nanfang Hospital, Southern Medical University.

### Mice

C57BL/6 mice were purchased from National Cancer Institute (Bethesda, MD, USA). SKG mice were kindly provided by Dr. Abul Abbas, University of California, San Francisco (UCSF), USA. All experiments and procedures involving mice have been reviewed and approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University, USA.

### Induction and evaluation of experimental arthritis

Collagen-induced arthritis (CIA) was conducted as previously described [12]. Chicken type II collagen (Sigma-Aldrich, MO, USA) was emulsified with CFA containing 4 mg/ml heat-killed *M. tuberculosis* H37 (BD Biosciences, CA, USA) at 1:1 ratio (vol:vol). Male C57BL/6 mice at 8–10 weeks old were subjected to a single intradermal injection of the emulsion (100  $\mu$ l) at the base of the tail. For *in vivo* depletion of MDSCs [19], mice were administrated intraperitoneally with 50 mg/kg 5-Fluorouracil (5-Fu, Sigma-Aldrich, MO, USA) or PBS every 3 days from day 30 after the first immunization ( $n=8$  per group). Signs of arthritis in mice were monitored according to the swelling and redness of each limb, the severity of which was scored in a blinded manner [20]. For SKG mice, 8-week-old female mice were intraperitoneally (*i.p.*) injected 2 mg zymosan (Sigma-Aldrich, MO, USA) to facilitate the onset of disease.

### Statistical analysis

The probability-probability plot (P-P plot) was used to examine data normality. For normally distributed data, differences between various groups were evaluated using unpaired two-tailed Student's *t* test or one-way ANOVA with Fisher Least Significant Difference (LSD) test. Mann-Whitney U or Kruskal-Wallis followed by Dunn's multiple comparisons test were used for non-normally distributed data. Spearman rank test was used for analysing correlation of non-normally distributed data. Generalized Estimating Equations was used for comparisons of repeated measurement data (i.e. arthritic scores). Fisher's exact test was used for categorical variables. Statistical analyses were performed using SPSS 20.0 (SPSS Inc., IL, USA) or SigmaPlot 12.5 (Systat Software Inc., CA, USA).  $P < 0.05$  were considered statistically significant.

Detailed experimental procedures are described in [supplementary materials](#), available at *Rheumatology* online

## Results

### M-MDSCs correlate with bone destruction in RA and promote a pro-osteoclastogenic phenotype of Th17 cells

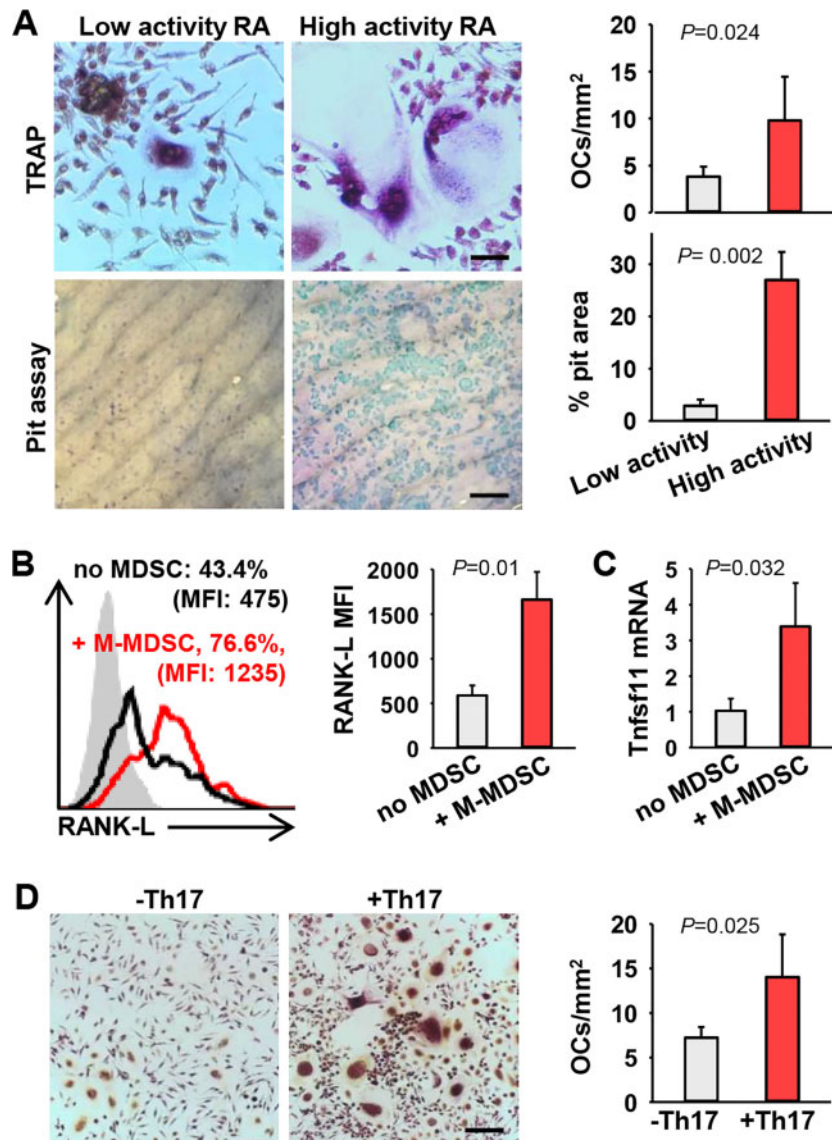
Our previous study reported that human MDSCs were significantly elevated in RA patients and correlated with the level of inflammatory cytokine IL-17A [12]. Given that IL-17-producing Th17 cells can promote RANK-L signalling dependent OC differentiation [21, 22], we analysed the frequency of MDSCs and joint destruction in RA patients to determine a potential association between these cells and bone erosion. Detailed clinical and laboratory characteristics and treatment information of included patients are presented in [Supplementary Tables S2 and S3](#), available at *Rheumatology* online. We

showed that the frequency of MDSCs or M-MDSCs, not G-MDSCs, positively correlated with bone erosion scores as assessed by joint ultrasound in RA patients ([Supplementary Table S1](#), available at *Rheumatology* online). This correlation was also supported by significantly higher levels of TRAP5b and  $\beta$ -CTX, two bone destruction markers [23, 24], in RA patients with more circulating M-MDSCs ([Supplementary Table S1](#), available at *Rheumatology* online). We further showed that CD11b<sup>+</sup>CD33<sup>+</sup>HLA-DR<sup>-/low</sup>CD14<sup>+</sup>CD15<sup>-</sup> M-MDSCs, isolated from peripheral blood of RA patients, were able to differentiate into TRAP<sup>+</sup> OCs that can induce bone resorption ([Fig. 1A](#)). Intriguingly, M-MDSCs from RA patients with high disease activity were more readily to differentiate into OCs as compared with those from patients with low disease activity ([Fig. 1A](#)), suggesting that the intrinsic OC-differentiating potential of M-MDSCs may be determined by the disease status.

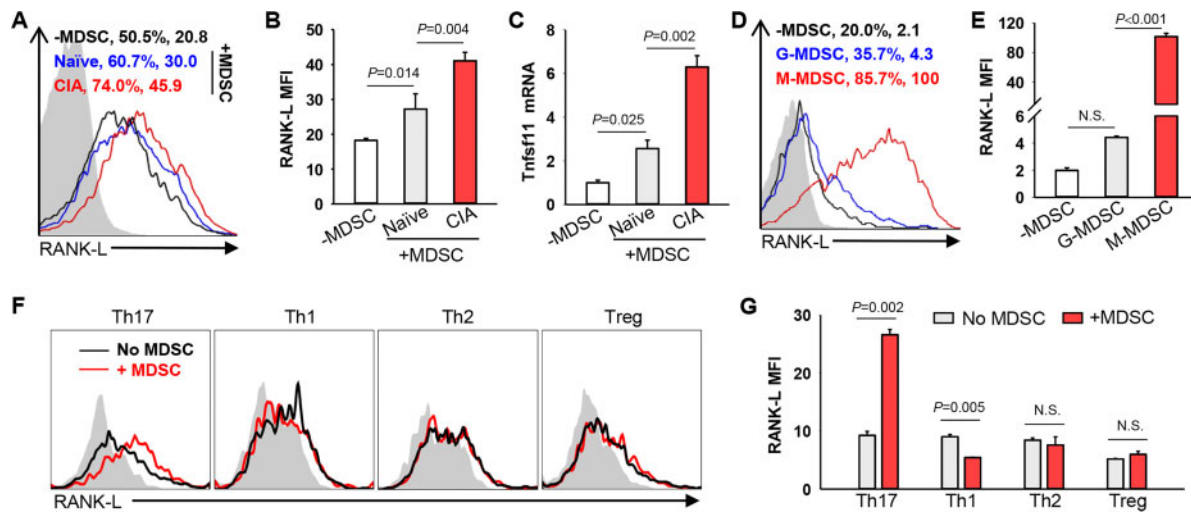
During the study of human MDSC-Th17 interaction, we made a surprising observation that M-MDSCs from RA patients upregulated membrane-bound RANK-L on autologous Th17 cells during co-culture ([Fig. 1B](#)). Upregulation of RANK-L was also indicated by increased gene transcription of *Tnfsf11* (i.e. *rankl*) in Th17 cells, assessed by quantitative real-time PCR (qRT-PCR) analysis ([Fig. 1C](#)). Our result suggests that, in addition to its intrinsic OC-differentiating capability, M-MDSCs in RA has acquired the capability to skew Th17 cells towards a pro-osteoclastogenic phenotype. Indeed, M-MDSC-conditioned human Th17 cells were highly efficient in supporting OC differentiation from M-MDSCs ([Fig. 1D](#)), suggesting that reciprocal actions of MDSCs and Th17 cells during their interaction may contribute to bone destruction in RA patients.

### MDSCs induce the expression of RANK-L preferentially by Th17 cells in mouse arthritis model

We next sought to use mouse model of RA, i.e. collagen-induced arthritis (CIA) that resembles the pathogenesis of human RA, to critically understand this previously unrecognized immune-osteoclastogenic pathway. We showed that the presence of MDSCs, especially those from arthritic mice (MDSC-CIA), significantly enhanced the expression of RANK-L on Th17 cells ([Fig. 2A and B](#)). Upregulation of RANK-L was also indicated by increased transcription of *Tnfsf11* gene in Th17 cells ([Fig. 2C](#)). Compared with G-MDSCs, M-MDSCs from arthritic mice are more efficient in stimulating RANK-L expression by Th17 cells ([Fig. 2D and E](#)), which is consistent with our previously reported superior Th17-promoting capacity of this subpopulation [12, 14]. Intriguingly, MDSCs failed to induce T-cell-associated RANK-L expression when co-cultured with Th1, Th2 or Treg cells ([Fig. 2F and G](#)), suggesting that arthritis-expanded MDSCs selectively stimulate the pro-osteoclastogenic activity in Th17 cells.

**Fig. 1** Human M-MDSCs from RA patients promote osteolysis

**(A)** M-MDSCs from peripheral blood of RA patients with low disease activity (28-joint disease activity score, DAS28  $\leq$  3.2) or high disease activity (DAS28  $>$  5.1) were cultured in the presence of M-CSF and RANK-L *in vitro* for 14 days. TRAP staining and pit assay were performed to assess differentiation and biological activity of OCs. Scale bars in the images of TRAP and pit assay represent 50  $\mu$ m and 200  $\mu$ m, respectively (TRAP,  $P=0.024$ ; Pit,  $P=0.002$ , unpaired *t* test). **(B)** Naïve CD4<sup>+</sup> T cells from RA patients were co-cultured with or without autologous M-MDSCs under Th17-polarizing conditions for 5 days. Membrane-bound RANK-L expression on Th17 cells was analysed by flow cytometry. Percentage of RANK-L positive Th17 cells and mean fluorescence intensity (MFI, difference between the fluorescence intensity and isotype control) of RANK-L are shown ( $P=0.01$ , unpaired *t* test). **(C)** Relative mRNA levels of *Tnfsf11* gene were analysed by qRT-PCR ( $P=0.032$ , unpaired *t* test). **(D)** Naïve CD4<sup>+</sup> T cells from RA patients were co-cultured with autologous M-MDSCs under Th17-polarizing conditions *in vitro* to generate M-MDSC-conditioned Th17 cells. M-MDSCs were then cultured with or without these Th17 cells in the presence of M-CSF, IL-2 and RANK-L for OC differentiation ( $P=0.025$ , unpaired *t* test). Scale bar, 200  $\mu$ m. Data are representative of three independent experiments.

**Fig. 2** MDSCs from arthritic mice augment expression of pro-osteoclastogenic RANK-L on Th17 cells

(A) and (B) MDSCs derived from naïve or CIA mice were co-cultured with naïve CD4<sup>+</sup>CD62L<sup>+</sup> T cells under Th17-polarizing conditions for 48h. Membrane-bound RANK-L expression on Th17 cells was analysed by flow cytometry. Representative flow charts showing the fluorescence intensity of RANK-L staining are presented (A) (Naïve vs -MDSC,  $P = 0.014$ ; CIA vs Naïve,  $P = 0.004$ ). One-way ANOVA with Fisher LSD test). (C) Relative mRNA levels of *Tnfsf11* gene were analysed by qRT-PCR (Naïve vs -MDSC,  $P = 0.025$ ; CIA vs Naïve,  $P = 0.002$ ). One-way ANOVA with Fisher LSD test). (D) and (E) M-MDSCs or G-MDSCs were cultured with naïve CD4<sup>+</sup>CD62L<sup>+</sup> T cells under Th17-polarizing conditions (G-MDSC vs -MDSC,  $P = 0.303$ ; M-MDSC vs G-MDSC,  $P < 0.001$ ). One-way ANOVA with Fisher LSD test). (F) *In vitro* polarized Th17, Th1, Th2 or Treg cells were co-cultured with or without MDSCs in the presence of plate-bound anti-CD3 Ab for 24h. RANK-L expression on T cells was analysed by flow cytometry (Th17,  $P = 0.002$ ; Th1,  $P = 0.005$ ; Th2,  $P = 0.496$ ; Treg,  $P = 0.152$ . Unpaired *t* test). The results shown are representative of three independent experiments. N.S., not significant.

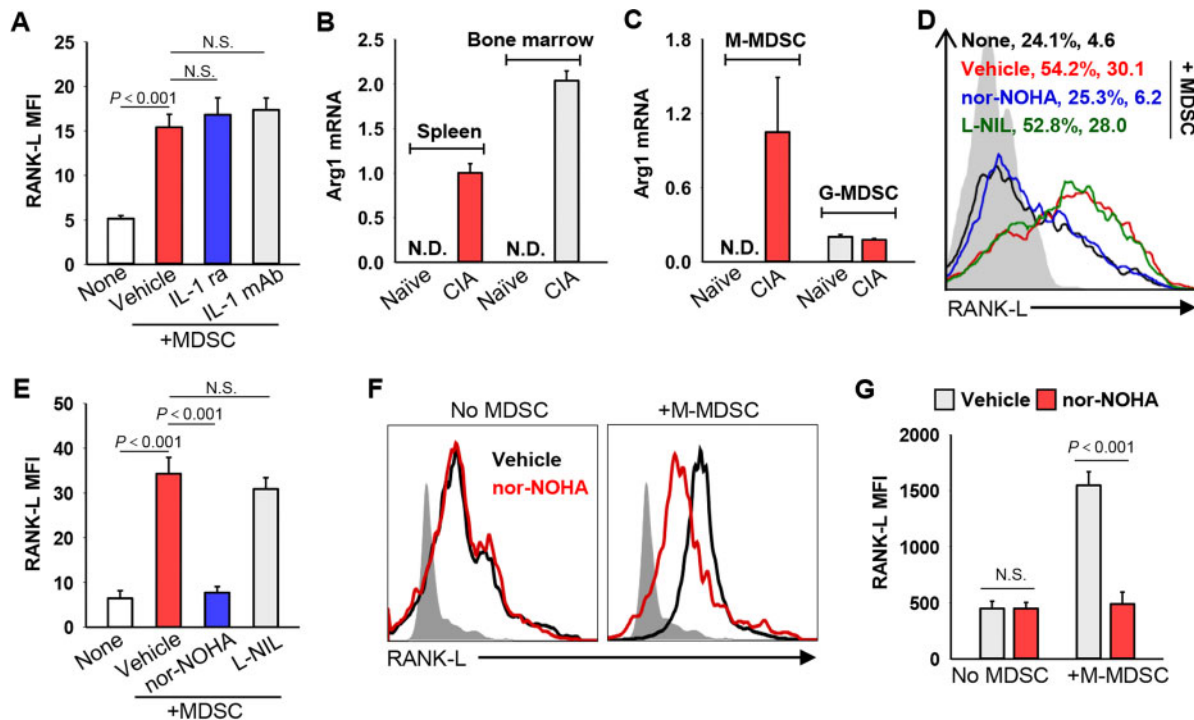
#### Arginase 1 is required for MDSC-enhanced RANK-L expression on Th17 cells

We examined the potential molecular factors that are responsible for MDSC-enhanced RANK-L expression. Considering that IL-1 $\beta$  is critical for Th17 polarization driven by MDSCs under autoimmune conditions [12, 14], we first assessed the role of IL-1 $\beta$  using either IL-1 $\beta$  mAbs or IL-1 receptor antagonist. However, the activity of MDSC-CIA in promoting RANK-L production from Th17 cells was not impaired by blockade of IL-1 $\beta$  signalling (Fig. 3A), suggesting that IL-1 $\beta$  is not required for MDSC-CIA enhanced RANK-L expression. In light of our recent report that the MDSC-associated effector molecule arginase 1 mediates the MDSC-Th17 interplay in SLE [13], we examined the expression of *arginase 1* (*Arg1*) in CIA-associated MDSCs by qRT-PCR. Gene transcription of *Arg1* was only detectable in MDSCs derived from arthritic mice, not those from naïve mice (Fig. 3B). The mRNA levels of *Arg1* were higher in M-MDSCs than in G-MDSCs in arthritic mice (Fig. 3C). To test the contribution of arginase 1 to MDSC-enhanced RANK-L expression, we blocked arginase 1 activity in MDSCs using the arginase inhibitor N-omega-hydroxy-nor-L-arginine (nor-NOHA) [25] during cell co-culture. The presence of nor-NOHA, not L-NIL that inhibits inducible nitric oxide synthase [26], abolished MDSC-CIA facilitated RANK-L upregulation (Fig. 3D and E).

Additionally, nor-NOHA reduced the capability of M-MDSCs from patients with RA to induce RANK-L production. However, it did not affect RANK-L expression on Th17 cells in the absence of MDSCs (Fig. 3F and G). Therefore, MDSCs enhance the pro-inflammatory and pro-osteoclastogenic phenotypes of Th17 cells in RA through independent pathways.

#### Arthritis-associated M-MDSCs not G-MDSCs exhibit the OC-differentiating capacity

Given that human MDSCs from patients with RA can differentiate into fully functional OCs, we also compared the OC-differentiating capacity of MDSCs from naïve or CIA mice in the presence of M-CSF and RANK-L. CIA-associated MDSCs displayed an increase in its potential of differentiation into OCs, indicated by elevated numbers of OCs (Fig. 4A), heightened transcription of *Acp5* (tartrate-resistant acid phosphatase), *Ctsk* (cathepsin k) or *Mmp9* (matrix metalloproteinase 9) genes (Fig. 4B), as well as enhanced activity in bone degradation (Fig. 4C). SKG mice, a genetic model of autoimmune arthritis [27], were also used to examine MDSC-initiated osteoclastogenesis and bone absorption. Consistent with our study in CIA model [12], we showed a substantial expansion of MDSCs including CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>high</sup> M-MDSCs and CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>low</sup> G-MDSCs in arthritic SKG mice (Supplementary Fig. S2A, available at *Rheuma*

**Fig. 3** Arginase 1 is involved in MDSC-enhanced RANK-L expression on Th17 cells

(A) Naïve CD4<sup>+</sup>CD62L<sup>+</sup> T cells were co-cultured with MDSCs from arthritic mice (i.e. CIA) under Th17 polarizing conditions in the presence of IL-1 receptor antagonist (IL-1ra) or IL-1 $\beta$  neutralizing antibodies (IL-1 mAb) for 48 h. RANK-L expression was examined by flow cytometry (Vehicle vs None,  $P < 0.001$ ; IL-1 ra vs Vehicle,  $P = 0.247$ ; IL-1 mAb vs Vehicle,  $P = 0.123$ ). One-way ANOVA with Fisher LSD test. (B) Relative mRNA levels of *Arg1* in MDSCs purified from indicated mice were analysed by qRT-PCR. (N.D., not detected). (C) Transcription of *Arg1* gene in M-MDSCs or G-MDSCs was determined by qRT-PCR. (D) and (E) RANK-L expression was examined after mouse Th17 polarization in the presence of L-NIL or nor-NOHA (Vehicle vs None,  $P < 0.001$ ; nor-NOHA vs Vehicle,  $P < 0.001$ ; L-NIL vs Vehicle,  $P = 0.131$ ). One-way ANOVA with Fisher LSD test. (F) and (G) Naïve CD4<sup>+</sup> T cells from RA patients were co-cultured in the presence of M-MDSCs and/or nor-NOHA under Th17-polarizing conditions for 3 days. RANK-L expression on Th17 cells was examined (nor-NOHA vs Vehicle: No MDSC,  $P = 0.986$ ; +M-MDSC,  $P < 0.001$ ). One-way ANOVA with Fisher LSD test). Data are representative of three independent experiments. N.S., not significant.

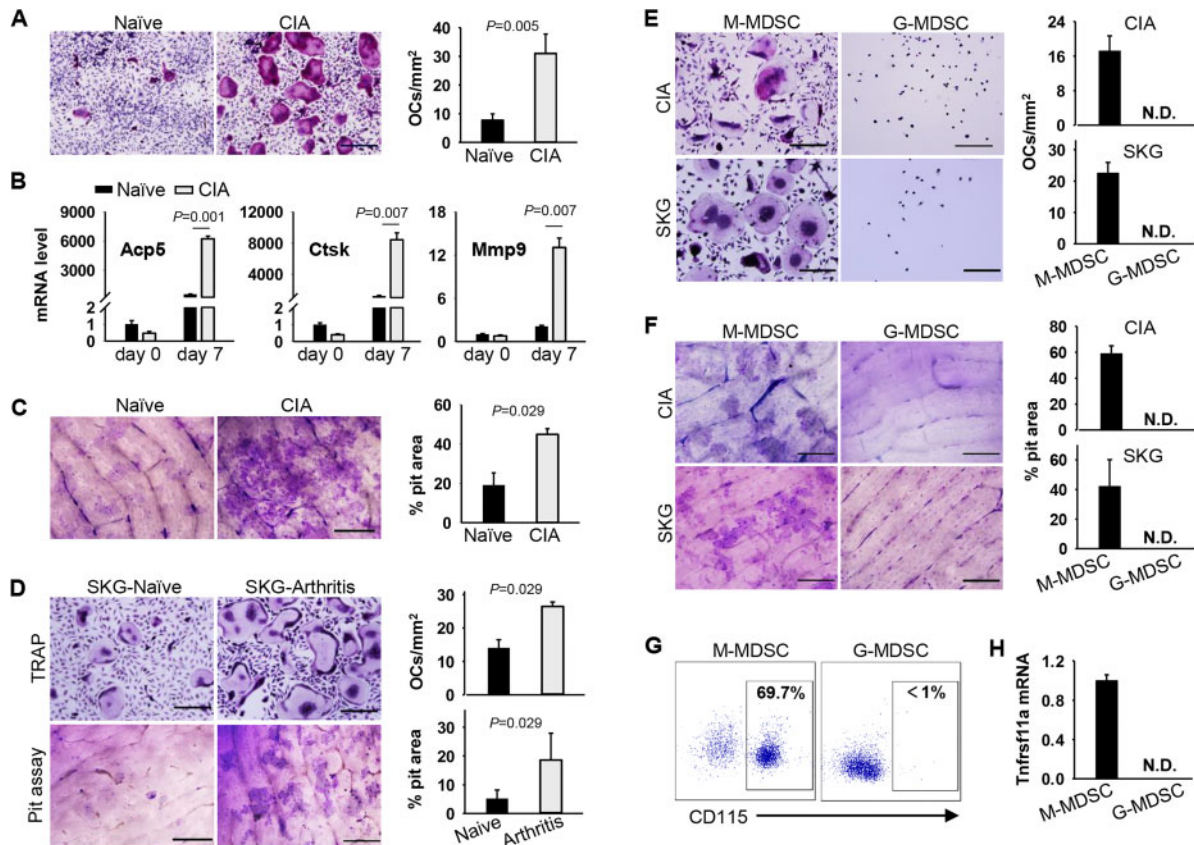
ology online). MDSCs from arthritic SKG mice inhibited the proliferation of and IFN- $\gamma$  production by T cells upon stimulation with anti-CD3/CD28 antibodies (Supplementary Fig. S2B and C, available at *Rheumatology* online), indicating these cells retain immunosuppressive activity. Similarly, MDSCs from arthritic SKG mice were also more readily to differentiate into TRAP-positive OCs and to induce bone resorption (Fig. 4D), which was associated with increased gene transcription of the OC signature genes *Acp5*, *Ctsk* or *Mmp9* (Supplementary Fig. S3, available at *Rheumatology* online).

Considering that M-MDSCs and G-MDSCs not only are phenotypically and morphologically distinct, but also have different functional characteristics [28], we further examined the OC-differentiating capacity of these two MDSC subsets. TRAP staining and pit assays showed that M-MDSCs derived from CIA mice or arthritic SKG mice, not G-MDSCs, were able to differentiate into OCs (Fig. 4E and F). Consistent with their differential OC-differentiating capacities, G-MDSCs displayed

significantly lower levels of the M-CSF receptor CD115 (Fig. 4G) and the RANK-L receptor RANK (Fig. 4H), as compared with M-MDSCs. These suggest that only arthritis-associated M-MDSCs exhibit a unique potential of differentiating into functionally active OCs.

#### MDSC-Th17 interaction potentiates MDSC differentiation into OCs

Because RANK-L expression on Th17 cells is upregulated by MDSCs with the intrinsic OC-differentiating compacity, we address the question of whether the MDSC-Th17 interplay further promotes the differentiation of MDSCs into OCs in RA. To test this, Th17 cells were polarized in the presence of MDSC-CIA to produce MDSC-conditioned Th17 cells, which were then co-cultured with MDSCs that have been primed with M-CSF for induction of RANK expression (Supplementary Fig. S4, available at *Rheumatology* online). Differentiation of MDSCs into TRAP<sup>+</sup> OCs was barely

**Fig. 4** MDSCs from arthritic mice display enhanced capacity in differentiation into functional OCs

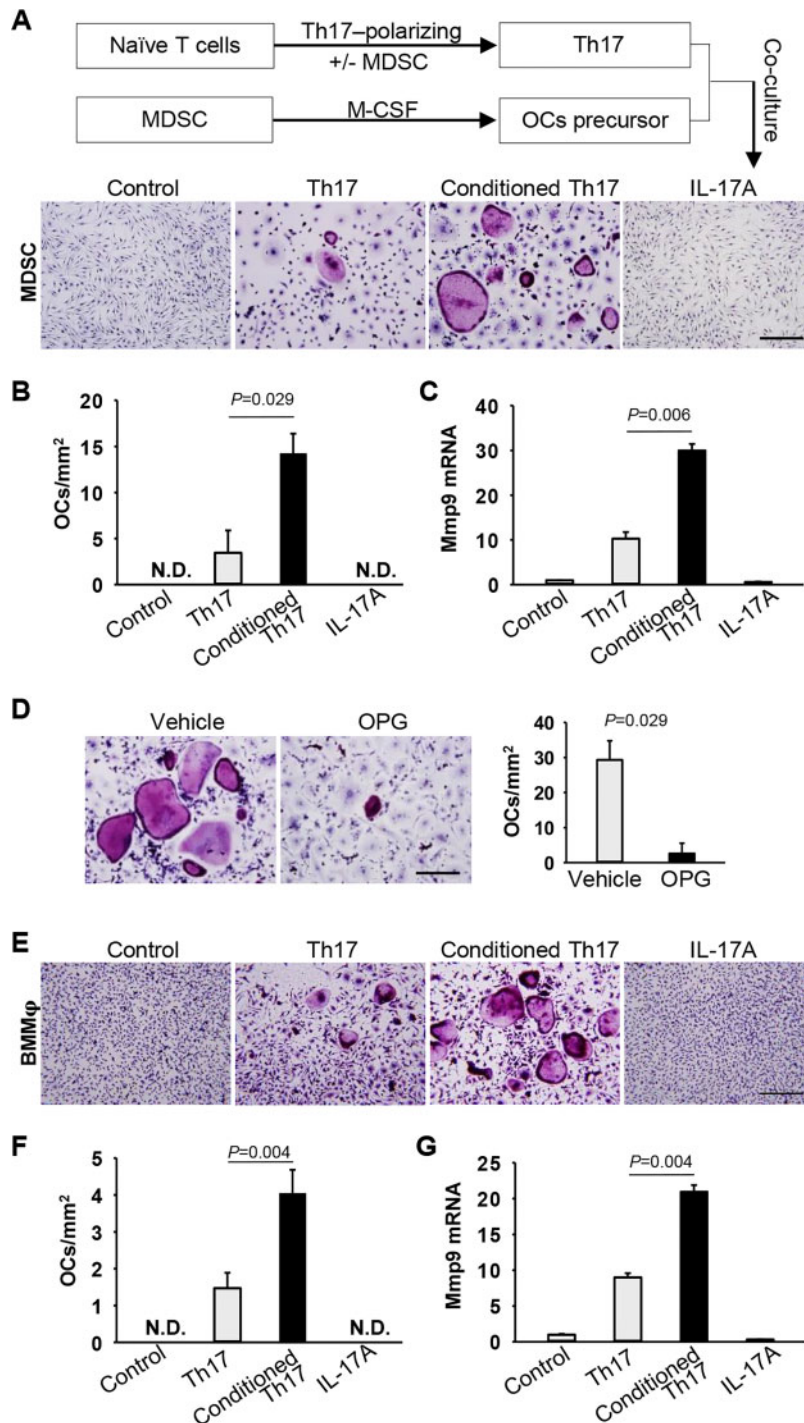
(A) MDSCs from naïve or CIA mice were cultured in the presence of M-CSF and RANK-L for OCs differentiation. Representative images of TRAP staining and OCs numbers are shown ( $P=0.005$ , Unpaired  $t$  test). (B) Relative mRNA levels of *Acp5*, *Ctsk*, and *Mmp9* genes were assessed using qRT-PCR (*Acp5*,  $P=0.001$ ; *Ctsk*,  $P=0.007$ ; *Mmp9*,  $P=0.007$ , Unpaired  $t$  test). (C) MDSCs were seeded on bone slices in the presence of M-CSF and RANK-L. Bone resorption pit was examined using pit assays ( $P=0.029$ , Mann-Whitney  $U$  test). (D) MDSCs sorted from naïve or arthritic SKG mice were differentiated into OCs as described above ( $P=0.029$ , Mann-Whitney  $U$  test). (E) and (F) OC-differentiating potential of M-MDSCs or G-MDSCs and bone resorption activity were assessed. (G) Expression of CD115 on MDSCs subsets was assayed by flow cytometry. (H) Transcription of *Tnfrsf11a* (*RANK*) was determined by qRT-PCR. Scale bar, 200  $\mu$ m. Results shown are representative of three independent experiments. N.D., not detected.

detected when co-culturing with non-conditioned Th17 cells. In sharp contrast, Th17 cells that had been pre-conditioned with MDSC-CIA significantly enhanced MDSC-derived OC differentiation (Fig. 5A–C). This increased OC formation was independent of IL-17A, as addition of exogenous IL-17A failed to induce OC differentiation from MDSCs (Fig. 5A–C). However, use of osteoprotegerin (OPG), a soluble decoy RANK-L receptor that inhibits the RANK-L/RANK signalling, abrogated the pro-osteoclastogenic activity of MDSC-CIA-conditioned Th17 cells (Fig. 5D), supporting a requirement of the RANK-L signal that is derived from Th17 cells. Additionally, this pro-osteoclastogenic activity of MDSC-conditioned Th17 cells was further validated using bone marrow-derived macrophages as OC precursors (Fig. 5E–G). Therefore, interaction with MDSCs confers Th17 cells with a pro-osteoclastogenic phenotype

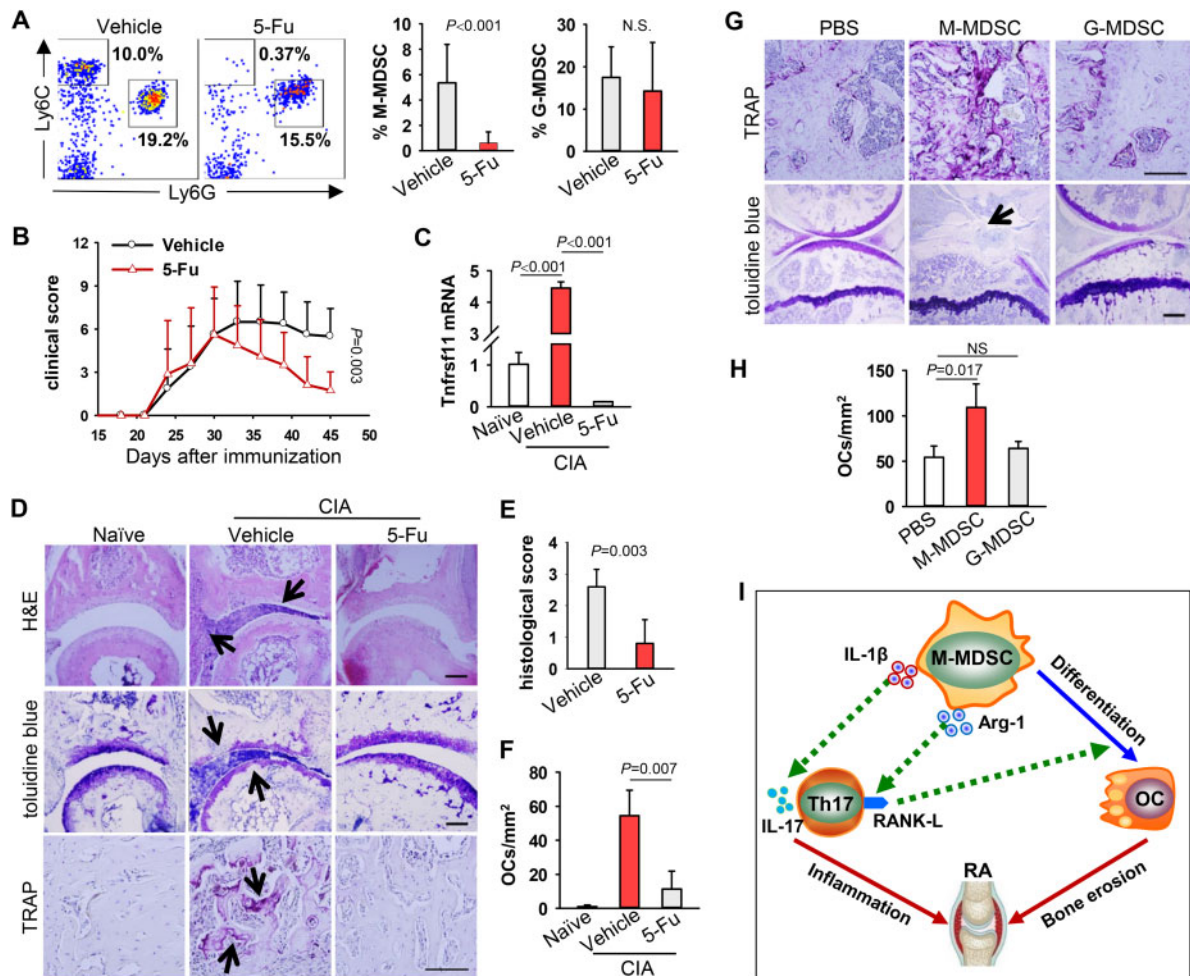
that in return supports RANK-L-dependent differentiation of MDSCs or other OC precursors into OCs.

#### M-MDSCs are involved in osteoclastogenesis and osteolysis in arthritic mice

We next examined the pathogenic role of MDSCs in inflammatory arthritis using 5-Fluorouracil (5-Fu) that can selectively remove MDSCs without affecting other immune cell types such as T cells, natural killer cells, dendritic cells or B cells [19]. Arthritic mice were administered with 5-Fu at a dose of 50 mg/kg every 3 days starting at the disease onset. We showed that 5-Fu treatment selectively depleted M-MDSCs, not G-MDSCs or other immune cells (Fig. 6A, Supplementary Fig. S5, available at *Rheumatology* online). Removal of M-MDSCs significantly reduced the severity of arthritis,

**Fig. 5** MDSC–Th17 interplay facilitates MDSC differentiation into OCs

**(A)–(C)** Th17 cells were polarized from naïve CD4<sup>+</sup>CD62L<sup>+</sup> T cells in the presence or absence of MDSCs from CIA mice. Th17 cells were then co-cultured with MDSCs in the presence of M-CSF and IL-2 without addition of exogenous RANK-L for 5 days. IL-17A was added as a control. Representative images of TRAP staining **(A)** and statistical analysis of OCs number **(B)** are shown (Th17 vs Conditioned Th17,  $P=0.029$ , Mann–Whitney *U* test). **(C)** Relative mRNA level of *Mmp9* gene was determined by qRT-PCR (Th17 vs Conditioned Th17,  $P=0.006$ , Unpaired *t* test). **(D)** MDSCs were co-cultured with MDSC-conditioned Th17 cells with or without osteoprotegerin (OPG) ( $P=0.029$ , Mann–Whitney *U* test). **(E)–(G)** Bone marrow derived macrophages (BMM $\phi$ ) were co-cultured with indicated Th17 cells in the presence of M-CSF and IL-2 for 5 days. OCs number **(F)** and relative *Mmp9* level **(G)** were determined [Th17 vs Conditioned Th17:  $P=0.004$  **(F)**;  $P=0.004$  **(G)**, Unpaired *t* test]. Scale bar, 200  $\mu$ m. Data shown represent three independent experiments. N.D., not detected.

**Fig. 6** Arthritis-associated M-MDSCs differentiate into functional OCs *in vivo*

(A) CIA mice ( $n=8$ ) were administrated *i.p.* with 50 mg/kg 5-Fu or PBS every 3 days from day 30 after the collagen immunization. Frequency of MDSC subsets in peripheral blood was assessed on day 50. N.S., not significant (M-MDSC,  $P<0.001$ ; G-MDSC,  $P=0.505$ , Mann-Whitney  $U$  test). (B) Clinical scores during arthritic development were recorded (Vehicle vs 5-Fu,  $P=0.003$ , Generalized Estimating Equations). (C) mRNA levels of *Tnfsf11* gene in inflamed paws were analysed by qRT-PCR (Vehicle vs Naive,  $P<0.001$ ; 5-Fu vs Vehicle,  $P<0.001$ , One-way ANOVA with Fisher LSD test). (D) Pathology of paws was assayed by H&E, toluidine blue or TRAP staining. Representative images of TRAP staining in proximal metatarsal epiphysis are shown. Arrows indicate infiltrated inflammatory cells (top panel, H&E), cartilage damage (middle panel, toluidine blue) and TRAP<sup>+</sup> OCs (bottom panel, TRAP<sup>+</sup>), respectively. (E) Pathology scores of bone destruction were assessed. (F) Numbers of TRAP<sup>+</sup> cells in the sections were quantified (vehicle vs 5-Fu,  $P=0.007$ , Mann-Whitney  $U$  test). (G) Arthritic mice depleted of MDSCs by 5-Fu were injected with PBS, M-MDSCs or G-MDSCs isolated from CIA mice into the tibia ( $n=5$ ). TRAP or toluidine blue staining were performed to evaluate OC differentiation and cartilage damage, respectively. Representative images of TRAP staining in proximal tibial epiphysis are shown. Arrow indicates cartilage damage as shown by toluidine blue staining. (H) Number of TRAP<sup>+</sup> cells in G was quantified. Scale bar = 100  $\mu$ m. (PBS vs M-MDSC,  $P=0.017$ ; PBS vs G-MDSC,  $P=0.865$ , Kruskal-Wallis test followed by Dunn's multiple comparisons). Data represent three independent experiments. (I) Proposed model for the role of MDSC-Th17 interplay in RA. M-MDSCs with inherent OC-differentiating capability display versatile functions during their interaction with Th17 cells. While promoting Th17 differentiation via pro-inflammatory IL-1 $\beta$  signalling, M-MDSCs can also upregulate RANK-L on Th17 cells in an arginine 1-dependent fashion. Th17-derived RANK-L, in return, further drives programming of M-MDSCs into bone-destructing OCs.

indicated by decreased swelling of inflamed paws (Fig. 6B). Consistent with our previous report [12], absence of M-MDSCs resulted in decreased infiltration of

Th17 cells (Supplementary Fig. S6, available at *Rheumatology* online). Ablation of M-MDSCs also caused a significant reduction of *Tnfsf11* gene

transcription in inflamed paws (Fig. 6C). Histology analysis of the paws in M-MDSC-depleted mice showed reduced infiltration of inflammatory cells, ameliorated cartilage damage, and decreased numbers of OCs (Fig. 6D–F). The reduced osteoclastogenesis in the absence of M-MDSCs was also supported by decreased transcription of *Acp5*, *Ctsk* or *Mmp9* genes in the inflamed paws (Supplementary Fig. S7, available at *Rheumatology* online).

Lastly, we performed adoptive transfer of MDSCs to confirm MDSC-promoted osteoclastogenesis and bone resorption *in vivo*. Type II collagen/CFA immunized mice were first treated with 5-Fu to remove endogenous MDSCs, followed by intramedullary injection of CIA mice-derived M-MDSCs or G-MDSCs into the tibia of recipient mice. Elevation in the numbers of OCs were only seen in the tibia of mice that had been transferred with M-MDSCs, not G-MDSCs (Fig. 6G and H). This increased osteoclastogenesis in mice receiving M-MDSCs correlated with significantly aggravated cartilage damage (Fig. 6G).

## Discussion

Despite their intrinsic immunosuppressive activity, our previous work established a highly pro-inflammatory feature of MDSCs in sustaining inflammation by amplifying a Th17 response in autoimmune disorders including RA [12–14]. Our research in the present study reveals a novel pro-osteoclastogenic activity of MDSCs in RA, highlighting their functional plasticity and versatility in disease pathogenesis. More importantly, we uncover a previously undefined signalling feedback loop engaged during the MDSC–Th17 interplay, which contributes to the dysregulation of osteoclastogenesis and bone destruction in RA.

To the best of our knowledge, this is the first report that MDSCs or M-MDSCs in patients with RA not only positively correlate with clinical bone erosion scores or markers, but also acquire the increased OC-differentiating capacity. This is further supported by our finding that M-MDSCs from patients with high disease activity were more readily to differentiate into bone resorption capable OCs than those from patients with low disease activity. It is likely that the inflammatory milieu governs the OC-differentiating potential of MDSCs. Our clinical data are consistent with the recent reports of MDSCs as OC progenitors in mouse models of cancer [29] and arthritis [30]. Furthermore, using two mouse arthritic models (i.e. CIA, SKG), we demonstrate that M-MDSCs display a superior OC-differentiating capacity as compared with G-MDSCs, which may be attributed to the high expression of CD115 and RANK required for OC programming. These results advance the understanding of the functional distinctions of these two MDSC subsets in RA-associated osteoclastogenesis.

In addition to the inherent OC-differentiating property of RA-associated human MDSCs, we have made an

important finding in this work that MDSCs, particularly M-MDSCs, can program human Th17 cells towards a pro-osteoclastogenic phenotype during their interaction, indicated by upregulation of RANK-L in autologous Th17 cells. Moreover, this Th17 cell-derived RANK-L signal is functionally significant, because Th17 cells conditioned by RA-associated MDSCs greatly potentiate the MDSC differentiation into OCs. Our extensive studies in arthritic mice (i.e. CIA, SKG) also similarly elucidate MDSC-enhanced production of RANK-L by Th17 cells, which can further promote the MDSC-derived OC differentiation dependent on the RANK signalling. Intriguingly, we show that the elevated pro-osteoclastogenic signal on Th17 cells appears to drive the differentiation of OCs from their other known precursors, such as macrophages [12]. Given that Th17 cells have been shown to promote arthritic development by exaggerating inflammation [31–33] and promoting bone erosion [21, 22], the results from this study may have broad implications in understanding of an intimate relationship between inflammation and osteoclastogenesis in RA or other autoimmune conditions.

While retaining immunosuppressive activity, mouse MDSCs display reduced suppressive activity on T cells during arthritic progression (i.e. CIA) [12]. These MDSCs associated with autoimmune arthritis [12] or other inflammatory disorder [13, 14] have acquired a pro-inflammatory phenotype indicated by their production of cytokines (e.g. IL-1 $\beta$ , TNF- $\alpha$ ) as well as pro-osteoclastogenic activities as reported in our current study. This functional plasticity is likely to be dictated by the distinct inflammatory signals present in the inflamed sites. We postulate that these pro-inflammatory and pro-osteoclastogenic functions become dominant over their intrinsic T-cell suppressive activity, and collaboratively perpetuate multiple pro-arthritic processes.

The IL-1 $\beta$  signalling was previously shown by us and others to primarily mediate MDSC-enhanced Th17 response under several autoimmune conditions [12, 14, 15]. Intriguingly, the ability of MDSCs to enhance the pro-osteoclastogenic activity of Th17 cells involves arginase 1 not IL-1 $\beta$  signalling. Although arginase 1 has been reported to mediate the immune suppressive function of cancer-associated MDSCs, the immune suppressive activity of MDSCs-CIA does not appear to be arginase 1 dependent [12]. The functional significance of arginase 1 is also supported by the fact that arginase 1 is mostly expressed by arthritis-associated MDSCs, not by those from naïve mice, which corresponds to their differential RANK-L-promoting capabilities. In view of the involvement of arginase 1 in regulating pro-osteoclastogenic and/or pro-inflammatory effect of MDSCs, therapeutically targeting arginase 1 may help alleviate joint inflammation and bone erosion in patients with RA. Given the well-documented role of the IL-6 signalling in pathogenesis of human RA [34] and IL-6-induced up-regulation of arginase 1 in MDSCs from the SLE patients [13], it is possible that the therapeutic

benefits of the IL-6 inhibitor in RA treatment may also be partially attributed to inhibition of arginase activity in MDSCs.

In the present study, we have also experimentally established a functional linkage of M-MDSCs with osteoclastogenesis and bone erosion in arthritic mice using both gain- and loss-of-function approaches. Depletion of M-MDSCs reduces numbers of OCs, cartilage damage and immune infiltration. However, transfer of M-MDSCs, not G-MDSCs, aggravates cartilage damage associated with elevation of OCs in inflamed paws. These striking results together with our recent reports [12–14] indicate that MDSCs, often expanded in auto-immune arthritis, not only promote polarization of inflammatory Th17 cells, but also define their pro-osteoclastogenic phenotype. However, Fujii *et al.* reported that adoptive transfer of MDSCs suppressed progression of collagen-induced arthritis in DBA/1 mice [35]. This discrepancy may be caused by the methodological differences (e.g. timing or number of cells for transfer). Indeed, the pathogenic role of MDSCs in promoting inflammation and arthritic progression has been confirmed by an independent study [15].

Our previous work established a highly pro-inflammatory role of MDSCs for exacerbating inflammation in autoimmune disorders [12–14]. Our current work identifies novel pro-osteoclastogenic function of MDSCs acquired in RA and elucidates an under-appreciated osteoimmune pathway involving complex interactions between M-MDSCs and Th17 cells (Fig. 6). In addition to driving Th17 polarization and IL-17 production, as we previously documented [12], MDSCs or M-MDSCs skew Th17 cells towards a pro-osteoclastogenic phenotype. Production of RANK-L by Th17 cells can serve as a feedback loop to program M-MDSCs or other OC precursors into OCs. These reciprocal and distinctive actions of MDSCs and Th17 cells collaboratively perpetuate multiple pathogenic processes, including but not limited to aggravated inflammation and osteolysis. Targeting the pro-arthritic MDSC-Th17 axis based on a precise understanding of the nature of this cellular crosstalk may help alleviate disease symptoms by concurrently antagonizing abnormal inflammation as well as osteoclastogenesis in RA.

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## Data availability statement

All data relevant to the present study are included in the article or uploaded as online supplementary information. All data are available upon request.

## Supplementary data

Supplementary data are available at *Rheumatology* online.

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