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Roles of JAK-STAT signaling and granulocyte–macrophage colony-stimulating factor in the development of osteitis and bone microstructure changes in rheumatoid arthritis

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Abstract

Background Osteitis on magnetic resonance imaging (MRI) and bone microstructure changes (BMC) on high-resolution peripheral quantitative computed tomography (CT) are the earliest signs of arthritis, preceding the development of bone erosion on X-ray in rheumatoid arthritis (RA). Recently, a Janus kinase (JAK) inhibitor, baricitinib, reportedly suppresses these early changes. This study aimed to elucidate the underlying molecular mechanism of osteitis and BMC using an animal model of RA and human samples.

Methods Osteitis and BMCs were assessed via MRI and micro-CT, respectively, in zymosan-treated SKG mice. Bone marrow (BM) cells were analysed via flow cytometry. JAK–signal transducer and activator of transcription (STAT) cytokine expression was measured via performing quantitative PCR. Zymosan-treated SKG mice received baricitinib, anti-granulocyte–macrophage colony-stimulating factor (GM-CSF) antibodies, or recombinant GM-CSF (r-GM-CSF). r-GM-CSF was also used in an in vitro osteoclast differentiation assay. Osteoclast differentiation was also investigated with human monocytes.

Results Osteitis and BMCs occurred prior to arthritis in SKG mice. Granulocyte–macrophage-lineage cells and osteoclast precursor cells (OCPs) expanded in the inflammatory BM. Receptor activator of nuclear factor kappa-B ligand-positive osteoclasts were observed at BMC sites. The expression of GM-CSF, a JAK–STAT cytokine, was upregulated in osteitic BM. Both baricitinib and anti-GM-CSF antibodies suppressed osteitis and BMCs, whereas r-GM-CSF exacerbated these changes. In vitro addition of r-GM-CSF to osteoclast differentiation assay markedly increased the number and size of osteoclasts, especially when added in the late phase of osteoclast differentiation both in mice and humans.

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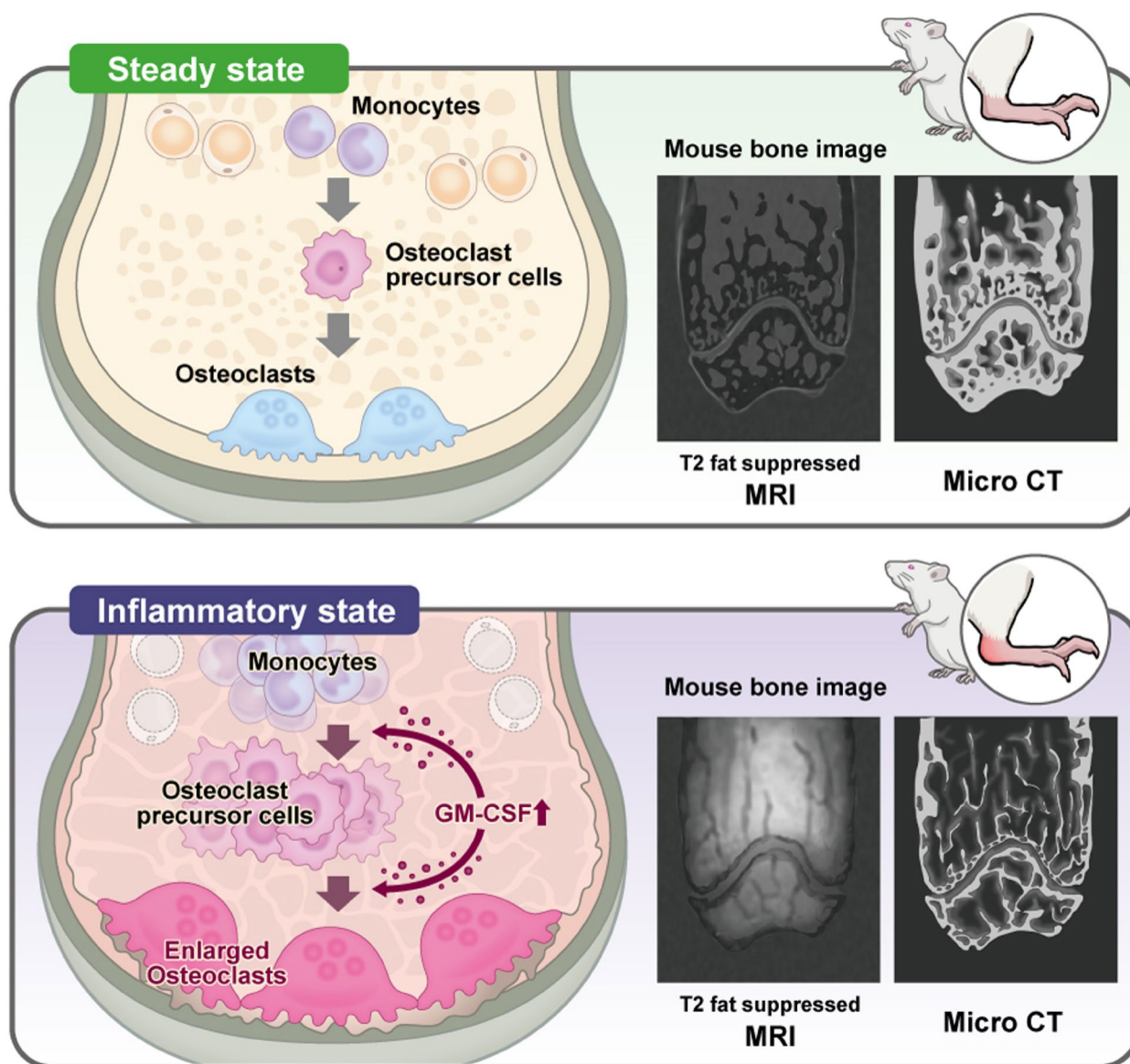
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Conclusions GM-CSF drives osteitis and BMCs by increasing granulocyte–macrophage-lineage cells and OCPs and promoting osteoclast differentiation. Targeting GM-CSF is a potential therapeutic strategy to prevent early radiographic changes in RA.

Graphical Abstract



Keywords Rheumatoid arthritis, Granulocyte–macrophage colony-stimulating factor, Osteitis, Bone microstructural changes, JAK–STAT signaling

Introduction

Rheumatoid arthritis (RA) is the most prevalent autoimmune inflammatory disease, characterized by persistent inflammation and progressive destruction of bone and joint structures [1]. Historically, periarticular osteoporosis observed by conventional radiography has been considered an early feature of RA [2]. However, emerging

imaging modalities have redefined early bone pathology: osteitis detectable by magnetic resonance imaging (MRI) and bone microstructural changes (BMCs) identified by micro-computed tomography (micro-CT) are now recognized as among the earliest radiographic features of RA. Critically, these bone lesions not only precede overt

erosions but also predict their progression [3–6], and have been observed even in the preclinical phases of RA [7–9].

Despite their clinical relevance, the cellular and molecular mechanisms linking osteitis to BMC formation remain poorly understood. Osteitis is associated with infiltration of monocyte-lineage cells, yet the cytokine networks orchestrating this process are still undefined [10, 11]. Osteitic lesions progress to BMCs and erosions more frequently than lesions dominated by synovitis or tenosynovitis [5, 12, 13] indicating that osteitis is an independent driver of bone destruction.

Janus kinase (JAK) inhibitors, which broadly suppress inflammatory cytokine signalling, have been shown effective in ameliorating osteitis and BMCs in recent clinical trials [14, 15]. While, JAK inhibition enhances osteoblast function by up-regulating osteocalcin and activating Wnt pathways [16], its impact on osteoclast-mediated bone resorption remains unclear. Because osteoclasts are pivotal to structural damage, we hypothesised that specific JAK–signal transducer and activator of transcription (STAT)-dependent cytokines link osteitis to BMCs by driving the differentiation of monocytes into osteoclasts within osteitic lesion, thereby accelerating BMC formation.

To overcome the limitations of human studies in accessing bone tissue from patients with very early RA, we employed a murine model to dissect how JAK–STAT signalling governs the transition from osteitis to BMCs. Clarifying this pathway should refine our understanding of RA pathogenesis and reveal opportunities to intercept structural damage at its inception.

Methods

A concise description of the methods is provided below, with detailed methodologies described in the Supplementary Methods (Supplementary Methods).

Animal model and arthritis induction

We conducted experiments using BALB/c-background SKG mice, a murine model for RA carrying a ZAP-70 point mutation (W163C) that predisposes them to autoimmune arthritis. The mice were obtained from Prof. Shimon Sakaguchi (Osaka University, Osaka, Japan) and maintained under specific pathogen-free conditions at the Institute of Laboratory Animals, Kyoto University, Kyoto, Japan. All experiments were approved by the Kyoto University Animal Ethics Committee (approval number Medkyo 16106–23104). Before the experimental interventions, age- and sex-matched littermates from various parental cages were mixed and assigned randomly to each treatment and control group. Arthritis was induced in 8- to 13-week-old female mice via intraperitoneal injection of 2 mg zymosan (Sigma-Aldrich), with

phosphate-buffered saline (PBS) serving as a control. Arthritis scores were evaluated every 3–4 days.

Treatment protocols

Baricitinib (ChemScene) was dissolved in 0.5% methyl cellulose (FUJIFILM Wako) and administered orally. For arthritis treatment, age and arthritis score-matched SKG mice at week 5 received daily baricitinib or methyl cellulose daily until week 8. For prevention, baricitinib was administered daily from weeks 2 to 5 post-zymosan injection. Additional experiments included administering recombinant murine granulocyte – macrophage colony-stimulating factor (GM-CSF) or interleukin 6 (IL-6) (2 µg, PeproTech) intraperitoneally every 2 days (four doses) starting at week 4 or anti-GM-CSF antibody (100 µg) weekly beginning at week 2 (Biolegend).

Magnetic resonance imaging (MRI)

Femur osteitis was assessed via 7T MRI (BioSpec, Bruker). Anesthetized mice were scanned via a circular polarized volume coil with water – fat separation imaging modified from the rapid acquisition with a relaxation enhancement sequence. The data were processed via MATLAB (MathWorks, MA, USA), and osteitis scores were calculated by dividing the average region of interest intensity of the distal femoral bone marrow by that of the gastrocnemius muscle.

Micro-computed tomography (micro-CT)

Trabecular bone parameters in femurs were analysed via a SkyScan 1275 micro-CT system (Bruker) with 7-µm voxel resolution. Three-dimensional reconstructions and analyses were performed via NRecon (Bruker Corp., Billerica, MA, USA) and CTAn software (Bruker Corp.).

Histology and osteoclast staining

Femurs were fixed in 10% formalin, decalcified with 5% neutral buffered ethylenediaminetetraacetic acid, embedded in paraffin, sectioned (4 µm), and stained with hematoxylin and eosin (H&E) or tartrate-resistant acid phosphatase (TRAP) solution.

Bone marrow cell isolation and flow cytometry

The BM was harvested by flushing femurs with D-PBS and processing them into single-cell suspensions. FcγR blocking was performed prior to staining for extracellular and intracellular antigens. Cell sorting (> 95% purity) was conducted on a FACS Melody system (BD Biosciences) and analyzed via FlowJo v10.8.1 (<https://www.flowjo.com/>).

Gene expression analysis

RNA was extracted from BM cells, converted to cyclic DNA, and subjected to quantitative PCR via TaqMan or

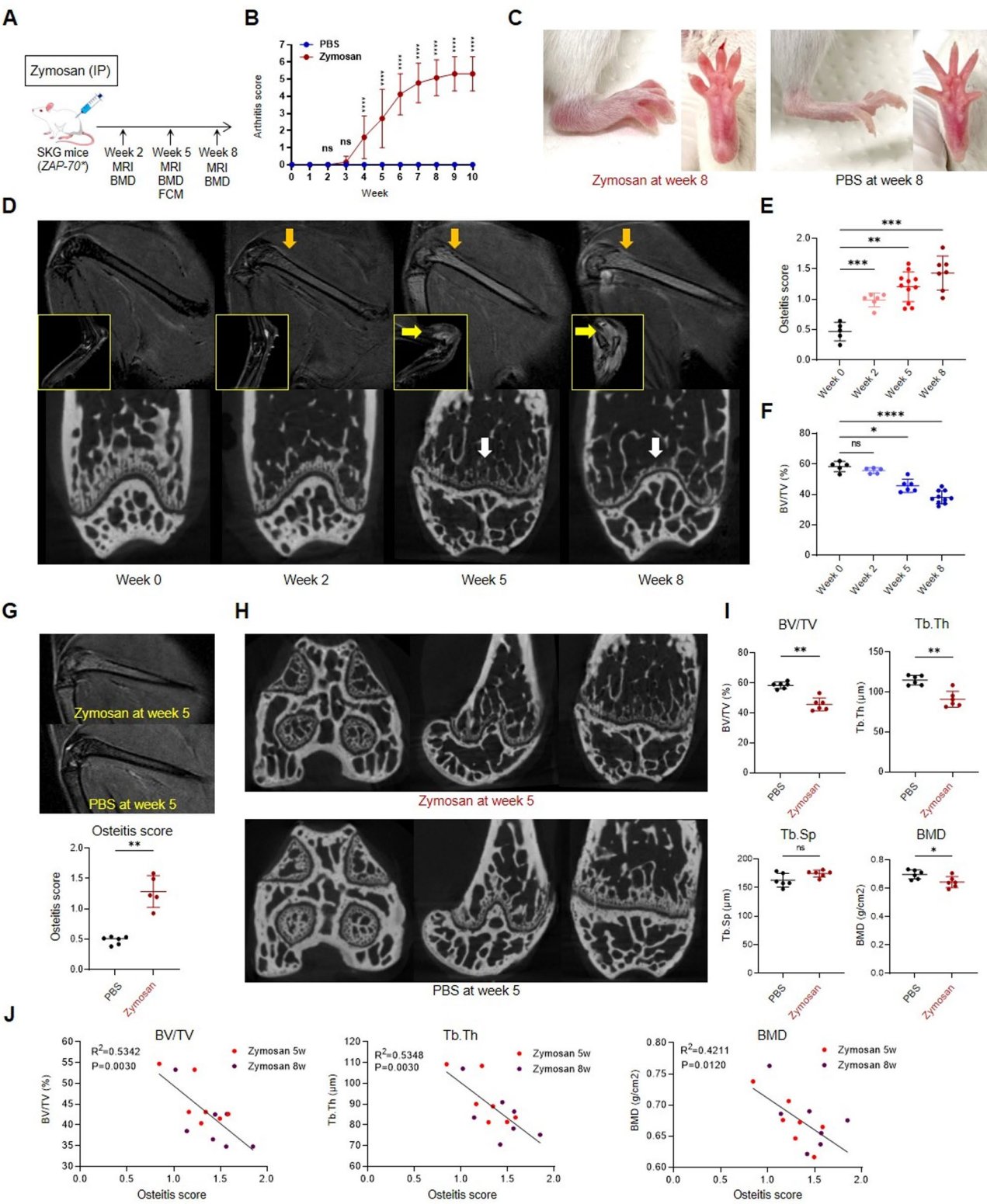


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Fig. 1 Osteitis precedes arthritis development and is correlated with bone microstructural changes. **(A)** Experimental schema showing rheumatoid arthritis induction (RA) in SKG mice. **(B)** Comparison of arthritis scores between PBS and zymosan-injected SKG mice ($n = 10$). **(C)** Representative images of the development of arthritis in zymosan-SKG and PBS-SKG mice (week 8). **(D)** Sequential T2 fat-suppressed magnetic resonance (MR) images and micro-CT imaging of the femurs from zymosan-SKG mice. The orange, yellow, and white arrows indicate osteitis, synovitis, and bone trabecular loss, respectively. **(E, F)** Sequential osteitis scores and bone volume/tissue volume (BV/TV). **(G)** Representative T2 fat-suppressed MR images and osteitis scores of femurs from zymosan-SKG and PBS-SKG mice at week 5. **(H)** Representative micro-CT images of femurs from zymosan-SKG and PBS-SKG mice at week 5. **(I)** Micro-CT quantification of trabecular bone mass in the femur, showing BV/TV, trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and bone mineral density (BMD). **(J)** Linear regression analysis of osteitis scores versus BV/TV, Tb.Th, and BMD. The data are the means $\pm$ SDs. Each data point represents an independent biological sample. Groups were compared via multiple Mann–Whitney test **(B)**, Kruskal–Wallis test with Dunn's post-hoc test **(E, F)**, and Mann–Whitney test **(G, I)**. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant

SYBR Green system. The expression of genes related to JAK – STAT signaling and osteoclastogenesis was normalized to Hprt expression.

Osteoclast differentiation assays

Mouse bone marrow-derived macrophages were differentiated into osteoclasts with macrophage colony-stimulating factor (M-CSF) (30 ng/mL) and receptor activator of nuclear factor κ B ligand (RANKL) (100 ng/mL) in the presence of GM-CSF or baricitinib. TRAP staining identified osteoclasts. Similarly, human CD14⁺ monocytes from healthy donors were differentiated into osteoclasts using M-CSF and RANKL with GM-CSF added in a dose – dependent manner. TRAP-positive cells were quantified under a BZ-X800L microscope (Keyence, Tokyo, Japan). All experiments involving human samples were approved by the ethics committee of Kyoto University Graduate School and the Faculty of Medicine (approval number R1166-3).

Public database analysis

The associations between genetic variants near CSF2 were evaluated using public genome-wide association study data. Gene expression in RA and osteoarthritis patients was compared via the limma package, and GM-CSF pathway activity scores were calculated.

Statistical analysis

The data were analyzed via Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA). Group comparisons were performed via Mann–Whitney or Kruskal–Wallis tests with Dunn's post-hoc correction. Relationships between continuous variables were assessed via linear regression, with p -values < 0.05 considered significant.

Results

Osteitis precedes arthritis development and is correlated with BMCs

Following the intraperitoneal administration of zymosan induced arthritis in SKG mice (zymosan-SKG), the arthritis score increased gradually from week 3, and the arthritis was fully developed by week 8 (Fig. 1A–C). Sequential T2-weighted, fat-suppressed MRI of the femur in zymosan-SKG mice revealed the onset of osteitis at

week 2, preceding arthritis development. Osteitis scores increased progressively through week 8 (Fig. 1D, upper panel; Fig. 1E). Micro-CT analysis of the same femoral site revealed a reduction in bone volume starting at week 2, with significant changes observed from week 5 to 8 compared with baseline (Fig. 1D, lower panel; Fig. 1F).

To explore the relationship between osteitis and BMCs before the full establishment of arthritis, we compared the osteitis and BMC scores between the zymosan-SKG and PBS-treated control groups in the early phase (week 5). The osteitis scores of zymosan-SKG-treated mice were significantly greater than those of the PBS-treated control mice (Fig. 1G). Consistently, micro-CT revealed reductions in bone volume/tissue volume, trabecular thickness, and bone mineral density in zymosan-SKG mice at week 5 (Fig. 1H–I). Statistical analysis revealed a significant correlation between increased osteitis scores on MRI and decreased bone volume ($R^2 = 0.5342$, $p = 0.003$), trabecular thickness ($R^2 = 0.5348$, $p = 0.003$), and bone mineral density on micro-CT ($R^2 = 0.4211$, $p = 0.012$) (Fig. 1J).

Skewed myelopoiesis in inflammatory BMs is correlated with osteitis severity

H&E staining of the distal femur exhibiting osteitis revealed an increase in BM cells, with a relatively brighter cytoplasm accompanying the depletion of adipocytes (Fig. 2A). Flow cytometry of femoral BM cells revealed increased total BM cell counts in zymosan-SKG mice at week 5, although the difference was not significant (Supplementary Fig. S1A). BM cells were categorized into developmental stages via established flow cytometry panels (Supplementary Fig. S1B–C) [17, 18].

In zymosan-SKG mice, the counts of upstream Lin[–], Sca-1⁺, c-Kit⁺ (LSK) populations, short-term hematopoietic stem cells, and multipotent progenitors were increased significantly (Supplementary Fig. S1D–E). Conversely, the number of long-term hematopoietic stem cells did not increase (Supplementary Fig. S1F). In downstream populations, counts of granulocyte–macrophage progenitors were elevated, whereas counts of megakaryocyte–erythroid progenitors were reduced significantly (Supplementary Fig. S1G). Granulocyte (Ly6G⁺) and monocyte (sum of Ly6C^{high}/CD11b^{high}, Ly6C^{mid}/CD11b^{high}, and Ly6C^{high}/CD11b^{low} as discussed below) counts

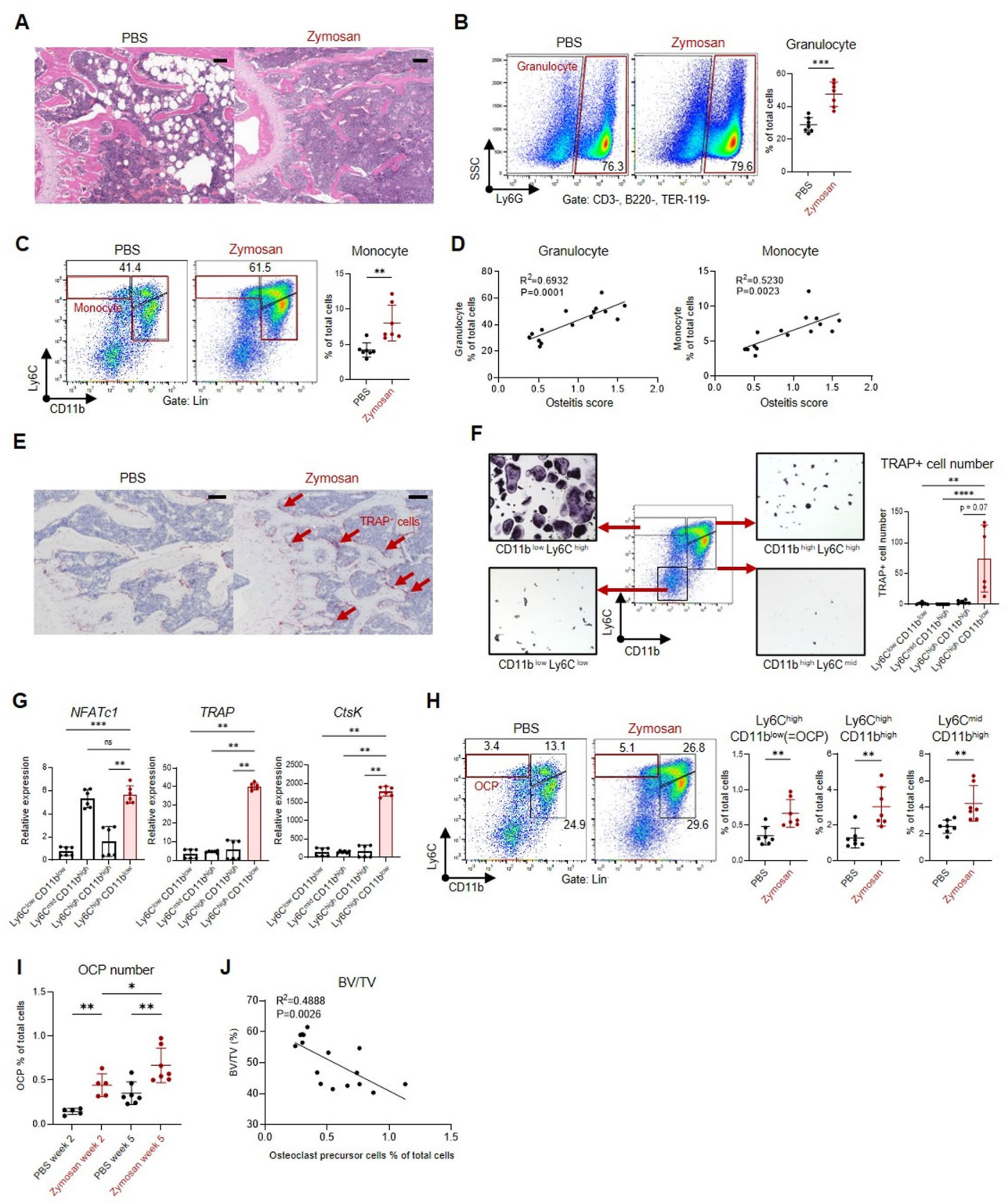


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Fig. 2 Skewed myelopoiesis in the inflammatory bone marrow is correlated with osteitis severity. **(A)** Representative histological sections of femurs from PBS-SKG and zymosan-SKG mice (week 5). Scale bar, 100 μ m. **(B)** Frequencies of granulocytes and **(C)** monocytes (sum of CD11b⁺ or Ly6C⁺ subsets) among lineage (CD3/TER-119/B220)-negative cells. **(D)** Linear regression analysis of osteitis scores versus granulocyte or monocyte frequencies. **(E)** Representative TRAP⁺ staining of distal femurs from PBS-SKG and zymosan-SKG mice (week 5). Red arrows indicate TRAP⁺ cells (osteoclasts). Scale bar, 100 μ m. **(F)** Osteoclasts differentiated from four monocyte subsets (classified by Ly6C and CD11b expression). **(G)** *NFATc1*, *TRAP*, and *CtsK* mRNA expression in monocyte subsets two days after RANKL supplementation. The results from two independent experiments are shown. **(H)** Bone marrow (BM) staining in PBS-SKG and zymosan-SKG mice (week 5), showing increased counts of osteoclast precursor cells (OCPs: OCP: Ly6C^{high}, CD11b^{low}) and other monocyte subsets. **(I)** Time-dependent increase in OCPs in the femoral BM of zymosan-SKG mice. **(J)** Linear regression analysis of OCP frequencies versus BV/TV. The data are the means $\pm$ SDs. Each data point represents an independent biological sample. Groups were compared via Kruskal–Wallis test with Dunn's post-hoc test (**F, G, I**) and Mann–Whitney test (**B, C, H**). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant

were also elevated (Fig. 2B–C). In contrast, T-cell (CD3⁺), B-cell (B220⁺), plasma cell (TAC1⁺, CD138⁺), and erythrocyte (TER-119⁺) counts were decreased significantly (Supplementary Fig. S1H–K). Notably, granulocyte and monocyte counts in the BM were significantly correlated with osteitis severity on MRI (granulocyte: $R^2 = 0.6392$, $p = 0.0001$, monocyte: $R^2 = 0.523$, $p = 0.0023$) (Fig. 2D).

Osteoclast precursor cells expand in the osteitic BM

To investigate the mechanistic link between osteitis and subsequent BMCs, we examined osteoclasts in the inflammatory BM of osteitic SKG mice (5 weeks after zymosan). TRAP staining revealed an increase in TRAP-positive cells in BM regions affected by osteitis (Fig. 2E). To identify the origin of these osteoclasts, we isolated three subsets of monocytes Ly6C and CD11b expression profiles: Ly6C^{high}/CD11b^{high}, Ly6C^{mid}/CD11b^{high}, Ly6C^{high}/CD11b^{low}, and control (Ly6C^{low} CD11b^{low}). These subsets were subjected to in vitro osteoclast differentiation assays with M-CSF and RANKL. Among the three subsets, only the Ly6C^{high}/CD11b^{low} subset successfully differentiated into osteoclasts (Fig. 2F). Furthermore, this subset expressed osteoclast-related genes, including *NFATc1*, *TRAP*, and *CtsK*, confirming their identity as osteoclast precursor cells (Fig. 2G).

In addition to the Ly6C^{high}, CD11b^{high}, and Ly6C^{mid} CD11b^{high} monocyte subsets, osteoclast precursor cells were increased in the BM of zymosan-SKG mice (Fig. 2H). Notably, the number of osteoclast precursor cells increased over time in the inflammatory BM, and this increase was significantly correlated with reduced bone volume ($R^2 = 0.4888$, $p = 0.0026$) (Fig. 2I–J).

Baricitinib suppressed osteitis and BMCs by reversing the skewed myelopoiesis

Recently, clinical trials have demonstrated that baricitinib, a JAK 1/2 inhibitor, suppresses osteitis and BMCs in patients with RA [14, 15]. To explore the underlying mechanism in our animal model, baricitinib was administered to arthritic zymosan-SKG mice beginning at week 5. Treatment resulted in a concentration-dependent reduction in arthritis severity (Fig. 3A, Supplementary Fig. S2A). Validation experiments confirmed that arthritis was more significantly reduced in baricitinib-treated

mice than in methyl cellulose-treated controls (Fig. 3B–C). Additionally, baricitinib significantly ameliorated osteitis and BMCs (Fig. 3D–F).

Histological analysis of distal femoral specimens revealed the reappearance of previously depleted adipocytes and a significant reduction in TRAP-positive cell counts following baricitinib treatment (Fig. 3G–H). Flow cytometric analysis revealed that baricitinib did not affect upstream hematopoietic cell populations, including LSK populations. However, it significantly reduced the counts of multipotent progenitors and downstream populations, such as granulocyte – macrophage progenitors, and monocyte subsets, including osteoclast precursor cells (Fig. 3I, Supplementary Fig. S2B–C). Megakaryocyte – erythroid progenitor and granulocyte counts were not significantly altered (Fig. 3J, Supplementary Fig. S2C).

Importantly, the prophylactic effects of baricitinib on arthritis, osteitis, and BMCs were also evident when it was administered early. Baricitinib treatment beginning at week 2 in zymosan-treated SKG mice tended to inhibit the emergence of osteitis and BMCs and suppress arthritis onset (Supplementary Fig. S2D–H).

GM-CSF promotes osteitis and BMCs under inflammatory conditions

Given that baricitinib ameliorated osteitis and BMCs by correcting skewed myelopoiesis, we hypothesized that inflammatory cytokines contribute to the development of osteitis and BMCs via the JAK – STAT signaling pathway. To test this hypothesis, we analyzed cytokine expression at both the mRNA and protein levels in the osteitic inflammatory BM (Fig. 4A). We found that the genes encoding GM-CSF (*Csf2*) and its receptor (*Csf2ra*) were first upregulated in the osteitic BM starting at week 2 after zymosan injection and were significantly upregulated by week 5 (Fig. 4B). The overexpression of GM-CSF and its receptor was also confirmed at the protein level (Fig. 4C, Supplementary Fig. 3A).

Although the expression of IL-6 gene (*Il6*) was not upregulated at week 2, it was slightly elevated and the protein levels were significantly increased by week 5 (Fig. 4B–C, Supplementary Fig. S3A). In contrast, the expression of TNF was not significantly elevated at either the mRNA or protein level throughout the time course

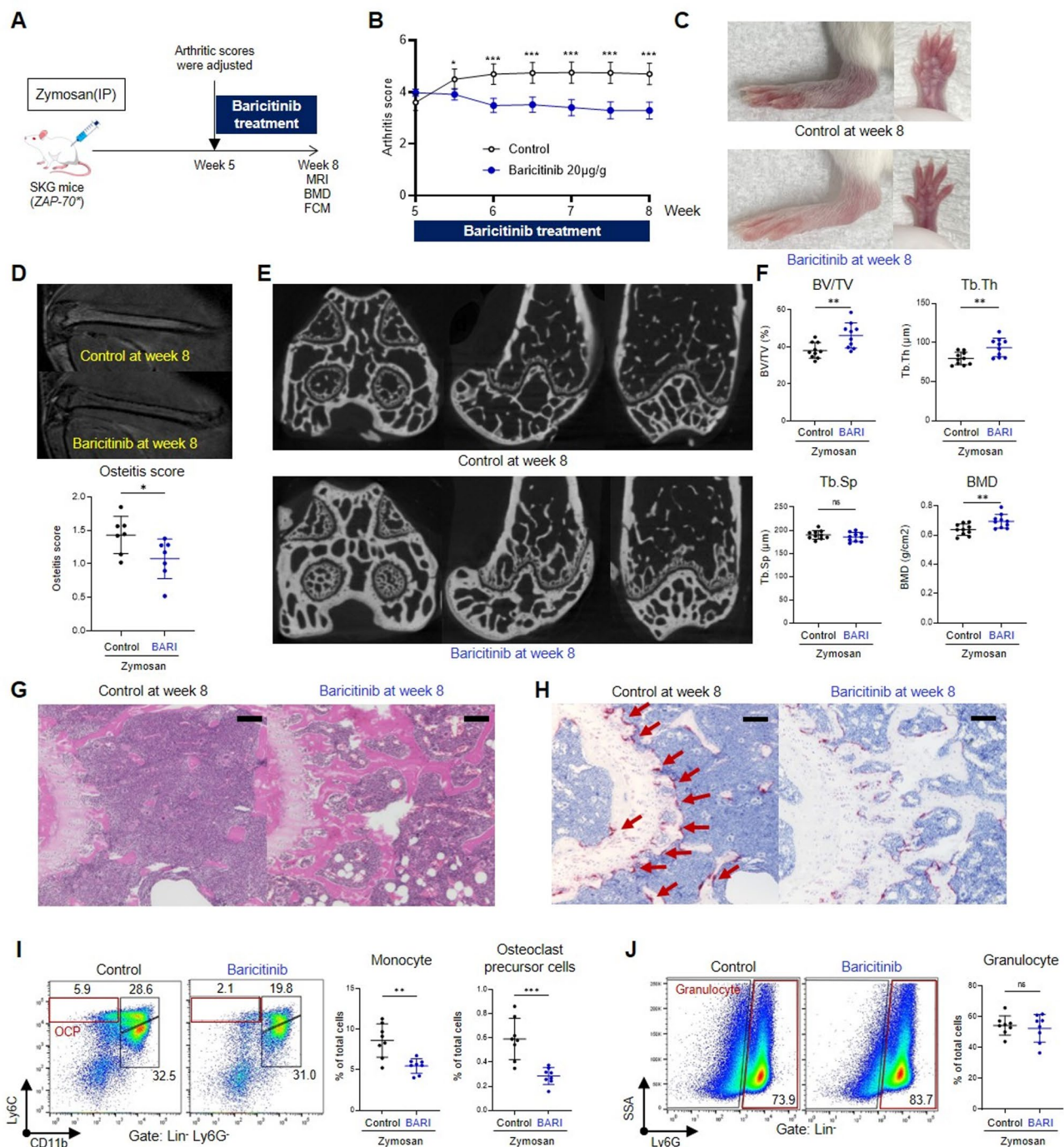


Fig. 3 The JAK inhibitor baricitinib suppresses osteitis and BMCs by reversing skewed myelopoiesis. **(A)** Experimental schema of baricitinib treatment of zymosan-SKG mice. Zymosan-treated SKG mice were treated with baricitinib or methylcellulose **(B)** Comparison of arthritis scores between the baricitinib-treated and vehicle control groups. **(C)** Representative images of arthritis in the baricitinib-treated and vehicle control groups at week 8. **(D-E)** Representative T2 fat-suppressed MR images, osteitis scores, and micro-CT images of femurs in the baricitinib-treated and vehicle control groups at week 8. **(F)** Micro-CT quantification of femoral trabecular bone mass, including BV/TV, Tb.Th, Tb.Sp, and BMD, in the baricitinib-treated and vehicle control groups at week 8. **(G, H)** Representative histological sections and TRAP⁺ staining of femurs from baricitinib-treated and vehicle control groups at week 8. Red arrows indicate TRAP⁺ cells (osteoclasts). Scale bar, 100 μm. **(I)** BM staining and graphs showing monocyte (CD3⁺, B220⁻, TER119⁻, Ly6G⁻ and CD11b⁺/Ly6C⁺) and osteoclast precursor cell (OCP; CD11b^{low}, Ly6C^{high}) frequencies. **(J)** BM staining shows granulocyte (Ly6G⁺) frequencies among lineage (CD3/B220/TER119) negative cells. The graph displays the granulocyte frequency within the BM population. The data are the means ± SEMs in **(B)** and means ± SDs in **(D, F, I-J)**. Each data point represents an independent biological sample. Groups were compared via multiple Mann–Whitney test **(B)** and Mann–Whitney test **(D, F, I-J)**. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant

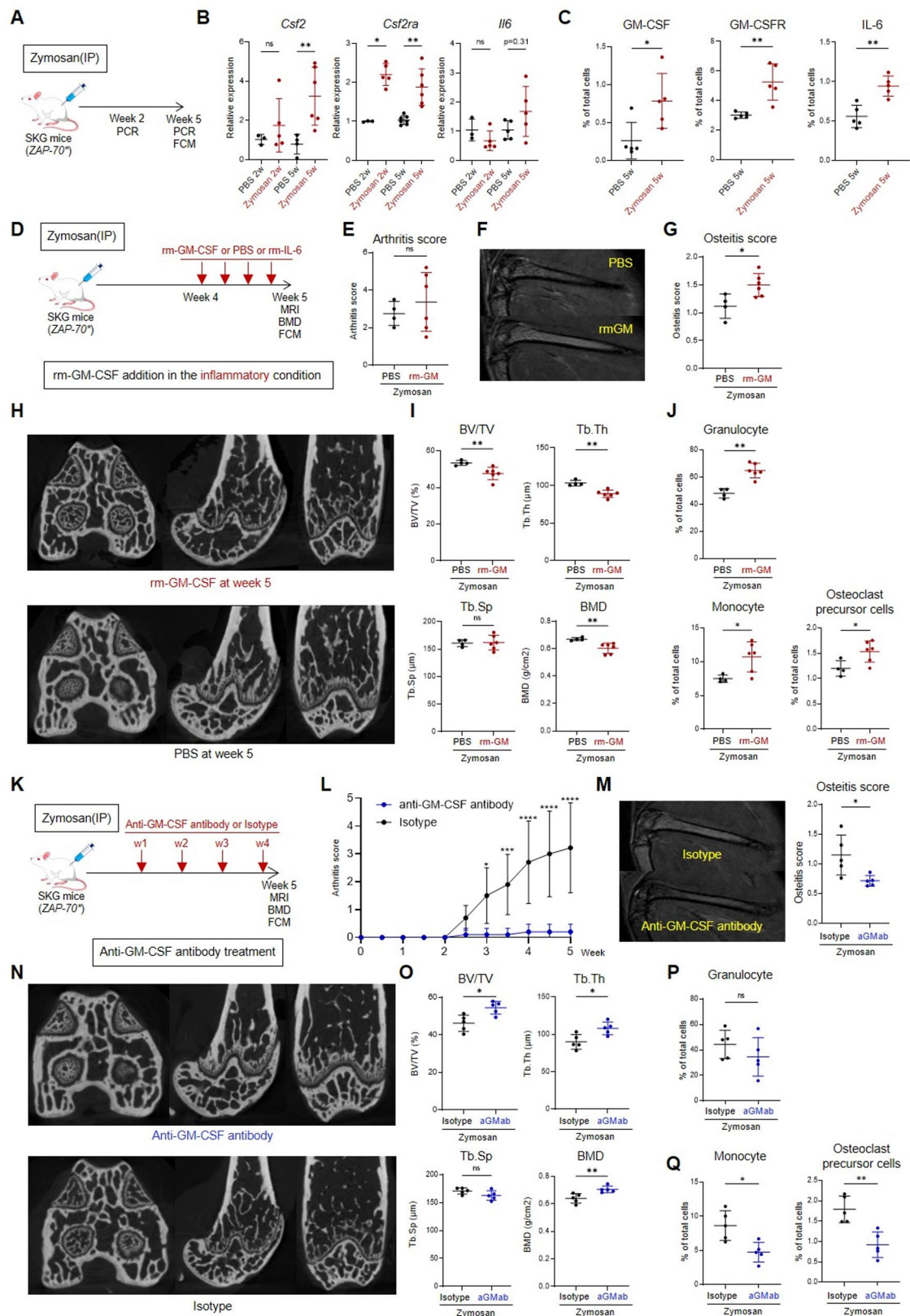


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Fig. 4 GM-CSF promotes osteitis and BMCs under inflammatory conditions and anti-GM-CSF treatment suppresses this process. **(A, D, K)** Experimental schema for measuring cytokine expression in BM **(A)**, administering recombinant murine GM-CSF (rm-GM-CSF), IL-6, or PBS to zymosan-SKG mice **(D)**; and anti-GM-CSF antibody treatment in zymosan-SKG mice **(K)**. **(B, C)** mRNA expression and frequencies of GM-CSF (*Csf2*), GM-CSF receptor (GM-CSFR, *Csf2ra*), and IL-6(*Il6*)-positive cells among total BM cells from zymosan-SKG mice. **(E)** Comparison of arthritis scores between PBS and rmGM-CSF-treated zymosan-SKG ($n=5$). **(F, G, M)** Representative T2 fat-suppressed MR images and osteitis scores of femurs from the rm-GM-CSF group (week 5) **(F, G)**, and the anti-GM-CSF antibody treatment group (week 5) **(M)**. **(H, N)** Representative femoral micro-CT images of the rm-GM-CSF group (week 5) **(H)**, and the anti-GM-CSF antibody treatment group (week 5) **(N)**. **(I, O)** Micro-CT quantification of femoral trabecular bone mass, including BV/TV, Tb.Th, Tb.Sp, and BMD, in the rm-GM-CSF group (week 5) **(I)**, and in the anti-GM-CSF antibody treatment group (week 5) **(O)**. **(J, P, Q)** Frequencies of granulocytes, monocytes, and OCPs in the BM population in each group. **(L)** Comparison of arthritis scores between anti-GM-CSF antibody and isotype control-treated zymosan-SKG mice ($n=5$). Lin⁻: Lineage negative. The data are the means $\pm$ SDs. Each data point represents an independent biological sample. Groups were compared via Mann–Whitney test. * $P < 0.05$; ** $P < 0.01$; ns, not significant

(Supplementary Fig. S3A–B). Other cytokine genes associated with type I (*Irf9*) and type II (*Ifng*) interferon signatures were not upregulated in the BM of zymosan-SKG mice (Supplementary Fig. S3B). To determine whether GM-CSF or IL-6 exacerbates osteitis and BMCs under inflammatory conditions, we administered recombinant murine GM-CSF (rm-GM-CSF) and rm-IL-6 to zymosan-SKG mice (Fig. 4D).

Although arthritis score was not significantly affected, administration of rm-GM-CSF exacerbated osteitis, whereas rm-IL-6 did not (Fig. 4E–G, Supplementary Fig. S3C). Similar results were observed for BMCs (Fig. 4H–I, Supplementary Fig. S3D – E). Flow cytometry revealed that rm-GM-CSF increased granulocyte and monocyte counts, including those of osteoclast precursor cells (Fig. 4J, Supplementary Fig. S3F – G). Interestingly, the administration of rm-GM-CSF to SKG mice under non-inflammatory conditions also induced osteitis, but it did not induce BMCs or arthritis (Supplementary Fig. S4A–F).

Anti-GM-CSF therapy suppresses osteitis and BMCs by reversing skewed myelopoiesis

To confirm the role of GM-CSF in promoting osteitis and BMCs, we administered anti-GM-CSF antibodies to zymosan-SKG mice beginning at the early phase week 1 (Fig. 4K). The progression of arthritis, osteitis, and BMCs was significantly suppressed in the early administration group compared with in the isotype control group (Fig. 4L–O). Bone marrow analysis revealed no significant difference in granulocyte counts; however, anti-GM-CSF antibody treatment significantly reduced monocyte counts, including those of osteoclast precursor cells (Fig. 4P–Q, Supplementary Fig. S5A–B).

GM-CSF plays a dual role in osteoclast differentiation

We demonstrated that GM-CSF exacerbates osteitis by promoting skewed myelopoiesis and contributes to BMCs by increasing the number of osteoclast precursor cells in the inflammatory BM. However, its effect on osteoclast differentiation remains unclear, as prior studies have reported contradictory findings [19, 20]. While some studies suggest that GM-CSF promotes osteoclast

differentiation, others indicate an inhibitory effect [21–24]. To clarify this discrepancy, we investigated the impact of GM-CSF on osteoclast differentiation. BM-derived macrophages were isolated from SKG mice and subjected to GM-CSF supplementation in vitro under different conditions (Fig. 5A). When GM-CSF was added alongside M-CSF and RANKL in the early phase, osteoclast differentiation was inhibited in a concentration-dependent manner (Fig. 5B). In contrast, priming with M-CSF or RANKL followed by the later addition of GM-CSF enhanced osteoclast differentiation and resulted in the production of large osteoclasts (Fig. 5C). Additionally, while baricitinib did not inhibit osteoclast differentiation induced by M-CSF and RANKL alone, it suppressed the osteoclastogenic effect of GM-CSF supplementation in a dose-dependent manner during the late phase (Fig. 5D).

GM-CSF gene expression in RA and osteoclast differentiation in humans

To investigate the role of GM-CSF in patients with RA, we examined the associations between *CSF2* variants and RA using data from the largest available genome-wide association study [25]. This analysis revealed that the A allele of the rs657075 variant increased the risk of RA development ($p = 4.5 \times 10^{-7}$) (Supplementary Fig. 6, Supplementary Table S1). Using an optimized single-nucleotide polymorphism (SNP)-to-gene approach, we found that this SNP resides within an enhancer region of *CSF2* [26]. Furthermore, analysis of the influence of the SNP on hematological traits suggested an association with increased monocyte counts ($p = 0.08$) (Supplementary Table S1) [27].

Next, we analyzed GM-CSF-related gene expression in the BM of patients with RA ($n = 9$) and osteoarthritis (OA) ($n = 10$) via a public database of total knee replacement samples [28]. A volcano plot revealed significant upregulation of GM-CSF-associated genes, including *STAT5B*, *CSF2RB*, and *STAT5A*, in the BM of patients with RA (Fig. 6A). GM-CSF scores, calculated as the average expression levels of defined GM-CSF-related gene sets (Supplementary Table S2) [29], were significantly greater in the BM of patients with RA than in the BM of patients with OA (Fig. 6B). Similarly, the myeloid

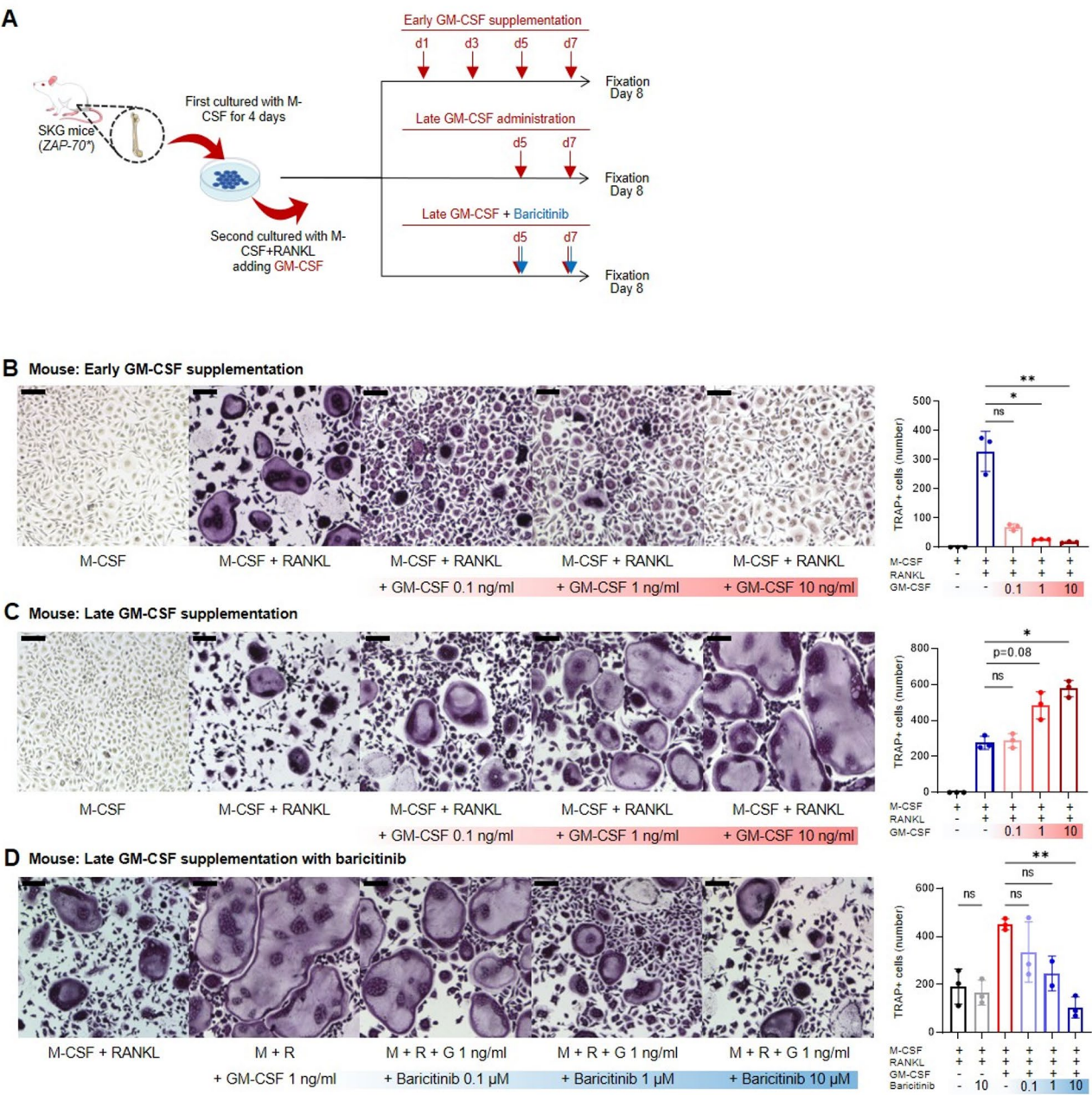


Fig. 5 GM-CSF plays a dual role in osteoclast differentiation. **(A)** Experimental schema for the in vitro supplementation with rm-GM-CSF in the early or late phase of osteoclast differentiation, with or without baricitinib treatment. **(B, C, D)** Representative images of osteoclast formation under each condition. Scale bar: 100 μ m. The graphs display the number of TRAP⁺ multinucleated cells (≥ 3 nuclei) under the indicated conditions. The data represent the results from two independent experiments. The data are the means $\pm$ SDs. Groups were compared via Kruskal–Wallis test with Dunn’s post-hoc test. * $P < 0.05$; ** $P < 0.01$; ns, not significant

progenitor score, derived from gene sets associated with myeloid progenitor cells affected by GM-CSF, was also significantly greater in patients with RA (Fig. 6C, Supplementary Table S3) [10].

Finally, we examined the influence of GM-CSF on human osteoclast differentiation. Peripheral blood monocytes were isolated from healthy individuals, and CD14-positive monocytes were extracted via magnetic

cell sorting. Culturing these cells with M-CSF and RANKL revealed that early GM-CSF addition suppressed osteoclast differentiation, whereas late GM-CSF addition enhanced osteoclast differentiation (Fig. 6D–E). Importantly, baricitinib suppressed GM-CSF-induced osteoclastogenesis in a dose-dependent manner, while having no inhibitory effect on osteoclast differentiation driven by M-CSF and RANKL alone (Fig. 6F).

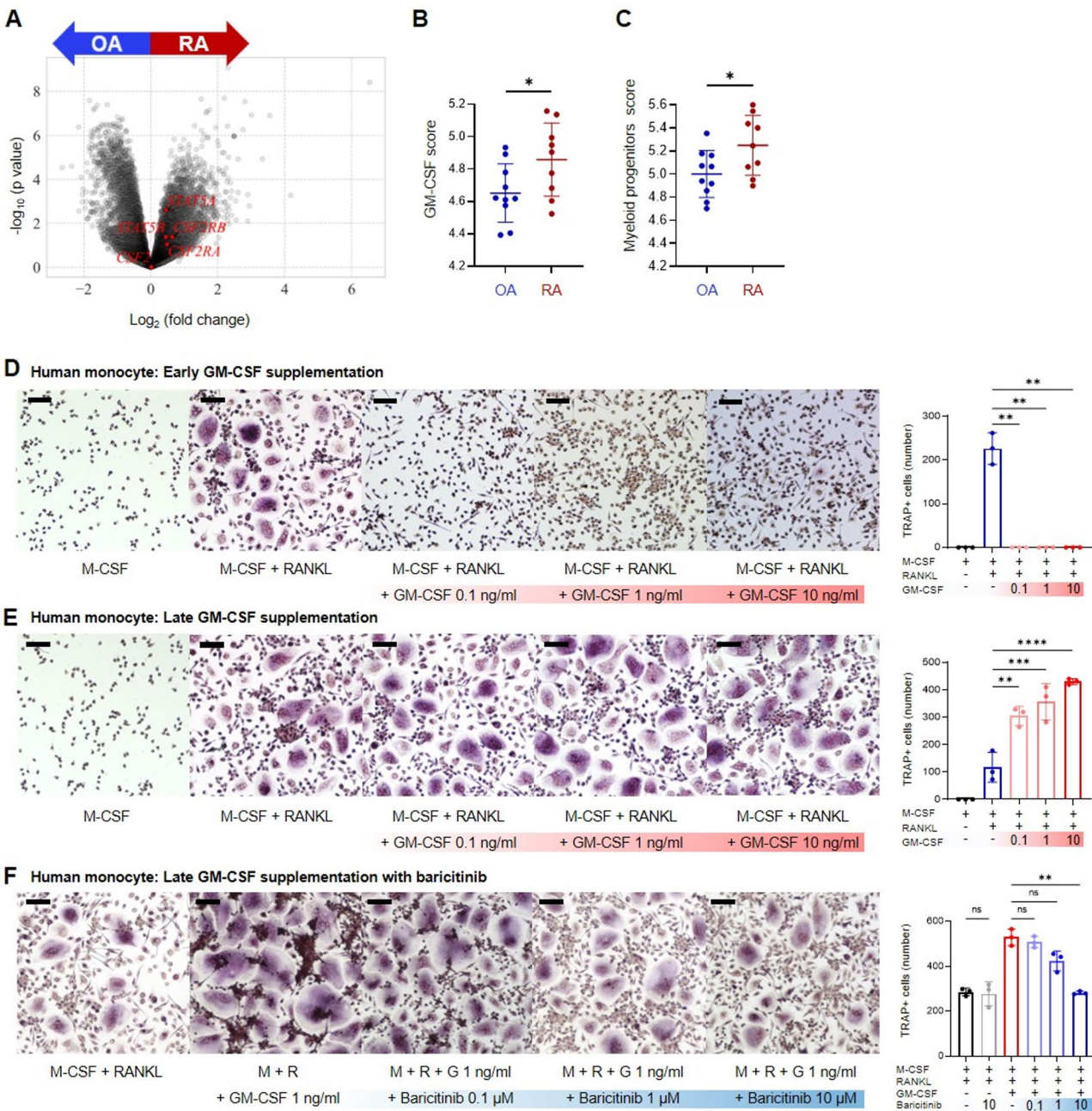


Fig. 6 GM-CSF gene involvement in patients with RA and its role in osteoclast differentiation in humans. **(A)** Volcano plot showing differential gene expression analysis of BM-derived mononuclear cells between RA patients and osteoarthritis (OA) patients. **(B, C)** Comparison of GM-CSF scores and myeloid progenitor scores in the BM of RA and OA patients. **(D, E, F)** Representative images of osteoclasts differentiated from peripheral CD14-positive monocytes from healthy individuals (TY and YM), supplemented with recombinant human GM-CSF in the early **(D)** or late **(E)** phase of osteoclast differentiation, with **(F)** or without baricitinib treatment **(D, E)**. Scale bar: 100 μm . The graphs display the number of TRAP⁺ multinucleated cells (≥ 3 nuclei) under each condition. The data represent the results from two independent experiments. The data are the means $\pm$ SDs. Groups were compared via Mann-Whitney test **(B, C)** or Kruskal-Wallis test with Dunn's post-hoc test **(D, E, F)**. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

Discussion

In this study, we delineated a marrow-centred pathway by which GM-CSF drives the earliest bone lesions of RA. Using the zymosan-SKG mouse model, we demonstrated that GM-CSF, via JAK2-STAT signalling, promotes

expansion of the monocyte-macrophage lineage and induces osteitis. Moreover, GM-CSF increased the pool of osteoclast precursors and accelerates their differentiation into bone-resorbing osteoclasts. These events occur prior to—and mechanistically facilitate—the cortical and trabecular damage that typifies BMCs.

Osteitis, visualised on fat-suppressed T2-weighted MRI as focal marrow edema, represents the earliest imaging hallmark that precedes bone destruction in patients with RA. In RA, *external* bone erosion is typically driven by pannus formation, where proliferating fibroblast-like synoviocytes invade cortical bone through high expression of RANKL, thereby promoting osteoclast-mediated resorption [30]. However, radiologic-pathologic studies have delineated an *internal* route: marrow regions that appear as osteitis on MRI are characterised by a loss of adipocytes, expansion of monocyte populations, and accumulation of RANKL-positive cells, anticipating BMCs and later erosions [10, 11, 31–33]. Our data extended these observations by demonstrating that GM-CSF markedly expands the monocytic compartment, including osteoclast precursors, within the marrow. These findings strongly implicate GM-CSF-driven myelopoiesis as a key initiator of osteitis and a critical trigger of marrow-based bone loss.

Our results demonstrate that GM-CSF regulates osteoclastogenesis through two distinct, time-dependent mechanisms both in vitro and in vivo. Early GM-CSF exposure in culture suppressed osteoclast formation, consistent with previous reports showing downregulation of the M-CSF receptor and inhibition of macrophage commitment to the osteoclast lineage [34]. In contrast, late-phase exposure promoted the formation of large, multinucleated osteoclasts, likely by enhancing precursor survival and terminal differentiation [35, 36]. These findings help resolve earlier discrepancies and emphasize the timing of GM-CSF signalling as a key determinant of osteoclast fate [37]. In vivo, GM-CSF induced osteitis under non-inflammatory conditions but did not lead to BMCs. Under inflammatory conditions, however, it exacerbated both osteitis and BMCs. Together, these results indicate that GM-CSF intrinsically drives myelopoiesis-associated osteitis, while progression to bone resorption requires an inflammatory environment that promotes osteoclast differentiation [38].

Clinically, inhibitors of JAK, IL-6, and TNF reduce osteitis and BMCs [39, 40]. These inhibitors block the inflammatory cytokine cascades associated with osteitis and bone destruction. Our findings highlight the central role of the JAK–STAT pathway, particularly GM-CSF, in bone inflammation dynamics. GM-CSF acts as a pivotal amplifier of inflammatory cytokine networks, markedly boosting the production of macrophage-derived TNE, a key driver of osteoclast differentiation [19, 41]. Thus, GM-CSF may act in concert with other inflammatory cytokines to cause osteitis and BMCs in RA patients [42]. Although clinical trials of anti-GM-CSF antibody therapy have shown partial efficacy in patients with treatment-resistant RA, our results suggest that GM-CSF inhibition in early RA could improve osteitis and BMCs [43]. Given

that GM-CSF transmits signals via the JAK2 homodimer followed by the JAK–STAT signaling pathway, JAK inhibitors such as baricitinib may alleviate osteitis and BMCs by targeting JAK2 and inhibiting GM-CSF. Further studies are needed to determine whether other JAK inhibitors have similar effects on osteitis and BMCs both in vivo and in vitro [44].

Finally, we acknowledge the limitations of our work and propose future research directions. First, the pattern of osteitis development differs between mice and humans. In humans, osteitis is typically observed in bone lesions adjacent to the inflammatory synovium [31], whereas in mice, MRI consistently shows edematous changes throughout the femoral BM, regardless of strain [45]. This discrepancy likely reflects differences in inflammation severity and progression between species. In humans, RA progresses years before arthritis onset, whereas in mice, inflammation accelerates over several weeks. The intense inflammation in murine BM may lead to more pronounced osteitis manifestations on MRI. Second, this study involved limited investigations in human patients; therefore, further research is needed to evaluate GM-CSF expression in the RA BM across different disease stages. Studies should also assess the effects of GM-CSF inhibition on osteitis and osteoclast differentiation in human subjects. Finally, although the present study focused on JAK inhibitors and the effects of GM-CSF on osteoclasts, we did not examine their effects on osteoblasts and bone formation, as these effects are already well-documented [16].

In conclusion, the present study elucidated a pathogenic mechanism linking early RA lesions—osteitis and BMCs—and identified GM-CSF, which acts via the JAK–STAT signaling cytokine, as a critical mediator. These findings could inform the development of new therapeutic strategies targeting early RA lesions, potentially improving patient outcomes.

Abbreviations

BM	Bone marrow
BMCs	Bone microstructural changes
CT	Computed tomography
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	Hematoxylin and eosin
JAK	Janus kinase
M-CSF	Macrophage colony-stimulating factor
MRI	Magnetic resonance imaging
OCPs	Osteoclast precursor cells
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor κB ligand
r-GM-CSF	Recombinant GM-CSF
rm-GM-CSF	Recombinant murine GM-CSF
STAT	Signal transducer and activator of transcription
TNF	Tumor necrosis factor
TRAP	Tartrate-resistant acid phosphatase

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13075-025-03597-6>.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

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Author contributions

Conceptualization: TY, MH. Data curation: TY. Formal analysis: TY, MH. Funding acquisition: MH. Investigation: TY, YM, HI, TI. Methodology: Tsuneyasu Yoshida. Project administration: TY, MH. Software: TI. Resources: TY, YM, TI, YN. Supervision: MH. Validation: TY, YM, HI. Visualization: TY. Writing—original draft: TY, MH. Writing—review & editing: All authors.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Kyoto University Animal Ethics Committee (approval number Medkyo 16106–23104). All experiments using human samples were approved by the ethics committee of Kyoto University Graduate School and Faculty of Medicine (approval number R1166–3).

Consent for publication

Not applicable.

Competing interests

TY has received speaker fees from AbbVie GK, Asahi Kasei Pharma Corp., and Eli Lilly Japan K.K. MH has received research grants from AbbVie GK, Asahi Kasei Pharma Corp., Astellas Pharma Inc., Bristol-Myers Squibb, Eisai Co. Ltd., Daiichi Sankyo Co. Ltd., Eli Lilly Japan K.K., Novartis Pharma Co. Ltd., Taisho Toyama Co. Ltd., and Towa Pharma Co. Ltd., and speaker fees from Bristol-Myers Squibb, Chugai Pharmaceutical Co. Ltd., Eisai Co. Ltd., Eli Lilly Japan K.K., and Mitsubishi Tanabe Pharma Corp. Ltd. YM has received honoraria from Asahi Kasei Pharma Corp. RW has received a grant from AbbVie GK, and has received honoraria from AbbVie GK, Chugai Pharmaceutical Co. Ltd., Asahi Kasei Pharma Corp., GlaxoSmithKline, Eli Lilly Japan K.K., and UCB Japan. KM has received honoraria from Chugai Pharmaceutical Co. Ltd., and Asahi Kasei Pharma Corp. AM has received research grants from AbbVie GK, Asahi Kasei Pharma Corp., Chugai Pharmaceutical Co. Ltd., Mitsubishi Tanabe Pharma Corp. Ltd., and Eisai Co. Ltd., and has received honoraria from AbbVie GK, Chugai Pharmaceutical Co. Ltd., Eli Lilly Japan K.K., Eisai Co. Ltd., Pfizer Inc., Bristol-Myers Squibb, Mitsubishi Tanabe Pharma Corp., Astellas Pharma Inc., and Gilead Sciences Japan.

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