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TGF-β dampens IL-6 signaling in articular chondrocytes by decreasing IL-6 receptor expression

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SUMMARY

Objective: Transforming growth factor-β (TGF-β) is an important homeostatic regulator of cartilage. In contrast, interleukin-6 (IL-6) is a pro-inflammatory cytokine implicated in cartilage degeneration. Cross-talk between TGF-β and IL-6 is reported in tissues other than articular cartilage. Here, we investigated regulation of IL-6 signaling by TGF-β in articular chondrocytes.

Design: Human primary chondrocytes and the human G6 chondrocyte cell line were stimulated with TGF-β1 or interleukin-1β (IL-1β). Expression of IL-6 and IL-6 receptor (IL-6R) was determined on mRNA and protein level. TGF-β regulation of IL-6 signaling via phosho-STAT3 (p-STAT3) was determined using Western blot, in presence of inhibitors for IL-6R, and Janus kinase(JAK)- and activin receptor-like kinase ALK)5 kinase activity. Furthermore, induction of STAT3-responsive genes was used as a read-out for IL-6 induced gene expression.

Results: TGF-β1 increased IL-6 mRNA and protein expression in both G6 and primary chondrocytes. Moreover, TGF-β1 stimulation clearly induced p-STAT3), which was abolished by inhibition of either IL-6R, JAK- or ALK5 kinase activity. However, TGF-β1 did not increase expression of the STAT3-responsive gene *SOCS3* and pre-treatment with TGF-β1 even inhibited induction of p-STAT3 and SOCS3 by rhIL-6. Interestingly, TGF-β1 potently decreased IL-6R expression. In contrast, IL-1β did increase IL-6 levels, but did not affect IL-6R expression. Finally, addition of recombinant IL-6R abolished the inhibitory effect of TGF-β1 on IL-6-induced p-STAT3 and downstream *SOCS3, BCL3, SAA1 and MMP1* expression.

Conclusions: In this study we show that TGF-β decreases IL-6R expression, thereby dampening IL-6 signaling in chondrocytes. This reveals a novel effect of TGF-β, possibly important to restrict pro-inflammatory IL-6 effects to preserve cartilage homeostasis.

Introduction

The main function of chondrocytes is to maintain cartilage homeostasis, which is a balance between cartilage matrix synthesis and degradation. This balance is tightly regulated by soluble anabolic and catabolic factors. During osteoarthritis (OA), the balance shifts towards catabolic processes, leading to cartilage damage. In OA cartilage, chondrocytes display a catabolic phenotype and produce matrix degrading enzymes such as matrix metalloproteinase (MMP)-13, leading to destruction of surrounding cartilage matrix[1–3]. It is unclear why OA chondrocytes display this catabolic phenotype, but both increased levels of catabolic mediators and a changed response to anabolic factors are thought to contribute to this process.

Transforming growth factor-β (TGF-β) is a crucial factor for the formation and maintenance of healthy articular cartilage[4,5]. Knock-out studies investigating loss of the TGF-β type II receptor or intracellular signaling mediator Smad3 in mice show the rapid development of severe cartilage damage[6,7]. Furthermore, TGF-β signaling is involved in protection against cartilage destruction, by counteracting the effects of catabolic, pro-inflammatory cytokines[8]. We have previously shown that TGF-β can effectively counteract pro-inflammatory IL-1β signaling and thereby prevent IL-1β induced cartilage destruction[9,10]. However, we have also shown that in OA, TGF-β protective effects in cartilage are impaired, which may be caused by a decrease in expression of TGF-β type I receptor (activin A receptor-like kinase)5 which signals via the protective

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Smad2/3 pathway[11,12]. This loss in responsiveness to TGF-β potentially contributes to the imbalance in cartilage homeostasis observed in OA.

Interleukin-6 (IL-6) is considered to be a catabolic factor for chondrocytes because several studies show a role for IL-6 in cartilage degradation[13–15]. IL-6 is present in increased levels in serum and synovial fluid of OA patients compared to unaffected individuals, and circulating IL-6 levels are even considered to be predictive for OA development[16,17]. *In vitro*, IL-6 enhances the expression of MMP-3 and -13 in bovine chondrocytes, as well as the expression of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 4 and -5[13]. Also in mice, intra-articular injection of IL-6 results in cartilage damage and increased production of MMP-3 and -13[14]. Moreover, therapeutically blocking IL-6 or its downstream mediator STAT3 during experimental OA evidently rescues cartilage damage and osteophyte formation[15].

Interestingly, regulation of IL-6 signaling by TGF-β has been reported in various cell types[18–21]. For example, TGF-β blocks IL-6-mediated phosphorylation of intracellular mediators STAT1 and STAT3 in intestinal epithelial cells, as well as the induction of ICAM expression[18]. Furthermore, TGF-β can induce the expression of suppressor of cytokine signaling (SOCS)3, a negative regulator of the IL-6 pathway, in macrophage/osteoclast precursors, but not in T-cells[20,22]. In contrast, TGF-β synergizes with IL-6 in T-cells by promoting the degradation of FOXP3[19]. This shows that TGF-β regulation of IL-6 signaling is context and cell type dependent. Although it is evident that TGF-β and IL-6 play important roles in regulating cartilage homeostasis and degeneration, a link between these two factors in chondrocytes remains to be identified. Therefore, the goal of this study was to investigate potential regulation of IL-6 signaling by TGF-β in articular chondrocytes. This will improve insights in the functional role of TGF-β in cartilage homeostasis.

Materials and methods

Patient material

Primary human chondrocytes were isolated from macroscopically intact cartilage obtained after surgery from seven anonymized OA patients undergoing total knee or hip arthroplasty (Radboud University Medical Center, Nijmegen, the Netherlands). Patients were informed about the anonymized use of this material for research and were able to refuse. According to Dutch law, informed consent is therefore not necessary.

Primary cell culture

To obtain chondrocytes, cartilage explants were digested overnight in 1.5 mg/ml Collagenase B (Roche Diagnostics) in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium (Gibco). Chondrocytes were seeded in a density of 1×10^5 cells/cm^2 in 6-well plates (Cellstar; Greiner Bio-one International) for protein experiments, or in 24-wells plates (Cellstar; Greiner Bio-one International) for gene expression experiments. Chondrocytes were cultured in monolayer for 1 week prior to experiments in DMEM/F12 supplemented with 10% fetal calf serum (FCS; Thermo Scientific), 100 mg/l pyruvate (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin in standard conditions [5% CO2 (v/v), 37 °C, 95% humidity]. Chondrocytes were serum-starved overnight in DMEM/F12 medium without FCS before start of the experiment.

Chondrocyte cell line culture

Human G6 chondrocytes are adult articular chondrocytes derived from femoral head cartilage of an anonymous OA donor, transduced with a temperature-dependent SV40 large oncogene[23]. Consequently, the G6 chondrocytes proliferate at 32°C, but not at 37°C. G6 chondrocytes were cultured at 32°C under the same conditions as primary chondrocytes, except with 5% FCS. Two days before experiments were performed, G6 chondrocytes were cultured at 37°C to stop proliferation and serum-starved overnight in DMEM/F12 medium supplemented with 0.5% FCS.

Culture with cytokines or pharmacological inhibitors

Serum starved primary chondrocytes and G6 chondrocytes were stimulated with rhTGF-β1 (Biolegend), rhIL-1β (R&D systems), rhIL-6 (Biolegend), rhIL-6R (Biolegend) or a combination of these proteins, for time periods and dosages indicated in figure legends. In experiments where inhibitors were used, dimethyl sulfoxide (DMSO) (0.05% (v/v)) was used as vehicle control. To inhibit ALK5 kinase activity, SB-505124 (Sigma Aldrich)[24] was used in a concentration of 5 μM. For inhibition of Janus-kinases (JAK-kinases), the pan-JAK inhibitor Tofacitinib (LC Laboratories) was used in a concentration of 1 μM. A humanized anti-IL-6R monoclonal antibody (Tocilizumab, RoActemra, Roche) was used to block IL-6 signaling at 1 μg/ml. Chondrocytes were pre-incubated with the inhibitors 1 h prior to addition of rhTGF-β1.

Protein measurement using Luminex technology

Luminex multianalyte technology on the Bio-Plex 200 system (Bio-Rad) in combination with multiplex cytokine kit (Bio-Rad) was used to study levels of IL-6 in 50 μl of chondrocyte culture supernatants after rhTGF-β1 stimulation. Samples below detection limit (0.40 pg/ml) were set at lowest measurable concentration to perform statistical analysis.

Protein isolation and Western blot

Chondrocytes were lysed using lysis buffer (Cell Signaling) containing protease inhibitor cocktail (Roche Diagnostics). Cell lysates were sonicated on ice using a Bioruptor (Diagenode)[25]. Protein concentration was normalized between samples using a bicinchoninic acid assay (Thermo Scientific). Samples were boiled for 10 min at 95°C in Laemmli Sample buffer (1% SDS and 100 mM Tris, pH 9.5). Proteins were separated on a 10% reducing bis-acrylamide SDS-PAGE gel, and transferred to a 0.45 μm pore nitrocellulose membrane using wet transfer (Towbin buffer, 2 h, 275 mA at 4°C). Non-specific antibody binding was blocked for 1 h with 5% non-fat dry milk (Campina) to detect IL-6R, β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or blocked in 5% bovine albumin serum to detect phospho-STAT3 (p-STAT3) or phospho-Smad2/3 (p-Smad2/3), in TBS-T (15 mM Tris-HCl, pH 7.4, 0.1% Tween). Membranes were incubated overnight at 4°C with primary antibodies directed against IL-6R (polyclonal rabbit antibody, 128,008, 1:1000, Abcam), p-STAT3 (polyclonal rabbit antibody, #9131, 1:1000, Cell Signaling), or p-Smad2/3 (polyclonal rabbit antibody, #3101, 1:1000, Cell Signaling) and afterwards labeled for 1 h with polyclonal Goat anti-Rabbit or Rabbit anti-Mouse coupled to horseradisch peroxidase (1:1500, Dako) at RT. Enhanced chemiluminescence (ECL) was used to visualize proteins with ECL prime kit (GE Healthcare) and ImageQuant LAS4000 (Leica). GAPDH was used as loading control (mouse monoclonal antibody, 1G5, 1:10,000 Sigma Aldrich). ImageJ was used for quantification of the signal.

Table 1
Template and sequence of the primers used in this study.

Gene	Forward sequence (5′➔ 3′)	Reverse sequence(5′➔ 3′)
GAPDH	ATCTTCTTTTGCGTCGCCAG	TTCCCCATGGTGTCTGAGC
IL-6	AGCCCACCGGGAACGA	GGACCGAAGGCGCTTGT
IL-6R	GTACCACTGCCCACATTCCT	CCACGTCTTCTTGAACCTCAGA
RPS27a	GTTAAGCTGGCTGTCCTGAAA	CATCAGAAGGGCACTCTCG
SOCS3	TCGGACCAGCGCCACTT	CACTGGATGCGCAGGTTCT
SAA1	GTGATCAGCGATGCCAGAGA	TCGGAAGTGATTGGGGTCTT
BCL3	GGAGGCCCGCAATTATGA	CTTAATGTCCACTGCGTCGAT
MMP1	ACTGCCAAATGGGCTTGAAG	TTCCCTTTGAAAAACCGGACTT

RNA isolation and quantitative real-time PCR

RNA was isolated using Tri-reagent (Sigma–Aldrich) following manufacturer's protocol. RNA concentrations were determined using a NanoDrop photospectrometer (Thermo Scientific). Thereafter, a maximum of 1 μg RNA was dissolved in ultrapure water and treated with 1 μl of DNAse (Life Technologies) for 15 min at room temperature to remove possible genomic DNA, followed by incubation at 65°C with 1 μl EDTA (Life Technologies). Samples were reverse transcribed into complementary DNA (cDNA) using 1.9 μl ultrapure water, 2.4 μl 10× DNase buffer, 2.0 μl 0.1 M dithiothreitol, 0.8 μl 25 mM dNTPs, 0.4 μg oligo dT primer, 1 μl 200 U/ml MMLv-reverse transcriptase (Life Technologies) and 0.5 μl 40 U/ml RNAsin (Promega) and incubated for 5 min at 25 °C, 60 min at 39 °C, and 5 min at 95 °C using a thermo-cycler. Gene expression was measured using SYBR Green Master Mix (Applied Biosystems) and 0.25 mM primers (see Table 1) (Biolegio) with a StepOnePlus real-time PCR system (Applied Biosystems). The amplification protocol was 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Melting curves were analyzed to confirm product specificity. To calculate the relative gene expression, the average of the reference genes GAPDH and ribosomal protein S27A (RPS27A) was used.

Statistical analysis

Quantitative data of gene expression analysis was expressed as column scatter or grouped category graphs and display mean values of separate experiments (G6 chondrocytes) or donors (primary chondrocytes) with corresponding 95% confidence interval (CI). Significance was tested using displayed means with repeated measures analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's post-test (see figure legends). Differences in IL-6 protein levels after rhTGF-β1 stimulation were tested using an unpaired two-tailed *t*-test. For each analysis, Gaussian distribution was tested using the D'Agostino-Pearson Omnibus K2 test. $P < 0.05$ was considered significant. All analyses were performed using Graph Pad Prism version 5.03 (GraphPad Software, La Jolla, CA).

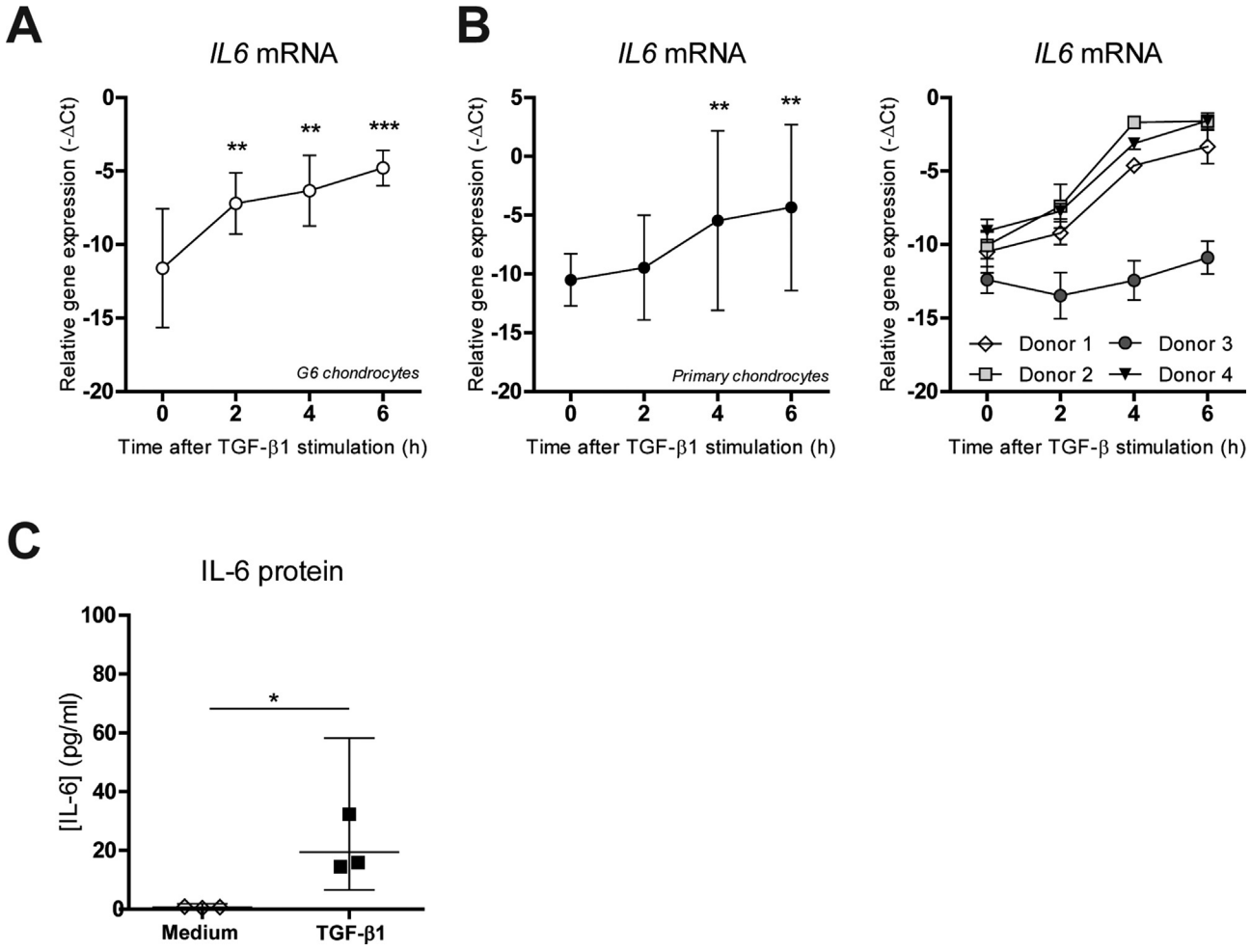

Fig. 1. TGF-β1 stimulation of human chondrocytes rapidly induces IL-6. The human G6 chondrocyte cell line and primary human chondrocytes of four donors were stimulated in triplo with 1.0 ng/ml of rhTGF-β1 for 2, 4 and 6 h to study TGF-β1-induced *IL6* gene expression using qPCR. (A) For G6 chondrocytes, the mean of three separate experimental repeats is shown with corresponding 95% confidence interval (CI). (B) For primary chondrocytes, the mean of four donors is shown with corresponding 95% CI, and individual donors are plotted showing mean ± SD of technical replicates (C) G6 chondrocytes were stimulated with 1.0 ng/ml of rhTGF-β1 for 24 h, after which the concentration of IL-6 in the supernatant was measured using Luminex technology. The means of three separate experiments are shown with corresponding 95% CI. * = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$ as measured by repeated measures ANOVA with Dunnett's post-test (A, B) or unpaired two-tailed *t*-test (C).

Results

TGF-β1 rapidly induces IL-6 in human chondrocytes

To examine if TGF-β1 can regulate IL-6 expression, G6 chondrocytes were stimulated with 1.0 ng/ml of TGF-β1. TGF-β1 significantly induced *IL6* mRNA expression after 2 h, 4 h and 6 h by respectively 21-, 38- and 112-fold (4.4, 5.3, 6.8 ΔC_t) [Fig. 1(A)]. To validate these results, we performed the same experiment in freshly isolated primary chondrocytes of four different donors. Although variation between donors was observed, TGF-β1 induced a significant increase in *IL6* expression of on average 32-fold (5.0 ΔC_t) at 4 h and 74-fold (6.2 ΔC_t) at 6 h [Fig. 1(B)]. Similar induction of *IL6* expression was observed when 0.1 ng/ml of rhTGF-β1 was used, corresponding to

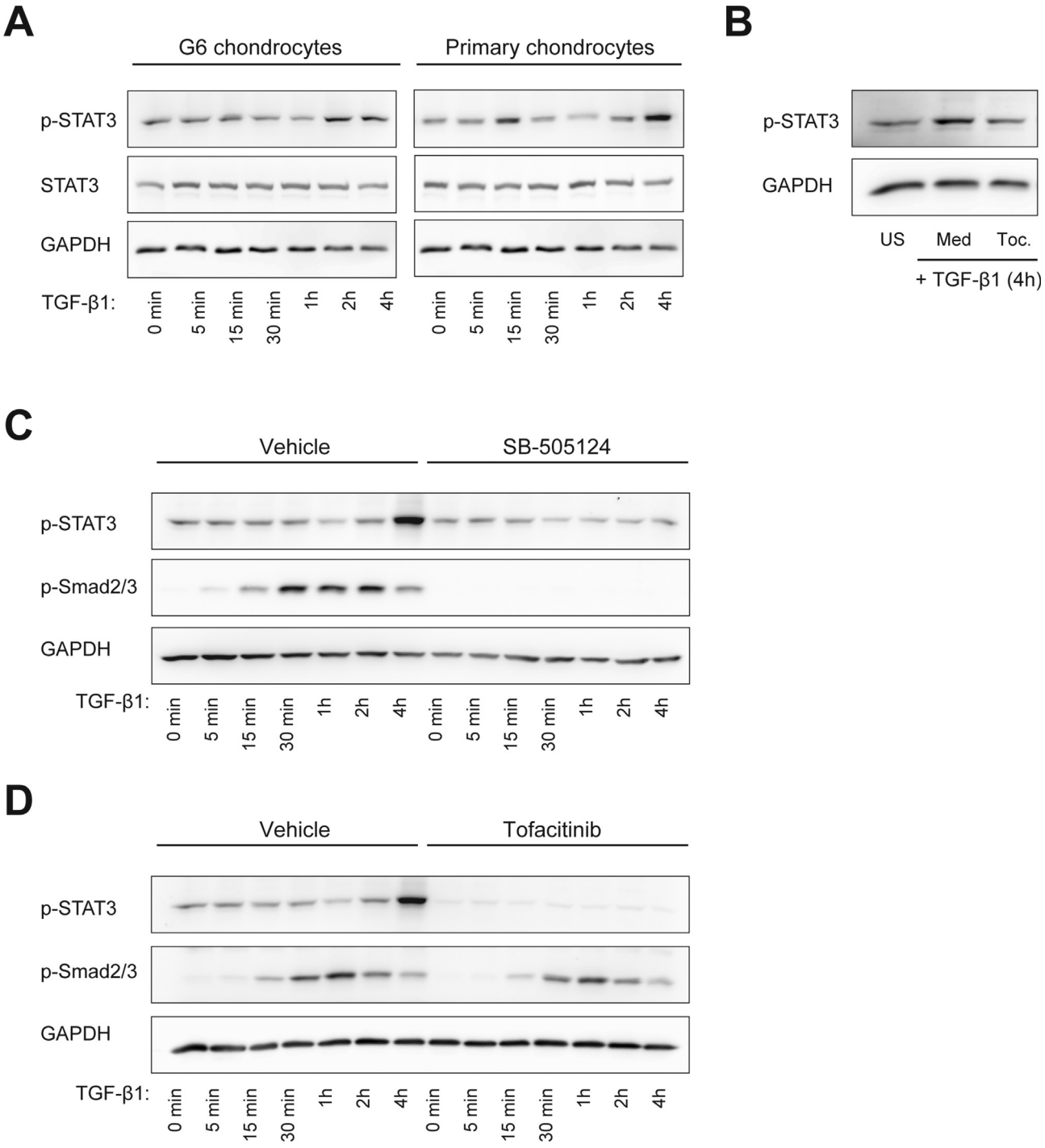

Fig. 2. TGF-β1 stimulation leads to STAT3 phosphorylation, dependent on the IL-6R, as well as ALK5- and JAK-kinase activity. (A) The human G6 chondrocyte cell line and primary human chondrocytes of three donors were stimulated with 1.0 ng/ml of rhTGF-β1 for 5, 15, or 30 min and 1, 2, and 4 h, to study TGF-β1-induced STAT3 phosphorylation with Western Blot. (B) Tocilizumab (anti-IL-6R antibody) was used to investigate if TGF-β1-induced STAT3 phosphorylation was dependent on IL-6R signaling. G6 chondrocytes were pre-incubated with 1 μg/ml of Tocilizumab for 1 h, and afterwards stimulated with 1 ng/ml of rhTGF-β1 for 4 h. Small molecule inhibitors for (C) TGF-β receptor kinase activity (SB-505124, 5 μM) and (D) JAK-kinase activity (Tofacitanib, 1 μM) were used to investigate if TGF-β1-induced STAT3 phosphorylation was dependent on ALK5- or JAK kinase activity respectively. G6 chondrocytes were pre-incubated with inhibitors or vehicle for 1 h, and stimulated with 1 ng/ml of rhTGF-β1 for indicated time points. Western Blots shown are representative of at least three independent experiments. Toc: Tocilizumab; Med: medium; US: unstimulated.

TGF-β levels found in synovial fluid of healthy donors (Supplementary Fig. 1(A))[26]. We further confirmed these results on protein level, and observed a significant 20-fold increase in IL-6 production after TGF-β1 stimulation of G6 chondrocytes [Fig. 1(C)].

TGF-β1 induces phosphorylation of STAT3 via IL-6 receptor signaling

To investigate if TGF-β1-induced IL-6 leads to autocrine signaling, we investigated the pattern of p-STAT3, an IL-6R downstream signaling protein. TGF-β1 stimulation of G6 chondrocytes induced a clear increase in p-STAT3 signal at 2 h and 4 h [Fig. 2(A)]. In contrast, total levels of STAT3 protein were not changed by stimulation with rhTGF-β1. When we studied the p-STAT3 pattern in primary chondrocytes from different donors, we found that p-STAT3 was consistently upregulated by TGF-β1 at 2 h and 4 h in all donors. In two out of four donors, we observed a minor increase at 5–15 min, but this was not consistent throughout experiments. Because G6 chondrocytes and primary chondrocytes showed similar patterns of p-STAT3 induction by TGF-β1, we used G6 chondrocytes for further inhibitor studies. To test if TGF-β1-induced p-STAT3 was IL-6R dependent, we stimulated G6 chondrocytes with TGF-β1 in presence of the IL-6R blocking antibody tocilizumab. As expected, induction of p-STAT3 by TGF-β1 was completely prevented by blockade of the IL-6R suggesting IL-6 dependency [Fig. 2(B)]. To further explore which signaling proteins are responsible for induction of IL-6 and p-STAT3 via TGF-β1 we used small molecule inhibitors. Inhibition of TGF-β receptor (ALK5) kinase activity with SB-505124 inhibited both induction of p-Smad2/3 and p-STAT3 at all time points after TGF-β1 stimulation [Fig. 2(C)]. Furthermore, addition of the pan-JAK inhibitor tofacitinib completely prevented induction of p-STAT3 by TGF-β1 and even decreased baseline levels [Fig. 2(D)]. This effect was not due to inhibition of the TGF-β signaling pathway, as the increase in p-Smad2/3 by TGF-β1 was not inhibited by tofacitinib. All together, this shows that TGF-β1-

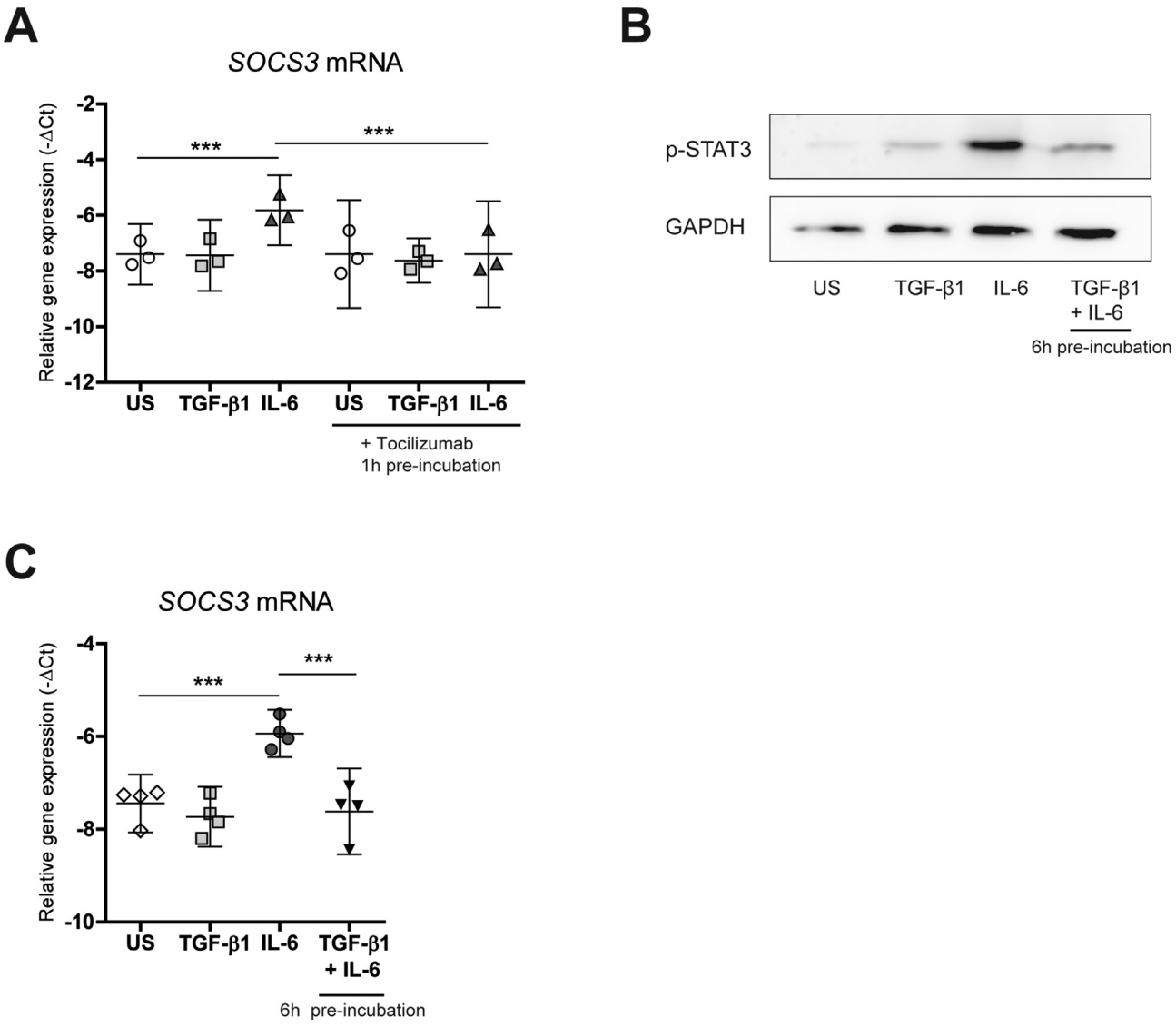

Fig. 3. TGF-β1 blocks IL-6 mediated gene expression of *SOCS3* and limits STAT3 phosphorylation. (A) To investigate the downstream effects of TGF-β1-induced IL-6 signaling, G6 chondrocytes were pre-incubated with 1 μg/ml of Tocilizumab to block IL-6R signaling, and stimulated with TGF-β1 for 6 h. Expression level of the STAT3 target gene *SOCS3* was measured as a read-out for IL-6-mediated gene expression. (B, C) To study the effect of TGF-β1 on signaling of exogenous rhIL-6, human G6 chondrocytes were pre-stimulated with or without 1.0 ng/ml of rhTGF-β1 for 6 h, and afterwards stimulated with rhIL-6 (10 ng/ml). The IL-6 response was measured by induction of STAT3 phosphorylation after 30 min (B) or induction of *SOCS3* mRNA after 6 h (C). Western Blot is representative of three independent experiments. For gene expression results, the means of three or four separate experimental repeates are plotted with corresponding ±95% CI. * = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$ as measured by repeated measurements ANOVA with Bonferroni's post-test. US: unstimulated.

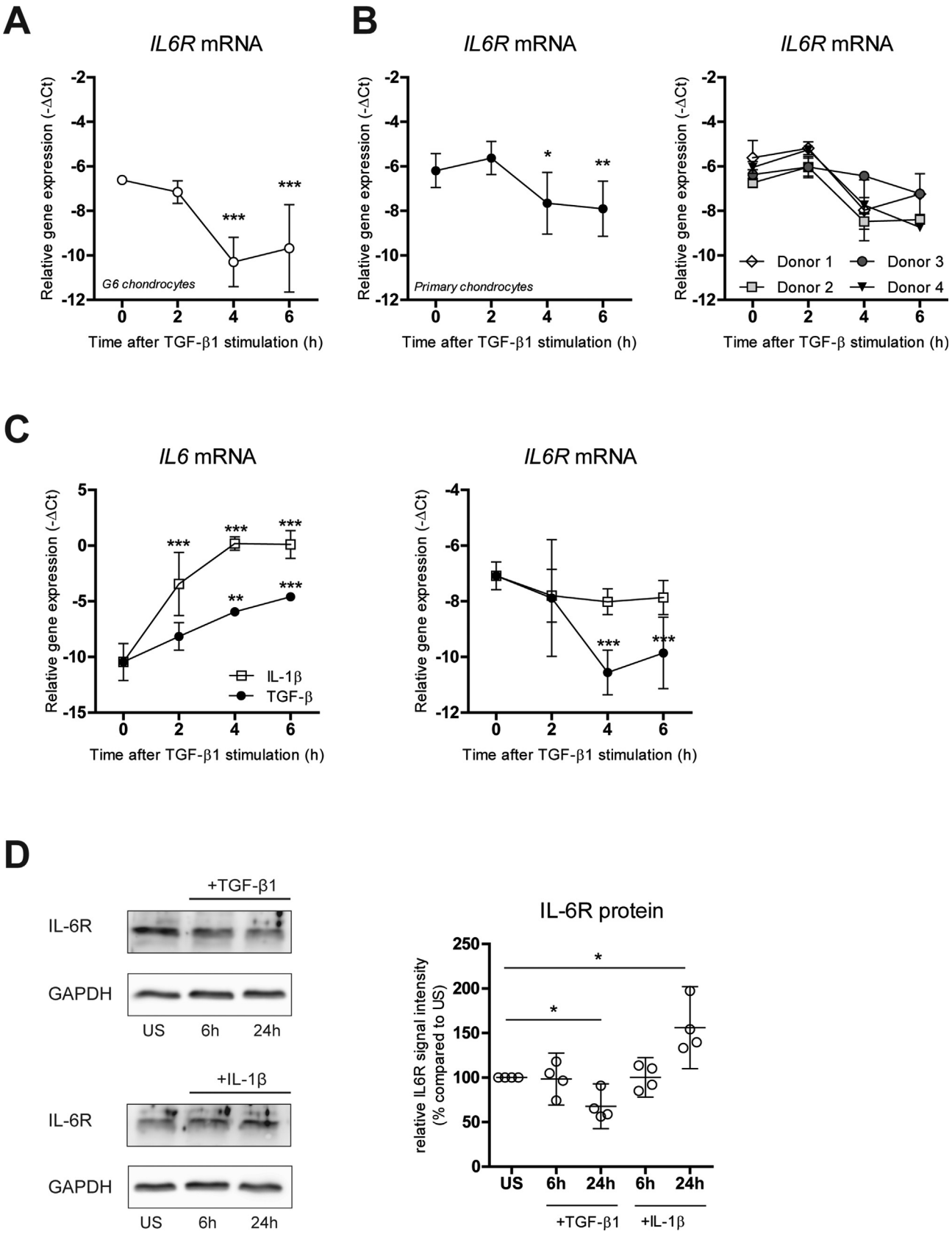

Fig. 4. TGF-β1 potently decreases IL-6 receptor levels in both G6 and primary chondrocytes. To investigate the effect of TGF-β1 on expression of the *IL6R*, G6 chondrocytes and primary human chondrocytes of four donors were stimulated in triplicate with 1.0 ng/ml of rhTGF-β1 for 2, 4 or 6 h. (A) For G6 chondrocytes, the mean of three separate experimental repeats is shown with corresponding 95% CI. (B) For primary chondrocytes, the mean of four donors is shown with corresponding 95% CI, and individual donors are plotted showing mean ± SD of technical replicates. (C) G6 chondrocytes were stimulated with 1.0 ng/ml of rhTGF-β1 or 1.0 ng/ml of rhIL-1β for 2, 4 or 6 h to study effects on *IL6* and *IL6R* expression. Mean of four separate experimental repeats is shown with corresponding 95% CI. (D) G6 chondrocytes were stimulated with 1.0 ng/ml of rhTGF-β1 or 1.0 ng/ml of

induced p-STAT3 depends on the IL-6R, and ALK5- and JAK-kinase activity.

TGF-β1 blocks IL-6-mediated gene expression of SOCS3 and limits STAT3 phosphorylation

We showed that TGF-β1 induces IL-6 signal transduction via activation of its downstream mediator STAT3. To investigate if the observed STAT3 phosphorylation results in gene transcription, mRNA expression of the well-known STAT3 target gene *SOCS3* was measured, which itself is an important negative regulator of IL-6 signaling[27]. Because *SOCS3* expression is immediately induced after p-STAT3 induction[28], we investigated the effect of TGF-β1 on *SOCS3* expression at 6 h, shortly after TGF-β1 induction of p-STAT3 (2–4 h). Surprisingly, we did not find any evidence of an increase in *SOCS3* mRNA expression by TGF-β1 [Fig. 3(A)]. Also addition of tocilizumab, which blocks TGF-β1-induced IL-6 signaling, did not significantly change *SOCS3* expression. In contrast, stimulation of G6 chondrocytes with 10 ng/ml of recombinant IL-6 did lead to a significant increase in *SOCS3* expression of 3.0-fold (1.6 ΔC_t), confirming that *SOCS3* is a IL-6/STAT3 target gene in our experimental setting [Fig. 3(A)]. Thus, stimulation with IL-6 alone increased *SOCS3* expression, but stimulation with TGF-β1 did not, while this ultimately also leads to IL-6 production. This suggests that TGF-β1 blocks IL-6-induced regulation of *SOCS3* expression. To further prove that TGF-β1 indeed inhibits IL-6 effects, we investigated if TGF-β1 could also block the effects of recombinant IL-6. We performed pre-treatment with TGF-β1 for a 6 h period, mimicking the setting where cells are first exposed to TGF-β1 and afterwards to IL-6. Interestingly, activation of STAT3 after rhIL-6 exposure was inhibited by TGF-β1 pre-treatment, but p-STAT3 levels were not fully reduced to baseline level [Fig. 3(B)]. Furthermore, TGF-β1 pre-treatment completely prevented rhIL-6-induced *SOCS3* expression [Fig. 3(C)]. These data demonstrate that TGF-β1 inhibits the IL-6 response in chondrocytes, despite upregulating IL-6 itself.

TGF-β1 potently decreases IL-6 receptor levels in articular chondrocytes

Next, we studied the effects of TGF-β1 on the IL-6R, a potential mechanism via which TGF-β could regulate IL-6 intracellular signaling. Interestingly, we observed that TGF-β1 stimulation led to a striking decrease in *IL6R* expression in G6 chondrocytes after 4 h (13-fold, 3.7 ΔC_t) and 6 h (8-fold, 3.0 ΔC_t) [Fig. 4(A)]. This effect of TGF-β1 also held true in primary chondrocytes derived from different donors, showing that the effect of TGF-β on *IL6R* expression is not donor specific. In primary chondrocytes, TGF-β1 significantly decreased *IL6R* expression after both 4 h and 6 h with an average of 2.75-fold (1.5 ΔC_t) and 3.2-fold (1.7 ΔC_t) respectively [Fig. 4(B)]. In addition, 0.1 ng/ml of rhTGF-β1, a concentration reflecting TGF-β levels in synovial fluid of healthy donors[26], decreased *IL6R* expression to the same extent (Supplementary Fig. 1(B)). To investigate if other factors stimulating IL-6 production can also regulate *IL6R* expression, we included stimulation with the pro-inflammatory cytokine IL-1β, a known inducer of IL-6. As expected, IL-1β strongly elevated *IL6* expression by 1024-fold (10.0 ΔC_t) after 4–6 h, even to a higher extent than TGF-β1 [Fig. 4(C)]. Interestingly, in contrast to TGF-β1, IL-1β did not significantly change the expression of *IL6R* in G6 chondrocytes. This suggests that IL-6 itself is not involved in regulation of *IL6R* expression. Also on protein level, we observed significantly lower IL-6R levels after 24 h of TGF-β1 stimulation of G6 chondrocytes, while IL1β even increased IL-6R levels [Fig. 4(D)]. These data show that TGF-β1 decreases IL-6R expression, which could explain why TGF-β1 inhibits IL-6 signaling.

TGF-β1 blocks the IL-6 response by decreasing IL-6 receptor levels

To determine the consequences of TGF-β1-mediated down regulation of IL-6R expression, we investigated if TGF-β1-mediated inhibition of IL-6 signaling was dependent on reduced IL-6R levels. We performed pre-treatment with TGF-β1 for a 6 h period, to ensure that IL-6R levels were decreased before stimulation with rhIL-6. In addition, we rescued IL-6R levels using a soluble form of the IL-6R. Strikingly, levels of p-STAT3 were dose-dependently increased after adding soluble IL-6R, showing that TGF-β1 inhibition of rhIL-6-induced p-STAT3 is dependent on the IL-6R [Fig. 5(A)]. On gene expression level, the addition of rhIL-6R also restored induction of *SOCS3* expression by rhIL-6 in the presence of TGF-β1 [Fig. 5(B)]. Moreover, the expression of additional IL-6-responsive genes, *MMP1*, *BCL3* and *SAA1*, was comparably regulated. Pre-treatment with rhTGF-β1 clearly inhibited the induction of these genes by rhIL-6, which was abolished by addition of rhIL-6R. All together, this indicates that TGF-β1 dampens IL-6 effects on chondrocytes by decreasing IL-6R expression (see Fig. 6).

Discussion

In this study we identified TGF-β as a key regulator of the IL-6 signaling pathway in articular chondrocytes. We show that articular chondrocytes can produce IL-6 in response to TGF-β1 but that downstream signaling is limited. We show that this inhibition is facilitated by down regulation of the IL-6R by TGF-β1, a unique mechanism which had never been reported until now.

IL-6 is a pleiotropic cytokine that has an essential role in regulating immune response and defense[29]. In OA, levels of IL-6 are enhanced in serum and the synovial fluid and correlate with disease progression[16,17]. Within the joint, both synoviocytes and chondrocytes can produce IL-6 and several inflammatory factors are known to increase IL-6 production[30–32]. We show that also stimulation with TGF-β1, a homeostatic regulator of cartilage, leads to IL-6 production in chondrocytes. Only Guerner *et al.* previously studied regulation of IL-6 production in chondrocytes in response to both catabolic and anabolic factors. They reported that TGF-β, but not epidermal-, insulin-, or platelet derived-growth factor, increased the production of IL-6 by chondrocytes[33]. This observation was surprising, because TGF-β is perceived as a homeostatic regulator of cartilage[8]. In contrast, IL-6 is mainly recognized as a catabolic mediator that activates MMPs and ADAMTSs enzymes, contributing to cartilage degradation[14,15,34,35]. Interestingly, we discovered that IL-6 induced by TGF-β1 did not lead to downstream induction of the STAT3 target gene *SOCS3*. Furthermore, TGF-β1 blocked the effects of recombinant IL-6, by limiting STAT3 phosphorylation and blocking induction of SOCS3. Together, this suggests that the presence of TGF-β can block downstream IL-6 signaling and thereby protect chondrocytes from the catabolic effects of IL-6.

Inhibition of IL-6 signaling by TGF-β had not yet been reported in articular chondrocytes. Also in other cell types only few studies investigated inhibitory effects of TGF-β on IL-6 signaling. In T-cells it has been shown that TGF-β inhibits IL-6-induced *SOCS3*

rhIL-1β for 6 h and 24 h to determine effects on IL-6R protein expression using Western Blot. Western blots are representative of four independent experiments. Quantification of the Western Blot was performed by Image J. Quantitative values of IL6R protein intensity were first corrected for GAPDH values, and then plotted as fold change (%) compared to US samples. * = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$ as measured by one-way ANOVA with Dunnett's (A, B, C) or Bonferroni's (D) post-test. US: unstimulated.

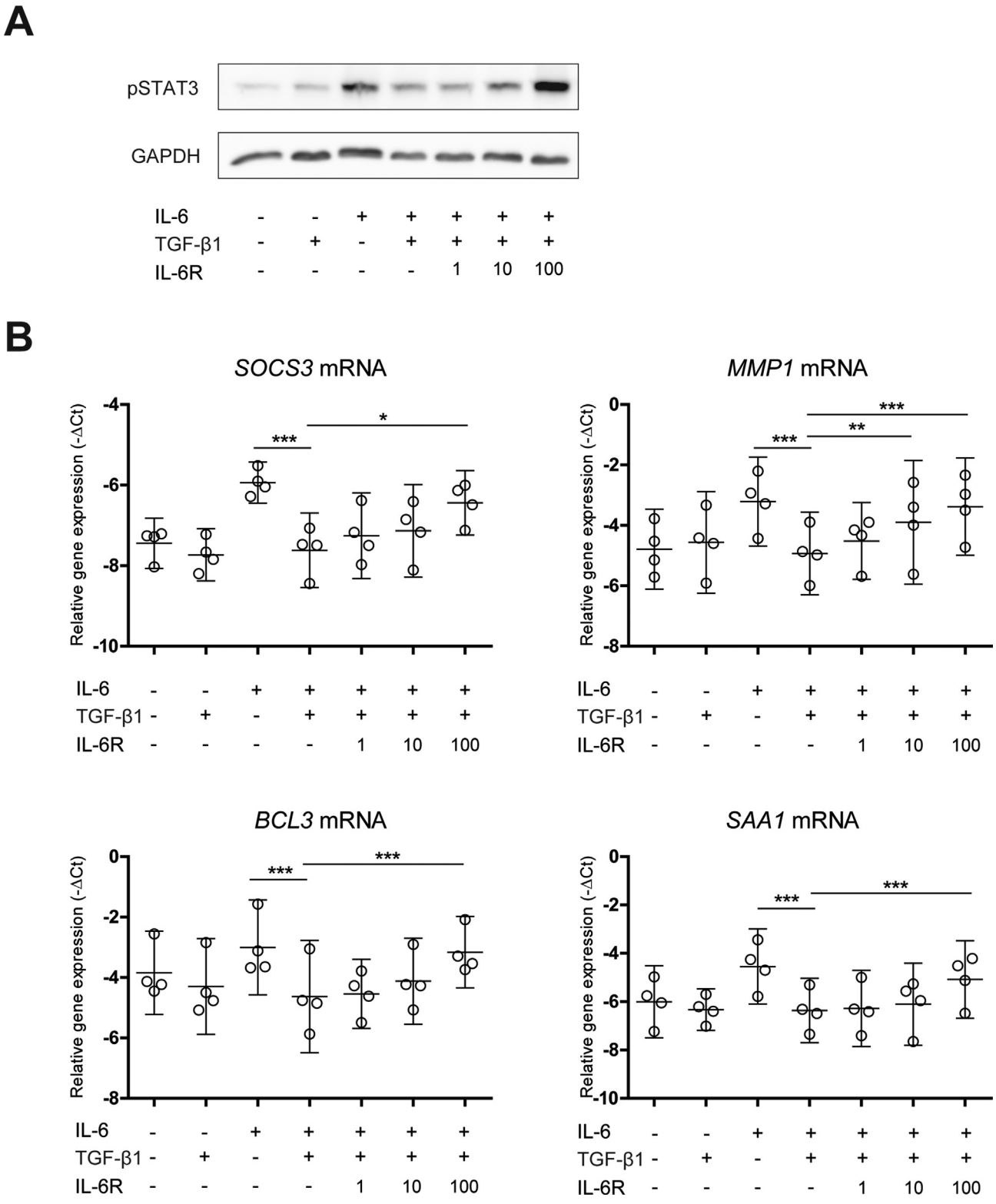

Fig. 5. TGF-β1-mediated inhibition of IL-6 signaling is dependent on decreased IL-6R levels. To study the effect of TGF-β1 on IL-6 signaling with or without recombinant IL-6R, human G6 chondrocytes were pre-stimulated with 1.0 ng/ml of rhTGF-β1 for 6 h, and afterwards stimulated with rhIL-6 (10 ng/ml) alone or in combination with rhIL-6R (1, 10 and 100 ng/ml). The IL-6 response was measured by induction of p-STAT3 after 30 min of IL-6 stimulation (A) or induction of *SOCS3*, *MMP1*, *BCL3 and SAA1* expression after 6 h (B). Western blots are representative of three independent experiments. For gene expression studies, the means of four separate experimental repeates are plotted with corresponding ±95% CI. * = $P < 0.05$; ** = $P < 0.01$, *** = $P < 0.001$ as measured by repeated measures ANOVA with Bonferroni's post-test.

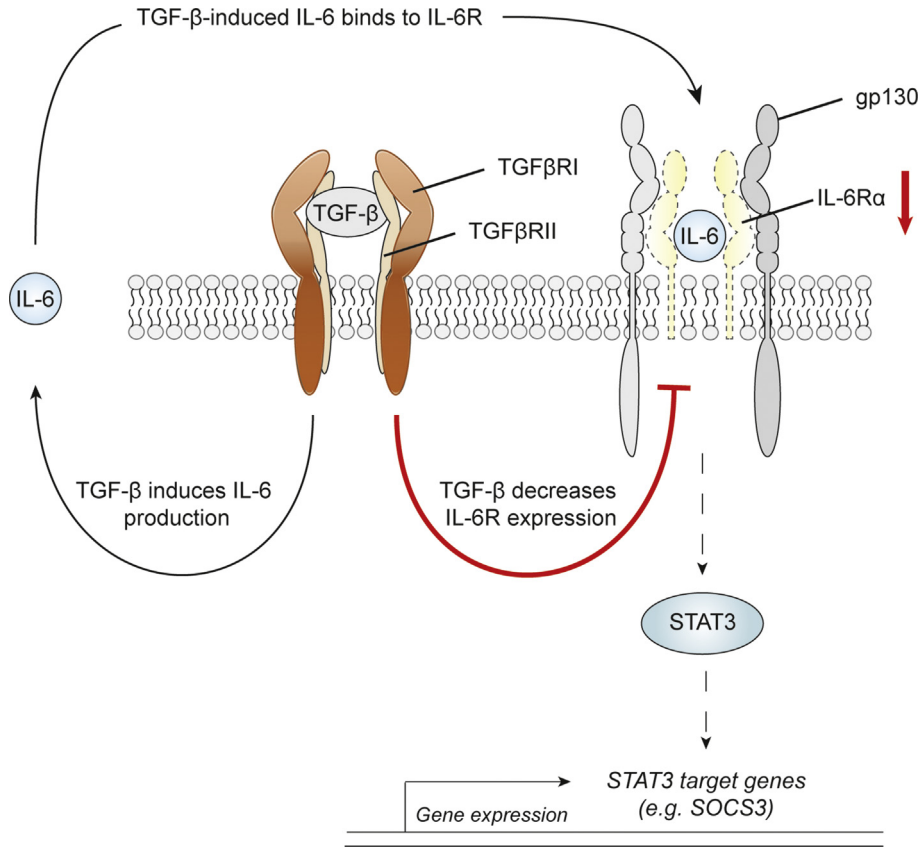

Fig. 6. Schematic overview of TGF-β1-mediated regulation of IL-6 signaling in chondrocytes. TGF-β1 induces the release of IL-6 in human articular chondrocytes. Released IL-6 binds in an autocrine manner to membrane-bound IL-6 receptor on chondrocytes and leads to phosphorylation of the intracellular signaling mediator STAT3. Simultaneously, TGF-β1 decreases IL-6 receptor expression on chondrocytes, resulting in limited STAT3 phosphorylation and inhibition of STAT3-responsive target genes.

expression, thereby promoting Th17 development[20]. However, TGF-β did not reduce IL-6-mediated p-STAT3, in contrast to our results. This implies that in T-cells there might be specific inhibition of SOCS3, and not a general inhibition of IL-6 effects. In intestinal epithelial cells, TGF-β inhibits IL-6 signaling in a similar manner as we observed in chondrocytes[18]. Pre-treatment with TGF-β inhibited IL-6-mediated induction of both p-STAT1 and p-STAT3, as well as IL-6-induced gene expression of ICAM[18]. Until now it was unclear via which mechanism TGF-β blocked IL-6 signaling.

In this study, we demonstrate that TGF-β1 can block IL-6 signaling *via* decreasing IL-6R levels. Because cartilage contains large amounts of inactive TGF-β, which can be activated upon loading, TGF-β mediated down regulation of IL-6R could be involved in homeostatic regulation of cartilage[4,11]. This is an entirely new concept, as there is no literature reporting that TGF-β decreases IL-6R levels in cartilage. This effect of TGF-β might be independent of IL-6, as we showed that IL-1β increased IL-6 production but did not affect IL-6R expression. In literature, inflammatory factors such as LPS and IL-1β are mainly known to increase *IL6R* expression[36,37]. However, this seems to be cell type specific as IL-1β decreases *IL6R* expression in monocytes, but increases its levels in hepatocytes[38]. Therefore, it is possible that TGF-β effects on IL-6R expression could be specific for cartilage and TGF-β differently affects the IL-6 signaling pathway in other tissues or cell types. Interestingly, multiple studies report that *IL6R* expression is directly regulated by various miRNA's (miR-34a, miR-218, miR-590-5p)[39–41]. Several of these miRNA's have been linked to the TGF-β signaling pathway, and direct regulation of miR-34a by TGF-β has been described[40]. Follow-up studies are needed to show that TGF-β suppression of IL6R expression is caused by TGF-β-induced miRNA's.

Besides regulation of IL-6R expression, it is possible that TGF-β blocks other mediators of IL-6 signaling downstream of the receptor as well. Interaction at the level of downstream mediators Smad3 and STAT3 has been extensively reported[42,43]. These studies demonstrate that complex formation between Smad3 and STAT3 can lead to either inhibition of TGF-β signaling or synergy with STAT3 signaling cytokines, dependent on cell type or context[44]. Complex formation between Smad3 and STAT3 has not been studied in chondrocytes until now, but it is possible that these complexes are formed within our experimental setting and contribute to regulation of IL-6 signaling by TGF-β. However, we showed that TGF-β-mediated down regulation of IL-6R expression was the rate limiting factor, as rescuing IL-6R levels with soluble IL-6R abolished the inhibitory effect of TGF-β on IL-6 signaling.

Next to a human chondrocyte cell line, we used human primary chondrocytes derived from macroscopically intact cartilage of OA patients in this study to investigate if our hypothesis held true in freshly isolated chondrocytes from different donors. Between the different individuals we observed diversity in the magnitude of TGF-β effect size, more specifically in the regulation of IL-6 and IL-6R mRNA expression by TGF-β1. Moreover, we observed rapid p-STAT3 activation (5–15 min) in two donors. Direct activation of STAT3 was previously detected in hepatic stellate cells, but not in normal hepatocytes, indicating that this pathway is strongly cell-context dependent[45]. In this study, early p-STAT3 activation was caused by direct binding of JAK3 to TGF-βRI. It is therefore possible that the basal levels of TGF-β receptors (ALK5 vs ALK1), determines the early p-STAT3 peak in our setting[12]. However, this can also be caused by different factors, such as gender, age, disease severity or OA phenotype, which we were unable to study due to the use of anonymized

material. It would be interesting for future research to establish whether rapid STAT3 activation represents a specific patient group.

Next to membrane-bound IL-6R present on the cell membrane, also a soluble form of the IL-6R exists. Soluble IL-6R results from shedding of membrane-bound IL-6R or from differential splicing of IL-6R mRNA and can form a complex with IL-6 and IL-6 receptor β (gp130), bypassing the need of membrane-bound IL-6R[46,47]. Interestingly, endogenous soluble IL-6R can be detected in synovial fluid of OA patients and contributes to enhanced activation of chondrocytes by IL-6[48–50]. This process, called trans-signaling, has been shown to negatively affect cartilage. For example, the IL-6/IL-6R complex can decrease transcription of the matrix components aggrecan and collagen type II in chondrocytes[34,35]. This implies that in OA, the presence of soluble IL-6R may bypass the need of membrane bound IL-6R, which would abolish the protective effects of TGF-β on membrane bound IL-6R levels similar to our experiments where we add exogenous rhIL-6R (Fig. 5). We propose that the presence of soluble IL-6R bypasses protective TGF-β effects, rendering cartilage sensitive again for catabolic IL-6 trans-signaling. These implications would highlight soluble IL-6R as an important target for therapies directed against IL-6.

In conclusion, we show that TGF-β1 dampens IL-6 signaling in chondrocytes, despite upregulating IL-6 levels. Moreover, we demonstrate that TGF-β1 inhibits IL-6 effects via down regulation of the IL-6R. This reveals a novel, protective effect of TGF-β potentially contributing to cartilage homeostasis. We suggest that when this protective effect is lost, due to the presence of soluble IL-6R during inflammation, cartilage is more sensitive to catabolic IL-6 signaling. This sheds light on the protective role of TGF-β in cartilage homeostasis and OA development.

Authors contributions

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No other contributors are involved next to the authors.

Conflict Of Interest

The authors have no conflict of interest.

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Supplementary data

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