

The recombinant Link module of human TSG-6 suppresses cartilage damage in models of osteoarthritis: A potential disease-modifying OA drug



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SUMMARY

Objective: To investigate the role of endogenous TSG-6 in human osteoarthritis (OA) and assess the disease-modifying potential of a TSG-6-based biological treatment in cell, explant and animal models of OA.

Design: Knee articular cartilages from OA patients were analyzed for TSG-6 protein and mRNA expression using immunohistochemistry and RNAscope, respectively. The inhibitory activities of TSG-6 and its isolated Link module (Link_TSG6) on cytokine-induced degradation of OA cartilage explants were compared. Human mesenchymal stem/stromal cell-derived chondrocyte pellet cultures were used to determine the effects of Link_TSG6 and full-length TSG-6 on IL-1 α -, IL-1 β -, or TNF-stimulated ADAMTS4, ADAMTS5, and MMP13 mRNA expression. Link_TSG6 was administered i.a. to the rat ACLTMMx model; cartilage damage and tactile allodynia were assessed.

Results: TSG-6 is predominantly associated with chondrocytes in regions of cartilage damage where high TSG-6 expression aligns with low MMP13, the major collagenase implicated in OA progression. Link_TSG6 is more potent than full-length TSG-6 at inhibiting cytokine-mediated matrix breakdown in human OA cartilage explants; > 50% of donor cartilages, from 59 tested, were responsive to Link_TSG6 treatment. Link_TSG6 also displayed more potent effects in 3D pellet cultures, suppressing ADAMTS4, ADAMTS5, and MMP13 gene expression, which was consistent with reduced aggrecanase and collagenase activities in explant cultures. Link_TSG6 treatment reduced touch-evoked pain behavior and dose-dependently inhibited cartilage damage in a rodent model of surgically-induced OA.

Conclusions: Link_TSG6 has enhanced chondroprotective activity compared to the full-length TSG-6 protein and shows potential as a disease modifying OA drug via its inhibition of aggrecanase and collagenase activity.

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Abbreviations: ACLTMMx, Anterior Cruciate Ligament Transection with partial Medial Meniscectomy; ADAMTS, A Disintegrin and Metalloproteinase with Thrombospondin motifs; AMG, Anteromedial Gonarthrosis; ANCOVA, Analysis of Covariance; ANOVA, Analysis of Variance; BMI, Body Mass Index; CTXII, C-terminal Cross-linked Telopeptide of Type II Collagen; CXCL8, C-X-C motif Chemokine Ligand 8; DMB, dimethylmethylene blue; DMOAD, Disease Modifying Osteoarthritis Drug; ELISA, Enzyme-linked Immunosorbent Assay; GAG, glycosaminoglycan; HA, hyaluronan; hMSC, human Mesenchymal Stem/Stromal Cell; i.a., intra articular; IL-1, Interleukin-1; JNK, c-Jun N-terminal Kinase; Link_TSG6, the Link module of human TSG-6; MMP, Matrix Metalloproteinase; mRNA, messenger Ribonucleic Acid; NF-kB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; OA, osteoarthritis; OSM, Oncostatin M; qPCR, quantitative Polymerase Chain Reaction; RA, rheumatoid arthritis; rhTSG-6, recombinant human Tumour Necrosis Factor-Stimulated Gene-6; TKA, total knee arthroplasty; TNAIP6, Tumour Necrosis Factor-Induced Protein 6; TNF, Tumour Necrosis Factor; TSG-6, Tumour Necrosis Factor-Stimulated Gene-6.

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Introduction

Osteoarthritis (OA) is the most common form of joint disease, where associated pain and functional decline represent a major and growing cause of long-term disability.¹ A large proportion of this is accounted for by knee OA, which is estimated to affect > 250 million people worldwide. OA is a complex disorder where constitutional (e.g., age, obesity), biomechanical (e.g., joint injury, occupational activity) and genetic factors contribute to structural deterioration and, potentially, failure of the synovial joint.² Whilst degeneration of the cartilage matrix is a major hallmark of OA, the whole joint is affected; for example, synovitis and pathological changes to subchondral bone are common features of the disease. At present there are no licensed disease-modifying OA drugs (DMOAD) and strategies for pain relief are inadequate.^{2,3} Ultimately, a large number of patients undergo joint arthroplasty for late stage OA with outcomes being unsatisfactory in a significant proportion of cases.⁴

Joint injury and obesity are major risk factors for knee OA, consistent with the contribution of adverse mechanical loading to disease onset and progression. What is not clear is why some individuals experience progressive, symptomatic OA while others with similar structural changes to the joint do not.² The balance between degenerative and protective mechanisms in cartilage is likely an important factor and these processes provide potential targets for new therapeutic strategies. The principle catabolic enzymes that drive cartilage degradation during OA are the collagenase MMP13 and the ADAMTS family of aggrecanases, particularly ADAMTS4 and ADAMTS5.^{2,3} These proteases are expressed by chondrocytes and mediate the loss of the proteoglycan aggrecan that is a pre-requisite for irreversible breakdown of type II collagen. Animal models where joints are surgically destabilized have been validated as recapitulating many aspects of the OA disease process.^{3,5} Studies in such models have identified that the immediate response to injury occurs primarily in the cartilage, is inflammatory in nature and involves elevated expression of both pro- and anti-catabolic factors.^{6,7} For example, genes encoding cytokines (e.g., *Il1*), *Adamts4* and *Adamts5*, are rapidly up-regulated following joint destabilization in mice; expression of *Tsg6*, which is associated with protective/reparative effects in other tissues, is also increased.⁶

TSG-6 gene expression is induced by inflammatory cytokines, for example in IL-1-treated chondrocytes from OA patients.⁸ Recent analyses of chondrocyte transcriptomes have consistently identified TSG-6 (also known as *TNFAIP6*) as one of the most highly up-regulated genes in damaged regions of OA cartilage compared to intact tissue from the same joint.^{9–11} Furthermore, TSG-6 is secreted by mesenchymal stem/stromal cells (MSC) in response to inflammatory signals and mediates tissue protective and immunomodulatory effects in a diversity of disease models.¹² Several mechanisms of action have been described for these activities. For example, we have shown that TSG-6 (and its isolated Link module domain; Link_TSG6) inhibits neutrophil migration^{13,14} via interaction with the chemokine CXCL8.¹⁵ This anti-inflammatory activity of TSG-6 was suggested to underpin its chondroprotective effects in models of rheumatoid arthritis (RA), where *Tsg6*-null mice develop much more severe joint damage with extensive neutrophil infiltration.¹⁶ Treatment with TSG-6 protein, or its overexpression, in RA models were found to significantly reduce joint damage.^{17,18} Moreover, TSG-6 can suppress inflammation by other mechanisms, for example, via down regulation of NF- κ B-, JNK-, and p38-mediated signaling pathways, reducing cytokine expression and promoting polarization of macrophages towards an M2 (anti-inflammatory) phenotype.¹²

The local expression of TSG-6 in OA joints^{9–11,19} and its protective effects in RA suggest that this protein is part of an intrinsic repair pathway that could be targeted in the treatment of OA. This is consistent with TSG-6 having a chondroprotective effect in surgically-induced OA.²⁰ Conversely, it has been hypothesized that TSG-6

contributes to the OA disease process²¹ and an enzymatic function of TSG-6 has been linked with airway hyperresponsiveness and some other lung pathologies.¹² However, there are no data on the direct effects of the TSG-6 protein on chondrocyte activity and there has been little exploration of its therapeutic potential in models of OA.

Methods

Full details of all methods (including statistical analyses) and reagents are provided in the [Supplementary Material](#).

Human tissue and cellular studies

Human tissues were used with UK NHS Research Ethics Committee approval and with subjects' informed consent. Medial tibial plateaus, from patients with anteromedial gonarthrosis (AMG) undergoing unicompartmental knee arthroplasty ([Supplementary Table 1](#)), were used for histological and fluorescent staining to determine the localization of TSG-6 protein in the context of OA-associated cartilage damage.

Full-thickness, macroscopically undamaged, cartilage was dissected from whole tibial plateaus of patients with symptomatic OA undergoing total knee arthroplasty (TKA; [Supplementary Table 1](#)) for use in explant assays. Cubes of cartilage were incubated with/without IL-1 β (10 ng/ml)/OSM (30 ng/ml) and with/without rhTSG-6 or Link_TSG6 at the concentrations indicated. Glycosaminoglycan (GAG) release into the media, as an indicator of cartilage degradation, was quantified using the Dimethylmethylene Blue (DMB) assay.²² The aggrecan ARGS neopeptide²³ and collagen II telopeptide (CTXII) were quantified in the explant media using ELISAs.

Damaged cartilage from the tibial plateaus of OA patients was used in RNAscope with fluorescent probes to quantify mRNA expression for *TSG-6*, *MMP13*, and *ADAMTS5*.

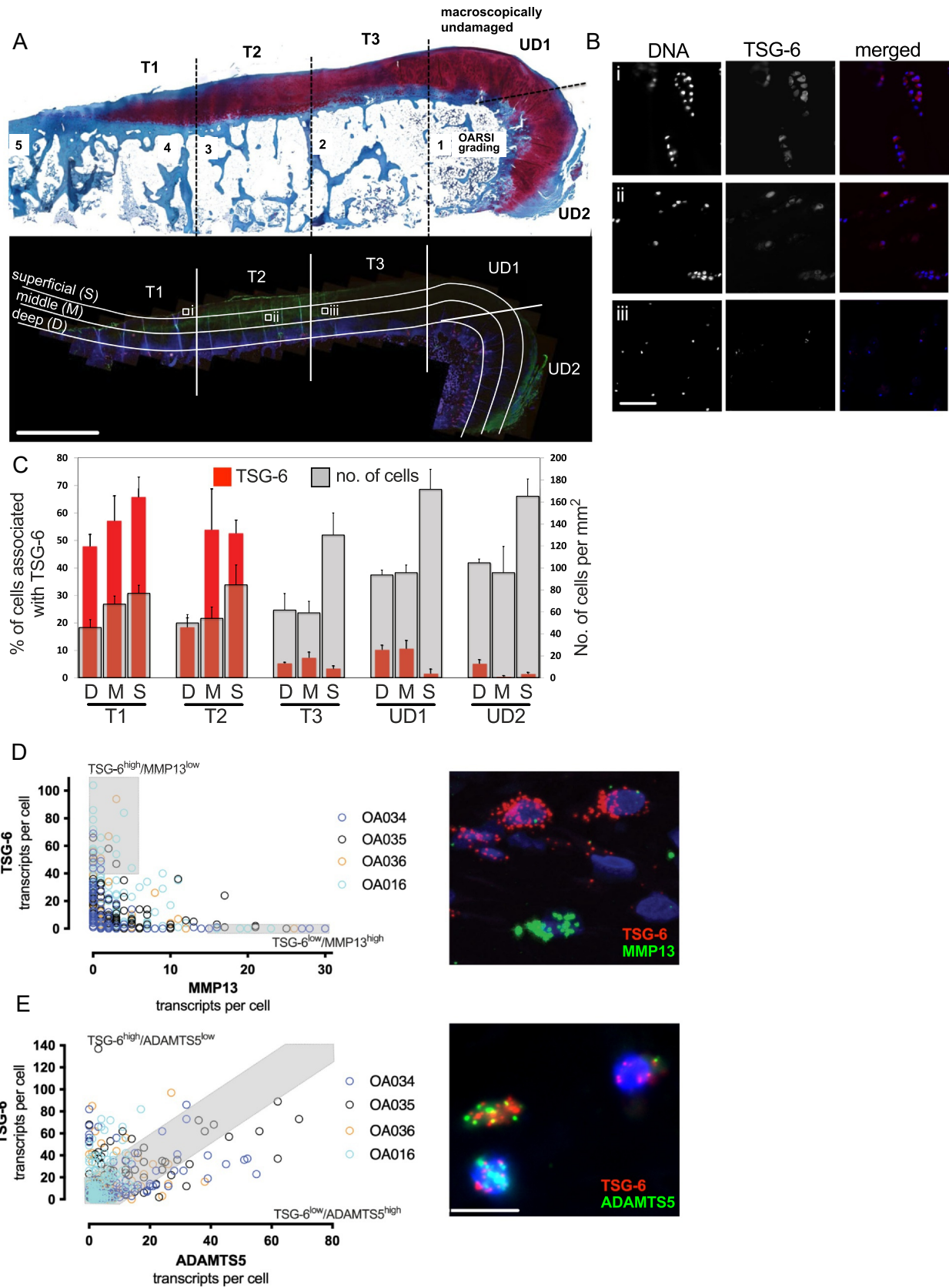
Chondrocyte 3D pellets were generated from human MSCs (hMSC) (StemCell Technologies). Fourteen-day pellet cultures were treated \pm 10 ng/ml cytokine (IL-1 α , IL-1 β , or TNF) and \pm rhTSG-6 or Link_TSG6 at the concentrations indicated for 24 h. mRNA was extracted and qPCR was performed using primer pairs for *ADAMTS4*, *ADAMTS5*, and *MMP13* and fold changes in gene expression were quantified.

Animal studies

All procedures involving experimentation on animals were performed according to German Animal Welfare Legislation and registered by the Competent Authority. OA was induced in 12-week old, male Lewis rats by anterior cruciate ligament transection (ACLT) with partial (30%) medial meniscectomy (pMMx).^{24,25} Treatments were administered by intra-articular (*i.a.*) injection at the doses and time points indicated; animals were euthanized at 28 days post-surgery for histological and macroscopic evaluations of the medial tibiae. Prior to euthanasia, tactile allodynia was assessed by withdrawal thresholds to von Frey filaments applied to the plantar surface of hind paws. In all investigations, operators were blinded.

Statistical methods

Statistical analyses were performed with GraphPad (Prism 9.2.1), StatsDirect or IBM (SPSS Statistics v28) software. Normality and homoscedasticity assumptions were evaluated using the Shapiro Wilk test and QQ plots, respectively. *p* values of < 0.05 were considered significant. All *p* values > 0.0001 and < 0.9999 are presented together with corresponding 95% confidence intervals (95% CI).



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Fig. 1

Localization of TSG-6 protein and mRNA in OA cartilage. (A) Representative section of medial tibial plateau (5 μ m) from a patient with AMG stained with (upper panel) Safranin O and Fast Green or (lower panel) a TSG-6-specific antiserum RAH-1 (red) and HA-binding protein (green, for visualization of cartilage matrix). Sections stained with Safranin O/Fast Green were graded according to the OARS scale and divided into regions for analysis: full-thickness cartilage was denoted ‘undamaged’ (UD1 and UD2) and cartilage between UD and the full-thickness lesion was segregated into T1, T2, and T3; all regions were subdivided longitudinally into superficial (S), middle (M) and deep (D) zones. Scale bar = 4 mm. (B) Representative images (corresponding to regions i–iii in (A)) showing cell populations associated with fluorescent staining of TSG-6 and DNA (DAPI). Scale bar = 50 μ m. (C) Cell density and the percentage of cells associated with TSG-6 staining (from semi-automated ‘object counting’) within each zone defined in (A). Data, which are from a single AMG donor and are representative of 3 distinct donors (see [Supplementary Fig. 2](#) for methodology and analysis of the other 2 donors), are presented as mean values from three sequential sections \pm SEM. (D,E) In the T1–T2 regions of degraded OA cartilages from 4 different donors, transcripts for (D) TSG-6 and *MMP13* and (E) TSG-6 and *ADAMTS5* were labeled using specific RNAscope probes; ≥ 100 cells per donor, expressing at least one transcript of either mRNA, were analyzed. Left hand panels show the numbers of transcripts per cell with gray shaded boxes indicating areas of the plot that correspond to cells in (D) with high levels of TSG-6 (≥ 40 transcripts) and low levels of *MMP13* (≤ 5) or high levels of *MMP13* (≥ 15) and low levels of TSG-6 (≤ 5) and in (E) with similar levels of TSG-6 and *ADAMTS5* transcripts. Representative images of cells labeled with the indicated probe pairs are shown at the same magnification (right hand panels); scale bar = 10 μ m. The data from these four donors, labeled with each probe pair, are presented individually in [Supplementary Fig. 4](#).

Results

TSG-6 protein is associated with damaged regions of OA cartilage

TSG-6 is expressed by chondrocytes in response to both mechanical and inflammatory stimuli^{6,8,26} and both cell- and matrix-associated protein has been detected in OA cartilage.^{19,21} Here, we analyzed cartilage from 3 patients with AMG ([Fig. 1A](#) and [Supplementary Fig. 2](#)), where the medial tibial plateau displays a characteristic pattern of erosion ([Supplementary Fig. 1](#)).²⁷ Fluorescent staining revealed discrete populations of chondrocytes, namely cells associated with relatively strong TSG-6 protein staining, cells with low TSG-6 staining and cells with little or no staining ([Fig. 1B](#)). TSG-6 immunoreactivity was predominantly associated with chondrocytes (including cell clusters) in the most damaged regions of the tibial plateau ([Fig. 1](#) and [Supplementary Fig. 2](#)). Use of an ‘object counting’ approach, to quantify fluorescent staining within individual zones of cartilage from three AMG patients (see [Supplementary Table 1](#)), confirmed the association of TSG-6 with cells in regions of significant cartilage loss (i.e., all zones within T1 and the superficial and middle zones of T2; [Fig. 1C](#) and [Supplementary Fig. 2](#)). Small percentages of TSG-6⁺ cells were also identified within macroscopically undamaged regions (UD1 and UD2) of all the donor cartilages analyzed ([Fig. 1](#) and [Supplementary Fig. 2](#)). Joint tissue from patients with early-stage OA is not readily available; however, immunohistochemistry of rat knee cartilage 4-weeks after surgical induction of OA (ACLTpMMx) revealed cell- and matrix-associated TSG-6 staining both concomitant with cartilage fibrillation and in regions where lesions had not yet developed ([Supplementary Fig. 3](#)). The expression of TSG-6 by chondrocytes in undamaged regions of human and rat OA cartilage led us to hypothesize that this protein may play a role in an attempted repair process.

RNAscope analysis of cartilage sections from 4 donors ([Supplementary Table 1](#)) revealed that individual chondrocytes with high numbers of TSG-6 transcripts typically have very few or no *MMP13* transcripts and vice versa ([Fig. 1D](#) and [Supplementary Fig. 4](#)). This is indicative of distinct chondrocyte phenotypes (e.g., with different catabolic activities), where we have observed TSG-6^{High}*MMP13*^{Low} and TSG-6^{Low}*MMP13*^{High} cells in close proximity to each other within cartilage ([Fig. 1D](#), right panel). In contrast, single cell RNAscope data from the same 4 donors indicated that TSG-6 and *ADAMTS5* are expressed at similar levels in most cells, that is, with relatively few cells being TSG-6^{High}*ADAMTS5*^{Low} or TSG-6^{Low}*ADAMTS5*^{High} ([Fig. 1E](#) and [Supplementary Fig. 4](#)).

Link_TSG6 suppresses breakdown of human OA cartilage ex vivo by down-regulating aggrecanase and collagenase activity

In OA, cartilage damage is mediated by aggrecanase and collagenase enzymes, for example, as a consequence of their elevated expression and/or reduced uptake by chondrocytes during inflammation.^{28,29} Here we investigated whether TSG-6 can act directly on chondrocytes to counter cytokine-mediated effects that drive cartilage breakdown. Cartilage explants from 59 OA patients undergoing TKA ([Supplementary Table 1](#)) were cultured with IL-1 β /OSM in the absence or presence of either rhTSG-6 or Link_TSG6 for 7 or 14 days, and the extent of cartilage degradation was determined by quantification of GAG release into the culture media. Due to its greater solubility and stability in solution, Link_TSG6 was tested at a wider range of concentrations than rhTSG-6 (≤ 33.3 μ M and ≤ 1 μ M, respectively). Only Link_TSG6 dose-dependently inhibited cartilage breakdown, for example, showing a significant inhibitory effect at concentrations between 0.1 and 33.3 μ M at the 7-day time point, whereas rhTSG-6 did not have a significant effect at any of the concentrations tested ([Fig. 2A,B](#)). This indicates that Link_TSG6 has much greater chondroprotective activity than the full-length protein. At the highest dose of Link_TSG6 (33.3 μ M) there was a substantial reduction (mean $\sim 30\%$) in GAG loss based on analysis of the entire sample population.

The inhibitory effect of Link_TSG6 on cytokine-induced cartilage breakdown varied greatly between donor cartilages (with a wide spectrum of responses; [Fig. 2A](#)). In this regard, 30 out of the 59 cartilages tested exhibited a statistically significant ($p < 0.05$) reduction in the percentage release of GAGs (relative to treatment with IL-1/OSM alone) when treated with ≤ 10 μ M Link_TSG6 for 7 days; these were defined as ‘Group I’ (colored blue in [Fig. 2A,B](#) and [Supplementary Table 2](#)). The other 29 did not show a significant reduction, and these were termed ‘Group II’ (red in [Fig. 2A,B](#) and [Supplementary Table 2](#)). For all of the donor cartilages in Group I, Link_TSG6 treatment reduced the cytokine-induced mean GAG loss by $> 15\%$, ranging up to $\sim 94\%$ inhibition; all of the Group II cartilages had $< 15\%$ reduction in mean GAG loss with the exception of 2 donors, which had large confidence intervals (see [Supplementary Table 2](#)). Quantification of lactate dehydrogenase in explant media (data not shown) indicated that the responsiveness to Link_TSG6 observed was not related to a difference in cell viability between Group I and Group II cartilages. Additionally, analysis of data from all cartilages treated with 10 μ M Link_TSG6 (for 7 days) revealed a significantly greater reduction in cytokine-mediated matrix breakdown in tissues from female donors compared to those from male donors ([Fig. 2D](#)); donor age and BMI exert little/no confounding bias in this analysis.

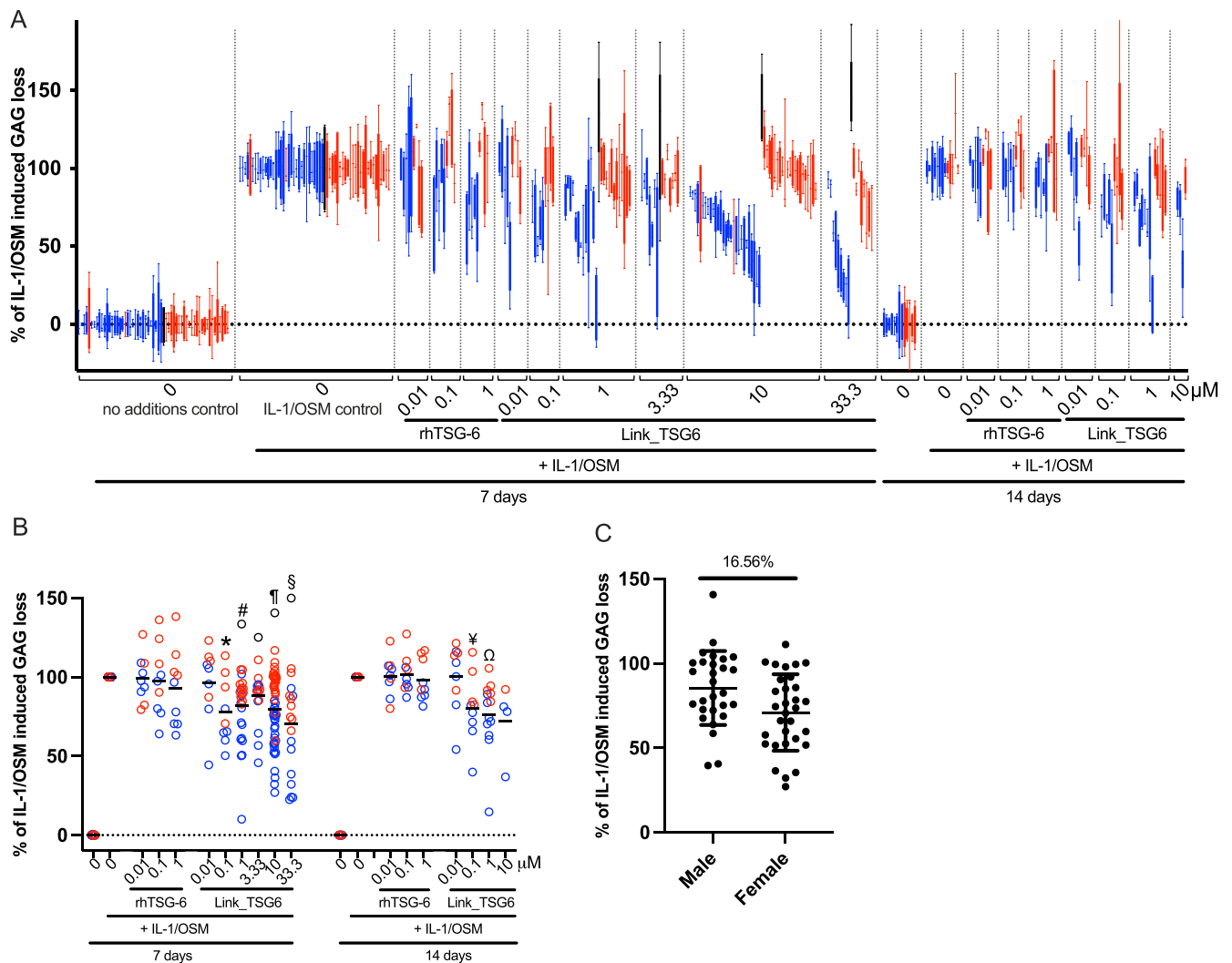


Fig. 2

TSG-6 proteins reduce cytokine-mediated breakdown of OA cartilage ex vivo. Cartilage explants were incubated \pm 10 ng/ml IL-1 β and 30 ng/ml Oncostatin M (OSM) \pm the indicated concentrations of rhTSG-6 or Link_TSG6. Due to the limited solubility of rhTSG-6 the maximum concentration was 1 μ M, whilst Link_TSG6 was tested at \leq 33.3 μ M. GAG release after 7 or 14 days was determined using the DMB assay and expressed as the percentage of GAG loss relative to the IL-1/OSM control, which was normalized to 100%. The GAG loss in the 'no additions' control was normalized to 0%. (A) Box and whisker plots (delineating 25th and 75th percentile (box; where determined) around median with whiskers showing minimum and maximum values) for DMB data (GAG loss; highest to lowest) from 59 donor cartilages incubated \pm IL-1/OSM and \pm Link_TSG6 or rhTSG-6 over 7 or 14 days; statistical analyses for each donor cartilage (for 7-day treatments with Link_TSG6 at 1 or 10 μ M) are presented in [Supplementary Table 2](#). (B) Mean percentage values of IL-1/OSM-induced GAG loss upon incubation with various concentrations of Link_TSG6 or rhTSG-6 relative to the IL-1/OSM control (normalized to 100%) after 7 and 14 days. In (A,B) data from the individual donor cartilages where Link_TSG6 had a significant inhibitory effect ($p < 0.05$) are shown in blue, whereas those that did not differ significantly from the cytokine alone control are shown in red; data shown in black are from OA027, the only donor cartilage where Link_TSG6 caused a statistically significant increase in GAG release relative to the IL-1/OSM control. Horizontal bars denote grand mean values (for that concentration of Link_TSG6/rhTSG-6). Two-tailed one-sample t -tests were used to compare grand means for each Link_TSG6 or rhTSG-6 treatment group with the 'IL-1/OSM' control normalized to 100%. * $p = 0.0195$ (95% CI: -36.71, -4.146), # $p = 0.0003$ (95% CI: -27.67, -9.085), $^{\dagger}p < 0.0001$ (95% CI: -27.61, 14.09), $^{\S}p = 0.0025$ (95% CI: -47.93, -11.92), $^{\Psi}p = 0.0329$ (95% CI: -32.68, -1.747), $^{\Omega}p = 0.0025$ (95% CI: -32.61, -8.701). (D) Percentage GAG loss from female (n = 31) and male (n = 28) donor cartilages treated with IL-1/OSM + 10 μ M Link_TSG6 for 7 days; circles denote mean values for each donor and horizontal bars show the overall mean (for male or female donors) \pm SD. One-way Analysis of Covariance (ANCOVA) comparing female and male donor cartilages revealed that when adjusted for age and BMI the sex-dependent size effect (F v M) was -16.56% (95% CI: -28.01, -5.01).

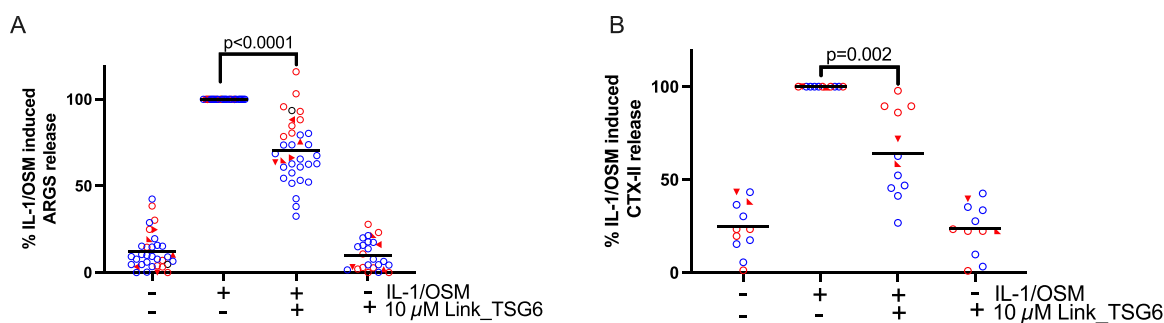


Fig. 3

Osteoarthritis and Cartilage

Link_TSG6 suppresses aggrecanase and collagenase activity in OA cartilage explants. Culture supernatants from cartilage explants, incubated \pm 10 ng/ml IL-1 β and 30 ng/ml Oncostatin M (OSM) \pm 10 μ M Link_TSG6 for 7 days, were analyzed by ELISA. The concentrations of the ARGs aggrecan neopeptide (A) and the collagen telopeptide CTX-II (B) were determined and plotted as the mean of replicate values for each donor relative to the IL-1/OSM control normalized to 100%; data are color coded as in Fig. 2 and Supplementary Table 2 (that contains statistical analysis of data from individual explants). The mean values for 'IL-1/OSM + 10 μ M Link_TSG6' and the 'IL-1/OSM' control were compared by two-tailed one sample t-tests: in (A) $p < 0.0001$ (95% CI: -35.72, -23.23); in (B) $p = 0.002$ (95% CI: -50.30, -21.40). Donor cartilages that showed a significant decrease in the release of ARGs or CTX-II, but did not exhibit a significant inhibition of GAG release as based on the DMB assay (in Fig. 2 and Supplementary Table 2) are indicated by solid symbols: in (A) \blacktriangledown OA017 $p = 0.0125$ (95% CI: -59.66, -12.94), \blacktriangleleft OA042 $p = 0.0123$ (95% CI: -39.83, -7.2470), \blacktriangle OA044 $p = 0.0496$ (95% CI: -58.98, -0.08818), \blacktriangle OA045 $p = 0.0157$ (95% CI: -40.89, -7.529) and \blacktriangleright OA055 $p = 0.0341$ (95% CI: -62.73, -4.082); in (B) \blacktriangledown OA17 $p = 0.0343$ (95% CI: -52.74, -3.373), and \blacktriangle OA044 $p = 0.0063$ (95% CI: -62.74, -19.35).

To further understand the mechanism of Link_TSG6's protective effect, media from cartilage explants were analyzed using ELISAs to quantify release of the aggrecan ARGs neopeptide (as an indicator of ADAMTS4/ADAMTS5 activity) and the collagen II telopeptide CTX-II (as a marker of collagenase activity); samples from 33 donors were analyzed for ARGs (21 from Group I and 12 from Group II, colored blue and red/black, respectively on Fig. 3), and samples from 12 donors were analyzed for CTX-II (6 each from Group I (blue) and Group II (red)). As can be seen from Fig. 3, Link_TSG6 mediated a statistically significant suppression of cytokine-induced ARGs and CTX-II release across all of the cartilage samples tested. This indicates that Link_TSG6 is acting via inhibition of aggrecanase (e.g., ADAMTS4 and ADAMTS5) and collagenase (e.g., MMP3 and MMP13) enzymes. Overall, these data reveal that a high proportion of OA donor cartilages are responsive to Link_TSG6 treatment (i.e., ~51% based on the DMB 'GAG release' assay and ~79% from the ARGs ELISA; see Supplementary Table 2).

The findings that Link_TSG6 inhibits cartilage breakdown *ex vivo* and suppresses the cleavage of both aggrecan and collagen, led us to further explore the regulation of catabolic pathways at a cellular level.

TSG-6 proteins suppress chondrocyte responses to pro-inflammatory cytokines

Here, we used qPCR to investigate the effects of Link_TSG6 and rhTSG-6 on cytokine-induced expression of the genes encoding aggrecanase (ADAMTS4 and ADAMTS5) and collagenase (MMP13) enzymes in 3D pellet cultures of chondrocytes, derived from hMSCs of three healthy individuals. After 14-days in culture the hMSCs had differentiated into cells displaying a chondrocyte-like gene expression profile and had deposited a cartilage-like matrix with little or no TSG-6 expressed at the gene or protein level (Supplementary Fig. 5). At this time point the chondrocytes were responsive to pro-inflammatory cytokines, where treatment for 24 h with 10 ng/ml IL-1 α , IL-1 β or TNF induced the expression of ADAMTS4, ADAMTS5 and MMP13 mRNAs (Fig. 4). The inclusion of exogenous rhTSG-6 or

Link_TSG6 protein suppressed IL-1 β -induced expression of ADAMTS4, ADAMTS5, and MMP13 in a dose-dependent manner; neither rhTSG-6 nor Link_TSG6 alone had any effect on gene expression. Whereas 0.1 and 1.0 μ M doses of Link_TSG6 reduced expression of MMP13 to basal levels, the corresponding doses of rhTSG-6 were less effective. This demonstrates that the isolated Link module has more potent anti-catabolic/anti-inflammatory effects on chondrocytes in the pellet cultures than full-length TSG-6. This was supported by the observations that the 0.1 μ M dose of Link_TSG6 was more effective than a 10-fold higher (1 μ M) dose of rhTSG-6 at inhibiting both IL-1 α - and TNF-induced expression of ADAMTS4, ADAMTS5 and MMP13 (Fig. 4D and 4E, respectively).

In a complementary approach, endogenous full-length TSG-6 was over-expressed in hMSCs using lentivirus. When these cells were differentiated into chondrocytes in 3D pellets their responses to IL-1 β , IL-1 α and TNF (Supplementary Fig. 6) were significantly reduced compared to controls. Collectively, this shows that expression of TSG-6 in human chondrocytes, or their treatment with TSG-6 proteins, effectively suppresses the expression of cartilage degrading proteases. Thus, we have identified a novel chondroprotective mechanism for TSG-6, via inhibition of inflammatory cytokine-mediated catabolic pathways, and shown that Link_TSG6 is considerably more potent than the full-length protein.

Link_TSG6 reduces cartilage damage and pain behavior in the ACLT/MMx rat OA model

Our *in vitro* and *ex vivo* data demonstrate that Link_TSG6 can potently suppress the cytokine-driven expression of proteolytic enzymes by chondrocytes and the cleavage of aggrecan and collagen in cartilage. We then evaluated the potential of Link_TSG6 to limit joint damage in the rat ACLT/MMx model, where surgically induced joint destabilization gives rise to rapid cartilage degeneration.^{30,31} Link_TSG6 (14, 40 and 120 μ g doses) was administered via intra-articular (i.a.) injections at 7-, 14-, and 21- days post-surgery. At the 28-day endpoint histopathological scoring across seven parameters

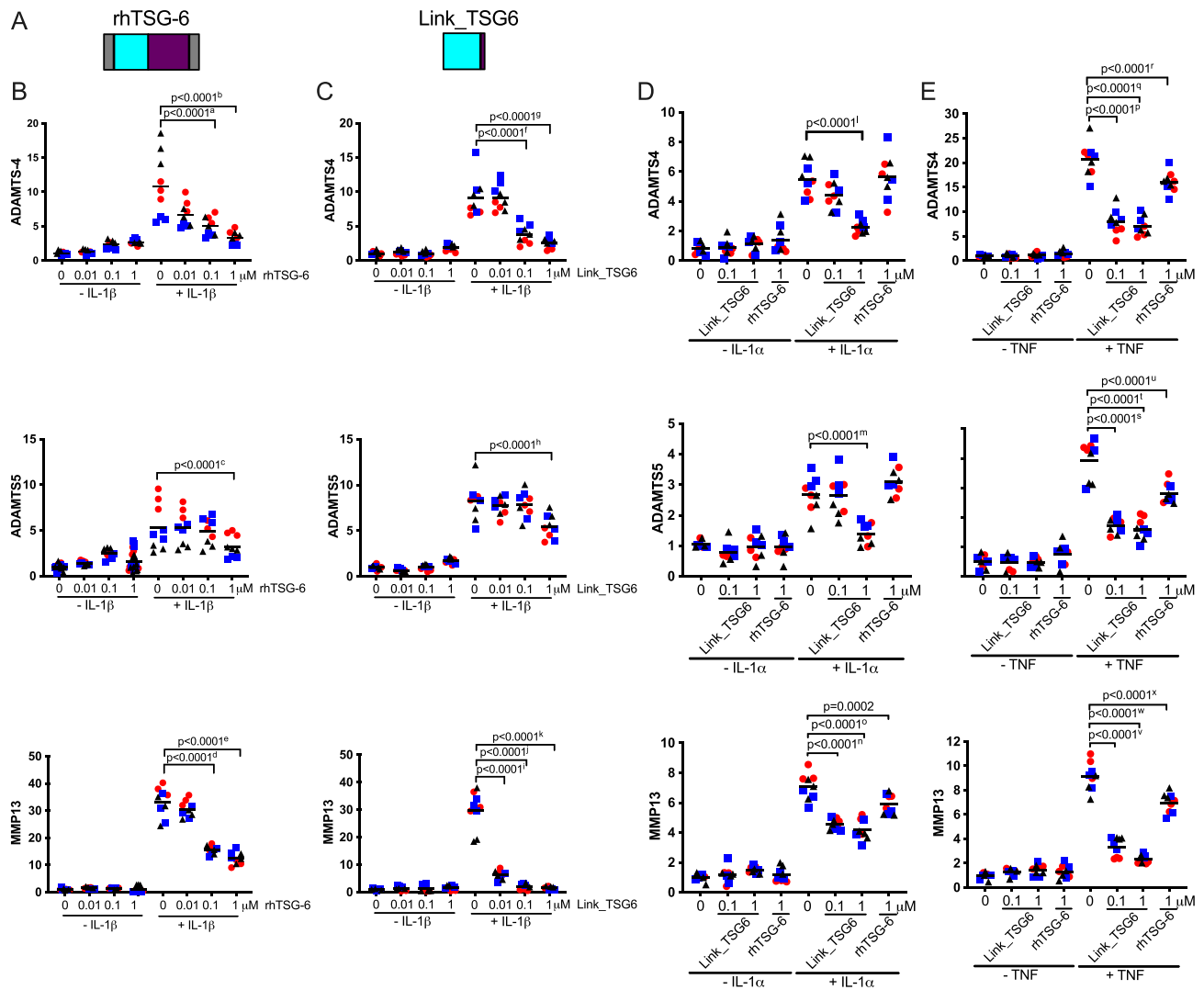


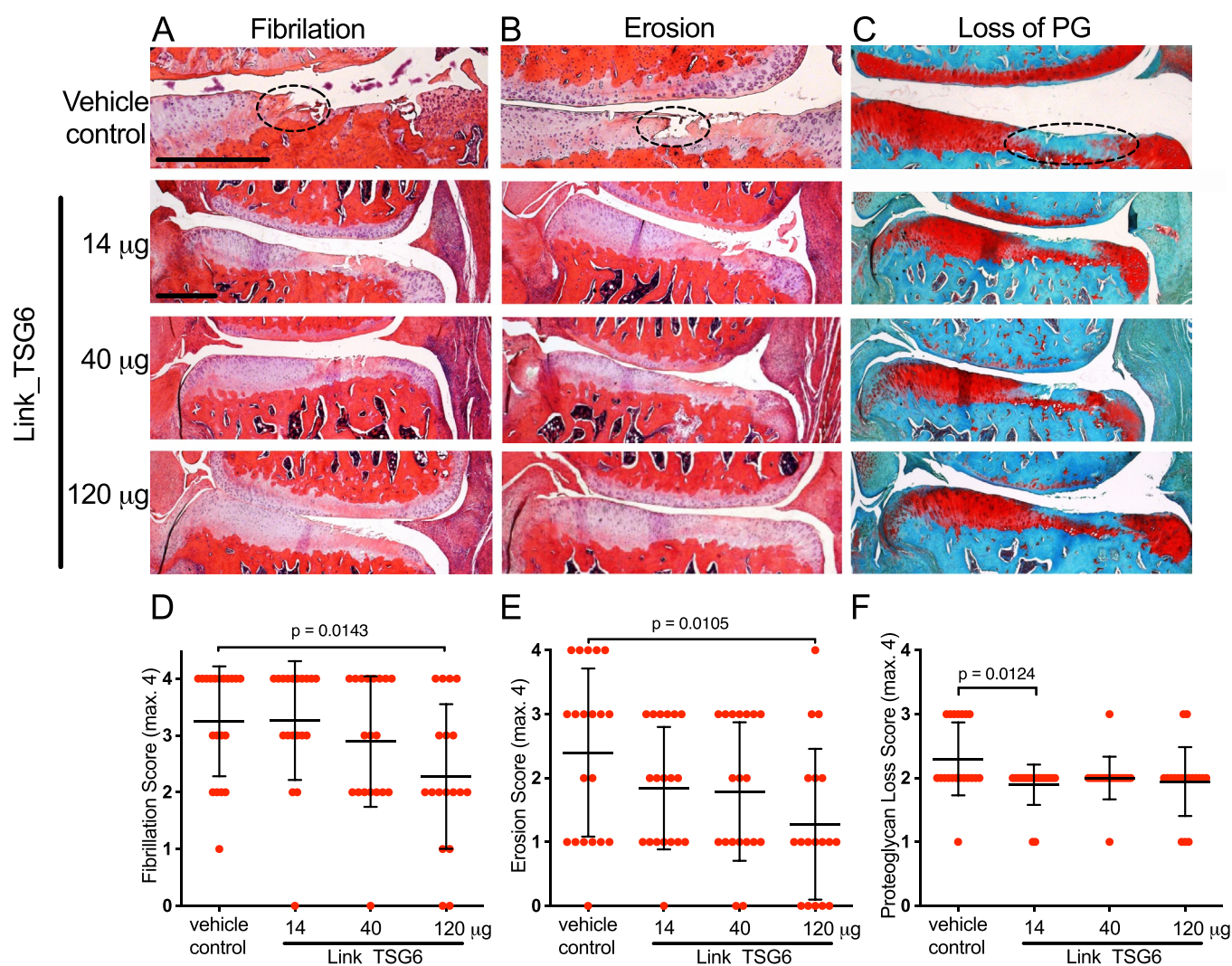
Fig. 4

Osteoarthritis and Cartilage

rhTSG-6 and Link_TSG6 suppress cytokine-induced expression of ADAMTS4, ADAMTS5 and MMP13 in chondrocytes. (A) Schematic representations of rhTSG-6 and Link_TSG6 proteins. (B–E) hMSCs were cultured as 3D pellets in chondrogenic media for 14 days and then treated for 24 h ± 10 ng/ml IL-1β (B,C), IL-1α (D) or TNF (E) and with/without rhTSG-6 (B,D,E) or Link_TSG6 (C,D,E) at the concentrations indicated. ADAMTS4, ADAMTS5, and MMP13 gene expression levels were determined by qPCR and are presented as fold-change relative to the no-addition control. Experiments were performed in triplicate with hMSCs from three donors (Donor A = red circles, Donor B = blue squares, Donor C = black triangles). Data from each experiment are shown (n = 9–12) with mean values indicated by horizontal lines. Data were analyzed using two-way ANOVA with Tukey's *post hoc* test; *p* values are relative to the corresponding control with cytokine alone. $p < 0.0001^a$ (95% CI: 4.731, 7.017), $p < 0.0001^b$ (95% CI: 6.369, 8.655), $p < 0.0001^c$ (95% CI: 1.142, 3.029), $p < 0.0001^d$ (95% CI: 14.53, 20.46), $p < 0.0001^e$ (95% CI: 17.74, 23.68), $p < 0.0001^f$ (95% CI: 3.732, 6.928), $p < 0.0001^g$ (95% CI: 5.029, 8.224), $p < 0.0001^h$ (95% CI: 1.134, 4.368), $p < 0.0001^i$ (95% CI: 19.74, 27.29), $p < 0.0001^j$ (95% CI: 23.67, 31.52), $p < 0.0001^k$ (95% CI: 24.32, 31.87), $p < 0.0001^l$ (95% CI: 1.940, 4.525), $p < 0.0001^m$ (95% CI: 0.7295, 1.838), $p < 0.0001^n$ (95% CI: 1.754, 3.252), $p < 0.0001^o$ (95% CI: 2.138, 3.636), $p < 0.0001^p$ (95% CI: 9.985, 15.44), $p < 0.0001^q$ (95% CI: 10.87, 16.33), $p < 0.0001^r$ (95% CI: 1.861, 7.313), $p < 0.0001^s$ (95% CI: 3.397, 5.449), $p < 0.0001^t$ (95% CI: 3.645, 5.697), $p < 0.0001^u$ (95% CI: 1.236, 3.289), $p < 0.0001^v$ (95% CI: 5.009, 6.533), $p < 0.0001^w$ (95% CI: 6.013, 7.537), $p < 0.0001^x$ (95% CI: 1.394, 2.917), $p = 0.0002$ (95% CI: 0.4370, 1.935).

(Supplementary Table 4) revealed a dose-dependent reduction in damage to the medial tibia (Fig. 5 and Supplementary Fig. 7). The effects of Link_TSG6 on cartilage degeneration were particularly striking. At the highest dose (120 µg) there were significant reductions both in the severity and number of fibrillation/fissures in the cartilage (Fig. 5A,D) and in the extent of cartilage erosion (Fig. 5B,E).

For example, in the latter, five of the vehicle-treated rats had a maximal erosion score of 4 (exposed bone) and one had a score of 0 (normal cartilage), whereas only one of the rats treated with 120 µg Link_TSG6 had a score of 4 and five had a score of 0. Furthermore, Link_TSG6 suppressed proteoglycan loss, where this effect was significant at the 14 µg dose (Fig. 5C,F). Overall, these data indicate that

**Fig. 5**

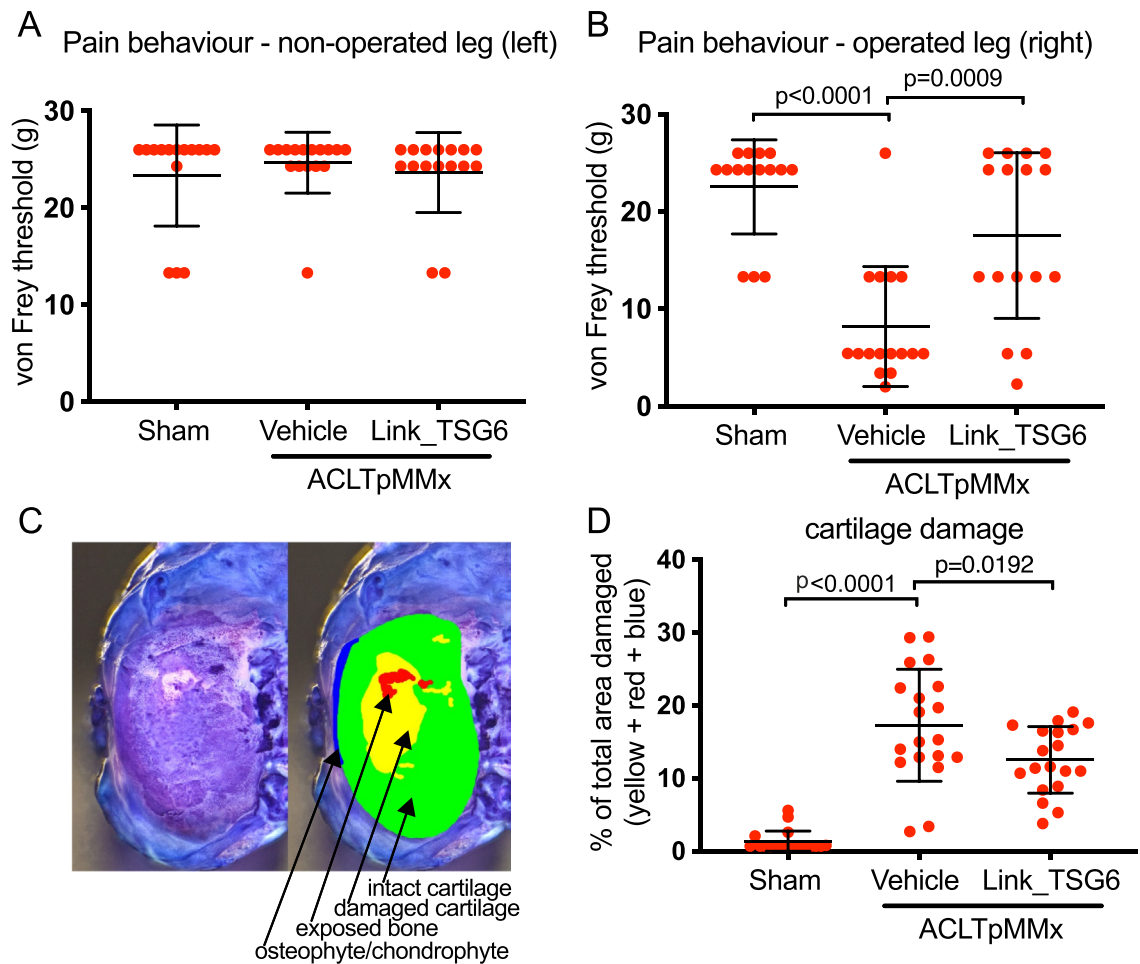
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Link_TSG6 reduces cartilage damage *in vivo* in the ACLTpMMx rat. OA was induced in the right knees of male Lewis rats by transection of the anterior cruciate ligament (ACL) and a partial (30%) medial meniscectomy (pMMx). Link_TSG6 treatment (or vehicle) was administered *i.a.* at 7-, 14-, and 21- days post-surgery, at the doses indicated. At the 28-day end point, joint tissues were fixed, decalcified and 7 μ m sections were stained with Safranin O/Fast Green or Alcian Blue (pH 2.5), prior to blinded histopathological scoring by two individuals. Representative stained sections for each of the treatment groups are shown to illustrate (A) cartilage fibrillation, (B) erosion, and (C) loss of proteoglycan (PG); pathological features are indicated with dashed ovals. The outcomes of scoring these parameters (on scales of 0–4; see [Supplementary Table 4](#)) for the medial tibiae are shown in (D), (E), and (F), respectively. Data are shown as scatter plots for each treatment group ($n = 18$ –20; with $n < 20$ due to losses under anesthesia), with the mean scores \pm SD indicated by horizontal bars. Data were analyzed using two-tailed *t* tests (Mann–Whitney), where *p* values < 0.05 are shown.

intra-articular administration of Link_TSG6 has the potential to slow the OA disease process.

In a separate study we investigated whether Link_TSG6 treatment suppressed pain behavior and whether this was associated with the reduction of cartilage damage. ACLTpMMx rats were treated with Link_TSG6 (40 μ g, *i.a.*, at 7-, 14-, and 21- days post-surgery) and, at the 4-week time point, touch-evoked pain (tactile allodynia) was assessed in the paws of operated (right) and un-operated control (left) legs as an indicator of secondary hyperalgesia. Paw withdrawal responses for the un-operated legs (with or without Link_TSG6 treatment) were essentially identical to those of sham

operated control animals ([Fig. 6A](#)). On the other hand, in ACLTpMMx-operated legs (vehicle treated) there was a significant increase in sensitivity to punctate mechanical stimulus. This response was significantly reduced by intra-articular injection of Link_TSG6 ([Fig. 6B](#)). Macroscopic evaluation of the medial tibiae (using digital segmentation to quantify cartilage damage, exposed bone and osteophyte/chondrophyte formation) at the 28-day study endpoint revealed extensive structural damage associated with ACLTpMMx surgery ([Fig. 6C,D](#)). In Link_TSG6-treated animals the mean total lesion area was reduced by $\sim 30\%$ ([Fig. 6D](#)), which is consistent with the histological analysis of cartilage fibrillation and

**Fig. 6**

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Link_TSG6 reduces tactile allodynia and cartilage lesion size in the ACLTpMMx rat. OA was induced in the right knees of male Lewis rats by ACLT and pMMx (30%). Link_TSG6 treatment (40 µg) or vehicle was administered *i.a.* at 7-, 14-, and 21- days post-surgery. After 28 days tactile allodynia was assessed by determining withdrawal thresholds to von Frey hairs applied to hind paws of the (A) un-operated control legs and (B) operated legs ($n = 15$ sham, $n = 16$ ACLTpMMx groups; chosen randomly). (C) Articular joint surfaces ($n = 19$ for all groups; with $n < 20$ due to loss under anesthesia or due to knee infection), from euthanized animals, were stained with India Ink (left panel) and digital macroscopy (right panel) was used to determine the areas of intact cartilage (green), damaged cartilage (yellow), exposed bone (red) and osteophyte/chondrophyte formation (blue). (D) The total damaged area was determined (yellow + red + blue) as a percentage of total tibial plateau area, for each animal. Data in A,B,D are presented as scatter plots for each treatment group, with mean values \pm SD indicated by horizontal bars. Data were analyzed by one-way ANOVA with Tukey's *post hoc* test. (B) $p < 0.0001$ (95% CI: 8.222, 19.84), $p = 0.0005$ (95% CI: -15.65, -4.029) and (D) $p < 0.0001$ (95% CI: -20.48, -10.70), $p = 0.0349$ (95% CI: 0.3048, 9.909), relative to vehicle control.

erosion in the dose-response study (Fig. 5). However, there was no correlation between the effects of Link_TSG6 on preserving joint structure and reducing tactile allodynia (data not shown). This indicates that Link_TSG6 may have an effect on pain that is unrelated to its effect on joint structure.

The above *in vivo* data support the potential of intra-articular Link_TSG6 as a protein biological treatment for OA with both disease modifying and analgesic effects.

Discussion

Here we have shown that TSG-6 proteins suppress inflammatory cytokine-induced expression and activity of the major aggrecanase

and collagenase enzymes implicated in OA pathology, thereby identifying a novel chondroprotective mechanism. Thus, our observation that endogenous TSG-6 is largely associated with the most damaged regions of OA cartilage may link to it having an intrinsic function in tissue protection²⁰ rather than driving the disease process.²¹ This aligns with a wealth of data on the reparative and anti-inflammatory effects of TSG-6 in a diverse range of animal models.¹² The role of inflammatory cytokines in OA pathogenesis has been an area of debate,^{2,32–35} but a recent *post hoc* analysis showing that Canakinumab reduces rates of knee and hip replacement³⁵ provides evidence that inhibiting the downstream effects of IL-1 β is a potential therapeutic target. The lack of success of anti-cytokine clinical trials in OA,³² which may be due in part to the heterogeneity of

the patients included,² suggests that anti-inflammatory approaches will only work alongside appropriate patient stratification.

Full-length TSG-6 protein lacks suitability as a biological drug due to its poor solubility and the fact that it aggregates even at low concentrations.³⁶ In addition, an enzymatic activity of TSG-6, that leads to the covalent modification of HA with heavy chains from members of the inter- α -inhibitor family, has been associated with certain lung pathologies.¹² Link_TSG6 has no such enzyme function and, moreover, can be used at much higher doses.³⁷ As shown here, it is also considerably more potent than rhTSG-6 at inhibiting the cytokine-induced breakdown of cartilage explants and suppressing the induction of *ADAMTS4*, *ADAMTS5* and *MMP13* expression in chondrocytes. Similarly, Link_TSG6 was found to have greater potency than rhTSG-6 in an *in vivo* model of dry eye disease.³⁷

Here, we found that intra-articular administration of Link_TSG6 reduced joint pathology in a model of surgically-induced osteoarthritis, which along with our *in vitro* and *ex vivo* data, underpins its potential as a DMOAD. Link_TSG6 also reduced touch-evoked pain behavior in the ACLT_{PMx} rat. At present the mechanism underlying this potential analgesic effect is unknown, but might involve the interaction of Link_TSG6 with chemokines;^{15,38–40} further work is needed to explore this. The lack of correlation seen here between Link_TSG6's effects on joint structure and pain is consistent with observations in human OA.⁴¹

Our finding that a substantial proportion of OA donor cartilages are responsive to Link_TSG6 treatment suggests that this protein biologic may have therapeutic utility for a large number of OA patients, e.g., by suppressing aggrecanase- and collagenase-mediated damage. Here cartilage explants from female donors exhibited a greater response compared to those from males, which is consistent with substantial evidence for sex differences in drug pharmacokinetics including in other arthritides.^{42,43} In the context of future clinical trials, *a priori* determination of patients that are most likely to be highly responsive to Link_TSG6 treatment will be important given the heterogeneous nature of the OA patient population; it has been shown previously that such stratification in OA is likely to be feasible.⁴⁴

Our analysis of endogenous TSG-6 in OA cartilage (at both protein and RNA levels) indicates that it is a marker of chondrocyte phenotype, being expressed by a subset of cells. The existence of chondrocytes with different phenotypes in OA cartilage is consistent with recent single cell analysis.⁴⁵ Importantly, TSG-6 expression in the most damaged regions of cartilage and the finding that cells with high numbers of TSG-6 transcripts have low levels of *MMP13* expression, suggest that it is associated with chondrocytes that have low catabolic activity against type II collagen. It is possible that TSG-6 actively suppresses collagenase production in this context, based on the 3D pellet culture data showing that rhTSG-6, or lentiviral overexpression of TSG-6, counter IL-1/TNF-induced *MMP13* expression. Currently, the mechanism by which TSG-6 inhibits cytokine-mediated pathways in OA chondrocytes is not understood. While there is no evidence that TSG-6 has a dedicated cell surface receptor, some of its anti-inflammatory activities have been found to be dependent on CD44 and may involve HA;¹² i.e. given that TSG-6-mediated HA crosslinking enhances its interaction with HA receptors.^{46–48} However, in cartilage this mechanism of action seems unlikely since Link_TSG6 (which is more potent than rhTSG-6) only poorly enhances HA-CD44 interactions compared to the full-length protein⁴⁶ and our observation that TSG-6⁺ chondrocytes have little or no HA staining (unpublished data).

Single cell analysis in OA cartilage revealed that many TSG-6 expressing cells also express similar numbers of *ADAMTS5* mRNA transcripts. Given that *ADAMTS5* plays a major role in OA pathology,⁴⁹ this finding was unexpected. However, our pellet culture and explant studies indicate that TSG-6 inhibits cytokine-induced

aggrecanase production in human tissues, which is consistent with a recent study showing that deletion of the *Tsg6* gene in mice is associated with the rapid upregulation of proinflammatory and catabolic gene expression (*Adamts5*, *Ccl2* and *Il1a*) following joint destabilization.²⁰ In human cartilage the level of *ADAMTS5* protein is, in part, dictated by its residence time in the tissue, which is dependent on LRP-1-mediated endocytosis by chondrocytes; in OA, uptake of *ADAMTS5* is impaired via cytokine-dependent shedding of LRP-1 leading to increased aggrecanase activity.²⁸ Further work is required to better understand how TSG-6 (and Link_TSG6) affect *ADAMTS5* expression and turnover.

In summary, this study has identified a potential new treatment for osteoarthritis based on the Link module from human TSG-6. Link_TSG6 mimics the intrinsic anti-inflammatory and chondroprotective properties of the endogenous TSG-6 protein, as well as having greater potency, making it an attractive and novel target as a DMOAD.

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Declaration of Competing Interest

AJD and CMM are founders of Link Biologics Limited and AJD, CMM, SPD, JLS and NK are shareholders in the company.

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Author contributions

CMM and AJD conceived the study and **AJD, CMM and LCB** obtained funding. **SPD, EB, AJP, SA, LCB, TL, MH, CMM and AJD** contributed to study design. **SPD, EB, NK, JLS, DPD and JMT** collected/analysed data. **SPD, NK, JLS, EB, CMM and AJD** interpreted data. **AJP, SA, LCB** provided patient samples. **SPD, CMM and AJD** drafted the manuscript and all authors provided critical revision for important intellectual content. All authors read and approved the submitted manuscript. **AJD** (anthony.day@manchester.ac.uk) and **CMM** (caroline.milner@manchester.ac.uk) take responsibility for the integrity of the work as a whole from inception to the finished article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.joca.2023.05.013](https://doi.org/10.1016/j.joca.2023.05.013).

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