American College of Rheumatology | Arthritis & Rheumatology

Interleukin 1 acts via c-jun N-terminal kinase-2 signalling pathway to induce aggrecan degradation by human chondrocytes.

Journal:	*Arthritis & Rheumatology*
Manuscript ID:	ar-14-1479.R3
Wiley - Manuscript type:	Full Length
Date Submitted by the Author:	17-Feb-2015
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Keywords:	Aggrecanase, Cartilage Degradation, Cell-signalling molecules, Chondrocyte, Interleukins

Cover page

Running Title: JNK2 mediates IL1-induced aggrecan degradation

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ACKNOWLEDGEMENTS: We thank Arthritis Research UK (grants 2025 and 20563), NIAMS (grant AR40994) and the Kennedy Trust for financial support, Lesley Rawlinson for making IL1 and Chris Murphy for supplying chondrocytes. LT and TV are Arthritis Research UK Fellows (grants 19466 and 20009). The content is solely the responsibility of the authors and does not necessarily represent the official views of NIAMS or NIH.

Abstract

Objectives: Aggrecan enables articular cartilage to bear load and resist compression. Aggrecan loss occurs early in osteoarthritis and rheumatoid arthritis and can be induced by inflammatory cytokines such as IL1. IL1 induces specific aggrecan cleavage characteristic of ADAMTS proteinases. We aimed to identify the intracellular signalling pathways by which IL1 causes aggrecan degradation by human chondrocytes, and investigate how aggrecanase activity is controlled by chondrocytes.

Methods: We developed a cell-based assay combining siRNA-induced knockdown with aggrecan degradation assays. Human articular chondrocytes were overlaid with bovine aggrecan after transfection with siRNAs against molecules of the IL1 signalling pathway. After IL1 stimulation, aggrecan fragments released were detected with AGEG and ARGS neo-epitope antibodies. Aggrecanase activity and TIMP3 levels were measured by ELISA. LRP-1 shedding was analysed by western blotting.

Results: ADAMTS5 is a major aggrecanase in human chondrocytes regulating aggrecan degradation in response to IL1. The TRAF6/TAK1/MKK4 signalling axis is essential for IL1 induced aggrecan degradation, while NFkB is not. Of the three MAPK kinases ERK, p38 and JNK, only JNK2 showed a significant role in aggrecan degradation. Chondrocytes constitutively secreted aggrecanase, which was continuously endocytosed by LRP-1 keeping the extracellular level low. IL1 caused aggrecanase activity to appear in the medium in a JNK2-dependent manner, possibly by reducing its endocytosis since IL-1 caused JNK2-dependent shedding of LRP-1.

Conclusion: The signaling axis TRAF6/TAK1/MKK4/JNK2 mediates IL1 induced aggrecanolysis. Aggrecanase level is controlled by its endocytosis which may be reduced upon IL1 stimulation because LRP-1 is shed.

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Introduction

Interleukin (IL) 1 is a major inflammatory cytokine with diverse biological actions[1]. Among these is its ability to cause local resorption of connective tissues such as articular cartilage and bone at inflammatory sites. The effect on articular cartilage has been much studied in organ culture because cartilage loss is a major feature and clinical problem in rheumatoid arthritis and osteoarthritis. When stimulated with IL1 the chondrocytes first resorb the proteoglycan aggrecan, the major soluble polymer of their extracellular matrix, and then more slowly degrade the insoluble network of collagen fibres, which mainly comprises type II collagen[2].

Aggrecan is a large proteoglycan whose protein core is heavily decorated with chondroitin and keratan sulphates. The hydrophilicity of these acidic polysaccharides creates the swelling pressure that enables cartilage to resist compression. The N-terminal G1 domain of aggrecan interacts with hyaluronic acid to form giant aggregates, which are trapped in the collagen fibre mesh[3].

The turnover of aggrecan is controlled extracellularly by proteinases of which the most important cleave it N-terminally to alanines 374 and 1820, releasing large C-terminal fragments from the aggregates [4]. These aggrecanases belong to the ADAMTS (standing for A Disintegrin And Metalloproteinase with ThromboSpondin domains) family[5, 6]. Cartilage from ADAMTS-5 -/- mice did not show accelerated aggrecan degradation in response to IL-1, and the animals had less cartilage damage in models of auto-immune inflammatory arthritis and osteoarthritis[7, 8]. ADAMTS-4, the first aggrecanase identified, may also be involved in cartilage degradation. Although it has not been found to play a role in murine cartilage it has been implicated in human cartilage aggrecan degradation in experiments employing RNA interference (RNAi)[9]. The activity of these enzymes is prevented by the tissue inhibitor of metalloproteinases (TIMP)-3 which is made by chondrocytes and many other cell types[10, 11].

Thus the evidence from experiments on ADAMTS-5 -/- mice strongly suggests that IL-1 works by increasing activity of ADAMTS-5. However the mechanism of this is unknown: expression of the enzyme might be increased, or TIMP-3 levels reduced, by IL-1 stimulation.

The intracellular signalling mechanisms by which IL-1 causes chondrocytes to degrade aggrecan are also unknown. The type 1 IL1 receptor signals via the IL1R-associated kinases (IRAK)-1 and 4 to activate the TNF receptor-associated factor (TRAF)6, an ubiquitinyl E3 ligase. TRAF6 forms lysine 63-linked

polyubiquitin chains which bind the TGF beta-activated kinase (TAK)1 via the TAK1-associated binding protein (TAB) causing activation of TAK1[12]. This in turn activates a core of four major pathways involving protein kinases: these are the canonical pathway that activates the transcription factor nuclear factor (NF) kappa B, and the three mitogen activated protein kinase (MAPK) cascades. The three MAPKS are the extracellularly regulated kinase (ERK), the c-jun N-terminal kinase (JNK) and p38. Genes whose transcriptional expression increases in inflammation are typically regulated by NFkB. Their promoters also commonly contain binding sites for activator protein (AP)-1 transcription factors such as c-jun, which is activated by JNK[13], and for C/EBP beta (also known as NF-IL6), which is activated by ERK[14]. The p38 MAPK controls the stability of many transcripts of the inflammatory response, but can also phosphorylate transcription factors[15].

We set out to understand the molecular mechanisms by which IL-1 stimulates human articular chondrocytes to degrade aggrecan. We found that aggrecan degradation was mainly dependent on ADAMTS-5, and this response was strikingly mediated by JNK2. IL1 did not cause increased production of ADAMTS-5; rather it apparently reduced its endocytic uptake via LDL receptor-related protein (LRP)-1 by causing increased shedding of LRP-1.

Materials and methods

Reagents and antibodies:

Anti-TRAF6(80285), anti-TAK1(D94D7), anti-MKK4(9152S), anti-ph-MKK4T261(9151S), anti-JNK2 (4672S), anti-IKB(4814S), anti-phospho-IkBα-Ser32/36(9246), and anti-phospho-p38-T180/Y182 (9211S), anti-phospho-TAK1(45085) from Cell Signalling. Anti-phospho-JNK-pTpY$^{183/185}$(44682G) from Invitrogen, anti-T-ERK(sc-94) from Santa Cruz, anti-LRP1 (8G1) and anti-ARGS(ab3773) from Abcam. TAK1 inhibitor 5z-7-oxozeanol from Tocris Bioscience, neddylation inhibitor MLN4924 from Active Biochem, MEK inhibitor U0126 from Promega , and p38 inhibitor SB203580 from Promega. Recombinant human C-terminally His-tagged RAP (human receptor-associated protein) was expressed in Escherichia coli using a pET3a-based expression vector and purified as described previously[17]. Bovine aggrecan was purified from bovine nasal cartilage [18]. TIMP3 ELISA was from R&D systems (DY973) and Aggrecanase Activity Assay ELISA (M046008) was from MD Bioproducts.

Cartilage tissue preparation and isolation of human chondrocytes

Normal articular cartilage was obtained from patients with informed consent and following local ethical committee guidelines. Cartilage was harvested from the femoral condyle and tibial plateau following surgery for tumours not involving the joint. Tissue was obtained from twenty- six donors (Supplementary table 1). Full-thickness (~1.5 mm^3) explants (3 per time point, 120 mg cartilage/ml medium) were cultured in 48-well plates containing serum-free DMEM (4.5g/L glucose with L-glutamine), 1% penicillin, streptomycin and amphotericin for 24 h. The medium was replaced with serum-free medium containing the inhibitor under test ± IL1 (50ng/ml) then incubated for a further 24 h. Conditioned medium was harvested and treated as below for detection of aggrecan cleavage. ***To prepare human articular chondrocytes***, freshly harvested human cartilage was chopped finely and placed in DMEM containing 10% fetal calf serum (Invitrogen) and 1.5 mg/ml collagenase type 2 (Roche). It was then incubated at 37°C for 18-24 h with shaking at 180 rpm. Chondrocytes were passed through a cell strainer, pelleted, and washed twice with medium. Cells were seeded at a density of 2 million cells/ 10 cm^2 tissue culture plate or 6 million cells in 15cm^2 tissue culture plate in full DMEM containing. Chondrocytes cultures (P0) were left undisturbed for 7 days then passaged at confluence. To ensure efficient transfection, passaged human chondrocytes (P2-P3) were used for siRNA experiments. Passaged chondrocytes may have undergone a degree of de-differentiation. The morphology of the cells was not changed by IL1 or siRNA treatments.

SiRNA Transfections

Human articular chondrocytes were transfected as described previously[19]. Briefly, chondrocytes were plated at 300,000 cells/well in 6 well plates. The following day, transfection with siRNA of interest was carried out at a final concentration of 10-30 nM using Lipofectamine 2000 (Invitrogen) for 4 h in serum-free OptiMEM I. Cells were then incubated in full medium for 72h to allow efficient knockdown. Following incubation, medium was changed to a serum-free medium containing the appropriate stimulus and / or inhibitor according to the experiment design. Gene-specific siRNAs were from Ambion (Life Technologies). siRNA oligos are as follows: ADAMTS-4 (s18227), ADAMTS-5 (s368), TRAF6(s14389), TAK1(s13767), MKK4(s12703), JNK1(s11152), JNK2 (s229708, s229709), JNK3 (s11161) and TIMP3 (s14146).

Detection of aggrecan degradation in chondrocytes culture medium: the aggrecan overlay assay.

Chondrocytes were overlaid with sterile filtered bovine aggrecan (25-50 µg/ml) in serum-free DMEM in the presence or absence of IL1 (50 ng/ml) according to the experimental set-up. Medium harvested from the cultures was processed to detect the aggrecanase-generated fragments as described previously[20]. Briefly, proteoglycans were extracted from the medium samples and deglycosylated in sodium acetate buffer with keratanase II (0.001 µ/µg GAG, Sigma) and chondroitinase ABC (0.001 U/µg GAG, Sigma). Proteins were then precipitated using ice-cold acetone and analysed by western blotting. mAb BC-3 which recognises N-terminal ARGS was used to detect fragments generated by cleavage of aggrecan at the Asn^{373}-Ala^{374} bond [21], and a polyclonal rabbit antibody directed at N-terminal AGEG was used to detect fragments generated by cleavage at the Glu^{1819}-Ala^{1820} bond[16].

ELISA for aggrecanase activity

The Aggrecanase Activity Assay (MD bioproducts) was used according to the manufacturer instructions to measure activity in cell culture medium. This assay consists of two modules, the Aggrecanase Module and the ELISA Module. A recombinant fragment of human aggrecan interglobular domain (aggrecan-IGD) is first digested with the sample of culture medium under test. Aggrecanase cleavage of the substrate releases a peptide with the N-terminal sequence ARGSVIL (ARGSVIL-peptide). The ARGSVIL-peptide is then quantified with two monoclonal anti-peptide antibodies.

Adenoviral infection of chondrocytes and NFkB Luciferase reporter assay

Chondrocytes in a 6-12 well plate were infected with adeno-IkBα non-phosphorylatable mutant, adeno-empty vector and/ or adeno-NF-κB luciferase reporter virus (AdvNF-κB-luc) as described previously[22]**.** Culture medium was harvested for analysis of aggrecan fragments AGEG and ARGS by western blotting as described above for the aggrecan overlay assay, while the cell layer was used for NFkB luciferase reporter assay.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from cells using a Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from total RNA High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Taqman probes for the following genes were from Applied Biosystems: ADAMTS4 (Hs00192708), ADAMTS5 (Hs00199841), JNK1 (Hs00177102), JNK2 (Hs001177012), JNK3 (Hs00373461), NFKBIA (Hs00355671), TIMP3 (Hs00 65949) and GAPDH (Hs0275899). Real-time PCR was performed ona Corbett Rotor-gene 6000 system (Corbett Research Ltd.) using 2X Taqman Master mix (Applied Biosystems) according to the manufacturer's instructions. Expression of the respective genes was normalized to GAPDH as an internal control using ddCT method.

Protein extracts and western blotting

Chondrocytes were lysed in RIPA buffer containing protease inhibitors. Proteins were resolved by SDS-PAGE and transferred membranes were incubated with antibodies against proteins of interest. **For LRP1 shedding,** media were collected and concentrated 10-fold using spin filters. LRP1 in the media was analyzed by western blotting using the 8G1 anti-LRP-1 heavy-chain antibody.

Statistical analysis

Data from experiment replicates were expressed as means ± S.E.M. The level of statistical significance was established using the unpaired, two-tailed Student's t-test at $p < 0.05$ using Prism 6 software (GraphPad).

Results

Aggrecan degradation by human chondrocytes is strongly dependent on ADAMTS-5.

Ideally one would wish to study molecular mechanisms of control of aggrecan turnover in tissue explants. However we needed to use RNAi and viral infection as well as pharmacological reagents and we found it difficult to achieve robust reproducible transfection of chondrocytes in intact tissue. We therefore set up a system in which isolated human chondrocytes in monolayer culture caused aggrecan degradation mirroring that seen in organ culture. Figure 1A is a simplified diagram of aggrecan interacting with hyaluronan, and the enlargement shows the location of the major aggrecanase cleavages immediately before alanines 374 and 1820 [23, 24]. Upon IL1 stimulation aggrecan breakdown increases and the fragments escape. They are detected, after deglycosylation by western blotting with antibodies to the ARGS and AGEG N-terminal neo-epitopes. ARGS fragments of Mr ~250, ~180 and 70 kDa (Figure 1B left upper panel) and an AGEG fragment of Mr ~130kDa (Figure 1B left lower panel) are seen. Passaged human chondrocytes did not secrete enough aggrecan for cleavage neoepitopes to be detected after IL1 stimulation. We therefore overlaid the cells with purified full length bovine aggrecan. When stimulated with IL1 they generated the 250kDa ARGS and the 130 kDa AGEG fragments (Figure 1B right hand panels), but not those of 180 and 70 kDa. The two latter may arise because some aggrecan in human articular cartilage is truncated at the C-terminus [24, 25]. The assay system is robust and reproducible although it does not analyse degradation in a true cell associated matrix. We next used siRNA to knock down either ADAMTS-4 or ADAMTS-5 in the chondrocytes before overlaying them with aggrecan substrate and stimulating them with IL1. Chondrocytes transfected with scrambled (CTRL) sequence oligonucleotides were always used as controls in RNAi experiments. Figure 1C shows that both spontaneous and IL1-induced generation of the 250kDa ARGS and 130kDa AGEG fragments were strongly reduced by knocking down ADAMTS-5. Combining results of three experiments, inhibition of AGEG generation by the knockdowns was 90% for ADAMTS-5 and 25% for ADAMTS-4 (Figure 1D, upper graph). Knockdown efficiencies were higher than 80% (Figure 1D, lower graph). We decided to use the 250kDa ARGS and 130kDa AGEG fragments as indicators of aggrecan degradation stimulated by IL-1.

Upstream components of IL-1 signalling pathways, TRAF6 and TAK1 are essential for IL-1 induced degradation of aggrecan.

We next investigated the intracellular signalling pathways controlling aggrecan degradation. The IL1R1 activates TAK1 via TRAF6. Knocking down either TRAF6 (Figure 2A) or TAK1 (Figure 2B) inhibited generation of the AGEG fragment by IL1.We tested a pharmacological inhibitor of TAK1 on cartilage explants in order to validate the chondrocyte experiments. Figure 2C shows that the inhibitor 5-z-7-oxozeanol [26] blocked IL-1 generating ARGS and AGEG aggrecan fragments in cultured cartilage.

NFkB, p38 or ERK pathways are not necessary for IL1-induced aggrecan degradation in human chondrocytes

TAK1 is involved in the activation of NFkB which drives transcription of many IL1-induced genes. We inhibited the canonical pathway of activation by transfecting chondrocytes with adenovirus expressing a non-phosphorylatable mutant form of the inhibitor of NFkB (IkB) alpha [22]. The ability of chondrocytes to generate the ARGS and AGEG fragments in response to IL-1 was unaffected by inhibiting NFkB , although the induction of cyclooxygenase-2 protein was prevented (Figure 3A). To verify that NFkB was being inhibited by the mutant IkB alpha we co-transfected the chondrocytes with an NFkB reporter (Figure 3C). To check that the IL-1-induced aggrecan degradation in cartilage tissue was independent of NFkB we used the neddylation inhibitor MLN9424 which blocks IkB degradation [27] (Figure 3D, left panel).

TAK1 also activates the p38 MAP kinase pathway. There are well characterized inhibitors for p38, so we tested one, SB201920, on cartilage tissue. Figure 3D, middle panel shows it did not affect IL1-induced aggrecan degradation. In addition we tested an inhibitor of ERK activation, U0126, since the ERK MAPK is also activated by IL1. This actually enhanced the generation of both fragments by cartilage treated with IL-1 (Figure 3D, right panel).

IL1- induced aggrecan degradation depends upon MKK4/JNK2.

Having established that NFkB, p38 and ERK were not mediating the IL-1 signal to degrade aggrecan we examined the JNK pathway. Knocking down MAPK activating kinase (MKK)4 which is a substrate of TAK1 and an activator of JNK prevented generation of the AGEG aggrecan fragment by IL-1 (Figure 4A). We also knocked down MKK7, which may also be involved in the activation of JNK, but this only caused about 30% reduction in production of AGEG neo-epitope(data not shown).

There are three JNK genes. Knocking down JNK2 prevented generation of the AGEG fragment by chondrocytes stimulated with IL1, while knocking down JNK1 or JNK3 had no discernible effect (Figure 4B). In a further experiment two different oligonucleotides were used to knock down JNK2. The generation of both ARGS and AGEG aggrecan fragments by IL1 stimulation of cells treated with scrambled oligonucleotides was suppressed in those subjected to JNK2 knockdown (Figure 4C, left versus right panels). Quantification of combined experiments showed 80-90% suppression of the aggrecan cleavages induced by IL-1 in the JNK2 knockdown cells(Figure 4C, bar graph). Knockdowns of JNK2 and 3 mRNAs were 90% efficient, but that of JNK1 only 50% (Figure 4D, graph). IL1 stimulation increased phosphorylation of JNK bands of Mr 54kDa and 46kDa (Figure 4D upper panel, lane 2). The 54kDa band was strongly reduced in the JNK2 knockdown (Figure 4D upper panel, lane 4). Western blotting of the 54kDa JNK protein confirmed the knockdown was efficient (Figure 4D middle panel).

IL1 via JNK2 increases aggrecanase activity and enhances LRP-1 shedding.

Having established the importance of the JNK pathway for IL1-induced aggrecan degradation we investigated the mechanism. The initial experiments (Figure 1) showed that the degradation was mainly due to ADAMTS-5 and to a lesser extent ADAMTS-4. There are no specific sensitive ELISAs or western blotting antibodies available for these enzymes, but their combined activity can be measured in the culture medium with an assay that uses a recombinant peptide substrate containing the ARGS cleavage site. The cleaved ARGS fragment is detected by ELISA. Aggrecanase activity was barely detectable in medium from resting cultures, but was present in that from IL1-stimulated chondrocytes. It was reduced 70% by knockdown of ADAMTS-5, and 20% by ADAMTS-4 knockdown (Figure 5A). When JNK2 was knocked down the activity was reduced by 80% (Figure 5B). Knocking down JNK2 had no effect on either ADAMTS-4 (Supplementary Figure 1A) or ADAMTS-5 basal mRNA levels (Supplementary Figure 1B). IL-1 did not alter ADAMTS-5 mRNA but increased ADAMTS-4 mRNA (Supplementary Figure 1A and B).

The IL1-induced aggrecanase activity was mainly due to ADAMTS-5 whose mRNA is not significantly increased. It was possible that IL1 increased the amount of ADAMTS-5 protein without regulating its mRNA. The absolute abundance of ADAMTS5 transcripts was markedly greater than those of ADAMTS4 even after IL1 stimulation (Supplementary Table 2). Given that ADAMTS-5 is a 25-fold stronger aggrecanase than ADAMTS4 [28], the knockdown result is not surprising.

Another possibility was that IL1 reduced TIMP3 levels so unmasking enzyme activity. Both ADAMTS enzymes and TIMP3 are endocytosed by the LDL receptor-related protein (LRP)-1 [17, 29, 30]. The steady state levels of TIMP3 and aggrecanases reflects the rates of both secretion and uptake by the cells. We reasoned that if we blocked their uptake by LRP-1 with the LRP-like receptor antagonist, receptor antagonist protein (RAP) [31], and in addition knocked down TIMP3 (to prevent any inhibition of aggrecanase), we should be able to measure the activity of the total amount of aggrecanase secreted by the cells, assuming there was no other major route of uptake. Adding RAP to the resting chondrocytes increased the aggrecanase activity in the medium by 10-50 fold (Figure 5C, bar 3 v 1), indicating that in resting cells 98% of secreted enzyme detected by the activity assay kit is endocytosed. Knocking down TIMP3 in addition to adding RAP did not significantly increase the amount of aggrecanase activity seen with RAP alone (Figure 5C bar 7 v 3). The efficiency of the knockdown can be seen by the reduction in TIMP3 protein levels shown in Figure 5D bars 5-8. The result was surprising because we expected removing TIMP3 from the system would increase the amount of aggrecanase activity detected. It suggested that in molar terms much less TIMP3 was being made than active aggrecanase, or conceivably other proteinases with higher affinity were making the TIMP3 unavailable. Nevertheless it could be concluded that most of the active aggrecanase detected by the assay kit (and probably aggrecanase protein) secreted was endocytosed via LRP-1.

We next investigated the effect of IL1: as before, stimulation increased the aggrecanase activity detected (Figure 5C bar 1 v 2). If RAP was added along with IL-1 the activity increased (Figure 5C bar2 v 4), but was not significantly more than was found in the medium of resting cells in the presence of RAP alone (Figure 5C bar 3 v 4). Combining TIMP3 knockdown with adding RAP did not further increase the activity produced in the presence of IL1 (Figure 5C bar 4 v 8). TIMP3 protein levels in the medium were not significantly changed by IL1 stimulation (Figure 5D bar1 v 2) and adding RAP increased the level 3-4 fold (Figure 5D bars 1 and 2 v bars 3 and 4).

We concluded (a) that virtually all the aggrecanase detectable by the aggrecanase kit (mainly ADAMTS-5) made by the chondrocytes is normally endocytosed by LRP-1 so keeping extracellular levels low and (b) that IL1 did not significantly increase the amount of active aggrecanase synthesized and secreted in the presence of RAP. We reasoned that IL1 was possibly reducing the amount of aggrecanase endocytosed and this was causing the increased activity in the medium. LRP-1 is shed from macrophages stimulated with bacterial lipopolysaccharide [32] and from fibroblasts stimulated with TNF [33]. Shedding might reduce surface expression of LRP-1 and so reduce endocytosis of its ligands. We therefore examined the effect of IL1 on expression and shedding of LRP-1. IL1 increased the amount of LRP-1 shed by chondrocytes and reduced the cellular LRP-1 by about 50% during 24 hours culture (Figure 6A). Cell surface LRP-1 measured by FACS showed a similar reduction in response to IL1 (data not shown). LRP-1 mRNA was not significantly altered by IL1 (Supplementary Figure 1C). The shedding of LRP-1 induced by IL1 was prevented by knockdown of JNK2 (Figure 6C and D). The knockdown did not affect LRP-1 mRNA levels (Supplementary Figure 1C). Thus JNK2-mediated shedding of LRP-1 could cause reduction in endocytosis of aggrecanase and increase its concentration in the culture medium during IL1 stimulation.

Discussion.

Aggrecan degradation is traditionally studied in explant cultures because this keeps the cells in their physiological extracellular matrix. However chondrocytes embedded in tissue are very difficult to manipulate by transfection or viral infection. To dissect the molecular mechanisms by which IL1 causes degradation of aggrecan we therefore set up a model system in which monolayer cultures of chondrocytes were transfected with oligonucleotide or virus of interest, then overlaid with a pure aggrecan substrate. The cells were then stimulated with IL-1 and the aggrecan fragments they generated could be analysed. Alternatively, the spent medium could be used for analysis of enzymes or other products. We adopted this approach because the control of aggrecan breakdown by chondrocytes is dynamic, tightly controlled and not understood. It is well known that the mRNA of a major aggrecanase implicated in mediating cartilage damage in murine models, ADAMTS-5, shows little or no increase in human chondrocytes stimulated with IL1. An additional difficulty in understanding aggrecan degradation lies in determining and measuring the different forms of aggrecanase proteins because of a lack of antibodies suitable for western blotting the low (picomolar) concentrations found in cell cultures. ADAMTS enzymes

have multiple domains which determine binding to extracellular matrix, substrate specificity and interaction with the cell surface [17, 34]. While we can measure total aggrecanase activity in the spent culture medium we do not understand which processed or modified forms are contributing to the measurement or which are most relevant to cleaving aggrecan in cartilage. Another factor confounding the analysis of aggrecan degradation is the presence of the inhibitor TIMP3, and the fact that TIMP3, and ADAMTS-4 and ADAMTS-5 are all internalized by cells via LRP-1[17, 29, 30]. LRP-1 is a promiscuous surface receptor which can bind and internalise at least forty different proteins [31].

The main conclusions from the experiments are that IL1 causes aggrecan degradation by human chondrocytes by signalling through the JNK pathway, and that a major aggrecanase responsible for the IL1-induced activity was ADAMTS-5, with ADAMTS-4 playing a minor role. Our work is limited to analysis to two major cleaveages of aggrecan: to what extent blocking JNK inhibits all aggrecan degradation is under investigation. The experiments in which LRP-1 was blocked with RAP showed that in resting cells aggrecanase was secreted but that 98% of this was endocytosed. Knocking down TIMP3 had no measurable effect on the amount of aggrecanase detected, suggesting that enzyme is made in larger amount than the inhibitor. Stimulation by IL1 also did not increase the amount of aggrecanase produced detectable by the assay kit. The amount found in the spent medium of the IL-1-stimulated cultures represented about 20% of the total being made. The lack of a clear increase in synthesis of active aggrecanase by stimulated cells, together with its efficient endocytosis in resting cells suggests a reduction in its endocytosis during IL1 stimulation as a possible means of regulation. The JNK2-dependent shedding of LRP-1 and the reduction in its cellular and surface expression are consistent with this possibility.Further investigation is therefore needed to establish the relationship between LRP-1 shedding, the aggrecanase(s) that accumulates in the culture medium and the form(s) of aggrecanase that cleave aggrecan in the overlay cell culture system and cartilage tissue.

The aggrecan cleavages in the overlay system were strikingly dependent on JNK2, and although we did not achieve complete knockdown of JNK1 we expected to see some reduction of generation of the fragments if it was involved. Quantification of the individual JNK proteins is not possible, but the human chondrocytes stimulated with IL1 appeared to have similar amounts of phosphorylated JNK2, the 54kDa band, and of the 46kDa band which is likely to be mainly JNK1. JNK1 and JNK2 are ubiquitous, while JNK3 expression is mainly confined to the CNS[35]. It is not known whether the different JNKs have

different downstream functions. Our understanding of the physiological functions of these kinases has been held up by the lack of good pharmacological inhibitors. The single knockouts of JNK1 and JNK2 have no marked phenotype, while the double knockouts are embryonically lethal[36]. Further work is required to determine whether the action of IL1 on aggrecan degradation is mediated specifically by JNK2. Another question is whether activity in the JNK pathway determines the level of aggrecan degradation under normal physiological conditions or whether it only comes into play in a stressful situation such as cytokine stimulation. Related to this question is whether JNK activity is sufficient for aggrecan degradation; our experiments show it is necessary in the context of IL1 stimulation, although we did not find activation of NFkB, p38 or ERK were contributing to degradation.

Our studies have been restricted to IL1, but many factors affect JNK activity[35]. Among inflammatory stimuli TNF, TLR ligands, IL-17 and IL-18 all activate the same set of pathways including JNKs. JNK is also activated by a variety of physical stressful stimuli[35] including simple mechanical injury of cartilage and synovium[37]. Thus in OA, for example, in which the role of IL1 is highly debatable, other JNK activators might determine pathology.

We speculated that the JNK2- dependent shedding of LRP-1 might be a mechanism of reducing its surface expression and thereby increase aggrecanase activity. Shedding of LRP-1 may be mediated by metalloproteinases(e.g. ADAM9, 10 and 12) as well as other enzymes [38], but how JNK2 might control this mechanism is unknown. JNKs characteristically activate AP-1 transcription factors which control expression of many genes.

References

1. Garlanda C, Dinarello CA, Mantovani A: **The interleukin-1 family: back to the future**. *Immunity* 2013, **39**(6):1003-1018.

2. Rowan AD, Hui W, Cawston TE, Richards CD: **Adenoviral gene transfer of interleukin-1 in combination with oncostatin M induces significant joint damage in a murine model**. *The American journal of pathology* 2003, **162**(6):1975-1984.

3. Kiani C, Chen L, Wu YJ, Yee AJ, Yang BB: **Structure and function of aggrecan**. *Cell research* 2002, **12**(1):19-32.

4. Sandy JD, Neame PJ, Boynton RE, Flannery CR: **Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain**. *The Journal of biological chemistry* 1991, **266**(14):8683-8685.

5. Tortorella MD, Burn TC, Pratta MA, Abbaszade I, Hollis JM, Liu R, Rosenfeld SA, Copeland RA, Decicco CP, Wynn R *et al*: **Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins**. *Science* 1999, **284**(5420):1664-1666.

6. Abbaszade I, Liu RQ, Yang F, Rosenfeld SA, Ross OH, Link JR, Ellis DM, Tortorella MD, Pratta MA, Hollis JM *et al*: **Cloning and characterization of ADAMTS11, an aggrecanase from the ADAMTS family**. *The Journal of biological chemistry* 1999, **274**(33):23443-23450.

7. Glasson SS, Askew R, Sheppard B, Carito B, Blanchet T, Ma HL, Flannery CR, Peluso D, Kanki K, Yang Z *et al*: **Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis**. *Nature* 2005, **434**(7033):644-648.

8. Stanton H, Rogerson FM, East CJ, Golub SB, Lawlor KE, Meeker CT, Little CB, Last K, Farmer PJ, Campbell IK *et al*: **ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro**. *Nature* 2005, **434**(7033):648-652.

9. Song RH, Tortorella MD, Malfait AM, Alston JT, Yang Z, Arner EC, Griggs DW: **Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5**. *Arthritis and rheumatism* 2007, **56**(2):575-585.

10. Brew K, Nagase H: **The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity**. *Biochimica et biophysica acta* 2010, **1803**(1):55-71.

11. Kashiwagi M, Tortorella M, Nagase H, Brew K: **TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5)**. *The Journal of biological chemistry* 2001, **276**(16):12501-12504.

12. O'Neill LA: **The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress**. *Immunological reviews* 2008, **226**:10-18.

13. Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J, Woodgett JR: **The stress-activated protein kinase subfamily of c-Jun kinases**. *Nature* 1994, **369**(6476):156-160.

14. Nakajima T, Kinoshita S, Sasagawa T, Sasaki K, Naruto M, Kishimoto T, Akira S: **Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for**

transcription factor NF-IL6. *Proceedings of the National Academy of Sciences of the United States of America* 1993, **90**(6):2207-2211.

15. Clark AR, Dean JL, Saklatvala J: **Post-transcriptional regulation of gene expression by mitogen-activated protein kinase p38**. *FEBS letters* 2003, **546**(1):37-44.

16. Troeberg L, Fushimi K, Khokha R, Emonard H, Ghosh P, Nagase H: **Calcium pentosan polysulfate is a multifaceted exosite inhibitor of aggrecanases**. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2008, **22**(10):3515-3524.

17. Yamamoto K, Troeberg L, Scilabra SD, Pelosi M, Murphy CL, Strickland DK, Nagase H: **LRP-1-mediated endocytosis regulates extracellular activity of ADAMTS-5 in articular cartilage**. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2013, **27**(2):511-521.

18. Hascall VC, Sajdera SW: **Proteinpolysaccharide complex from bovine nasal cartilage. The function of glycoprotein in the formation of aggregates**. *The Journal of biological chemistry* 1969, **244**(9):2384-2396.

19. Lafont JE, Talma S, Murphy CL: **Hypoxia-inducible factor 2alpha is essential for hypoxic induction of the human articular chondrocyte phenotype**. *Arthritis and rheumatism* 2007, **56**(10):3297-3306.

20. Sawaji Y, Hynes J, Vincent T, Saklatvala J: **Fibroblast growth factor 2 inhibits induction of aggrecanase activity in human articular cartilage**. *Arthritis and rheumatism* 2008, **58**(11):3498-3509.

21. Hughes CE, Caterson B, Fosang AJ, Roughley PJ, Mort JS: **Monoclonal antibodies that specifically recognize neoepitope sequences generated by 'aggrecanase' and matrix metalloproteinase cleavage of aggrecan: application to catabolism in situ and in vitro**. *Biochem J* 1995, **305 (Pt 3)**:799-804.

22. Sanlioglu S, Williams CM, Samavati L, Butler NS, Wang G, McCray PB, Jr., Ritchie TC, Hunninghake GW, Zandi E, Engelhardt JF: **Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor-alpha secretion through IKK regulation of NF-kappa B**. *J Biol Chem* 2001, **276**(32):30188-30198.

23. Tortorella MD, Pratta M, Liu RQ, Austin J, Ross OH, Abbaszade I, Burn T, Arner E: **Sites of aggrecan cleavage by recombinant human aggrecanase-1 (ADAMTS-4)**. *The Journal of biological chemistry* 2000, **275**(24):18566-18573.

24. Struglics A, Hansson M, Lohmander LS: **Human aggrecanase generated synovial fluid fragment levels are elevated directly after knee injuries due to proteolysis both in the inter globular and chondroitin sulfate domains**. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2011, **19**(8):1047-1057.

25. Sandy JD, Verscharen C: **Analysis of aggrecan in human knee cartilage and synovial fluid indicates that aggrecanase (ADAMTS) activity is responsible for the catabolic turnover and loss of whole aggrecan whereas other protease activity is required for C-terminal processing in vivo**. *The Biochemical journal* 2001, **358**(Pt 3):615-626.

26. Ninomiya-Tsuji J, Kajino T, Ono K, Ohtomo T, Matsumoto M, Shiina M, Mihara M, Tsuchiya M, Matsumoto K: **A resorcylic acid lactone, 5Z-7-oxozeaenol, prevents inflammation by inhibiting**

the catalytic activity of TAK1 MAPK kinase kinase. *The Journal of biological chemistry* 2003, **278**(20):18485-18490.

27. Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, Brownell JE, Burke KE, Cardin DP, Critchley S *et al*: **An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer**. *Nature* 2009, **458**(7239):732-736.

28. Fushimi K, Troeberg L, Nakamura H, Lim NH, Nagase H: **Functional differences of the catalytic and non-catalytic domains in human ADAMTS-4 and ADAMTS-5 in aggrecanolytic activity**. *The Journal of biological chemistry* 2008, **283**(11):6706-6716.

29. Yamamoto K, Owen K, Parker AE, Scilabra SD, Dudhia J, Strickland DK, Troeberg L, Nagase H: **Low-density lipoprotein receptor-related protein 1 (LRP1)-mediated endocytic clearance of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4: Functional differences of non-catalytic domains of ADAMTS-4 and ADAMTS-5 in LRP1 binding**. *The Journal of biological chemistry* 2014.

30. Scilabra SD, Troeberg L, Yamamoto K, Emonard H, Thogersen I, Enghild JJ, Strickland DK, Nagase H: **Differential regulation of extracellular tissue inhibitor of metalloproteinases-3 levels by cell membrane-bound and shed low density lipoprotein receptor-related protein 1**. *The Journal of biological chemistry* 2013, **288**(1):332-342.

31. Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK: **LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies**. *Physiological reviews* 2008, **88**(3):887-918.

32. Gorovoy M, Gaultier A, Campana WM, Firestein GS, Gonias SL: **Inflammatory mediators promote production of shed LRP1/CD91, which regulates cell signaling and cytokine expression by macrophages**. *Journal of leukocyte biology* 2010, **88**(4):769-778.

33. Wygrecka M, Wilhelm J, Jablonska E, Zakrzewicz D, Preissner KT, Seeger W, Guenther A, Markart P: **Shedding of low-density lipoprotein receptor-related protein-1 in acute respiratory distress syndrome**. *American journal of respiratory and critical care medicine* 2011, **184**(4):438-448.

34. Gendron C, Kashiwagi M, Lim NH, Enghild JJ, Thogersen IB, Hughes C, Caterson B, Nagase H: **Proteolytic activities of human ADAMTS-5: comparative studies with ADAMTS-4**. *The Journal of biological chemistry* 2007, **282**(25):18294-18306.

35. Kyriakis JM, Avruch J: **Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update**. *Physiological reviews* 2012, **92**(2):689-737.

36. Kuan CY, Yang DD, Samanta Roy DR, Davis RJ, Rakic P, Flavell RA: **The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development**. *Neuron* 1999, **22**(4):667-676.

37. Watt FE, Ismail HM, Didangelos A, Peirce M, Vincent TL, Wait R, Saklatvala J: **Src and fibroblast growth factor 2 independently regulate signaling and gene expression induced by experimental injury to intact articular cartilage**. *Arthritis and rheumatism* 2013, **65**(2):397-407.

38. Emonard H, Theret L, Bennasroune AH, Dedieu S: **Regulation of LRP-1 expression: make the point**. *Pathologie-biologie* 2014, **62**(2):84-90.

Figure Legends

Figure 1: IL1-induced aggrecan cleavage by human chondrocytes depends mainly upon the aggrecanase ADAMTS-5. (A) A simplified diagram of a proteoglycan aggregate formed by aggrecan monomers (enlargement) binding to hyaluronic acid. The arrows indicate aggrecanase specific cleavages that generate ARGS and AGEG fragments. **(B)** Human cartilage explants (left panels) were adapted to culture for 24h at 37ºC, then left or treated with IL1(50ng/ml). Medium was then processed and blotted for ARGS and AGEG neo-epitopes. Human articular chondrocytes (right panels) were cultured in 6 well plates (3 x 10^5 cells/well) at 37°C for 24h. Cells were then overlaid with bovine aggrecan (50 µg/ml) ± IL1(50ng/ml) for 24h. Medium was western blotted as for cartilage. **(C)** Chondrocytes were cultured as for (B). Cells were transfected with 20nM siRNA against ADAMTS-4, ADAMTS-5 or with scrambled (CTRL) siRNA as in Methods. After 72 h, cells were overlaid with bovine aggrecan (50µg/ml) ±IL1 (50ng/ml) for 24h. Medium was processed and western blotted as for (B). **(D)** upper bar graph shows densitometry of IL1 panels of AGEG blots in (C). ***P*<0.01 and ****P*<0.001 are for un-paired t-test comparing test siRNA with scrambled siRNA. Lower graph shows knockdown efficiency of ADAMTS-4, and ADAMTS-5 measured by qRT-PCR.

Figure 2: Essential role for TAK1 and TRAF6, the key upstream components of IL-1 signalling pathways, in IL-1 induced degradation of aggrecan. (A&B) Chondrocytes (passage 2-4) were cultured as described in Methods. Cells were transfected with 20nM siRNA against TRAF6, TAK1 or with scrambled (CTRL) siRNA. After 72 h. one set of cells **(top panels)** were overlaid with aggrecan (50 µg/ml) ± IL1(50ng/ml) in serum free DMEM for 24h. Medium was blotted for the AGEG aggrecan fragment. Bar graphs are densitometry of IL-1 panels (n=3) for each siRNA. AGEG fragments generated were compared by un-paired t-test. **P<0.01, ***P<0.001, ****P<0.0001. The second set of cells **(lower panels)** were treated ± IL1 (50ng/ml) for indicated short time points and cell lysates were analysed for knockdown efficiency by western blotting for TRAF6 **(A)** or TAK1 **(B)**, and for phosphorylated TAK1 **(A&B)** as indicated. Total ERK was used as a loading control. **(C)** Human cartilage explants were adapted to culture for 24h, then pre-treated with or without TAK1 inhibitor 5-z-7-Oxozeanol for 1hr. Cartilage explants were then treated with ± IL1 (50ng/ml) for 24 hours. Medium was then blotted for AGEG or ARGS neo-epitopes. **Graph in (C)** shows quantification of AGEG and ARGS blots.

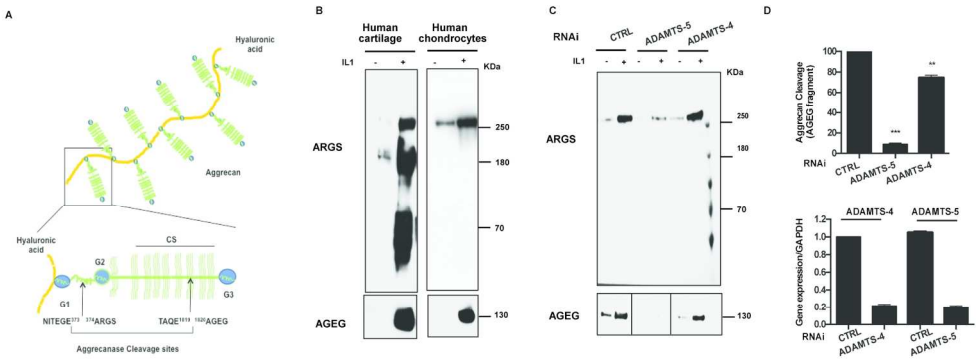
Figure 3: NFkB, p38 or ERK pathways are not essential for IL1-induced aggrecan degradation in human chondrocytes and human articular cartilage. (A-C) Chondrocytes were cultured then infected with empty adenovirus or adenovirus expressing a non phosphorylatable mutant of IkB**α** as in Methods. **(A)** After 24h**,** cells were overlaid with aggrecan (50µg/ml) ±IL1 (50ng/ml) for 24h. Medium was then blotted for ARGS and AGEG neo-epitopes. The cells were lysed in RIPA buffer and western blotted for cyclooxygenase(Cox)-2 or ERK. **(B)** After infection as in **(A)**, cells were stimulated with IL1 for the indicated times then lysates were western blotted for phosphorylated IkB**α**, IkB**α**, phosphorylated JNK, and also ERK to check equal protein loading. **(C)** Cells were infected as in **(A)** for 2h, then with an NFkB reporter for another 2h. Luciferase activity was measured by a luminometer as in Methods. **(D)** Human cartilage explants were adapted to culture for 24h, then pre-treated with or without inhibitors for 1h at the indicated concentrations. NFkB inhibitor (MLN9424), p38 inhibitor (SB201920), and ERK inhibitor (U0126). Cartilage explants were then treated ± IL1 (50ng/ml) for 24h. After incubation, culture medium was blotted for aggrecan fragments containing AGEG and ARGS neo-epitopes.

Figure 4: MKK4/JNK2 pathway regulates IL1-induced aggrecan degradation in human chondrocytes : Chondrocytes (passages 2-3) were cultured as described in Methods. Cells were then transfected with 20-50nM siRNA against MKK4, JNK1, JNK2, JNK3 or with scrambled (CTRL) siRNA as indicated. Cells were left for 72h to allow knockdown then overlaid with aggrecan (50 µg/ml) ± IL1 (50ng/ml) for 24h. Medium was blotted for the aggrecan AGEG fragment **(A&B)**. Lower panel blots in **(A)**

show knockdown efficiency of MKK4. **(C**) as for **(B)** except that two oligonucleotides (labelled oligo1 and oligo 2) directed at JNK2 were used and western blotting was for both ARGS and AGEG aggrecan fragments. Bar graphs are densitometry of IL-1 panels from at least three independent experiments. **P<0,01, ***P,<0.001, ****P<0.0001) are for un-paired t-test of AGEG**(A)** or AGEG and ARGS fragments generated**(C)**. **(D)** Cells were cultured and transfected as in **(C)**. After 72h, cells were left or treated with IL1 (50ng/ml) for 5min. Cell lysates western blotted phosphorylated-JNK, JNK2 and ERK as indicated. Graph in **(D)** shows mRNA of JNKs and GAPDH were measured by RT-PCR in extracts of cells in C and D. Results are means of triplicate cultures ± SEM.

Figure 5 : IL1, via JNK2, reduces endocytosis of aggrecanase. Human articular chondrocytes were cultured as described in Methods and transfected with 20nM siRNA against ADAMTS-4, ADAMTS-5, JNK2, TIMP-3 as indicated.. Cells were left for 72h to allow efficient knockdown. Cells were overlaid with bovine aggrecan then left or treated with IL-1 (50ng/ml) for 24h as indicated. **(A & B)** Aggrecanase activity in the culture medium was measured using an aggrecanase activity kit. The activity in the medium from cells treated with scrambled siRNA and IL1 was set as 100%. Results are means of three independent experiments ± SEM. **P<0,01, ****P<0.0001). (**C&D)** Cells were treated with siRNA to TIMP3 or with scrambled siRNA. Cells were then left or treated with RAP (500 nM), in the presence or absence of IL1 (50 ng/ml. After 24h, the aggrecanase activity of the culture medium was measured. Results are mean of four independent experiments ± SEM. **(D)** TIMP3 in the culture medium harvested from **(C)** was measured by ELISA.

Figure 6: IL1 stimulation of LRP1 shedding is dependent on JNK2. (A) Human articular chondrocytes (passages 2-4) were cultured as described in Methods. Cells were treated with or without IL1 for 24 h. LRP1 levels in cells (Cellular LRP1) or in culture medium (Shed LRP1) were analyzed by western blotting. (B) Graph shows quantification of western blotting of three independent experiments. **(C)** Cells were transfected with 20nM siRNA against JNK2 or with scrambled siRNAs. Cells were left for 72 h Cells then left or treated with IL-1 (50 ng/ml) for 24 h as indicated. Shed LRP1 levels in culture medium were analyzed by western blotting. The knockdown efficiency of JNK2 was analyzed in cell lysates by Western blotting and membrane re-blotted for total ERK as a loading control. **(D)** Graph shows quantification of western blotting of three independent experiments in **(C)**.



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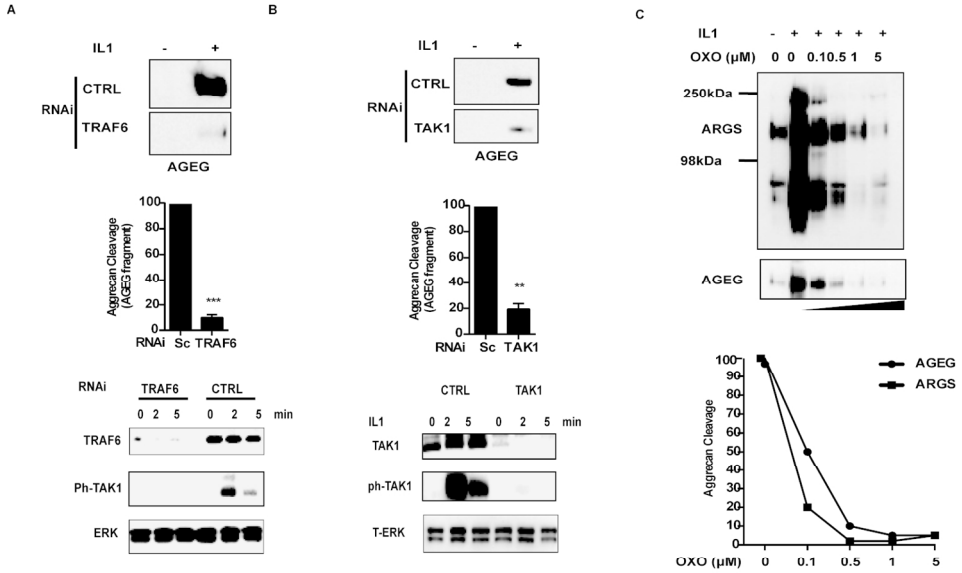

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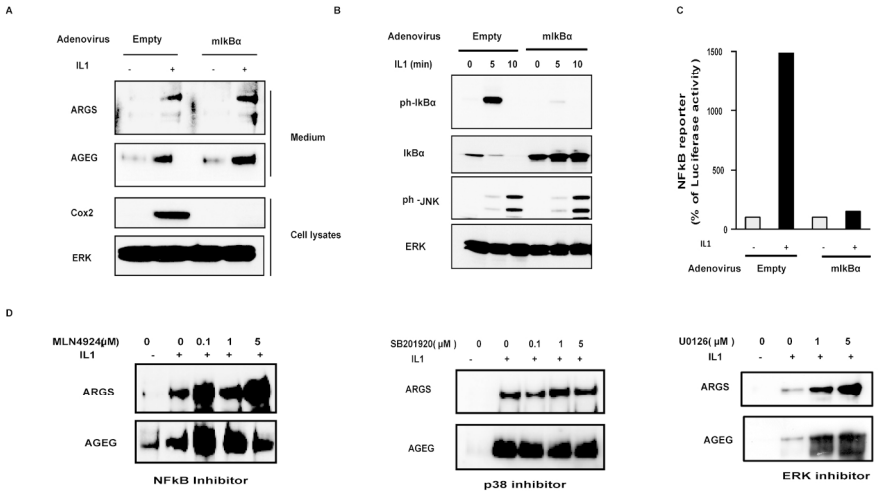

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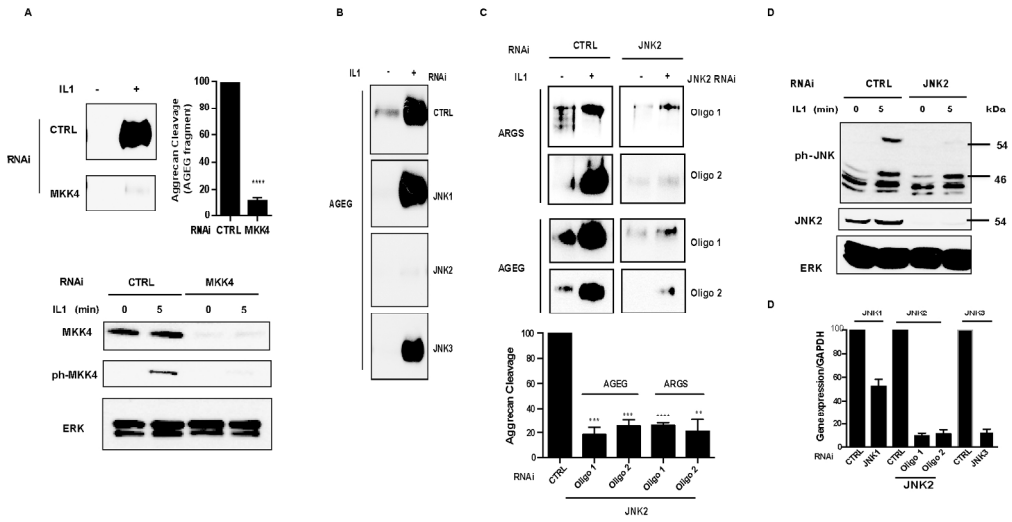

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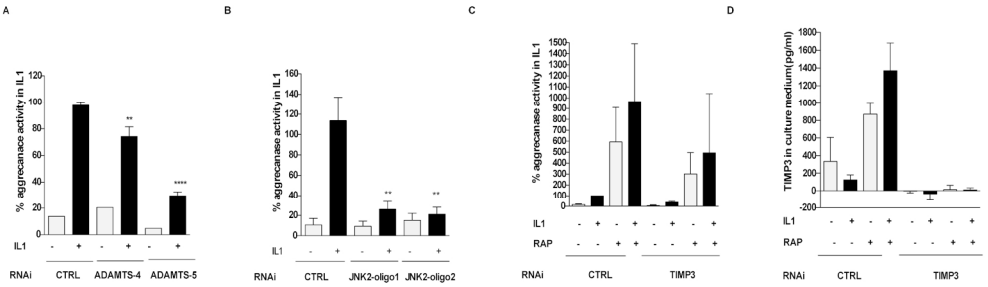

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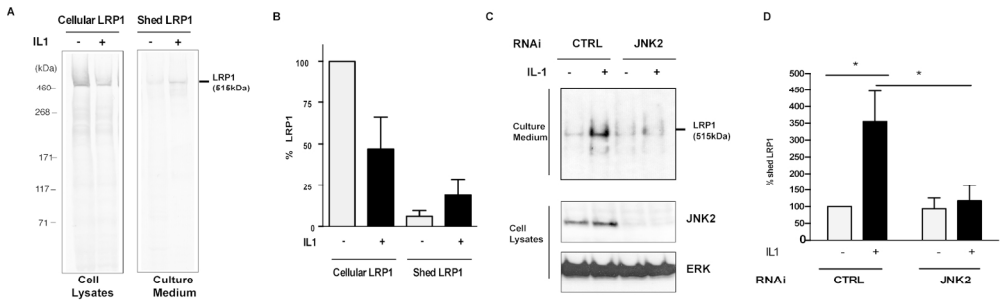

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156x75mm (300 x 300 DPI)

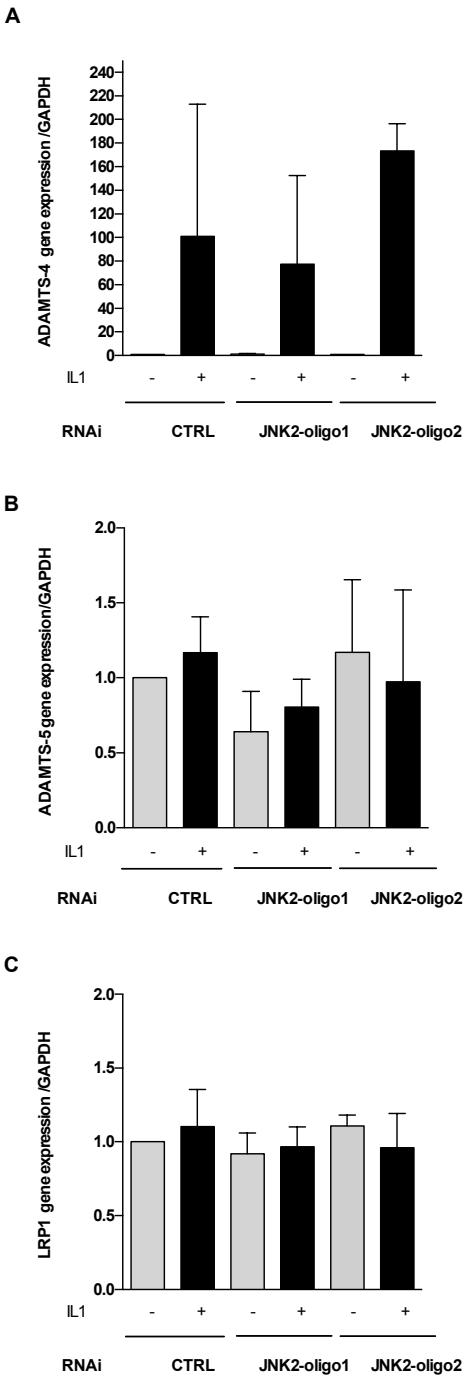
Supplementary data

Supplementary Table 1: Details of cartilage tissue donors in the study

Patient ID	Age	Specimen	Sex	Diagnosis
N10*	19	Femoral condyle	F	Osteosarcoma
N15	16	tibial plateau, patella	M	Knee amputation for Osteosarcoma
N16	15	Femoral condyle, tibial plateau	M	?
N20	8	tibial plateau,patella, femoral condyle	M	Osteosarcoma
N21	12	Femoral condyle and tibial plateau	M	
N30	55	Femoral condyle, tibial plateau	M	Osteosarcoma?
N31	54	Femoral condyle, tibial plateau	M	Osteosarcoma?
N45	55	tibial plateau and distal femur	M	Myxofibrosarcoma
N48	28	tibial plateau and distal femur	M	
N94	13	Fem condyle	M	Fibromatosis
N99	16	Knee	M	Osteosarcoma
N101	41	Knee	M	Chondrosarcoma
N107	21	Distal femur only	M	Ewing's sarcoma
N108				
N109				
N110	47	Above knee amp (+syn fluid)	M	Chondrosarcoma
N111	58	Distal fem and tibial plateau	F	
N112	14		M	Osteosarcoma
N117	38		M	Chondrosarcoma
N118	9		M	
N119	16	Distal Femur	F	Ewings sarcoma
N120	66		M	High grade chondrosarcoma
N121	46		M	Synovial sarcoma
N122	15		F	Osteosarcoma
P1	16		M	
P2	54		F	
P3	56		M	

Supplementary Table 2: CT values of ADAMTS4 and ADAMTS5 genes and their relative abundance in human chondrocytes unstimulated and stimulated with IL1.

	CT Values-CTRL cells		CT values- IL1 treated cells		Relative abundance of ADAMTS5:ADAMTS4 (=2^{-dct})	
Patient ID	**ADAMTS-4**	**ADAMTS-5**	**ADAMTS-4**	**ADAMTS-5**	**ADAMTS5:ADAMTS4 CTRLcells**	**ADAMTS5:ADAMTS4 IL1 treated cells**
N15	30.3	22.3	27.1	22.7	256	21.11212657
N45	30.7	22.3	24.7	22.5	337.7940252	4.59479342
N48	30.9	22.7	28	21.5	294.0667789	90.50966799
N31	32.1	25.7	27	24.9	84.44850629	4.28709385
N12	30.4	23.9	22.4	22.7	90.50966799	0.812252396
P2	30.7	24.9	23.7	22.3	55.71523605	2.6
N10	28.6	21.1	24.755	22.990	181.019336	3.39873855867
N120	33.524	28.264	24.628	23.544	38.31927397	2.11990502868
P3	33.801	25.795	23.692	22.448	257.066643650284	2.36854237197
P4	31.381	24.248	24.111	23.464	140.361292494464	1.565907896911
P5	35.336	26.969	23.487	22.962	330.154768371553	1.438933199536



Supplementary Figure 1: Effect of JNK2 Knockdown on mRNA levels of ADAMTS-4 (A) , ADAMTS-5(B) and LRP1(C).Cells were transfected with 20nM siRNA against JNK2 or with scrambled siRNAs, by using lipofectamine 2000. After 4h, transfection medium was replaced with DMEM supplemented with 10% FBS and antibiotics. Cells were left for 72h to allow efficient knockdown. Cells were then left or treated with IL-1 (50ng/ml) for 24h as indicated. mRNA levels of ADAMTS-4 , ADAMTS-5 , LRP1and GAPDH were measured by RT-PCR in extracts of cells.