# Interleukin 34 Upregulation Contributes to the Increment of MicroRNA 21 Expression through STAT3 Activation Associated with Disease Activity in Rheumatoid Arthritis

Sisi Yang, Shujun Jiang, Yu Wang, Shenghao Tu, Zhigang Wang, and Zhe Chen

*ABSTRACT.* ***Objective.*** Interleukin 34 (IL-34) and microRNA 21 (miR-21) were found to be involved in the pathological process of rheumatoid arthritis (RA), but the details were unclear. In this study, we aimed to clarify the relationship between IL-34 and miR-21 in RA.

***Methods.*** IL-34 concentrations in serum and synovial fluid (SF) of patients with RA were measured by ELISA. Fibroblast-like synovial cells (FLS) were cultured for evaluation of STAT3 activation, miR-21, and Bax/Bcl-2 expression by Western blot and real-time PCR. Correlations were analyzed between clinical features and detectable variables including SF IL-34 levels and miR-21 expression.

***Results.*** SF IL-34 levels were significantly higher in patients with RA who had a high 28-joint Disease Activity Score (DAS28 ≥ 3.2) than in those with a lower DAS28 (DAS28 < 3.2). DAS28 scores and miR-21 expression in FLS had a significant positive correlation with the SF IL-34 levels. In addition, IL-34 stimulation strengthened the activation of p-STAT3, resulting in the increment of miR-21 expression. Inhibiting of miR-21 expression contributed to decreased Bcl-2/Bax ratio, suggesting that miR-21 was involved in the resistance to apoptosis. With the blocking of the colony-stimulating factor-1 receptor (CSF1R), decreased protein expressions including CSF1R, p-STAT3/STAT3, and Bcl-2/Bax were shown, suggesting that CSF1R participated in the biological functions of IL-34 in RA.

***Conclusion.*** The IL-34/STAT3/miR-21 pathway is crucial for the survival of synovial fibroblasts in RA, which might be candidate therapeutic targets for RA treatment. (J Rheumatol First Release April 15 2016; doi:10.3899/jrheum.151253)

*Key Indexing Terms:*
INTERLEUKIN 34 MICRORNA 21 STAT3
COLONY-STIMULATING FACTOR-1 RECEPTOR RHEUMATOID ARTHRITIS

*From the Department of Geriatrics, and Department of Integrated Chinese Traditional and Western Medicine, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology; Department of Pathogen Biology, Basic Medical College, Hubei University of Chinese Medicine, Wuhan, China.*

*Supported by grants from the National Natural Science Foundation of China (No. 81503426).*

*S. Yang, PhD, Department of Geriatrics, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology; S. Jiang, PhD, Department of Integrated Chinese Traditional and Western Medicine, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology; Y. Wang, PhD, Department of Integrated Chinese Traditional and Western Medicine, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology; S. Tu, PhD, Department of Integrated Chinese Traditional and Western Medicine, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology; Z. Wang, PhD, Department of Pathogen Biology, Basic Medical College, Hubei University of Chinese Medicine; Z. Chen, PhD, Department of Integrated Chinese Traditional and Western Medicine, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology.*

*Address correspondence to Dr. Z. Wang, Department of Pathogen Biology, Basic Medical College, Hubei University of Chinese Medicine, No. 1 Huangjia Lake West Road, Hongshan District, Wuhan 430065, China. E-mail: wangzhigang1979@hotmail.com; or Dr. Z. Chen, Department of Integrated Chinese Traditional and Western Medicine, Tongji Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430030, China. E-mail: zhepi2006@163.com.*

*Accepted for publication March 1, 2016.*

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized mainly by chronic synovial inflammation[1]. Synovial fibroblasts, the major cell population in synovial tissue, are involved and actively participate in the pathogenesis of RA[2]. In more recent years, interleukin 34 (IL-34), as a newly discovered cytokine, has shown that it can be locally expressed in the synovial tissue and is associated with synovitis severity of RA[3]. The positive correlation between IL-34 levels and rheumatoid factor (RF) and anticyclic citrullinated peptide antibody titers was also indicated[4,5]. However, for IL-34, the precise mechanism by which it carries out its biological functions in RA is still unknown.

MicroRNA (miRNA), small noncoding RNA molecules, serve as key mediators of gene silencing at the posttranscription level in fundamental biological and pathological processes[6]. A report showed that miRNA 21 (miR-21) was involved in the imbalance of Th17/Treg cells by regulating STAT3/STAT5 in patients with RA[7]. In addition, high expressions of Bcl-2 and miR-21 in synovial fluid (SF) Treg cells of patients with RA were found, suggesting that miR-21 might participate in the apoptotic progress and regulation of RA pathogenesis[8]. Regrettably, few details are known about the clinical significance of miR-21 in RA, and the expression

and function of miR-21 in fibroblast-like synovial cells (FLS) also remain unclear.

In our study, we found that stimulation with IL-34, binding to colony-stimulating factor-1 receptor (CSF1R), induced increment of miR-21 expression through p-STAT3 activation, which was associated with disease activity of RA. Inhibiting of miR-21 resulted in increased apoptosis in the FLS, suggesting that miR-21 was involved in the pathogenesis of RA. Our findings suggested that the IL-34/STAT3/miR-21 pathway was crucial for the survival of synovial fibroblasts, which might be candidate therapeutic targets for the treatment of RA.

## MATERIALS AND METHODS

*Patients*. Thirty patients with RA were enrolled from the Department of Integrated Chinese Traditional and Western Medicine at the Tongji Hospital, affiliated with the Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China)[9]. Twenty patients with osteoarthritis (OA) and 20 age- and sex-matched healthy individuals served as controls. Our study was approved by the Ethics Committee of the Tongji Medical College according to the Declaration of Helsinki and written informed consent was given by all patients.

The 4 variables used to calculate the 28-joint Disease Activity Score (DAS28) included the number of swollen joints, number of tender joints, C-reactive protein or erythrocyte sedimentation rate, and the patient's global assessment. A DAS28 score of 3.2 and above indicated moderate to high disease activity whereas a value of below 3.2 referred to remission to low disease activity[10].

*Isolation and culture of FLS*. FLS were isolated by enzymatic digestion of synovial tissue from patients with RA and OA undergoing total joint replacement surgery. Briefly, synovial tissue was minced and incubated for 2 h at 37°C with 1 mg/ml collagenase (Sigma-Aldrich Co.) in Dulbecco modified Eagle's medium (Hyclone) containing 10% fetal bovine serum (Hyclone). The cell suspension was filtered through a 70-μm nylon cell strainer (BD Falcon). Cells were sequentially subcultured for 3–6 passages and used as the source of FLS.

Different concentrations of IL-34 (0, 10, 20, and 50 ng/ml; R&D Systems) were used at indicated times as stimulators of FLS. Unless specifically mentioned, STA21 (STAT3 inhibitor, 50 μM; Santa Cruz Biotechnology) and human CSF1R-blocking antibody (20 ng/ml; Novus Biologicals) were used in our study.

*ELISA*. IL-34 levels in serum and SF samples were measured by ELISA according to the manufacturer's directions (R&D Systems).

*Flow cytometry detection of apoptosis*. The apoptotic cells were detected using an FITC Apoptosis Detection Kit (BD Pharmingen). FLS were collected, washed, and resuspended in buffer containing 5 $\mu$L of FITC annexin V and 5 $\mu$L of propidium iodide (PI). Following incubation of 15 min in the dark at room temperature, fluorescence of the cells was analyzed by BD FACSCanto II using DiVa software (BD Bioscience). Apoptosis cells contained the early apoptotic cells (annexin V-positive and PI-negative) and the late apoptotic or necrotic cells (both annexin V-positive and PI-positive).

*RNA isolation and real-time PCR*. Total RNA was extracted using Trizol reagent (Invitrogen) and reverse transcribed using M-MLV-RTase (Promega) according to the manufacturer's instructions. Real-time PCR was performed using the SYBR-Green Master PCR Mix (Applied Biosystems) on the TP800 qPCR System (Takara). Real-time PCR of cDNA was performed using the forward (F) and reverse (R) primer sequences: miR-21: F, 5′-TGT CGG GTA GCT TAT CAG ACT-3′; R, 5′-TGT CAG ACA GCC CAT CGA CT-3′; U6: F, 5′-CTC GCT TCG GCA GCA CA-3′; R, 5′-AAC GCT TCA CGA ATT TGC GT-3′. The expression of U6 was used as endogenous control for miRNA analysis. β-actin: F, 5′-TCA TGA GGT AGT CAG TCA GG-3′; R, 5′-CTT CTA CAA TGA GCT GCG TG-3′; Bcl-2: F, 5′-TCC GAT CAG GAA GGC TAG AGT T-3′; R, 5′-TCG GTC TCC TAA AA GCA GGC-3′; Bax: F, 5′-CCG CCG TGG ACA CAG AC-3′; R, 5′-CAG AAA ACA TGT CAG CTG CCA-3′. The relative Bax or Bcl-2 mRNA levels were normalized to those of β-actin for mRNA analysis. For relative quantification, $2^{-(Ct-Cc)}$ was used to calculate the relative expression (Ct and Cc are the mean threshold cycle differences after normalizing to control that was calculated and used as an indication of the relative expression levels[11]).

*Western blot analysis*. FLS from patients with RA was collected and washed 3 times with sterile phosphate buffered saline, and then lysed with 0.1–0.2 ml RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% NaN3, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM sodium orthovanadate, and 1 mM NaF). Equal amounts of proteins were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories) presoaked with 100% methanol. This was followed by incubation with mouse anti-human CSF1R monoclonal antibody (Novus Biologicals) and the rabbit anti-human antibody against Bcl-2, Bax, STAT3, p-STAT3 (Tyr705), and β-actin (Cell Signaling Technology Inc). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin G secondary antibodies were used, respectively. Finally, immunoblot signals were visualized using a scanner (HP Scanjet 7400C, Hewlett-Packard), and then imaged and quantitated using a ChemiScope 3400 Mini (CLINX Science Instruments).

*Transfection*. Transfection was performed using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. MiR-21 inhibitor (5'-UCA ACA UCA GUC UGA UAA GCU A-3') and negative control (5'-CAG UAC UUU UGU GUA GUA CAA-3') were synthesized by Genepharma. All of the oligonucleotides were transfected at a final concentration of 50 mM.

*Statistical analysis*. All data are presented as mean ± SD. Paired Student t test was used to compare variables between groups. Pearson correlation coefficient was used to test the correlations analysis. $P < 0.05$ was considered statistically significant.

## RESULTS

*SF IL-34 levels and its association with miR-21 expression in FLS from patients with RA with moderate to high disease activity*. First, we aimed to learn the relationship of SF IL-34 levels and miR-21 expression in FLS. Clinical characteristics of patients with RA are shown in Table 1. As shown in Figure 1A, serum IL-34 levels were much higher among the patients with RA (n = 30) compared with the healthy controls (n = 20, $p < 0.001$). SF IL-34 levels were markedly higher in patients with RA (n = 30) than in patients with OA (n = 20, $p < 0.001$; Figure 1B). Patients with RA with moderate to high disease activity (DAS28 ≥ 3.2, n = 17) had significantly higher mean SF IL-34 levels than did those with low disease activity (DAS28 < 3.2, n = 13, $p < 0.05$; Figure 1C). The miR-21 expression of FLS samples from patients with RA with moderate to high disease activity (DAS28 ≥ 3.2, n = 12) was elevated compared with patients with OA (n = 6) and patients with RA with low disease activity (DAS28 < 3.2, n = 6, $p < 0.05$; Figure 1D).

On linear regression analysis, SF IL-34 levels had a significant positive relationship with miR-21 expression ($r = 0.909$, $p < 0.01$; Figure 1E). Additionally, miR-21 expression was significantly associated with disease activity in patients with RA ($r = 0.95$, $p < 0.01$; Figure 1F). The

*Table 1*. Clinical characteristics of patients with RA and healthy controls. Values are mean ± SD or n (%).

| Characteristics | Patients with RA, n = 30 | Healthy, n = 20 |
|---|---|---|
| Age, yrs | 56.6 ± 10.2 | 49.3 ± 12.8 |
| Female | 26 (86.7) | 18 (90) |
| Disease duration, yrs | 6.2 ± 3.5 | — |
| DAS28 scores | 3.1 ± 1.4 | — |
| DAS28 ≥ 3.2 | 4.1 ± 1.7 | |
| DAS28 < 3.2 | 1.9 ± 0.9 | |
| RF, IU/ml | 420.5 ± 86.4 | 38.2 ± 4.9 |
| ESR, mm/h | 65.8 ± 24.3 | 9.1 ± 3.6 |
| CRP, mg/l | 42.5 ± 11.3 | 1.4 ± 0.2 |
| Anti-CCP, RU/ml | 41.8 ± 3.2 | 6.2 ± 1.7 |
| Treatment | | |
| Methotrexate | 24 (80) | — |
| Sulfasalazine | 15 (50) | — |
| Leflunomide | 7 (23.3) | — |
| Hydroxychloroquine | 9 (30) | — |
| Prednisolone | 18 (60) | — |
| Anti-TNF agent | 3 (10) | — |

RA: rheumatoid arthritis; DAS28: Disease Activity Score in 28 joints; RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; anti-CCP: anticyclic citrullinated peptide antibodies; anti-TNF: anti-tumor necrosis factor.

correlation between the clinical features and detectable variables including SF IL-34 and miR-21 expression are also shown in Table 2.

*STAT3 activation contributes to IL-34–dependent miR-21 expression*. Based on the correlation analysis, we wanted to indicate which 1 gene activation was involved in the continuous IL-34 stimulation. As shown in Figure 2A, treatment with IL-34 significantly decreased apoptosis of FLS by flow cytometric analysis. A representative Western blotting showed that expression of p-STAT3 was evidently increased in a dose-dependent manner upon IL-34, and miR-21 expression in FLS was also raised in response to stimulation of IL-34 (Figure 2B, Figure 2C). Of note, treatment with STA21, a STAT3 inhibitor, abrogated IL-34–induced p-STAT3 activation and miR-21 expression, suggesting that STAT3 activation contributed to IL-34–dependent miR-21 expression (Figure 2D).

*MiR-21 is involved in the resistance to apoptosis of IL-34*. Considering the involvement of miR-21 in apoptosis, we continued to examine its effect on apoptosis-associated genes in synovial fibroblasts. As shown in Figure 3A, expression of antiapoptotic Bcl-2 was higher in FLS from patients with RA than patients with OA, and expression of proapoptotic Bax was lower (Figure 3B). The similar tendency was discovered in the patients with RA with different disease activity (Figure 3A, Figure 3B). Following the stimulation of IL-34, the expression of Bcl-2 increased in a dose-dependent manner, but the expression of Bax was decreased (Figure 3C, Figure 3D). Interestingly, transfection of miR-21 inhibitor resulted in decrement of the Bcl-2/Bax ratio, suggesting that miR-21 was involved in the regulation of Bcl-2/Bax (Figure 3E).

*CSF1R participates in the biological activities of IL-34 in RA*. We aimed to clarify whether CSF1R was involved in the biological functions of IL-34. With IL-34 stimulation, increased CSF1R expression in the FLS was found (Figure 4A). Simultaneously, miR-21 expression was enhanced in the IL-34–dependent manner, which was noticeably limited in the presence of CSF1R-blocking antibody (Figure 4B, Figure 4C). Importantly, the declining protein expressions including CSF1R, p-STAT3/STAT3, and Bcl-2/Bax were displayed with the treatment of CSF1R-blocking antibody, suggesting that CSF1R participated in the biological function of IL-34 in RA (Figure 4D).

## DISCUSSION

In our study, we demonstrate that upregulated IL-34 can strengthen STAT3 activation, resulting in increments of miR-21 expression by binding to CSF1R, which regulate the change of Bcl-2/Bax ratio and contribute to the resistance to apoptosis of FLS in RA.

IL-34, produced by synovial fibroblasts in the rheumatoid joints, might be involved in the pathogenesis of RA[12]. Our results showed that serum and SF IL-34 levels were increased significantly in patients with RA, as a previous paper described (Figure 1A, Figure 1B)[4]. In our study, patients with RA were divided into 2 groups according to the DAS28 scores: moderate to high disease activity (DAS28 ≥ 3.2) and low disease activity (DAS28 < 3.2). Patients with RA with a higher DAS28 (DAS28 ≥ 3.2) had significantly higher mean SF IL-34 levels and miR-21 expression than those with a lower DAS28 (DAS28 < 3.2; Figure 1C, Figure 1D). Additionally, miR-21 expression had a significant positive relationship with the SF IL-34 levels in patients with RA with moderate to high disease activity (Figure 1E). To our knowledge, ours is the first study to show the difference of SF IL-34 levels based on the DAS28 scores in patients with RA. The positive relationship of DAS28 scores and SF IL-34 levels suggest that elevated SF IL-34 might be a useful marker to evaluate active RA (Table 2).

The relation between miR-21 and STAT3 has been described in a paper[13]. In RA synovial tissue, STAT3 increased in proportion to the severity of synovitis[14]. In Sézary syndrome, miR-21 expression in CD4+ T cells was regulated by STAT3, and the silencing of miR-21 resulted in increased apoptosis[15]. In our current study, treatment with IL-34 significantly decreased apoptosis of FLS by flow cytometric analysis (Figure 2A). However, IL-34 stimulation enhanced the activation of STAT3 in a dose-dependent manner (Figure 2B). Consistent with the result, the miR-21 expression was increased upon IL-34, but was hardened in the presence of STAT3 inhibitor (Figure 2C, Figure 2D). These findings suggested that IL-34 contributed to the increment of miR-21 expression through STAT3 activation. On the contrary, Dong, *et al* reported that the decreased expression of miR-21 was correlated with the imbalance of

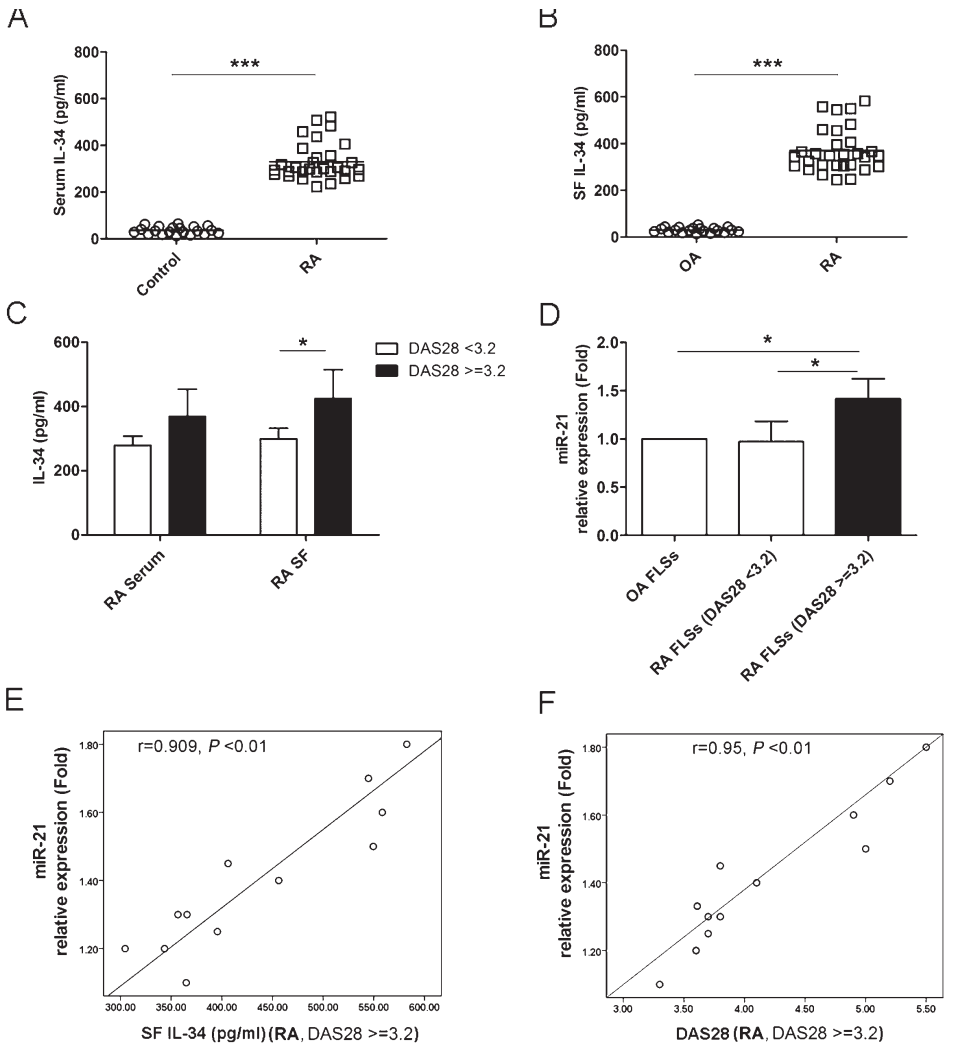


*Figure 1*. Relationship of SF IL-34 levels with miR-21 expression in FLS samples from patients with RA. A. Serum IL-34 levels in healthy controls (n = 20) and patients with RA (n = 30). B. SF IL-34 levels in patients with OA (n = 20) and patients with RA (n = 30). C. Serum and SF IL-34 levels in patients with RA with different disease activity (DAS28 ≥ 3.2, n = 17; DAS28 < 3.2, n = 13). D. Mir-21 expression in FLS from patients with OA (n = 6) and patients with RA (DAS28 ≥ 3.2, n = 12; DAS28 < 3.2, n = 6) undergoing total joint replacement surgery. E. Correlation analysis between SF IL-34 levels and miR-21 expression in FLS from patients with RA (DAS28 ≥ 3.2, n = 12). F. Correlation analysis between DAS28 scores and miR-21 expression in FLS from patients with RA (DAS28 ≥ 3.2, n = 12). Data are presented as mean ± SD. * P < 0.05. *** P < 0.001. SF: synovial fluid; IL-34: interleukin 34; miR-21: microRNA 21; FLS: fibroblast-like synovial cells; RA: rheumatoid arthritis; OA: osteoarthritis; DAS28: 28-joint Disease Activity Score.

Th17 and Treg cells in patients with RA[7]. Although accompanied by STAT3 activation, the levels of miR-21 expression were significantly lower in peripheral blood mononuclear cells (PBMC) and CD4+ T cells of patients with RA. Compared with Dong, *et al*[7], there were some differences in our study. First, we enrolled patients with RA who had moderate to high disease activity. The individual differences including duration time of RA, drugs for treatment such as methotrexate, and baseline health status might affect the results. Second, FLS were separated and cultured as detected samples, but not PBMC and CD4+ T cells. Additionally, there was no positive correlation between SF IL-34 and miR-21 expression in CD4+ T cells (or PBMC) from patients with RA in our study (data not shown). Third, FLS were subsequently stimulated with different concentration of IL-34, unlike lipopolysaccharide used by Dong, *et al*.

T cells from synovial tissue of patients with RA were resistant to apoptosis because of the high expression of Bcl-2[16]. SF Treg cells displayed high transcription levels of Bcl-2 and miR-21, which induced little apoptosis in comparison with SF-conventional T cells and peripheral blood Treg cells[17]. Our results also showed that the proapoptotic Bax was decreased and antiapoptotic Bcl-2 was increased, but the change can be reversed by transfection with

*Table 2*. Correlation analysis between clinical characteristics and detectable variables including SF IL-34 levels and miR-21 expression in FLS.

| Characteristics† | IL-34 | | miR-21 | |
|---|---|---|---|---|
| | Correlation Coefficient, r | p | Correlation Coefficient, r | p |
| Age, yrs | –0.006 | 0.968 | –0.005 | 0.875 |
| Female | 0.103 | 0.402 | 0.114 | 0.381 |
| Disease duration, yrs | –0.221 | 0.012* | –0.178 | 0.024* |
| DAS28 scores | 0.304 | 0.006* | 0.264 | 0.009* |
| RF, IU/ml | 0.421 | < 0.001 | 0.282 | < 0.001 |
| ESR, mm/h | 0.313 | 0.002* | 0.215 | 0.019* |
| CRP, mg/l | 0.362 | < 0.001 | 0.249 | 0.002 |
| Anti-CCP, RU/ml | 0.398 | < 0.001 | 0.325 | < 0.001 |

† Patients with RA with moderate to high disease activity (DAS28 ≥ 3.2). * P < 0.05. SF: synovial fluid; IL-34: interleukin 34; miR-21: microRNA 21; FLS: fibroblast-like synovial cells; DAS28: Disease Activity Score in 28 joints; RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; anti-CCP: anticyclic citrullinated peptide antibodies; RA: rheumatoid arthritis.

miR-21 inhibitor (Figure 3). We speculate that IL-34 stimulation induced the STAT3 activation, and subsequently increased miR-21 expression, leading to an upregulated Bcl-2/Bax ratio, which contributed to limited apoptosis in FLS of RA. Further study needs to be designed to explain the details, which would help to clarify the significance and function of IL-34 and miR-21 in RA.

IL-34 was identified as an alternative CSF1R ligand and shared functional similarities with macrophage colony stimulating factor (M-CSF)[18]. It was discovered that CSF1R expression was increased in the synovium of patients with RA, and the blocking of CSF1R protected against bone and cartilage destruction in the collagen-induced arthritis model[19]. In our paper, IL-34 stimulation mediated p-STAT3 activation by binding to CSF1R and induced the increased expression of miR-21, which contributed to the upregulated Bcl-2 and downregulated Bax expressions. Inhibition of miR-21 restricted the IL-34/STAT3/miR-21 pathway and effectively promoted the expression of Bax, resulting in

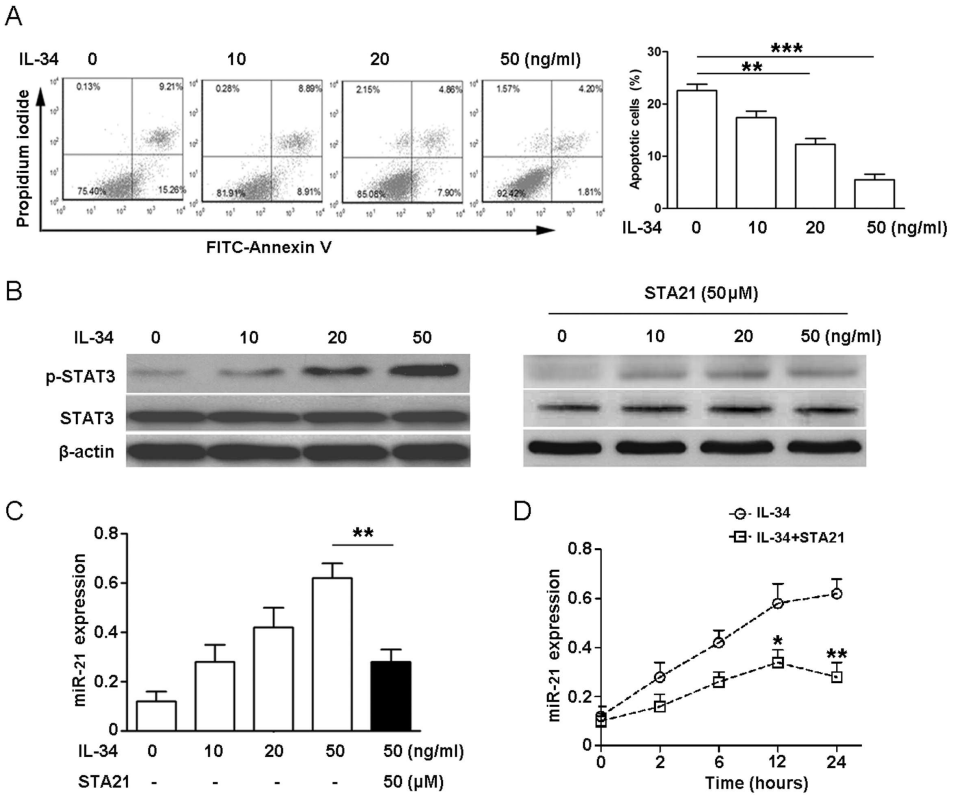


*Figure 2*. IL-34 stimulation contributes to the increment of miR-21 expression by STAT3 activation. FLS from patients with RA (DAS28 ≥ 3.2) were cultured and treated with IL-34 in the presence or absence of STA21 for 24 h. A. Cell apoptosis was detected by flow cytometric analysis following staining with annexin V and PI, and statistical analysis of apoptotic cells was displayed. A representative result is shown. B. The protein expressions of STAT3 and p-STAT3 were detected with different concentrations of IL-34, and 1 representative Western blotting is shown. C. miR-21 expression was evaluated with different concentrations of IL-34 by real-time PCR. D. miR-21 expression was evaluated following IL-34 stimulation (50 ng/ml) for different times. All experiments were performed in triplicate. Data are presented as mean ± SD (n = 6). * P < 0.05 and ** p < 0.01, comparison with IL-34 stimulation group without STA21. *** P < 0.001. IL-34: interleukin 34; miR-21: microRNA 21; FLS: fibroblast-like synovial cells; RA: rheumatoid arthritis; DAS28: 28-joint Disease Activity Score; PI: propidium iodide.

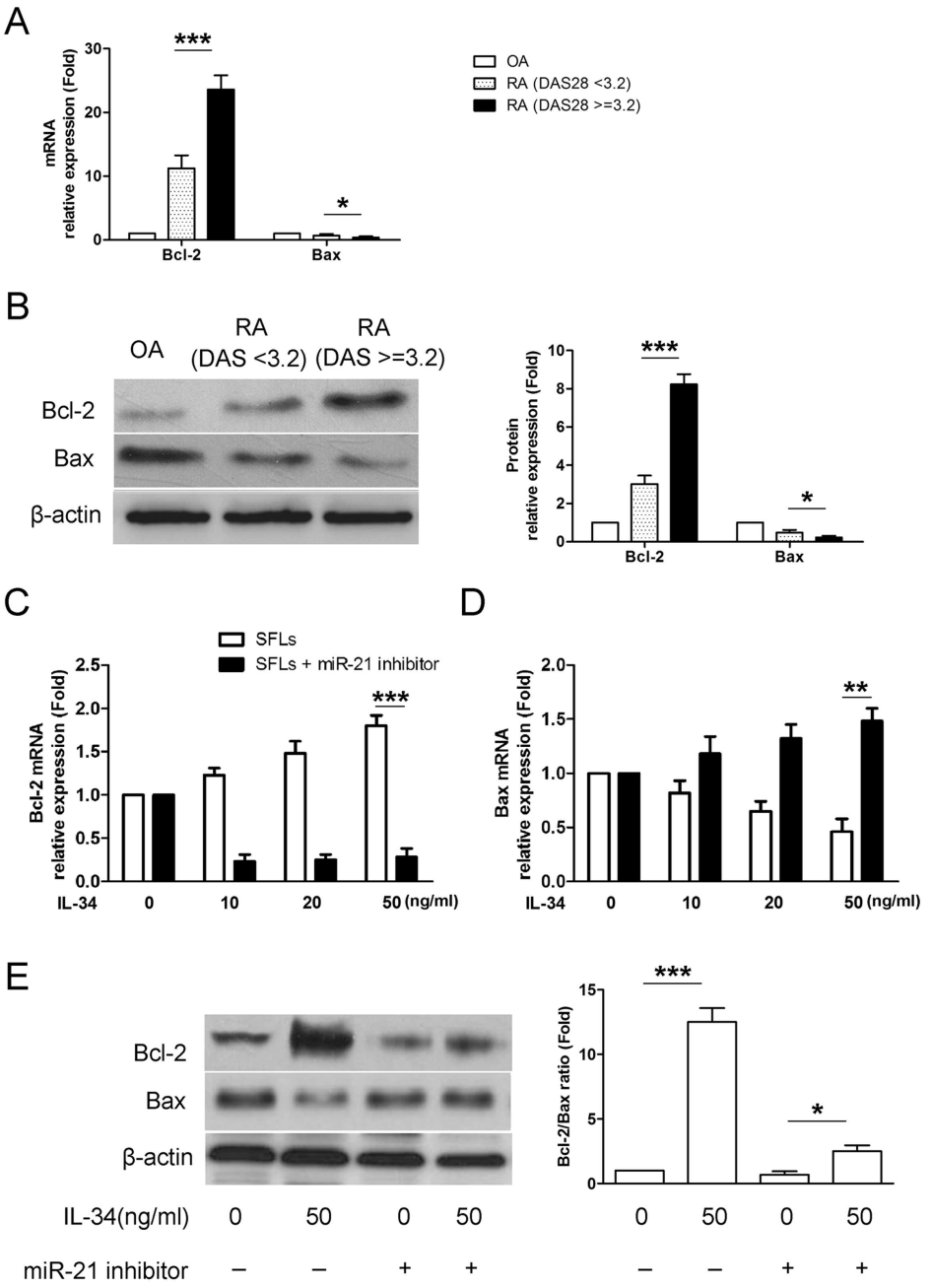


*Figure 3*. MiR-21 participates in the regulation of Bcl-2/Bax upon IL-34. FLS from patients with RA (DAS28 ≥ 3.2, n = 12; DAS28 < 3.2, n = 6) and OA (n = 6) were collected. (A and B) The expressions of Bcl-2 and Bax mRNA were evaluated by real-time PCR and Western blotting, respectively. (C–E) FLS were transfected with or without miR-21 inhibitor, and subsequently were cultured with different concentration of IL-34 for 24 h. The mRNA and protein expressions of Bcl-2 and Bax were evaluated, respectively. Data are presented as mean ± SD (n = 6), and 1 representative experiment is shown. * $P < 0.05$. ** $P < 0.05$. *** $P < 0.001$. MiR-21: microRNA 21; IL-34: interleukin 34; FLS: fibroblast-like synovial cells; RA: rheumatoid arthritis; DAS28: 28-joint Disease Activity Score; OA: osteoarthritis.

increased apoptosis. Compared with the miR-21 inhibitor, treatment with CSF1R-blocking antibody restrained the expression of miR-21 and Bax, suggesting that CSF1R participated in the IL-34/STAT3/miR-21 pathway (Figure 4). Other researchers reported that protein tyrosine phosphatase-ζ (PTP-ζ), a novel IL-34 receptor, increased the biological effects of IL-34[20]. In addition, IL-34 could induce the migration of syndecan-1–expressing cells, indicating syndecan-1 as a new molecular actor in the M-CSF/IL-34/M-CSFR triad[21]. More studies are required for further

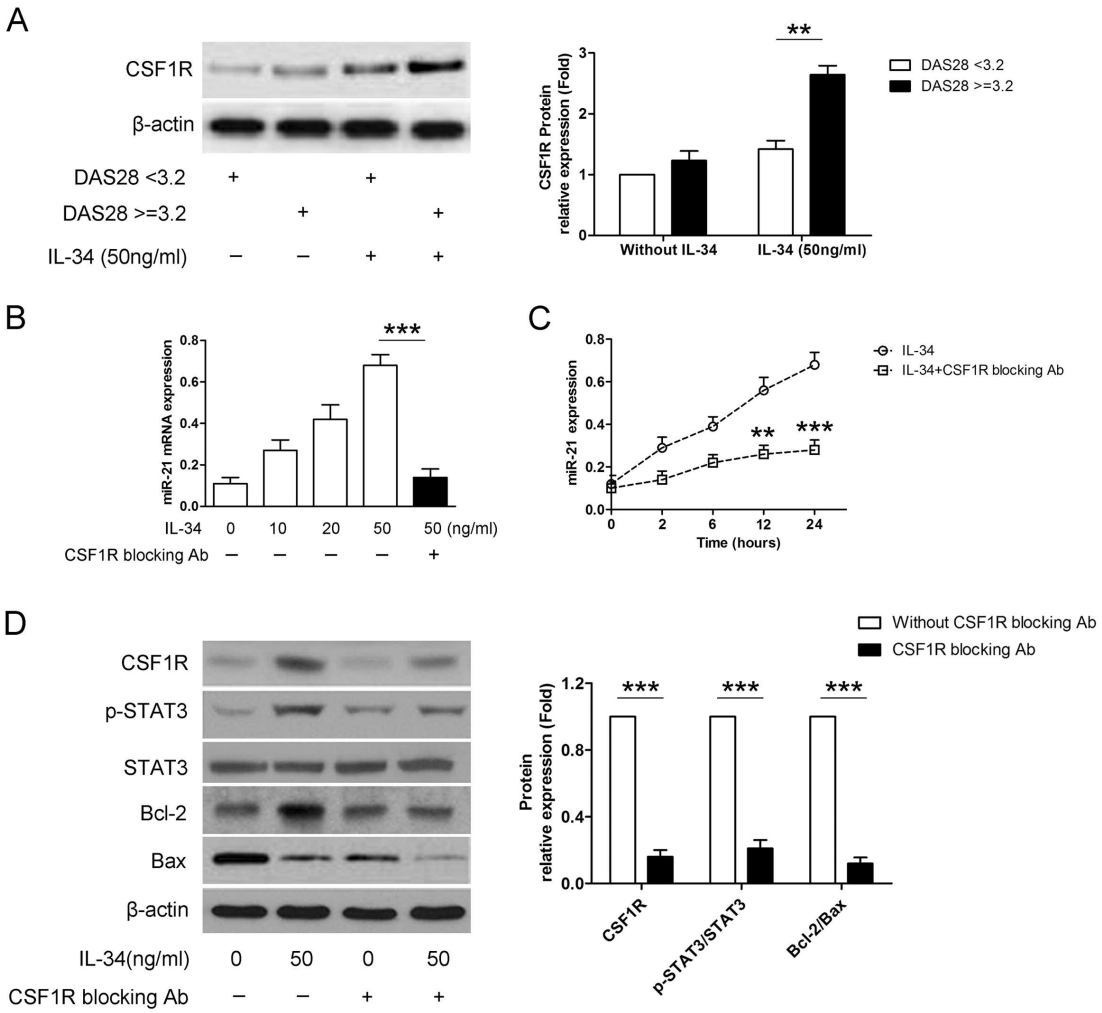


*Figure 4*. CSF1R is involved in the biological activities of IL-34 in RA. A. CSF1R expression in patients with RA (DAS28 ≥ 3.2, n = 17; DAS28 < 3.2, n = 13). FLS from patients with RA (DAS28 ≥ 3.2) were isolated and cultured in the presence or absence of CSF1R-blocking antibody for 24 h. B. MiR-21 expression was evaluated with different concentrations of IL-34 by real-time PCR. C. MiR-21 expression was evaluated following IL-34 stimulation (50 ng/ml) for different times. D. The proteins expressions of CSF1R, p-STAT3/STAT3, and Bcl-2/Bax were evaluated by Western blotting. All experiments were performed in triplicate. Data are presented as mean ± SD (n = 6), and 1 representative experiment is shown. ** $P < 0.05$. *** $P < 0.001$. CSF1R: colony-stimulating factor-1 receptor; IL-34: interleukin 34; RA: rheumatoid arthritis; DAS28: 28-joint Disease Activity Score; FLS: fibroblast-like synovial cells; miR-21: microRNA 21.

clarification of whether other IL-34 receptors, PTP-ζ and syndecan-1, are involved in the binding activity of IL-34 or present a cross-reaction to affect miR-21 expression.

Our results showed that IL-34 could induce STAT3 activation by binding to CSF1R and contributing to the increment of miR-21 expression, which was associated with disease activity of RA. Further, increased miR-21 expression resulted in an improved Bcl-2/Bax ratio, which was involved in limited apoptosis of FLS. These findings suggest that the IL-34/STAT3/miR-21 pathway is crucial for the survival of synovial cells, which might be alternative targets for the treatment of RA.

## REFERENCES


1. Kirino Y, Remmers EF. Genetic architectures of seropositive and seronegative rheumatic diseases. Nat Rev Rheumatol 2015; 11:401-14.
2. Leech MT, Morand EF. Fibroblasts and synovial immunity. Curr Opin Pharmacol 2013;13:565-9.
3. Chemel M, Le Goff B, Brion R, Cozic C, Berreur M, Amiaud J, et al. Interleukin 34 expression is associated with synovitis severity in rheumatoid arthritis patients. Ann Rheum Dis 2012;71:150-4.
4. Tian Y, Shen H, Xia L, Lu J. Elevated serum and synovial fluid levels of interleukin-34 in rheumatoid arthritis: possible association with disease progression via interleukin-17 production. J Interferon Cytokine Res 2013;33:398-401.
5. Moon SJ, Hong YS, Ju JH, Kwok SK, Park SH, Min JK. Increased

levels of interleukin 34 in serum and synovial fluid are associated with rheumatoid factor and anticyclic citrullinated peptide antibody titers in patients with rheumatoid arthritis. J Rheumatol 2013;40:1842-9.
6. Kumarswamy R, Volkmann I, Thum T. Regulation and function of miRNA-21 in health and disease. RNA Biol 2011;8:706-13.
7. Dong L, Wang X, Tan J, Li H, Qian W, Chen J, et al. Decreased expression of microRNA-21 correlates with the imbalance of Th17 and Treg cells in patients with rheumatoid arthritis. J Cell Mol Med 2014;18:2213-24.
8. van der Geest KS, Smigielska-Czepiel K, Park JA, Abdulahad WH, Kim HW, Kroesen BJ, et al. SF Treg cells transcribing high levels of Bcl-2 and microRNA-21 demonstrate limited apoptosis in RA. Rheumatology 2015;54:950-8.
9. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO 3rd, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis 2010; 62:1580-8.
10. Welsing PM, Landewé RB, van Riel PL, Boers M, van Gestel AM, van der Linden S, et al. The relationship between disease activity and radiologic progression in patients with rheumatoid arthritis: a longitudinal analysis. Arthritis Rheum 2004;50:2082-93.
11. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.
12. Hwang SJ, Choi B, Kang SS, Chang JH, Kim YG, Chung YH, et al. Interleukin-34 produced by human fibroblast-like synovial cells in rheumatoid arthritis supports osteoclastogenesis. Arthritis Res Ther 2012;14:R14.
13. Ju JH, Heo YJ, Cho ML, Jhun JY, Park JS, Lee SY, et al. Modulation of STAT-3 in rheumatoid synovial T cells suppresses Th17 differentiation and increases the proportion of Treg cells. Arthritis Rheum 2012;64:3543-52.
14. Lee SY, Yoon BY, Kim JI, Heo YM, Woo YJ, Park SH, et al. Interleukin-17 increases the expression of Toll-like receptor 3 via the STAT3 pathway in rheumatoid arthritis fibroblast-like synoviocytes. Immunology 2014;141:353-61.
15. van der Fits L, van Kester MS, Qin Y, Out-Luiting JJ, Smit F, Zoutman WH, et al. MicroRNA-21 expression in CD4+ T cells is regulated by STAT3 and is pathologically involved in Sézary syndrome. J Invest Dermatol 2011;131:762-8.
16. Tischner D, Woess C, Ottina E, Villunger A. Bcl-2-regulated cell death signalling in the prevention of autoimmunity. Cell Death Dis 2010;1:e48.
17. Firestein GS, Yeo M, Zvaifler NJ. Apoptosis in rheumatoid arthritis synovium. J Clin Invest 1995;96:1631-8.
18. Chihara T, Suzu S, Hassan R, Chutiwitoonchai N, Hiyoshi M, Motoyoshi K, et al. IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation. Cell Death Differ 2010;17:1917-27.
19. Toh ML, Bonnefoy JY, Accart N, Cochin S, Pohle S, Haegel H, et al. Bone- and cartilage-protective effects of a monoclonal antibody against colony-stimulating factor 1 receptor in experimental arthritis. Arthritis Rheumatol 2014;66:2989-3000.
20. Nandi S, Cioce M, Yeung YG, Nieves E, Tesfa L, Lin H, et al. Receptor-type protein-tyrosine phosphatase ζ is a functional receptor for interleukin-34. J Biol Chem 2013;288:21972-86.
21. Segaliny AI, Brion R, Mortier E, Maillasson M, Cherel M, Jacques Y, et al. Syndecan-1 regulates the biological activities of interleukin-34. Biochim Biophys Acta 2015;1853:1010-21.