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Lumican is upregulated in osteoarthritis and contributes to TLR4-induced pro-inflammatory activation of cartilage degradation and macrophage polarization

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SUMMARY

Objective: Lumican (LUM) is a major extracellular matrix glycoprotein in adult articular cartilage and its expression is known to be upregulated upon cartilage degeneration. LUM is associated with the pathogen-associated molecular pattern (PAMP) activation of the TLR4 signalling cascade, with TLR4 being highly associated with inflammation in rheumatic diseases. However, the main role of the LUM structural molecule in osteoarthritis (OA) remains elusive. The aim of this study was, therefore, to understand the role of LUM during TLR4-mediated activation in OA.

Methods: After measuring LUM levels in synovial fluid (SF) of OA patients and lipopolysaccharide (LPS)-induced TLR4 activation, the role of LUM in the expression of pro-inflammatory molecules and cartilage degradation was assessed *in vitro* and ex vivo in a cartilage explant model. Primary macrophage activation and polarization were studied upon LUM co-stimulation with LPS.

Results: We demonstrate that LUM is not only significantly upregulated in SF from OA patients compared to healthy controls, but also that LUM increases lipopolysaccharide (LPS)-induced TLR4 activation. Furthermore, we show that a pathophysiological level of LUM augments the LPS-induced TLR4 activation and expression of downstream pro-inflammatory molecules, resulting in extensive cartilage degradation. LUM co-stimulation with LPS also provided a pro-inflammatory stimulus, upregulating primary macrophage activation and polarization towards the M1-like phenotype.

Conclusions: These findings strongly support the role of LUM as a mediator of PAMP-induced TLR4 activation of inflammation, cartilage degradation, and macrophage polarization in the OA joint and potentially other rheumatic diseases.

Introduction

Osteoarthritis (OA), is a leading cause of disability worldwide[1] with incidence levels reaching 36% of the US adult population[2]. Treatment options are not only limited by a lack of understanding of the molecular events involved in OA disease stages but also because OA has multiple disease phenotypes. While trauma is often an early traceable event leading to symptomatic OA, current theories on OA pathogenesis also propose an intrinsic interaction between joint damage and chronic inflammation[3]. The innate immune system and associated inflammation have been shown to

Abbreviations: DAMPs, danger-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; PRRs, pattern-recognition receptors; LUM, lumican; OA, osteoarthritis; LPS, lipopolysaccharide; TLR4, toll-like receptor 4; HEK-hTLR4, human embryonic kidney cells with inserted human TLR4; MMP, matrix metalloproteinase; TNF-α, tumor necrosis factor alpha; CD197, cluster of differentiation 197; IL-4, interleukin 4; SEAP, secreted embryonic alkaline phosphatase; FITC, Fluorescein isothiocyanate; CD44, antigen precursor; CD90, antigen; CC3, cleaved caspase 3.

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participate in the onset and development of OA[4,5]. Moreover, key events during OA are associated with the activity of the innate immune system, including infiltration and inflammatory activation of macrophages[6] and activation of the Toll-like receptor (TLR) pathway[7] and complement system[8].

As part of the inflammatory pathways driving OA progression, initial inflammatory responses to cartilage damage are mediated by receptors, including TLRs. TLRs are a group of membrane-associated pattern-recognition receptors (PRRs) that recognize endogenous and exogenous danger-associated molecular patterns (DAMPs) such as cartilage matrix fragments from fibronectin9, hyaluronan[10], biglycan[11], among others, while also responding to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS)[12]. In OA, activation of TLRs via the NF-κB pathway leads to the secretion of cytokines and chemokines such as TNF, interleukin 6 (IL-6) and matrix metalloproteinases (MMPs), as well as the downregulation of collagen and proteoglycan synthesis[13]. When tissue is damaged and degraded during OA, extracellular matrix (ECM) proteins may be released, fragmented and turned into signaling molecules capable of interacting with TLRs to trigger an innate immune response[14,15].

Small leucine-rich proteoglycans (SLRPs) are a group of important biologically active ECM components found in all tissues[16]. Once thought to be purely structural matrix-bound components, SLRPs have now been associated with pathogenic mechanisms of several diseases and disorders such as osteoporosis, muscular dystrophy, among others[17]. Intriguingly, SLRPs are also known to activate and regulate the activity of the immune system[18,19]. The expression of SLRPs is altered with disease state during OA progression, which consequently affects key structural, organizational and biological properties such as cartilage collagen network assembly as well as growth factor signalling, e.g., TGF-β[20]. Moreover, SLRPs such as biglycan and fibromodulin are increasingly fragmented and released into the synovial fluid (SF), ultimately resulting in their depletion from OA cartilage[21,22]. Once released, SLRPs can exert biological effects such as immune responses on synovial tissue resident cells, including synoviocytes, fibroblasts, and macrophages, among others[7].

Lumican (LUM) is a member of the SLRP family best known for its interaction with fibrillar collagens[23] and has recently emerged as a participant in host–pathogen interactions. LUM is known to interact with TLR4-ligand LPS, and LUM KO mice are hyporesponsive to LPS-induced septic shock[24,25]. Strikingly, LUM expression is significantly fragmented and reduced in OA cartilage (with fragmentation accounting for less than 50% of total protein) but is also upregulated in serum and SF of OA patients[21,22]. The increased presence of LUM in SF thus raises the question if dysregulated levels of LUM impact immune responses in the OA joint, particularly together with OA pathophysiological molecules such as LPS[26].

Macrophages are key players of the innate immune system and are associated with inflammation and pathogenesis of many diseases, including OA[27]. In response to various stimulators, macrophages may undergo classical M1 activation or alternative M2 activation[28]. Although it is known that macrophages are activated during OA[29], the role of overexpressed LUM in this process is not understood.

The goal of this study was to characterize how overexpression of LUM contributes to LPS-induced low-grade inflammation, chondrocyte-mediated cartilage destruction and regulation of macrophage polarization and immune system activation in OA. Our data demonstrate that overexpressed LUM contributes to the innate immune-mediated pathogenesis of primary OA, with potential implications in other rheumatic diseases.

Materials and methods

Synovial fluid collection

SF samples from knee meniscectomy, end-stage knee OA, knee rheumatoid arthritis (RA), and carpometacarpal thumb joint (CMC-I) OA patients were collected prior to surgical incision. SF collection, and patient inclusion and exclusion criteria details are described in the supplementary material.

Enzyme-linked immunosorbent assay

Non-fragmented LUM was measured in SF (1:50) and chondrocyte culture media (1:1) by sandwich enzyme-linked immunosorbent assay (ELISA) (cat. DY2846-05, R&D Systems, US), according to manufacturer instructions.

SEAP NF-κB activity assays

The TLR4/NF-κB activity was measured using the HEK-hTLR4 and THP-1 dual™ cells-based reporter gene assays according to the manufacturer's instructions (cat. hkb-htlr4, thpd-nfis, InvivoGen, CA, USA). Upon recognition of an agonist, TLR4 activation results in NF-κB activation and SEAP reporter gene activation. This leads to the secretion of alkaline phosphatase, which was quantified by a colorimetric assay (cat. QUANTI-Blue; InvivoGen).

HEK-hTLR4 cells were cultured according to the manufacturer's instructions (InvivoGen) and as previously described. Cells were stimulated with a dose series of recombinant LUM (cat. 2846-LU, R&D Systems) and with 10 ng/ml LPS from *Escherichia coli* (strain K12) as a positive control. In a separate experiment, cells were stimulated with 1 μg/ml LUM in combination with a dose series of LPS from *E. coli* (strain K12) (cat. tlrl-peklps, Invivogen) or *Bacteroides dorei* (isolated as previously described[30]) for 24 h. To study if increased amounts of LUM augment the OA SF induction of TLR4 activation, HEK-hTLR4 cells were stimulated with OA SF, or OA SF spiked with LUM. LPS stimulation was used as a positive control.

THP1-Dual™ cells were cultured and differentiated into Mϕ (naïve macrophages) as previously described[31]. Mϕ were stimulated with 10 ng/ml LPS, or 1 μg/ml LUM or a combination of the two for 24 h. In selected conditions, 1 μM of TLR4 inhibitor CLI095 (cat. tlrl-cli95, InvivoGen) was added for 30 min prior to the addition of LPS or LUM.

Flow cytometry

LUM was labelled with FITC using a commercially available labelling kit according to the manufacturer's recommendation (cat. 46950, Sigma). Association of LUM with TLR4 was studied after incubation of HEK-293 and HEK-Blue™ hTLR4 cells (InvivoGen) with FITC-LUM (1 μg/ml). Cell-associated fluorescence was analyzed using BD FACSAria™ III (BD Biosciences) with identical acquisition parameters in all experiments. Human articular chondrocytes were analyzed for the expression of LC3B cytosolic marker of autophagy (cat. 8899S, Cell Signalling) by flow cytometry using the BD FACSVerse™ (BD Bioscience). Briefly, cells were washed with PBS, centrifuged (300 rcf, 5 min), and the supernatant discarded. Cells were incubated with the chondrocyte positive marker antibodies CD44 (cat. 17-0441-81, eBioscience), CD90 (cat. 561558, BD Bioscience) (15 min, RT), followed by 15 min of permeabilization (cat. 554714, BD Biosciences) and staining with LC3B (15min, RT), followed by washing with PBS, and resuspension in PBS-EDTA-BSA. All data was processed in Flowjo software (Flowjo LLC).

Tissue acquisition, primary chondrocyte culture and OA cartilage explant culture maintenance

Patient recruitment, participation, and sample collection were approved by the local cantonal ethics committee, Zürich, Switzerland (BASEC Nr. PB_2017-00510) with patient signed informed consent. Samples were collected from patients who underwent arthroscopic reconstruction ($N = 4$), and OA patients who underwent total knee arthroplasty (TKA) ($N = 4$).

Human chondrocytes and OA cartilage explants were isolated from the lateral tibial plateau region. Cartilage extraction and chondrocytes culture details are provide in the supplementary material.

Stimulation of primary chondrocytes and cartilage explant cultures

Chondrocytes were stimulated with 10 ng/ml LPS, 1 μg/ml LUM, or a combination of both for 24 h. In selected conditions, 1 μM of TLR4 inhibitor CLI095 was added for 30 min prior to the addition of LPS or LUM. Cartilage explants were maintained for 3–4 days and stimulated in identical conditions to chondrocytes during 48 h.

Lactate dehydrogenase activity assay

Cytotoxicity was assessed 24 h post-stimulation of chondrocytes. Briefly, lactate dehydrogenase (LDH) activity in the conditioned medium of monolayer chondrocyte cultures was determined after 24 h, according to the manufacturer's instructions (cat. 4744926001, Roche, Penzberg, Germany).

Macrophage differentiation and polarization studies

To study the effect of LUM on macrophage polarization, human blood-derived monocytes were used. Peripheral blood was obtained from donors under informed consent according to approval from the local cantonal ethics committee, St. Gallen, Switzerland (BASEC Nr. PB_2016-00816) and peripheral blood mononuclear cells (PBMC) were isolated via ficoll gradient separation and subsequent negative selection using monocyte isolation kit II (cat. 130-091-153, Miltenyi Biotec). Purified monocytes were suspended in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin–neomycin mixture (cat. 15640, ThermoFisher) and 2 mM l-glutamine. Seeded at a cell density of 1×10^5 cells/cm^2, monocytes were differentiated with 20 ng/ml human macrophage colony stimulating factor (M-CSF, cat. PHC9501, Invitrogen, Switzerland) for 6 days. The medium was refreshed on day 3. To induce polarization, 50 ng/ml LPS (cat. L7770, Sigma) were used for M1-like polarization, whereas 20 ng/ml IL-4 (cat. 130-093-921, Miltenyi Biotec) were used for M2-like polarization for 24 h. To test the effect of LUM, 10 ng/ml of LUM were added with or without polarization substance. Thereafter, total RNA (RNAeasy micro kit, Qiagen) and supernatants were collected. cDNA was produced (ThermoScript real-time-PCR System, Invitrogen) and quantitative real-time PCR was performed (iCycler iQ Real-Time PCR Detection System, Bio-Rad) using SYBR Green I (Applied Biosystems). Primer sequences are provided in Supplementary Table 1.

Protein measurements using luminex xMAP® technology

Protein levels were measured using the xMAP® technology (Luminex, Austin, TX, USA). Chondrocyte and macrophage culture supernatants were harvested and MMP-1, MMP-13, IL-6, IL-10, and TNF-α were measured using ProcartaPlex immunoassays (Thermofisher) and read by a MAGPIX® system (ThermoFisher) according to the manufacturer's instructions.

Histology and immunofluorescence

Cartilage explant samples were formaldehyde fixed and sectioned to a thickness of 5 μm, as previously described[31]. Tissue sections were then pre-treated with 0.1% hyaluronidase and blocked with 5% BSA. For staining of LUM, sections were incubated with lumican antibody (cat. AF2846, 1:20, R&D Systems) followed by secondary antibody rabbit anti-goat IgG Alexa Fluor 488 (cat. A27012, 1:2000, Invitrogen) and DAPI nuclear staining. For Col2 and Col10 stainings, the sections were incubated with anti-Col2 (cat. 600-401-104S, 1:200, Rockland) and anti-Col10 (cat. ab49945, 1:300, Abcam) antibodies followed by secondary antibodies AlexaFluor 488 goat anti-rabbit IgG (cat. A11008, 1:200, Invitrogen,) and AlexaFluor 594 goat anti-mouse IgG (cat. A110058, 1:200, Invitrogen) and DAPI nuclear staining. Safranin-O stainings were performed as previously described[31].

Primary chondrocytes were fixed with 4% PFA and permeabilized by 0.1% Triton-X. 1% BSA was used for blocking non-specific binding. To stain for apoptotic marker cleaved caspase-3 (CC3), chondrocytes were incubated with antibody CC3 (cat. MAB835, 1:200, R&D Systems) followed by incubation with secondary antibody AlexaFluor 488 goat anti-rabbit IgG (cat. A11008, Life Technologies) and DAPI nuclear staining.

All immunofluorescence stainings were imaged using a Zeiss Axio Observer (Carl Zeiss Microscopy GmbH, Thuringia, Germany). Only Safranin-O stained samples were imaged using a Slide Scanner (Pannoramic 250, 3D Histech).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 4.0 or higher versions. Kruskall–Wallis test was used to analyzed the LUM ELISA (LUM levels in SF, and LUM secretion by chondrocytes), LDH activity and macrophage polarization protein markers, followed by Dunn's post hoc test. One-way ANOVA was used to analyze chondrocyte catabolic markers, and HEK-hTLR4 stimulation with OA SF, followed by Tukey's post hoc test. Two-way ANOVA was used to analyze RT-PCR, remaining HEK-TLR4 related experiments, and THP1 NF-kB activation, followed by Tukey's post hoc test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

The choice of $N = 4$ biological replicates with $n = 3$ technical replicates in general for the experiments was considered to have sufficient power (calculated by post-hoc power analysis) for exploratory analyses.

Results

The concentration of secreted LUM in the SF of OA patients, either with knee or CMC-I OA were compared to “healthy” controls (meniscectomy patients), as well as to RA as a positive control with inflammatory arthritic condition [Fig. 1(a)]. Interestingly, LUM levels of knee OA and RA SF were significantly upregulated vs meniscectomy control, but also higher vs CMC-I OA. As cartilage is a major source of LUM in joints, we analyzed LUM expression in osteoarthritic cartilage tissue and chondrocytes upon LPS stimulation [Fig. 1(b)]. Strikingly, LPS-induced inflammation led to a drastic reduction of LUM in articular cartilage, particularly in the cartilage surface zone. Moreover, chondrocyte secretion of LUM was significantly increased by LPS and pro-inflammatory cytokine IL-1β stimulation, although not significantly in IL-1β condition [Fig. 1(c)]. Together, these data suggest that LUM secretion is significantly increased in an OA inflammatory environment.

Since the LUM structure contains a leucine-rich repeat (LRR) domain that may be recognized by TLR4, LUM alone may act as a TLR4-DAMP, triggering and/or binding to TLR4[32,33]. We, therefore,

examined the activation of TLR4 by LUM. By using the HEK-hTLR4 reporter cell line, it could be observed that LUM alone does not trigger TLR4 activation independent of the dose [Fig. 2(a)]. Furthermore, FACS analysis confirmed that LUM-FITC does not bind with a higher degree to the HEK-TLR4 cell line in comparison to the TLR4-absent parental cell line HEK-293T [Fig. 2(b)], neither was the binding degree affected by TLR4 antibody [Fig. 2(c)].

To confirm that LPS-induced TLR4 activation is mediated by LUM, we studied LPS-induced TLR4-activation in the presence of LUM. Interestingly, increasing levels of LUM (as observed in OA synovial fluid) augmented LPS-induced TLR4 activation in the HEK-hTLR4 reporter cell line, independent of the gram-negative bacterial origin of LPS [Fig. 3(a)]. Given the presence of LPS, but also other known DAMPs in OA SF, we also studied if spiking OA SF with LUM would increase TLR4 activation. Notably, OA SF-induced TLR4 activation was significantly upregulated upon spiking with LUM [Fig. 3(b)].

In order to understand how chondrocytes respond to LPS co-stimulation with LUM, typical features observed in OA cartilage such as the release of pro-inflammatory molecules, cell death, apoptosis, and cartilage degradation and remodeling were assessed. LUM co-stimulation with LPS significantly upregulated chondrocyte secretion of key OA molecules IL-6 and metalloproteinases 1 and 13 (MMP-1, MMP-13) in a TLR4-dependent manner [Fig. 4(a)]. TLR4 inhibitor CLI095 co-stimulation demonstrated that LUM does not trigger TLR4 activation or that LUM recombinant protein is not contaminated with endotoxins. Interestingly, not only inflammation but also cytotoxicity was significantly increased upon LUM co-stimulation with LPS [Fig. 4(b)]. When staining chondrocytes with the apoptotic marker CC3, a marked upregulation could be observed upon LUM co-stimulation with LPS [Fig. 4(c)]. Autophagy was also a cell death mechanism taking place as evident by the upregulation of autophagy marker LC3B by LUM co-stimulation with LPS upon Flow Cytometry measurements (Supplementary Fig. 1).

Cartilage degradation was analyzed using an OA cartilage explant culture model. When explants were stimulated with the same concentrations of LPS and LUM as in 2D culture, cartilage degradation, as a result of OA disease, could be observed in all donors [Fig. 5(a)]. LPS stimulation resulted in increased proteoglycan depletion, but also surface fibrillation. More importantly, LUM co-stimulation with LPS had a striking effect on proteoglycan depletion and surface fibrillation in all donors. Collagen type II degradation and collagen type X were also studied [Fig. 5(b)], showing markedly downregulated collagen type II upon LUM co-stimulation with LPS and LPS alone when compared to control. In parallel, the hypertrophy marker collagen type X was upregulated by LUM co-stimulation with LPS, when compared to LPS alone and control.

To determine whether LUM has an effect on macrophage polarization, human macrophages were treated with a pro-inflammatory stimulus (i.e., LPS) or an anti-inflammatory stimulus (i.e., IL-4) in presence or absence of LUM and gene expression profiles of pro-inflammatory TNF-α and CD197 as well as anti-inflammatory CCL22 and CD206 (Fig. 6) were assessed. RT-PCR results showed significantly increased TNF-α and CD197 levels upon LUM co-stimulation with LPS [Fig. 6(a) and (b)]. Conversely, LUM co-stimulation suppressed the expression of CD206 and CCL22 in IL-4 treated macrophages [Fig. 6(c) and (d)]. While LUM dramatically affected polarization in LPS or IL-4 treated macrophages, LUM alone did not show a significant difference in the expression of polarization markers with the exception of CD197 expression.

The effect of LUM co-stimulation with LPS on the gene expression of pro-inflammatory and anti-inflammatory cytokines was further confirmed by monitoring their protein levels as well as NF-κB activation in macrophages. Macrophage polarization by LPS lead to elevated levels of pro-inflammatory cytokine TNF-α, and co-stimulation with LUM resulted in a further increase of TNF-α levels [Fig. 7 (a)]. Protein expression of the anti-inflammatory marker IL-10 corroborated the RT-PCR results. While IL-4 stimulation showed a significant increase in IL-10 expression, co-stimulation with LUM resulted in lower IL-10 cytokine expression [Fig. 7(b)]. THP1 derived macrophages showed a dose-dependent NF-κB activation in response to LPS, which was further enhanced by co-stimulation with LUM [Fig. 7(c)], and was TLR4-dependent (CLI095).

Discussion

Given the increasing incidence and prevalence of OA worldwide, a detailed understanding of the molecular mechanism underlying OA pathogenesis is crucial for the discovery of effective treatments.

Matrix biology is an essential part of OA pathogenesis research, ranging from cartilage ECM matrix degeneration studies, to the biological activity of major matrix proteins, and to the development of OA biomarkers[7,34]. Here, we demonstrated that cartilage matrix protein LUM is significantly upregulated in knee OA SF relative to knee meniscectomy patients, and is comparable to RA patient levels. As LUM is synthesized by chondrocytes and deposited in the articular cartilage matrix[35], cartilage degradation may also release SLRPs like LUM from the matrix, which leads to their accumulation

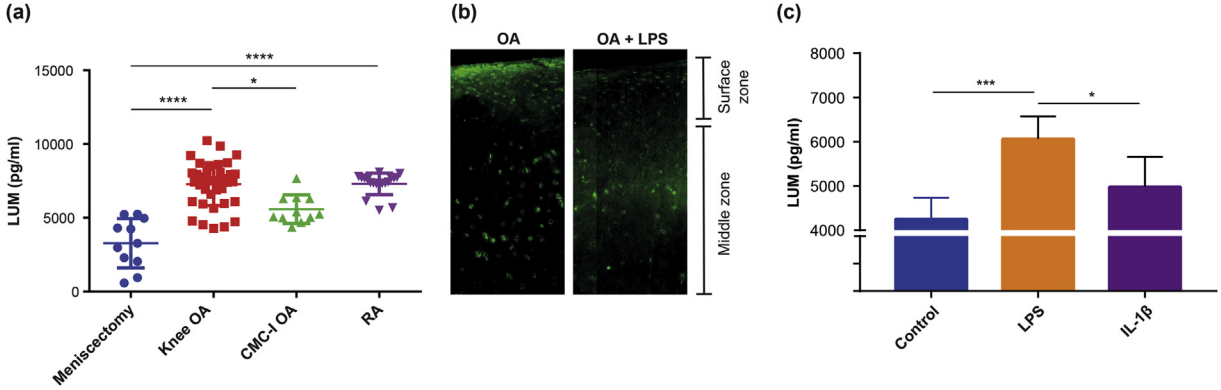

Fig. 1. Presence of LUM in OA synovial fluid, cartilage and LUM secretion by chondrocytes. **(a)** LUM levels in synovial fluid from arthritic conditions. Meniscectomy SF ($N = 11$), OA SF ($N = 40$), CMC-I OA SF ($N = 11$), and RA SF ($N = 20$) **(b)** LUM content in OA cartilage with and without LPS-stimulation **(c)** Amount of LUM secretion by chondrocytes under inflammatory stimulation by LPS and IL-1β. Values are mean ± SD ($n > 3$).

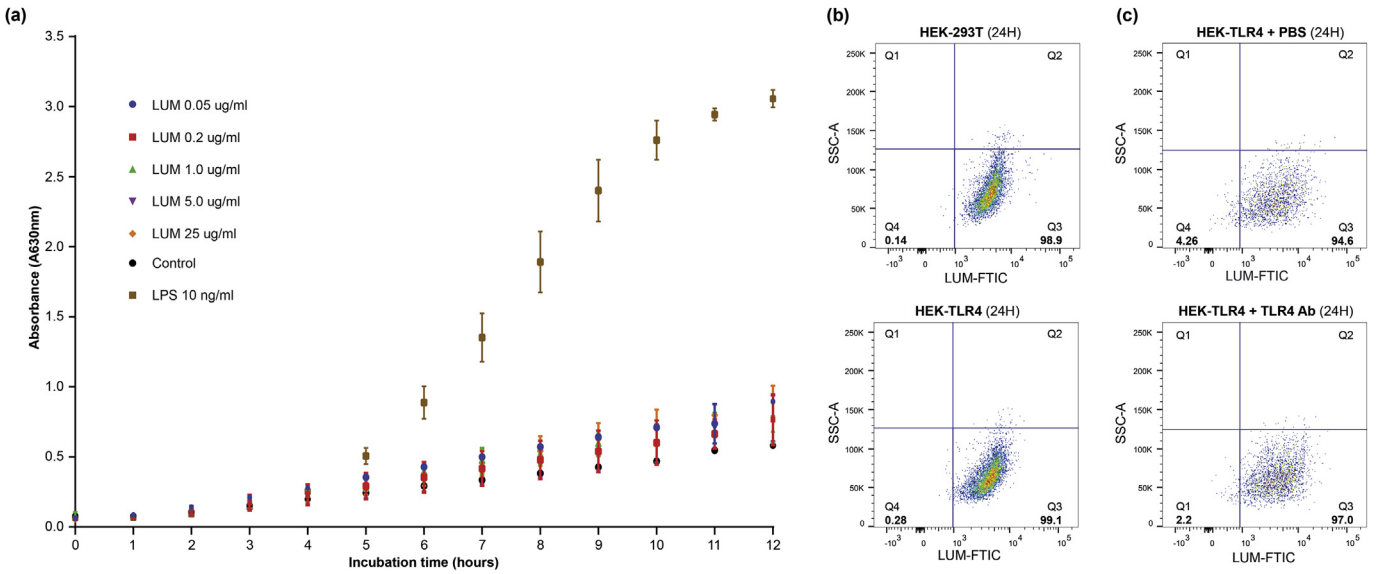

Fig. 2. LUM-induced TLR4-activation and binding of LUM to TLR4 receptor (a) SEAP reporter gene expression by TLR4/NF-κB activation upon stimulation with increasing doses of LUM. LPS is the positive control. (b) FACS measurements of LUM-FITC signal upon binding to TLR4 and non-TLR4 HEK 293 cells after 24 h incubation, in order to study the LUM binding to TLR4. (c) LUM-FITC binding to HEK-TLR4 cells in PBS or in the presence of TLR4 antibody, after 24 h incubation. Values are mean ± SD ($n > 6$).

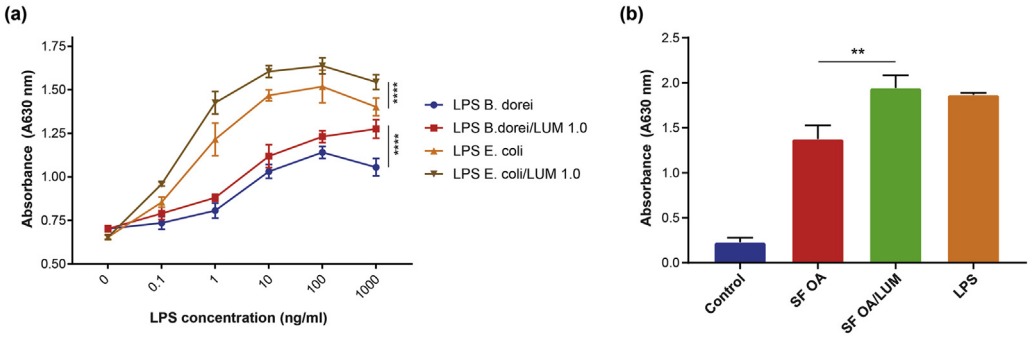

Fig. 3. Lumican co-stimulation with LPS and OA synovial fluid and ensuing TLR4/NF-KB activation. **(a)** SEAP reporter gene activation by TLR4/NF-κB activity upon LUM co-stimulation by a dose-series of LPS of different bacterial origin. **(b)** LUM spiking of OA synovial fluid and ensuing TLR4/NF-κB activation, measured by a SEAP reporter gene assay. Values are mean ± SD ($n > 4$).

in SF at supraphysiological levels and thereby contributes to numerous molecular interactions and inflammatory responses[7]. Several studies have reported an upregulation of SLRP fragments, including LUM, in both human OA and OA animal models as potential OA biomarkers[21,36]. Our results are in line with previous studies, where LUM was found to be upregulated in OA cartilage at the mRNA expression level[36]. Strikingly, RA and OA synovium and cartilage have been shown to contain LUM antigen complexed to IgG, which further suggests that LUM may contribute to arthritic inflammation[37].

During OA, TLRs expression is upregulated in cartilage and synovium cells[13]. In this study, we demonstrate that intact LUM in SF augments LPS-induced inflammatory responses in a TLR4 dependent manner. This is of particular importance since LPS is associated with OA disease activity and proposed to be a mediator of OA pathogenesis[26,38]. Strikingly, LPS stimulation of cartilage explants led to a drastic reduction of LUM in the cartilage surface zone. LPS-induced cartilage degradation might explain the observed depletion of LUM from cartilage and its concomitant increase in OA SF. Intriguingly, LUM was overexpressed in the pericellular matrix surrounding chondrocytes. This can potentially be explained by the renewed LUM synthesis upon inflammatory stress, as reported previously[39,40]. Moreover, LPS and IL-1β stimulation, two important OA pathophysiological molecules, led to increased secretion of LUM, providing further evidence of LUM association with inflammatory events in OA pathogenesis.

Although LUM was shown to interact with LPS, LUM alone did not bind to TLR4, nor did it trigger an inflammatory response. This is supported by flow cytometry analysis, where FITC-labelled LUM did not change binding rates to HEK-TLR4 when compared to parental HEK-293 cell line, or when TLR4-binding was inhibited by TLR4 antibody.

In order to better understand the impact of LUM overexpression on LPS-induced inflammatory responses, we used the HEK-TLR4 reporter cell line for monitoring NF-κB activity, as well as human primary chondrocytes and macrophages, two essential OA joint cells. In line with previous reports where LUM KO cells inhibited LPS-induce NF-κB activation[24], we observed that the presence of LUM also regulates and augments LPS-induced NF-κB activity, independent of the LPS dose, LPS bacterial origin and hence LPS inflammatory nature. This is especially relevant since multiple LPS types might be present in the OA joint[41]. The levels of LPS in SF can be considerable (~5 ng/ml)[26], and when spiking OA SF with

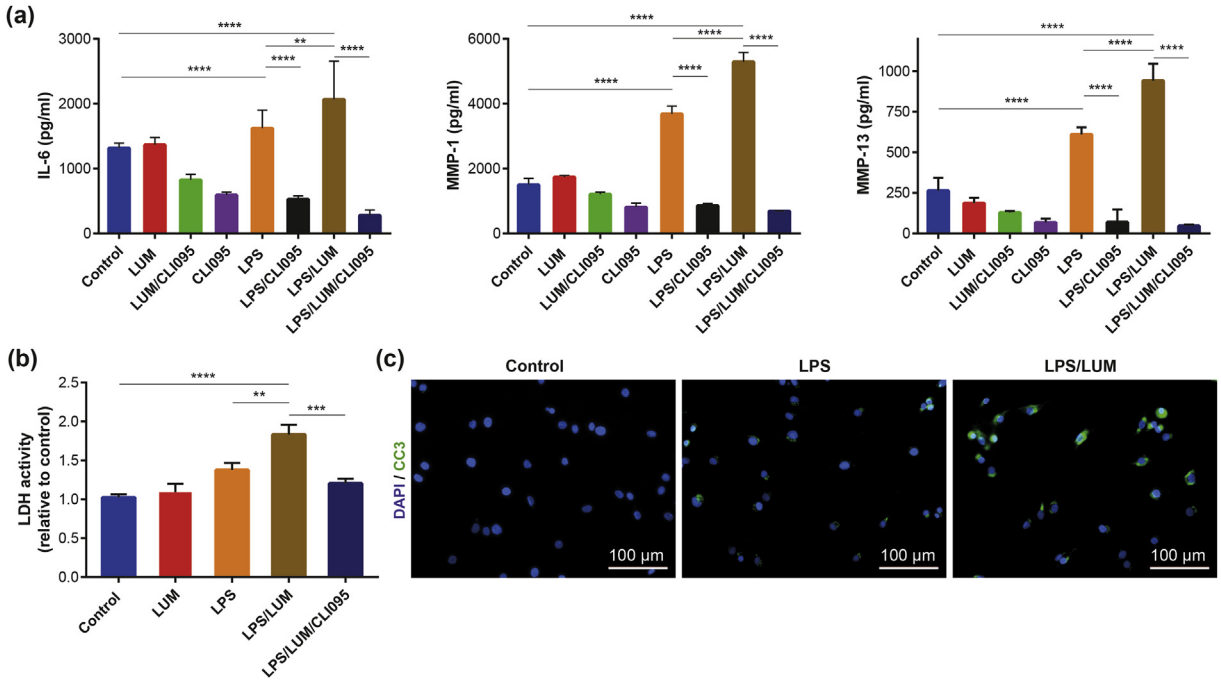

Fig. 4. Pro-inflammatory marker secretion, cell death, and apoptosis by co-stimulation of LUM and LPS in chondrocytes. **(a)** IL-6, MMP-1, and MMP-13 induced secretion upon stimulation by LUM, LPS, or both. CLI095 was used to observe TLR4 dependent effects. **(b)** Chondrocyte cell death, measured by LDH activity, after LUM co-stimulation with LPS. **(c)** Apoptotic marker cleaved caspase three immunostaining after LUM co-stimulation with LPS. Values are mean ± SD ($n > 3$) from four biological replicates ($N = 4$).

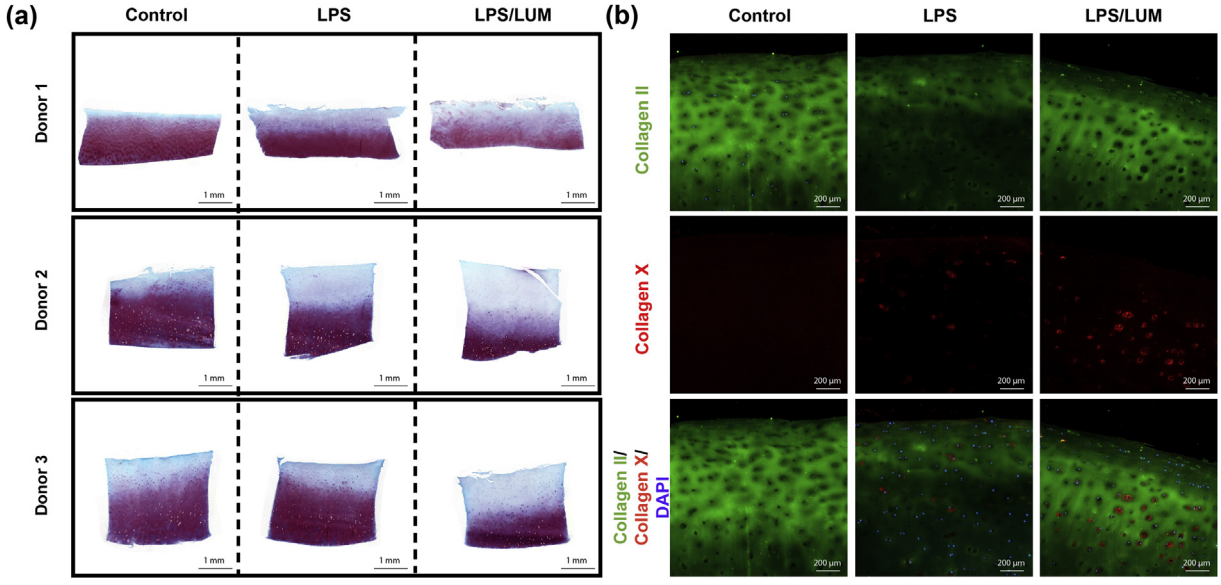

Fig. 5. Cartilage degradation and remodeling upon LUM co-stimulation with LPS. **(a)** Cartilage proteoglycan content, indicated by Safranin-O staining, upon LUM co-stimulation with LPS after 24 h. **(b)** Collagen type II (green) and X (red) immunostaining reflecting their distribution in cartilage explants upon LUM co-stimulation with LPS after 24 h.

supraphysiological LUM levels, we observed significantly upregulated NF-κB activation when compared to OA SF alone. This demonstrated *in vitro* that LUM overexpression in OA SF causes an exacerbated inflammatory activation, further supporting the pathophysiological role of LUM in OA. Besides, we also show a novel function of LUM, not only as being essential for LPS-induced pro-inflammatory responses, as shown previously with the abrogation of LPS-induced effects in LUM KO cells by Wu *et al.*[24], but also that overexpression of LUM exacerbates LPS-induced inflammatory responses.

Given the potential relevance of LUM in OA disease activity, we further studied its effects on chondrocytes, given their essential contribution for cartilage homeostasis, but also for OA progression[42]. In line with the results on NF-κB activity, LUM significantly upregulated LPS-induced secretion of catabolic markers IL-6, MMP-1 and MMP-13, when compared to control and LPS alone. These responses were TLR4-dependent, as indicated by the lack of response upon addition of TLR4 inhibitor CLI095. The upregulation of catabolic markers is an important observation, as MMP-1 and MMP-13 play a major role in cartilage degradation in OA, while IL-6

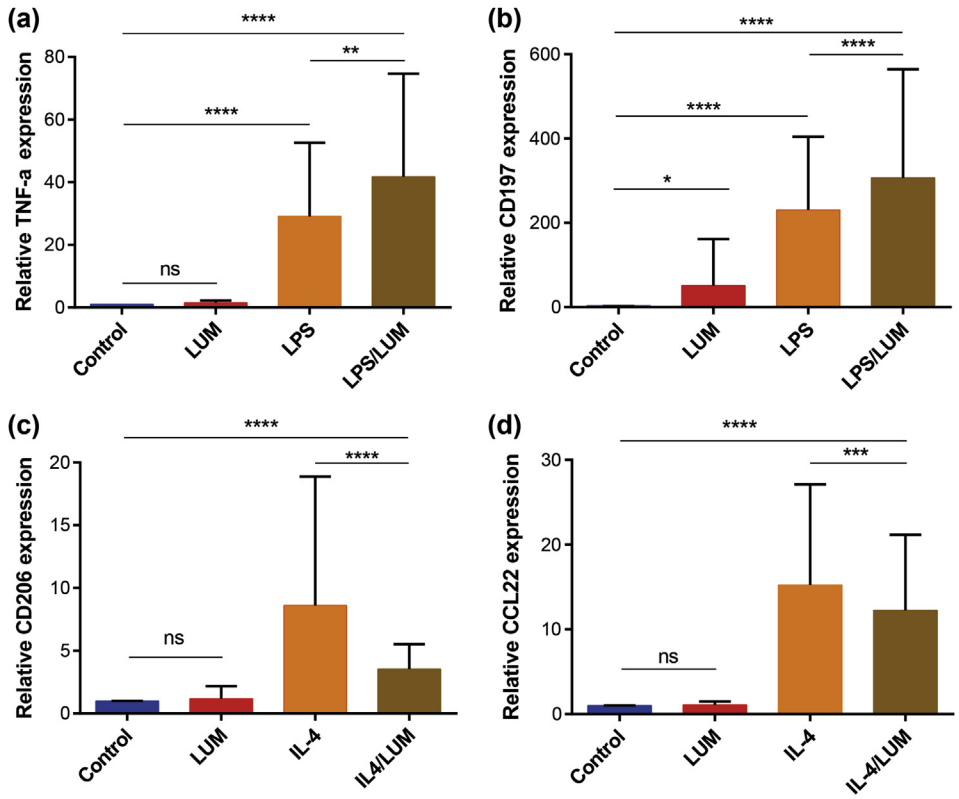

Fig. 6. Effect of LUM on macrophage polarization. Human macrophages were cultivated in the presence or absence of LUM and expression profiles were assessed for pro-inflammatory TNF-α after 6 h of treatment **(a)** and CD197 after 24 h of treatment **(b)** as well as for anti-inflammatory CD206 **(c)** and CCL22 **(d)** after 24 h. Values are mean ± SD ($n > 5$) from three biological replicates ($N = 3$).

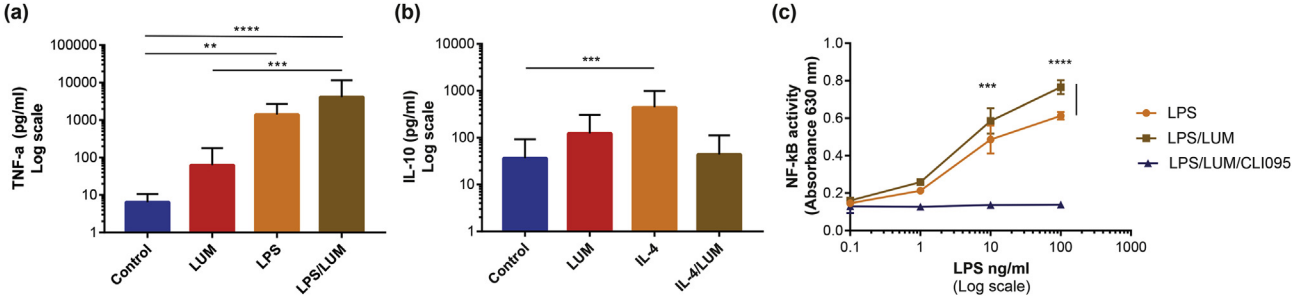

Fig. 7. Secretion of inflammatory proteins and NF-κB activity of macrophages upon LUM co-stimulation with LPS. **(a)** Protein levels of pro-inflammatory TNF-α and **(b)** anti-inflammatory IL-10 after 24 h of treatment. **(c)** NF-κB activity upon LUM co-stimulation with increasing dose of LPS using a THP1 reporter cell line. CLI095 inhibitor of TLR4 was used to observe TLR4 dependent effects. Values are mean ± SEM ($n > 3$) from three biological replicates ($N = 3$).

is a central cytokine in OA, whose primary function is to activate and recruit professional immune cells to the synovium[43]. Paralleling the increased pro-inflammatory response by LUM co-stimulation with LPS, cell death was also significantly upregulated in a TLR4 dependent manner when compared to control and LPS. Chondrocyte death is a feature of OA and is known to be associated with TLR4 activation, often through apoptosis and autophagy mechanisms[44,45]. Strikingly, we observed that both apoptotic biomarker CC3 and autophagy LC3B marker were increased in chondrocytes that were LUM co-stimulated with LPS when compared to the reduced expression of both makers in LPS treated or control cells, respectively. This is in good agreement with previous reports[46].

To demonstrate the catabolic influence of LUM on resident chondrocytes in a matrix-regulated environment, we used an *ex vivo* cartilage explant model[47]. LUM co-stimulation with LPS or LPS alone lead to a loss of collagen type II immunostaining in cartilage explants when compared to control. This can potentially be explained by the observed upregulation of MMP-1 and MMP-13 by LUM co-stimulation with LPS. Furthermore, collagen type X expression increased upon LUM co-stimulation with LPS, which is of particular importance since collagen type X is synthesized by hypertrophic chondrocytes and represents a hallmark of OA[48]. Together, the downregulation of collagen type II and upregulation of type X are in line with current theories where cartilage degradation by proteases and a dysregulated chondrocyte phenotype result in a vicious cycle of OA disease[49]. Notably, LUM co-stimulation with LPS led to severe degradation of proteoglycans (which are tightly intercalated with the collagen network) when compared to controls, as indicated by the reduction of Safranin-O

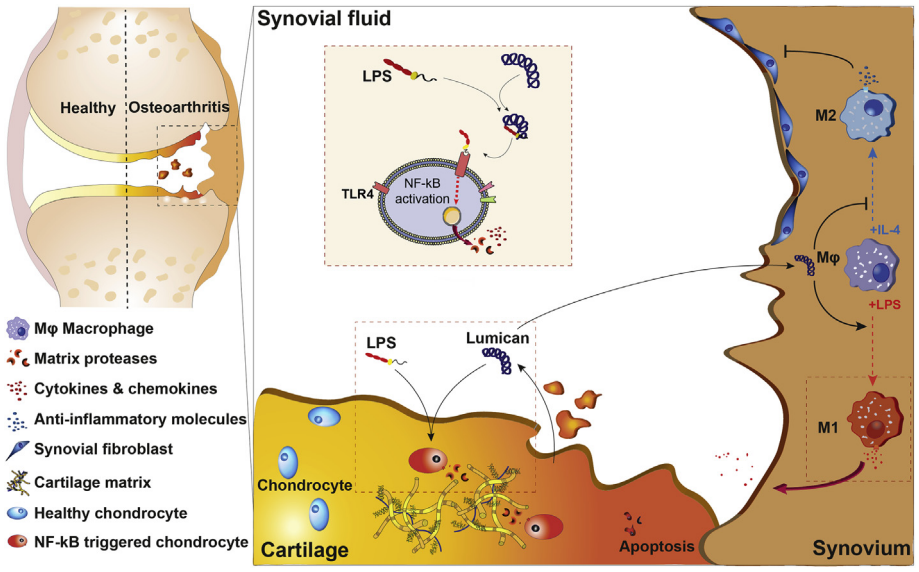

Fig. 8. Pathogenic role of lumican in the osteoarthritic joint. Upon degeneration of articular cartilage, matrix proteins such as the lumican glycoprotein are released in increased amounts which left unchecked may have potentially deleterious effects. Soluble lumican (LUM) may interact with pathogenic molecules such as lipopolysaccharide (LPS) upregulating it's proinflammatory properties, particularly through NF-κB activation. LUM-LPS has a broad impact in the joint physiology by triggering chondrocytes catabolic response, characterized by the secretion of matrix proteases and cytokines, leading to further degradation of articular cartilage. Furthermore, LUM-LPS may penetrate the synovium and impact infiltrating and resident MΦ macrophages by dysregulating their polarization towards the pro-inflammatory M1 macrophage population and ensuing burst cytokine release.

staining. LPS stimulation has been demonstrated previously to cause a loss of proteoglycan content in cartilage explants[46].

Besides chondrocytes, macrophages are key cells involved in the regulation of joint inflammation and erosion. It has been shown previously that macrophages transition from a pro-inflammatory (M1) to an anti-inflammatory (M2) state in OA[29,50]. However, the role of LUM in this process is currently unknown. To the best of our knowledge, this is the first study showing that LUM has a regulatory effect on M1/M2 macrophage polarization in the presence of a stimulator. To better understand the modulatory effects of LUM on macrophage polarization, gene expression profiles were studied with LPS-induced M1-like or IL-4 mediated M2-like macrophages in the presence or absence of LUM. Gene expression analysis of pro-inflammatory markers TNF-α and CD197 upon LUM co-stimulation with LPS showed enhanced M1-like macrophage activation whereas anti-inflammatory markers CD206 and CCL22 were downregulated upon LUM co-stimulation with IL-4, indicating a suppressed M2-like macrophage phenotype activation. These results were consistent with the protein secretion levels of IL-10 and TNF-α, albeit IL-10 secretion failed to reach statistical significance. Notably, LUM alone had no effect on TNF-α, CD206, and CCL22 expression, with only a small effect on CD197 expression, which is in line with previous reports[51].

Our study does have limitations. Although we were able to show differences in LUM concentration among arthritic groups, the selected patients in this study were not stratified with respect to disease activity or severity. Nevertheless, all selected arthritic patients were at end-stage joint arthroplasty phase.

In conclusion, this study demonstrates that LUM is upregulated in the SF of OA and RA patients, and that supraphysiological levels of LUM exacerbate the LPS-induced chondrocyte inflammatory response, mediated by TLR4, which leads to upregulated secretion of catabolic molecules, cell death and apoptosis, but also to the degradation of cartilage essential anabolic factors, such as collagen type II and GAGs (Fig. 8). LUM co-stimulation also interfered with macrophage polarization, leading to a reduced polarization into the anti-inflammatory phenotype, while increasing the polarization into the pro-inflammatory phenotype and ensuing production of inflammatory molecules. Importantly, LUM alone did not induce any inflammatory response, further supporting the fact that LUM acts as an immunologic adjuvant in OA (Fig. 8). These results highlight the potential of LUM as an OA biomarker, but also as a therapeutic target, particularly via suppressing LUM/LPS exacerbation of TLR4-mediated immune responses in the OA joint.

Author contributions

Study conception and design: Gonçalo Barreto, Marcy Zenobi-Wong, Markus Rottmar. Acquisition of data: Gonçalo Barreto, Berna Senturk, Lorenzo Colombo, Oscar Brück, Philipp Neidenbach, Gian Salzmann. Analysis and interpretation of data: Gonçalo Barreto, Berna Senturk, Lorenzo Colombo, Oscar Brück, Marcy Zenobi-Wong, Markus Rottmar. All authors made substantial contributions revising this manuscript for intellectual content and approved the final version to be published.

Markus Rottmar (markus.rottmar@empa.ch) and Marcy Zenobi-Wong (marcy.zenobi@hest.ethz.ch) had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflicts of interest

None.

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Supplementary data

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