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ORIGINAL ARTICLE

# Effects of IL-34 on the secretion of RANKL/OPG by fibroblast-like synoviocytes and peripheral blood mononuclear cells in rheumatoid arthritis

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**ABSTRACT.** ***Objective:*** **To detect the effect of interleukin (IL)-34 on the secretion of Receptor activator of nuclear factor kappa-B ligand (RANKL)/Osteoprotegerin (OPG) and Matrix metalloproteinase (MMP)-3 by fibroblast-like synoviocytes (FLS) and peripheral blood mononuclear cells (PBMCs) of rheumatoid arthritis (RA) patients and to investigate whether the effect is mediated by IL-17.** ***Method:*** **RA-FLS and RA-PBMCs were stimulated with recombinant human (rh) IL-34, with or without the IL-17 inhibitor Plumbagin. The supernatant of the culture medium was collected and the levels of RANKL, OPG, and MMP-3 were detected by enzyme-linked immunosorbent assay (ELISA).** ***Results:*** **RhIL-34 promoted RANKL secretion and inhibited OPG secretion in RA-FLS. The effect was weakened by the addition of the IL-17 inhibitor. In contrast, rhIL-34 had no significant effect on MMP-3 secretion by FLS. RhIL-34 elevated the secretion of RANKL by RA-PBMCs but not by healthy-PBMCs. Furthermore, the secretion of RANKL by RA-PBMCs reduced after the addition of the IL-17 inhibitor. OPG secretion by both RA-FLS and FLS from healthy controls was inhibited by rhIL-34, but were elevated after the addition of the IL-17 inhibitor. RhIL-34 had no significant effect on MMP-3 secretion by both RA-PBMCs and healthy-PBMCs.** ***Conclusion:*** **IL-34 enhances RANKL/OPG expression by RA-FLS and RA-PBMCs, and this effect is, indirectly, mediated by IL-17. This cytokine is therefore likely to to play an important role in local joint destruction and systemic osteoporosis in RA, and is therefore a potential therapeutic target for the treatment of this disease.**

**Key words:** rheumatoid arthritis, interleukin-34, interleukin-17, receptor activator of nuclear factor kappa-B ligand, osteoprotegerin, matrix metalloproteinase

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovitis, bone, and cartilage damage of multiple joints. Periarticular osteopenia, joint space narrowing, and bony erosions are characteristics of bone involvement in RA [1]. Bone and cartilage damage in RA are mostly caused by osteoclasts and fibroblast-like synoviocytes (FLSs) [2, 3]. Bone damage is caused by osteoclast, while cartilage damage is caused by protease secreted by FLSs. FLSs produce abundant amount of protease which bind to chondrocytes and invade into extracellular matrix [4].

Various inflammatory cytokines and immune cells are involved in the osteoporosis and bone destructive process of RA, among which RANKL is most important [5]. Serum RANKL levels in RA patients are significantly elevated. Some groups reported that concentration of RANKL in the synovial fluids is significantly higher in patients with RA, while the level of OPG is decreased [6, 7]. RANKL knock-out mice did not appear bone destruction. Moreover, Denosumab, a RANKL antibody, reduced bone destruction in RA, which demonstrates the function interaction between the RANK-RANKL system and bone damage [8]. The previous study suggested that the predominant cells producing RANKL in rheumatoid arthritis joints are FLSs [6, 9]. OPG acts as the natural inhibitor of RANKL, preventing RANKL binding to its receptor, RANK. OPG knock-out mice exhibit severe osteoporosis and bone erosion [10]. The RANKL/OPG ratio in RA synovial fluid is significantly higher than that of osteoarthritis, which is consistent with the fact that RA is more invasive than the other.

Matrix metalloproteinase is a group of enzymes whose function is to degrade extracellular matrix of chondrocytes. MMP-3 is one of the MMPs which shows

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strongest association with RA. Serum levels of MMP-3 are higher in RA patients than osteoarthritis patients and PBMCs are important source of MMP-3 [11]. Serum MMP-3 levels have been shown to be promising markers for the prediction of subsequent cartilage damage in RA. MMP-3 is abundantly expressed in active rheumatoid synovium. In RA, increased expression of MMP-3 in synovial fluid can aggravate cartilage erosion [12-14]. Given that MMP-3 expression by FLSs is induced by many inflammatory cytokines, it would be of interest to know whether there are other inducers of MMP-3 expression in FLSs of RA patients.

FLSs are the main cell type located in the intimal lining layer of in flamed synovial tissue, found to exhibit multipotent pathogenic properties in RA by contributing to the inflammatory micro-environment through directly producing pro-inflammatory factors or indirectly activating or recruiting other immune cells [15]. In patients with RA, Th17 cells are present in the inflamed synovial tissue and synovial fluid, and Th17 cell frequencies are increased in peripheral blood of RA patients [16, 17]. IL-17 is capable of inducing synovial cell to secrete MMPs and pro-inflammatory cytokines [18, 19]. IL-17 level is significantly associated with RANKL level in RA [20].

IL-34 was proved to be a crucial cytokine in the pathology of rheumatoid arthritis. Studies reported that the levels of IL-34 in synovial fluid, serum, and FLSs are increased compared with healthy control [21]. Overexpression of IL-34 is associated with the existence of autoantibodies such as Rheumatoid Factor, and the severity of synovitis and disease progression [22]. The IL-34 levels in synovial fluid of patients with high disease activity were significantly increased and positively associated with the ESR and CRP levels [23]. Levels of IL-34 decreased after anti-rheumatoid arthritis treatment. Tian et al demonstrated that levels of IL-34 in synovial fluid were much higher than that in serum. IL-34 plays an important role in RANKL-induced osteoclastogenesis, as it can substitute for M-CSF and support osteoclast differentiation. Serum level of IL-34 is positively correlated with serum level of MMP-3, which indicates possible interaction between IL-34 and MMP-3. IL-34 receptor, CSF-1R, is highly expressed on RA-FLS. The IL-34/CSF-1R axis promoted a dramatic production of IL-6 by FLSs. Furthermore, IL-6 secreted by IL-34 stimulated RA-FLSs was found to upregulate the number of Th17 [24]. IL-34 induced IL-17 production by RA-PBMC in one study [25]. These results suggest that IL-34 probably function through IL-17 expression. Plumbagin (IL-17 Inhibitor) suppresses IL-17A production in human PBMC stimulated with anti-CD3/CD28 [26]. Our study aims to investigate the effect of IL-34 on RA-FLS and RA-PBMC to induce RANKL/OPG and MMP-3 production, and whether the effect is mediated by IL-17 with rhIL-34 and IL-17 inhibitor.

## MATERIALS AND METHODS

### *Patients*

PBMCs were obtained from 20 RA patients (male to female ratio 6:14) of mean age 54.07 ± 11.58 years, who fulfilled the American College of Rheumatology criteria for RA [27] and the median duration of the disease was 3.75 years (range 1-15). In addition, 10 age and sex-matched healthy persons served as controls. All the patients were hospitalized for the first time and did not receive systemic therapy before. The present study was approved by the ethics committee in our institution according to the Declaration of Helsinki and written informed consent was given by all the patients.

Human Rheumatoid Arthritis Fibroblast-like Synoviocytes (MH7A cells) were purchased from Shanghai Hongshun Company, cell source was Riken cell bank (Ibaraki, Japan). Cells were cultured with H-DMEM, 10%FBS, and 1% antibiotics solution. The 3rd-5th generations were used for experiments.

### *Preparation, stimulation of PBMCs*

PBMCs were purified from peripheral blood of 20 RA patients by centrifugation, using a Ficoll-Hypaque gradient (Amersham Pharmacia Biotech). For the measurement of cytokine secretion, PBMCs were seeded into the wells of 96-well culture plates (Nunc) at $10^5$/200 mL/well in triplicate suspended with culture medium (89%RPMI1640 + 10%FBS + 1%antibiotics) and stimulated with anti-CD3/CD28 antibodies. RhIL-34 (0, 10, 20, 50,100 ng/mL) (R & D Systems) with or without IL-17 inhibitor (Plumbagin, 5 nmol/mL) was added to the media. IL-17 inhibitor (Plumbagin) was purchased from Novusbio (NOVUS, USA). The stimulating concentration of Plumbagin was selected according to the manufacturer's instructions (NOVUS). After incubation for 72 hours, cell-free supernatants were collected.

### *Stimulation of RA-FLS*

The cells of 3rd-5th generation were used for the experiment. The cells were seeded on a 24-well plate at a density of $5 \times 10^4$/ml. The cells were cultured in H-DMEM supplemented with 10%FBS at 37 °C in 5% $CO_2$ for 12 h. RhIL-34 (0, 10, 20, 50,100 ng/mL) with or without Plumbagin (4 nmol/mL) was added to the media. The optimal stimulating concentration was selected by pre-experiments. After 48 hours of stimulation, cell-free supernatants were collected.

### *Detection of RANKL, OPG, MMP-3 levels in supernatants*

The concentrations of RANKL, OPG, and MMP-3 in both PBMCs and FLSs cell culture supernatant were assessed using ELISA kits, according to the manufacturer's instructions (R & D Systems).

### *Statistical analysis*

All data are presented as the mean ± SD. Unpaired Student's t-test or paired t-test was used to compare variables between groups. All analyses were performed using Graph-Pad Prism 7 software. $P < 0.05$ is considered as reaching statistical significance.

## RESULTS

### *IL-34 promotes RANKL secretion and this promoting effect was blocked by IL-17 inhibitor. IL-34 inhibits OPG secretion by RA-FLS and this inhibiting effect was attenuated by the addition of IL-17 inhibitor*

RhIL-34 stimulates RANKL secretion by RA-FLSs. The levels of RANKL at 50 ng/ml and 100 ng/mL rhIL-34 are significantly higher than 0 ng/ml of rhIL-34 ($P < 0.01$, $P < 0.01$) (*figure 1A*). After the addition of IL-17 inhibitor (Plumbagin, 4 nmol/ml) in 0 ng/mL IL-34, 50 ng/mL IL-34 group, and 100 ng/mL IL-34 group, RANKL secretion by RA-FLS significantly decreased ($P < 0.01$, $P < 0.01$, $P < 0.01$, respectively) (*figure 1B*). RhIL-34 inhibited OPG secretion by RA-FLS. 50 ng/mL and 100 ng/mL rhIL-34 showed strongest inhibitory effect on OPG secretion compared with 0 ng/ml IL-34 group ($P < 0.01$, $P < 0.01$) (*figure 1C*). After the addition of IL-17 inhibitor (Plumbagin, 4 nmol/ml) in 0 ng/mL, 50 ng/mL, and 100 ng/mL IL-34 groups, levels of OPG significantly increased. ($P < 0.01$, $P < 0.01$, $P < 0.01$, respectively) (*figure 1D*).

### *IL-34 promotes RANKL secretion by RA-PBMC which were blocked by IL-17 inhibitor*

RhIL-34 stimulated RA-PBMC to produce RANKL. The level of RANKL at 50 ng/mL IL-34 was significantly higher than that of 0 ng/mL IL-34 ($P < 0.01$) (*figure 2A*). After the addition of IL-17 inhibitor (Plumbagin, 4 nmol/mL), RANKL expression by RA-PBMC was significantly decreased ($P < 0.01$) (*figure 2B*).

In contrast, rhIL-34 had no effect on RANKL secretion by healthy control (*figure 2C*).

### *IL-34 inhibited OPG secretion by both RA-PBMCs and healthy-PBMCs. The inhibitory effect was reduced by IL-17 inhibitor*

After treatment with rhIL-34OPG secretion by RA-PBMCs was significantly decreased. The levels of OPG at 50 ng/mL and 100 ng/mL rhIL-34 were significantly lower than 0 ng/mL rhIL-34 ($P < 0.01$, $P < 0.01$ respectively) (*figure 3A*). OPG secretion in healthy control was also significantly reduced by 100 ng/mL rhIL-34 stimulation ($P < 0.01$) (*figure 3A*). Furthermore, the levels of OPG at 100 ng/mL rhIL-34 were significantly elevated by IL-17 inhibitor ($P < 0.01$) (*figure 3B*).

### *IL-34 had no significant effect on MMP-3 secretion in RA-FLS and RA-PBMCs*

After being treated with different concentrations of rhIL-34, MMP-3 secretion by RA-FLS showed no significant difference (*figure 4A*). MMP-3 secretion

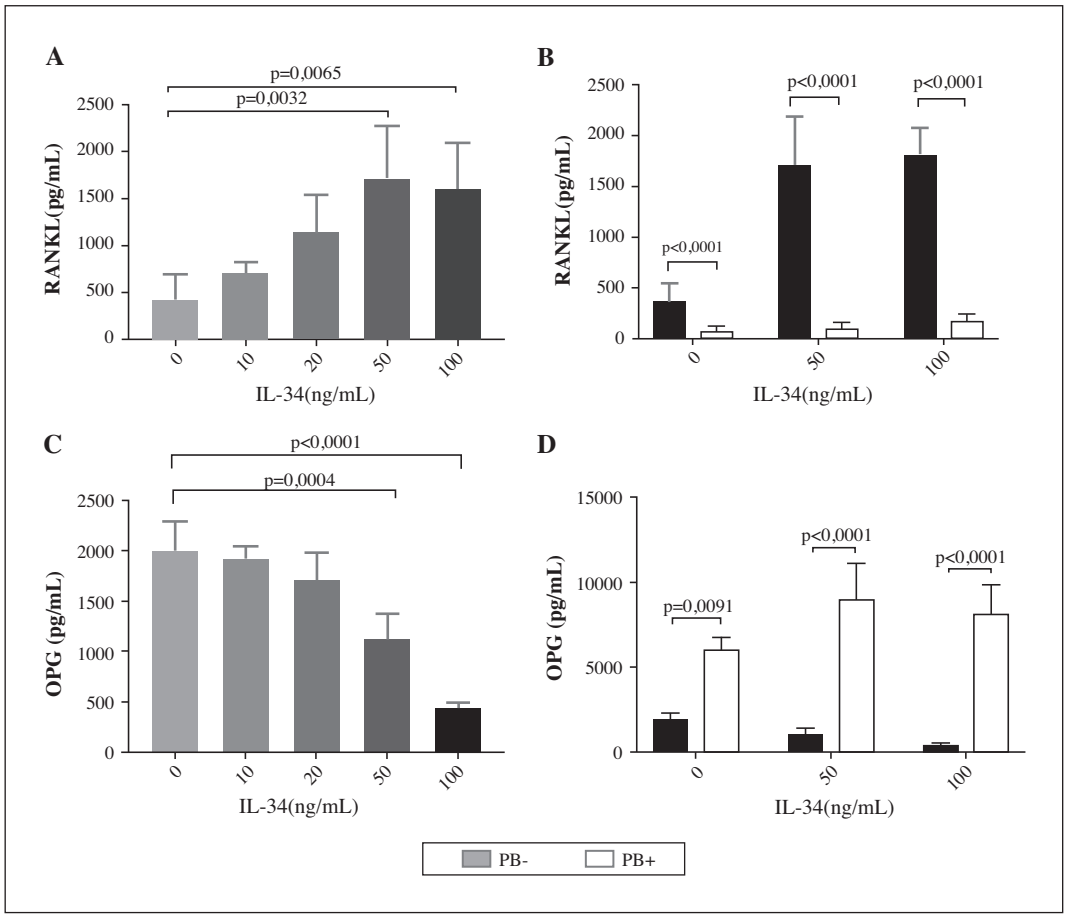


**Figure 1**
Effect of rhIL-34 on RANKL/OPG secretion by RA-FLS. **A)** RhIL-34 stimulates RANKL secretion in RA-FLS; **B)** After the addition of an IL-17 inhibitor (Plumbagin, 4 nmol/mL), RANKL secretion by FLS significantly decreased; **C)** RhIL-34 inhibited OPG secretion in RA-FLS; **D)** After the addition of IL-17 inhibitor (Plumbagin, 4 nmol/mL), OPG secretion by RA-FLS was significantly increased.

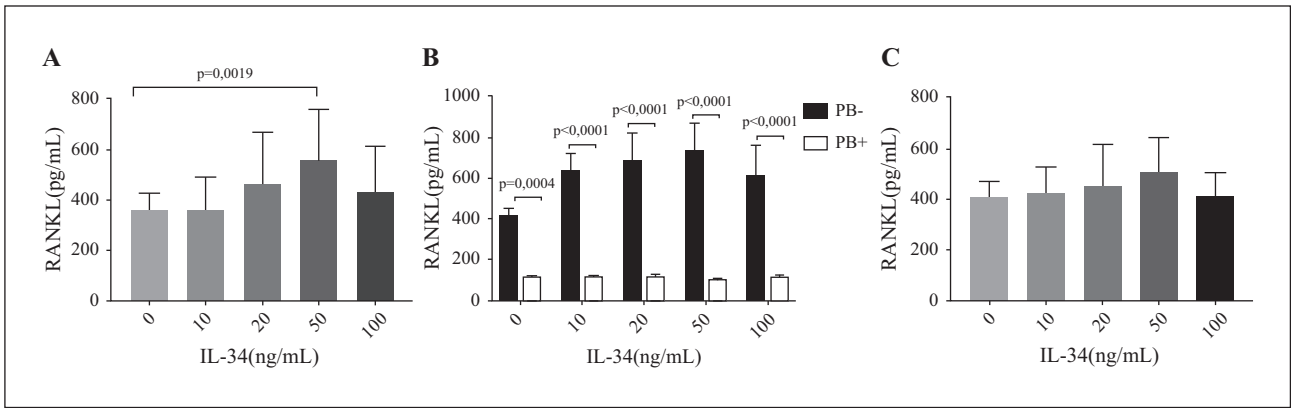


**Figure 2**
Effect of rhIL-34 on RANKL secretion by PBMC. **A)** RhIL-34 stimulated RA-PBMC to secrete RANKL; **B)** IL-17 inhibitor inhibited RANKL secretion by rhIL-34 stimulated RA-PBMC; **C)** RhIL-34 had no significant effect on RANKL secretion by healthy PBMC.

after rhIL-34 treatment in RA-PMNCs and healthy-PBMCs also showed no significant difference (*figure 4B*).

## DISCUSSION

Bone destruction plays an important role in pathology of RA. The RANKL/OPG system and MMP-3 plays a crucial role in occurrence and progression of bone remodeling and bone destruction. Our study demonstrates that IL-34 affected RANKL and OPG secretion by RA-FLS as well as RA-PBMC via IL-17 regulation, indicating the potential role of IL-34 in progression of bone destruction in RA.

In our study, L-34 promoted secretion of RANKL in RA-FLS. The promoting effect of IL-34 was reduced by the IL-17 inhibitor. Previous studies demonstrated that IL-34 is closely related to osteoclastogenesis [28-30]. It can substitute for M-CSF to work together with RANKL for osteoclastogenesis [28]. However, we believe that the function of IL-34 in osteoclastogenesis is not limited to promote osteoclastogenesis. Abundant evidence suggests that IL-34 can promote production of pro-inflammatory cytokines in synovium tissue, and these cytokines play important roles in osteoclastogenesis. In the presence of rhIL-34, stimulation of PBMC from RA patients resulted in increased production of IL-17 [25]. Moreover, IL-17 upregulated RANKL expression in FLSs and directly induced osteoclastogenesis [20, 31, 32]. One crucial regulator for RANKL expression by FLSs is IL-17 [33]. IL-17 can significantly upregulate RANKL production and down-regulate OPG production by FLSs [17, 34]. One of the important regulatory factors for IL-17 production is IL-34 [35, 36]. These findings suggest that FLS may be the primary cells producing RANKL after stimulation with IL-34, and this effect is mediated by IL-17.

Our results show that IL-34 inhibited expression of OPG in RA-FLSs. The inhibition effect of IL-34 on OPG expression is reduced by the IL-17 inhibitor. OPG is produced by osteoblasts and acts as a decoy receptor that competes with RANKL for RANK. This interaction inhibits osteoclastic proliferation and

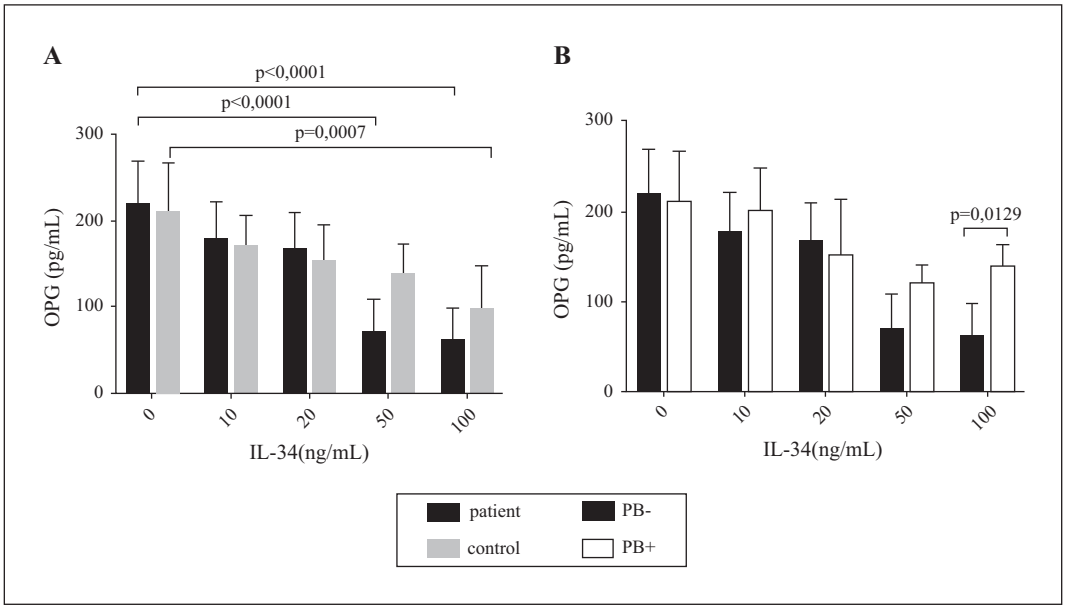


**Figure 3**
Effect of rhIL-34 on OPG secretion by PBMC. **A)** RhIL-34 inhibited OPG secretion by RA-PBMC and healthy PBMC; **B)** IL-17 inhibitor increased OPG secretion by rhIL-34 stimulated RA-PBMC.

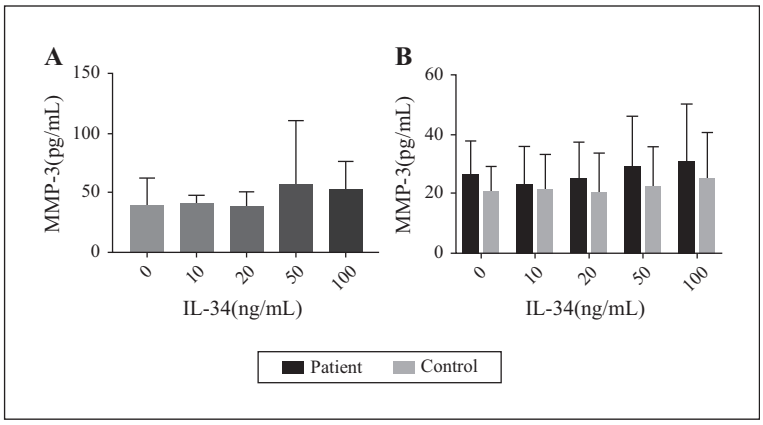


**Figure 4**
Effect of rhIL-34 on MMP-3 secretion by RA-FLS and RA-PBMC. **A)** RhIL-34 had no significant effect on MMP-3 secretion by RA-FLS. **B)** RhIL-34 had no significant effect on MMP-3 secretion by RA-PBMC and healthy PBMC.

differentiation and consequently prevents bone resorption. Therefore, OPG expression is usually negatively correlated with RANKL expression [37, 38]. This increased RANKL/OPG ratio is correlated with their capacity to support osteoclast formation and activation [39-41]. The results of this study are consistent with this fact. RANKL expression in FLSs is elevated after IL-34 stimulation, while OPG expression is decreased. Inhibitory effect of IL-34 on OPG expression is attenuated, which is consistent with the inhibitory effect of IL-17 on OPG expression. Blocking IL-34 may not only cause a decrease in RANKL level but also increase in OPG level, which may lead to an unexpected side effect. More study is needed to investigate the function of OPG in rheumatoid arthritis.

Effects of IL-34 on the expression of MMP-3 in RA-FLS and RA-PBMC are not significant. In the present study, the MMP-3 level of the supernatant of FLSs culture increased after stimulation with IL-34, but did not reach significant difference. One possibility is that IL-34 does not affect the expression of MMP-3 on FLS and PBMC. One study reported that blocking GM-CSF, but not IL-17, inhibited production of MMPs [42]. Previous studies demonstrated that the major source of MMP-3 is chondrocyte rather than FLS and lymphocyte. Moreover, it is possible that IL-34 affects the active MMP-3 but not the total MMP-3. One study reported that serum active MMP-3 but not total MMP-3 level decreased after anti-rheumatic treatment [43]. One important possibility that the difference of MMP-3 expression by PBMCs did not reach statistical significance is that there may be heterogeneity among the baseline MMP-3 levels in different patients. In our subjects, the distribution of baseline MMP-3 levels without IL-34 stimulation was skewed, and the range was wide. It is possible that PBMCs of patients whose serum MMP-3 level is below one standard increase MMP-3 expression after IL-34 stimulation, and those with MMP-3 level higher than standard decrease MMP-3 expression after stimulation. Further study of a more detailed investigation after dividing the patients into two groups with higher and lower baseline MMP-3 levels is needed to further investigate the MMP-3 expression in PBMCs.

IL-34 promoted expression of RANKL in PBMC of rheumatoid arthritis patients. IL-34 does not affect the expression of RANKL in PBMC of healthy control. IL-34-mediated RANKL expression reduced after addition of IL-17 inhibitors to RA-PBMC. Emerging findings indicate that IL-34 levels are abnormally increased in serum and SF and strongly associated with rheumatoid factor and anti-cyclic citrullinated peptide antibody [21, 25, 44]. It is possible that IL-34 is associated with bone-damaging factor in peripheral blood. It was reported that serum RANKL level is significantly elevated, and is positively correlated with bone damage and osteoporosis [7, 45, 46]. However, the source of the cytokine is not elucidated. Our study demonstrated that IL-34 promoted RANKL production and inhibited OPG production in PBMCs, increasing the RANKL/OPG ratio. These results are consistent with the fact that IL-34 is positively associated with disease progression [22, 23, 47]. The results that RANKL level was elevated to a higher extent when stimulated with 50 ng/mL IL-34 than 100 ng/ml indicate that the number of receptors and binding sites of RANKL on FLS and PBMC may be restricted. A dose-dependent effect is exhibited at a concentration below 50 ng/mL of rhIL-34, while at higher concentrations, there may be a different binding mode between the ligand and the receptor, resulting in a decrease in the effect of RANKL production. It was reported that one major regulator of RANKL/OPG production in PBMCs is IL-17 [20]. Expression of IL-17 in PBMCs is upregulated by cytokines including IL-34 [25]. Conclusively, IL-34 may modulate RANKL/OPG production in PBMCs through regulating IL-17. This indicates that IL-34 may play a pivotal role in osteoclastogenesis and bone destruction in rheumatoid arthritis but not in healthy control, and this effect of IL-34 in rheumatoid arthritis is mediated by IL-17 secretion by PBMC. This differential effect of IL-34 on PBMCs of rheumatoid arthritis and healthy control needs further investigation.

IL-34 inhibited secretion of OPG in PBMC of patients and healthy control. There is no significant difference between RANKL expression in PBMC of patients and healthy control (IL-34 = 50 ng/mL, $P = 0.1095$). The inhibitory effect of IL-34 on OPG secretion in PBMC

is reduced after IL-17 inhibitor treatment. OPG is also expressed in PBMCs [48]. IL-34 presented the same regulatory function on RANKL/OPG in PBMCs as in FLSs. Serum level IL-34 was reported to be associated with bone damage in RA. The differential expression of OPG may be caused by alteration of phenotype of PBMCs. The inhibitory effect is reduced after the administration of IL-17 inhibitor. This result indicated that IL-17 participated in the regulation of OPG expression in PBMCs by IL-34. Conclusively, IL-34 may also play an important role in systemic bone remodeling and bone destruction. And this effect is mediated by IL-17. This suggests that IL-34 can be a potential target for the treatment of bone destruction in RA patients.

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