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Original Article

Wear particle-induced interleukin-34 expression contributed to osteoclastogenesis and involved in aseptic loosening

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Abstract: Purpose: This study explored the expression of interleukin-34 (IL-34) on the peri-implant membranes of patients with aseptic loosening and whether its expression is induced by wear particles, and moreover whether IL-34 could contribute to osteoclastogenesis. Methods: IL-34 was detected in the periprosthetic membranes of patients with aseptic loosening by real-time polymerase chain reaction (RT-PCR) assay and its expression was studied by qPCR and ELISA in human fibroblasts after stimulation by $TiAl_6V_4$ particles (TiPs), CoCrMo metal particles (CoPs), and treatment with inhibitors of intracellular pathways. Tartrate-resistant acid phosphatase (TRAP) staining was performed to determine the formation of osteoclasts after bone marrow macrophages treated with IL-34 or M-CSF in the presence of receptor activator of nuclear factor kappa-B ligand (RANKL). Results: IL-34 was expressed in peri-implant membranes of patients with aseptic loosening. The expression was stimulated by wear particles via the JNK and p44/42 MAPK signaling pathways in vitro. Furthermore, the administration of IL-34 and RANKL was able to increase osteoclastogenesis in vitro. Conclusions: Our results reveal that IL-34 is involved in the pathological process of particle-induced aseptic loosening and may be a potential therapeutic target for inhibiting osteoclastogenesis.

Keywords: Wear particles, IL-34, osteolysis, osteoclastogenesis, fibroblasts

Introduction

Aseptic loosening, which has been ascribed to periprosthetic osteolysis, is a serious long-term complication of total joint replacement. This disorder may lead to implant instability and surgical revision. The balance of bone formation and resorption is broken by the complex response to phagocytosis of wear debris, which leads to particle-induced osteolysis [1]. Although numerous studies have demonstrated that activated osteoclasts are responsible for aseptic loosening, the specific impacts of particles on osteoclastogenesis have not been clearly investigated. The formation of osteoclasts depends mainly on RANKL and on macrophage-colony stimulating factor (M-CSF), which is also known as CSF-1. It has been demonstrated that M-CSF binds to its receptor CSF1R on osteoclast progenitor cells in concert with RANKL binding to the RANK-receptor on the same cell, thus leading to the differentiation and activation of osteoclasts [2]. Thus, based on their role in osteoclastogenesis, it is clear that M-CSF and RANKL are involved in the pathogenesis of aseptic loosening. M-CSF can modulate inflammatory osteolysis and plays an important role in bone destruction [3]. The depletion of M-CSF attenuates the inflammatory response in rheumatoid arthritis animal models, whereas the administration of M-CSF aggravates inflammation and bone resorption [4, 5]. Hence, M-CSF inhibition has been considered a potential means of treating inflammatory osteolysis. However, it has been observed that mice deficient in CSF1R exhibit more severe phenotypic characteristics than *Csf1*^{op}/*Csf1*^{op} mice, whose *Csf1* gene was inactivated, which indicates that there is another ligand for CSF1R and that inhibiting M-CSF alone is not sufficient to block the effect through CSF1R [6]. Besides, due to the accompanying significant

Table 1. Clinical data on patients

Case	Gender	Age (years)	Years after implantation	Type of fixation	Specimen collection site	Type of prosthesis
CON 1	M	89	-	-	Acetabular	-
CON 2	M	57	-	-	Acetabular	-
CON 3	F	63	-	-	Acetabular	-
AP 1	F	68	9	Cementless	Cup	SP II
AP 2	F	58	7	Cementless	Cup	DURALOC
AP 3	F	64	5	Cementless	Cup	ABG
AP 4	M	67	5	Cement	Cup	Rejuvenat

CON, control; AP, aseptic loosening; F, female; M, male; Cup, acetabular cup. The AP 3 prosthesis was made from CoCrMo alloy, and the others were made from $TiAl_6V_4$ alloy. Control specimens were collected from the capsule samples, and the loose tissues were collected from the interface of the bone and the artificial femoral head. SP II, Waldemar Link GmbH & Co SP II implants. DURALOC, DePuy Synthes DURALOC implant. ABG, Stryker ABG implant. Rejuvenat, Stryker Rejuvenat implant.

complications of the anti-cytokine therapy [7], it is necessary to find another substitute cytokine.

Recently, a new cytokine interleukin-34 (IL-34) was identified as an alternative ligand for CSF1R [8]. Previous studies have confirmed the expression of IL-34 in gingival fibroblasts, osteoblasts and the synovial tissue of patients with arthritis, and it can promote osteoclastogenesis with the same efficiency as M-CSF [9-13]. As a downstream effector of TNF-α and IL-1β [10], IL-34 may contribute to inflammation and bone destruction in inflammatory bone diseases. However, the role of IL-34 in the pathogenesis of aseptic loosening has never been discussed yet. Accordingly, we hypothesized that IL-34 might play a key role in wear particle-induced osteoclastogenesis and be a part of the pathogenesis of aseptic loosening.

The aim of this study was to explore whether IL-34 is expressed by the periprosthetic membranes of patients with aseptic loosening and whether this expression is induced by wear particles. Next, we sought to analyze whether IL-34 can be substituted for M-CSF in RANKL-induced osteoclastogenesis in vitro, and we studied the mechanism by which IL-34 can support osteoclastogenesis.

Material and methods

Reagents

DMEM and α-MEM were purchased from Hyclone (Logan, UT, USA), and fetal bovine serum (FBS) was obtained from Gibco (Carlsbad, CA, USA). RIPA lysis buffer was purchased from Beyotimme (Nantong, China). JNK inhibitor and p44/42 MAPK inhibitor were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Specimens from patients

Periprosthetic interface membranes were obtained during revision operations of patients with aseptic loosening, after the absence of infection was confirmed. The control specimens were obtained from patients with femoral neck fracture during their primary total hip replacement. Clinical data on these patients are shown in **Table 1**.

Particle preparation

The $TiAl_6V_4$ and CoCrMo particles, which were kindly provided by Dr Zhenzhong Zhang (The College of Materials Science and Engineering of Nanjing University of Technology), had a mean particle diameter of 51.7 nm. Particles were suspended in phosphate buffered saline (PBS, pH 7.2-7.4) at a concentration of 50 mg/ml as stock solutions. The particles were autoclaved for 15 min at 121°C and 15 psi for sterilization. For the in vitro experiments, particles were further diluted in cell culture medium to attain concentrations ranging from 10 to 500 μg/ml and ultrasonicated for 10 min before exposing them to cells. All particles were free of endotoxin, as determined using a quantitative Limulus Amebocyte Lysate (LAL) Assay (Charles River, Grand Island, UK) at a detection level of 0.25% EU/ml.

Fibroblast cultures

Synovial fibroblasts (SF) were isolated from specimens of patients with femoral neck fractures without any evidence of synovial reaction.

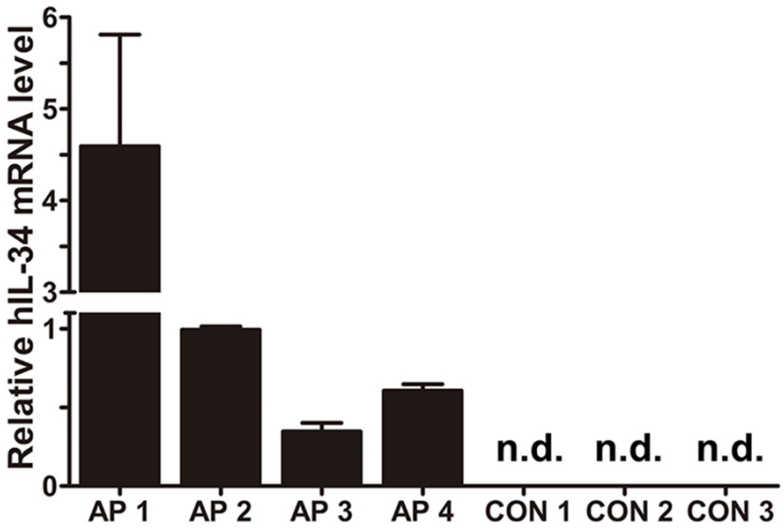

Figure 1. Expression of hIL-34 mRNA in periprosthetic membrane of patients with aseptic loosening. Relative levels of hIL-34 mRNA measured by real-time PCR in interface membranes of patients with aseptic loosening. CON, control; AP, aseptic loosening; n.d., not detected. Data are represented as the means ± S.D. for each group.

Written consent was received before the isolation of fibroblasts. According to conventional protocols, cells were obtained by enzymatic digestion and cultured in DMEM medium supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The fibroblasts were then maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For in vitro experiments, cells were cultured in the absence (controls) or presence of wear particles with different concentrations (0, 50, 100, 500 μg/ml) for 24 h. For time-course experiments, cells were incubated with particles (100 μg/ml) for different time periods (1 h, 3 h, 6 h, 12 h, 24 h).

RNA isolation and quantitative polymerase chain reaction (qPCR) assay

Total RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY, USA), and 1 μg total RNA was used for first strand cDNA synthesis using the PrimeScript RT Master Mix kit (TaKaRa Bio, Shiga, Japan). Real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol. Primers for β-actin were used as internal controls. The primer sequences were as follows: Forward: 5'-GGACACACTTCTGGGGACA-3' and reverse: 5'-CCAAAGCCACGTCAAGTAGG-3' for mouse IL-34; and forward: 5'-GACCTCTATGCCAACACAGTGC-3' and reverse: 5'-GTACTCCTGCTTGCTGATCCAC-3' for mouse β-actin.

ELISA assay for IL-34

The cytokines IL-34 were quantified using specific ELISA kits (Senbeijia Biotech, Nanjing, China). All processes were conducted according to the manufacturer's instructions.

In vitro osteoclastogenesis assay

Bone marrow macrophages were isolated from the whole bone marrow of 6-week-old C57BL/J6 mice as previously described [14]. Briefly, cells were isolated from the femoral bone marrow and cultured in α-MEM medium supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37°C and 5% CO_2 until reaching 90% confluence. The cells were then seeded into 24-well plates in the presence of 30 ng/mL IL-34 (R&D Systems, Minneapolis, MN) and 20 ng/ml RANKL (R&D Systems, Minneapolis, MN) or 30 ng/mL M-CSF (R&D Systems, Minneapolis, MN) and 20 ng/ml RANKL. The culture media were replaced every 2 days until mature osteoclasts were formed. Then, the cells were analyzed for TRAP activity using a commercial kit (Jiancheng Biotech, Nanjing, China). TRAP-positive cells with more than three nuclei were considered osteoclasts and counted under a microscope.

Western blotting

Cells prepared for western blotting were lysed in RIPA lysis buffer including a cocktail of protein inhibitors on ice for 30 min. The lysates were centrifuged at 12,000 g for 10 min at 4°C; then, the supernatants were collected, and the protein concentrations were determined using a BCA protein assay kit (Biocolor Bioscience and Technology Co., Shanghai, China). Proteins (30 μg of each group) were run on 10% SDS-PAGE before being transferred to polyvinylidene fluoride membrane (Pall Co., East Hills, NY, USA), then incubated with the following primary antibodies: anti-Phospho-JNK (4668, 1:1000,

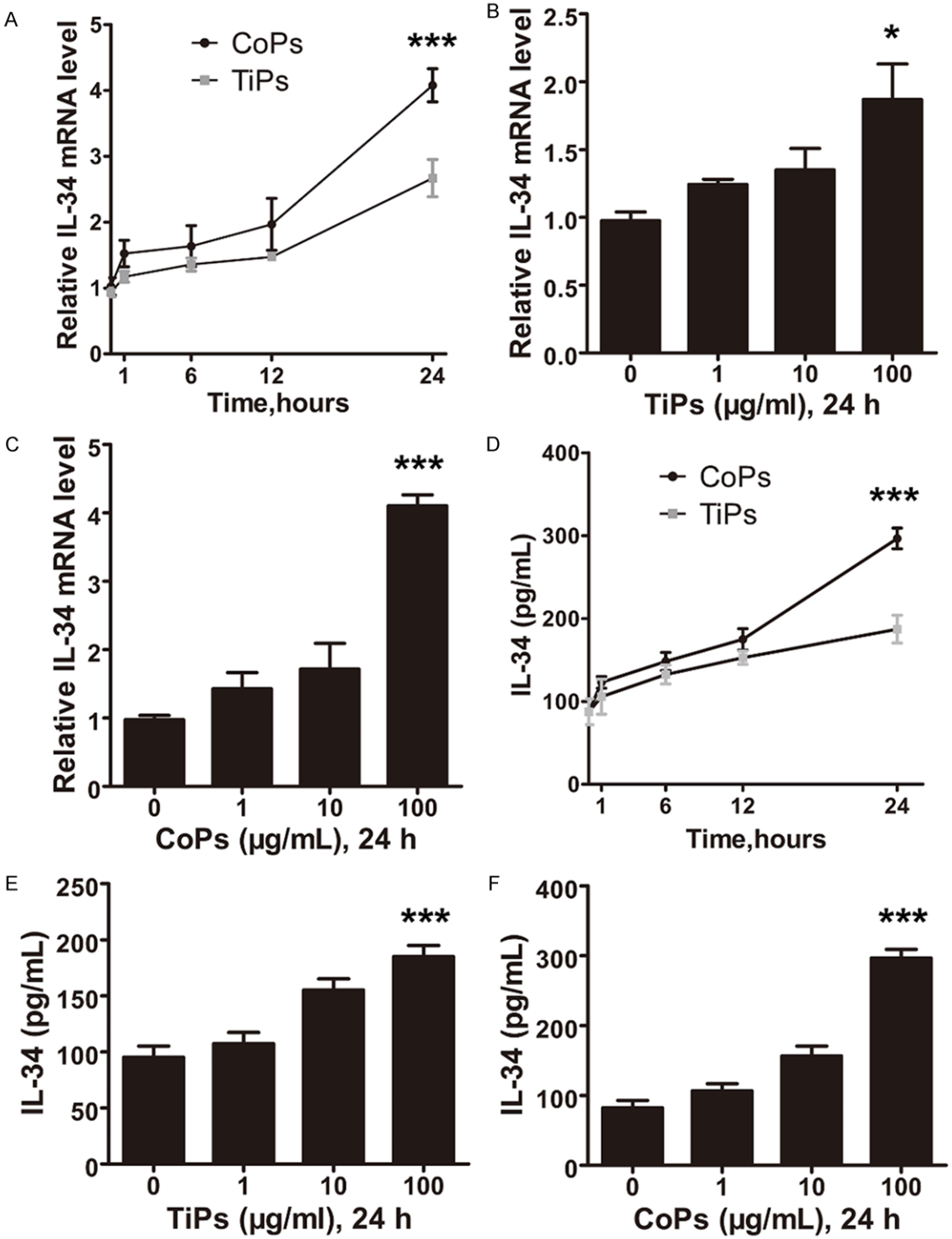

Figure 2. Upregulation of IL-34 expression by wear particles in human fibroblasts. Real-time PCR performed after fibroblasts were treated with (A) particles (100 μg/ml) for different time periods (1, 3, 6, 12, and 24 h) or with increased doses of (B) TiPs (0, 50, 100, and 500 μg/ml) or (C) CoPs (0, 50, 100, and 500 μg/ml) for 24 h. ELISA analysis performed after fibroblasts were treated with (D) particles (100 μg/ml) for different time periods (1, 3, 6, 12, and 24 h) or with increased doses of (E) TiPs (0, 50, 100, and 500 μg/ml) or (F) CoPs (0, 50, 100, and 500 μg/ml) for 24 h. Data are represented as the means ± S.D. from three independent experiments. *P < 0.05, ***P < 0.001 compared with the control.

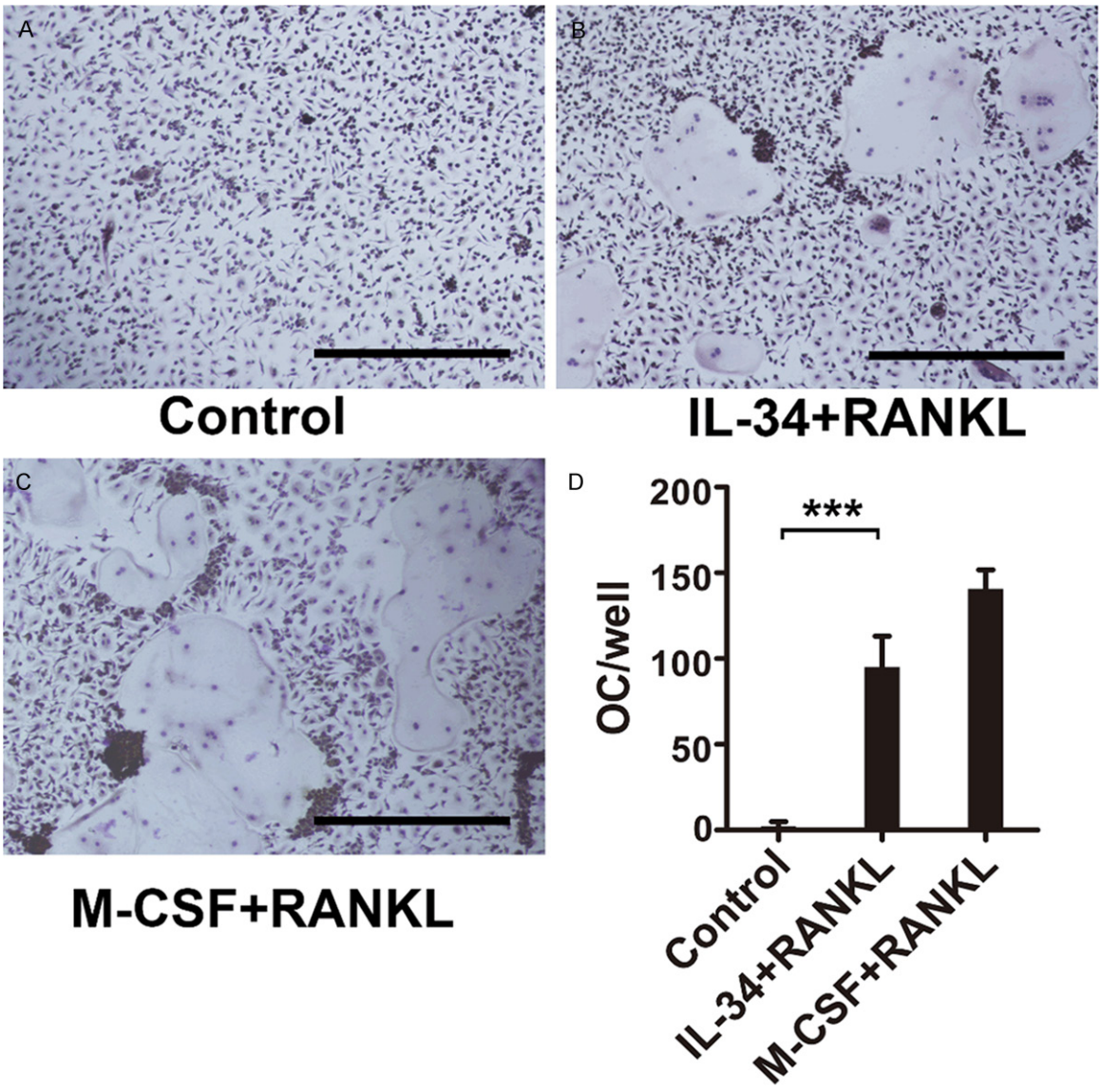

Figure 3. IL-34 upregulated RANKL-induced osteoclastogenesis. Bone marrow macrophages (BMMs) were cocultured with (A) RANKL (20 ng/ml), (B) RANKL (20 ng/ml) & IL-34 (30 ng/mL), (C) RANKL (20 ng/ml) & M-CSF (30 ng/mL) for 8-10 days. Scale bar, 500 μm. (D) The number of TRAP-positive osteoclasts was analyzed. Data are represented as the means ± S.D. from three independent experiments. ***P < 0.001 compared with the control.

Cell Signaling Technology), anti-Phospho-ERK1/2 (4370, 1:1000, Cell Signaling Technology) and the following secondary antibodies: horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (7074, 1:2000, Cell Signaling Technology). The protein bands were then detected, and the band density was analyzed using ImageJ 1.41 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The date was expressed as the mean values ± standard deviation (S.D.). Statistical analysis was performed by one-way ANOVA test. The results were considered to show a significant difference at p values < 0.05.

Results

Expression of IL-34 in the peri-implant membrane of patients with aseptic loosening

Previous studies have reported that IL-34 can promote osteoclastogenesis [15]. In this study, we were interested in whether IL-34 was involved in aseptic loosening. Specimens of periprosthetic membranes from patients with

aseptic loosening were obtained for real-time PCR. The data showed that IL-34 mRNA was expressed in periprosthetic membranes, but it could not be detected in the control tissue (**Figure 1**). The results suggested that IL-34 was differentially expressed by periprosthetic membranes and control tissues, which might be involved in the pathological process of aseptic loosening.

Upregulation of IL-34 by wear particles in human fibroblasts

As fibroblasts compose nearly 70% of the cells in the peri-implant membrane and respond directly to wear particles [16], we next investigated whether wear particles could induce IL-34 expression in human fibroblasts. RT-PCR analyses showed that both TiPs and CoPs enhanced IL-34 mRNA expression in a dose- and time-dependent manner in fibroblasts (**Figure 2A-C**). To further confirm that IL-34 can be induced by wear particles, ELISA analyses were also performed. The expression of IL-34 was induced in a dose-dependent manner by TiPs with a 2-fold change at 100 μg/ml and by CoPs with a 3-fold change at 100 μg/ml (**Figure 2D**, **2E**). In addition, the expression of IL-34 protein was markedly elevated by wear debris in a time-dependent manner (**Figure 2F**).

Upregulation of osteoclastogenesis by IL-34

To further determine the role of IL-34 in osteoclastogenesis, the combined effect of IL-34 and RANKL on the formation of osteoclasts was assessed in vitro using BMMs. As shown in **Figure 3A-C**, IL-34 cotreatment with RANKL efficiently increased the formation of osteoclasts. The quantification of TRAP-positive osteoclasts revealed that the effect of IL-34 was comparable to that of M-CSF at the same concentration (**Figure 3D**). These data indicated that IL-34 upregulated RANKL-induced osteoclastogenesis.

JNK and p44/42 MAPK mediated wear particle-induced IL-34 expression

It has been reported that mitogen-activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK) and p44/42 MAPK (also known as ERK-1/2) are important intracellular mediators of inflammatory cytokine-mediated IL-34 expression [17]. To determine the mechanism through which wear particles induce IL-34 expression, the levels of phosphorylated JNK and ERK 1/2 in wear debris-stimulated cells were examined by western blot. **Figure 4A-D** showed that although wear debris upregulated the expression of IL-34, it also induced the phosphorylation of JNK and ERK 1/2. More importantly, the increased IL-34 expression was rescued by both JNK inhibitor and p44/42 MAPK inhibitor (**Figure 4E**, **4F**). The results suggested that both the JNK and p44/42 MAPK pathways were involved in wear particle-induced IL-34 expression in fibroblasts.

Discussion

Total joint arthroplasty is commonly used to treat end-stage joint diseases. However, periprosthetic bone resorption and subsequent aseptic loosening induced by wear particles remain the major causes of failed arthroplasty. Although numerous efforts have been made to improve the biomaterials and the implant design as well as the surgical techniques, it has remained impossible to eliminate particle generation from bearing surfaces [1]. Hence, the inhibition of osteoclast formation is considered a potential target for therapy.

IL-34 is a newly discovered cytokine that binds to CSF1R and has similar functions to M-CSF in promoting monocyte viability and osteoclast formation [8, 9]. The critical role of IL-34 in osteoclastogenesis has been proved both in giant cell tumours of bone (GCTs) and rheumatoid arthritis. But it still remains unknown whether IL-34 is involved in the pathogenesis of aseptic loosening. In this study, we observe for the first time that IL-34 is highly expressed in the periprosthetic membranes of patients with aseptic loosening, which could not be detected in the control tissue. However, there are still some limitations of this study such as the small number of samples. Protein detection of IL-34 in the tissues along with cellular localization in tissue should have been performed in the following research to further confirm the relationship between IL-34 and aseptic loosening.

It has been reported that IL-34 is mostly expressed in osteoblasts and fibroblasts, and its expression can be mediated by the pro-inflammatory cytokines TNF-α and IL-1β via JNK and NF-κB signaling [13, 15]. Interestingly, in this study, we show for the first time that the

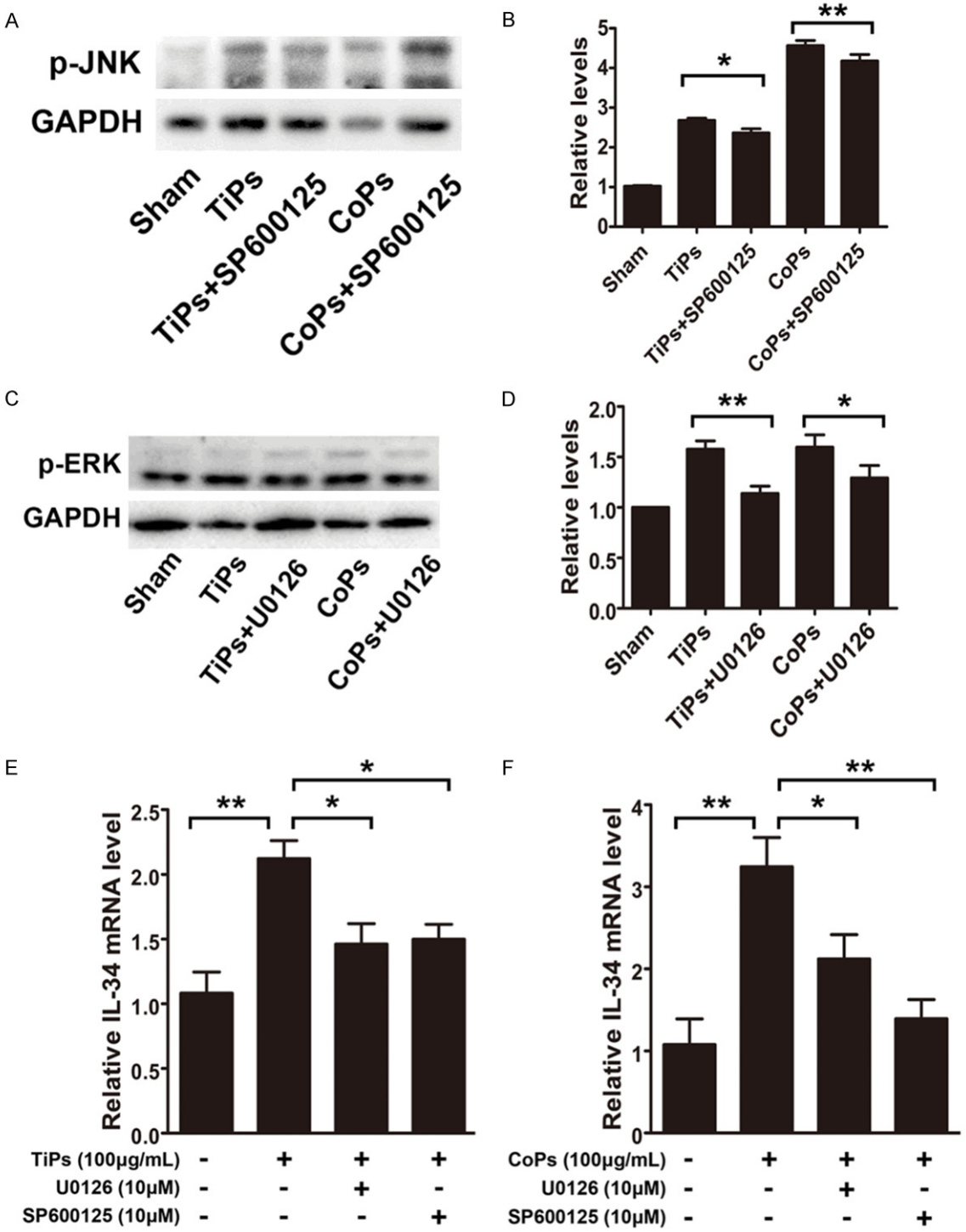

Figure 4. JNK and p44/42 MAPK pathways mediated wear particle-induced IL-34 expression. (A) Western blots performed after fibroblast cells were treated with JNK inhibitor (SP600125, 10 μM) before stimulation with particles (100 μg/ml) for 24 h. (B) The density of western blot bands shown in (A) was quantified using ImageJ software. (C) Western blots performed after fibroblast cells were treated with ERK 1/2 inhibitor (U0126, 10 μM) before stimulation with particles (100 μg/ml) for 24 h. (D) The density of western blot bands shown in (C) was quantified using ImageJ software. The expression of IL-34 in fibroblasts incubated with U0126 (10 μM) or SP600125 (10 μM) before stimulation with (E) TiPs (100 μg/ml) or (F) CoPs (100 μg/ml) for 24 h. Data are represented as the means ± S.D. from three independent experiments. *P < 0.05, **P < 0.01.

expression of IL-34 in fibroblasts can be upregulated by wear particles. TiPs and CoPs have been proved to activate MAPK signaling pathways [18]. We show in this article that the activation of these pathways is required for the wear particle-induced expression of IL-34. However, we have to point out that this study has examined only the expression of IL-34 induced by wear particles in human Synovial fibroblasts from patients with femoral neck fractures, the same experiments should be carried out on cells from loosening patients in the future.

Previous studies showed that IL-34 not only plays a critical role in regulating osteoclastogenesis but also improves the bone resorbing activity of osteoclasts both *in vitro* and *in vivo* [15]. We also show in this paper that the administration of IL-34 and RANKL increases the number of multinucleated osteoclasts. Therefore, together with the previous conclusions, our data suggest that IL-34 may play a predominant role in particle-induced osteoclastogenesis.

In conclusion, we demonstrated in this study for the first time that the newly discovered cytokine IL-34 is expressed by the periprosthetic membranes of patients with aseptic loosening and that the implant-derived wear particles, TiPs and CoPs, enhance IL-34 expression in fibroblasts via JNK and p44/42 MAPK signaling pathways. Furthermore, the administration of IL-34 upregulated RANKL-induced osteoclastogenesis. Our findings suggest that IL-34 may play a role in particle-induced osteolysis, and further explorations are needed to determine whether it is a potential therapeutic target for aseptic loosening.

Acknowledgements

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Disclosure of conflict of interest

None.

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