TGFβ DRIVEN INTERLEUKIN-6 PRODUCTION BY HUMAN FIBROBLAST-LIKE SYNOVIOCYTES IS OA PATIENT SPECIFIC

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INTRODUCTION: Osteoarthritis (OA) is a total joint disease associated with an imbalance in secretion of cytokines and chemokines into the synovial fluid (SF). The synovial membrane is a key factor in determining a healthy SF composition and provides nutrients for the articular cartilage, but can also contribute to OA development by secreting catabolic factors in the SF such as IL-6 [1,2]. IL-6 is a major predictor of structural changes in OA and post-traumatic OA, has also been associated with inflammatory pain and may serve as a critical effector connecting OA or post-traumatic OA-associated joint changes and pain [3,4]. Recently, it has been demonstrated that transforming growth factor-beta (TGFβ), which is important for healthy joint homeostasis, but also increased in OA synovial fluid, can induce IL-6 production in chondrocytes [5]. Currently it is unknown whether other cell types in the joint are also competent to induce IL-6 production upon TGFβ stimulation, and at which molecular level this is regulated. We hypothesized that TGFβ induces IL-6 production by fibroblast-like synoviocytes (FLS) and that this TGFβ-driven IL-6 production is patient-specific.

METHODS: FLS were obtained from synovium of patients with end-stage knee OA (K&L 3-4) who underwent total knee arthroplasty (METC approval number 2017-0183). Synovium was cut into small pieces and digested overnight in Collagenase II (300 units/ml in HEPES buffered Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 1% antibiotics). The resulting cell suspension was rinsed with 0.9% NaCl solution over a 70 µm cell strainer. Cells were cultured until confluence at passage one in DMEM/F12 supplemented with 10% fetal calf serum, 1% antibiotics, and 1% non-essential amino acids in a humidified atmosphere at 37°C and 5% CO_2. FLS were plated in replicates at a seeding density of 30.000 cells/cm^2 and stimulated with different concentrations TGFβ3 for 24 hours. For probing the involvement of molecular pathways, we used either 0.5 µM SB505124 (ALK5 inhibitor), 0.5 µM Oxozeanol (TAK1 inhibitor), 10 µM SB203580 (P38 inhibitor) or 10 µM Parthenolide (NFκB inhibitor). IL-6 protein secretion in cell culture supernatant was measured by IL-6 ELISA according to manufacturer's protocol (R&D systems). Total RNA was extracted using TRIzol. The quantity and purity of extracted RNA was determined by UV-spectrometry. Total RNA was reverse transcribed to cDNA using standard procedures and random hexamer priming. IL-6 mRNA levels were measured by RT-qPCR and normalized to 28S rRNA as a housekeeping gene. To determine IL-6 gene promotor activity, the NLuc and IL-6 gene -600 – +121 promotor sequence was cloned into the lentiviral pLVX vector and virus was produced using HEK293T cells. Bioluminescence luciferase measurements after lentiviral transduction were used as a readout for IL-6 promoter reporter activity. To classify FLS donors in responders and non-responders, a power calculation to calculate sample size for this experiment was performed based on a previous experiment with FLS and their exposure to 0.1 ng/ml TGFβ3. The formula to calculate the sample size was based on independent samples with continuous outcome, which turned out to be a minimum of 17 FLS donors for this specific experiment. All reported data compared a condition to the unstimulated control conditions, unless indicated otherwise. Statistical analysis was performed using Graphpad Prism 10, determining statistical significance ($P < 0.05$) by unpaired two-tailed student t-tests.

RESULTS: To examine whether TGFβ3 can induce IL-6 protein secretion in FLS, FLS from five different donors were stimulated with either 0.1, 1, or 10 ng/ml TGFβ3 for 24 hours. At a concentration of 1 and 10 ng/ml TGFβ3, FLS significantly upregulated IL-6 in all tested donors. We tested different TGFβ isoforms (TGFβ1, TGFβ2, and TGFβ3) and all TGFβ isoforms provoked a statistically significant upregulation of secreted IL-6 protein levels from FLS after 24h of stimulation. However, TGFβ3 appeared to be the most potent IL-6 inducer in FLS. To acquire a deeper understanding if this upregulation was caused at the gene transcription level, IL-6 mRNA expression was measured and a TGFβ3 dose-dependent upregulation of the IL-6 mRNA gene was observed in FLS. In addition, TGFβ3-driven IL-6 gene promoter reporter activity in FLS was dose- and time-dependent. To explore molecular routes that may be involved in the TGFβ-dependent IL-6 production by FLS, we used specific small-molecule inhibitors for a number of key intra-cellular pathways. We found a statistically significant inhibition of TGFβ-dependent IL-6 production by FLS when cells were treated with inhibitors for either ALK5, TAK1, P38 or NFκB. These results indicate that TGFβ-induced production of IL-6 requires the ALK5–TAK1–P38–NFκB signaling axis. Next, we investigated the potential role of donor variability in the TGFβ-dependent IL-6 production by FLS. FLS of 24 individual end-stage OA donors were exposed to either 0.1 or 1 ng/ml TGFβ3. FLS from all donors showed a statistically significant upregulation of IL-6 production when exposed to 1 ng/ml TGFβ3. However, at a concentration of 0.1 ng/ml TGFβ3, patient-specific differences in upregulation of IL-6 production became significantly detectable, separating the FLS in responders (n=9) and non-responders (n=15) (Figure 1).

DISCUSSION: TGFβ induces IL-6 production by FLS and we discovered that this is regulated at the level of IL-6 transcription. Signaling via the ALK5–TAK1–P38–NFκB axis is responsible for this TGFβ-driven IL-6 production. To investigate this into further molecular detail, we are currently probing the transcription factor-binding sequences within the proximal IL-6 gene promoter that are involved in its TGFβ-responsivity. While our data show that higher concentrations of TGFβ faithfully induce IL-6 secretion by FLS, our data clearly show OA patient-specific differences in the FLS's competence to activate TGFβ-driven IL-6 production at lower TGFβ concentrations. This is relevant for OA-related synovial inflammatory processes and may provide a way to endotype OA patients. We are therefore planning to analyze the patient-specific FLS secretomes, in order to inflammatory characterize the non-responder and responder OA FLS endotype.

SIGNIFICANCE/CLINICAL RELEVANCE: We found that FLS respond to TGFβ in an inflammatory fashion by producing IL-6, which is regulated transcriptionally by the ALK5–TAK1–P38–NFκB signaling axis. Of clinical significance, this inflammatory response is OA patient-specific and provides a novel basis for endotyping based on inflammatory processes in OA and corresponding future disease-modifying treatment options.

REFERENCES: [1]G. Livshits, et al., 2009, Arthritis Rheumatology. [2]S. Larsson, et al., 2015, Osteoarthritis Cartilage. [3] D. Harvanova, et al., 2022, Int J Mol Sci. [4] R. Miller, et al., 2014, Cytokine. [5]R. Wiegertjes, et al., 2019, Osteoarthritis and Cartilage.

IMAGES AND TABLES:

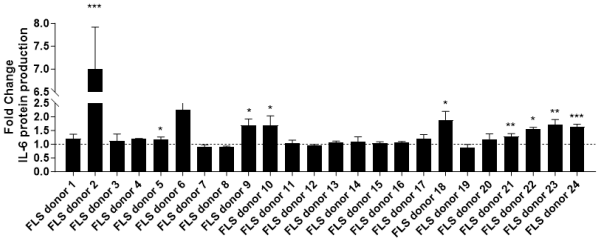

Figure 1. Patient-specific TGFβ3-driven IL-6 secretion by individual FLS donors. To identify responders and non-responders, we stimulated 24 FLS donors individually with 0.1 ng/ml TGFβ3 for 24 hours. Bars represent IL-6 responses of individual FLS donors exposed to 0.1 ng/ml TGFβ3 relative to corresponding individual controls (dotted line) and show mean ± SEM. Statistics are represented as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.