

RESEARCH ARTICLE

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Interleukin-34 produced by human fibroblast-like synovial cells in rheumatoid arthritis supports osteoclastogenesis

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Abstract

Introduction: Interleukin-34 (IL-34) is a recently defined cytokine, showing a functional overlap with macrophage colony stimulating factor (M-CSF). This study was undertaken to address the expression of IL-34 in rheumatoid arthritis (RA) patients and to investigate its regulation and pathogenic role in RA.

Methods: IL-34 levels were determined in the RA synovium, synovial fluid (SF) and fibroblast-like synovial cells (FLS) by immunohistochemistry, real-time PCR, enzyme-linked immunosorbent assay and immunoblotting. RA activity was assessed using Disease Activity Score 28 (DAS28) activity in the plasma collected at baseline and one year after treatment. Conditioned media (CM) were prepared from RA FLS culture with tumor necrosis factor alpha (TNF α) for 24 hours and used for functional assay.

Results: IL-34 was expressed in the synovium, SF, and FLS from RA patients. The production of IL-34 in FLS was up-regulated by TNF α in RA samples compared with osteoarthritis (OA) patients. Importantly, the preferential induction of IL-34 rather than M-CSF by TNF α in RAFLS was mediated by the transcription factor nuclear factor kappa B (NF- κ B) and activation of c-Jun N-terminal kinase (JNK). IL-34 elevation in plasma from RA patients was decreased after the administration of disease-modifying anti-rheumatic drugs (DMARDs) in accordance with a decrease in DAS28. CM from RAFLS cultured with TNF α promoted chemotactic migration of human peripheral blood mononuclear cells (PBMCs) and subsequent osteoclast (OC) formation, effects that were attenuated by an anti-IL-34 antibody.

Conclusions: These data provide novel information about the production of IL-34 in RA FLS and indicate that IL-34 is an additional osteoclastogenic factor regulated by TNF α in RA, suggesting a discrete role of IL-34 in inflammatory RA diseases.

Introduction

Rheumatoid arthritis (RA) is an autoimmune chronic inflammatory disease characterized by bone and cartilage destruction that is mediated by bone-resorbing osteoclasts (OCs) [1,2]. OCs differentiate from the monocyte/macrophage lineage of hematopoietic myeloid progenitors in response to macrophage colony-stimulating factor (M-CSF) and RANKL (receptor activator of NF- κ B ligand) [3,4] and participate in a variety of

inflammatory bone degenerative diseases. OC differentiation correlates with the severity of the inflammatory condition [2]. OCs mediate erosive bone resorption at the bone-pannus interface of the synovium in RA joints resulting from chronic inflammation of multiple synovial joints [5]. Synovial fluid (SF) produced by the inflamed synovium in joints, hyperplastic synovial fibroblasts, and activated synovial T cells increases the production of RANKL and several inflammatory cytokines [6,7]. These inflammatory conditions lead to enhanced OC formation and the subsequent increase in resorbing activity [2].

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Tumor necrosis factor alpha (TNF α) is well established as a critical OC differentiation-enhancing factor that acts by mediating mobilization of osteoclast precursors (OCPs) from bone marrow into the inflamed joint, where they appear to contribute to inflammatory erosive arthritis [8]. TNF α -stimulated fibroblast-like synovial cells (FLS) increase cytokine production [6], which accelerates OC formation in the inflamed synovium of RA [9]. Thus, the administration of TNF α blocking agents results in a decrease in the pathological changes indicative of RA inflammatory responses, and as such provides a potential clinical benefit [10]. Recent studies have shown that administration of an antibody (Ab) against the M-CSF receptor, c-Fms or inhibitor, selectively and completely blocks osteoclastogenesis and bone erosion induced by TNF α injection or inflammatory arthritis [11,12], suggesting a link between TNF α and c-Fms under pathological inflammatory conditions. Accordingly, identifying factors involved in TNF α -induced OCPs mobilization and subsequent differentiation that contribute to erosive arthritis is a matter of considerable interest.

The recently discovered cytokine IL-34 binds to the M-CSF receptor c-Fms [13]. The functional similarity of IL-34 and M-CSF is demonstrated by their role in osteoclastogenesis [14-18]. Although IL-34 and M-CSF share the c-Fms receptor, their signal transduction mechanisms and biological activity are not identical [16]. Functional overlap [16], but differential expression, of M-CSF and IL-34 has been observed in the context of M-CSF receptor-mediated regulation of myeloid cells [18]. However, whether IL-34 is involved in RA pathogenesis is still unknown.

Materials and methods

Patients and reagents

All RA patients enrolled in this study fulfilled the 1987 revised criteria of the American College of Rheumatology [19]. Patients were compared with age- and sex-matched control patients with OA.

Informed consent was obtained from all patients and the experimental protocol was approved by the Human Research Ethics Committee of the University of Ulsan College of Medicine (2010-871). SF was collected from the knee joint (with or without swelling) of each patient by sterile aspiration and centrifuged at 250g for 10 minutes. Cell-free RA synovial fluid (RA SF) was stored at -70°C until used. Synovia were isolated from seven patients with RA (mean age, 59.9 \pm 12.3; range, 42 to 75 years) and seven patients with OA (mean age, 62.5 \pm 8.2; range, 46 to 77 years). All patients were undergoing knee replacement surgery. For the one-year follow-up study, sera were collected from the patients listed in Additional file 1 at baseline and one year after treatment

from ten patients with RA. RA activity was simultaneously assessed using Disease Activity Score 28 (DAS28) activity [20].

Pyrrolidine dithiocarbamate (PDTTC), SP600125, SB203580, PD98059, and anti- β -actin antibody was purchased from Sigma (St. Louis, MO, USA). Rabbit anti-human IL-34 for immunoblotting was from ProSci Incorporated (Poway, CA, USA). Mouse anti-human IL-34 for blocking IL-34 activity, mouse polyclonal immunoglobulin G (IgG), IL-17, TNF α , and IL-1 β were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant M-CSF, RANKL, and IL-34 were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA).

Immunohistochemistry

OA and RA synovia for immunohistochemistry analysis were collected in accordance with experimental protocols approved by the Stanford University Institutional Review Board and with the patients' informed consent as described [12]. Sections of optimal cutting temperature (OCT) compound-embedded synovial tissue samples were cut on a cryostat (Thermo Scientific, Kalamazoo, MI, USA) and fixed in 4% paraformaldehyde for 10 minutes at 4°C. The slides were then washed twice (5 minutes each) with PBS and permeabilized with 0.1% Triton X-100 for 3 minutes at room temperature. After washing with PBS, endogenous peroxidase activity was quenched with 0.03% H₂O₂ solution. The slides were blocked with 7.5% normal goat serum for 20 minutes and thereafter incubated with 4 μ g/ml of IL-34 antibody (LSBio, Seattle, WA, USA) or control rabbit IgG (Abcam, Cambridge, MA, USA) for 20 minutes at room temperature. Following two washes with PBS, the slides were incubated with 1:200 diluted biotinylated secondary antibody (Vectastain EliteABC Kit; Vector Laboratories, Orton Southgate, Peterborough, UK) for 30 minutes. After washing with PBS, the slides were incubated with complexed avidin-biotin horseradish peroxidase ABC reagent (Vectastain Elite ABCkit; Vector) for 30 minutes, and then exposed to 3,3-diaminobenzidine (DAB) substrate according to the manufacturer's instructions (Vector). The nuclei were subsequently counterstained with hematoxylin (Vector). Tissues were examined and photographed with an Olympus BX51 microscope outfitted with an Olympus DP72 digital camera (Olympus).

Isolation of FLS and OCPs from human peripheral blood mononuclear cells (PBMCs)

FLS were isolated by enzymatic digestion of synovial tissues obtained from RA and OA patients undergoing total joint replacement surgery, as previously described [21]. Blood was collected from healthy volunteers and PBMCs were obtained from whole blood by

centrifugation over Ficoll gradients (Pharmacia Biotech, Uppsala, Sweden) at 400g for 30 minutes and erythrocytes were removed by lysing in ACK buffer (0.15 M NH₄Cl, 1 mM KHCO₃ and 0.1 mM EDTA, pH 7.2).

Conventional reverse transcriptase-polymerase chain reaction (RT-PCR) PCR and quantitative real-time RT-PCR

Total RNA was extracted from synovial tissues obtained from three RA patients, as indicated, using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA was synthesized from total RNA using murine Moloney leukemia virus reverse transcriptase (MMTV-RT, Gibco BRL, Gaithersburg, MD, USA). The cDNA was amplified using the Takara PCR amplification kit (Takara Biotechnology, Shiga, Japan); PCR was carried out in a Perkin Elmer thermal cycler. Quantitative RT-PCR (qRT-PCR) was performed using Power SYBR Green 1-Step Kit and an ABI 7000 Real Time PCR System (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. The sequences of primers used were as follows: IL-34, 5'-TAC AGG AGC CGA CTT CAG-3' (sense) and 5'-CTC ACC AAG ACC CAC AGA-3' (antisense); M-CSF, 5'-GCT GCT TCA CCA AGG ATT ATG-3' (sense) and 5'-GGG TCA CTG CTA GGG ATG-3' (antisense); and GAPDH (glyceraldehyde 3-phosphate dehydrogenase), 5'-AGC CAC ATC GCT CAG ACA-3' (sense) and 5'-GCC CAA TAC GAC CAA ATC C-3' (antisense). Results are expressed as the ratio of target PCR product relative to GAPDH product. The amplification protocol consisted of an initial reverse transcription step at 48°C for 30 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute.

Enzyme-linked immunosorbent assay (ELISA)

IL-34 concentration was measured using an IL-34-specific sandwich ELISA (R&D systems, Minneapolis, MN, USA) in accordance with the protocols of the manufacturer. Recombinant human IL-34 serially diluted in culture media was used as a standard. ELISA plates were incubated with mouse anti-human IL-34 Ab and then blocked with 3% BSA in PBS for 1 hour at room temperature. SF from patients with RA and OA, or supernatants from FLS cultures were added to the plates. After washing with PBS containing 0.05% Tween 20, each well was incubated with biotinylated mouse anti-human IL-34 (0.5 µg/ml) for 2 hours at room temperature. Plates were then washed with PBS containing 0.05% Tween 20 and incubated with streptavidin conjugated to horseradish peroxidase (HRP) for 1 hour at room temperature. A color reaction was developed with tetramethylbenzidine solution, and then stopped with 0.1 M H₂SO₄. The absorbance at 450 nm was measured using a Bio-Rad microtiter plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblotting

RA FLS were collected and proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper (GE Healthcare, Piscataway, NJ, USA). The membranes were immunoblotted with an anti-human IL-34 rabbit polyclonal IgG or β-actin antibody. Bound antibodies were visualized with HRP-conjugated antibodies against rabbit or mouse IgG using an enhanced chemiluminescence Western blotting system (Millipore, Billerica, MA, USA).

PBMCs migration assay

Transmigration assays were performed as follows using PBMCs: conditioned media (CM) was prepared by collecting supernatant from RA FLS cultured in the presence of TNFα (10 ng/ml) for 24 hours. CM from RA FLS cultures, recombinant IL-34 (100 ng/ml), or CM plus anti-IL-34 Ab was added to the lower compartments of a 5-µm pore Transwell system (Costar, Corning, NY, USA). PBMCs (1.2 × 10⁶) were added to the upper chamber of each transwell and allowed to migrate for 6 hours. The number of PBMCs in the lower chamber was then counted.

OC differentiation

For osteoclastogenesis, PBMCs were plated on 48-well plates and cultured in α-MEM supplemented with 10% fetal bovine serum (FBS) and cultured with RANKL (100 ng/ml) plus M-CSF (100 ng/ml) as a control as described [21] or RANKL (100 ng/ml) plus IL-34 (100 ng/ml). RANKL was added at a final concentration of 100 ng/ml to CM obtained from RA FLS cultured in the presence of TNFα (10 ng/ml) for 24 hours. Osteoclastic differentiation was maintained for 12 to 14 days prior to tartrate resistant acid phosphatase (TRAP) staining and scoring of multinucleated OCs, as previously described [22].

Statistical analysis

Means ± standard deviations (SDs) were calculated for all conditions, and differences between means were analyzed using Student's *t*-test. A *P*-value < 0.05 was considered significant. Patient data were tested by the Mann-Whitney U test.

Results

High levels of IL-34 in synovia and SF from patients with RA

To demonstrate the involvement of IL-34 in RA pathogenesis, we first determined whether synovia and SF from patients with RA (RA SF) displayed high levels of IL-34. IL-34 mRNA expression was detected in synovia obtained from three RA patients at levels similar to those of M-CSF (Figure 1A); high levels of IL-34 mRNA

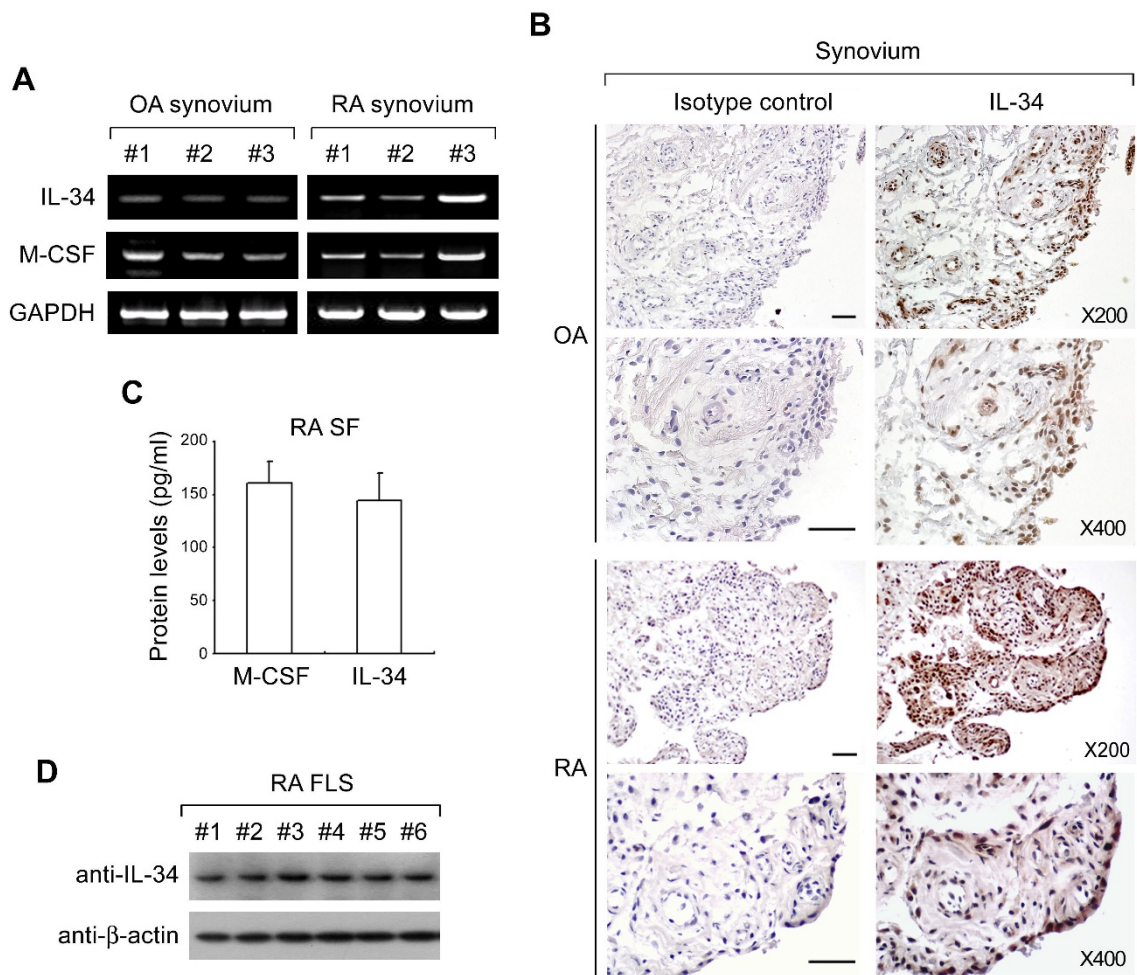


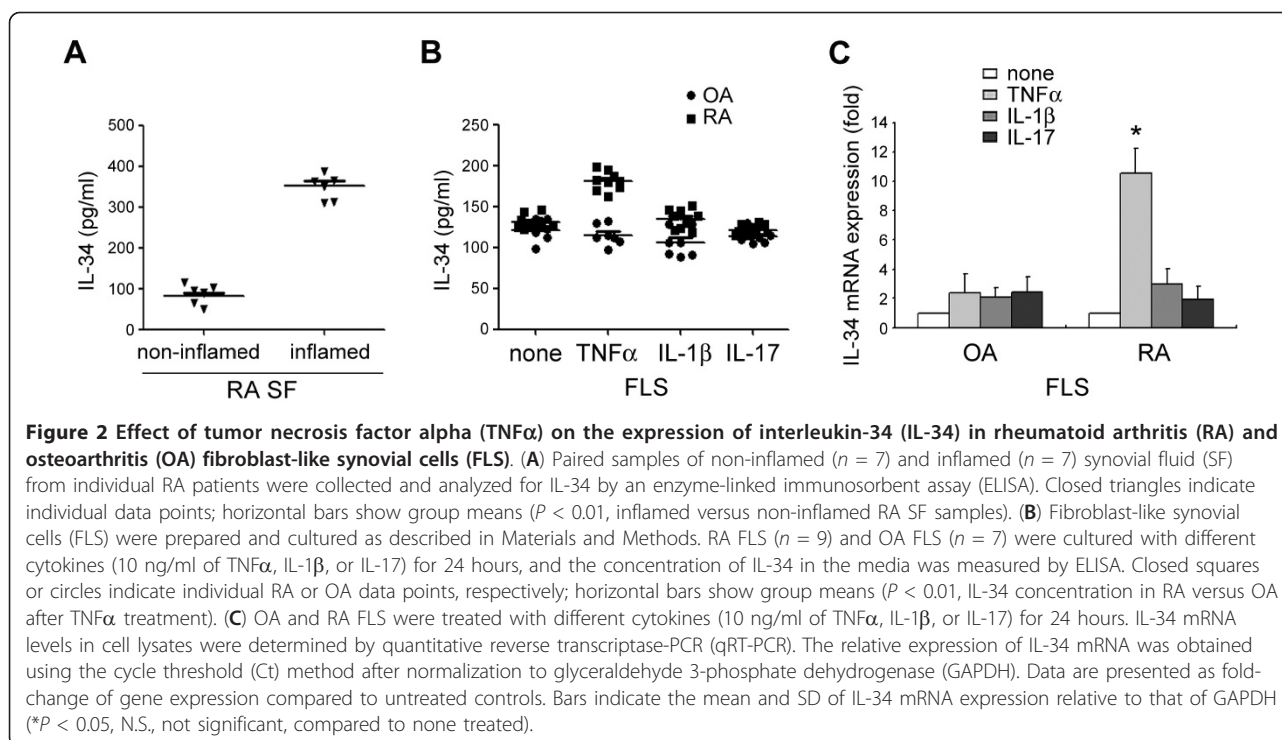
Figure 1 Expression of interleukin-34 (IL-34) in human rheumatoid arthritis (RA) synovium and synovial fluid (SF). (A) IL-34 and macrophage colony-stimulating factor (M-CSF) mRNA levels in synovia from patients with RA ($n = 3$) and osteoarthritis (OA) ($n = 3$) were determined by reverse transcriptase PCR (RT-PCR). Synovium were homogenized and lysed, and total RNA was extracted as described in Materials and Methods. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were detected as a control. (B) Representative immunohistochemical images of OA or RA synovia stained with antibodies against IL-34 or isotype controls. Images are shown at 200 \times (upper) and 400 \times (lower) magnification. Scale bars = 100 μ m. (C) Synovial fluid (SF) from RA patients ($n = 7$) (RA SF) was collected and the concentrations of M-CSF and IL-34 were measured using an enzyme-linked immunosorbent assay (ELISA) assay. (D) Expression of IL-34 protein in fibroblast-like synovial cells (FLS) from RA patients ($n = 6$) was determined by immunoblotting against human IL-34. Whole-cell lysates of RA FLS cells were resolved by SDS-PAGE and followed by immunoblotting with anti-human IL-34 and anti- β -actin antibodies.

(Figure 1A) and protein (Figure 1B) were observed in human RA synovium compared to OA synovium. The levels of IL-34 protein in RA SF were comparable to those of M-CSF (Figure 1C). In addition, we found that IL-34 protein was expressed in FLS from RA patients (Figure 1D). These observations indicate that IL-34 is expressed in synovial tissue, synovial fluid, and FLS from RA patients, suggesting that IL-34 is an additional factor involved in the pathogenesis of RA.

TNF α -induced IL-34 production by FLS of RA patients

FLS act as the main cell mediating the cytokine-rich environment of the inflamed synovium in RA pathogenesis [23]

and stimulate OC differentiation and activity in the inflammatory bone destruction by regulating RANKL expression [24,25]. Interestingly, we found that the level of soluble IL-34 in RA SF from inflamed RA synovium (351.7 ± 30.7 pg/ml) with joint swelling was significantly higher than that in RA SF from non-inflamed RA synovium (81.1 ± 24.5 pg/ml; $P < 0.01$) (Figure 2A). We therefore determined whether RA FLS produced IL-34 in response to pro-inflammatory cytokines. RA FLS ($n = 9$) or OA FLS ($n = 4$), used as a disease control, were cultured with TNF α , IL-1 β , or IL-17 (each at 10 ng/ml) for 24 hours. Interestingly, the increase in IL-34 concentration was more marked in TNF α -stimulated RA FLS than in IL-1 β - or IL-



17-stimulated RA FLS (Figure 2B). In contrast, OA FLS showed no significant changes in those responses (Figure 2B). Moreover, an analysis of IL-34 mRNA levels using quantitative real-time RT-PCR showed that IL-34 mRNA expression was markedly increased in TNF α -stimulated RA FLS compared to controls ($P < 0.01$) (Figure 2C). IL-1 β and IL-17 treatment induced a smaller increase in IL-34 mRNA expression (Figure 2C). However, the level of IL-34 expression in control OA FLS was not significantly modulated by these cytokines (Figure 2C). These results provide the first demonstration that RA FLS produce IL-34 and that this production is enhanced by TNF α .

Differential induction of IL-34 by RA FLS treated with TNF α

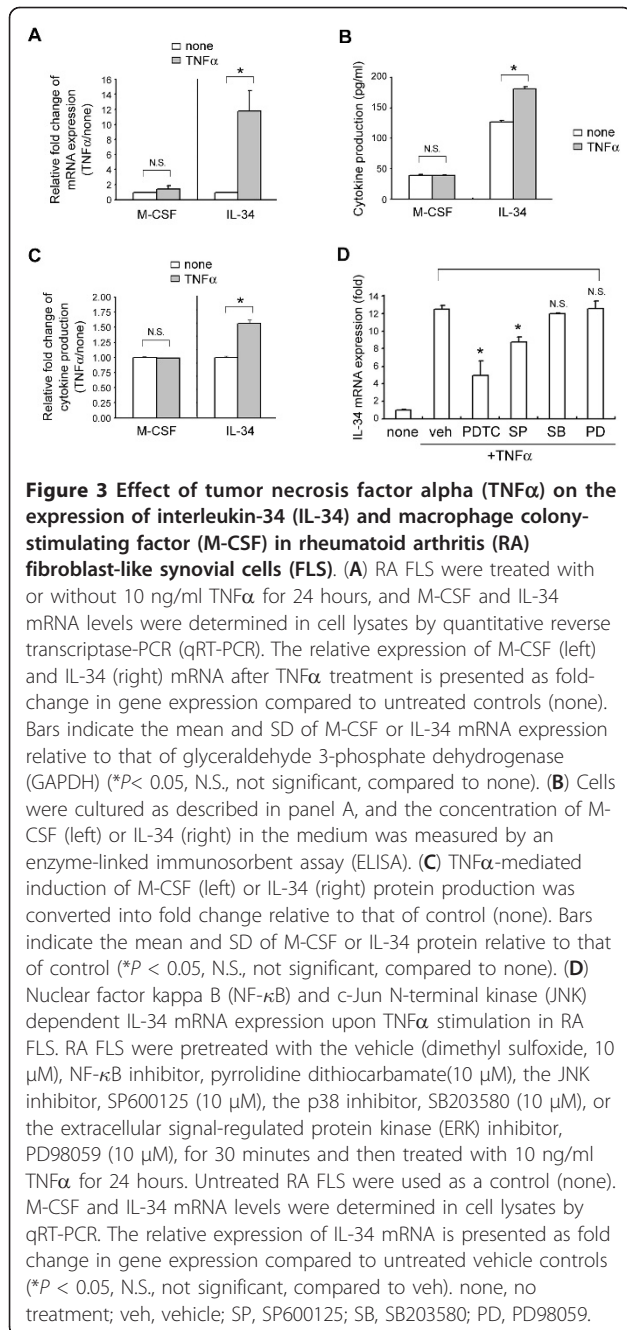
IL-34 and M-CSF were recently reported to share the same receptor, c-Fms [13]. Our data showed that IL-34 expression was enhanced after treatment with TNF α (Figure 2), suggesting that differential production of IL-34, regulated by the pathological condition of RA, can replace M-CSF function. To test this possibility, we next examined whether TNF α differentially regulates IL-34 and M-CSF expression. RA FLS constitutively expressed and produced both M-CSF and IL-34 (Figure 3A). TNF α treatment caused a 12-fold increase in IL-34 mRNA expression above this basal level (Figure 3A, right), but had little effect on M-CSF mRNA expression (Figure 3A, left). Together, the level of IL-34 protein in RA FLS was much greater than M-CSF production in

the presence of TNF α (Figure 3B), showing approximately a 1.5-fold increase in TNF α -treated RA FLS over that of the control RA FLS (Figure 3C). Thus, these data demonstrate the differential regulation of IL-34 and M-CSF expression by TNF α .

TNF α activates NF- κ B and JNK, which are important for TNF α -mediated gene expression and are involved in the activation of FLS [26]. To understand better the mechanism by which TNF α increases IL-34 production in RA FLS, we examined the effects of inhibitors of NF- κ B or JNK on IL-34 expression. RA FLS were pretreated with the vehicle, NF- κ B inhibitor, PDTC, or the JNK inhibitor, SP600125, at a concentration of 10 μ M for 30 minutes followed by incubation with TNF α for 24 hours. As shown in Figure 3D, the NF- κ B inhibitor, PDTC, effectively reduced TNF α -induced IL-34 mRNA expression; moreover, JNK inhibition modestly decreased the TNF α -induced elevation of IL-34. However, p38 or extracellular signal-regulated protein kinase (ERK) inhibition did not affect TNF α -induced IL-34 mRNA expression (Figure 3D). Collectively, these results suggest that the TNF α -induced increase in IL-34 expression in RA FLS is mediated by NF- κ B and JNK pathways.

Clinical determination of IL-34 in plasma from patients with RA

Given that IL-34 is up-regulated in inflamed synovium and in RA FLS in response to TNF α , (Figure 2) the levels of IL-34 may reflect the pathogenesis of



inflammation in RA patients. To explore this correlation, we performed a comparative analysis of plasma IL-34 levels in RA patients ($n = 10$) using ELISAs. The patients with RA had higher plasma IL-34 levels than normal or OA controls (Figure 4A). One group of patients with rheumatoid arthritis, defined by a plasma DAS28 score at baseline of 2.9 to 7.4, had undergone a 1-year follow-up treatment with DMARDs, including methotrexate, sulfasalazine, leflunomide, minocycline, and/or hydroxychloroquine. Patients in this group ($n =$

10) showed an apparent decrease in DAS28 score of approximately 50% (to 1.7 to 3.4) after taking DMARDs (Figure 4B). Importantly, high levels of soluble IL-34 (6.573 ± 2.003 ng/ml) were detected in plasma from RA patients before DMARDs treatment (Figure 4C). However, a significant decrease ($P < 0.01$) in plasma IL-34 levels was observed after taking DMARDs in this group (Figure 4C). These data suggest that the plasma level of IL-34 correlates with the level of inflammation in RA patients.

Effect of IL-34 on PBMCs migration and subsequent OC formation

Having shown that RA FLS produce IL-34 (Figure 2A and 3), we thus questioned whether IL-34 produced by RA FLS has functional activities to induce chemotactic migration of OCPs and to subsequently induce osteoclastogenesis as a substitute for M-CSF in RANKL-induced osteoclastogenesis. Human PBMCs as OCPs [27] in the upper chamber and CM from TNF α -treated RA FLS or recombinant IL-34 (positive control) in the lower chamber were incubated. Approximately 7.5×10^5 human PBMCs migrated in the presence of recombinant IL-34; a similar level of cell migration was observed in the presence of CM, which contains soluble IL-34 secreted from RA FLS (Figure 5A). However, addition of a blocking Ab against IL-34 to CM reduced the number of migrated human mononuclear cells in a dose-dependent manner.

To further assess the effects of IL-34 produced by RA FLS on RANKL-induced OC differentiation, we cultured isolated human PBMCs with RANKL in the presence or absence of M-CSF, IL-34, CM, or an anti-IL-34 Ab for 12 to 14 days. M-CSF plus RANKL treatment induced OC differentiation (851.5 ± 17.7 ; Figure 5B). IL-34 or CM from RA FLS cultures containing IL-34 supported RANKL-induced OC formation to an extent similar to that of M-CSF. The addition of anti-IL-34 antibody to block functional IL-34 in CM (CM + R + IL-34 Ab) significantly reduced OC formation (472.4 ± 39 ; $P = 0.042$) (Figure 5C). These data demonstrate that IL-34 secreted by RA FLS is functionally active enough to stimulate the chemotactic migration of OCPs and subsequent OC formation.

Discussion

Although RANKL is absolutely required for OC differentiation *in vivo* [28,29], several cytokines, including vascular endothelial growth factor and hepatocyte growth factor, can be substituted for M-CSF in promoting osteoclastogenesis [30-32]. The inflamed synovium of RA patients is the major source of overproduction of cytokines, and these cytokines may contribute to osteoclastic activation [33]; thus, understanding the specific

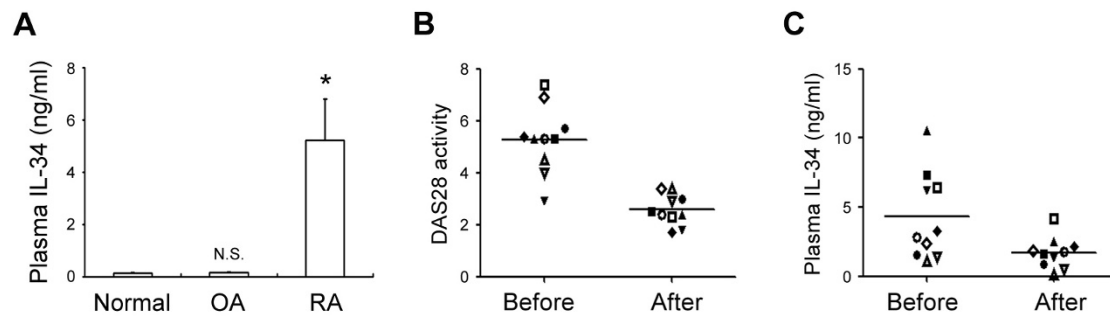


Figure 4 Correlation between interleukin-34 (IL-34) levels and inflammation in rheumatoid arthritis (RA) patients. (A) IL-34 levels were determined in plasma of healthy controls (normal), OA or RA patients by enzyme-linked immunosorbent assay (ELISA) (* $P < 0.05$, N.S., not significant, compared to normal). (B) and (C) Plasma was collected from individual RA patients ($n = 10$) and the same patients were administered disease-modifying anti-rheumatic drugs (DMARDs) and followed-up for 1 year. Disease Activity Score 28 (DAS28) activity (A) and IL-34 levels (ELISA) (B) were determined for each patient in plasma samples collected before and after administration of DMARDs. Symbols indicate individual data points; horizontal bars show group means ($P < 0.01$, after versus before DMARDs administration).

factors capable of replicating the effect of M-CSF is of considerable research interest.

Inflammatory cytokines, including IL-1 β , IL-17 and TNF α , are elevated in the SF of RA patients [34] and are known to stimulate osteoclastogenesis through induction of M-CSF [35-37]. In this study, we demonstrated that both IL-34 mRNA and protein expression were up-regulated by TNF α to a greater extent in RA FLS than in OA FLS, a pattern distinct from that of M-CSF expression (Figure 2). Thus, this is the first report describing the stimulation of IL-34 expression by TNF α in FLS of RA patients, suggesting that RA FLS are much more susceptible to the production of IL-34 than M-CSF in response to TNF α . Further study implies a discrete role of IL-34 in the pathological inflammatory setting of RA.

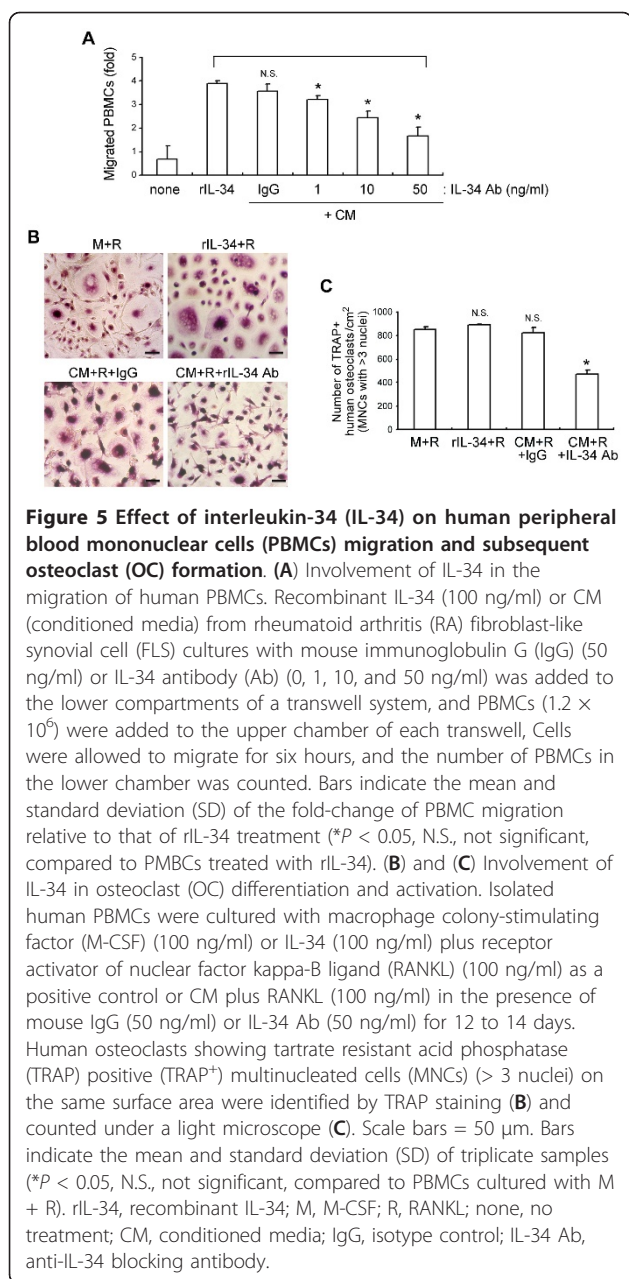
A recent report demonstrated that TNF α induces IL-34 mRNA via JNK and NF- κ B, but not the p38 pathway, in osteoblasts [38]. NF- κ B, in particular, is responsible for TNF α -induced M-CSF expression in primary osteoblast cells [39]. In our experiments, TNF α -mediated induction of IL-34 expression was inhibited by an NF- κ B inhibitor, indicating that IL-34 expression is regulated, at least in part, by NF- κ B. Moreover, JNK inhibition modestly decreased the elevation of IL-34 expression, indicating that JNK may also be involved in IL-34 expression in FLS of RA patients (Figure 3D).

Several combinations of DMARDs used for the treatment of arthritis conditions [40], such as RA or psoriatic arthritis (PsA), have been demonstrated to abrogate inflammation [41]. IL-34 was detected in plasma samples of RA patients and it was effectively reduced by DMARDs treatment in RA patients (Figure 4A). Our data provide an initial indication of the importance of the clinical correlation between plasma IL-34 levels and inflammatory responses in RA patients, suggesting the

significance of IL-34 as a plasma factor in RA patients representing inflammatory arthritis in the pathogenesis of RA. In our study population, individual variations in combination therapy were not controlled; thus, additional clinical studies with a larger number of patients divided into subgroups is needed to validate the clinical implications of elevated IL-34 in the pathogenesis of RA.

IL-34, like M-CSF, has been shown to promote osteoclastogenesis in combination with RANKL [14,18] and induce bone resorption activity [18]. We also found that IL-34 produced from TNF α -stimulated RA-FLS promoted chemotactic migration of human PBMCs and RANKL-induced osteoclastogenesis (Figure 5), demonstrating a biologically active function of IL-34. Therefore, TNF α -stimulated up-regulation of IL-34 in RA FLS may play a pathological role in RA, possibly at the site of inflammatory erosive bone resorption and local bone destruction. In PsA, an inflammatory joint disease characterized by extensive bone resorption, OCPs arise from TNF α -activated PBMCs that migrate to the inflamed synovium and subchondral bone, where they are exposed to unopposed RANKL and TNF α [42]. This leads to osteoclastogenesis at the erosion front and in subchondral bone, resulting in a bidirectional assault on psoriatic bone [42]. A recent report showed that M-CSF levels are not elevated in SF from patients with PsA to the same extent as in RA patients [12]. Accordingly, IL-34 may replace this M-CSF function and contribute to the pathogenesis of PsA; however, the role of IL-34 in PsA remains to be investigated.

Genetic ablation of M-CSF in *op/op* (M-CSF-deficient) mice and inhibition of M-CSF by administration of anti-M-CSF mAbs exerts protective effects in a number of inflammatory and/or autoimmune conditions [43,44]. Moreover, blocking c-Fms inhibits macrophage infiltration, TNF α production by macrophages, and OC



formation and activation in several mouse arthritis models [12,45], and reduces TNF α -induced inflammatory arthritis [11], suggesting a therapeutic benefit in RA treatment. The increase in IL-34 in RA reported here supports the concept that IL-34 regulates OCP migration and subsequent OC differentiation, suggesting the possibility that IL-34 can functionally substitute for M-CSF and is involved in the RA disease process elicited by TNF α . It is important to note in this context that elevated IL-34 in RA SF can activate c-Fms, even in the absence of M-CSF. Thus, inhibiting c-Fms may be a more promising strategy than targeting M-CSF in the

treatment of RA where the disease pathogenesis involves TNF α .

Conclusions

We propose that IL-34 is elevated in RA SF and is produced by RA FLS. IL-34 expression is enhanced by TNF α through the NF- κ B and JNK pathway and is a biologically active stimulator of OC differentiation that exerts synergistic effects with RANKL. Further investigations to define the pathological implications of this cytokine in osteolysis *in vivo* are warranted.

Additional material

Additional file 1: Characteristics of patients with rheumatoid arthritis (RA).

All patients were 30 years or older at the time of RA diagnosis and their mean \pm SD age was 53.3 ± 16.0 years. All patients were positive for rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) antibody and had a diagnosis of RA with a median baseline Disease Activity Score 28 (DAS28) of 5.27 (range 2.9 to 7.4), a median 1 year DAS28 of 2.58 (1.7 to 3.4), a baseline median erythrocyte sedimentation rate of 69.6 (28 to 120), and a median baseline C-reactive protein (CRP) of 1.05 (0.1 to 2.04). With regard to medications used for the treatment of RA, patients were treated with prednisolone and disease-modifying antirheumatic drugs (DMARDs) including methotrexate, sulfasalazine, leflunomide, FK506, and/or hydroxychloroquine. SD, standard deviation.

Abbreviations

Ab: antibody; BSA: bovine serum albumin; CM: conditioned media; DAS28: disease activity score 28; DMARDs: disease-modifying antirheumatic drugs; ERK: extracellular signal-regulated protein kinase; FLS: fibroblast-like synovial cells; HRP: horseradish peroxidase; IL-34: interleukin-34; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; M-CSF: macrophage colony-stimulating factor; NF- κ B: nuclear factor kappa B; OA: osteoarthritis; OC: osteoclast; OCPs: osteoclast precursors; PBS: phosphate-buffered saline; PDTC: pyrrolidine dithiocarbamate; PsA: psoriatic arthritis; PBMCs: peripheral blood mononuclear cells; qRT-PCR: quantitative reverse transcriptase -polymerase chain reaction; RA: rheumatoid arthritis; RANKL: receptor activator of NF- κ B ligand; RT-PCR: reverse transcriptase-polymerase chain reaction; SF: synovial fluid; TNF α : tumor necrosis factor alpha; TRAP: tartrate-resistant acid phosphatase.

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Authors' contributions

SH, BC, and SK performed experiments, analyzed data, and wrote the manuscript. JC and YC performed FLS and PBMCs isolations. DS performed immunohistochemistry. WHR was involved in study design and data analysis. YK and CL, as clinical supervisors, provided RA SF and synovial tissues. MS

performed statistical analysis on the patients' plasma samples. EC designed the study, contributed to data analysis, and wrote the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

- McInnes IB, Schett G: Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol* 2007, **7**:429-442.
- Takayanagi H: Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat Rev Immunol* 2007, **7**:292-304.
- Teitelbaum SL: Bone resorption by osteoclasts. *Science* 2000, **289**:1504-1508.
- Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD, Nishikawa S: The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* 1990, **345**:442-444.
- Schwarz EM, Looney RJ, Drissi MH, O'Keefe RJ, Boyce BF, Xing L, Ritchlin CT: Autoimmunity and bone. *Ann N Y Acad Sci* 2006, **1068**:275-283.
- Huber LC, Distler O, Tärner I, Gay RE, Gay S, Pap T: Synovial fibroblasts: key players in rheumatoid arthritis. *Rheumatology (Oxford)* 2006, **45**:669-675.
- Dai SM, Nishioka K, Yudoh K: Interleukin (IL) 18 stimulates osteoclast formation through synovial T cells in rheumatoid arthritis: comparison with IL1 β and tumour necrosis factor α . *Ann Rheum Dis* 2004, **63**:1379-1386.
- Zhang Q, Guo R, Schwarz EM, Boyce BF, Xing L: TNF inhibits production of stromal cell-derived factor 1 by bone stromal cells and increases osteoclast precursor mobilization from bone marrow to peripheral blood. *Arthritis Res Ther* 2008, **10**:R37.
- Schett G: Review: Immune cells and mediators of inflammatory arthritis. *Autoimmunity* 2008, **41**:224-229.
- Bradley JR: TNF-mediated inflammatory disease. *J Pathol* 2008, **214**:149-160.
- Kitaura H, Zhou P, Kim HJ, Novack DV, Ross FP, Teitelbaum SL: M-CSF mediates TNF-induced inflammatory osteolysis. *J Clin Invest* 2005, **115**:3418-3427.
- Paniagua RT, Chang A, Mariano MM, Stein EA, Wang Q, Lindstrom TM, Sharpe O, Roscow C, Ho PP, Lee DM, Robinson WH: c-Fms-mediated differentiation and priming of monocyte lineage cells play a central role in autoimmune arthritis. *Arthritis Res Ther* 2010, **12**:R32.
- Lin H, Lee E, Hestir K, Leo C, Huang M, Bosch E, Halenbeck R, Wu G, Zhou A, Behrens D, Hollenbaugh D, Linnemann T, Qin M, Wong J, Chu K, Doberstein SK, Williams LT: Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. *Science* 2008, **320**:807-811.
- Baud'huin M, Renault R, Charrier C, Riet A, Moreau A, Brion R, Gouin F, Duplomb L, Heymann D: Interleukin-34 is expressed by giant cell tumours of bone and plays a key role in RANKL-induced osteoclastogenesis. *J Pathol* 2010, **221**:77-86.
- Eda H, Zhang J, Keith RH, Michener M, Beidler DR, Monahan JB: Macrophage-colony stimulating factor and interleukin-34 induce chemokines in human whole blood. *Cytokine* 2010, **52**:215-220.
- Chihara T, Suzu S, Hassan R, Chutiwittoonchai N, Hiyoshi M, Motoyoshi K, Kimura F, Okada S: IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation. *Cell Death Differ* 2010, **17**:1917-1927.
- Wei S, Nandi S, Chitu V, Yeung YG, Yu W, Huang M, Williams LT, Lin H, Stanley ER: Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. *J Leukoc Biol* 2010, **88**:495-505.
- Chen Z, Buki K, Vääränen J, Gu G, Väänänen HK: The critical role of IL-34 in osteoclastogenesis. *PLoS One* 2011, **6**:e18689.
- Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, et al: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988, **31**:315-324.
- van der Kooij E, Klarenbeek NB, Güler-Yüksel M, Kerstens PJ, van der Lubbe PA, Westedt ML, ten Wolde S, Huizinga TW, Dijkmans BA, Allaart CF: A decrease in disease activity score (DAS) level is associated with a decrease in health assessment questionnaire (HAQ) score, independent of follow-up duration, during 5 years of tightly controlled treatment: results from the BeSt study. *Ann Rheum Dis* 2011, **70**:168-171.
- Kim YG, Lee CK, Oh JS, Kim SH, Kim KA, Yoo B: Effect of interleukin-32gamma on differentiation of osteoclasts from CD14+ monocytes. *Arthritis Rheum* 2010, **62**:515-523.
- Chang EJ, Kim HJ, Ha J, Kim HJ, Ryu J, Park KH, Kim UH, Lee ZH, Kim HM, Fisher DE, Kim HH: Hyaluronan inhibits osteoclast differentiation via Toll-like receptor 4. *J Cell Sci* 2007, **120**:166-176.
- Pap T, Muller-Ladner U, Gay RE, Gay S: Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis. *Arthritis Res* 2000, **2**:361-367.
- Kim KW, Cho ML, Lee SH, Oh HJ, Kang CM, Ju JH, Min SY, Cho YG, Park SH, Kim HY: Human rheumatoid synovial fibroblasts promote osteoclastogenic activity by activating RANKL via TLR-2 and TLR-4 activation. *Immunol Lett* 2007, **110**:54-64.
- Hashizume M, Hayakawa N, Mihara M: IL-6 trans-signalling directly induces RANKL on fibroblast-like synovial cells and is involved in RANKL induction by TNF- α and IL-17. *Rheumatology* 2008, **47**:1635-1640.
- Dulos J, Kaptein A, Kavelaars A, Heijnen C, Boots A: Tumour necrosis factor-alpha stimulates dehydroepiandrosterone metabolism in human fibroblast-like synoviocytes: a role for nuclear factor-kappaB and activator protein-1 in the regulation of expression of cytochrome p450 enzyme 7b. *Arthritis Res Ther* 2005, **7**:R1271-1280, *Immunol Lett* 2010;127:100-107.
- Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, Koga T, Martin TJ, Suda T: Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. *Proc Natl Acad Sci USA* 1990, **87**:7260-7264.
- Heymann D, Guicheux J, Gouin F, Passuti N, Daculsi G: Cytokines, growth factors and osteoclasts. *Cytokine* 1998, **10**:155-168.
- Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM: OPG is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 1999, **397**:315-323.
- Niida S, Kaku M, Amano H, Yoshida H, Kataoka H, Nishikawa S, Tanne K, Maeda N, Nishikawa S, Kodama H: Vascular endothelial growth factor can substitute for macrophage colony-stimulating factor in the support of osteoclastic bone resorption. *J Exp Med* 1999, **190**:293-298.
- Aldridge SE, Lennard TW, Williams JR, Birch MA: Vascular endothelial growth factor receptors in osteoclast differentiation and function. *Biochem Biophys Res Commun* 2005, **335**:793-798.
- Adamopoulos IE, Xia Z, Lau YS, Athanasou NA: Hepatocyte growth factor can substitute for M-CSF to support osteoclastogenesis. *Biochem Biophys Res Commun* 2006, **350**:478-483.
- Romas E, Gillespie MT, Martin TJ: Involvement of receptor activator of NFkappaB ligand and tumor necrosis factor-alpha in bone destruction in rheumatoid arthritis. *Bone* 2002, **30**:340-346.
- Kutukculer N, Caglayan S, Aydogdu F: Study of pro-inflammatory (TNF-alpha, IL-1alpha, IL-6) and T-cell-derived (IL-2, IL-4) cytokines in plasma and synovial fluid of patients with juvenile chronic arthritis: correlations with clinical and laboratory parameters. *Clin Rheumatol* 1998, **17**:288-292.
- Hamilton JA: Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol* 2008, **8**:533-544.
- Hamilton JA, Filonzi EL, Lanches G: Regulation of macrophage colony-stimulating factor (M-CSF) production in cultured human synovial fibroblasts. *Growth Factors* 1993, **9**:157-165.
- Campbell IK, Lanches G, Hamilton JA: Production of macrophage colony-stimulating factor (M-CSF) by human articular cartilage and chondrocytes. Modulation by interleukin-1 and tumor necrosis factor alpha. *Biochim Biophys Acta* 1993, **1182**:57-63.
- Eda H, Shimada H, Beidler DR, Monahan JB: Proinflammatory cytokines, IL-1beta and TNF-alpha, induce expression of interleukin-34 mRNA via JNK- and p44/42 MAPK-NF-kappaB pathway but not p38 pathway in osteoblasts. *Rheumatol Int* 2011, **31**:1525-1530.

39. Yao GQ, Sun BH, Insogna KL, Weir EC: Nuclear factor-kappaB p50 is required for tumor necrosis factor-alpha-induced colony-stimulating factor-1 gene expression in osteoblasts. *Endocrinology* 2000, **141**:2914-2922.
40. Dale J, Alcorn N, Capell H, Madhok R: Combination therapy for rheumatoid arthritis: methotrexate and sulfasalazine together or with other DMARDs. *Nat Clin Pract Rheumatol* 2007, **3**:450-458.
41. Scott DL, Wolfe F, Huizinga TW: Rheumatoid arthritis. *Lancet* 2010, **376**:1094-108.
42. Ritchlin CT, Haas-Smith SA, Li P, Hicks DG, Schwarz EM: Mechanisms of TNF-alpha- and RANKL-mediated osteoclastogenesis and bone resorption in psoriatic arthritis. *J Clin Invest* 2003, **111**:821-831.
43. Campbell IK, Rich MJ, Bischof RJ, Hamilton JA: The colony-stimulating factors and collagen-induced arthritis: exacerbation of disease by M-CSF and G-CSF and requirement for endogenous M-CSF. *J Leukoc Biol* 2000, **68**:144-150.
44. Lenda DM, Kikawada E, Stanley ER, Kelley VR: Reduced macrophage recruitment, proliferation, and activation in colony-stimulating factor-1-deficient mice results in decreased tubular apoptosis during renal inflammation. *J Immunol* 2003, **170**:3254-3262.
45. Irvine KM, Burns CJ, Wilks AF, Su S, Hume DA, Sweet MJ: A CSF-1 receptor kinase inhibitor targets effector functions and inhibits pro-inflammatory cytokine production from murine macrophage populations. *FASEB J* 2006, **20**:1921-1923.

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