LINK N SUPPRESSES INTERLEUKIN-1β-INDUCED BIOLOGICAL EFFECTS ON HUMAN OSTEOARTHRITIC CARTILAGE

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Abstract

Osteoarthritis (OA) is a disease of diarthrodial joints associated with extracellular matrix proteolytic degradation under inflammatory conditions, pain and disability. Currently, there is no therapy to prevent, reverse or modulate the disease course. The present study aimed at evaluating the regenerative potential of Link N (LN) in human OA cartilage in an inflammatory milieu and determining if it could affect pain-related behaviour in a knee OA mouse injury model.

Osteo-chondro OA explants and OA chondrocytes were treated with LN in the presence of interleukin-1β (IL-1β) to simulate an osteoarthritic environment. Quantitative von Frey polymerase chain reaction and Western blotting were performed to determine the effect of LN on matrix protein synthesis, catabolic enzymes, cytokines and nerve growth factor expression. Partial medial meniscectomy (PMM) was performed on the knee of C57BL/6 mice and, 12 weeks post-surgery, mice were given a 5 µg intra-articular injection of LN or phosphate-buffered saline. A von Frey test was conducted over 24 h to measure the mechanical allodynia in the hind paw.

LN modulated proteoglycan and collagen synthesis in human OA cartilage through inhibition of IL-1β-induced biological effects. LN also supressed IL-1β-induced upregulation of cartilage-degrading enzymes and inflammatory molecules in OA chondrocytes. Upon investigation of the canonical signalling pathways IL-1β and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), LN resulted to significantly inhibit NF-κB activation in a dose-dependent manner. In addition, LN suppressed mechanical allodynia in an OA PMM mouse model. Results supported the concept that LN administration could have therapeutic potential in OA.

Keywords: Osteoarthritis, cartilage repair, bioactive peptides, link N, nuclear factor kappa-light-chain-enhancer of activated B cells, interlukin-1.

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List of Abbreviations		ANOVA	analysis of variance
		BSA	bovine serum albumin
AC	articular cartilage	*COL2A1*	collagen type II (gene)
ACAN	aggrecan (gene)	Col II	collagen type II
ADAMTS	a disintegrin and metalloproteinase	DMEM	Dulbecco's modified Eagle medium
	with thrombospondin motifs	DMMB	1,9-dimethylmethylene blue

ECM	extracellular matrix
FBS	foetal bovine serum
GAG	glycosaminoglycan
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HRP	horseradish peroxidase
IL	interleukin
IVD	intervertebral disk
LN	Link N
MAP	mitogen-activated protein
MMP	matrix metalloproteinase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NSAIDs	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
PMM	partial medial meniscectomy
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride or polyvinylidene difluoride
PWT	paw weight threshold
RA	rheumatoid arthritis
RIPA	radio immuno-precipitation assay
TBS	tris-buffered saline
TNF-α	tumour necrosis factor-alpha

Introduction

The AC is a unique connective tissue, covering the bony parts of diarthrodial joints, which, in conjunction with lubricating functions provided by the synovial fluid, allows the frictionless motion of the joint. It has no blood supply, lymphatics or nerves and its properties are related to the composition and structure of an abundant ECM. The main matrix proteins in cartilage are proteoglycans and collagen. Aggrecan is the key proteoglycan responsible for the resiliency of the tissue, while Col II provides tensile strength (Roughley, 2006). Cartilage responds to a complex multitude of autocrine and paracrine factors, either anabolic or catabolic, that regulate gene expression and protein synthesis in chondrocytes and are responsible for ECM synthesis and maintenance. When the catabolic processes overtake the anabolic activities, cartilage degeneration occurs with the onset of OA (Lotz and Loeser, 2012). Unfortunately, unlike most tissues in the body, cartilage does not regenerate.

OA is a chronic incurable degenerative joint disorder characterised by two fundamental pathological changes: inflammation, which may be driven by cytokines (Kapoor *et al.*, 2011), and destruction of the AC (mediated by proteolytic enzymes). Pain is the major symptom in OA and one of the leading causes of impaired mobility in the elderly population. Ageing, obesity and joint injuries are associated with increased risk of OA (Felson *et al.*, 2000; Loeser, 2010). The most recent update by the Global Burden of Disease declared OA the 11th most debilitating disease around the world. By 2050, 130 million people will suffer from OA worldwide, of whom 40 million will be severely disabled by the disease (Lawrence *et al.*, 2008). OA is resistant to effective therapy and the relief from severe OA pain remains an unmet medical need and a major reason for seeking surgical intervention.

Several cytokines have been implicated in OA pathogenesis including TNF-α, IL-1β, IL-6 and other common c-chain cytokines (IL-2, IL-7, IL-15 and IL-21) (Goldring and Berenbaum, 2004; Kapoor *et al.*, 2011). These factors produced by synovial cells and chondrocytes result in the upregulation of members of the MMP and ADAMTS families of enzymes. IL-1β is considered one of the key cytokines involved in OA progression by disrupting chondrocyte metabolism.

There is a general agreement that, since inflammatory processes play a fundamental role in the pathogenesis of various rheumatic diseases such as OA and RA, selective inhibition of inflammatory activities is vital for therapy and that the family of NF-κB transcription factors (Marcu *et al.*, 2010; Poulet *et al.*, 2012; Rigoglou and Papavassiliou, 2013) plays a prominent role in this process. Thus, several studies have been directed towards the pharmacological modulation of NF-κB pathways using non-steroidal anti-inflammatory drugs, corticosteroids, nutraceuticals, antisense DNA therapy, RNA interference and anti-rheumatic drugs. However, there is a need for new generations of NF-κB-targeting anti-inflammatory agents that are specific, efficacious and cost-effective. Despite significant progress in understanding the molecular mechanisms involved in the pathogenesis of OA, there are currently no disease-modifying agents for its treatment. Current treatment methods are generally not effective and involve either symptomatic relief with non-steroidal anti-inflammatory drugs and physical therapy or surgery, when conservative treatments fail. The lack of an adequate analgesia for OA pain contributes to the opioid epidemic.

Link N (DHLSDNYTLDHDRAIH, LN) is the naturally occurring N-terminal peptide of Link protein that stabilises the proteoglycan aggregates in both disc and cartilage. This peptide is generated *in vivo* by MMPs during tissue turnover (Nguyen *et al.*, 1989). There is an abundance of pre-clinical data confirming that LN can stimulate synthesis of proteoglycans and collagens in explant cultures of normal human articular cartilage (McKenna *et al.*, 1998; Liu *et al.*, 1999; Liu *et al.*, 2000). LN can also preferentially stimulate the synthesis of proteoglycan over collagen by bovine IVD cells *in vitro*, without any effect on cell division (Mwale *et al.*, 2003).

Two studies have confirmed the repair potential of LN in the IVD in a rabbit model of disc degeneration (Mwale *et al.*, 2018; Mwale *et al.*, 2011). LN inhibits the expression of neurotrophic factors in human disc cells (Noorwali *et al.*, 2012). LN also modulates inflammation and stimulates the repair of arthritis-

mediated temporomandibular joint disruption *in vivo* (Yang *et al.*, 2019). Delivery of synthetic LN-encoding mRNA into primary human chondrocytes and mesenchymal stromal cells results in an enhanced expression of aggrecan, Sox9 and Col II (Tendulkar *et al.*, 2019). Similarly, LN stimulates the chondrogenic differentiation of cartilage stem/progenitor cells (He *et al.*, 2018).

To date, there have been no reports on the effect of LN on OA cartilage under inflammatory conditions and on pain-related behaviours associated with progressive joint damage. The purpose of the present study was to determine whether LN could regulate human OA cartilage under inflammatory conditions and if LN could affect pain-related behaviour in an established inflammatory-mechanical murine model of OA (Glasson *et al.*, 2007).

Materials and Methods

Peptide synthesis
LN was synthesised with a purity > 98 % by CanPeptide (Pointe Claire, QC, Canada).

Antibodies
Anti-Collagen II antibody (ab34712, Abcam), anti-phospho-NF-κB p65 antibody (3033, Cell Signaling Technology), anti-GAPDH (G8795, Sigma-Aldrich) and anti-G1 aggrecan antibody (generated by the late Dr Peter Roughley) (Roughley and Mort, 2012) were used. Anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch (111-035-144, West Grove, PA, USA).

OA cartilage
OA cartilage was obtained, with informed consent, from 10 donors 45-65 years old undergoing total knee arthroplasty. Samples were collected immediately after surgery and classified as grade IV based on X-ray observations using the Kellgren Lawrence grading system. Chondrocytes and osteochondral explants were processed from non-weight bearing areas of the condyle (mostly the lateral condyle).

Cartilage explant preparation and cell culture
Osteochondral explants were prepared from donors and included cartilage with the cortical bone. Explants were cut using a circular saw and bone cutters to approximately 1 cm^2 in size. Explants were washed thrice in PBS containing penicillin-streptomycin (Wisent Bioproducts, Montreal, QC, Canada) prior to incubation with 0.125 % trypsin (Wisent Bioproducts) for 30 min in DMEM. Explants were washed twice in regular culture medium (low-glucose DMEM supplemented with 10 % FBS serum, 1 % penicillin-streptomycin and 1 % amphotericin) (Wisent Bioproducts) and maintained for 6 d in regular culture medium under standard culture conditions (humidified atmosphere and 5 % CO$_2$). Culture medium was changed every 3 d.

OA chondrocytes were recovered from the cartilage of each knee by sequential digestion with 0.125 % pronase followed by 0.2 % collagenase. After isolation, cells were expanded in DMEM supplemented with 10 % heat-inactivated FBS and 1 % streptomycin, with culture medium changed every 3 d. Cells were used until passage 3.

Osteochondral explant treatments
After 6 d, explants were exposed to either 5 ng/mL IL-1β (Sigma-Aldrich), 1 µg/mL LN or co-exposed to IL-1β + LN for 21 d. Explants cultured in DMEM without supplementation were used as control. Culture medium was changed every 3 d.

Histological assessment
After 21 d of treatment, explants were fixed in Accustain (Millipore) and decalcified in Osteosoft (Millipore) before paraffin-wax embedding. Sections were rehydrated prior to immunohistochemistry or staining by deparaffinisation in xylene followed by sequential incubation in decreasing concentrations of alcohol and water. Then, 5 µm-thick sections were stained with 0.1 % safranin O for 5 min, rinsed in water, dehydrated by sequential alcohol concentrations and xylene and mounted in Permount (Thermo Fisher Scientific). Images were captured using an Optika light microscope (FroggaBio, Toronto, ON, Canada).

Aggrecan extraction
Cartilage plugs of 3 mm diameter (approximately 30-60 mg) obtained from different sites for each explant were extracted with 15 volumes (v/w) guanidinium chloride buffer (4 M guanidinium chloride, 50 mM sodium acetate, 10 mM ethylenediaminetetraacetic acid, pH 7.4) for 72 h, as previously described (Roughley and Mort, 2012). Purified tissue extracts were digested for 1 h at 37 °C with keratanase (0.2 mU/µL buffer: 50 mM Tris-HCl, pH 7.4) followed by chondroitinase digestion (10 mU/uL buffer: 100 mM sodium acetate and 100 mM Tris-HCl, pH 7.4) for 16 h at 37 °C.

Aggrecan and Col II content in the extracts was assessed by Western blotting. Briefly, extracts were electrophoresed on 4-20 % gradient gels (Bio-Rad) and transferred to PVDF membrane as previously described (Grant *et al.*, 2016). Blots were blocked in 5 % BSA in PBS and 0.1 % Tween 20 for 1 h and probed with anti-G1 (1 : 2,000) and anti-Collagen II (1 : 5,000) for detection of aggrecan and Col II, respectively, in antibody solution (TBS, 1 % BSA and 0.1 % Tween 20). Blots were developed by incubation with anti-rabbit-HRP secondary antibodies (1 : 20,000) and Amersham ECL Prime chemiluminescent detection reagent (GE Healthcare). Images were captured on a Molecular Imager VersaDoc (Bio-Rad).

Proteoglycan and collagen content
Cartilage plugs were digested with proteinase K solution (1.5 mg/mL proteinase K, 50 mM Tris,

Table 1. Primers for gene expression. F: forward. R: reverse.

Genes	Primer sequence
ACAN	F: 5'-TGAGTCCTCAAGCCTCCTGT-3' R: 5'-CCTCTGTCTCCTTGCAGGTC-3'
COL1A1	F: 5'-GAGAGCATGACCGATGGATT-3' R: 5'-CCTTCTTGAGGTTGCCAGTC-3'
COL2A1	F: 5'-ATGACAATCTGGCTCCCAAC-3' R: 5'-CTTCAGGGCAGTGTACGTGA3'
ADAMTS4	F: 5'-TCCTGCAACACTGAGGACT-3' R: 5'-GGTGAGTTTGCACTGGTCC-3'
ADAMTS5	F: 5'-ACAAGGACAAGAGCCTGGAA-3' R: 5'-ATCGTCTTCAATCACAGCACA-3'
NGF	F: 5'-TCAGCATTCCCTTGACACTG-3' R: 5'-TGCTCCTGTGAGTCCTGTTG-3'
MMP-3	F: 5'-GGCAGTTTGCTCAGCCTATC-3' R: 5'-GAGTGTCGGAGTCCAGCTT-3'
MMP-13	F: 5'-TAAGGAGCATGGCGACTTC-3' R: 5'-GGTCCTTGGAGTGGTCAAG-3'
IL-1	F: 5'-ACCTATCTTCTTCGACACATG-3' R: 5'-ACCACTTGTTGCTCCATATCC-3'
IL-6	F: 5'-AACCTTCCAAAGATGGCTGAA-3' R: 5'-TGTACTCATCTGCACAGCTCT-3'

pH 7.4) in a ratio of 10 µL/mg tissue and incubated overnight at 55 °C. To determine proteoglycan content in explant cartilage, sulphated GAG (predominantly aggrecan) content in the tissue was quantified using the DMMB dye-binding assay (Farndale *et al.*, 1986). Shark chondroitin-6-sulphate (Sigma-Aldrich) dissolved at a concentration of 0-40 mg/mL in serum-free culture medium was used to generate the standard curve.

Hydroxyproline assay was used to determine collagen content. Briefly, 10 µL of digested sample were incubated for 30 min at 120 °C in an equal volume of sodium hydroxide (7 N). An equal volume of sulphuric acid (3.5 N) was added along with PBS to a final volume of 100 µL. Then, samples were incubated for 20 min at room temperature with 50 µL of chloramine T solution. Perchloric acid was added to the samples followed by 50 µL of p-dimethylaminobenzaldehide solution and incubated for 20 min at 60 °C. Samples were transferred to a 96-well plate and analysed by a spectrophotometer at an absorbance of 570 nm (BMG LABTECH, Ortenberg, Germany).

NF-κB signalling
OA human chondrocytes (n = 4 donors) were transferred to 6-well plates and seeded at a cell density of 2.5×10^6 cells per well. Cells were serum-deprived overnight and incubated for 10 min at 37 °C in culture medium containing IL-1β (5 ng/mL), LN (1 µg/mL) or combination of the two. Cells were lysed in RIPA buffer supplemented with protease cocktail II (Sigma-Aldrich) and phosphatase (Thermo Fisher Scientific) inhibitors. The lysate was electrophoresed on a 4-20 % gradient gel (Bio-Rad) under reducing conditions and transferred to 0.2 µm PVDF membranes. Blots were probed with anti-phospho-NF-κB p65 monoclonal rabbit antibody (1 : 1,000) and GAPDH (1 : 20,000) (Sigma-Aldrich) for normalisation.

RNA extraction and quantitative real-time PCR
OA human chondrocytes (n = 4 donors) were cultured as micro-pellets at a density of 3×10^5 cells/pellet and treated for 6 d with 0.5 mL medium supplemented with 1 µg/mL LN, 5 ng/mL IL-1β, 1 µg/mL LN + 5 ng/mL IL-1β or with vehicle (PBS) alone as a control. Total RNA was extracted using a total RNA mini-kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) following manufacturer instructions. Complementary DNA was synthesised using a superscript Vilo cDNA synthesis kit (Thermo Fisher Scientific). Quantitative real-time PCR was quantified using an ABI 7500 fast light cycler using CYBR green master mix (Thermo Fisher Scientific) and specific primers (Table 1). Relative mRNA expression level was normalised against *GAPDH* as previously described (Antoniou *et al.*, 2012). One-way ANOVA followed by Tukey multiple comparisons test was used to assess differences in gene expression. $p \leq 0.05$ was deemed statistically significant.

Induction of OA in mouse knee joint by PMM
The protocols described in the present study were approved by the Institutional Animal Care and Use Committee at Rush University Medical Center (Glasson *et al.*, 2007). Mice were anaesthetised by 5 % isoflurane (Abbott Laboratories) in oxygen, the left hind limb was shaved and swabbed with 70 % ethanol, the animal positioned on a dissecting microscope and the leg draped. A medial para-patella arthrotomy was performed and the patella luxated laterally. The anterior fat pad was dissected to expose the anterior

medial meniscotibial ligament, which was elevated and severed using a microsurgical knife. For PMM surgery, the cranial horn of the medial meniscus was resected following the severance of the medial meniscotibial ligament. The cartilage was kept moist with sterile saline throughout surgery. The patella was repositioned, the knee was flushed with saline and the incision closed in 3 layers – simple continuous 8/0 vicryl in the joint capsule, simple continuous 8/0 vicryl subcutaneously and tissue glue for the skin. In sham operations, the meniscotibial ligament was exposed but not cut and the joint flushed and closed as above (Glasson *et al.*, 2007).

LN injections and von Frey assay

Mice were given a 5 µg injection of LN or PBS intra-articularly 12 weeks post-surgery and monitored for pain-related behaviours longitudinally over 24 h using the von Frey assay. Hind-paw mechanical allodynia (secondary allodynia) was determined with von Frey fibres using the up-down staircase method and expressed as PWT (Chaplan *et al.*, 1994; Das *et al.*, 2018). Mice were placed on a perforated metal grid floor within small Plexiglas® cubicles and a set of 8 calibrated von Frey fibres (Stoelting Touch Test Sensory Evaluator Kit #2 to #9, Stoelting Co., Wood Dale, IL, USA) were applied to the plantar surface of the hind paw until the fibre bowed and held for 3 s. The threshold force required to elicit paw withdrawal (median 50 % withdrawal) was determined twice per hind paw per testing day (Van de Weerd *et al.*, 2001).

Results

To determine if LN could supress IL-1β-induced biological effects on human OA cartilage, proteoglycan content in osteo-chondro explants from OA donors cultured for 3 weeks with LN, IL-1β or LN and IL-1β was investigated (Fig. 1). Treatment of explants with IL-1β significantly decreased ($p = 0.0418$) GAG content by approximatively 50 %, compared to control (explants in regular culture media), indicative of proteoglycan loss.

Proteoglycan deposition and distribution in the accumulated cartilage matrix was visualised by staining with safranin O. Staining of tissue sections confirmed a loss of proteoglycans in the cartilage of IL-1β-treated explants, where little safranin O (red) staining was found in the superficial and middle zones (Fig. 2). In contrast, LN supplementation led to an enhanced safranin O staining throughout the osteochondral explants. This pattern was also present when LN was administered alone. Thus, it seemed that LN could mitigate the effects of an inflammatory milieu on proteoglycan synthesis in cartilage.

Maintaining proteoglycan content also requires aggrecan retention. To address this, the effect of LN on aggrecan synthesis and retention in the tissue was determined using an antibody against the G1 domain. In the absence of treatment, OA cartilage showed a depletion and a multi-component pattern of aggrecan (Fig. 3). After culturing the osteochondral explants for 21 d in the presence of LN, the content of aggrecan fragments bearing the G1 domain was increased significantly ($p = 0.0005$). When explants were treated with IL-1β, the content of aggrecan fragments bearing the G1 domain was comparable to control. Analysis of LN co-administered with IL-1β revealed that proteoglycan fragments containing the G1 domain were enhanced and comparable to LN alone.

The function of articular cartilage is dependent mainly on proteoglycan and collagen composition in the ECM. During OA progression, degradation of collagen impairs cartilage integrity. To determine if LN could maintain or restore collagen content following IL-1β exposure, hydroxyproline was quantified. When osteochondral explants were treated with IL-1β, hydroxyproline content was significantly lower ($p = 0.0384$) as compared to controls (Fig. 4). In contrast, LN supplementation in the presence of IL-1β blunted the decrease in hydroxyproline content observed by treatment of IL-1β alone. There were no significant changes in hydroxyproline content when explants were incubated with LN.

Col II is the most abundant collagen in cartilage. IL-1β decreases Col II content through downregulation of gene expression and upregulation of catabolic enzymes, collagenases such MMP-3 and MMP-13. To further determine whether LN could affect Col II content in an OA environment, a Western blotting was performed on extractable collagen from osteochondral explants treated with LN, IL-1β and IL-1β + LN (Fig. 5). When explants were treated with IL-1β, Col II content was significantly decreased ($p = 0.0331$). Although no significant changes in the
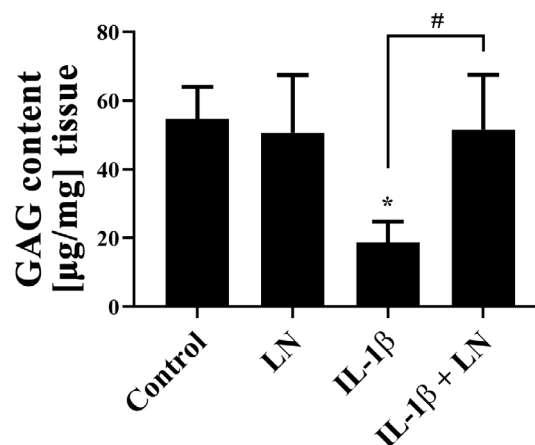

Fig. 1. GAG content in human OA cartilage treated with IL-1β and LN. Osteochondral explants were cultured for 21 d in medium supplemented with LN (1 µg/mL), IL-1β (5 ng/mL), IL-1β + LN or vehicle (control). GAG content was measured by DMMB assay and quantified as µg/mg of cartilage tissue wet weight. Results are represented as mean ± SD, $n = 4$ donors; ANOVA *post-hoc* Tukey's multiple comparison test; * $p < 0.05$ (compared to control); # $p < 0.05$.
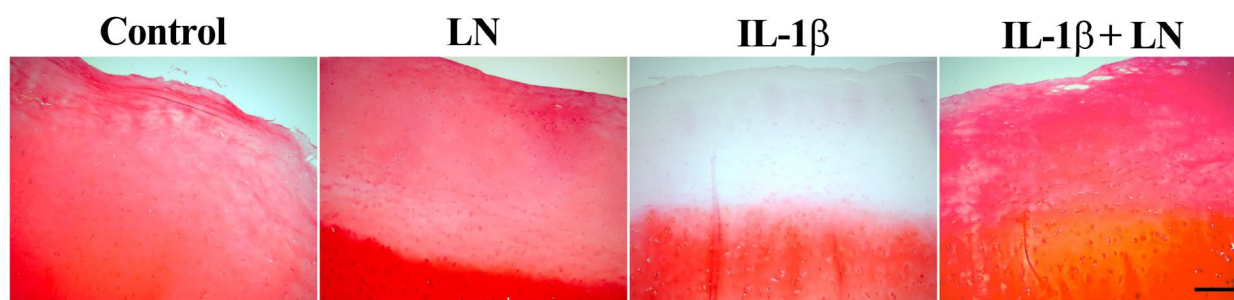
Fig. 2. Proteoglycan content in human OA cartilage treated with IL-1β and LN. Osteochondral explants were cultured for 21 d in medium supplemented with LN (1 µg/mL), IL-1β (5 ng/mL) or IL-1β + LN. Histological sections demonstrating safranin O staining for proteoglycan content. Scale bar: 350 µm.
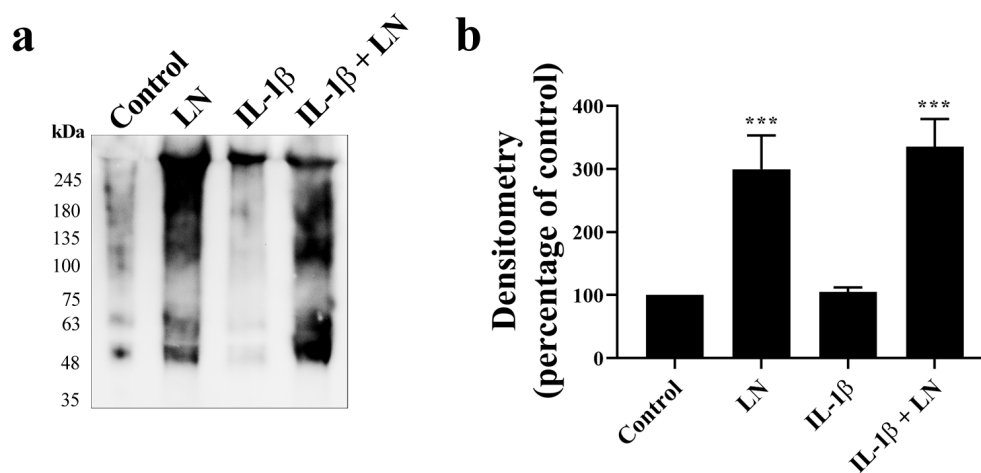

Fig. 3. Western blot of aggrecan in human OA cartilage. Osteochondral explants in medium supplemented with LN (1 µg/mL), IL-1β (5 ng/mL), IL-1β + LN and control cartilage. (**a**) Representative blot demonstrating intact aggrecan core protein with a molecular weight of about 250 kDa and aggrecan fragments. (**b**) Densitometry of blots presented in **a**. Results are represented as mean ± SD; $n = 3$ donors. ANOVA *post-hoc* Tukey's multiple comparison test. *** $p < 0.001$ as compared to control.

cartilage of LN-treated explants were observed, LN significantly reversed the effects of IL-1β on Col II content ($p = 0.0088$).

To determine if LN could supress IL-1β-induced biological effects on human OA chondrocytes, qPCR was performed to quantify changes in the expression of matrix proteins, catabolic enzymes, inflammatory cytokines and NGF, known factors regulated by IL-1β. After culturing OA chondrocytes as pellet cultures for 6 d supplemented with LN, cells showed significantly increased *ACAN* mRNA when compared to control ($p = 0.0001$) (Fig. 6). Although, IL-1β treatment did not increase expression of *ACAN*, in combination with LN, *ACAN* expression was significantly enhanced ($p = 0.0013$). Surprisingly, mRNA levels for *COL2A1* were not significantly altered by any of the treatments, despite the effect of LN on IL-1β induced changes in Col II content (Fig. 5,6).

To determine if LN could regulate catabolic factors associated with collagen and proteoglycan degradation, changes in the aggrecanases ADAMTS-4 and ADAMTS-5 and collagenases MMP-3 and MMP-13 were measured. IL-1β significantly upregulated the expression of *ADAMTS-4* ($p = 0.0045$) and *MMP-3*

($p = 0.0001$) in OA chondrocytes. In addition, there was a trend towards an increased expression of catabolic enzymes *ADAMTS-5* and *MMP-13* (Fig. 6). When chondrocytes were co-incubated with IL-1β and LN, upregulation of these catabolic factors was blunted.

In addition to catabolic enzymes, IL-1β is also known to upregulate inflammatory markers such as IL-6 and itself (Kushner, 1993). Similar to its effect on catabolic enzymes, LN also inhibited IL-1β-induced upregulation of *IL-1* ($p = 0.0001$) and *IL-6* ($p = 0.0311$) (Fig. 6). IL-1β is known to upregulate NGF (Manni and Aloe, 1998), one of the main factors associated with pain. *NGF* was significantly upregulated in IL-1β-treated chondrocytes ($p = 0.0467$), however, its expression was blunted when LN was present. Treatments of chondrocytes with LN alone had no effect on *NGF* expression.

NF-κB is a transcription factor that regulates the inducible expression of a wide range of proinflammatory mediators (Goldring and Marcu, 2009; Kapoor *et al.*, 2011; Rigoglou and Papavassiliou, 2013; Saklatvala, 2007; Yasuda, 2011). Since enhanced abnormal NF-κB activation is one of the characteristic features of OA pathophysiology
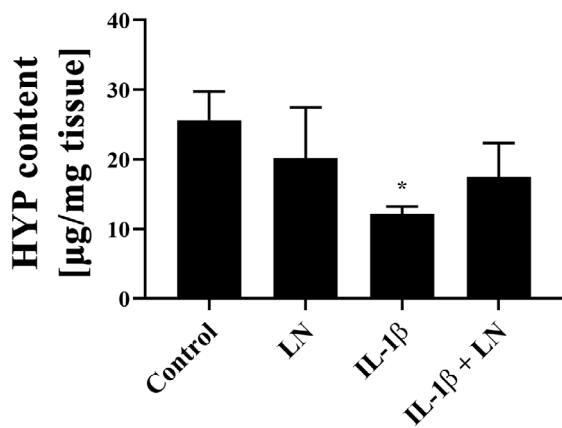
Fig. 4. Hydroxyproline content in human OA cartilage treated with IL-1β and LN. Osteochondral explants were cultured for 21 d in medium supplemented with LN (1 μg/mL), IL-1β (5 ng/mL) or IL-1β + LN. Hydroxyproline (HYP) was measured by the hydroxyproline assay and quantified as μg/mg of cartilage tissue wet weight. Mean ± SD, $n = 4$ donors; ANOVA *post-hoc* Tukey's multiple comparison test; * $p < 0.05$ as compared to control.
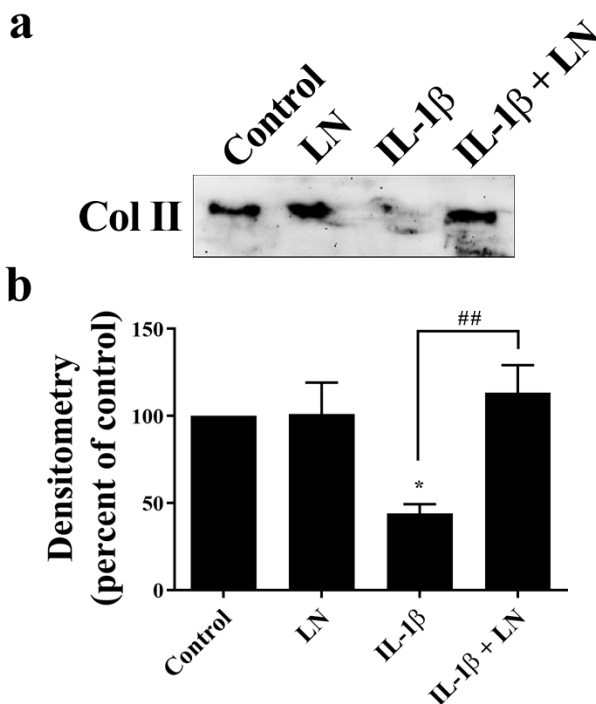
a

b

Fig. 5. Western blot of Col II in human OA cartilage. Osteochondral explants were treated with LN (1 μg/mL), IL-1β (5 ng/mL) or IL-1β + LN. (**a**) Representative blot of Col II. (**b**) Densitometry on blots presented in **a**. Mean ± SD, $n = 4$ donors; ANOVA *post-hoc* Tukey's multiple comparison test. * $p < 0.05$ as compared to control; ## $p < 0.01$ as compared to IL-1β.

(Rigoglou and Papavassiliou, 2013), the effect of various concentration of LN on NF-κB activation by IL-1β was examined in OA chondrocytes seeded in 6-well plates at a density of 2.5×10^6 cells (Fig. 7). Stimulation of phosphorylated NF-κB (P-NF-κB) was

determined by Western blotting using antibodies specific to P-NF-κB. After culturing chondrocytes for 10 min in the absence of IL-1β, cells showed no P-p65 protein (Fig. 7, CTL). With LN supplementation, no effect on P-p65 protein was observed either (Fig. 7, lane 2). As expected, P-p65 was prominent after stimulation with IL-1β ($p < 0.0001$) (Fig. 7, lane 3). LN significantly inhibited IL-1β-stimulated P-p65 in a dose-dependent manner. 100 ng of LN had a similar effect to 1,000 ng LN.

As cytokine-induced activation of NF-κB pathway has been associated with pain (Ahmed *et al.*, 2019; Yan *et al.*, 2019) and LN was found to suppress neurotrophins (Noorwali *et al.*, 2012), von Frey test was performed to determine whether LN could affect pain-related behaviours in a mouse model of knee OA, induced by PMM (Fig. 8). PMM resulted in OA-induced symptoms, showing mechanical allodynia after 12 weeks. In the sham group, mouse withdrew the paws after 44.13 mN force. In the PMM group, pain threshold was significantly decreased as mice withdrew the paws with less than 10 mN (~ 80 % decrease in pain threshold). In contrast, a single intra-articular injection of 5 μg LN into the mouse knee joint, caused a fairly rapid increase in pain threshold that was statistically significant after only 3 h. The threshold continued to increase 6 h post injection and was maintained throughout the 24 h-assessment period, suggesting LN pharmacological properties and therapeutic efficacy in chronic OA pain.

Discussion

AC architecture is kept intact and functional through anabolic and catabolic factors, which act on chondrocytes that in turn maintain tissue homeostasis by balancing AC synthesis and degradation. Degradation and loss of collagen and aggrecan, subchondral bone remodelling and inflammation of the synovial membrane characterise OA, as the balance shifts towards catabolism. Moreover, inflammatory mediators are thought to inhibit the compensatory synthesis of matrix macromolecules by chondrocytes, thereby exacerbating the problem (Goldring, 2006).

LN promotes matrix production in explant cultures of normal human AC (Liu *et al.*, 1999; Liu *et al.*, 2000; McKenna *et al.*, 1998). A recent *in vivo* study has provided evidence that LN can prevent OA lesion development or progression *in vivo* in a rabbit model of OA (Antoniou *et al.*, 2019). LN was also found to modulate inflammation and stimulate the repair of arthritis-mediated temporomandibular joint disruption *in vivo* (Yang *et al.*, 2019). Delivery of synthetic LN-encoding mRNA into primary human chondrocytes and mesenchymal stromal cells results in an enhanced expression of aggrecan, Sox9 and Col II (Tendulkar *et al.*, 2019). Similarly, LN stimulates the chondrogenic differentiation of cartilage stem/progenitor cells (He *et al.*, 2018). However, it is not

known if LN can restore proteoglycan and collagen content in OA cartilage in an inflammatory milieu.

The present work intended to determine whether LN could stimulate matrix production in an inflammation-induced human cartilage ECM and determine the potential mechanisms involved. Findings indicated that LN stimulated aggrecan expression when it was administered to human chondrocytes. In addition to stimulating *ACAN* expression, LN was also able to downregulate *ADAMTS-4*, *MMP-3*, *IL-1* and *IL-6* expression in human OA chondrocytes stimulated with IL-1β. LN also maintained total proteoglycan and collagen content in osteochondral explants following prolonged IL-1 exposure. When LN was co-administered with IL-1β, the decrease in GAG content was abolished. This suggested that during cartilage degeneration, LN had the potential to maintain the proteoglycan content in an inflammatory environment.

Interestingly, data indicated that LN suppressed the activation of NF-κB, the canonical signalling pathway of IL-1β. LN also supressed IL-1β-induced upregulation of *NGF*, a neurotrophic factor regulated by NF-κB activation. Furthermore, since NF-κB signalling has been associated with pain (Ahmed *et al.*, 2019; Cao *et al.*, 2019; Xu *et al.*, 2018) and LN suppressed the upregulation of neurotrophins (Noorwali *et al.*, 2012), a von Frey test was performed

to determine whether LN could affect nociception in a PMM mouse model of knee OA. *In vivo* studies demonstrated that a single intra-articular injection of LN significantly reduced advanced stage of chronic OA pain within 3 h and this reduced pain symptom was sustained over 24 h, suggesting its dual effects: (i) improvement of joint pathology and (ii) rapid pain reduction.

LN, a bioactive factor, has the potential to stimulate disc repair (AlGarni *et al.*, 2016; Bach *et al.*, 2017; Gawri *et al.*, 2013; Gawri *et al.*, 2014; Mwale *et al.*, 2003; Mwale *et al.*, 2018; Mwale *et al.*, 2011; Mwale *et al.*, 2014; Wang *et al.*, 2013; Yeh *et al.*, 2018). It has been identified using isolated IVD cells *in vitro*, where it induces collagen and proteoglycan mRNA synthesis, and it has been reported to increase incorporation of radioactive $^{35}SO_4$ into newly synthesised proteoglycans (Gawri *et al.*, 2013; Gawri *et al.*, 2014). Indeed, LN injection into intact human IVDs *ex vivo* (Gawri *et al.*, 2013) results in increased incorporation of radioactive $^{35}SO_4$ in newly synthesised proteoglycans and leads to partial restoration of disc height when injected into rabbit discs in a stable model of disc degeneration (Mwale *et al.*, 2018; Mwale *et al.*, 2011). Extending upon previous studies, the present work reported that LN could stimulate proteoglycan and collagen expression in chondrocytes from OA patients, consistent with a functional role in restoring the functional properties of cartilage.
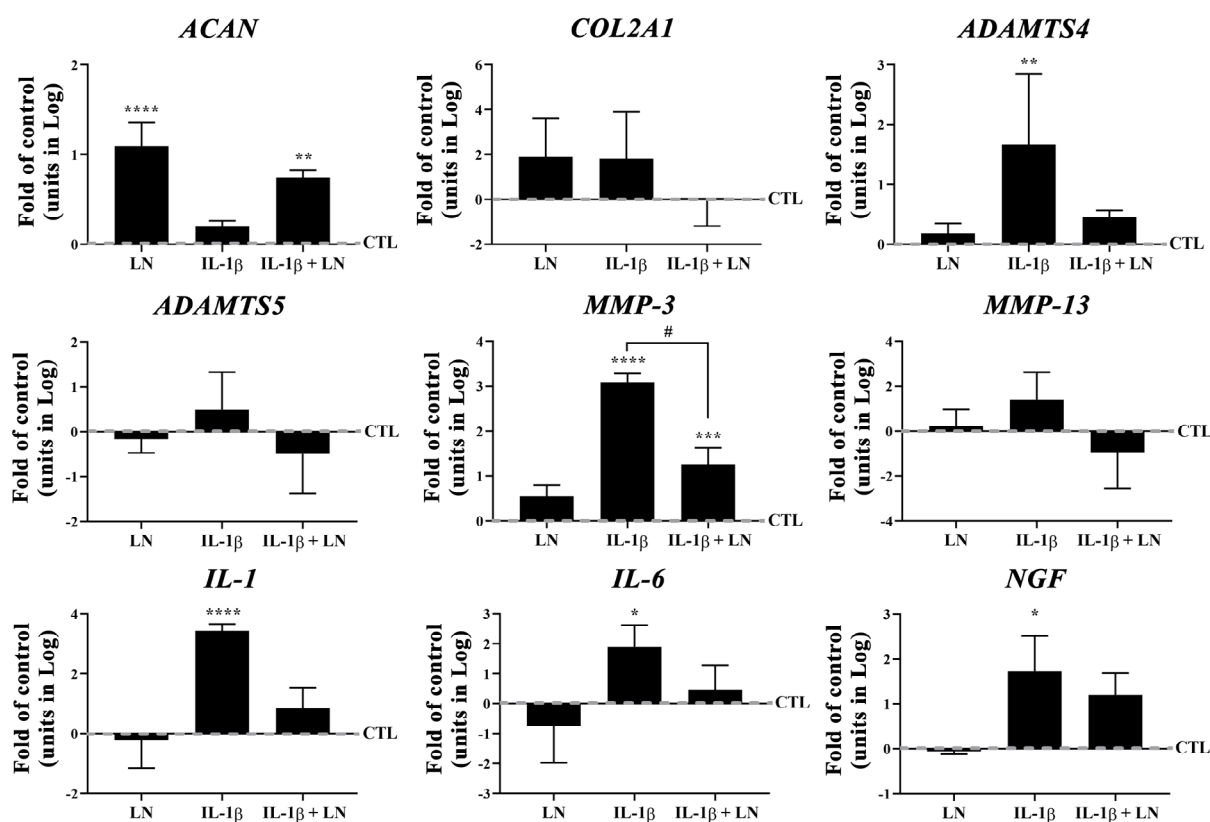

Fig. 6. Effect of LN on IL-1β-induced gene expression in human OA chondrocytes. Chondrocyte pellets were treated with LN (1 μg/mL) or PBS (CTL: control) for 6 d in the absence or presence of IL-1β (5 ng/mL). Gene expression was measured by qPCR. Mean ± SD; *n* = 4 donors; ANOVA, *post-hoc* Tukey's multiple comparison test. **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ as compared to control; # $p < 0.05$ as compared to IL-1β.
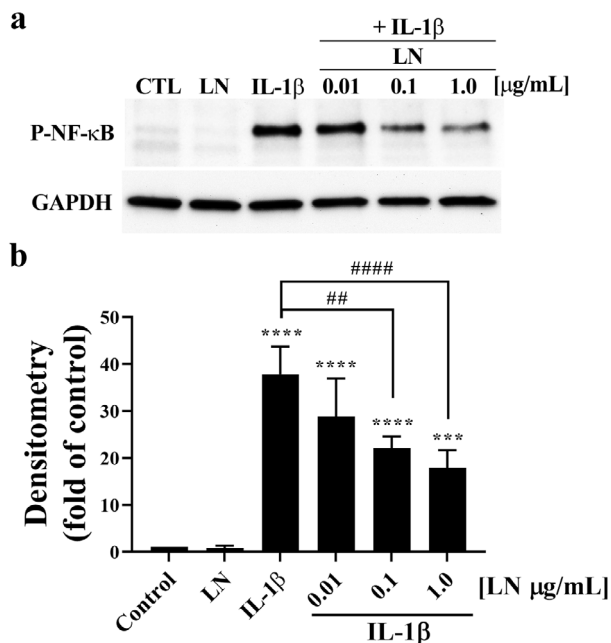
Fig. 7. Dose-dependent effect of LN on IL-1β signalling in human chondrocytes. Human osteoarthritic chondrocytes were treated with LN (1 μg/mL), IL-1β (5 ng/mL) or IL-1β with the indicated concentrations of LN (0.01, 0.1 or 1.0 μg/mL). (**a**) Representative blot demonstrating phosphorylated NF-κB (P-NF-κB) and GAPDH as a loading control. (**b**) Densitometry on blots presented in **a**. Mean ± SD, *n* = 4 donors; ANOVA *post-hoc* Tukey's multiple comparison test. *** *p* < 0.001, **** *p* < 0.0001 as compared to control; ## *p* < 0.01, #### *p* < 0.0001 as compared to IL-1β.
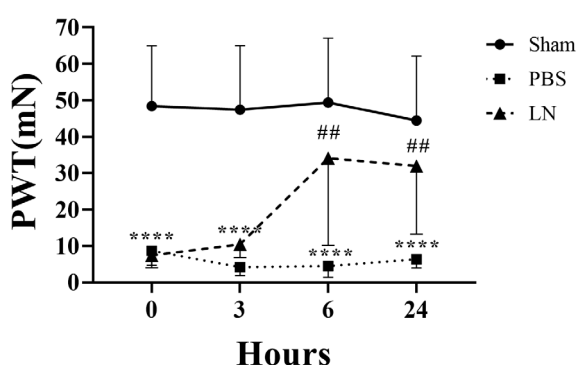

Fig. 8. Effect of LN on pain behaviour in a mouse model of knee OA. 12 weeks post-surgery, mice were injected intra-articularly with PBS or LN (5 μg) and monitored for pain-related behaviours longitudinally over 24 h using the von Frey assay. PWT in mN. Mean ± SD; *n* = 10 Sham, 8 PBS and 8 LN animals. ANOVA *post-hoc* Tukey's multiple comparison test. **** *p* < 0.0001 (PBS and LN *vs.* Sham); *** *p* < 0.001 (PBS *vs.* Sham); ## *p* < 0.01 (PBS *vs.* LN).

NF-κB signalling pathways play active roles in the development and progression of arthritis *in vivo* (Marcu *et al.*, 2010; Rigoglou and Papavassiliou, 2013). Indeed, the present study showed the activation of NF-κB in articular chondrocytes following stimulation with IL-1β. However, this activation was suppressed by the coincubation with LN.

NF-κB expression correlates with collagenase-3 (MMP-13) and stromelysin 1 (MMP-3) levels (Liacini *et al.*, 2003). Also, a shift to nuclear NF-κB localisation is shown in chondrocytes during cartilage destruction in the early stage of arthritis in DBA/1 mice immunised with Col II (Eguchi *et al.*, 2002). The present study showed that stimulation with IL-1β caused stimulation of NF-κB activation in articular chondrocytes. In such chondrocytes, NF-κB and MAP kinase pathways mediate inhibition of Col II and Link protein gene expression by TNF-*α* (Saklatvala, 2007). Other studies have also shown that NF-κB, as well as MAP kinases, mediates MMP-1, -3 and -13 expression induced by TNF-*α* or IL-1β in human OA chondrocytes (Liacini *et al.*, 2003; Liacini *et al.*, 2002). With regard to this, MAP kinase pathway is described as a partner signalling pathway for NF-κB. In the present study, MAP kinase pathway was not studied. However, LN decreases the phosphorylation of p38 in nucleus pulposus cells (Petit *et al.*, 2011).

The potential use of inhibitors of NF-κB to reduce AC degradation by MMPs in arthritis has been described (Liacini *et al.*, 2002; Vincenti and Brinckerhoff, 2002). Results using NSAIDs (Tegeder *et al.*, 2001) and glucocorticoids (Yamamoto and Gaynor, 2001) demonstrate decreased NF-κB activation. However, the use of NSAIDs can result in gastrointestinal side effects and the lack of specificity in antisense and transcription factor decoy strategies represents a big challenge when targeting gene expression in only a single organ. The problems of protein delivery, immunogenicity and cost of treatment have limited the realistic prospect of whole proteins for therapy. Being a synthetic peptide, LN has considerable financial benefits for clinical use over recombinant proteins.

Conclusions

LN could stimulate proteoglycan and collagen production in OA cartilage when it was administered in an inflammatory milieu. In addition, LN could suppress MMP activity and NF-κB expression in degenerated cartilage explants and chondrocytes from OA patients. These features are vital for any agent designed to become an effective therapy for joint diseases. In principle, systemic therapy by LN supplementation could be a viable option for treating cartilage degeneration and OA pain management.

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Discussion with Reviewer

Laura Creemers: Given that LN does not reverse inflammation-induced proteoglycan loss but prevents it, at what stage of the disease is LN treatment suggested?

Authors: LN was co-administered with the pro-inflammatory cytokine in the *in vitro* experiments (*i.e.* a prevention model). There were no experiments or data suggesting LN's inability to reverse inflammation-induced PG loss. In fact, administering LN after the onset of an inflammatory insult in both intervertebral disc or articular cartilage can either prevent further damage or may, in fact, reverse some of the damage (*i.e.* a treatment model).

LN stimulated proteoglycan synthesis in OA cartilage in an inflammatory environment and has pain relieving qualities. Recently, Antoniou *et al.* (2019) showed in an *in vivo* rabbit model of OA that LN has the features of a disease-modifying OA drug,

capable of preventing, or partially reversing, further progression of OA lesion development when dosed 3 weeks after the OA-inducing surgical procedure. Several other *in vitro* studies have demonstrated that LN promotes matrix production in explant cultures of normal human articular cartilage (Liu *et al.*, 1999; Liu *et al.*, 2000; McKenna *et al.*, 1998). Other studies have demonstrated the repair potential of LN in a rabbit model of interverbal disc degeneration (Mwale *et al.*, 2011; Mwale *et al.*, 2018) and that LN can increase proteoglycan synthesis in IVD cells (Gawri *et al.*, 2014) and aggrecan in whole-disc organ culture (AlGarni *et al.*, 2016) when administered 1 week after a trypsin injection induced PG loss. Thus, we would suggest that LN could be used both as a protective and therapeutic agent for mild to moderate degenerative conditions. For example, dosing during an acute phase of arthritis at the early stages of the arthritic process and possibly supplementing with an additional dose once the acute phase has subsided to continue to rebuild any damaged or lost cartilage that might have occurred.

Kandel Rita: How would you anticipate LN to be administered clinically? How do you think LN inhibits NF-κB activation?

Authors: The route of administration that we are currently studying is intra-articular injection. This would be given as needed on a monthly or perhaps even yearly basis depending on its formulation. How LN may be inhibiting NF-κB activation remains unknown. It is possible that LN may either interact directly with IL-1β or with its cognate receptor, IL-1R1, to interfere with its signalling pathway.