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IL-37 diminishes proteoglycan loss in human OA cartilage: donor-specific link between IL-37 and MMP-3

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SUMMARY

Objective: A hallmark of osteoarthritis (OA) is degradation of articular cartilage proteoglycans. In isolated human OA chondrocytes, the anti-inflammatory cytokine Interleukin-37 (IL-37) lowers the expression of the proteolytic MMP and ADAMTS enzymes, which mediate this degradation. Therefore, we investigated if IL-37 protects against proteoglycan loss in freshly obtained human OA explants.

Material and methods: Human OA cartilage explants were incubated with IL-37. Release of sulphated proteoglycans (sGAGs) was measured with the dimethylmethylene-blue assay. Production and degradation of newly synthesized proteoglycans was measured using ^{35}S-sulphate. Proteoglycan and proteolytic enzyme expression were analyzed by qPCR and Western Blot. Proteolytic activity was determined by measuring MMP- and ADAMTS-generated aggrecan neo-epitopes with ELISA and by using MMP-3-, MMP-13- or ADAMTS-5-inhibitors.

Results: Over time, a linear release of sGAGs from OA cartilage was measured. IL-37 reduced this release by 87 μg/ml (24%) 95%CI [21.04–141.4]. IL-37 did not affect ^{35}S-sulphate incorporation or proteoglycan gene expression. In contrast, IL-37 reduced loss of ^{35}S-sulphate labeled GAGs and reduced MMP-3 protein expression, indicating that IL-37 inhibits proteoglycan degradation. Remarkably, we observed two groups of patients; one group in which MMP-3-inhibition lowered sGAG release, and one group in which ADAMTS5-inhibition had this effect. Remarkably, IL-37 was only functional in the group of patients that responded to MMP-3-inhibition.

Conclusion: We identified a relationship between IL-37 and reduced sGAG loss in OA cartilage. Most likely, this effect is mediated by inhibition of MMP-3 expression. These results suggest that IL-37 could be applied as therapy in a subgroup of OA patients, in which cartilage degradation is mediated by MMP-3.

Introduction

Proteoglycans are essential structural components of the cartilage extracellular matrix. Two types of proteoglycans exist, large proteoglycans and small leucine rich proteoglycans (SLRPS). Based on weight, the most abundant proteoglycan in articular cartilage is the large proteoglycan aggrecan[1]. Aggrecan molecules consist of a core protein with covalently attached glycosaminoglycans (GAGs). These GAGs are highly negatively charged due to presence of sulphate groups (sGAGs). The negative charge, allows sGAGs to attract water to the joint which provides swelling pressure to cartilage. This swelling is needed to counteract compressive forces on the cartilage[2–4].

A hallmark of osteoarthritis (OA) is degradation of cartilage proteoglycans[5–7]. Proteoglycan degradation is mediated by proteolytic enzymes including matrix metalloproteinases (MMPs) and a disintegrin-like and metalloproteinase-like domain (ADAMTSs) (Supplementary Fig. 1). In OA cartilage and synovium, the expression of these MMPs and ADAMTSs is elevated[8–11]. Furthermore, the activity of MMP and ADAMTS enzymes positively correlates with

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cartilage destruction[12,13]. The involvement of both types of proteolytic enzymes in proteoglycan loss and cartilage degradation is additionally supported by mice studies. For example, postnatal overexpression of MMP-13 in cartilage of mice induces degradation of proteoglycans as measured by loss of safranin O staining[14]. Furthermore, cartilage from ADAMTS-5 knock-out mice demonstrates a significant reduction in proteoglycan release compared to wild type mice[15].

Both ADAMTSs and MMPs can degrade aggrecan, but cleavage of aggrecan by these enzymes is spatially and temporally different. ADAMTS enzymes predominantly cleave aggrecan pericellularly, whereas the majority of MMP activity occurs in the interterritorial matrix[16]. Furthermore, ADAMTSs can cleave intact aggrecan molecules, while MMPs have a higher affinity for aggrecan molecules which have already been C-terminally cleaved[17].

Inflammatory factors are known to induce expression and activity of these proteolytic enzymes. For example, incubation of *ex vivo* cartilage explants with IL1β induces the release of ADAMTS- and MMP-cleaved aggrecan neo-epitopes[18,19]. Furthermore, TNFα increases ADAMTS-5 expression levels in bovine and porcine cartilage explants[20]. Blocking the expression of inflammatory mediators and proteolytic enzymes is therefore a promising approach to prevent this enzyme activity and thus proteoglycan loss.

The anti-inflammatory cytokine IL-37 is a suppressor of inflammatory and immune responses both *in vitro* and *in vivo*. IL-37 protects mice against lung inflammation, arthritis, psoriasis and colitis[21–24]. Previously, we have found that in isolated human OA chondrocytes IL-37 not only reduces IL1β-induced expression of pro-inflammatory cytokines, but also the expression of proteolytic enzymes such as MMP-3, MMP-13 and ADAMTS-5[25]. Therefore, the goal of this study was to investigate if exogenously added recombinant human IL-37 (rhIL-37) protects against sGAG loss in freshly obtained human *ex vivo* cartilage OA explants and to explore the mechanism behind this protective effect.

Material and methods

Tissue acquisition and human cartilage explant culture

Human cartilage was obtained from 60 anonymous OA donors undergoing total knee ($n = 28$) or hip arthroplasty ($n = 32$). These donors were divided along the different sub studies (Figs. 1–4). In each sub study independent individuals were used.

From each individual donor cartilage explants were isolated with a biopsy punch (4 mm diameter) (Kai Medical, Japan). In sub study 1 (Fig. 1), we studied the conditions: concentration (four levels: control; 1; 10; 100 ng/ml rhIL-37) and time (six levels: 24, 48, 72, 96, 120, 144 hours). Material from 10 independent individual donors was used. Of each individual 24 biopsy punches were collected and randomly distributed over the 24 combinations. The dependent variable was the sulphated GAG release. The same approach was used for the sub studies regarding ^{35}S-synthesis, gene expression and protein expression. RNA and proteins were extracted from a total of six explants. In Fig. 4, we used 20 independent individual donors. Of each individual 42 biopsy punches were collected and randomly distributed among seven conditions (control, rhIL-37, DMSO (1 μM), MMP-3-inhibitor, MMP-13-inhibitor, DMSO (50 μM) and ADAMTS-5-inhibitor (50 μM). For each time point a sample was taken out to measure sGAG release.

Per condition, the six explants were added to a well of a 12-well plate and cultured at 37°C, 5% CO_2 and 95% humidity in 1 ml DMEM/Ham's F12-medium (1:1), supplemented with 10% fetal calf serum, 100 mg/l sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin.

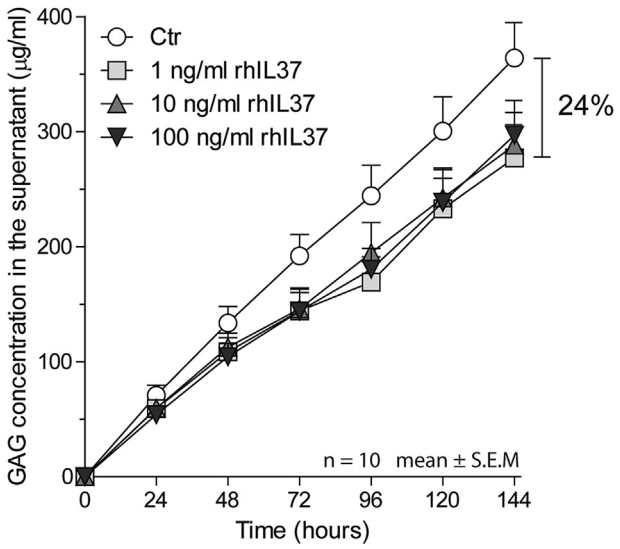


Fig. 1. Decrease in sGAG release from human OA cartilage explants by rhIL-37. Quantification of the sGAG release in supernatant of cartilage explants cultures in presence of rhIL-37 (1, 10, 100 ng/ml) by the DMMB-assay. Data are represented as mean ± (S.E.M), $n = 10$; * = $P < 0.05$, ** = $P < 0.001$ as determined via a one-way analysis of variance with Bonferonni multiple comparison test on day 6 time point.

Of note, it cannot be excluded that some patients have undergone more than one joint surgery and are thus included twice in our studies. However, this is deemed highly unlikely by the orthopedic surgeons involved in our study, since only 10 patients per year receive two joint surgeries.

Ex vivo stimulation of cartilage explants

After culturing overnight, explants were incubated with 1, 10 or 100 ng/ml rhIL-37 (R&D) in medium without serum for up to 48 h or 6 days, with new addition of rhIL-37 every other day. Additionally, inhibitors against MMP-3 (1 μM, Batimastat CAS 130370-60-4, MedChem Express), MMP-13 (1 μM, CAS 544678-85-5, Calbiochem) or ADAMTS-5 (50 μM, CAS 929634-33-3, EMD Millipore) were added following the same protocol. The functionality of these inhibitors on GAGs release was first confirmed, see Supplementary Fig. 2.

Histological analysis

To visualize proteoglycans in cartilage explants, explants were fixed either immediately or after 6 days of culture using 4% phosphate-buffered formalin (pH 7.0), dehydrated with an automated tissue-processing apparatus (Sakura Tissue Tek VIP, USA) and embedded in paraffin. Tissue sections of 7 μM were prepared, mounted on Superfrost plus glass slides (Thermo Scientific, Waltham, MA), stained with safranin O/fast green (Brunschwig Chemie, the Netherlands) and mounted with Permount.

Sulphated glycosaminoglycans (sGAGs) measurement

To measure the concentration of released sGAGs from the cartilage explants in the culture medium, the 1,9-dimethylmethylene blue (DMMB) assay was used. The sGAG content was measured by adding 200 μl DMB solution (0.05 mM DMMB, 41 mM NaCl, 45 mM glycin and pH = 3.0) to 40 μl supernatant sample (10 times pre-diluted in water) in a 96-well plate. The absorbance was measured at 595 nm using an iMark Reader (Bio-Rad, CA, USA).

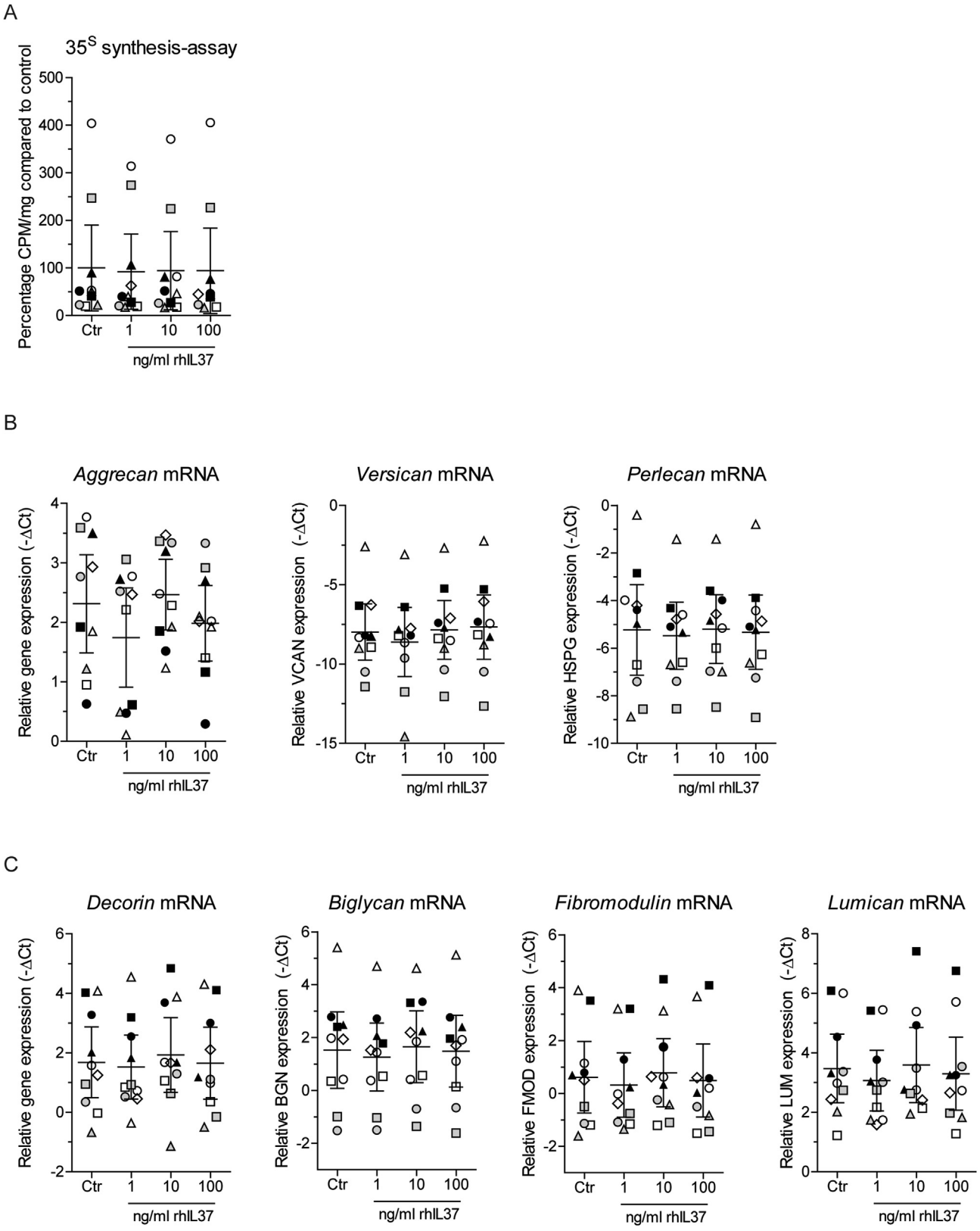


Fig. 2. No effect of rhIL-37 on ^{35}S-sulphate incorporation and proteoglycan expression in human OA cartilage explants. A) Quantification of the ^{35}S content of cartilage explants as measured by liquid scintillation counting. Explants were incubated for 48 h with 1, 10 or 100 ng/ml rhIL-37 and subsequently labeled for 4 h with ^{35}S-sulphate. Data are represented as individual data points with unique markers for each individual with 95% CI $n = 10$. **B**) Relative gene expression of the large proteoglycans *aggrecan*, *versican* and *perlecan* in human OA cartilage explants after incubation for 48 h with rhIL-37 (1, 10 or, 100 ng/ml) as determined by qPCR. Data are represented as individual data points with unique markers for each individual with 95% CI + = mean, $n = 10$. **C**) Relative gene expression of the small leucine-rich proteoglycans (SLRPs): *decorin*, *biglycan*, *fibromodulin* and *lumican* in human OA cartilage explants after incubation for 48 h with rhIL-37 (1, 10 or 100 ng/ml) as determined by qPCR. No changes were observed. Data are represented as individual data points with unique markers for each individual with 95% CI, $n = 10$.

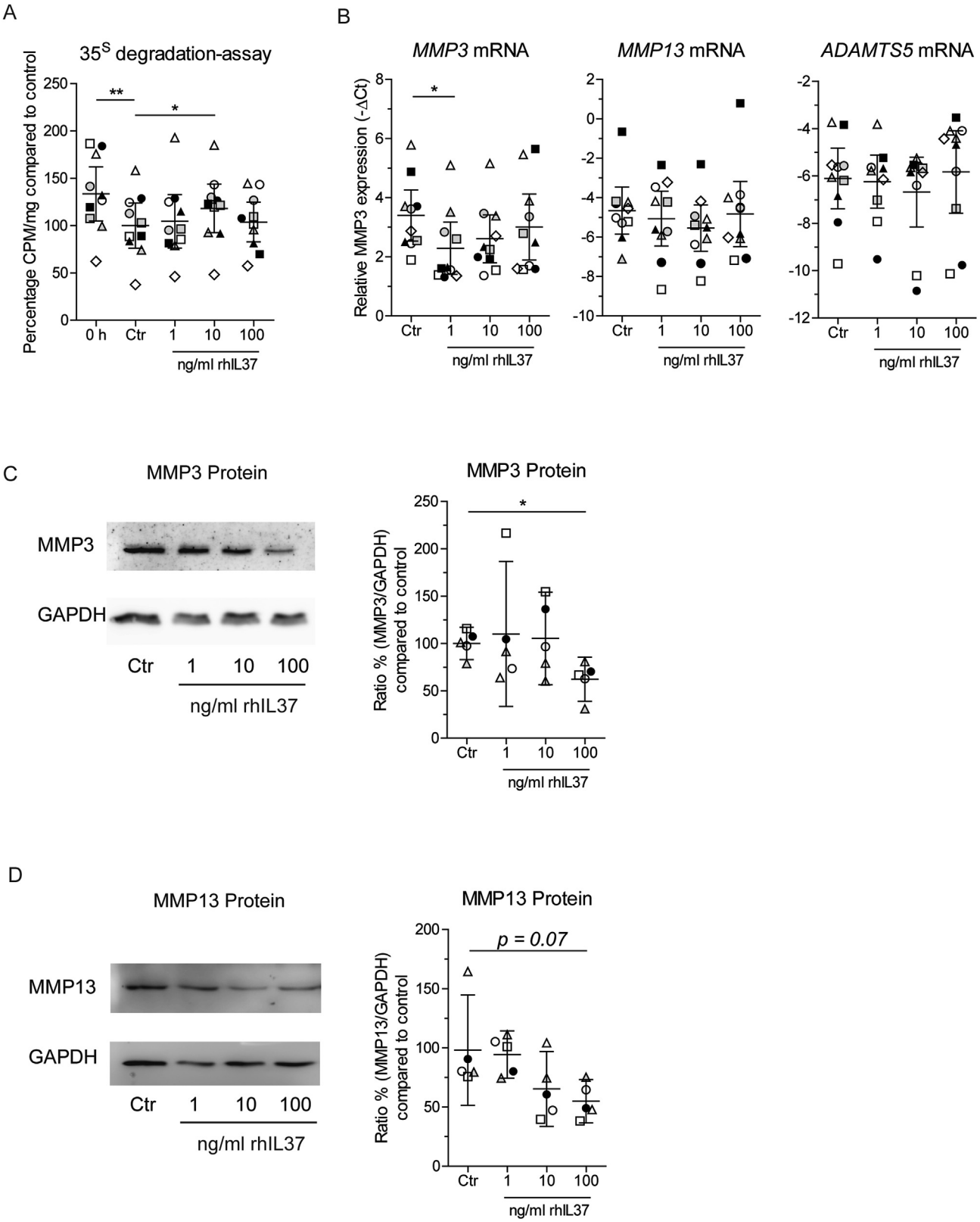
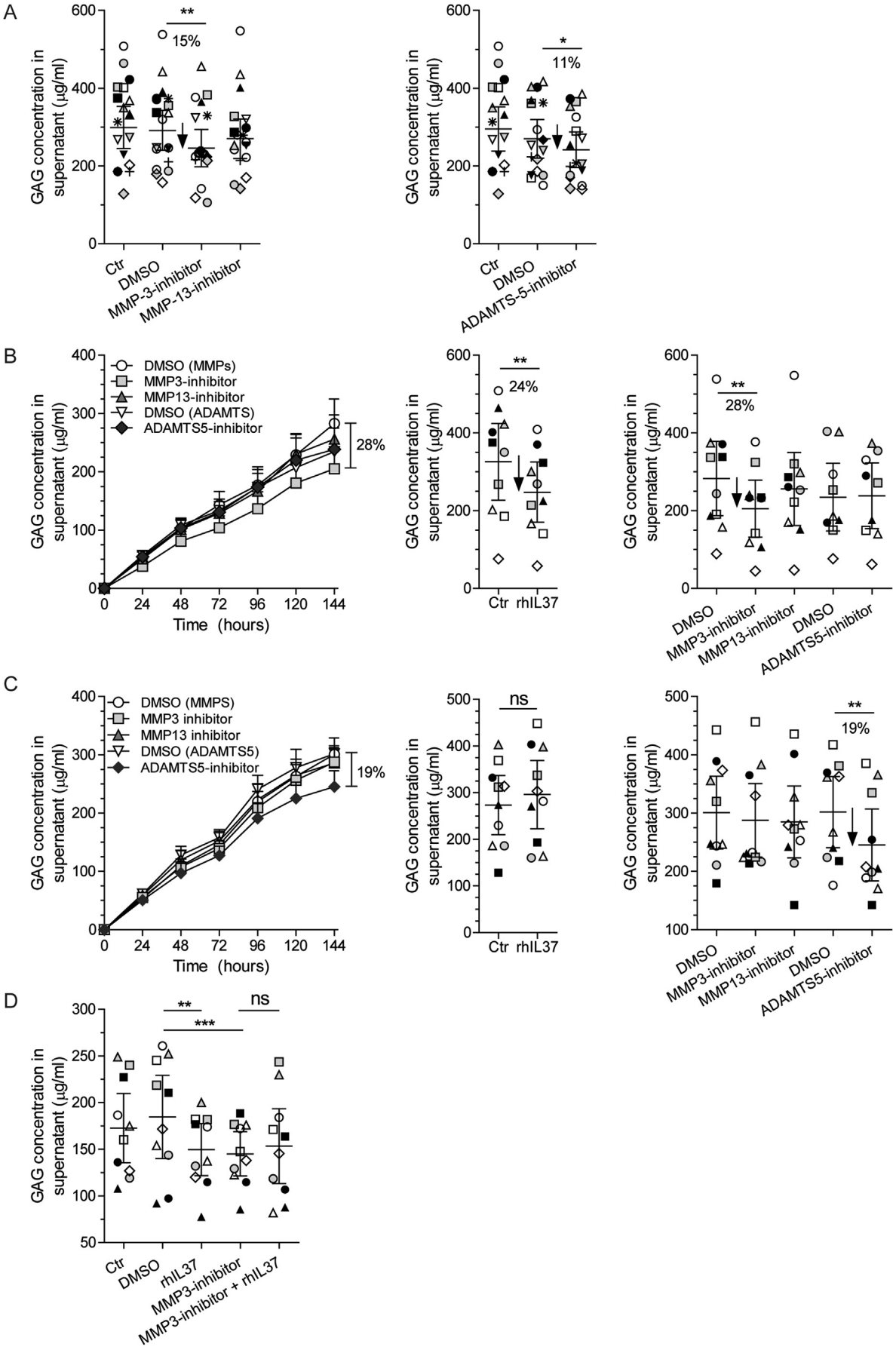


Fig. 3. RhIL-37 decreases loss of ^{35}S-sulphate labeled sGAGs and MMP expression in human OA cartilage explants. A) Quantification of the ^{35}S content of cartilage explants pre-labeled for 4 h with ^{35}S-sulphate and subsequently incubated for 48 h with rhIL-37 (1, 10 or 100 ng/ml) as determined by liquid scintillation counting. Data are represented as individual data points with unique markers for each individual with 95% CI, $n = 10$. **B)** Relative gene expression of the proteolytic enzymes *MMP-3*, *MMP-13* and *ADAMTS-5* in human OA cartilage explants after incubation for 48 h with rhIL-37 (1, 10 or 100 ng/ml) as determined by qPCR. Data are represented as individual data points with unique markers for each individual with 95% CI, $n = 10$. **C and D**) Protein expression of MMP-3 and MMP-13 in cartilage explants after 48 h incubation with rhIL-37 (1, 10 or 100 ng/ml) as determined by Western blot. Quantification of the Western Blot was performed by Image J. Data are represented as individual data points with unique markers for each individual with 95% CI, $n = 5$; $^{*} = P < 0.05$ as determined via one-way analysis of variance with Bonferonni multiple comparison test.


A
GAG concentration in supernatant (µg/ml)
600
400
200
0
**
15%
Ctr
DMSO
MMP-3-inhibitor
MMP-13-inhibitor
*
11%
Ctr
DMSO
ADAMTS-5-inhibitor
B
DMSO (MMPs)
MMP3-inhibitor
MMP13-inhibitor
DMSO (ADAMTS)
ADAMTS5-inhibitor
28%
0 24 48 72 96 120 144
Time (hours)
**
24%
Ctr rhIL37
**
28%
DMSO
MMP3-inhibitor
MMP13-inhibitor
DMSO
ADAMTS5-inhibitor
C
DMSO (MMPS)
MMP3 inhibitor
MMP13 inhibitor
DMSO (ADAMTS5)
ADAMTS5-inhibitor
19%
0 24 48 72 96 120 144
Time (hours)
ns
Ctr rhIL37
**
19%
DMSO
MMP3-inhibitor
MMP13-inhibitor
DMSO
ADAMTS5-inhibitor
D
**
***
ns
Ctr
DMSO
rhIL37
MMP3-inhibitor
MMP3-inhibitor + rhIL37

RNA isolation and quantitative real-time polymerase chain reaction

Explants were homogenized using a micro-dismembrator (2 min, 1800 rpm) (B. Braun Biotech International, Melsungen, Germany). Total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen Inc., Valencia, CA, USA) according to manufacturers protocol. Subsequently, RNA was reverse transcribed with single step RT reaction and quantitative real-time PCR was performed with validated primers, as previously described (Table I)[25]. To calculate the relative gene expression, the reference gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used.

Protein isolation and Western blot

Explants were homogenized using a micro-dismembrator (2 min, 1800 rpm) (B. Braun biotech International, Melsungen, Germany). Samples were incubated with 1 ml ice cold RIPA buffer, containing 1% NP-40, 1 mM Na_3VO_4 and protease inhibitor cocktail (Complete, Roche), for 1 h on a roller bench at 4°C. Subsequently, samples were centrifugated for 3 min at 13,500× *g* at 4°C and supernatant was collected. Samples were boiled after addition of standard 6× Laemmli sample buffer. Proteins were separated on a 10% reducing bisacrylamide SDS-PAGE gel, and transferred onto a 0.1 μm pore nitrocellulose membrane (Life Sciences, Amersham) using wet transfer (25 mM *Tris*–HCl, 192 mM glycine, 20% methanol, pH 8.3), 2 h, 275 mA on ice. Non-specific protein binding was blocked for 1 h with 5% non-fat dry milk (Campina, the Netherlands) in TBS-T (15 mM *Tris*–HCl, pH 7.4, 0.1% Tween). Membranes were incubated overnight at 4°C with primary antibodies against MMP-3 (1:200, sc-6839, Santa-Cruz) or MMP-13 (1:1000, AF511, R&D), followed by incubation with polyclonal rabbit anti-goat antibody labeled with horseradish perioxidase (1:1500, DAKO, Belgium) for 2 h at RT. Enhanced chemiluminescence using ECL prime kit (GE Healthcare, UK) was used to visualize proteins with ImageQuant LAS4000 (Leica, Germany). GAPDH (1:1500, G8795, mouse anti-human, Sigma–Aldrich) was used as loading control. ImageJ was used to quantify the Western Blots.

Neo-epitope ELISA

Aggrecan neo-epitopes (^{373}NITEGE, ^{374}ARGS and ^{342}FFGV) were measured in the culture medium by ELISA, as previously described[26,27].

Synthesis of sulphated proteoglycans

Explants were placed in 1 ml of incubation medium consisting of DMEM/HAMS-F12 (1:1) without serum and incubated with rhIL-37 (R&D) for 48 h. Subsequently, cartilage explants were labeled with 3.7×10^5 Bq ^{35}S-sulphate (^{35}S, NEX041H001MC, PerkinElmer). After labeling for 4 h at 37°C, the explants were rinsed thrice in saline and dissolved in Luma Solve (Hicol, Oud-Beijerland, The Netherlands) overnight at 60°C. The ^{35}S-sulphate content of the explants, which is a reliable measure of cartilage proteoglycan production[28], was measured in Lipoluma (Hicol, Oud-Beijerland, The Netherlands) by liquid scintillation counting.

Degradation of newly synthesized sulphated proteoglycans

To study the *in vitro* degradation of human OA cartilage, cartilage explants were pre-labeled with 3.7×10^5 Bq ^{35}S-sulphate radionuclide (^{35}S, NEX041H001MC, PerkinElmer) for 4 h at 37°C. Subsequently, the explants were rinsed thrice with saline, cultured overnight and incubated with rhIL-37 (R&D) for 48 h. Next, explants were dissolved in Luma Solve (Hicol, Oud-Beijerland, The Netherlands) overnight at 60°C. The amount of ^{35}S-sulphate labeled proteoglycans was determined by liquid scintillation counting.

Statistics

Quantitative data were expressed as curve with S.E.M, as individual data points with unique markers for each individual with 95% Confidence Intervals. Datasets were checked for normality using histograms. Statistics were performed on the absolute measured values.

Repeated measurements ANOVA with two within subjects factors (concentration and time) and the interaction between these two were used. This was followed by a Bonferroni multiple comparison post test to estimate the effect of rhIL-37 dosages on the final time point compared to control: the number of dosages determines the number of tests and hence the size of the correction: for three dosages a *p*-value of 0.017 was used. In the inhibitor sub study, the MMP-3-inhibitor (1 μM) treated group and the MMP-13 inhibitor (1 μM) treated group determine the number of tests and hence the size of correction: for two treated groups a *p*-value of 0.025 was used. A paired one-tailed *t*-test was used to estimate the effect of the ADAMTS-5-inhibitor (50 μM) and rhIL-37 on sGAG release. A *p*-value <0.05 was considered significant. Lastly, a repeated measurements ANOVA with a Bonferroni correction was used to compare rhIL-37, MMP-3-inhibitor and MMP-3-inhibitor with DMSO (*P*-value 0.017).

All analyses were performed using Graphpad Prism 5.03 (Graphpad Software, San Diego, USA). 95% CI intervals of differences with Bonferroni correction are represented in Supplementary Fig. 6.

Results

RhIL-37 decreases sGAG release from human OA cartilage explants

First, we started by investigating sGAG loss from human OA cartilage *in vitro*. To do this, we quantified the release of sGAG in the culture media of explants (Fig. 1). We observed a clear linear increase in sGAG content in the culture medium over time; after 6 days, on average 350 μg/ml sGAGs were measured. Because per condition six explants were cultured, this indicates that each explant released approximately 10 μg/ml sGAGs per day. We also visualized this loss of GAGs in explants by safranin O/fast green staining, which is a measure for proteoglycan content (Supplementary Fig. 3). Next, we investigated if rhIL-37 could inhibit this sGAG release. Therefore, we incubated explants for 6 days with either 1, 10 or 100 ng/ml rhIL-37. From day two and onwards, rhIL-37 significantly reduced sGAG release ($P < 0.05$; 95% CI [21.04–141.4], [11.06–131.4], [1.32–121.6] for 1, 10 and 100 ng/ml rhIL-37 respectively). On day 6, a significant reduction in sGAG

Fig. 4. Positive correlation between rhIL-37 and MMP-3-inhibition, but not ADAMTS-5-inhibition, in sGAG release from human OA cartilage explants. A) Quantification of the sGAG release from human OA explants incubated for 6 days with the MMP-3-, MMP-13-, and ADAMTS-5-inhibitor as determined by the DMMB-assay. **B)** Quantification of the sGAG release from cartilage explants that do respond to both MMP-3-inhibitor and rhIL-37 (1 ng/ml) as determined by the DMMB-assay. **C)** Quantification of the sGAG release from cartilage explants on day 6 of culture, that do respond to the ADAMTS-5-inhibitor as determined by the DMMB-assay. **D)** Quantification of the sGAG release from human OA explants incubated for 6 days with rhIL-37 (1 ng/ml) and the MMP-3-inhibitor as determined by the DMMB-assay. Data are represented as individual data points with unique markers for each individual with 95% CI $n = 10$; $^*P < 0.05$ as determined via one-way analysis of variance with Bonferonni multiple comparison.

Table I
Sequence of the human primers used for RT-qPCR

Name	Gene symbol	Forward 5′→3′	Reverse 5′→3′
ADAM metallopeptidase with thrombospondin type 1 motif 5	*ADAMTS-5*	GCTCACGAAATCGGACATTTACTT	ACCAAGGTCTCTTCACAGAATTTG
Aggrecan	*ACAN*	GCCTGCGCTCCAATGACT	ATGGAACACGATGCCTTTCAC
Biglycan	*BGN*	AAGCTCCTCCAGGTGGTCTA	SGAGGCTGATGCCGTTGTAGT
Decorin	*DCN*	CCTCCTTTCCACACCTGCAA	TTTCACAACCAGGGAACCTTT
Fibromodulin	*FMOD*	GCAGCCTCCTTSGAGCTAGAC	GCTGCTGATGSGAGAACTCATTG
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	ATCTTCTTTTGCGTCGCCAG	TTCCCCATGGTGTCTSGAGC
Lumican	*LUM*	GCAGTGTCAAGACAGTAAGGATTC	GGCCACTGGTACCACCAATC
Matrix metalloproteinase 3	*MMP-3*	SGAGGCATCCACACCCTAGGTT	TCAGAAATGGCTGCATCGATT
Matrix metalloproteinase 13	*MMP-13*	ATTAAGSGAGCATGGCGACTTCT	CCCAGSGAGGAAAAGCATSGAG
Perlecan	*HSPG2*	ACACCTGTSGAGGCCATGAAC	GGGCCTGCTCGTTGTG
Versican	*VCAN*	CCAGTGTGSGAGGTGGTCTAC	TGGTTGTAGCCTCTTTAGGTTT

release of 24% was observed. This effect of rhIL-37 was already observed at the lowest dose of 1 ng/ml, indicating that this dose already reached maximum effects on sGAG release. Of note, the data are the mean of 10 individual donors. We observed that out of the 10 included donors, only two donors did not respond to rhIL-37.

No effect of rhIL-37 on ^{35}S-sulphate incorporation and proteoglycan expression in human OA cartilage explants

A reduction in proteoglycan release can occur when the balance between proteoglycan synthesis and degradation is disturbed. To investigate if rhIL-37 negatively affects proteoglycan synthesis, we incubated cartilage explants with rhIL-37 and ^{35}S-sulphate. The ^{35}S-sulphate incorporation in cartilage explants is a reliable measure of proteoglycan production due to its inclusion into the newly synthesized sulphate groups of proteoglycans[28]. However, no significant increase or decrease in ^{35}S-sulphate incorporation in explants was observed after addition of rhIL-37. This suggests not for a relationship between rhIL-37 and the new production of sulphated proteoglycans (sGAGs) [Fig. 2(A)]. Furthermore, we also measured gene expression of the large proteoglycans: *aggrecan*, *perlecan* and *versican* in cartilage explants treated for 48 h with rhIL-37; but we found no significant difference in gene expression for either gene [Fig. 2(B)]. Finally, we also measured gene expression of the SLRPs: decorin, biglycan, fibromodulin and lumican, but also for these genes no significant difference in their expression was observed after addition of rhIL-37 [Fig. 2(C)]. In conclusion, our results suggest not for a relationship between rhIL-37 and the proteoglycan synthesis of cartilage explants. The numerical results are presented in Supplementary Fig. 6.

RhIL-37 reduces loss of ^{35}S-sulphate labeled GAGs and MMP expression in human OA cartilage explants

We next investigated if we could find a relationship between rhIL-37 and the degradation of newly synthesized proteoglycans. Therefore, cartilage explants were pre-labeled with ^{35}S-sulphate and subsequently incubated with either 1, 10 or 100 ng/ml rhIL-37 for 48 h. In control samples, we observed a loss of 25% of incorporated ^{35}S-sulphate over time (48 h) [Fig. 3(A)]. No significant differences on ^{35}S-sulphate incorporation were observed after addition of 1 ng/ml or 100 ng/ml rhIL-37. However, a significant decrease in ^{35}S-labeled sGAGs loss was observed in the group treated with 10 ng/ml rhIL37 as measured by an increase in the ^{35}S-content of explants by on average 13% (mean of 10 donors) compared to ^{35}S-content of control explants ($P < 0.05$; 95% CI [−35.34 to −0.9195]). This result suggests that rhIL-37 can inhibit of ^{35}S-sulphate labeled sGAGs degradation.

To support such a relationship, aggrecan degradation fragments were measured in the supernatant. After addition of rhIL-37, no significant differences in both ADAMTS-generated ARGS and NITEGE neo-epitope levels and MMP-generated FFGV neo-epitope levels were measured after addition of rhIL-37 (Supplementary Fig. 4). Furthermore, gene expression of *MMP-13* and *ADAMTS-5* was not significantly different in presence of rhIL-37 [Fig. 3(B)]. In contrast, a dose of 1 ng/ml rhIL-37 significantly reduced MMP-3 gene expression by 2-fold (with 1 PCR-cycle) ($P < 0.01$, 95%CI [0.2158–1.992]). Also on protein level, a significant reduction in MMP-3 expression was observed after addition of 100 ng/ml rhIL-37 ($P < 0.05$; 95%CI [23.21–52.24]) [Fig. 3(C)]. MMP-13 protein expression was not significantly affected by rhIL-37 ($P = 0.07$) [Fig. 3(D)]. Unfortunately, we were not able to detect ADAMTS-5 protein levels on Western Blot, making us unable to draw conclusions about the effect of rhIL-37 on this enzyme on protein level.

In conclusion, these results, suggests for a relationship between rhIL-37 and proteoglycan degradation of cartilage explants, possibly via inhibition of MMP-3 expression.

Link between rhIL-37 and MMP-3 in sGAG release in human OA cartilage explants

Because our previous results suggests for a relationship between rhIL-37 and proteolytic enzyme expression, we wanted to investigate the involvement of these enzymes in our *ex vivo* culture system. Therefore, we added inhibitors of MMP-3, MMP-13 and ADAMTS-5 activity to the explants and measured sGAG release. We observed a significant reduction of the sGAG release by both the MMP-3-inhibitor and the ADAMTS-5-inhibitor of 15% ($P < 0.05$; 95% CI [17.13–73.77]) and 11% ($P < 0.05$; 95%CI [4.367–51.94]) respectively [Fig. 4(A)], supporting for a role of both MMP-3 and ADAMTS-5 enzyme in sGAG release. In contrast, sGAG release was not significantly affected by the MMP-13-inhibitor.

Most remarkably, when analyzing the results, we noticed the existence of two groups; in one group rhIL-37 significantly reduced sGAG release (by ±24%; $P < 0.01$; 95%CI [31.2–124.4])) [Fig. 4(B)], whereas in the second group no significant differences with rhIL-37 was observed. Surprisingly, in the group where rhIL-37 significantly reduced sGAG release, only the MMP-3-inhibitor significantly reduced the sGAG release (by ±28%; $P < 0.01$; 95%CI [36.12–119.1]) whereas the ADAMTS-5-inhibitor did not [Fig. 4(B)]. In the second group only the ADAMTS-5-inhibitor significantly reduced sGAG release (by ±19%; $P < 0.01$; 95%CI [18.19–94.77]) whereas the MMP-3-inhibitor did not [Fig. 4(C)]. These results suggest the presence of different OA subtypes in which proteoglycan degradation is mediated by different proteolytic enzymes. Furthermore, the observation that rhIL-37 is only protective against sGAG release in the same patients as those in which MMP-3-inhibition is protective, suggests that MMP-3 inhibition is part of IL-37 function.

To further investigate the hypothesis that MMP-3-inhibition is important for IL-37 function, explants were incubated with both

rhIL-37 and the MMP-3-inhibitor. If the MMP-3-inhibitor and rhIL-37 act via a similar mechanism, no additional effect on sGAG release is expected, whereas a different mechanism would result in additional effects. Indeed, no statistically significant additional effect of MMP-3-inhibition on the effect of rhIL-37 was observed, even though both single stimulations significantly lowered sGAG release by ±22% ($P < 0.01$; 95%CI [14.24–69.25]) and ±14% ($P < 0.01$; 95%CI [0.3385–55.35]) respectively [Fig. 4(D)]. This result suggests that rhIL-37 and the MMP-3-inhibitor act via the same mechanism, and thus further supports the relationship between MMP-3 inhibition and rhIL-37 function in OA cartilage.

Discussion

IL-37 is a suppressor of the innate and adaptive immune system[21–24]. Earlier, we have shown that overexpression of IL-37 in isolated human OA chondrocytes in monolayer decreases not only the expression of pro-inflammatory cytokines but also the expression of cartilage degrading enzymes[25]. These enzymes are known to mediate the degradation of sGAGs in *ex vivo* cartilage cultures. Therefore, in this study we investigated the effects of exogenously added rhIL-37 on sGAG release of OA cartilage explants. As a result, we here observed for the first time a relationship between rhIL-37 and a reduction in sGAGs release from human OA cartilage explants and suggest that most likely a MMP-3-related mechanism is responsible for this effect.

Culturing human OA cartilage explants for up to 6 days led to a gradual release of sGAGs, with approximately the same amount of sGAGs lost per day. The amount of released sGAGs was therefore similar between the early phase just after obtaining the explants and the later time points. Previously, a difference in GAG release between this early and late phase has been reported for healthy rabbit and bovine cartilage; an initial extensive release from day 0–3 and a slow release period from day 4 to day 15[29,30]. This difference between both phases has been attributed to a difference in proteolytic enzyme activity, with the first phase characterized by ADAMTS activity and the second phase by MMP activity. That we do not observe these two phases in our culture system, may be due to the tissue used; instead of healthy cartilage we used OA affected human cartilage. Possibly, the increased expression and activity of proteolytic enzymes in OA cartilage[8–11] prevents a clear temporal difference in ADAMTS and MMP activity as observed in healthy cartilage.

To inhibit sGAG release from cartilage, we use three different concentrations of rhIL-37; 1, 10 or 100 ng/ml rhIL-37. Remarkably, these three doses had a similar effect on sGAG release. Previously, it has also been observed that a low concentration of rhIL-37 can be as effective as or even more effective than high concentrations[31,32]. In bone marrow-derived dendritic cells, low concentrations of rhIL-37 are able to reduce *IL1* mRNA expression, whereas high concentrations are not[31], and in HUVEC cells, rhIL-37 in concentrations up to 1 ng/ml stimulates proliferation, but higher concentrations are less effective[32]. A possible explanation for these observations is the tendency of IL-37 to spontaneously form homodimers with increasing concentrations. The formation of these dimers limits the bioactivity of IL-37, as demonstrated by the observation that IL-37 monomers are 13-fold more effective in suppressing immune responses than IL-37 dimers[33]. Because less dimers formed at lower concentrations of IL-37, the bioactivity of IL-37 does not necessarily increase with increasing concentrations[34]. To find an explanation for the observed reduction in sGAG release from OA cartilage after addition of rhIL-37 we measured both synthesis and degradation of sGAGs. We did not observe a relationship between rhIL-37 and proteoglycan synthesis, but did find a relationship between rhIL-37 and a decreased degradation of these proteoglycans. We suggest that this effect of rhIL-37 is due to inhibition of MMP-3 expression because we observed a statistically significant downregulation of MMP-3 mRNA and protein expression by rhIL-37, and because an MMP-3-inhibitor does not have an additive inhibitory effect on GAG release when combined with rhIL-37 compared to the effect of rhIL-37 alone. Furthermore, in some donors we found an indication for lower ^{342}FFGV and ^{373}NITEGE neo-epitope levels in the culture media after addition of rhIL-37, suggesting for a relationship between rhIL-37 and decreased MMP activity; both these markers are released from the cartilage matrix after enzymatic cleavage of the G1 domain of aggrecan by MMPs[1].

A limitation of this study is our inability to measure all our different parameters on cartilage derived from one and the same individual because this required a too large amount of cartilage derived from one and the same donor. Between individuals, we observed differences in their response to the different concentrations of rhIL-37. This may have affected experimental outcomes, as we possibly may have unwillingly and accidentally included individuals with different responses to the different experiments. This can be the reason for inconsistencies regarding the response to the different concentration of rhIL-37 between the different readout parameters. A desirable experiment would be to measure all parameters in one and the same individual. However, due to shortage of cartilage material this was impossible.

Our results suggest an important role for MMPs in sGAG loss from human OA cartilage. The MMP-3-inhibitor Batimastat inhibited sGAG release even more than the ADAMTS-5-inhibitor (CAS 929634-33-3) we used. Previously it has been suggested that MMPs only have minor contribution to aggrecan catabolism in cartilage compared to ADAMTS-5 in view of their relative efficiency in cleaving the aggrecan core protein[35]. However, this concept derives from results obtained in healthy bovine cartilage explants[35]. In healthy cartilage, relatively low amounts of active MMPs are present[9], and therefore their relative contribution to aggrecan cleavage is most likely limited. In OA cartilage explants, MMP expression and activity is increased by the inflammatory environment[8–11], allowing for increased importance in proteoglycan degradation. An increased importance of MMPs in aggrecan degradation in damaged cartilage is illustrated by the observation that addition of the MMP-3 inhibitor CGS 27023A to uninjured cartilage samples does not result in a protective effect on GAG release, whereas addition of this inhibitor to injured cartilage samples does[36].

Surprisingly, analyzing the results of the MMP-3- and ADAMTS-5-inhibitors, we observed two groups. In one group, both the MMP-3-inhibitor and rhIL-37 significantly decreased sGAG release, whereas in the other group only the ADAMTS-5-inhibitor had an effect. These observations support the concept that (at least) two distinct pathways are responsible for aggrecan degradation in human OA cartilage; one via MMP-3 and one via ADAMTS-5. The presence of these groups can have multiple causes. First, there is the possibility that cartilage derived from hip or knee joint respond differently to the inhibitors. However, we did not observe any differences in response to the MMP-3-inhibitor or ADAMTS-5-inhibitor between cartilage explants derived from hip or knee joints (Supplementary Fig. 5). Another possibility is, that these two groups are a reflection of disease duration. In the literature it is hypothesized that ADAMTS-mediated cartilage degradation occurs at early stages of OA, whereas during later stages MMP-mediated degradation becomes more important[13,36–38]. However, as we used anonymized patient material, a limitation of our study is that we were unable to verify disease duration for our samples. Furthermore, there is lack of detailed information about the included donors regarding age,

gender, and severity of cartilage degeneration. These factors may explain the presence of two groups. It would be very interesting for future research to distinguish these results based on donor characteristics. Other possibilities for the presence of two groups derive from the multifactorial etiology of OA. For example, the involvement of inflammation, age-related factors, obesity, trauma and genetics have been shown to contribute to development of OA[39]. Although all these factors can facilitate cartilage loss, the underlying mechanism may be different, with different roles for MMP and ADAMTS enzymes. Identifying and distinguishing the different subtypes of OA and specifying which OA subtype is driven by MMP-3 activity, is important for applying IL-37 as OA therapy in the future.

In conclusion, we have found for the first time a relationship between rhIL-37 and a reduction in sGAG release from human OA cartilage explants, at least in a subgroup of OA patients. These findings, indicate that IL-37 supports matrix integrity in human OA cartilage. Moreover, we show that MMP-3 might be a pivotal downstream target of rhIL-37 in this process. This supports for the use of subgroup specific IL-37 therapy targeting MMP-3-mediated proteoglycan loss in human OA cartilage.

Contributorship

Conception and design: Ellen van Geffen, Arjan van Caam, Esmeralda Blaney Davidson, Peter van der Kraan.

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No other contributors are involved than the authors.

Disclosers

The authors have nothing to disclose; there is no conflict of interest.

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Ethical approval information

Not applicable, the material we received was anonymised, meaning that nor the patients or anyone else could identify the patients with certainty. In the Netherlands we are making use of the following policy: patients are informed that their residual tissue can be used for research purposes. Instead of given informed consent (opt-in regime), they can explicitly object to it (opt-out regime). In addition, it is not allowed to obtain personal data of the patients and to perform genetic research studies. These requirements are not applicable for our study. Due to this policy, we do not need further ethical approval for use of tissues from living individuals.

Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.joca.2018.08.016.

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