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RESEARCH

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IL-40 is up-regulated in the synovial fluid and cartilage of osteoarthritis patients and contributes to the alteration of chondrocytes phenotype in vitro

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Abstract

Introduction IL-40 is a novel cytokine associated with autoimmune connective tissue disorders such as rheumatoid arthritis (RA) or Sjögren syndrome. We have previously shown an accumulation of IL-40 in the RA joint and its expression by immune cells and fibroblasts. Therefore, we aimed to assess the role of IL-40 in association with hyaline cartilage and chondrocyte activity.

Methods Immunohistochemistry was employed to detect IL-40 in paired samples of loaded and unloaded regions of osteoarthritis (OA) cartilage (n=5). Synovial fluid IL-40 was analysed by ELISA in OA (n=31) and control individuals after knee injury (n=34). The impact of IL-40 on chondrocytes was tested *in vitro*.

Results IL-40 was found in chondrocytes of the superficial zone of the OA cartilage, both in loaded and unloaded explants. Additionally, only biopsies from loaded explants showed significant IL-40 positivity in transitional zone chondrocytes. Levels of IL-40 were significantly elevated in the synovial fluid from OA patients compared to controls (p<0.0009) and correlated with synovial fluid leukocyte counts in OA (r=0.444, p=0.014). Chondrocytes exposed to IL-40 dose dependently increased in the secretion of pro-inflammatory cytokines IL-6 (p<0.0001) and IL-8 (p=0.004). Moreover, a dose dependent up-regulation of matrix degrading metalloproteinases MMP-1 (p=0.004), MMP-3 (p=0.031) and MMP-13 (p=0.0002) upon IL-40 treatment was observed in contrast to untreated chondrocytes.

Conclusion This study is the first to demonstrate the accumulation of IL-40 in OA cartilage and its up-regulation in the synovial fluid of OA patients compared to controls. In addition, extracellular IL-40 appears to play a role in promoting inflammation and cartilage destruction by driving chondrocyte behaviour towards a more aggressive phenotype.

Keywords Interleukin 40, Osteoarthritis, Cartilage, Chondrocytes

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Introduction

Osteoarthritis (OA) is a chronic degenerative joint disease highly prevalent worldwide, characterized by the degradation of articular cartilage leading to cascade of symptoms including pain, diminished function and eventual disability [1]. The pathogenesis of OA is multifactorial, encompassing a spectrum of biomechanical,

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metabolic, and inflammatory factors [1]. Physiological alterations coupled with the avascular nature of articular cartilage compromise the viability and overall metabolism of chondrocytes [2]. Despite extensive research, our comprehension of the molecular interplay underlying the shift towards pathological chondrocyte metabolism remains incomplete, and a definitive therapy to effectively impede or prevent OA development remains elusive. Therefore, the regeneration of cartilage and restoration of chondrocyte function persist as paramount biomedical challenges [3].

IL-40 is a newly discovered cytokine [4] related to malignancies [4] and autoimmune rheumatic diseases [5–7]. Described in 2017, IL-40 is encoded by the uncharacterized gene C17orf99 and possesses a unique structure that distinguishes it from any known cytokine family [4]. Functionally, IL-40 is linked to activated B cells [4], neutrophils [5, 6] and T-lymphocytes [7]. Its overexpression has been detected in organs and tissues related to the immune system like the fetal liver, bone marrow [4], as well as in other tissues such as the synovial membrane [6] or salivary glands [7]. Although the physiological roles of this cytokine remain incompletely understood, IL-40 was proposed to be involved in B cell development and homeostasis or lactation [4]. Over the past few years, mounting evidence has linked IL-40 to various pathologies [4–7]. An association of IL-40 with malignancies was suggested based on differential expression of IL-40 found in B-cell lymphomas [4]. Involvement of IL-40 in the pathogenesis of several autoimmune rheumatic diseases such as rheumatoid arthritis [5, 6] or Sjogren's syndrome [7] was described. Our research group has recently demonstrated an accumulation of IL-40 within the joint environment in RA, in particular in the RA synovial tissue and fluid [6], and revealed its expression in both immune cells and RA synovial fibroblasts [5, 6]. We have also shown that IL-40 enhances the secretion of cytokines and matrix degrading enzyme MMP-13 in RA synovial fibroblasts [6]. Altogether, these findings prompted our current study focusing on the role of IL-40 in association with hyaline cartilage and chondrocyte activity.

Methods

Patient recruitment

Synovial fluid samples were obtained from 31 patients with OA (23 females and 8 males; mean age ± SD: 70 ± 9 years) and 34 non-OA control individuals (16 females and 18 males; mean age ± SD: 42 ± 11 years) during clinically indicated knee arthrocentesis. OA was diagnosed based on EULAR recommendations for knee OA [8]. The control non-OA group consisted of subjects who underwent arthrocentesis within 2-24 weeks after knee injury, who no longer had early acute swelling, or who had no clinical or radiographic evidence of post-injury OA. The characteristics of all participants are summarized in Table 1. For ethical reasons, it was not possible to obtain synovial fluid samples from healthy subjects and cadaveric control synovial fluid was also not available. The levels of IL-40 in the synovial fluid samples were quantified using the Human chromosome 17 open reading frame 99 ELISA Kit, cat.n. MBS9337680 (MyBioSource, San Diego, USA). Radiographs were scored using the Kellgren–Lawrence (KL) grading scale: grade 1, doubtful narrowing of joint space and possible osteophytic lipping; grade 2, definite osteophytes and possible narrowing of joint space; grade 3, moderate multiple osteophytes, definite narrowing of joints space, some sclerosis and possible deformity of bone contour; grade 4, large osteophytes, marked narrowing of joint space, severe sclerosis and definite deformity of bone contour [9]. Severity of pain, stiffness, and physical disability in OA patients were assessed by the Western Ontario and MacMaster University (WOMAC) score [10].

Immunohistochemistry

For immunohistochemical analysis, paired samples from mechanically loaded and unloaded regions of the articular cartilage from OA patients (n=5; 2 males and 3 females, mean age ± SD: 66 ± 5 years) were harvested by experienced orthopaedic surgeons during total knee arthroplasty. All samples were embedded in paraffin, cut into 5-mm-thick serial sections, deparaffinised and rehydrated. To assess the general cartilage morphology and proteoglycan content, conventional staining with hematoxylin and Safranin O – Fast Green (all from Sigma Sigma-Aldrich, St. Louis, MO, USA) was performed as described elsewhere [11]. To detect IL-40 in the cartilage, the following staining protocol was employed: Inhibition of endogenous peroxidase activity was achieved by incubating the sections with DAKO Dual Endogenous Enzyme Block (Agilent, Santa Clara, CA, USA) for 10 min in room temperature (RT). After rinsing with PBS

Table 1 Characteristics of patients with osteoarthritis (OA) and non-OA controls

	OA (*n*=31)	non-OA control (*n*=34)
Age (years)	72 (63-85)	46 (41-56)
Gender (F/M), n	23/8	16/18
Disease duration (years)	3.5 (1.0-12.5)	-
BMI (kg/m^2)	27.4 (25.0-30.7)	25.5 (23.4-26.9)
WOMAC	39.5 (28.0- 58.3)	-

BMI Body mass index, *OA* Osteoarthritis, *WOMAC* The Western Ontario and McMaster Universities Arthritis Index. Data are expressed as median [interquartile range (Q1–Q3)] if not stated otherwise

buffer, antigen retrieval was performed by use of Trypsin enzymatic antigen retrieval solution (Genetex, CA, USA) for 10 min in RT, followed by another PBS rinse. Subsequently, 2% bovine serum albumin (BSA, Thermo Fisher Scientific, Waltham, Massachusetts, USA) diluted in PBS was added to block potential non-specific binding sites (60 min, RT). For the detection of IL-40, the cartilage sections were subjected to immunoprobing with a primary rabbit polyclonal C17orf99 antibody (Abcam, Cambridge, UK) diluted 1:50 in 2% BSA in PBS, incubated overnight at 4°C. Isotype IgG Universal Negative Control for IS-Series Rabbit Primary Antibodies (Agilent, Santa Clara, CA, USA) served as the negative control. After rinsing with PBS, HRP conjugated polyclonal goat anti-rabbit secondary antibody (Agilent, Santa Clara, CA, USA) diluted 1:200 in 2% BSA in PBS was applied for 1h at RT. Liquid DAB + Substrate (Dako Cytomation, Glostrup, Denmark) was utilized as the chromogen, followed by counterstaining with Mayer's Haematoxylin solution. Evaluation of results was conducted using an Olympus BX53 microscope with DP80 Digital Microscope Camera and CellSens Standard V1.18 imaging software (Olympus, Philadelphia, PA, USA). The immunohistochemistry staining of IL-40 was analysed semi-quantitatively by Fiji software [12].

In vitro experiments on chondrocytes

In vitro experiments were conducted on CloneticsTM Normal Human Articular Chondrocytes, NHAC-kn (Lonza, Basel, Switzerland). Chondrocytes were expanded according to the manufacturer's protocol and cultured in CBM™ Chondrocyte Growth Basal Medium (CC-3217, Lonza, Basel, Switzerland) supplemented with Chondrocyte ReagentPack™ Subculture Reagents (CC-4409, Lonza, Basel, Switzerland) at 37°C in a 5% CO2 humidified atmosphere. Cells in the second passage were seeded on 6-well plates (1.0×10^5 cells/well) and exposed to recombinant IL-40 (Aviva Systems Biology, San Diego, CA, USA) at concentrations ranging from 5 to 200 ng/ml for 24 hours (n=7-9). The selected concentrations of IL-40 tested negative for cytotoxicity (Supplementary data). Interleukin (IL)-1β (10 ng/mL, R&D Systems, Minneapolis, MN, USA) was used as the positive control for the *in vitro* experiments. Cell culture supernatants were collected and levels of selected proteins were measured using commercially available ELISA kits (Ray Biotech, Norcross, GA, USA). Informed consents were obtained from all participants.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA) and data were expressed as median value and interquartile range (Q1–Q3). For analysis of differences between groups, Mann-Whitney U-test for non-paired data was performed. Analyses of differences between the groups were further adjusted for confounders (sex and age) using logistic regression. The association of IL-40 with selected parameters was determined by Spearman correlation. Wilcoxon matched-pairs signed rank test was used for the *in vitro* data. The results were considered significant when p was less than 0.05 ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$).

Results

IL-40 is upregulated in the synovial fluid and cartilage of OA patients

We demonstrated the accumulation of IL-40 in chondrocytes located in lacunae of hyaline cartilage (Fig 1A-D). The immunoreactivity of IL-40 was particularly intense in chondrocytes of the superficial and transitional zone in the loaded regions of OA cartilage, although some patients exhibited positivity even in radial zone chondrocytes (Fig. 1A, C). In unloaded regions, superficial zone chondrocytes exhibited positive IL-40 staining, whereas only a few cells in the transitional and radial zones tested positive for IL-40. (Fig. 1B, D). Moreover, we observed a significant elevation of IL-40 levels in the synovial fluid of patients with OA compared to controls [552.0 (329.0-2002.0) vs. 264.0 (100.0-743.0) pg/ml; p=0.008]. As age and sex represent commonly acknowledged confounding variables in OA patients [13], logistic regression analysis was conducted to account these factors. Upon adjustment for sex and age, the contrast in IL-40 levels between

(See figure on next page.)

Fig. 1 IL-40 was highly expressed in the superficial and transitional zone chondrocytes in the loaded areas of OA cartilage. In the radial zone of loaded explants, IL-40 positive chondrocytes were found only in some patients (**A**, **C**). **A**. In the unloaded areas, superficial zone chondrocytes tested positive for IL-40, whereas only random IL-40 staining was found in transitional and radial zones (**B**, **D**). Semi-quantitative analysis was performed to evaluate the percentage of the IL-40 positive cells in all analysed samples of loaded (**C**) and unloaded (**D**) cartilage explants (*n*=5). Rabbit IgG was used as isotype control (**A**, **B**). Safranin O – Fast Green and haematoxylin were used to assess the cartilage morphology (**A**, **B**). Representative images are shown at x40 and x200 magnification. Levels of IL-40 in the synovial fluid were significantly up-regulated in osteoarthritis (OA) patients compared to controls (**E**). No relation between synovial fluid IL-40 levels and radiographic severity (KL grade) was observed in OA patients (**F**). Synovial fluid IL-40 levels were positively associated with synovial fluid leukocyte counts in OA patients (**G**) The data are represented as the median; the error bars show 1st and 3rd quartiles. ***, *p*<0.001

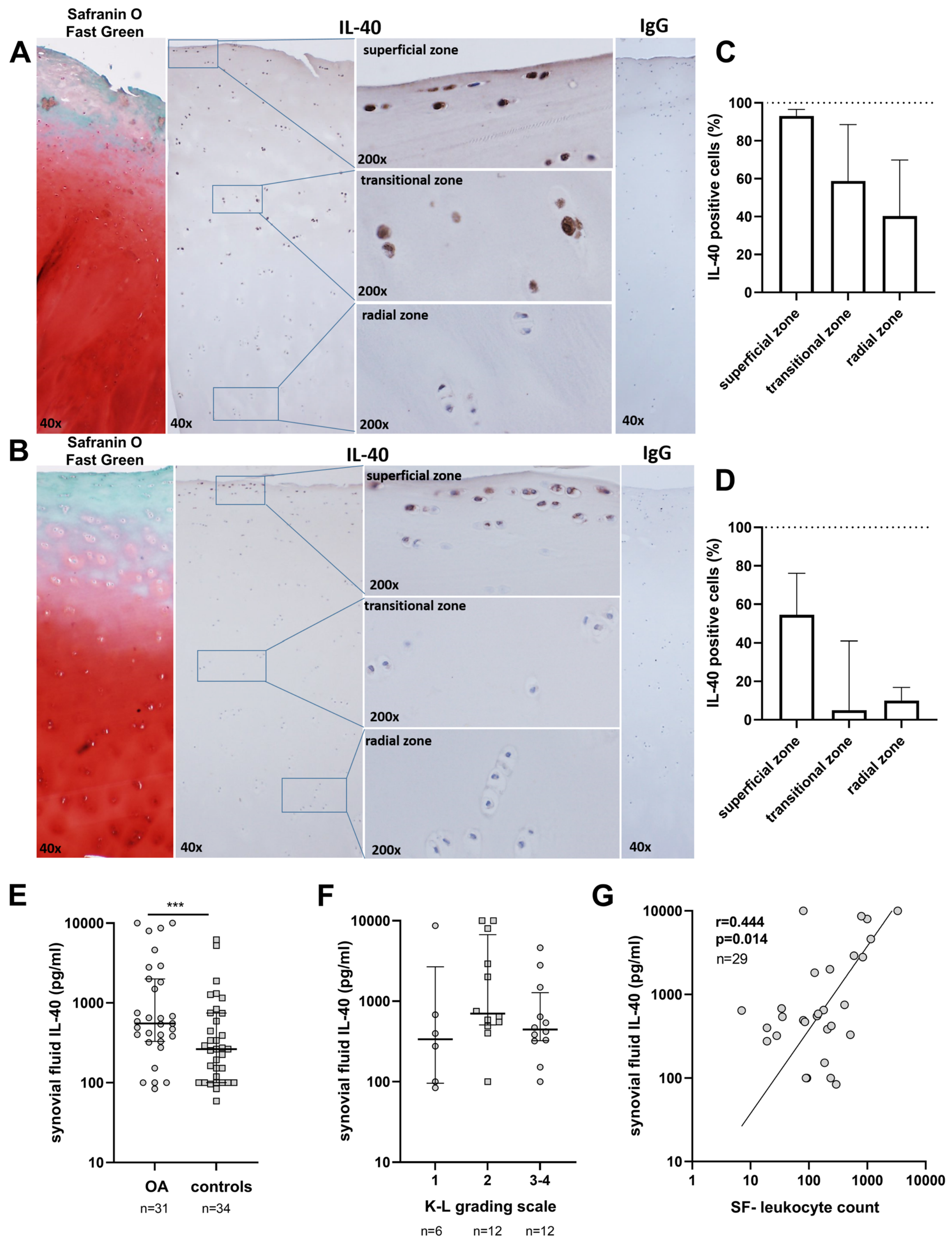

Fig. 1 (See legend on previous page.)

the OA and controls became notably more pronounced (p=0.0009) – (Fig. 1E). Dividing OA patients into three subgroups based on radiographic severity (KL grade scoring) revealed elevated levels of IL-40 in synovial fluid in KL 2 patients [700.5 (507.0-6723.0) pg/ml] compared with KL 1 [337.0 (96.0-2676.0) pg/ml] or KL 3-4 [445.0 (323.0-1280.0) pg/ml] patients. However, the number of patients in the subgroups was insufficient to draw statistically significant conclusions (Fig. 1F). Synovial fluid IL-40 levels were positively associated with synovial fluid leukocyte counts in OA patients (r=0.444, p=0.014) but not in controls (r=-0.020, p=0.921) (Fig. 1G). There was no correlation of synovial fluid IL-40 with the total WOMAC score (r=0.202, p=0.322) or with its individual parameters: pain (r=0.256, p=0.207), stiffness (r=-0.212. p=0.300) and physical function (r=0.215, p=0.291).

IL-40 promotes inflammation and aggressive phenotype in chondrocytes *in vitro*

Given the accumulation of IL-40 we have found in OA synovial fluid, we further sought to investigate the effect of extracellular IL-40 on normal human articular chondrocytes (n=7-9). Our experimental design included a range of IL-40 concentrations corresponding to physiological levels found in the OA joint (up to 10 ng/ml) as well as concentrations reported in inflamed joints of patients with rheumatoid arthritis (100-200 ng/ml) [6]. While the treatment by IL-40 did not increase IL-1β production by chondrocytes (Fig. 2A), other pro-inflammatory cytokines and matrix-degrading enzymes showed a significant up-regulation following exposure to IL-40. (Fig. 2B-F). We found that recombinant IL-40 dose-dependently potentiates the secretion of IL-6 in chondrocytes [for 200 ng/ml: 4104.0 (3086.0-5072.0) pg/ml, p<0.0001; for 100 ng/ml: 3090.0 (1923.0-3643.0) pg/ml, p=0.0002; for 10 ng/ml: 807.9 (612.7-850.4) pg/ml, p=0.002 compared to the unstimulated control: 392.2 (265.4-529.9) pg/ml] (Fig. 2B). Similarly, IL-8 secretion was enhanced in chondrocytes exposed to IL-40 [for 200 ng/ml: 851.5.0 (573.6-886.2) pg/ml, p=0.004; for 100 ng/ml: 769.5 (402.7-830.0) pg/ml, p=0.004; for 10 ng/ml: 157.5 (92.4-188.7) pg/ml, p=0.004 compared to the unstimulated control: 36.2 (25.6-57.9) pg/ml] (Fig. 2C). Furthermore, IL-40 significantly increases the secretion of matrix degrading metalloproteinase 13 (MMP-13) by chondrocytes [for 200 ng/ml: 1008.0 (744.5-1191.0) pg/

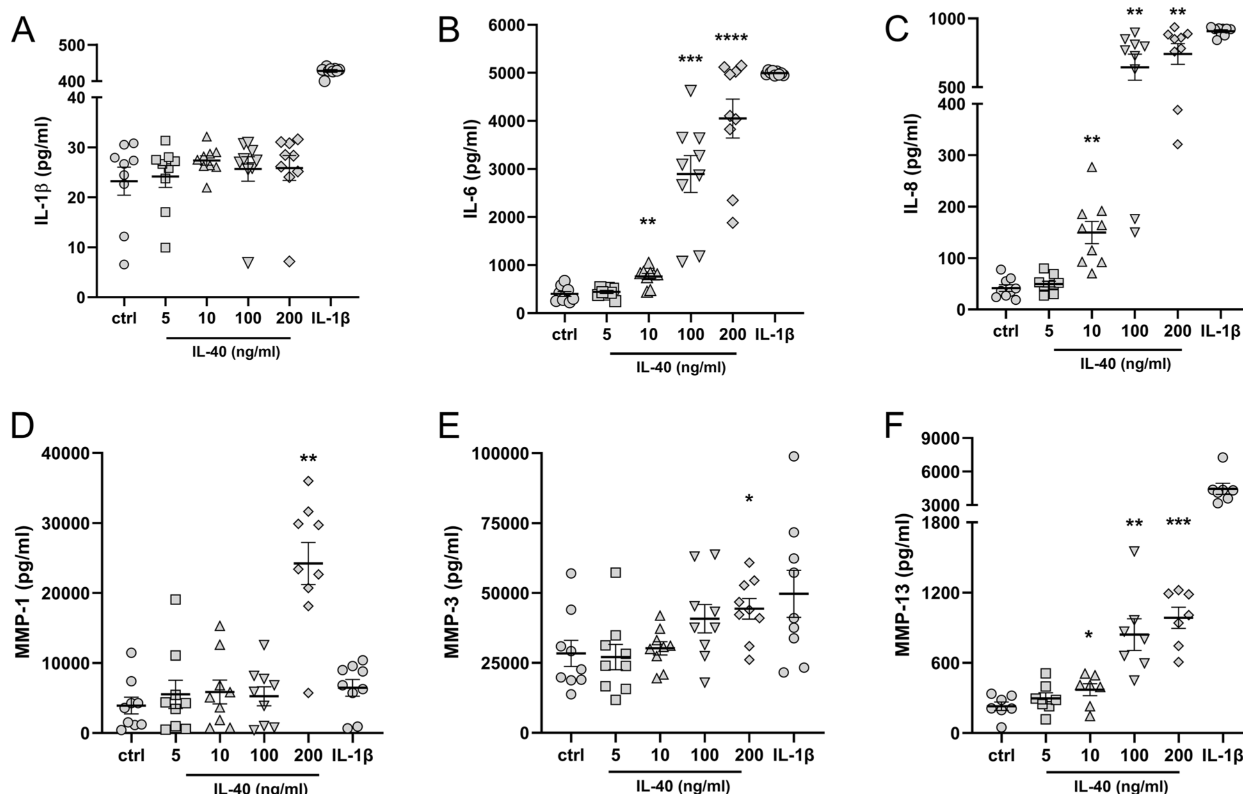

Fig. 2 Normal human articular chondrocytes (n=7-9) significantly increased the secretion of cytokines IL-6, IL-8 a matrix degrading metalloproteinases (MMPs) but not IL-1β upon the exposure to recombinant IL-40 (5-200 ng/ml) for 24h in *in vitro* conditions (**A**-**F**). The data are represented as the median; the error bars show 1st and 3rd quartiles. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001

ml, p=0.0002; for 100 ng/ml: 801.8 (595.3-963.4) pg/ml, p=0.003; for 10 ng/ml: 409.3 (228.9-492.9) pg/ml, p=0.016 compared to the unstimulated control: 211.3 (196.7-318.9) pg/ml]. Only at high concentration, IL-40 stimulated MMP-1 and MMP-3 secretion by chondrocytes (p=0.004 and p=0.031) (Fig. 2D-F).

Discussion

Our study is the first to demonstrate the local up-regulation of IL-40 in the synovial fluid and cartilage of OA patients. Moreover, IL-40 appears to be involved in the regulation of inflammation and cartilage destruction.

Here, we show that IL-40 accumulates in the chondrocytes of OA cartilage. The finding of significant staining positivity for IL-40 in the superficial zone of both loaded and unloaded areas of the cartilage suggests that the presence of IL-40 in the superficial zone of OA cartilage may not solely be attributed to mechanical load. It raises the question of whether superficial zone IL-40 is produced by activated chondrocytes or absorbed from the synovial fluid. The restricted expression of L-40 by transitional zone chondrocytes and in some patients even in the radial zone of in loaded regions of cartilage may be explained by the altered phenotype induced by long-term mechanical stress and structural changes in cartilage. However, further research is required to elucidate the biological processes underlying the expression pattern of IL-40 in cartilage. In addition, our findings regarding the elevation of IL-40 in the OA synovial fluid are consistent with our previously published data for RA [6] and suggest a potential role of IL-40 in joint pathologies. This is further supported by the positive correlation of IL-40 with the synovial fluid leukocyte count found in OA patients but not in control individuals (data not shown). As IL-40 has been proposed as a neutrophil-derived cytokine [5], we believe that its elevation in OA synovial fluid may be related to activated neutrophils - the most abundant cells in OA synovial fluid [14]. Altogether, IL-40 is elevated locally in the joint but not in the circulation of OA patients, in accordance with our prior report [6]. However, no association of synovial fluid IL-40 levels with radiographic severity and overall disability of OA patients was found. Given the increased molecular diffusion described in pathological cartilage [15], we hypothesized that the synovial fluid IL-40 may affect chondrocytes. Based on our in vitro experiments, chondrocytes exposed to IL-40 exhibited increased production of cytokines and MMPs, consistent with our previous research on RA synovial fibroblasts [6]. Although IL-40 did not increase the secretion of IL-1β, one of the major inflammatory and catabolic cytokine in OA [16], in chondrocytes, it significantly enhanced other pro-inflammatory cytokines such as IL-6 and IL-8. Since IL-6 and IL-8 are among the main chemoattractants for neutrophils, it is plausible to speculate that in the joint, chondrocytes surrounded by synovial fluid rich in IL-40 may enhance IL-6 and IL-8 production, leading to increased neutrophil recruitment and potentially exacerbating inflammation in the OA joint. In addition, the up-regulation of MMPs by IL-40 treated chondrocytes indicate that IL-40 could contribute to driving chondrocyte behaviour towards a more aggressive phenotype. Taken together, our study combined with existing data on synovial fibroblasts and immune cells [4–7], underscores the potential implication of IL-40 in inflammation-mediated tissue damage. This positions IL-40 as a promising subject for future research in other chronic degenerative and autoimmune diseases.

The present study has some limitations. First, the association between IL-40 expression in cartilage and synovial fluid could not be evaluated because the cartilage biopsies were collected from a different group of patients than those who underwent knee effusion aspiration. Second, the analysis of the synovial fluid and cartilage from healthy individuals was not performed due to ethical considerations.

Conclusions

We are the first to evidence the presence of IL-40 in chondrocytes and its up-regulation in synovial fluid from OA patients compared to control subjects. Additionally, it suggests that extracellular IL-40 may play a role in promoting inflammation and cartilage destruction by driving chondrocyte behaviour towards a more aggressive phenotype.

Abbreviations

BMI	Body mass index
ELISA	Enzyme immunoassay
EULAR	European League Against Rheumatism
IL	Interleukin
Q	quartile
KL	Kellgren-Lawrence
LPS	Lipopolysaccharide
MMP	Matrix degrading metalloproteinase
OA	Osteoarthritis
RA	Rheumatoid arthritis
SD	Standard deviation
WOMAC	The Western Ontario and McMaster Universities Arthritis Index

Supplementary Information

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Supplementary Material 1.

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Authors' contributions

LAC was responsible for the study concept and design and drafted the manuscript. LŠ supervised the study implementation and manuscript preparation.

LŠ, JV, KP, PF and RB were responsible for patient recruitment and clinical assessment. DV and SP provided the cartilage biopsies during total knee arthroplasty. MK, LAC and TK carried out the immunohistochemistry. LAC conducted the in vitro experiments. AN and AP performed the ELISA measurements. JB carried out the statistical analysis and IHC quantification. LAC and LŠ are responsible for data interpretation. All authors reviewed the manuscript and gave their final approval of the version to be published.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study involves human participants and was approved by the local Ethics Committee of the Institute of Rheumatology in Prague, Czech Republic. Reference numbers of the ethics approvals are 6485/2020 and 9906/2021. Written informed consent was obtained from all participants. Participants gave informed consent to participate in the study before taking part.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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