

# Interleukin-18-induced inflammatory responses in synoviocytes and chondrocytes from osteoarthritic patients

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**Abstract.** The major pathological changes of osteoarthritis (OA) include cartilage degeneration and synovial inflammation. Previous studies confirmed that interleukin-1 (IL-1) stimulates the secretion of multiple inflammatory factors in synoviocytes and chondrocytes. IL-18 is a member of the IL-1 superfamily. In this study, the pro-inflammatory effects of IL-18 on synoviocytes and chondrocytes in patients with OA were investigated. Knee synovial membrane and cartilage samples were obtained from OA patients, then primary cells were cultured. Synoviocytes and primary chondrocytes at different generations (primary, secondary and tertiary), were stimulated with IL-18, then inflammatory marker levels, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and cyclooxygenase-2 (COX-2), were measured using reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay. IL-1 receptor antagonist (IL-1Ra) was applied to interfere with the IL-18 stimulation of chondrocytes, and then the COX-2 expression in chondrocytes and the PGE<sub>2</sub> levels in the medium were measured. The expression of IL-18 receptor  $\alpha$  (IL-18R $\alpha$ ) and IL-18 receptor  $\beta$  (IL-18R $\beta$ ) in synoviocytes and chondrocytes was assessed, using RT-PCR. Our results showed that IL-18 stimulated the COX-2 and TNF- $\alpha$  expressions in primary synoviocytes, while increasing PGE<sub>2</sub> and TNF- $\alpha$  levels in the supernatant ( $P<0.05$ ) of the culture medium in primary synoviocytes. IL-18 also induced high PGE<sub>2</sub> level production in second-generation synoviocytes ( $P<0.05$ ). Moreover, IL-18 upregulated COX-2 and TNF- $\alpha$  mRNA in chondrocytes, while promoting PGE<sub>2</sub> and TNF- $\alpha$  ( $P<0.05$ ) secretions in a dose-dependent manner. The induced effects were not attenuated by the addition of

IL-1Ra ( $P<0.05$ ). IL-18R $\alpha$  was expressed in the chondrocytes and synoviocytes of 4/8 patients, while IL-18R $\beta$  was expressed in the chondrocytes of 4/8 patients and in the synoviocytes of 2/8 patients. We conclude that IL-18 induces inflammatory responses in synoviocytes and chondrocytes and that this effect was correlated with, although not entirely dependent on, IL-1 $\beta$ .

## Introduction

The major pathological changes in osteoarthritis (OA) include synovial proliferation and cartilage degeneration that involve several inflammatory factors. Cartilage damage is one of the primary pathological characteristics of OA. Cartilage fragments enter the synovial fluid, irritating the synovial membrane and leading to synovitis. The inflamed synovial membrane subsequently releases inflammatory mediators, which further degenerate cartilage, thereby forming a vicious circle. Thus, synovitis is the main cause for knee cartilage damage and chronic degeneration in OA (1). Elevated expression of interleukin-1 (IL-1), a crucial pro-inflammatory cytokine, is often observed in the synovial membrane in patients with OA. The imbalance between the IL-1 receptor (IL-1R) and the IL-1 receptor antagonist (IL-1Ra) is correlated with the severity of OA (2). IL-1 $\beta$  has the potential to stimulate chondrocytes to produce several inflammatory factors, including nitric oxide (NO), inducible nitric oxide synthase (iNOS), prostaglandin-E<sub>2</sub> (PGE<sub>2</sub>) or matrix metalloproteases (MMPs) (3), leading to the inflammatory response of chondrocytes as well as the degradation of the cartilage matrix (4-6).

IL-18 has a structure similar to that of IL-1 and is therefore acknowledged as a member of the IL-1 super family (7). Initially, T-cell-produced IL-18 was found to be able to induce interferon- $\gamma$  (IFN- $\gamma$ ) production, and was subsequently named IFN- $\gamma$ -inducing factor. IL-18 is derived from an inactive precursor. The mature and functional 18 kDa IL-18 protein molecule is generated through enzymatic cleavage (7,8). IL-18 has multiple biological activities, while being crucial for the pathogenesis of rheumatoid arthritis, especially synovitis. IL-18 can be detected in the synovial fluid in the knees in patients with rheumatic arthritis (RA). IL-18 receptor (IL-18R) is also expressed in the synovial membrane in RA-affected joints (9-11). A continuous low-level IL-18 expression, tumor

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necrosis factor- $\alpha$  (TNF- $\alpha$ ), granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-1 $\beta$  were reported in fibroblast-like synoviocytes (FLS) in the synovial membrane of RA and OA patients as well as in the synovial fluid of spondyloarthropathy (SpA) patients (12). A study analyzing the impact of high-level IL-18 on synoviocytes and peripheral blood monocytes in RA patients demonstrated that IL-18 had a more prominent pro-inflammatory effect on peripheral blood monocytes (12).

The response of synoviocytes in OA patients to IL-18 remains unclear. In OA, destruction of articular cartilage mainly results from the imbalance between the synthesis and the degradation of cartilage. IL-1 and TNF- $\alpha$  are essential in stimulating chondrocyte catabolism (13). IL-1 triggers PGE<sub>2</sub> production as well as the expression of COX-2 and iNOS (14). However, IL-18 also induces the expression of genes, such as iNOS, COX-2 and IL-6, leading to the degeneration and inflammation of chondrocytes (14). IL-18 inhibited the *in vitro* proteoglycan production in the patella of young rats in the presence of IL-1 (15). Nevertheless, the effect of IL-18 on OA chondrocytes and the role of IL-1 $\beta$  and IL-18 in these processes remains unclear.

In this study, we investigated the effect of IL-18 on the synoviocytes and chondrocytes of patients with OA. We also detected the expression of IL-18 receptors in the two types of cells, and examined the relationship between IL-18 and IL-1 in the pro-inflammatory process.

## Materials and methods

**Cell culture.** Synoviocytes and chondrocytes were collected from 8 patients with OA (5 females and 3 males; average age, 62.3 years). The patients provided informed consent prior to sample collection. This study was monitored and approved by the Medical Ethics Committee of the Nanfang Hospital.

Synoviocytes were cultured *in vitro* in accordance with the methods described by Zimmermann *et al* (16). The synovial tissue was washed twice using sterile phosphate-buffered saline (PBS). The sample was cut into sections in PBS and digested with 0.1% trypsin at 37°C for 30 min. The tissue was then placed in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% type I collagenase (Sigma, St. Louis, MO, USA) and 10% fetal bovine serum (FBS) for 2 h. After filtration and centrifugation, the cells were collected and cultured in medium with penicillin and streptomycin, for 7 days at 37°C in a 5% carbon dioxide environment and transferred to a 6-well plate. Synoviocytes were classified as described in the study by Franke *et al* (17). Identical numbers of primary, secondary and tertiary cells were starved in DMEM for 24 h. The cells were then transferred and cultured in fresh medium containing IL-18 (10 mM/ml) for an additional 24 h. The cultured cells and supernatants were harvested for additional analysis.

Articular cartilage collected from the femur condyle and tibial plateau in the surgeries of total knee joint arthroplasty was cut into 2-3 mm<sup>3</sup>-sized pieces and then washed with DMEM three times. The sample was digested in 10% trypsin at 37°C for 15 min. The digested tissue was then collected and mixed with 0.2% type II collagenase (Gibco, Carlsbad, CA, USA) and 0.1% hyaluronidase (Sigma) at 37°C in a shaker

for 6 h. Cells were washed with DMEM to remove proteases and filtered through a sterile cell strainer. Primary cells were suspended and cultured in DMEM supplemented with 10% FBS. The expression of type II collagen was then analyzed. Selected primary cells were seeded onto 6-well plates and cultured in medium with different concentrations of IL-18 (0, 50, 100 and 200 ng/ml) for 48 h.

**Immunohistochemistry.** Sterilized coverslips (8x8 mm) were placed onto 6-well plates. Cells were seeded and cultured for 24 h, allowing cells to attach to coverslips. After incubation, coverslips were washed three times with PBS, for 3 min/wash, and treated with 3% hydrogen peroxide and normal serum to block intrinsic peroxidases and proteins possibly generating non-specific cross reactions. After the removal of residual serum, coverslips were incubated with rabbit polyclonal antibodies against type I and type II collagen (Boster, China) in a wet box at 4°C overnight. The following day, polyclonal goat anti-rabbit secondary antibodies (Boster) were applied to the coverslips at 37°C for 30 min. After washing, the coverslips were incubated with horseradish peroxidase substrates and diaminobenzidine (DAB) and then examined under an inverted microscope.

**Cytokine detection.** Supernatants from cultured cells were collected and centrifuged in 1.5 ml microcentrifuge tubes at 3,000 x g for 10 min and then stored in a -80°C freezer. The concentration of cytokines in the samples was assayed simultaneously. Standard curves were plotted following the manufacturer's instructions (PGE<sub>2</sub>; Usen Life Science, Inc., Wuhan, China) (TNF- $\alpha$ ; Cusabio Biotech Co., Ltd., Wuhan, China) and the PGE<sub>2</sub> and TNF- $\alpha$  concentrations in the samples were then determined using enzyme-linked immunosorbent assay (ELISA).

**RT-PCR analysis.** Total-mRNA was extracted from synoviocytes and chondrocytes using TRIzol (Invitrogen, Carlsbad, CA, USA). The concentration mRNA concentration was determined using a NanoDrop Spectrophotometer (Wilmington, NC, USA). Reverse transcription was performed using a reverse transcription kit (Toyobo, Japan). The reaction conditions were as follows: 30°C for 10 min, 42°C for 20 min and 95°C for 5 min. The ΔΔCt method was applied for the quantitative analysis (18). Primer sequences used in this study were: aggrecan, forward: 5'-TGA GTC CTC AAG CCT CCT GT-3' and reverse: 5'-GTC CCT CTG TCT CCT TGC AG-3'; TNF- $\alpha$ , forward: 5'-TCT CTT CAA GGG ACA AGG CTG-3' and reverse: 5'-ATA GCA AAT CGG CTG ACG GT-3'; COX-2, forward: 5'-TGA ACC CAC TCC AAA CAC A-3' and reverse: 5'-CAG CAA ACC GTA GAT GCT CA-3'. PCR products were analyzed using agarose gel electrophoresis. The levels of IL-18R $\alpha$  and IL-18R $\beta$  expressions were determined as described in the study by Moller *et al* (19).

**Statistical analysis.** Statistical analysis was performed using the SPSS 13.0 software. Cytokine concentrations in the supernatant from cultured cells were shown as the mean ± SE. P<0.05 was considered to indicate a statistically significant difference. Two independent-sample t-tests were conducted to analyze the effects of IL-18 on synoviocytes. The effects of

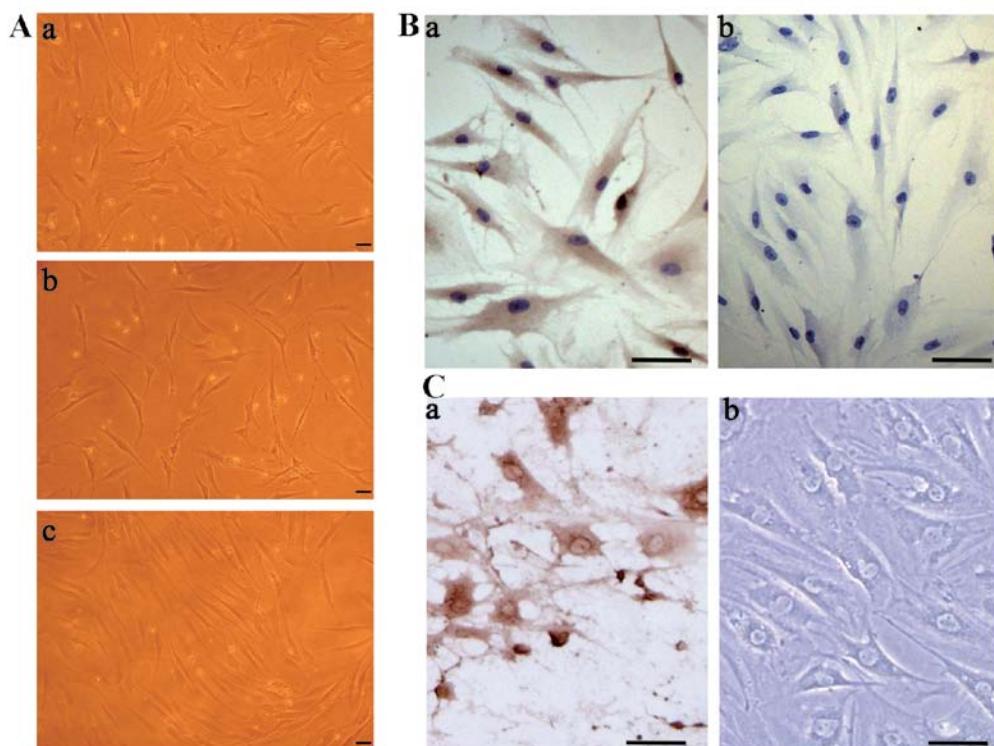


Figure 1. Morphological observation and characterization of synoviocytes and chondrocytes. (Aa-c) Most of the cells in the 1st-3rd generation synoviocytes derived from OA patients were spindle-shaped. Synoviocytes were stained with (Ba) type I collagen antibody, or with (Bb) rabbit serum as a negative control. Chondrocytes were stained with (Ca) type II collagen antibody, or with (Cb) rabbit serum as a negative control. Bars, 100  $\mu$ m.

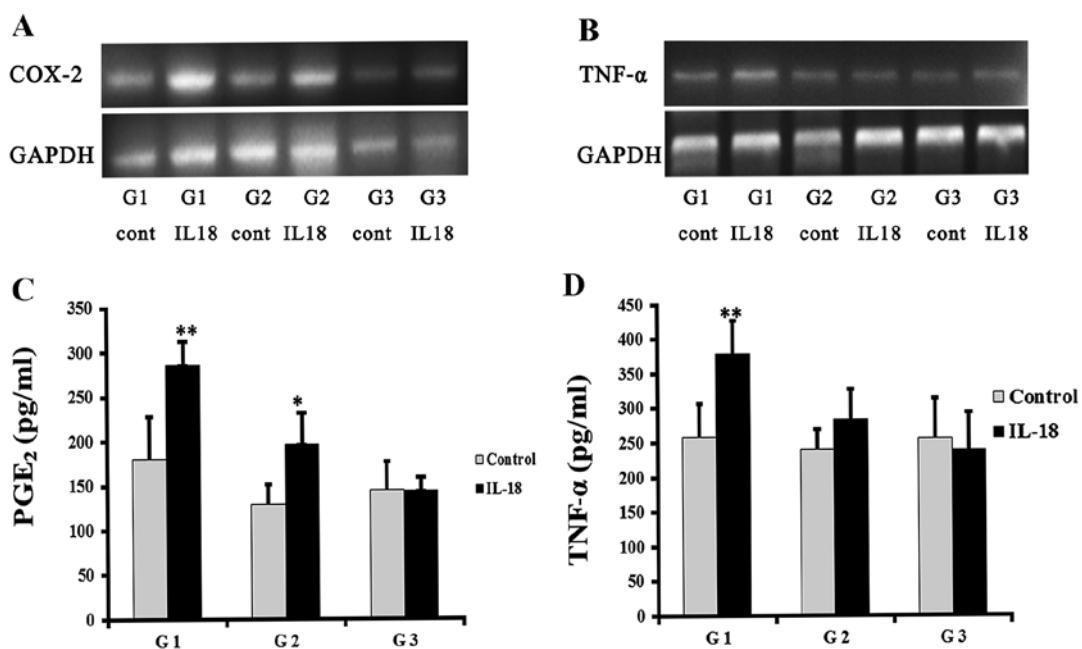


Figure 2. Expression of TNF- $\alpha$  and PGE<sub>2</sub> in synoviocytes is shown. (A) COX-2 and (B) TNF- $\alpha$  mRNA expression in synoviocytes was determined by RT-PCR. GAPDH was used as an internal control. The levels of (C) PGE<sub>2</sub> and (D) TNF- $\alpha$  in the supernatant were measured using ELISA. \*P<0.05, compared to the control group; \*\*P<0.01, compared to the control group. Cont, control group; IL-18, IL-18-treated group; G1, the first generation synoviocytes.

different IL-18 concentrations on synoviocytes were analyzed using one-way analysis of variance (ANOVA). When statistically significant difference was obtained, multiple comparisons were carried out, using the LSD test or Dunnett's T3 test (unequal variances).

## Results

*Characterization and morphology of synoviocytes and chondrocytes.* There were 2 morphological forms of primary synoviocytes (G1-synoviocytes): spindle-shaped fibroblast-like

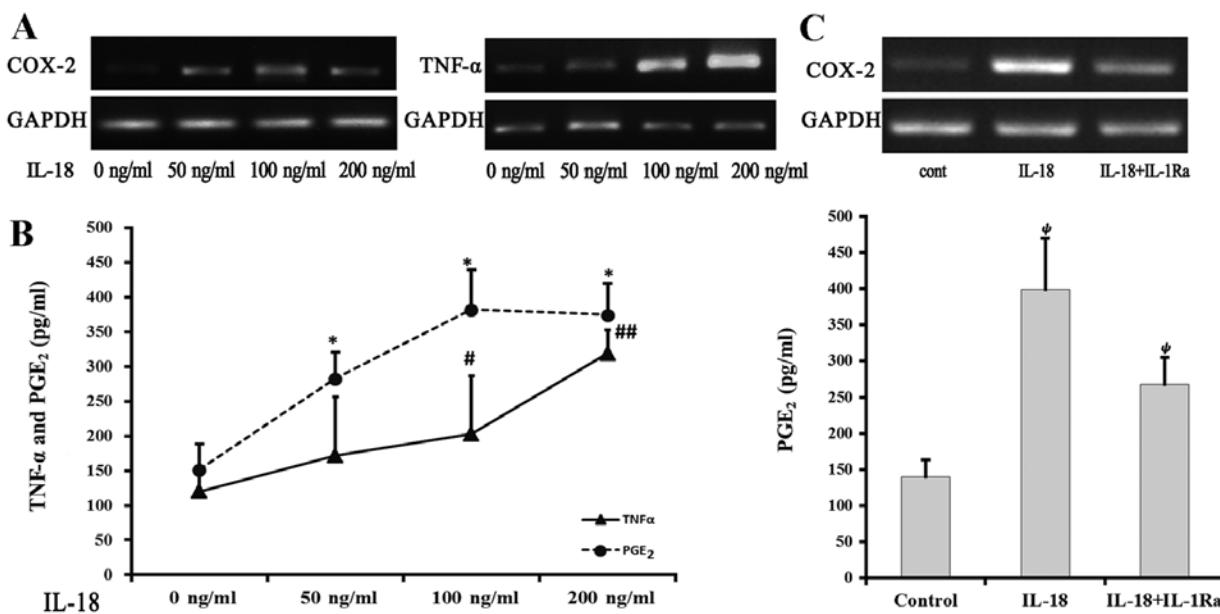


Figure 3. Effect of IL-18 on chondrocytes is shown. Chondrocytes were cultured in media containing different concentrations of IL-18 (0, 50, 100 and 200 ng/ml). (A) COX-2 and TNF- $\alpha$  expression was assessed using RT-PCR. GAPDH was used as an internal control. (B) The level of PGE<sub>2</sub> in the supernatant was determined using ELISA. (C) The levels of COX-2 mRNA and PGE<sub>2</sub>, after being treated with IL-1Ra, were measured. \*P<0.01, compared to the 0 ng/ml group; #P<0.05, compared to the 0 ng/ml group; \*\*P<0.01, compared to the 0 ng/ml group. \*\*P<0.01, compared to the control group.

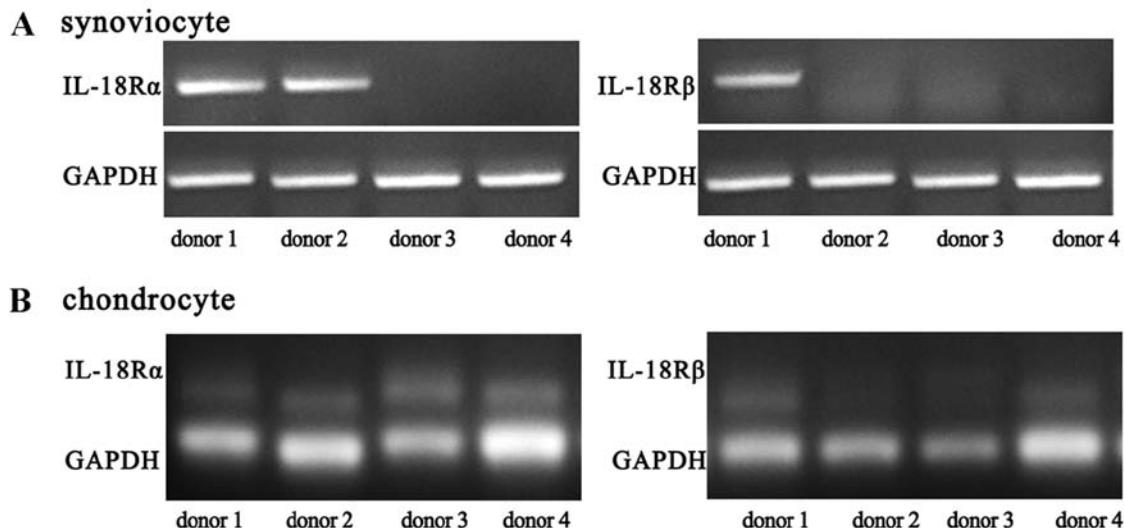


Figure 4. Expression of IL-18R in chondrocytes and synoviocytes is shown. The expression of IL-18R $\alpha$  and IL-18R $\beta$  mRNA in (A) synoviocytes and (B) chondrocytes from 4 donors was assessed by RT-PCR. GAPDH was used as the internal control.

synoviocytes and polygonal macrophage-like synoviocytes (Fig. 1Aa). The number of polygonal macrophage-like synoviocytes decreased in the secondary generation of synoviocytes (G2-synoviocytes) (Fig. 1Ab). The majority of cells in the third generation (G3-synoviocytes) were spindle-shaped, i.e., they were fibroblast-like synoviocytes (Fig. 1Ac). The numbers of the macrophage-like cells decreased with increasing passage numbers. Type I collagen expression in synoviocytes was detected by immunohistochemistry (Fig. 1B). The cytosol of the synoviocytes was stained brown, indicating a high level of type I collagen expression. Type I collagen expression in cells with the two forms was similar. Chondrocytes were stained with an antibody against type II collagen, while

reticular type II collagen was visible in the intracellular space (Fig. 1Ca).

*IL-18-induced inflammatory response in synoviocytes.* G1- and G2-synoviocytes demonstrated an elevated COX-2 expression subsequent to IL-18 induction, while G3-synoviocytes showed no significant change (Fig. 2A). All the synoviocytes demonstrated *in vitro* PGE<sub>2</sub> production. G1- and G2-synoviocytes in the IL-18-treated groups had markedly higher levels of PGE<sub>2</sub> expression, compared to the control group (286.21±26.24 vs. 180.31±47.69 pg/ml, 196.60±34.79 vs. 128.72±22.37 pg/ml; P=0.005 and P=0.011, respectively) (Fig. 2C). Similarly, IL-18 induced mRNA expression in G1-synoviocytes, although not

in G2-synoviocytes (Fig. 2B). The results were consistent with the protein assay results. There was a statistically significant difference in the TNF- $\alpha$  concentration in the G1-synoviocyte supernatant in the IL-18-treated and the control group ( $379.20 \pm 47.60$  vs.  $257.90 \pm 48.76$  pg/ml;  $P=0.007$ ) (Fig. 2D). However, G3-synoviocytes were less sensitive to IL-18 induction, whereas the TNF- $\alpha$  and PGE<sub>2</sub> levels were not increased ( $P>0.05$ ).

**Response of the chondrocytes to IL-18 stimulation.** An increase was observed in COX-2 and TNF- $\alpha$  mRNA expression with increasing concentrations of IL-18 (Fig. 3A). PGE<sub>2</sub> levels in the 50, 100 and 200 ng/ml IL-18 groups were  $282.83 \pm 52.16$ ,  $381.85 \pm 84.15$  and  $374.51 \pm 34.14$  pg/ml, respectively, which were significantly higher compared to the control group ( $151.72 \pm 23.76$  pg/ml;  $P=0.003$ ,  $P<0.001$  and  $P<0.001$ , respectively). In addition, the 100 and 200 ng/ml IL-18 groups showed significantly higher TNF- $\alpha$  levels compared to the control group ( $202.48 \pm 57.46$  and  $318.63 \pm 45.23$  pg/ml;  $P=0.002$  and  $P<0.001$ , respectively) (Fig. 3B). The induction of PGE<sub>2</sub> and TNF- $\alpha$  by IL-18 was dose-dependent.

To elucidate the correlation between IL-18 and IL-1 $\beta$ -induced inflammatory responses in chondrocytes, chondrocytes in the IL-18 group were treated with 100 ng/ml IL-18, while those in the IL-1Ra group were treated with 100 ng/ml IL-18 and IL-1Ra. After 24-h incubation, the IL-18 group demonstrated a significantly higher COX-2 mRNA expression, compared to the other 2 groups (Fig. 3C). The IL-18 group also had a markedly higher level of PGE<sub>2</sub>, compared to the control group ( $P=0.003$ ). The PGE<sub>2</sub> level decreased following addition of the inhibitor, although this level was still higher when compared to the control group ( $P=0.002$ ).

**Expression of IL-18R.** The primary synoviocytes of 8 patients were obtained and analyzed. The synoviocytes of two of the 8 donors had detectable IL-18R $\alpha$  expression, and those of one donor had IL-18R $\beta$  expression (Fig. 4A). After passage, no receptor expression was detected. Chondrocytes from 4 donors expressed IL-18R $\alpha$ , whereas those from 2 donors expressed IL-18R $\beta$  (Fig. 4B).

## Discussion

The present study investigated the impact of IL-18 on synoviocytes and chondrocytes. The results showed that high levels of IL-18 induced the expression of inflammatory factors, such as TNF- $\alpha$ , PGE<sub>2</sub> and COX2 in primary synoviocytes. With the increase of passage numbers, fibroblast-like cells became more dominant, IL-18R expression decreased, while the synoviocytes became less responsive to IL-18 stimulation. IL-18 had the potential to induce the expression of PGE<sub>2</sub>, TNF- $\alpha$  and COX-2 in human OA chondrocytes, which express IL-18R $\alpha$  and IL-18R $\beta$ .

IL-18 induced inflammatory responses in OA synoviocytes. The knee synovial membrane is composed of two types of cells: the macrophage-like synoviocytes known as A cells, and the fibroblast-like synoviocytes known as B cells. Goto *et al* (21) have differentiated and purified these two types of cells, using cell cloning (20). Type A cells proliferate slowly, are less active and lose activity after 10 days of culture (22).

Primary synoviocytes from OA patients contained two cell types expressing IL-18. They produced high TNF- $\alpha$ , PGE<sub>2</sub> and COX-2 levels when induced by IL-18. Such a pro-inflammatory response is also noted in RA patient-derived synoviocytes. In a mouse model of collagen-induced arthritis (CIA), mature IL-18 induced synovial proliferation, synovial inflammation and cartilage damage (23). It has even been attempted to neutralize intrinsic IL-18 in RA patients with a view to treat the disease (24). Synoviocytes from OA patients express higher levels of IL-18 than those from healthy individuals. The elevated levels of typical inflammatory factors, such as PGE<sub>2</sub> and TNF in OA in the joint fluid is closely associated with the clinical presentations of OA (25).

However, not all the synoviocytes are equally sensitive to IL-18. Primary cells secrete more inflammatory factors upon IL-18 stimulation. As passage numbers increased, the production of PGE<sub>2</sub> and TNF- $\alpha$  was less affected by IL-18 treatment. In the present study, only IL-18R $\alpha$  was detected in primary OA synoviocytes, while IL-18R $\beta$  was virtually absent. This finding suggested that IL-18 targets A cells, instead of B cells. Since the number of A cells decreased or even disappeared with increasing culture time, IL-18R expression disappeared along with the pro-inflammatory effects of IL-18 on synoviocytes. This is similar to RA synoviocytes: IL-18 induces inflammation and joint destruction through the stimulation of T-cells and macrophages, instead of fibroblast-like synoviocytes, which lack IL-18R.

Excessive IL-18 triggers inflammatory responses in chondrocytes. In this study, IL-18 was found to be able to induce TNF- $\alpha$  and PGE<sub>2</sub> expressions and to trigger excessive COX-2 production in chondrocytes. COX-2 degrades arachidonic acid to generate PGE<sub>2</sub>. An elevated level of PGE<sub>2</sub> has the potential to generate the metabolic imbalance and degradation of cartilage (26). TNF- $\alpha$  is a contributing factor of inflammation in OA, and its level in the joint fluid is closely correlated with the progression of OA (27-29). In addition, in this study, all the OA chondrocytes expressed IL-18R $\alpha$ , while certain cells expressed IL-18R $\beta$ . IL-18 stimulates cells through the two receptors, triggering signal transduction via the NF- $\kappa$ B pathway thus inducing cellular inflammatory responses (30).

IL-18 and IL-1 $\beta$  affect chondrocytes in an interactive yet independent way. IL-1 $\beta$  promotes the secretion of inflammatory factors and MMPs in chondrocytes, leading to the degradation of cartilage matrix. IL-1 $\beta$  is considered to be a typical pro-inflammatory factor in OA pathogenesis (31,32). IL-18 has similar functions to IL-1 $\beta$ , while both promote the production of inflammatory factors in chondrocytes. Our results have demonstrated that IL-18 induced inflammatory responses in chondrocytes despite the blockage of the IL-1 $\beta$  function by IL-1Ra, although the PGE<sub>2</sub> and TNF- $\alpha$  levels were lowered, indicating that the effect of IL-18 is only partially dependent on IL-1 $\beta$ , possibly due to their distinct signaling transduction pathways. IL-18 induces COX-2 through the MAPK-p38-AP1 and NF- $\kappa$ B pathways (33,34), whereas IL-1 $\beta$  induces MMP through the p38 APK/c-Fos/AP-1 and JAK2/STAT1/2 pathways. The utilization of different signaling pathways by IL-18 and IL-1 $\beta$  led to their disparate effects on chondrocytes.

In conclusion, synoviocytes and chondrocytes from OA patients express IL-18R. IL-18 were able to induce

inflammatory responses in the two types of cells. This pro-inflammatory effect was partially independent on IL-1 in chondrocytes.

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