Osteoarthritis and Cartilage 32 (2024) 561–573

Osteoarthritis and Cartilage

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Importance of IL-6 trans-signaling and high autocrine IL-6 production in human osteoarthritic chondrocyte metabolism

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ARTICLE INFO

Article history:
Received 10 August 2023
Accepted 7 February 2024

Keywords:
Osteoarthritis
Chondrocytes
Interleukin-6
IL-6 neutralization
IL-6 signaling

SUMMARY

Objective: Neutralization of Interleukin (IL)-6-signaling by antibodies is considered a promising tool for the treatment of osteoarthritis (OA). To gain further insight into this potential treatment, this study investigated the effects of IL-6-signaling and IL-6 neutralization on chondrocyte metabolism and the release of IL-6-signaling-related mediators by human chondrocytes.
Design: Chondrocytes were collected from 49 patients with advanced knee/hip OA or femoral neck fracture. Isolated chondrocytes were stimulated with different mediators to analyze the release of IL-6, soluble IL-6 receptor (sIL-6R) and soluble gp130 (sgp130). The effect of IL-6 and IL-6/sIL-6R complex as well as neutralization of IL-6-signaling on the metabolism was analyzed.
Results: OA chondrocytes showed high basal IL-6 production and release, which was strongly negatively correlated with the production of cartilage-matrix-proteins. Chondrocytes produced and released sIL-6R and sgp130. The IL-6/sIL-6R complex significantly increased nitric oxide, prostaglandin E_2 and matrix metalloproteinase 1 production, decreased Pro-Collagen Type II and mitochondrial ATP production, and increased glycolysis in OA chondrocytes. Neutralization of IL-6-signaling by antibodies did not significantly affect the metabolism of OA chondrocytes, but blocking of glycoprotein 130 (gp130)-signaling by SC144 significantly reduced the basal IL-6 release.
Conclusion: Although IL-6 trans-signaling induced by IL-6/sIL-6R complex negatively affects OA chondrocytes, antibodies against IL-6 or IL-6R did not affect chondrocyte metabolism. Since inhibition of gp130-signaling reduced the enhanced basal release of IL-6, interfering with gp130-signaling may ameliorate OA progression because high cellular release of IL-6 correlates with reduced production of cartilage-matrix-proteins.

Introduction

Currently, no disease modifying drugs are available for the treatment of osteoarthritis (OA). Recent studies have found associations between Interleukin (IL)-6 in the joint and OA pathology as well as OA pain,[1–6] thus IL-6-signaling is an interesting target for OA treatment.[1] IL-6 levels in synovial fluid are elevated in OA patients and are 30- to 1000-fold higher in synovial fluid than in serum, suggesting production and release by cells in the joint.[1,3,4,7] Intra-articular application of IL-6 sensitizes joint nociceptors to mechanical stimuli.[8] In OA patients, the concentration of IL-6 in synovial fluid correlates with the intensity of joint pain.[3–5] Since chondrocytes release IL-6,[9] they may contribute significantly to OA pain.

In general, IL-6-signaling is complex and IL-6 levels alone are not sufficient to assess its biological role. Classic IL-6-signaling requires a

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https://doi.org/10.1016/j.joca.2024.02.006

membrane-bound IL-6 receptor (IL-6R). After IL-6-binding, IL-6R activates the receptor-associated glycoprotein 130 (gp130) unit in the membrane to transduce the signal into the cell. However, most IL-6-responsive cell types do not express membrane-bound IL-6R. In these cells, the ubiquitously expressed gp130 is activated by so-called IL-6-trans-signaling. Here, IL-6 forms a complex with soluble IL-6R (sIL-6R), which is present e.g. in serum and synovial fluid.[1] Soluble gp130 (sgp130) can bind and neutralize IL-6/sIL-6R complexes and inhibit IL-6-trans-signaling. Activation of gp130 in the membrane induces phosphorylation of Janus kinases (JAK), which can then activate canonical signaling via signal transducer and activator of transcription 3 (STAT3) and non-canonical signaling via mitogen-activated protein kinase cascade or phosphatidylinositol 3-kinase/protein kinase B (PKB/Akt).[1]

Chondrocytes express membrane-bound IL-6R[10] but few data have shown sIL-6R and sgp130 in synovial fluid of healthy individuals or OA patients.[1] Thus, IL-6 may stimulate chondrocytes by both IL-6 classic- and trans-signaling, but the functional importance of both pathways should be evaluated in more detail. IL-6-induced sensitization of joint nociceptors is mainly mediated by IL-6-trans-signaling and prevented by sgp130.[8]

Both chondrodegenerative and chondroprotective effects of IL-6 have been reported. IL-6 induces the expression of metalloproteinases and aggrecanases in chondrocytes that promote cartilage degeneration.[1] IL-6 levels in synovial fluid correlate with progression and severity of OA.[1] In a murine OA model, systemic blockade of IL-6 or IL-6 signal transduction reduced cartilage lesions, osteophyte formation and synovial inflammation.[11] However, especially in healthy chondrocytes, IL-6 increased GAG production, a chondroprotective effect.[12] Little is known about the release of sIL-6R and sgp130 by chondrocytes. Chondrocytes express enzymes for shedding of sIL-6R from membrane-bound IL-6R, but no sIL-6R release has been detected under normal conditions.[7] Reports about a release of sgp130 by chondrocytes could not be found.

The present study quantified the production and release of IL-6, sIL-6R and sgp130 by human chondrocytes from patients with advanced knee and hip OA. Because of reported opposing effects of IL-6 on chondrocytes, the effects of IL-6 classic- and trans-signaling on chondrocyte metabolism and the release of pain-related mediators were evaluated. Furthermore, the effects of selective blockade of IL-6-trans-signaling and neutralization of IL-6 were investigated. The data obtained are necessary to evaluate the effects and consequences of targeting IL-6-signaling for OA and OA pain treatment.

Methods

Reagents for cell culture experiments

We purchased human IL-6, sIL-6R, IL-8, IL-11, Transforming growth factor β (TGF-β), Nerve growth factor (NGF) from Prospec (Ness-Ziona, Israel), IL-1β, Tumor necrosis factor (TNF) from PeproTech (Rocky Hill, NJ, USA), Prostaglandin E_2 and D_2 (PGE_2 and PGD_2), 2-(7-Fluoropyrrolo[1,2-a]quinoxalin-4-yl) 2-pyrazinecarboxylic acid hydrazide hydrochloride (SC144) from Cayman Chemical (Ann Arbor, MI, USA), TNF protease inhibitor-2 (TAPI2) from Tocris Bioscience (Bristol, UK), sgp130, anti-IL-6R antibody (IL-6R AB) from R&D Systems (Minneapolis, MN, USA), anti-IL-6 antibody (IL-6 AB) from InvivoGen (Toulouse, France), pronase E from Merck KGaA (Darnstadt, Germany), and collagenase P from Roche Diagnostics GmbH (Mannheim, Germany). Substances were diluted to the final concentration in chondrocyte culture medium consisting of Chondrocyte Basal Medium + 10% Chondrocyte Growth Medium SupplementMix (PromoCell GmbH, Heidelberg, Germany) + 1% penicillin/streptomycin solution (Life Technologies Europe BV, NN Bleiswijk, Netherlands).

Patient group	OA knee	OA hip	Non-OA hip
No. of patients	29	10	10
Age, y	68.45 ± 8.9	69.30 ± 6.8	73.10 ± 8.8
Sex (female/male)	12/17	8/2	7/3
BMI, kg/m^2	31.08 ± 4.5	28.14 ± 3.9	22.95 ± 3.6

BMI, body mass index.

Table I Osteoarthritis and Cartilage

Demographic and laboratory parameters of included patients, values are expressed as number of patients or mean ± standard deviation.

Patient material

Human hip and knee chondrocytes were obtained from 49 patients with end-stage knee or hip OA or femoral neck fracture who underwent joint arthroplasty (patient parameters in Table I). The non-OA control group included only patients with femoral neck fractures who had no evidence of osteoarthritic cartilage alterations and pain before the fracture. All patients were informed about the purpose of tissue sampling and gave written consent. The study was approved by the Ethics Committee for Clinical Trials of the Friedrich Schiller University of Jena and the Sächsische Landesärztekammer (2020-1630-BO, 5208-07/17, EK-BR-81/17-1) and conducted in accordance with the Declaration of Helsinki.

Isolation of human chondrocytes

To isolate hip and knee chondrocytes, cartilage was cut into pieces and treated with 0.01 mg/mL Pronase E in Dulbecco´s modified Eagles´s medium for 30 min at 37 °C following collagenase P treatment (1.3 mg/mL in chondrocyte culture medium) for 16 hours at 37 °C. Isolated cells were filtrated, washed, and seeded in cell culture plates for stimulation experiments or frozen for analysis of intracellular protein expression.

Stimulation of human chondrocytes

To analyze released substances, chondrocytes were plated on 24-well culture plates at a density of 4×10^4 cells/cm^2 and cultured in chondrocyte culture medium. The medium was renewed after 3 days. After another 2 days, chondrocytes were stimulated with IL-1β (0.1 ng/mL), TNF, NGF, IL-8, IL-11, TGF-β (all 0.1 µg/mL), PGD_2, PGE_2 (10 µg/mL), IL-6 (0.1 µg/mL), or a IL-6 (0.1 µg/mL)/sIL-6R (0.3 µg/mL) complex for 48 h. To block IL-6-trans-signaling or to neutralize IL-6-signaling, chondrocytes were additionally treated with IL-6R AB, IL-6 AB, sgp130 (all 0.6 µg/mL) or TAPI2 (20 µM). Supernatant was collected and stored at −80 °C until use. Cells were stimulated and analyzed in duplicate for each condition. Experiments were performed with a minimum of 6 replicates (donors) to ensure reproducibility.

Analysis of produced mediators and cartilage-matrix-proteins

We used enzyme-linked immunosorbent assay kits for human IL-6, sIL-6R, sgp130, PGE_2, matrix-metalloproteinase 1 (MMP1), Pro-Collagen-Type II, tissue inhibitor of metalloproteinases-1 (TIMP1), and glucose and lactate specific assays (see Supplementary Table I) according to the manufacturer's instructions. Absorbance was analyzed at 450 nm using the Epoch microplate spectrophotometer and Gen5 software (both BioTek Instruments GmbH, Bad Friedrichshall, Germany).

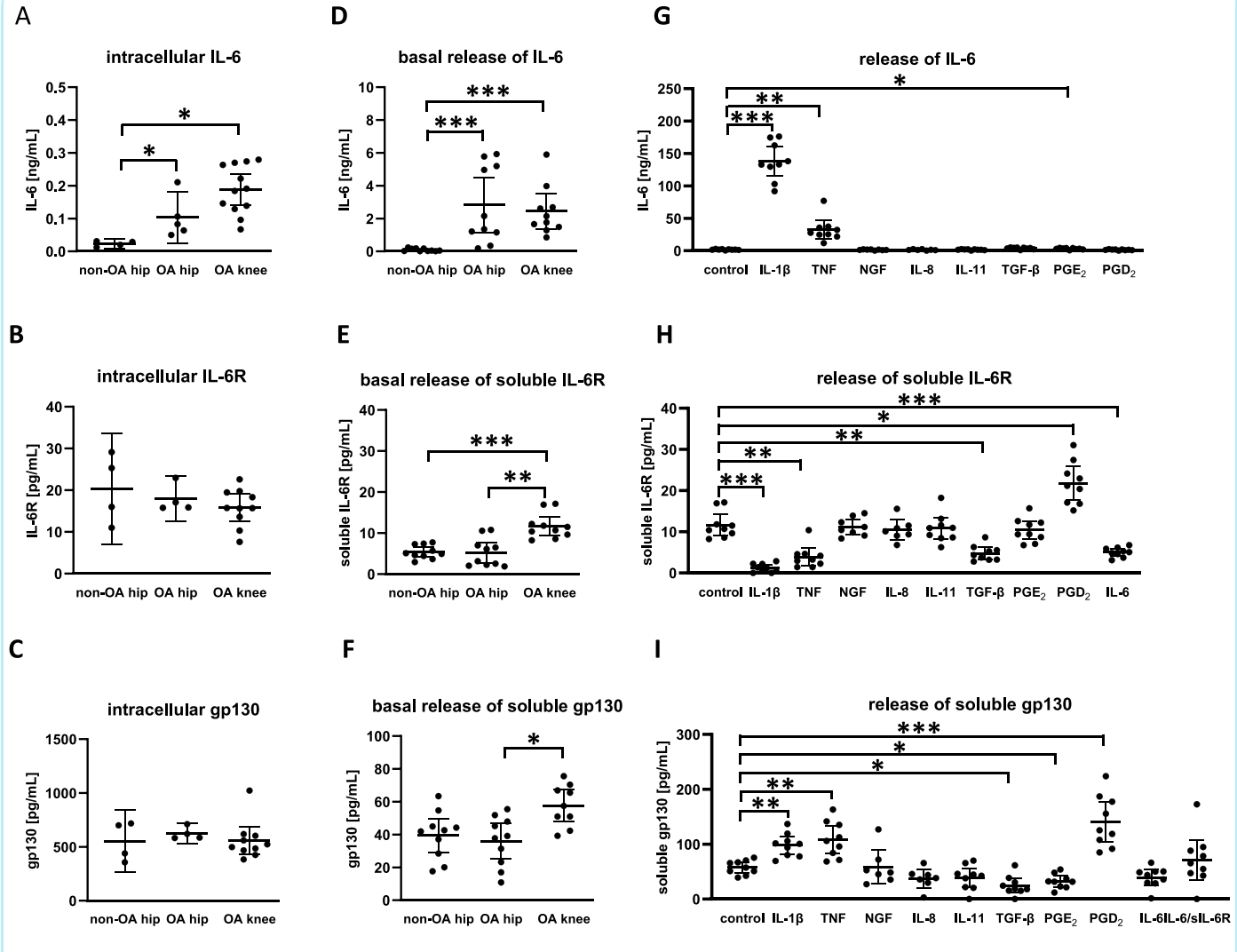

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Fig. 1 Osteoarthritis and Cartilage

Expression and release of IL-6, IL-6R and gp130 by human chondrocytes. Comparison of intracellular expression of (A) IL-6 in non-OA hip (n = 4), OA hip (n = 5) and OA knee (n = 12) chondrocytes, (B) IL-6R in non-OA hip (n = 4), OA hip (n = 4) and OA knee (n = 10) chondrocytes and (C) gp130 in non-OA hip (n = 4), OA hip (n = 4) and OA knee (n = 10) chondrocytes. Comparison of basal release of (D) IL-6 in non-OA hip (n = 10), OA hip (n = 10) and OA knee (n = 10) chondrocytes, (E) sIL-6R in non-OA hip (n = 10), OA hip (n = 10) and OA knee (n = 10) chondrocytes and (F) sgp130 in non-OA hip (n = 10), OA hip (n = 10) and OA knee (n = 9) chondrocytes. Release of (G) IL-6, (H) sIL-6R and (I) sgp130 by OA knee chondrocytes (all n = 9) after stimulation with different mediators. Possible IL-6-signaling activations (J). Statistical differences between groups or stimulated vs. unstimulated cells (control), respectively: Mann-Whitney U test with Bonferroni adjustment for multiple comparisons, *** $p < 0.005$, ** $p < 0.01$, * $p < 0.05$. The patients included in each experiment and their demographic parameters are listed in Supplementary Table III. gp130, glycoprotein 130.

For intracellular protein analysis, cells were physically disrupted by repeated freezing and thawing to extract cellular components.

To measure nitrite in the supernatant as an indicator of nitric oxide (NO) production we used the Griess Reagent Kit (#G7921, Invitrogen) and analyzed absorbance (spectrophotometry see above) at 548 nm.

Glycosaminoglycan (GAG) was measured spectrophotometrically (see above) using 1,9-dimethylmethylene blue (DMB, Sigma-Aldrich, Taufkirchen, Germany), absorbance was analyzed at 525 nm.

Analysis of mitochondrial function and glycolysis

Mitochondrial function and glycolysis were calculated from oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measured with the Seahorse XF Analyzer (Agilent, Santa Clara, CA, USA) using the Seahorse XF Real-Time ATP Rate Assay (#103592-100, Agilent), Cell Mito Stress Test (#103015-100, Agilent) or Glycolysis Stress Test Kits (#103017-100, Agilent). Assays include modulators of the mitochondrial respiratory chain to determine mitochondrial adenosintriphosphat (ATP)-linked oxygen consumption, glycolytic ATP-linked acidification, non-mitochondrial oxygen consumption and non-glycolytic acidification. Total OCR and total ECAR were measured before application of modulators. Parameters were calculated using Seahorse XF Report Generator software (Agilent).

Chondrocytes, plated on Seahorse cell culture plates (Agilent) at a density of 3×10^4 cells/well and cultured in chondrocyte culture medium at 37 °C for 6 days, were stimulated with IL-6 or IL-6/sIL-6R complex for 24 h. Experiments were performed with a minimum of 6 replicates (donors).

Impact of mediators on the viability of chondrocytes

Chondrocytes, plated on 96-well culture plates at a density of 4×10^4 cells/cm^2 and cultured in chondrocyte culture medium (see above), were stimulated with all mediators to test their effect on viability. We used the Invitrogen LIVE/DEAD Viability/Cytotoxicity Kit (#L3224) which determines the percentage of live and dead cells in stimulated and unstimulated cells. Fluorescence intensity was measured using the microplate reader CLARIOstar (BMG LABTECH GmbH, De Meern, Netherlands).

Analysis of activation of IL-6-signaling pathways using western blot

Chondrocytes, plated on 12-well culture plates at a density of 4×10^4 cells/cm^2 and cultured in chondrocyte culture medium (see above) were stimulated with IL-6 or IL-6/sIL-6R complex for 30 min or 48 h. Cells were lysed on ice using RIPA lysis buffer (Cell Signaling, Danvers, MA, USA) freshly supplemented with protease inhibitor cocktail tables (Roche, Mannheim, Germany), and frozen at −80 °C.

Immunoblotting was performed with primary antibodies against total STAT3, phospho-STAT3-Tyr705, phospho-PKB/AKT-Ser473, phospho-ERK1/2-Thr202/Tyr204, phospho-STAT3-Ser727, suppressor of cytokine signaling 3 (SOCS3), vinculin and peroxidase-labeled secondary antibodies (antibody information in Supplementary Table I). Signals were visualized with an enhanced chemiluminescence reaction (#34075, Thermo Fisher, Darmstadt, Germany) according to the manufacturer, using a camera system with GeneSnap 7.12 software (Synoptics, Cambridge, UK). Densitometry of related Western blot band intensities from the same blot was performed using Image J 1.52a software (https://imagej.nih.gov/ij/). Phosphorylated proteins were calculated relative to unphosphorylated proteins or to reference vinculin.

Statistical analysis

SPSS statistics 27 software (SPSS, Inc, Chicago, IL, USA) was used for statistical analysis. Demographic parameters of included patients were expressed as mean ± standard deviation. Experimental results were presented as scatter plots including mean with 95% confidence interval (CI) which were generated in GraphPad Prism 10. Unstimulated cells were defined as control. Groups were compared with Mann-Whitney U test. Bonferroni adjustment was performed for multiple comparisons. Effect sizes between groups were expressed as Cohen's $|d|$ and calculated as the difference between means divided by the pooled standard deviation. For normalized data, the one-sample Wilcoxon signed rank test was used. Correlation analysis was performed using Spearman's rank correlation. Effect sizes were expressed as the correlation coefficient R, and R uncertainty was expressed as the 95% CI. Significance was accepted at $p < 0.05$.

Results

This study included 29 patients with advanced knee OA, 10 patients with advanced hip OA and 10 patients with femoral neck fracture (non-OA) who underwent arthroplasty. Demographic parameters of all patients are shown in Table I. Age and body mass index of the knee and hip OA patients were comparable, but patients of the non-OA hip group were older and had a lower body mass index than OA groups (Table I).

Expression and release of IL-6, IL-6R and gp130 by non-OA and OA chondrocytes

Intracellular expression and basal release of IL-6 were significantly higher in OA than in non-OA chondrocytes (Fig. 1A,D), but comparable in hip and knee OA chondrocytes. Between non-OA and OA hip chondrocytes, the effect size was very large for intracellular IL-6 ($|d| = 1.64$, $p = 0.043$) and basal release of IL-6 ($|d| = 1.65$, $p < 0.001$). Correlation analysis including chondrocytes from non-OA and OA patients revealed a strong correlation between basal IL-6 release and intracellular IL-6 expression ($N = 15$; $R = 0.786$ CI: 0.368, 0.960; $p < 0.001$).

Both non-OA and OA chondrocytes exhibited quantitatively similar intracellular expression of IL-6R and gp130 (Fig. 1B,C). Non-OA

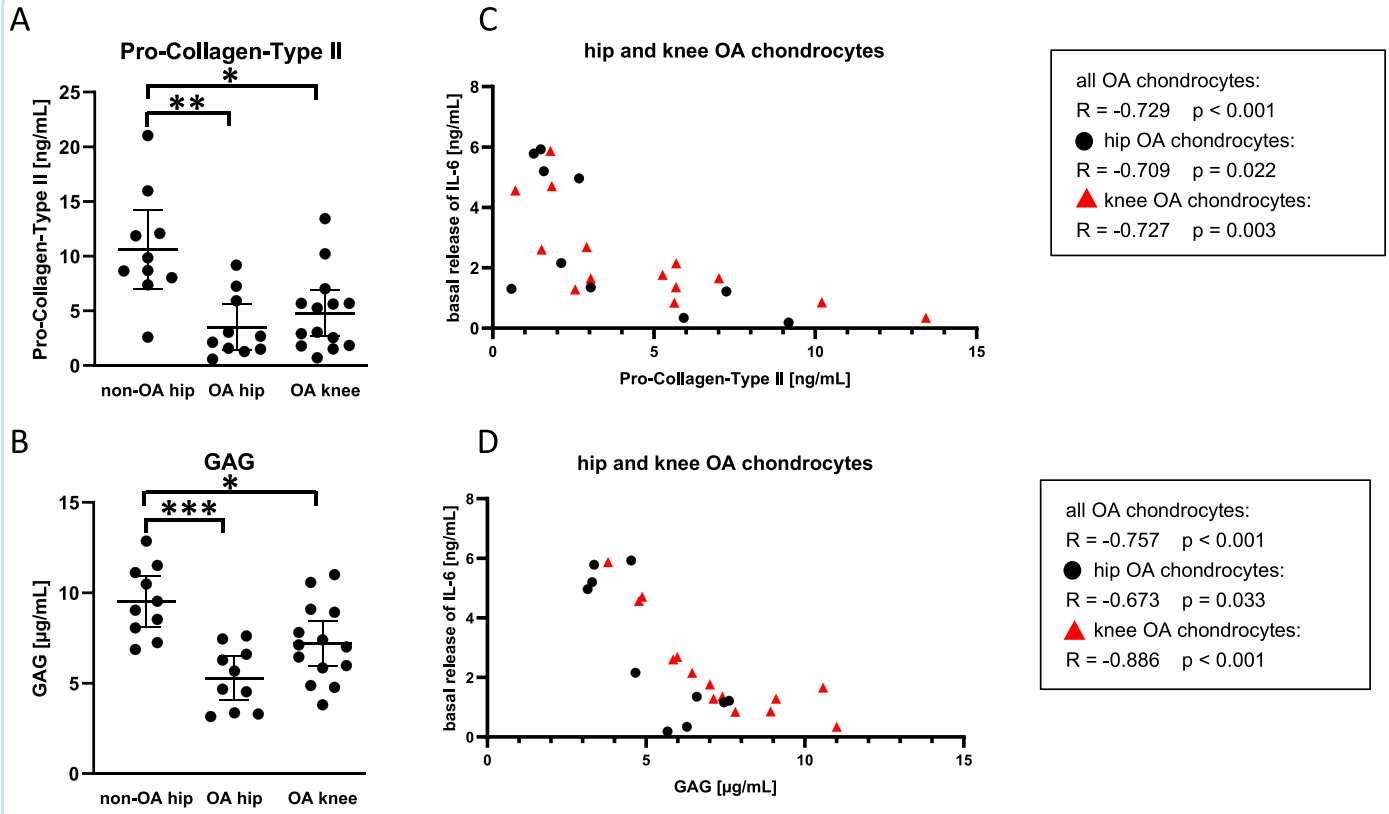

Fig. 2

Osteoarthritis and Cartilage

Production and release of the cartilage-matrix-proteins by human non-OA and OA chondrocytes. (A) Comparison of basal release of Pro-Collagen Type II in human non-OA hip (n = 10), OA hip (n = 10) and OA knee (n = 14) chondrocytes. (B) Comparison of basal release of GAG in human non-OA hip (n = 10), OA hip (n = 10) and OA knee (n = 14) chondrocytes. Statistical differences between groups (A, B): Mann-Whitney U test with Bonferroni adjustment for multiple comparisons. ***p < 0.005, **p < 0.01, *p < 0.05. (C) Correlation analysis between the basal release of IL-6 and Pro-Collagen-Type II of hip and knee OA chondrocytes (n = 24, R = Spearman's Rank correlation coefficient). (D) Correlation analysis between the basal release of IL-6 and GAG of hip and knee OA chondrocytes (n = 24, R = Spearman's Rank correlation coefficient). The patients included in each experiment and their demographic parameters are listed in Supplementary Table III.

and OA hip chondrocytes showed a quantitatively similar release of sIL-6R and sgp130, but the release from OA knee chondrocytes was significantly higher (Fig. 1E,F).

Pro- and anti-inflammatory mediators known to be present in synovial fluid, modified the release. IL-6 secretion was increased by stimulation with IL-1β, TNF, and PGE_2, and unaffected by stimulation with NGF, IL-8, IL-11, TGF-β, and PGD_2 (Fig. 1G). The sIL-6R release was decreased after stimulation with IL-1β, TNF, TGF-β, IL-6, and upregulated after stimulation with PGD_2 (Fig. 1H). The release of sgp130 was increased by stimulation with IL-1β, TNF, PGD_2 and decreased by TGF-β and PGE_2 (Fig. 1I). Calculated $|d|$ are presented in Supplementary Table II.

Since OA chondrocytes can produce and release IL-6, IL-6sR and sgp130, IL-6 classic- and trans-signaling and inhibition of IL-6-signaling are possible (Fig. 1J).

Association of basal IL-6 and cartilage-matrix-protein production in OA chondrocytes

To evaluate whether basal IL-6 production affects the production of cartilage-matrix-proteins in OA chondrocytes, a correlation analysis was performed including all values of GAG and Pro-Collagen-Type II production in OA hip and OA knee chondrocytes, as shown in Fig. 2. The production of both cartilage-matrix-proteins was similar between OA hip and OA knee chondrocytes (Fig. 2A,B), whereas non-OA hip chondrocytes produced significantly more Pro-Collagen-Type II ($|d| = 1.72$, $p = 0.0058$) and GAG ($|d| = 2.33$, $p < 0.001$) than OA hip chondrocytes. However, basal IL-6 release showed a strong negative correlation with the production of Pro-Collagen-Type II (Fig. 2C, $R = -0.729$ CI: −0.892, −0.376; $p < 0.001$) and GAG (Fig. 2D, $R = -0.757$ CI: −0.918, −0.399; $p < 0.001$) in hip and knee OA chondrocytes.

Impact of IL-6-signaling on pain mediators and cartilage-matrix-proteins in knee OA chondrocytes

To evaluate the potency of IL-6 classic- and trans-signaling in the induction and release of pain-related mediators and cartilage-matrix-components, knee OA chondrocytes were stimulated with either IL-6 alone or IL-6/sIL-6R complex. Stimulation with IL-6 alone did not significantly affect the production of NO, PGE_2, GAG, Pro-Collagen-Type II, MMP1 and TIMP1 (Fig. 3). The IL-6/sIL-6R complex significantly increased the release of NO (Fig. 3A, $|d| = 3.14$, $p < 0.001$) and PGE_2 compared to unstimulated control (Fig. 3C, $|d| = 2.69$, $p < 0.001$). The IL-6/sIL-6 complex only slightly reduced

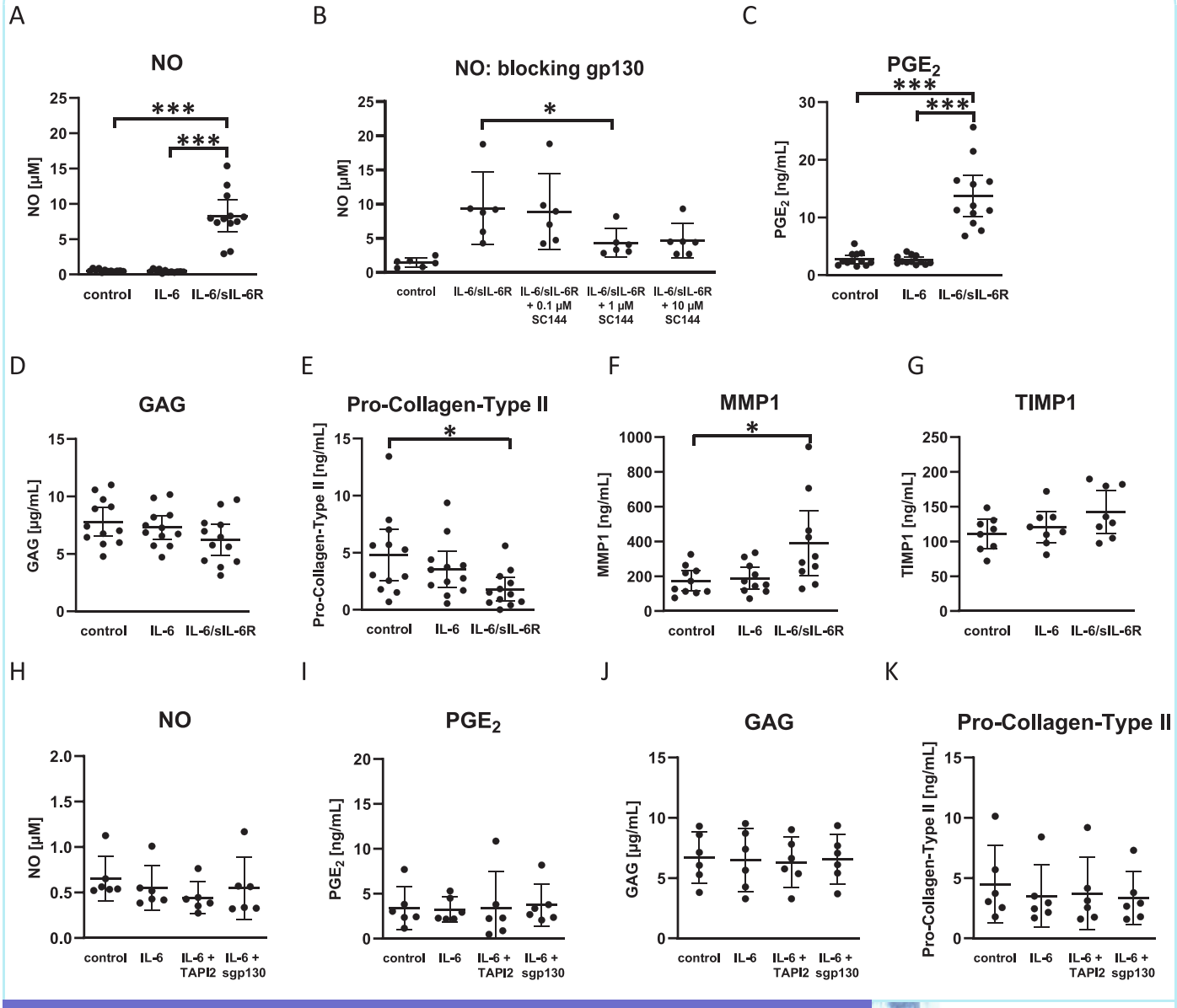

Fig. 3

Osteoarthritis and Cartilage

Effect of IL-6 classic- and trans-signaling on the release of mediators and cartilage-matrix-proteins from human knee OA chondrocytes. Effect of IL-6 and IL-6/sIL-6R complex on the release of the pain mediators (A, B) NO (A: n = 12; B: after blockade of gp130-signaling by SC144, n = 6) and (C) PGE_2 (prostaglandin E2) (n = 12), the cartilage-matrix-proteins (D) GAG (glycosaminoglycan) (n = 12) and (E) Pro-Collagen-Type II (n = 12), the matrix-metalloproteinase (F) MMP1 (matrix-metalloproteinase 1) (n = 10) and (G) the metallopeptidase inhibitor TIMP1 (n = 8). Effect of IL-6 classic-signaling via simultaneous blockade of IL-6 trans-signaling by TAPI2 or sgp130 on the production/release of (H) NO, (I) PGE_2, (J) GAG and (K) Pro-Collagen-Type II (H-K: n = 6). Statistical differences between groups or stimulated vs. unstimulated cells (control), Mann-Whitney U test with Bonferroni adjustment for multiple comparisons, ***$p < 0.005$, *$p < 0.05$. The patients included in each experiment and their demographic parameters are listed in Supplementary Table III. gp130, glycoprotein 130; NO, nitric oxide; SC144, 2-(7-Fluoropyrrolo[1,2-a]quinoxalin-4-yl) 2-pyrazinecarboxylic acid hydrazide hydrochloride; TAPI2, TNF protease inhibitor-2; TIMP1, tissue inhibitor of metalloproteinases-1.

the production of GAG (Fig. 3D), but significantly reduced the release of Pro-Collagen-Type II (Fig. 3E, $|d| = 1.08$, $p = 0.033$) and upregulated the production of MMP1 (Fig. 3F, $|d| = 1.12$, $p = 0.038$). IL-6/sIL-6 complex only slightly increased the production of the MMP-inhibitor TIMP1 (Fig. 3G). The upregulation of NO evoked by IL-6/sIL-6R complex was reduced by the gp130 inhibitor SC144 at a concentration of 1 µM (Fig. 3B, $p = 0.049$).

IL-6 stimulation had no effect on NO, PGE_2, GAG or Pro-Collagen-Type II production when IL-6-trans-signaling was blocked by either TAPI2 (inhibits shedding of sIL-6R from membrane-bound IL-6R by a disintegrin and metalloproteinase), or sgp130 (neutralizes IL-6/sIL-6R complexes) (Fig. 3H-K). Thus, only IL-6-trans-signaling but not classic-signaling mediates IL-6 effects in OA chondrocytes.

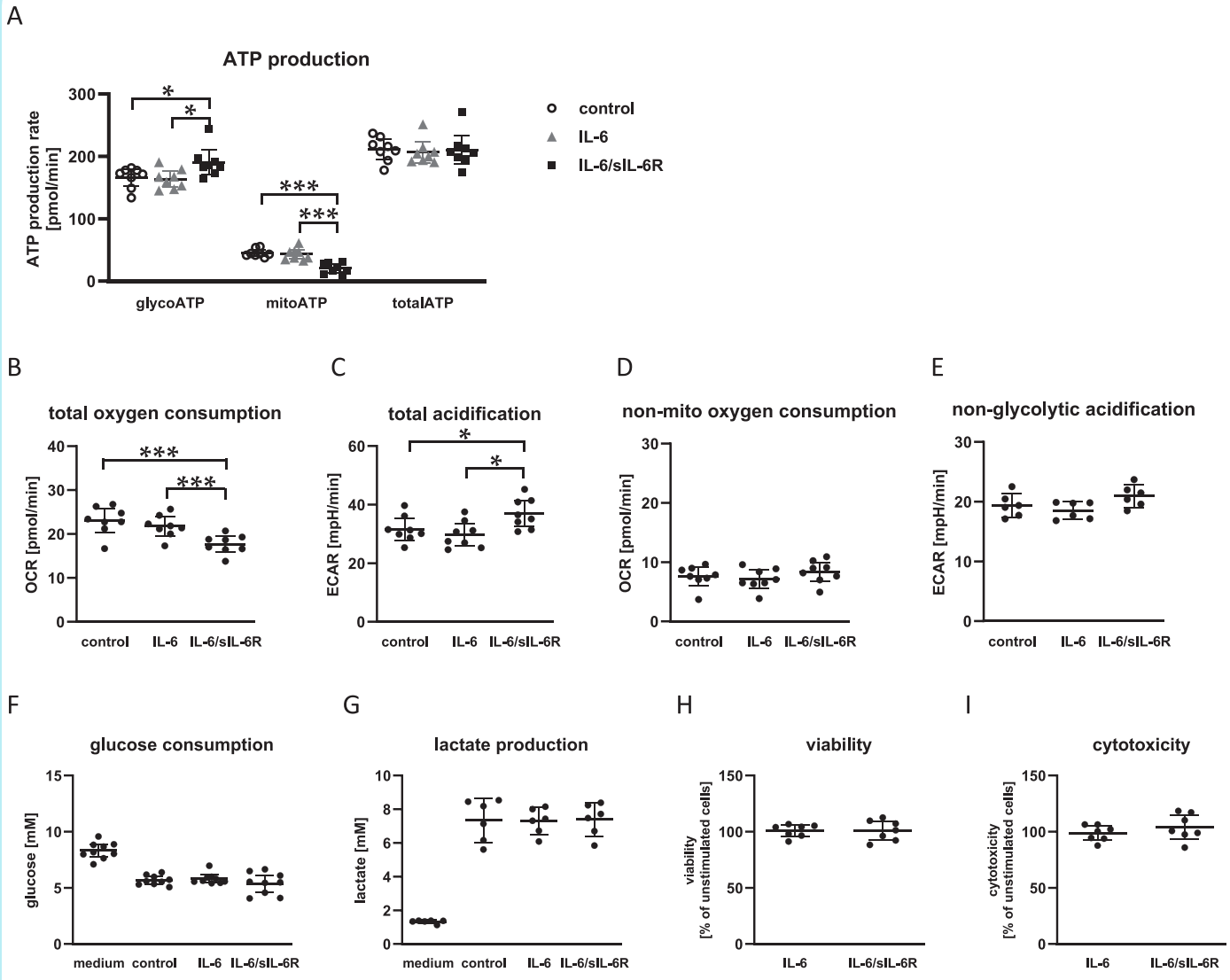

Fig. 4

Osteoarthritis and Cartilage

Effect of IL-6 and IL-6/sIL-6R complex on ATP production, metabolic parameters and viability of human knee OA chondrocytes. Effect of IL-6 and IL-6/sIL-6R complex on (A) mitochondrial, glycolytic and total ATP production rate (n = 8), (B) total oxygen consumption rate (n = 8), (C) glycolytic acidification rate (n = 8), (D) non-mitochondrial oxygen consumption rate (n = 8), (E) non-glycolytic acidification rate (n = 6), (F) glucose concentration in the supernatants of unstimulated and stimulated cells compared to the concentration of the original medium (without cells) (n = 9), (G) lactate concentration in the supernatants of unstimulated and stimulated cells compared to the concentration of the original medium (without cells) (n = 6), (H) viability (n = 7) and (I) cytotoxicity (n = 7). Statistical differences between groups or stimulated vs. unstimulated cells (control): Mann-Whitney U test with Bonferroni adjustment for multiple comparisons. Statistical analysis of normalized data vs. control value (H, I): one-sample Wilcoxon signed rank test with Bonferroni adjustment for multiple comparisons. ***$p < 0.005$, *$p < 0.05$. The patients included in each experiment and their demographic parameters are listed in Supplementary Table III. ATP, adenosintriphosphat.

Impact of IL-6-signaling on ATP production, metabolism and viability in knee OA chondrocytes

Since OA affects mitochondria and glycolysis, the effect of IL-6-signaling on both was determined by measuring oxygen consumption rate (OCR) and ECAR using Seahorse analyzer (Fig. 4, all $|d|$ in Supplementary Table II). IL-6/sIL-6R complexes increased glycolytic ATP production ($p = 0.047$) and decreased mitochondrial ATP ($p = 0.002$), whereas total ATP production was comparable in all groups (Fig. 4A). Total OCR was significantly decreased ($p = 0.0046$) and total ECAR was significantly increased ($p = 0.036$) after stimulation with IL-6/sIL-6R complex, but not after stimulation with IL-6 alone (Fig. 4B,C). Non-mitochondrial oxygen consumption was similar in stimulated and unstimulated chondrocytes (Fig. 4D). Non-glycolytic acidification was slightly (but not significantly) increased after stimulation with IL-6/sIL-6R complex (Fig. 4E). Glucose

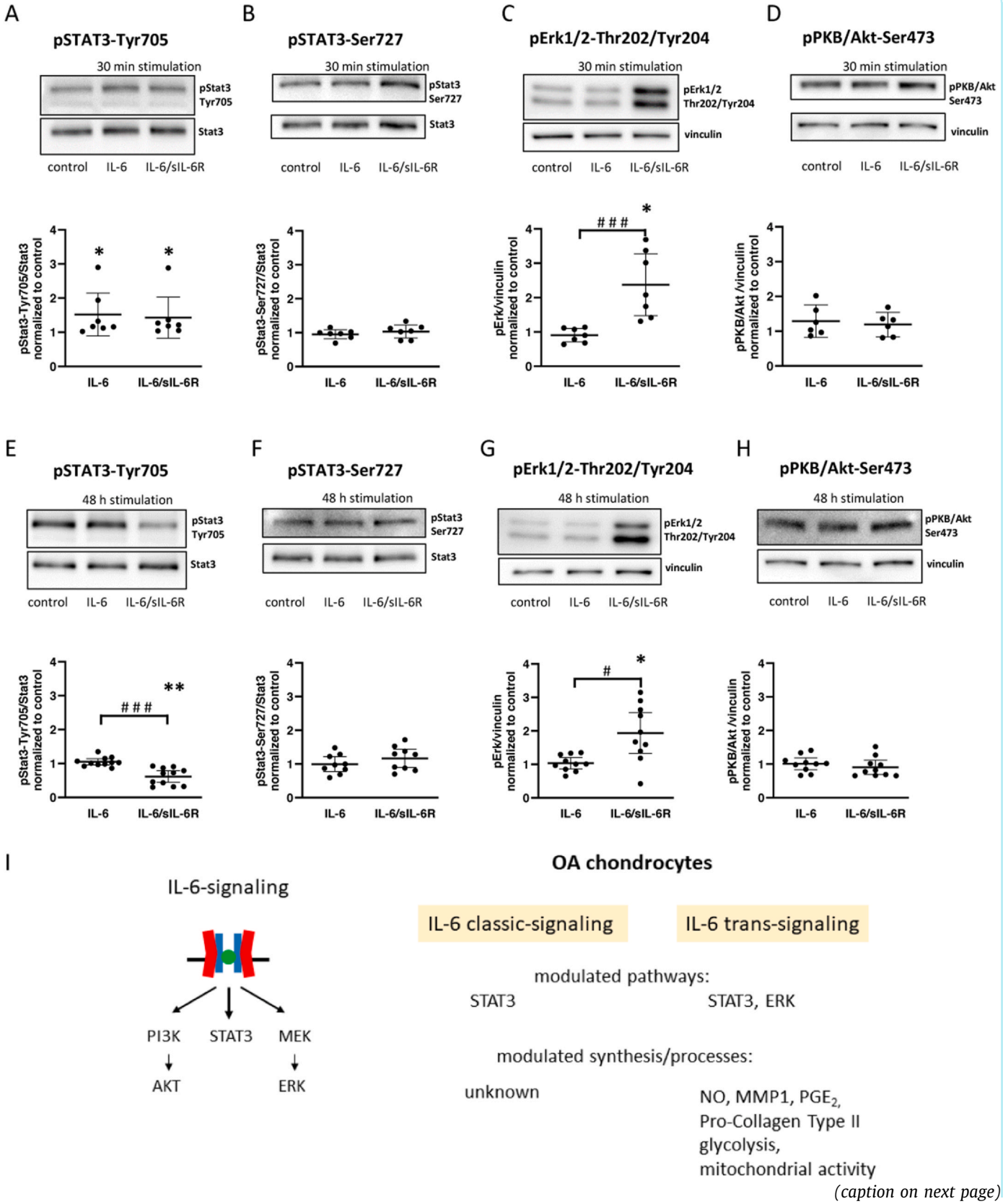

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Fig. 5 Osteoarthritis and Cartilage

Effect of IL-6 and IL-6/sIL-6R complex on IL-6-signaling in human knee OA chondrocytes. Representative Western blots and densitometric quantification of signaling molecules after stimulation with IL-6 or IL-6/sIL-6R complex for 30 min: (A) phosphorylation of STAT3 at tyrosine 705 (n = 7), (B) phosphorylation of STAT3 at serine 727 (n = 7), (C) phosphorylation of Erk1/2 (n = 7), (D) phosphorylation of PKB/Akt (n = 6), and after stimulation with IL-6 or IL-6/sIL-6R complex for 48 h: (E) phosphorylation of STAT3 at tyrosine 705 (n = 11), (F) phosphorylation of STAT3 at serine 727 (n = 9), (G) phosphorylation of Erk1/2 (extracellular signal-regulated kinase) (n = 10), (H) phosphorylation of PKB/Akt (n = 10). The effects of IL-6 classic- and trans-signaling in OA chondrocytes are summarized in Fig. 5I. Statistical analysis: one-sample Wilcoxon signed rank test was used to test for differences between stimulated vs. normalized control value (1), $^{*}p < 0.05$, $^{**}p < 0.01$; Mann-Whitney U test was used to test for differences between IL-6-stimulated vs. IL-6/sIL-6R-stimulated cells, $^{\#\#\#}p < 0.005$, $^{\#}p < 0.05$. Bonferroni adjustment was performed for multiple comparisons. The patients included in each experiment and their demographic parameters are listed in Supplementary Table III. PKB/Akt, protein kinase B; STAT3, signal transducer and activator of transcription 3.

consumption was approximately 2.6-2.9 mM after 48 h of cell culture and not affected by IL-6 or IL-6/sIL-6R complex (Fig. 4F). Similarly, lactate was increased by approximately 6 mM within 48 h and not affected by IL-6-signaling (Fig. 4G). Neither IL-6 nor IL-6/sIL-6R complexes altered chondrocyte viability or were cytotoxic (Fig. 4H,I).

IL-6-signaling in knee OA chondrocytes

The activation of typical IL-6-pathway molecules (see Fig. 5I) was evaluated in unstimulated OA chondrocytes and after stimulation with IL-6 or IL-6/sIL-6R complexes. Unstimulated knee OA chondrocytes exhibited phosphorylated STAT3 at tyrosine 705 and serine 727 residues (Fig. 5A,B). Stimulation with either IL-6 or IL-6/sIL-6R complexes for 30 min slightly increased the amount of phosphorylated STAT3 at tyrosine 705 (by approximately 17% and 21%, respectively), but only IL-6/sIL-6R complexes activated the Erk1/2-pathway (Fig. 5A,C). After stimulation with IL-6/sIL-6R complex for 48 h, the amount of phosphorylated STAT3 at tyrosine 705 was reduced, whereas the Erk1/2 pathway was still activated (Fig. 5E,G, Supplementary Fig. 1). Stimulation with IL-6 or IL-6/sIL-6R complex did not significantly change the level of phosphorylated STAT3 at serine 727 or PKB/Akt (Fig. 5B,D,F,H) or SOCS3 expression (Supplementary Fig. 1). Fig. 5I visualizes the observed effects of IL-6 classic- and trans-signaling in OA chondrocytes.

Impact of neutralization of IL-6-signaling in knee OA chondrocytes

To evaluate whether IL-6 neutralization as a potential OA therapy affects chondrocyte metabolism, neutralizing antibodies against IL-6 and IL-6R, or sgp130, or TAPI2 were applied to OA chondrocytes. The antibody against IL-6 reduced IL-6 levels in the supernatant by 96% ($p = 0.009$), and the antibody against IL-6R reduced sIL-6R levels in the supernatant by 70% ($p = 0.009$), indicating effective neutralization (Fig. 6A,B). Application of IL-6R AB, sgp130 or TAPI2 had no significant effect on the basal release of IL-6 (Fig. 6A). Neither the viability of chondrocytes nor the release of NO and PGE_2 or the production of cartilage-matrix-proteins were affected by the neutralization of IL-6-signaling with these compounds (Fig. 6C-H).

However, blockade of gp130-signaling by SC144 significantly reduced the basal release of NO ($|d| = 1.53$, $p = 0.037$) and IL-6 ($|d| = 1.29$, $p = 0.037$) with no effect on GAG production and cytotoxicity (Fig. 6I-L, all $|d|$ in Supplementary Table II).

Discussion

IL-6 is known to play a role in OA progression and pain, and could be a potential target for a therapeutic approach. Interestingly, we found high autocrine IL-6 production and persistent STAT3 activation in human OA chondrocytes. The amount of autocrine IL-6 was significantly negatively correlated with Pro-Collagen-Type II and proteoglycan production. Both non-OA and OA chondrocytes express and release sIL-6R and sgp130, which can be modulated by various mediators. Induced IL-6-trans-signaling by IL-6/sIL-6R complexes, but not IL-6 classic-signaling, increased the production of NO, PGE_2 and MMP1, glycolytic ATP, and decreased the production of cartilage-matrix-proteins and mitochondrial ATP. Neutralization of IL-6-signaling by antibodies against IL-6 or IL-6R for 48 h had no negative or positive effects on OA chondrocyte metabolism. Only blockade of IL-6 signal transduction by a gp130 inhibitor reduced NO and IL-6 release.

The tonic release of IL-6 by OA chondrocytes is an important finding. IL-6 from chondrocytes contributes to the IL-6 concentration in synovial fluid, which is much higher than the serum concentration.[3,7] Since IL-6-signaling activates STAT3, the strong STAT3 activation in the absence of exogenous IL-6 or IL-6/sIL-6R complexes may reflect persistent endogenous IL-6-signaling (but see below). In general, STAT3 activation up-regulates IL-6 autocrine production through IL-6 gene expression. Cancer cells and osteoblastic cells may show a feed-forward autocrine feedback loop with persistent high STAT3 activation, which cannot be further up-regulated by IL-6.[13–16] Since IL-6 production was negatively correlated with cartilage-matrix-protein production in OA chondrocytes, high autocrine IL-6 production and persistent STAT3 activation may reflect an osteoarthritic chondrocyte phenotype, indicating a deregulation of chondrocyte metabolism and a switch to catabolic processes.

Our results provide the first evidence that non-OA and OA chondrocytes produce and release sIL-6R (essential for IL-6-trans-signaling) and sgp130 (may limit IL-6-trans-signaling). Little is known about the regulation of sIL-6R and sgp130, which is based on differential messenger ribonucleic acid splicing and proteolytic cleavage.[17] Numerous clinical disorders are characterized by altered levels of sIL-6R and sgp130.[17]

The basal release of IL-6, sIL-6R and sgp130 was modified by pro- and anti-inflammatory mediators, in some cases in opposite directions. The strongest stimulators of IL-6 release were IL-1β and TNF both of are important in OA pathogenesis. Interestingly, both cytokines decreased the release of sIL-6R and increased the release of sgp130, thus possibly limiting IL-6 effects to some extent.

An important question of the study was whether the induced IL-6 effects are mediated by IL-6 classic- or trans-signaling. We found that all evoked IL-6 effects were mediated by IL-6/sIL-6R complexes and not by IL-6 alone, including increased release of NO, PGE_2, MMP1, reduced production of cartilage-matrix-proteins, and metabolic switch to aerobic glycolysis. All of these effects contribute to OA pathology, suggesting that the degenerative effects of IL-6 reported in previous studies[11,18–20] are produced by IL-6 trans-signaling.

In OA chondrocytes of the present study, the protective effects of IL-6 classic-signaling observed in regenerative and healthy chondrocytes[12] were not found. It is possible that IL-6 classic-signaling is no longer functional in OA chondrocytes. This is important because

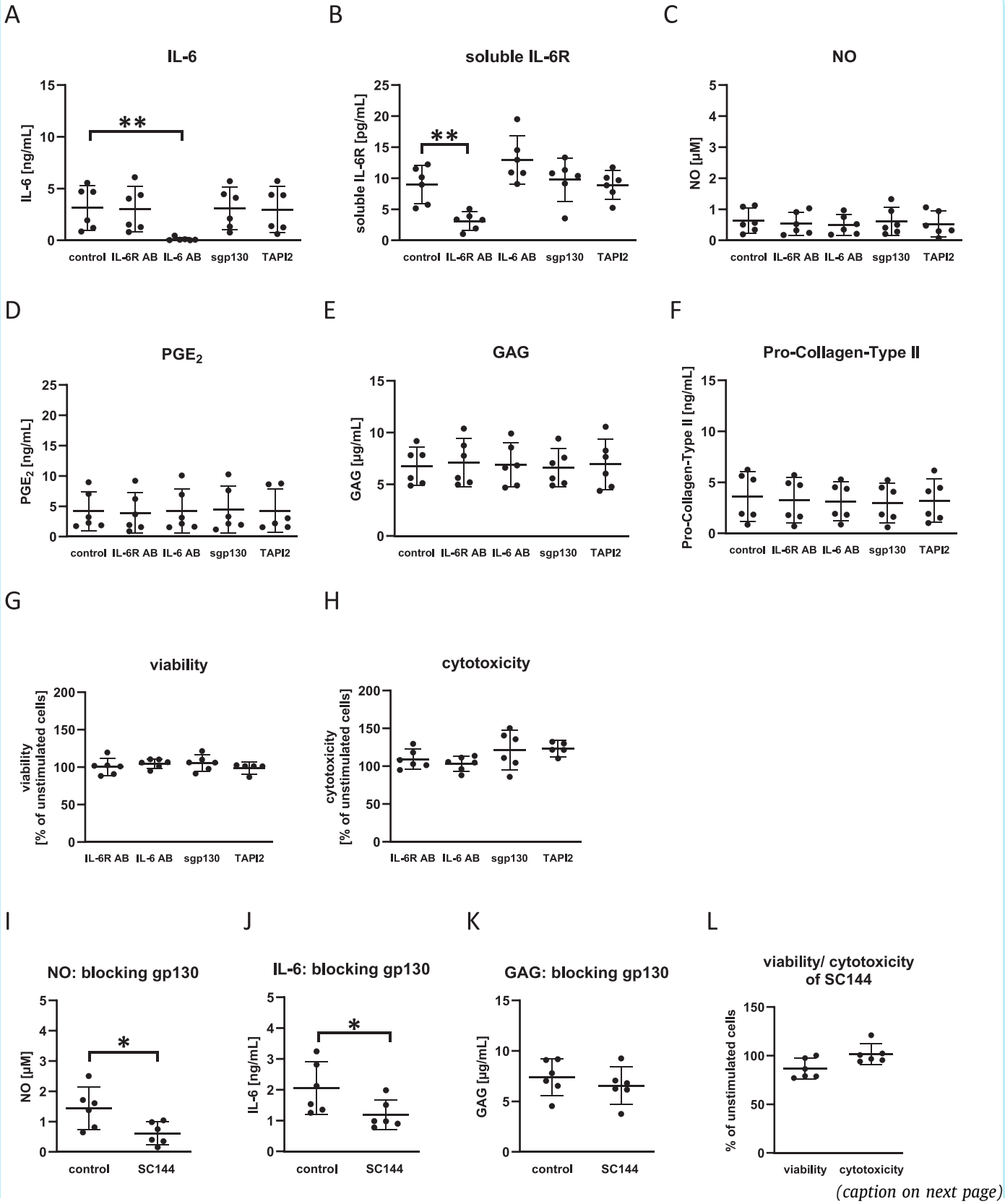

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Fig. 6 Osteoarthritis and Cartilage

Effect of neutralization of IL-6 signaling on the basal release of (A) IL-6, (B) sIL-6R, on the production/release of (C) NO, (D) PGE_2 (prostaglandin E2), (E) GAG (glycosaminoglycan) and (F) Pro-Collagen-Type II, and on (G) viability and (H) cytotoxicity of human chondrocytes (all n = 6). Effect of blocking gp130 signaling by SC144 on the basal release of (I) NO, (J) IL-6, (K) GAG, and on (L) viability and cytotoxicity (all n = 6). Statistical differences between stimulated vs. unstimulated cells (control): Mann-Whitney U test with Bonferroni adjustment for multiple comparisons. Statistical analysis of normalized data vs. control value (G, H, L): one-sample Wilcoxon signed rank test with Bonferroni adjustment for multiple comparisons. $^{**}p < 0.01$, $^{*}p < 0.05$. The patients included in each experiment and their demographic parameters are listed in Supplementary Table III. gp130, glycoprotein 130; NO, nitric oxide; SC144, 2-(7-Fluoropyrrolo[1,2-a]quinoxalin-4-yl) 2-pyrazinecarboxylic acid hydrazide hydrochloride.

then therapeutic neutralization of IL-6-signaling, e.g. with the aim of treating OA pain, does not additionally harm chondrocytes.

The STAT3 pathway is essential for the effects of IL-6. In tumor cells, STAT3-mediated metabolic transformation leads to a metabolic switch towards aerobic glycolysis by transcriptional up-regulation of glycolytic proteins and by direct regulation of mitochondrial function independent of transcriptional activity.[21] Similarly, we found an increased aerobic glycolysis in OA chondrocytes after stimulation with IL-6/sIL-6R complexes. Furthermore, STAT3 activation in chondrocytes, also found by Latourte et al.,[11] mediates IL-6-induced down-regulation of Collagen-Type II and proteoglycan.[22,23] We clearly show that autocrine IL-6 production is strongly negatively correlated with the production of cartilage-matrix-proteins. Therefore, the amount of autocrine IL-6 production and release may reflect the transformation process of OA chondrocytes. This may explain why IL-6 concentration in synovial fluid is associated with increased risk of OA progression in patients after meniscectomy,[24] and why IL-6 is a significant predictor of OA.[25,26]

In our study, application of IL-6 or IL-6/sIL-6R complex further increased the high level of tyrosine phosphorylation of STAT3, and additionally, the IL-6/sIL-6R complexes activated the extracellular signal-regulated kinase (ERK) pathway. Interestingly, long-term activation of the ERK pathway corresponds to decreased tyrosine phosphorylation of STAT3 in our experiments. Erk1/2-mediated inhibition of STAT3 has also been found in cancer cells, suggesting that ERK activation provides negative feedback by limiting STAT3 activation.[27,28] Modulation of SOCS3 expression by IL-6-induced STAT3 activation, another negative feedback mechanism which down-regulates the JAK/STAT pathway, was not found in OA chondrocytes.

Neutralization of IL-6 or blocking of IL-6R by antibodies is considered a promising OA therapy. In animals, IL-6 neutralization had a protective effect in experimental OA.[11,19] However, the first clinical trial, which applied systemically tocilizumab to neutralize IL-6R, failed to improve OA pain in hand OA.[29] Here we found that antibodies against IL-6 or IL-6R successfully neutralized IL-6 and sIL-6R released from chondrocytes of patients with late-stage OA, but conspicuously these antibodies did not improve chondrocyte metabolism and cartilage-matrix-protein production. One reason may be that STAT3 activation is not only induced by IL-6-signaling at the outer cell membrane. Autocrine production of IL-6 can intracellularly induce IL-6 classic- and trans-signaling by activation of intracellular gp130 and IL-6R, which may be located in endosomes, endoplasmic reticulum or Golgi bodies.[30] Lamertz et al. showed that neutralization of IL-6-signaling by extracellular inhibitors or by antibodies had no effect on intracellular induced IL-6-signaling or STAT3 activation.[31] Since an intracellularly active JAK inhibitor prevented IL-6-signaling, they concluded that autocrine signaling may be initiated from intracellular compartments.[31] Also in liver carcinoma, neutralizing gp130 antibodies were found to fail to inhibit STAT3 activation in cells, which showed gp130 mutations with persistent high gp130-signaling and ligand-independent STAT3 phosphorylation.[32] Blocking gp130-signaling with an intracellularly active gp130 inhibitor such as SC144 is a promising approach for cancer therapy.[33,34] First experiments with a gp130 modulator in a surgically-induced OA model showed promising results.[1] In our experiments, application of a gp130 inhibitor reduced the high basal IL-6 release from OA chondrocytes. The protective effects of IL-6 neutralization in experimental OA models[11,19] may result from early blocking of IL-6-signaling during OA induction. Once OA is established, systemically applied neutralizing IL-6R antibodies may not sufficiently inhibit the continuous high production of IL-6 nor may they be able to affect the ligand-independent intracellular mechanisms. Thus, in advanced OA, blocking intracellular IL-6-signaling may be a better target, in combination with a local application of drugs. Whether long-term inhibition of IL-6-signaling can abolish autocrine IL-6 release and STAT3 activation, increase cartilage-matrix-protein production and finally ameliorate OA should be evaluated.

A limitation of the present study is the limited number of experiments with healthy chondrocytes. Thus, the effects of IL-6-signaling were analyzed in knee OA chondrocytes, but not in healthy chondrocytes. For evaluation of IL-6 neutralization for OA treatment, additional experiments on healthy chondrocytes are necessary. Additional limitations of the study include: a) study design may have introduced donor-specific effects, b) results were not validated at the tissue level, c) results were not tested for effects of sexual dimorphism. Sexual dimorphism can influence the onset and development of OA, and recent work has also shown sex differences in chondrocyte biology.[35] Hernandez et al. found sexual dimorphism in the pericellular matrix, but gene expression of proteoglycan, collagen type II, and IL-6 were not significantly different between females and males.[36] In addition, Jain et al. found sexual dimorphism in phenotype and energy metabolism, including higher Collagen-Type II release in male vs. female chondrocytes, but similar Collagen-Type II gene expression.[37]

Funding source

This research was supported by the Deutsche Forschungsgemeinschaft (grant numbers: SCH 404/18-1, EI 1172/2-1).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank Ms. Lemser and Dr. Müller for excellent technical support and Dr. Lehmann from the Institute of Medical Statistics, Computer Sciences and Documentation (Jena University Hospital) for excellent advice on biostatistics.

Author contribution

AE, GOH and HGS designed the study. FCK and MA provided study materials and were involved in data collection; AE and CK performed experiments and analyzed data; AE, CK, BW and HGS

were involved in data interpretation; AE and HGS drafted the manuscript; all authors critically revised the manuscript and approved the final version for publication.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.joca.2024.02.006.

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