

IL-34 modulates rheumatoid synovial fibroblast proliferation and migration via ERK/AKT signalling pathway

A. Elkhider¹, J. Wei¹, M. AL-Azab¹, Y. Tang¹, W. Walana¹,
W. Li¹, B. Yuan¹, Y. Ye¹, G. Wang¹, Y. Zhang², X. Li¹

¹Department of Immunology, College of Basic Medical Science, Dalian Medical University, Liaoning, China; ²Department of Rheumatology and Immunology, The Second Affiliated Hospital of Dalian Medical University, Liaoning, China.

Abstract Objective

The interface between pro-inflammatory cytokines and rheumatoid synovial fibroblast (sFLS) has central effects on rheumatoid arthritis (RA). The present study aimed to explore the role of IL-34 expression as one of major cytokine implicated in RA.

Methods

We examined the expression of IL-34 after RA sFLS stimulated by IL-1 β and TGF- β 1 separately by reverse transcription polymerase chain reaction (RT-PCR). Transwell and wound closure techniques were used to detect whether IL-34 is involved in promoting cell migration. Cellular viability was determined via CCK-8 and cultural morphology assays between IL-34 downregulated group and non-transfected counterpart. We also tested the expression of VEGF gene with RT-PCR analysis and activation of the major signalling pathways by western blot in IL-34 down-regulated group, IL-1 β or TGF- β 1 treated groups. Propidium iodide (PI) staining and fluoresceine isothiocyanate (FITC) Annexin V and propidium iodide apoptosis assay were used to analyse cell cycle arrest and apoptosis separately in IL-34 down-regulated cells.

Results

We found that IL-1 β significantly enhanced IL-34 expression, while contrarily, TGF- β 1 restrained IL-34 gene expression. Transwell and wound closure techniques showed that IL-34 was involved considerably in promoting cell migration. However, IL-34 knock-down restricted sFLS migration possibly through the diminishing of MMP2 and MMP9 expression. Interestingly, IL-34 down-regulated cells exhibited significantly low cellular viability compared with the non-transfected counterpart via CCK-8 and cultural morphology assays. We found that IL-34 down-regulated cells have low VEGF gene expression compared with treated cells. PI staining showed a G0/G1 cell cycle arrest in IL-34 down-regulated cells. FITC Annexin V and propidium iodide apoptosis assay verified that IL-34 down-regulated cells induced massive apoptosis through apoptotic signalling caspase3, while IL-1 β treated cells presented termination of cellular apoptosis signalled by BCL-2. Furthermore, we observed IL-34 induced activation of ERK1/2 and AKT pathways while IL-34 down-regulation significantly decreased the activation of these pathways.

Conclusion

Our data add novel insights into the pathogenesis of RA and we suggest that IL-34 plays a dominant role in controlling migration and proliferation of sFLS. Consequently, therapeutic strategies targeting IL-34 could be a potent therapy for RA.

Key words

IL-34, rheumatoid arthritis, ERK/AKT, synovial fibroblast proliferation, synovial fibroblast migration

Abdalkhalig Elkhider*
 Jing Wei*
 Mahmoud AL-Azab*
 Yawei Tang
 Williams Walana
 Weiping Li
 Bo Yuan
 Yunshan Ye
 Guan Wang
 Yan Zhang
 Xia Li

*These authors contributed equally to this study.

Please address correspondence to:
 Xia Li,

Department of Immunology,
 College of Basic Medical Science,
 Dalian Medical University,
 Lvshun South Road,
 Dalian 116044, China
 E-mail: lixia0416@dlmedu.edu.cn

and

Yan Zhang,
 (address as above)
 E-mail: zhangy1971@163.com

Received on February 4, 2019; accepted
 in revised form on July 11, 2019.

© Copyright CLINICAL AND
 EXPERIMENTAL RHEUMATOLOGY 2020.

Funding: the research was supported by
 The National Natural Science Foundation
 of China (grant no. 81671606),
 The College Scientific Research Project
 of Education Department of Liaoning
 Province (grant no. LQ2017004),
 Liao Ning Science and Technology
 Department (grant no. 20180550789)
 and The Key Laboratory of Human
 Microecology, Homeostasis and Disease
 Immune Mechanisms (Dalian).
 Competing interests: none declared.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by chronic inflammation of synovial layers, resulting in progressive articular destruction, which eventually leads to restricted joint movement and disability. Among the cells located in rheumatic joints, synovial fibroblasts are the most critical, driving local production of cytokines and tissue-destroying proteolytic enzymes which destroy the extracellular matrix and cartilage in a pathological condition. Recent studies have reported that there exist active communication between cytokines and cells of RA disease, and most of these inflammatory cytokines, particularly TNF- α , IL-1, and IL-34 are critical in the pathogenesis of RA as they are involved in the regulation of proliferation, migration, and apoptosis of synovial fibroblast-like synoviocytes (sFLS) (1).

IL-34 has been found constitutively in many tissues, and it is most abundant in the spleen (2), but also expressed in the synovial tissue of RA patients. Additionally, the pathological phase of RA is characterised with elevated IL-34 in serum and synovial fluid (3). The expression of IL-34 is regulated by TGF- β 1, TNF- α and IL-1 β , and recent evidence show that it support the myeloid cells' differentiation and proliferation. IL-34 has been detected in oral inflammation involving the expression of TNF- α , IL-1 β , IL-17 and IL-23, and in other conditions which accompany autoimmune disorders such as RA, and lupus (4, 5). TGF- β 1 is a multifunctional cytokine that contributes to the proliferation and differentiation of numerous cell types, and IL-1 β cytokine is a key player in the establishment of chronic joint inflammation. The major characteristics largely responsible for the development of RA are the aberrant proliferation and migration of sFLS, hence, the optimum regulation of these characteristics are critical for the treatment of RA. Against this background, we hypothesised that IL-34 expressed by synovial fibroblasts, the most abundant cell type in RA, could be modulated by TGF- β 1 and IL-1 β , and subsequently influence negatively the aggressive proliferation and migration of sFLS. Our findings suggest that

IL-34 transcription is significantly enhanced by IL-1 β , while TGF- β 1 inhibits IL-34 mRNA and protein expression in sFLS. IL-34 targeted therapy via the optimum regulation of TGF- β 1 and IL-1 β could provide alternative diagnostic and therapeutic strategies in RA treatment.

Materials and methods

Patients

The synovial tissues were collected from the Orthopaedics Department of the Second Hospital of Dalian Medical University, Dalian, China. Patients were diagnosed according to the RA 1987 revised classification criteria of the American College of Rheumatology. Synovial tissues were collected from 7 patients who were undergoing total joint replacement surgery. The approval to conduct this study was granted by the ethical committee of human and animal research of the Dalian Medical University.

RA-FLS Isolation

Enzymatic digestion of synovial tissues was performed to get FLS. The collected synovial tissues were finely minced into pieces and treated with collagenase type I from *Clostridium histolyticum* (Biotopped, China) at a concentration of 3 mg/mL in Dulbecco's modified Eagle's medium (DMEM) (Biological Industries, USA) in a petri dish. The dish was incubated at 37°C on a shaker for 2 hours. The digested tissues were then filtered through nylon mesh of pore diameter 70 μ M to get desired FLS. The filtrate was centrifuged for 5 minutes at 300g. Finally, the sediment was re-suspended in an inadequate volume of DMEM supplemented with 20% fetal bovine serum (FBS) (Biological Industries, USA), 100 U/ml penicillin, and 100 μ g/ml streptomycin, and cultured in humidified environment incubator at 37°C and 5% CO₂. Three to five passages of RA-FLS were used for all experiments.

Cell culture and stimulation

At approximately 80–100% confluency, RA-FLS were trypsinised, harvested, re-suspended, divided for cell propagation in DMEM with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin, and incubated at 37°C, and 5% CO₂

in humidified environment. Three to five passages of RA-FLS showed typical morphological characteristics under phase contrast microscope. To study the effect of IL-34 on RA-FLS, RA-FLS were starved for 24h, and then stimulated with TGF- β 1 (10 ng/mL), IL-1 β (10 ng/mL) in serum-free DMEM for 8 h, while the incubation time was extended to 24 h for western blotting.

Total mRNA extraction

RA-FLS cells were washed twice with sterile PBS and harvested in RNAiso Plus (Takara Bio, Dalian, China). Total RNA was extracted from the cell as directed by the manufacturer. Briefly, the cells in RNAiso Plus were put on ice for five minutes and centrifuged at 4°C at 12000g for 15 minutes. The supernatant was added to equal volume of chloroform, incubated on ice for 10 minutes and centrifuge as above. The aqueous phase was added to 550 μ l isopropanol and mixed gently, and centrifuge at 4°C at 12000g for 15 minutes. The sediment was washed in 1mL 75% ethanol in DEPC treated H₂O, and centrifuge for 10 minutes. The pellet was air dried, dissolved in 10 μ l of RNase free H₂O, and the concentration determined by NanoDrop 2000 full-spectrum (Thermo Scientific™, USA).

Reverse transcription polymerase chain reaction (PCR)

Less than 1 μ g of the RNA extracted was used for cDNA synthesis, using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio, Japan). A mixture consisting of oligo dT primer, dNTP mixture, RNase Free ddH₂O and our extracted RNA templates was incubated at 65°C for five minutes and immediately cooled on ice. 5X prime-Script buffer, RNase inhibitor, RTase, and RNase Free dH₂O were added to the previous mixture to get 20 μ l in a micro-tube according to the manufacturer's instructions. The mixture was homogenised gently by micro-centrifuge and incubated for 60 minutes at 42°C. The enzyme was inactivated by incubating at 95°C for 5 minutes and then cooled on ice. Real-time PCR was performed to amplify the genes, and GAPDH was used as an internal con-

trol to evaluate the cDNA synthesis efficiency. The primers used in this study were obtained Takara Bio, Japan, and are indicated in Table I.

Protein extraction

RA-FLS culture to 100% confluence was scraped using ice-cold phosphate buffer saline (PBS) and cold plastic scraper and then harvested by centrifugation at 3000 RPM for 3 minutes at 4°C. Lysis buffer containing proteinase inhibitor, phosphate inhibitor, and phenylmethanesulfonyl fluoride (PMSF) as prescribed by the kit protocol (KeyGen Biotech, China) was added to the cells. The resulting mixture was vortexed for 40 minutes and centrifuged at 12000 RPM for 15 minutes at 4°C. The supernatant was used as the total protein extract. The concentration of the total protein was estimated with bicinchoninic acid (BCA), (KeyGen Biotech, China), in accordance with the manufacturer's instruction. Standard absorbance curve was established using standard protein, and concentrations of the samples extrapolated from the curve. Protein concentration of 20 μ g was used for western blotting.

Western blot

20 μ g of the total protein extracted from RA-FLS were subjected to 12% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and to Hybond-C nitrocellulose membranes, and blocked with 5% skimmed milk in Tris-buffered saline tween 20 (TBST) at room temperature for 1 h. The membranes were incubated with rabbit IgG (primary Ab), (1:1000, Abcam, USA) and β -actin (1:1000, Abcam, USA) at 4°C for overnight. After washing thrice with TBST, the membranes were incubated with H.R peroxidase-conjugated goat anti-rabbit IgG (secondary Ab) for 1 h at room temperature. The proteins were visualised by Enhanced chemiluminescent (ECL) detection system (Bio-Rad, USA). The band density quantification was performed by Image Lab software (Bio-Rad, USA).

IL-34 gene silencing

siRNA against hIL-34 and silencer negative siRNA control were purchased

Table I. Clinical and laboratory features of rheumatoid arthritis patients involved in this study.

Characteristics	Values
Age (years)	64.57 \pm 3.51
Men/Female	1/6
Disease duration (years)	8.43 \pm 5.04
ESR, mm/h	27.57 \pm 15.59
CRP, μ g/ml	21.02 \pm 19.69
RF-IgM, IU/ml	92.13 \pm 93.50
Anti-CCP, U/ml	336.75 \pm 492.64

ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, RF: rheumatoid factor; Anti-CCP: anti-cyclic citrullinated peptide.

from GenePharma Technologies, Shanghai, China, sequence indicated in Table II. They were dissolved in siRNA buffer 125 μ L DEPC (supplied by GenePharma Technologies) and transfected into RA-FLS of 70–90% confluence as described by the manufacturer. The effect of the transfection was confirmed by RT-PCR, western blot, and fluorescent microscopy 48 h post-transfection.

Transwell migration assay

Transwell migration assay was performed to measure cell migration. Transwell chambers (8 μ m pores; Corning, New York, USA) in 24-well plates were used. RA-FLS were added to the upper chambers, 100 μ l (1 \times 10⁵ cells/mL), and the lower chambers filled with 350 μ l (20% FBS in DMEM medium) as the attracting agent. After 24 h incubation, the Non-migrated cells were removed from the upper side and migrated cells were fixed with methanol for 10 min and stained with 0.1% crystal violet. The cells were visualised by an inverted microscope (Olympus IX71, Tokyo, Japan).

Wound-healing assay

RA-FLS were cultured into a six-well plate, and then scratches were made in the midst of each well using a 200 μ L pipette tip. Scratched layers were washed with PBS to remove non-adherent cells and replaced medium with serum-free DMEM. Wound healing was observed with an inverted microscope (Olympus IX71, Tokyo, Japan) and evaluated by compared the cell-free area with the initial wound region.

Table II. Primer sequences used for cDNA amplification.

Gene	Type	Sequences
IL-34	Forward	sense 5- AAGTCCTCAGTCTTGCAGCC -3
	Reverse	antisense 5- CCTATCCGAGTCACCATCC -3
MMP-2	Forward	sense 5- CCGTCGCCATCATCAAGTT -3
	Reverse	antisense 5- CTGTCTGGGGCAGTCCAAAG -3
MMP-9	Forward	sense 5- GGGACGCAGACATCGTCATC-3
	Reverse	antisense 5- TCGTCATCGTCGAAATGGGC -3
VEGF	Forward	sense 5- TTCTGGGCTGTTCTCGCTTC -3
	Reverse	antisense 5- CTCCTCTTCTCTTCTTCTCC -3
GAPDH	Forward	sense 5 - TGACCACAGTCCATGCCATCAC-3
	Reverse	antisense 5 - CGCCTGCTTCACCACCTTCTT-3

Table III. Sequences of siRNA used for IL-34 down-regulation.

Gene	Type	Sequences
IL-34-homo-578	Forward	sense 5_-UGCGCUAUCUUGGGAUUCUUTT-3
	Reverse	antisense 5_-AAGAUCCCAAGAUAGCGCATT-3
IL-34-homo-702	Forward	sense 5_-CCGACUUCAGUACAUGAAATT-3
	Reverse	antisense 5_-UUUCAUGUACUGAAGUCGGTT-3
IL-34-homo-1091	Forward	sense 5_GCUGUAAAACAAAGCUCGGUTT-3
	Reverse	antisense 5_-ACGGAGCUUUGUUUACAGCTT-3
Negative siRNA	Forward	sense 5_-UUCUCCGAACGUGUCACGUTT-3
	Reverse	antisense 5_-ACGUGACACGUUCGGAGAATT-3

Gelatin zymography assay

Gelatin zymography was performed to determine MMP-2 and MMP-9 activities in the supernatants of RA-FLS. Briefly, supernatants of treated cells (2x10⁴ cells) were collected and 20 µg of protein was loaded on electrophoresis polyacrylamide gel containing 0.2% gelatin (type A, G-8150, Sigma-AI-

drich). The gel was washed with washing buffer and stained by Coomassie Brilliant Blue.

CCK-8 cell proliferation assay

sFLS proliferation was demonstrated by the CCK-8 assay. To determine the effect of IL-34 expression on the proliferation of synovial fibroblast, 3x10³

of sFLS cells suspended in 100 ml of DMEM per well were seeded in 96-well plate and 24 h incubation to allow cells to attach on well bottoms. Thereafter, the cells were treated with (10 ng/mL IL-1β and 10 ng/mL TGF-β1) for 24 h. Next, 10 µl of CCK-8 reagent was added to treated, untreated (control) and IL-34 down-regulated cells. The cells were incubated for 1 h at 37°C and 5% CO₂, and the absorbance detected at an optical density (OD) of 450 nm using Multi-skanGo Spectrophotometer (USA).

Cultural morphological assay

The RA-FLS cells were seeded into 6-well plate (1000 cells/wells) in triplicates, containing 2 ml DMEM supplemented with 20% (FBS). After 48 h RA-FLS were treated with appropriate cytokines concentration, and further incubated for 7 days. The cells were then fixed with 25% acetic acid in ethanol and stained with 0.5% crystal violet. The cells were observed by inverted microscope (Olympus IX71, Tokyo, Japan).

Cell cycle analysis assay

To explore the influence of IL-34 on cell cycle progression of synovial fibroblast, sFLS were detached using trypsin and incubated for 24 h in a culture medium in 6 wells plate. After the 24 h, the medium was replaced and stimulated with IL-1β10 ng/mL and TGF-β1 10 ng/mL and then incubation in 37°C,

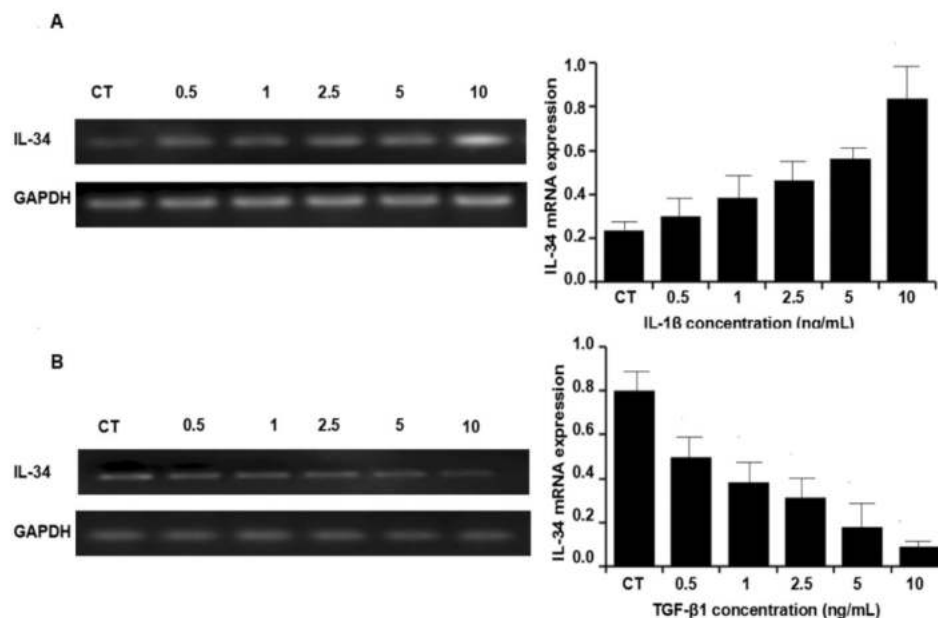


Fig. 1. IL-1β and TGF-β1 variably affect the expression IL-34.

A: sFLS from RA patients were incubated in the presence or absence of IL-1β (0.5, 1, 2.5, 5, 10 ng/mL) for 8 hours. IL-34 mRNA levels were determined by RT-PCR.

B: sFLS from RA patients were incubated in the presence or absence of TGF-β1 (0.5, 1, 2.5, 5, 10 ng/mL) for 8 hours. IL-34 mRNA levels were determined by RT-PCR.

p<0.002, *p<0.0003.

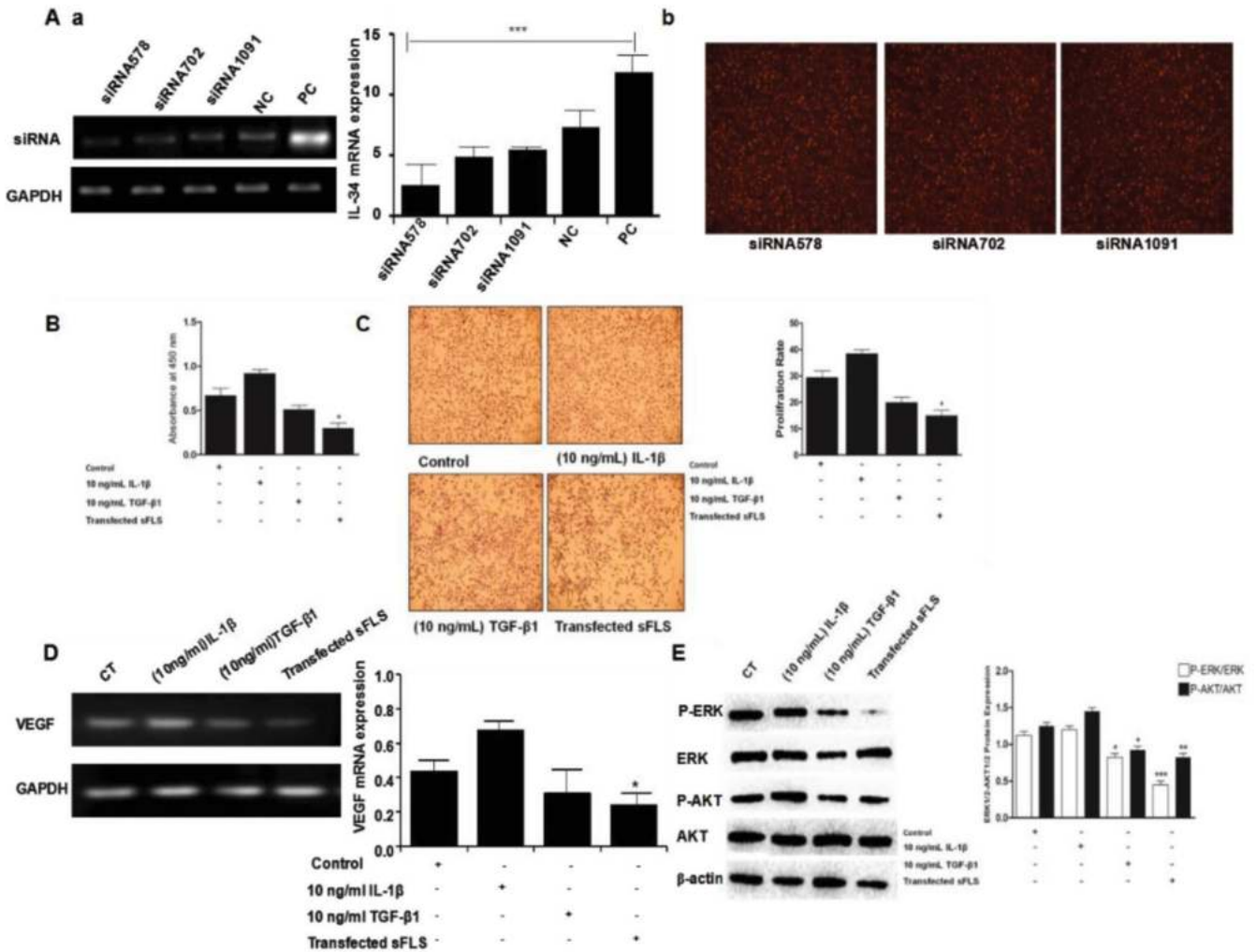


Fig. 2. IL-34 knock-down suppresses VEGF expression and sFLS proliferation via ERK1/2 and AKT1/2 pathways. **A/a:** Transfected sFLS with different siRNAs, IL-34 expression determined by RT-PCR. **A/b:** Transfected sFLS, images were taken by fluorescence microscope following transfection with different siRNAs. **B:** Cell growth was assessed using CCK-8 assay and analysed by MultiscanGo photospectrometer. **C:** Cell proliferation rate was examined using cultural proliferation assay. **D:** IL-34 down-regulation inhibits expression of VEGF; VEGF mRNA levels were determined by RT-PCR. **E:** RA-FLS were stimulated with different concentrations of cytokines (in an appropriate concentration for regulating IL-34 expression) and determined by immunoblotting, β -actin proteins expression used as internal control. **** p <0.001, *** p <0.0002, ** p <0.003, * p <0.006, *** p <0.0001.**

and 5% CO₂ in a humidified environment for 8 h. The cells were harvested at the log phase of growth, washed two times with ice-cold PBS and then fixed in 75% cold ethanol at 4°C overnight. After fixation, the cells were washed twice with ice-cold PBS, 50 μ g/ml RNase (Sigma, USA) was added to the cells and incubated for 30 minutes. The cells were stained with 20 μ g/ml of propidium iodide (Sigma USA) in darkness and analysed by flow cytometry (Beckman counter, USA). The data was analysed using Multicycle software (Phoenix Flow Systems, San Diego, USA) to substantiate the cell cycle population.

Cell apoptosis analysis

Apoptosis assay was used to evaluate the effect of IL-34 on RA-FLS as well as, IL-34 down-regulation on sFLS. Propidium iodide and Annexin V-FITC apoptosis test kit (KeyGEN, China) were used. 1 \times 10⁶ cells were seeded into 6 cm dish, incubated in humidified atmosphere under 5% CO₂ at 37°C for 24 h. Following 24 h, culture medium was replaced with either complete medium only for control and transfected cells or complete medium with (10 ng/mL IL-1 β and 10 ng/mL TGF- β 1) as treatment groups and incubated for 24 h. The cells were harvested and washed with cold

PBS for three times. Next, the cells were resuspended in 500 μ l binding buffer and incubated in 5 μ l PI and 5 μ l Annexin V-FITC in the dark area for 15 min at room temperature and, subsequently, examined by fluorescence-activated cell sorting (FACS) analysis (FACS Calibur, BD Biosciences, USA). The results were analysed using the Cell Quest software (BD Biosciences, USA).

Statistical analysis

GraphPad Prism v. 6.07 (San Diego, USA) was used for all statistical analysis. One-way analysis of variance (ANOVA) was employed to compare

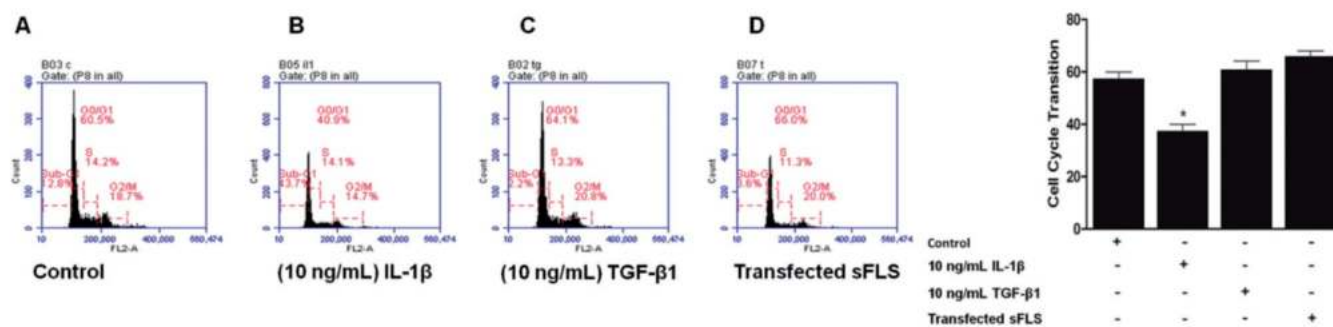


Fig. 3. IL-34 knock-down inhibits sFLS proliferation through inducing G0/G1 arrest. IL-34 down-regulation represses G0/G1-S cell cycle transition of sFLS. Flow cytometry analysis of treated, untreated and transfected sFLS. *** $p < 0.005$.

difference among groups. The results shown are representatives of three independent experiments, and p -value < 0.05 was considered statistically significant.

Results

IL-34 up-regulation and down-regulation by IL-1 β and TGF- β 1

Several studies reported that the RA-FLS expressed a variety of cytokines which are involved in cellular activities. Here, we found that the IL-34 mRNA was expressed in RA-FLS manifested by RT-PCR analysis which elucidated as endogenous control. (Fig. 1 A-B) We assessed the effects of IL-1 β (suggested as synergistic for IL-34) and TGF- β 1 (suggested as antagonistic for IL-34) with variable concentrations on IL-34 expressed with FLS. As Fig. 1A and B showed, 10 ng/ml of IL-1 β obviously promoted IL-34 expression on FLS. On the contrary, in the presence of TGF- β 1 (10 ng/ml), RA FLS showed significant decreased IL-34 gene expression.

IL-34 expression promotes RA-FLS proliferation via ERK1/2 and AKT1/2 pathways

The sFLS cells were transfected with three different sequences of IL-34 siRNA, and siRNA 578 exhibited optimum down-regulation of IL-34 gene, manifested by RT-PCR (Fig. 2A/a) and fluorescent imaging (Fig. 2A/b). CCK-8 proliferation assay revealed that IL-1 β showed increased proliferation compared to TGF- β 1 and untreated cells. However, IL-34 down-regulated cells had a significant decrease in proliferation (Fig. 2B). Although, increased sFLS proliferation in cell culture was observed in IL-1 β treated cells after 7 days from cell seeding, the transfected

cells and TGF- β 1 treated cells showed a significant decrease in proliferation. Cell proliferation was observed after 7 days and cells were stained with crystal violet for visualisation (Fig. 2C). We measured the gene expression of VEGF as a marker for sFLS proliferation. VEGF was significantly elevated in IL- β 1 treatment cells (Fig. 2D). To investigate the mechanisms involved in IL-34 enhanced proliferation of RA-FLS, we evaluated the effect of IL-34 protein expression on signalling pathways that contribute to cell proliferation particularly extracellular signal regulated kinase 1/2 (ERK1/2) and protein Kinase (AKT1/2) pathways. We stimulated RA-FLS with IL-1 β , TGF- β 1 and also used IL-34 down-regulated cells. These cytokine concentrations could variably affect the proliferation of RA-FLS via enhancing and inhibiting IL-34 expression. Western blot analysis showed that ERK1/2 and AKT1/2 pathways mediated IL-34 levels were significantly activated (Fig. 2E).

IL-34 knock-down induces G0/G1 arrest in RA-FLS

Depending on the observed continuation in synovial fibroblast proliferation upon IL-34 stimulation, the effect of IL-34 on cell cycle was assessed. Significant differences were observed in cell accumulation at G0/G1. IL-34 exhibited suppressed cell growth in the G0/G1 phase of cell cycle an average of 40.9%, 64.1%, 66.0%, for IL-1 β , TGF- β 1 and IL-34 down-regulated cells respectively. The results showed knock-down IL-34 exposed maximum cell cycle arrest in the cell population compared with other groups of the cell population (Fig. 3).

IL-34 decreases RA-FLS apoptosis

The apoptosis assay conducted showed that, IL-1 β treated cells had significant decreased initiated apoptosis compared with other treated cells (Fig. 4A/b). The total percentage of apoptotic cells (early stage and late stage) was 12.6% for IL1 β treated cells (Fig. 4A/b) and that TGF β 1 treated cells was 11.4% (Fig. 4A/c). This percentage increased remarkably to 34.2% for IL-34 transfected cells (Fig. 4A/d). To determine the apoptotic mechanism(s) fundamental to this observation, we investigate particular apoptotic proteins that could be induced cell death. The expression levels of BCL-2 and caspase3 were examined in sFLS treated with different cytokines concentration, untreated cells (control) and transfected cells. We observed significant differences in BCL-2 protein expression. IL-1 β treated cells showed a significant increase in expression level, while IL-34 transfected cells showed diminished BCL-2 protein expression. Contrarily, IL-1 β treated cells exhibited a significant decrease in caspase3 compared with transfected cells which demonstrated a significant increase in caspase3 protein expression compared with other groups (Fig. 4B).

IL-34 promotes RA-FLS migration

Wound-healing and transwell assays were used to evaluate sFLS migration. The sFLS cells treated with IL-1 β with high IL-34 gene expression had significantly accelerated migration compared with cells treated with TGF- β 1, and down-regulated IL-34 cells, which had low IL-34 gene expression, showed delay in closure of wound gap and percentage of cell migration across transwell membrane after 24 h. (Fig. 5A-B)

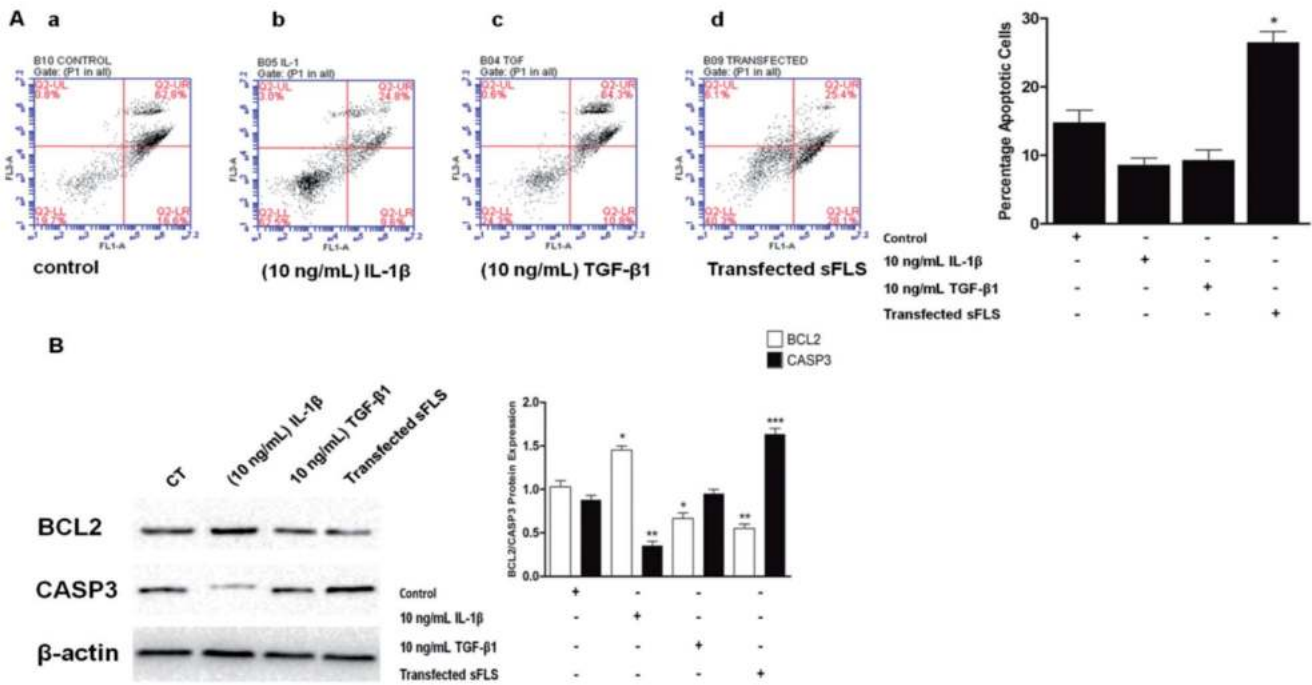


Fig. 4. IL-34 knock-down induces apoptosis in sFLS
A: sFLS cells were treated with different concentrations of cytokines for 8 h and analysed by apoptosis assay. FACS representative flow cytometry dot plots of analysis output of sFLS. **B:** BCL-2, caspase3 and β-actin proteins expression (internal control) of treated, untreated and transfected sFLS after western blot experiment. ***p*<0.003, ****p*<0.0001.

The expression of MMP-2 and MMP-9 were examined in a synovial fibroblast. Our RT-PCR results showed that both MMP-2 and MMP-9 are expressed by sFLS. Our findings demonstrate that MMP-2 and MMP-9 are significantly expressed in the presence of IL-1β treated cells, but less expressed in TGF-β1 treated or down-regulated IL-34 cells. (Fig. 5C) In the same context, the gelatinases zymography revealed a high activity of MMP-2 and MMP-9 in IL-1β treated cells, furthermore, low activity was observed in the supernatants of TGF-β1 and IL-34 down-regulated cells. (Fig. 5D) Collectively, these results indicate that IL-34 plays a contributory role in sFLS migration and restricting its expression could be a potent therapeutic target.

Discussion

Scientific studies have concentrated on the crucial role of cytokines in the development and progression of RA. Previous studies demonstrated that pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-21 play diverse roles in the pathophysiology of RA, and serve as therapeutic targets in RA patient. These cytokines certainly involve and promote

inflammation and osteoclastogenesis in the arthritic joints motivate the destruction of cartilage and subsequently the joint bone (1, 5, 6). In this study, we focused on IL-34, which substantially controls RA in many aspects. We demonstrated a downstream effect of IL-34 regulated by TGF-β1 and IL-1β in sFLS. The understanding of the mechanisms which regulate the production of this cytokine could contribute to the development of new drugs or treatment regimens. We demonstrated that two cytokines, IL-1β and TGF-β1, are capable to promoting and inhibiting IL-34 gene expression respectively. We confirmed that IL-1β promotes significantly IL-34 expression, while TGF-β1 inhibits IL-34 expression in synovial fibroblasts. Previous studies have shown that IL-34 expression could be controlled directly through IL-1β and TGF-β (4, 5). Synovial fibroblast is the major cell population in synovial tissues and plays an important role in the progress of RA. The relation between inflammatory cytokines, cellular proliferation, and angiogenesis, invasion, and metastasis of cancer cells are considered to be a new concept for diagnosis and treatment (6-8). In this study, we demonstrated that,

in RA-FLS, the expression of IL-34 significantly enhances RA-FLS proliferation and its knockdown is accompanied with significant diminishing of RA-FLS proliferation compared with control cells. To support our finding, we used IL-1β and TGF-β1 as enhancer and suppressor of IL-34 expression respectively, and we found similar results. Several reports have shown that IL-34 has a role in promoting fibrocyte proliferation and activation during arthritis (9). Furthermore; IL-34 is strongly associated with synovial inflammation and motivates a synovial angiogenesis and cell proliferation potentiated through hypoxia (10). Recently, several studies have shown that, VEGF is a new member in progression of RA, and that, it increases osteoclastogenesis and prompts destruction of joint bone in RA patients (11). Juan Zhang *et al.* reported a strong correlation between TGF-β1 and VEGF expression in supernatants of a RA-FLS compared with normal control FLS (12). Here, we report that IL-1β (up-regulates IL-34) increases expression of VEGF, we hypothesised that IL-34 could be performing as a potential link between TGF-B1 and VEGF expression.

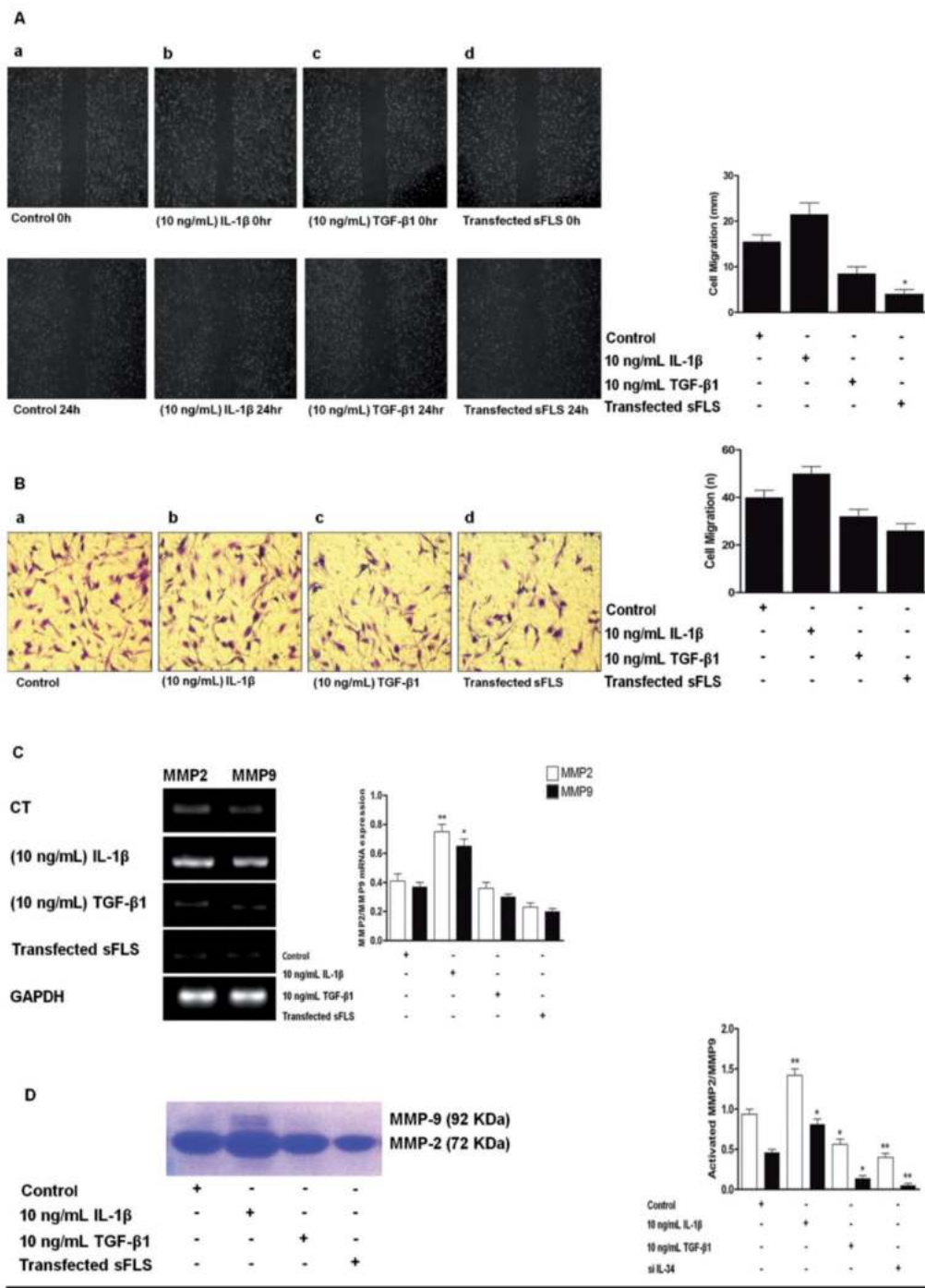


Fig. 5. Effect of IL-34 down-regulation on sFLS migration. **A:** Wound-healing assay associated with treated, non-treated and transfected sFLS migration. Phase-contrast micrographs of the scratched zone at 0 and 24 h after scratching. **B:** Treated, non-treated and transfected sFLS migration across transwell membrane at 24 h. **C:** MMP-2 and MMP-9 genes expression associated with different sFLS stimulation, transfected and control cells, MMP-2 and MMP-9 mRNA levels were determined by RT-PCR. **D:** Inverse picture of gelatin zymography gel for detected of MMP-2 and MMP-9 activists. The analysis was demonstrated in culture supernatants of RA-FLS cells post stimulation for 24h. **p*<0.02 ***p*<0.001 ****p*<0.0001 ****p*<0.0002

The cell-division cycle of mammalian cells is controlled by a sequence of positive and negative regulatory factors in the cell cycle. Certain elements play important roles in the protection of cells from cell cycle arrest. However, the inhibition of these elements could induce a remarkable arrest of cell cycle. Cyclin-dependent kinase 4/6 (CDK4/6) inhibition promote G0/G1 cycle arrest through CDK4/6 inhibition in cultured human renal proximal tubule cells after cell injury (13). Notably, we found that

the inhibition of IL-34 in sFLS markedly increases G0/G1 cell cycle. arrest. Our results partially explain IL-34 probably mediates the sFLS survival and the underlying molecular mechanism(s) still ambiguous and need further elucidation. Some studies have indicated that several cytokines might contribute to the behaviour of cells, such as cell migration (14). Qingli Bie and his colleagues demonstrated that the supernatant of mesenchymal stem cells treated with exogenous IL-17β promotes migration

of gastric cancer cells (15). From another perspective, the IL-33 and osteopontin coordinate neutrophil and monocyte migration in RA (16, 17). (MMPs) are the key enzymes implicated in RA progression, and their activity is highly controlled with proinflammatory cytokines (18). In this study, we exhibited IL-34 prompted the migration of RA-FLS, also enhanced the expression of endogenous MMP2 and MMP9. This agrees with previous study which showed down-regulation of endog-

enous MMP-2 or MMP-9 reduced RA-FLS migration and invasion. Moreover, MMP-9 activates RA-FLS mediated inflammation and degradation of cartilage in RA (19).

Cell suicide (apoptosis) is a programmed cell death that occurs in multiple cells due to biochemical reaction, which leads to characteristic changes and eventually cell death. Many reports have shown that several cytokines could induce apoptosis. Yan and others reported that TGF- β triggers apoptosis response in human endothelial cells through up-regulation of Nox4 NADPH oxidase and production of reactive oxygen species (ROS) (20-22). Here, we report that sFLS treated with TGF- β 1 showed significant apoptotic rate compared with IL-1 β . Therefore, we hypothesise that TGF-1 β induce the sFLS apoptosis. Moreover, in previous study, IL-33 improved neurological function activity by impeding the apoptosis (23). In contrast, we showed that IL-34 significantly decreased sFLS apoptosis. Conversely, knock-down of IL-34 enhances undoubtedly sFLS apoptosis. To highlight more on the mechanism that underlies the above observation, the expression of fundamental proteins that regulate apoptosis were determined. BCL-2 exhibited over-expression in response to IL-34 treatment. Also, caspase3 expression was increased in transfected cells (down-regulated IL-34) compared with untreated sFLS. However, this mechanism requires further investigations. IL-34 activates several signalling pathways including the MAPK, PI3K-Akt, JAK and NF- κ B. (24) We found that IL-34 through supporting cytokines, stimulate RA-FLS, and activate ERK1/2 and AKT1/2 signalling pathways. We suggest that these stimulations are partly triggered by IL-34. Also, we observed that blocking of IL-34 decreased significantly the expression of kinase pathways and inhibit IL-34 promoted proliferation. The ERK pathway has been reported to contribute to inflammatory response of RA-FLS. Moreover, ERK inhibition reduced the proliferation of sFLS (25, 26). Our findings import new evidence, proposing IL-34 as a promising therapeutic target for the treatment of RA.

Conclusion

In summary, our data demonstrate that the pro-inflammatory cytokine IL-34 modulates the aggressive sFLS pathological behaviour and protects the cells from apoptosis. In this context, significant diminishing of migration and proliferation, and induction of cell apoptosis and anti-growth was observed in down-regulated IL-34 cells. Based on the above findings, we suggest that IL-34 perhaps could be a promising therapeutic target for RA patients.

References

1. WANG B, MA Z, WANG M *et al.*: IL-34 Up-regulated Th17 Production through increased IL-6 expression by rheumatoid fibroblast-like synoviocytes. *Mediators Inflamm* 2017; 2017: 1567120.
2. CHEMEL M, LE GOFF B, BRION R *et al.*: Interleukin 34 expression is associated with synovitis severity in rheumatoid arthritis patients. *Ann Rheum Dis* 2012; 71: 150-4.
3. TIANY, 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.
4. BOSTRÖM EA, LUNDBERG P: The newly discovered cytokine IL-34 is expressed in gingival fibroblasts, shows enhanced expression by pro-inflammatory cytokines, and stimulates osteoclast differentiation. *PLoS One* 2013; 8: e81665.
5. CHEMEL M, BRION R, SEGALINY A-I *et al.*: Bone morphogenetic protein 2 and transforming growth factor β 1 inhibit the expression of the proinflammatory cytokine IL-34 in rheumatoid arthritis synovial fibroblasts. *Am J Pathol* 2017; 187: 156-62.
6. XING R, YANG L, JIN Y *et al.*: Interleukin-21 induces proliferation and proinflammatory cytokine profile of fibroblast-like synoviocytes of patients with rheumatoid arthritis. *Scand J Immunol* 2016; 83: 64-71.
7. NOLD-PETRY CA, RUDLOFF I, BAUMER Y *et al.*: IL-32 promotes angiogenesis. *The J Immunol* 2014; 192: 589-602.
8. ZENG Q, LI S, ZHOU Y *et al.*: Interleukin-32 contributes to invasion and metastasis of primary lung adenocarcinoma via NF-kappaB induced matrix metalloproteinases 2 and 9 expression. *Cytokine* 2014; 65: 24-32.
9. GALLIGAN CL, FISH EN: Interleukin-34 promotes fibrocyte proliferation. *J Interferon Cytokine Res* 2017; 37: 440-8.
10. BALOGH E, CONNOLLY M, BINIECKA M, MCCORMICK J, VEALE DJ, FEARON U: Interleukin-34 regulates angiogenesis and cell proliferation in inflammatory arthritis, this effect is potentiated by hypoxia. *Arthritis Rheumatol* 2013; 65: S405.
11. KIM HR, KIM KW, KIM BM, CHO ML, LEE SH: The effect of vascular endothelial growth factor on osteoclastogenesis in rheumatoid arthritis. *PLoS One* 2015; 10: e0124909.
12. ZHANG J, LI C, ZHENG Y, LIN Z, ZHANG Y, ZHANG Z: Inhibition of angiogenesis by arsenic trioxide via TSP-1-TGF- β 1-CTGF-VEGF functional module in rheumatoid arthritis. *Oncotarget* 2017; 8: 73529-46.
13. DIROCCO DP, BISI J, ROBERTS P *et al.*: CDK4/6 inhibition induces epithelial cell cycle arrest and ameliorates acute kidney injury. *Am J Physiol Renal Physiol* 2013; 306: F379-F88.
14. SEGALINY AI, BRION R, MORTIER E *et al.*: Syndecan-1 regulates the biological activities of interleukin-34. *Biochim Biophys Acta* 2015; 1853: 1010-21.
15. BIE Q, ZHANG B, SUN C *et al.*: IL-17B activated mesenchymal stem cells enhance proliferation and migration of gastric cancer cells. *Oncotarget* 2017; 8: 18914.
16. VERRI WA, SOUTO FO, VIEIRA SM *et al.*: IL-33 induces neutrophil migration in rheumatoid arthritis and is a target of anti-TNF therapy. *Ann Rheum Dis* 2010; 69: 1697-703.
17. TSAI C-H, LIU S-C, WANG Y-H *et al.*: Osteopontin inhibition of miR-129-3p enhances IL-17 expression and monocyte migration in rheumatoid arthritis. *Biochim Biophys Acta* 2017; 1861: 15-22.
18. ANGELOTTI F, PARMA A, CAFARO G, CAPECCHI R, ALUNNO A, PUXEDDU I: One year in review 2017: pathogenesis of rheumatoid arthritis. *Clin Exp Rheumatol* 2017; 35: 368-78.
19. XUE M, MCKELVEY K, SHEN K *et al.*: Endogenous MMP-9 and not MMP-2 promotes rheumatoid synovial fibroblast survival, inflammation and cartilage degradation. *Rheumatology* 2014; 53: 2270-9.
20. YAN F, WANG Y, WU X *et al.*: Nox4 and redox signaling mediate TGF- β -induced endothelial cell apoptosis and phenotypic switch. *Cell Death Dis* 2015; 5: e1010.
21. DAS R, XU S, QUAN X *et al.*: Upregulation of mitochondrial Nox4 mediates TGF- β -induced apoptosis in cultured mouse podocytes. *Am J Physiol Renal Physiol* 2013; 306: F155-F67.
22. FERRARI G, TERUSHKIN V, WOLFF MJ *et al.*: TGF- β 1 induces endothelial cell apoptosis by shifting VEGF activation of p38MAPK from the pro-survival p38 β to pro-apoptotic p38 α . *Mol Cancer Res* 2012; 10: 605-14.
23. GAO Y, MA L, LUO C-L *et al.*: IL-33 exerts neuroprotective effect in mice intracerebral hemorrhage model through suppressing inflammation / apoptotic / autophagic pathway. *Mol Neurobiol* 2017; 54: 3879-92.
24. ZHOU J, SUN X, ZHANG J, YANG Y, CHEN D, CAO J: IL-34 regulates IL-6 and IL-8 production in human lung fibroblasts via MAPK, PI3K-Akt, JAK and NF- κ B signaling pathways. *Int Immunopharmacol* 2018; 61: 119-25.
25. PILLINGER MH, DINSELL V, APSEL B *et al.*: Regulation of metalloproteinases and NF- κ B activation in rabbit synovial fibroblasts via E prostaglandins and Erk: contrasting effects of nabumetone and 6MNA. *Br J Pharmacol* 2004; 142: 973-82.
26. PELLETIER JP, FERNANDES JC, BRUNET J *et al.*: In vivo selective inhibition of mitogen-activated protein kinase kinase 1/2 in rabbit experimental osteoarthritis is associated with a reduction in the development of structural changes. *Arthritis Rheum* 2003; 48: 1582-93.