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Type II collagen peptide Coll2-1 is an actor of synovitis

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SUMMARY

Objective: We evaluated the ability of Coll2-1, a type II collagen peptide, to activate pro-inflammatory pathways in synovial cells and to induce arthritis in Lewis rats.
Method: Human synoviocytes and chondrocytes from knee OA patients were cultured for 24 h with/without Coll2-1 and/or purified immunoglobulin G (AS0619) binding specifically this peptide, and/or CLI-095, a TLR-4 signaling inhibitor and/or apocynin and diphenyleneiodonium, Reactive oxygen species (ROS) production inhibitors. The Interleukin (IL)-8 and Vascular Endothelium Growth Factor (VEGF) expression, the IL-8 production, the IκB-α and p65 phosphorylation and ROS were evaluated. Coll2-1 peptide, bovine type II collagen (CIA), streptococcal cell wall (SCW) or saline solution were injected into Lewis rats. The Coll2-1 peptide was injected subcutaneously (SC; 20–200μg/100μl/animal) or intra-articularly (IA; 0.5–5μg/50μl/animal) and compared to CIA injected in SC (200μg/100μl/animal) and SCW in IA (5μg/50μl/animal). The animals were injected on day 0 and monitored for 28 days. Histological lesions assessment was performed using an arthritis score.
Results: Coll2-1 peptide significantly increased IL-8 gene expression and production by synoviocytes. AS0619 and CLI-095 significantly decreased IL-8 expression. Coll2-1 induced p65 and IκBα phosphorylation and oxidative stress inhibitors decreased it. In human chondrocytes culture, Coll2-1 significantly increased MMP-3 and VEGF gene expression. In Lewis rats, CIA, SCW or Coll2-1 injection triggered arthritis. Like CIA or SCW, Coll2-1 induced synovitis, loss of cartilage proteoglycans, cartilage structure lesion and subchondral bone remodeling.
Conclusions: Coll2-1 activates synoviocytes to produce IL-8 and induces arthritis in rat. These findings suggest that neutralizing Coll2-1 could be a therapeutic approach of arthritis.

Introduction

Recently, Osteoarthritis (OA) was defined by the Osteoarthritis Research Society International (OARSI) as a disorder characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of immunity[1]. Innate and adaptive immune systems activation is closely associated with the low grade systemic inflammation in OA[2–4]. This process was initiated and driven in the synovial membrane, especially by synovium cells activated by damage-associated molecular patterns (DAMPs) released from cartilage during its degradation[5,6]. Tenascin, fibronectin fragments, biglycan, laminins, aggrecan-derived fragments are members of the DAMPs family directly involved in OA physiopathology[6]. DAMPs binds pattern-recognition receptors (PRRs) including membrane-bound receptors such as Toll-like receptors (TLRs) or Receptor for advanced glycation end products (RAGE) receptors and cytoplasmic receptors like NOD-like receptors (NLRs)[7]. These receptors are either localized on cells

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surface (immune cells, chondrocytes and synoviocytes; TLRs - RAGE) or in cell cytoplasm (NLRs). Receptors activation initiates downstream signalling cascades leading to the activation of transcription factors such as nuclear factor-κB (NF-κB), a key regulator of the inflammatory response. NF-κB activation leads to the release of various factors involved in OA pathogenesis like catabolic factors (MMP-1, -3, -9 and -13), cytokines (TNF-α and IL-1β), chemokines (CCL-7, -8, IL-8) and complement factors. This also leads to macrophage and T cell infiltration in the synovial membrane and to vascular permeability increase. Some recent papers also reported B cells activation and production of autoantibodies specific for cartilage cell surface proteins (collagen, osteopontin)[6,8–10].

In OA process, type II collagen is degraded in cartilage by collagenases particularly collagenase-3 (MMP-13), exposing cryptic epitopes that are suspected to be DAMPs, as supported by several *in vitro* studies on chondrocytes[11]. In this context, Klatt *et al.* observed a collagen II-dependent induction of the cytokines (IL-1β, −6 and −8) as well as MMPs (MMP-1, -3, -13 and −14), involved p38 and NF-kB signalling[12]. In the same culture model, Ruettger *et al.* demonstrated that a N-terminal fragment 29-mer fragment of type II collagen (named Ntelo) enhanced cathepsins B, L and K through activation of protein kinase C and p38 MAP kinase[13]. Fichter *et al.* demonstrated that mRNA and protein levels of MMP-2, -3, -9 and -13 are also up-regulated by Ntelo[14]. In cartilage explant culture model, Poole *et al.* also reported that a 24-mer synthetic peptide of type II collagen (named CB12-II) stimulated type II collagen cleavage with MMP-13 induction[15]. Subsequently, in a study conducted by Yasuda *et al.* demonstrated that CB12-II stimulated PI3K/Akt leading to NF-κB activation[16]. Recently, clinical trial for septic arthritis conclusions reported that collagen type II cleavage products (C2C) were associated to an up-regulation of growth factors (bFGF, BMP-2 and -7)[17]. Finally, by multiplexed high throughput selected reaction monitoring (SRM), Ritter *et al.* were able to identify peptides from 16 proteins in both synovial fluid and serum among which interestingly, type II collagen (COL2A1) was present[18]. These data suggesting that type II collagen peptides and Coll2-1, a peptide (^{108}HRGYPGLDG^{116}) located in triple helical part of type II collagen molecule and used as a biomarker of cartilage degradation could act as “DAMPs” and could be initiators of synovitis. This peptide was found to elevated in the serum of human OA patient and in the early phase of OA in guinea pigs developing spontaneously this disease[1,19].

This study investigated for the first time the effect of Coll2-1, on human synoviocytes and chondrocytes, and *in vivo* on rat joint inflammation.

Methods

Patients

OA human synovial tissue samples and cartilage were obtained from different patients (8 women - two men; mean age 70 ± 6 years for synovial biopsy samples and six women; mean age 61 ± 9 years for cartilage biopsy samples) with OA of the knee at the time of total knee joint replacement surgery. An ethical approval (ethics committee agreement of Catholic University of Louvain, no. B403201111664) was granted for this study and all subjects provided informed consent.

Isolation and culture of synovial cells

Synovial biopsies were cleaned from fat, cut into small pieces and subjected to digestion with type IA collagenase from Clostridium histolyticum (1 mg/mL; Sigma–Aldrich, Bornem, Belgium) in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10mM HEPES, 100 units/mL penicillin, 100 μg/mL streptomycin, 2mM glutamine and 10% fetal calf serum (Lonza) for 4 h at 37°C. This medium was named “complete medium” (CM). The cell suspension was passed through a 70-μm filter to remove any undigested tissue. The filtered cell suspensions were then collected by 800 g centrifugation and cultured in T25 cell culture flask with 10 mL of complete medium, at 37°C in a 5% CO_2 humidified atmosphere. After 2 days, medium was changed to remove non-adherent cells, and cells were cultured in complete medium renewed twice a week. For the experiments, synoviocytes at passage four were used.

Treatment of synovial fibroblast cells

Cells were seeded into a six-well plate at the density of 2×10^5 cells/well in two mL of CM.At confluence, the CM was replaced with 1% serum medium for 24 h. Cells were then incubated for 24 h with or withoutColl2-1 peptide (^{108}HRGYPGLDG^{116}; synthesized by Bachem, Bubendorf, Swiss with a purity (HPLC) > 98%) at the concentration of 0.45 and 4.5 nmol, according to the concentrations range commonly found in osteoarthritis patients blood[20,21]. For inhibition of TLR-4 receptor experiments, synoviocytes were pre-treated 1 h with CLI-095 (500 nM, 1 and 2.5 μM; InvivoGen, Toulouse, France), a cyclohexene derivative that specifically suppresses TLR-4 signaling, before a 24 h treatment with Coll2-1 at 4.5 nmol. For Reactive Oxygen Species (ROS) evaluation, synoviocytes were pre-treated 1 h with apocynin (0.2mM) and diphenyleneiodonium ($6.35\ 10^{-2}$ mM), two ROS production inhibitors before a 24 h treatment with Coll2-1 at 4.5 nmol.

Cartilage processing and chondrocytes culture

Full-depth articular cartilage was excised and immersed in DMEM with phenol red and 4.5 g/L glucose supplemented with N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) 10mM, penicillin (100 U/mL) and streptomycin (0.1 mg/mL) (all from Lonza, Belgium). After three washes, chondrocytes were released from cartilage by sequential enzymatic digestions with 0.5 mg/mL hyaluronidase type IV S (Sigma–Aldrich) for 30 min at 37 °C, 1 mg/mL pronase E (Merck, Leuven, Belgium) for 1 h at 37 °C and 0.5 mg/mL clostridial collagenase IA (Sigma–Aldrich) for 16–20 h at 37 °C. The enzymatically isolated cells were then filtered through a nylon mesh (70-μm), washed three times, counted and filled to the density of 0.1×10^6 cells/mL of DMEM (with phenol red and 4.5 g/L glucose) supplemented with 10 % fetal bovine serum, 10mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2mM glutamine (all from Lonza), 20 μg/mL proline and 50 μg/mL ascorbic acid (Sigma–Aldrich). Chondrocytes were seeded in six well plates at the density of 2×10^5 cells/well and cultured in monolayer for 5–7 days until 95% confluence. Only chondrocytes primary cultures were used in this study. Chondrocytes were then cultured 24 h in DMEM supplemented with 1 % fetal bovine serum, 10mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2mM glutamine, 20 μg/mL proline and 50 μg/mL ascorbic acid. Afterwards the culture medium was removed and was replaced by fresh culture medium in the presence or absence of Coll2-1 peptide (4.5 nmol). Cells were incubated for 24 h.

RNA extraction and real time reverse Transcriptase-Polymerase Chain Reaction (RT PCR)

Total RNA were extracted by combining three wells per treatment condition, using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and were reverse transcribed with Sensiscript Reverse Transcriptase (Qiagen) according to the manufacturer's

instructions. The cDNAs were quantified by quantitative real time Polymerase Chain Reaction (qPCR) using Rotor Gene instrument (Qiagen) and were performed with the SYBR Premix Ex Taq kit (Takara, Verviers, Belgium). The level of gene expression was determined by interpolation with a standard curve. To standardize mRNA levels, HPRT or GAPDH were amplified as internal control. The oligonucleotide primers sequences were as follow: HPRT forward, 5′-TGTAATGACCAGTCAACAGGG-3'; HPRT reverse, 5′-TGCCTGACCAAGGAAAGC-3'; GAPDH forward, 5′-TTGGTATCGTGGAAGGACTCA-3'; GAPDH reverse, 5′-TGTCATCATATTTGGCAGGTTT-3'; IL-8 forward, 5′-GGAACCATCTCACTGTGTGTAA-3'; IL-8 reverse, 5′-TGGAAAGGTTTGGAGTATGTCT-3'. Vascular Endothelium Growth Factor (VEGF) forward, 5′-TGCCTTGCTGCTCTAC-3'; VEGF reverse, 5′-CACACAGGATGGCTTGAA-3'; ADAMTS-5 forward, 5′-ATCACCCAATGCCAAGG-3'; ADAMTS-5 reverse 5′-AGCAGAGTAGGAGACAAC-3'; MMP-3 forward, 5′-CCCAAGAGGCATCCAC-3'; MMP-3 reverse 5′-GGGTCAAACTCCAACTGT-3'.

Immunoassay for interleukin-8 and KC/GRO

IL-8 was measured using specific enzyme amplified sensitivity immunoassay (Invitrogen, Merelbeke, Belgium). IL-8 productions were measured in triplicate in culture supernatants ($n = 3$/treatment condition) and reported to the corresponding amount of DNA. Serum KC/GRO was determined in the serum of Lewis rats using a V-Plex kit from Meso Scale Discovery (Rockville, USA) on the MESO QuickPlex SQ 120 imager.

DNA assay

DNA content was quantified in the cell extracts using Hoechst stain fluorimetric method[22].

Competitive inhibition of Coll2-1 peptide with an antiserum AS0619

AS0619 is an antiserum obtained after rabbit immunization with the sequence Coll2-1 peptide (^{108}HRGYPGLDG^{116}). This antiserum is specific for this peptide (kindly provided by Artialis S.A., Liège, Belgium). AS0619 was purified on Protein A Columns (Pierce, Gent, Belgium) to retain only the serum Immunoglobulin G (IgG). The absorbance of the IgG eluate at 280 nm was 1.974 and estimated IgG concentration was2.6649 mg/mL (1.974 X molar extinction coefficient (IgG = 1.35)). A Competition assay demonstrated that AS0619 at 1.33 μg/mL was able to bind500 nM of Coll2-1 peptide. Before addition tosynoviocytes culture medium, Coll2-1 peptide (4.5 nmol) was pre-incubated overnight at 4°C under constant agitation with AS0619 (1.33 μg/mL) in low glucose DMEM supplemented with 10mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 2mM glutamine.

Immunoblotting procedure

Cells were collected at 4°C and lysed on ice in 50 μL of buffer (25 mM Hepes, 150mM NaCl, 0.5% Triton X-100, 10% glycerol, and 1 mM dithiothreitol) containing protease and phosphatase inhibitors. After incubation for 30 min, lysates were centrifugated at 14,000 g for 30 min at 4°C. Protein concentrations were assayed using bicinchoninic acid (BCA) assay. Total protein extracts (10 μg) were fractioned by electrophoresis on polyacrylamide gel (10%) and transferred onto a polyvinylidene difluoride (PVDF) membrane that was blocked for 1 h in TBS-Tween containing 5% nonfat dried milk (HSC70) or in 2% bovine serum albumin (phospho−NF−κB p65 and phospho-IκBα). Membrane was incubated overnight at 4°C with primary antibodies. Anti-rabbit phospho−NF−κB p65 (1:1,000 dilution), anti-rabbit phospho-IκBα (1:1,000 dilution), anti-rabbit IkBα (1:500 dilution), anti-rabbit HSC70 (1:1,000 dilution) (Cell signaling, Boston, USA) and anti-rabbit NF-kB p65 (1:500 dilution, Upstate Biotechnology, NY, USA) were used. Horse-radish peroxidase (HRP)-linked anti-rabbit IgG antibody (1:2000 dilution) was used as secondary antibody (Cell signaling). The reaction was revealed with the Enhanced chemiluminescence (ECL) Western blotting substrate (Thermo Fisher Scientific, Erembodegem, Belgium). Densitometry analysis from three independent experiments was performed using ImageJ software to quantify Western blot images. The densitometry quantification for each protein was normalized to the appropriate loading control (NF-kB p65, IkBα).

Intracellular hydrogen peroxide (H_2O_2) production

Levels of intracellular hydrogen peroxide (H_2O_2) were assessed spectrofluorimetrically by oxidation of H_2DCFH-DA (Molecular Probes, Leiden, The Netherlands). Synovial cells seeded on 96-well plates (8×10^3 cells per well) were washed once with phosphate-buffered saline and then incubated with 50 μL of PBS containing either 200μM H_2DCFH-DA. The fluorescence intensity was measured every hour for 6 h H_2O_2 levels were expressed in arbitrary unit/living cell. Adherent cells were counted using crystal violet assay.

GSH assay

Levels of intracellular glutathione (GSH) were assessed by monoclorobimane staining. Synovial cells seeded on 96-well plates (8×10^3 cells/well) were washed once with phosphate-buffered saline and then incubated with 50μM monoclorobimane diluted in phosphate-buffered saline. The fluorescence intensities were measured at 3°C using excitation and emission wavelengths of 380 and 485 nm, respectively. GSH levels were expressed as arbitrary units of fluorescence intensity/living cell. Adherent cells were counted using crystal violet assay.

In vivo study design

Fifty-four 6-week-old female Lewis rats, purchased from Charles River Laboratories (Paris, France) were used[23]. The study protocol was submitted and approved by an independent ethical committee (CE/AG/16/012). All included animals were identified using unique chip and acclimated for 12−14 days (depending on study groups and randomization scheme established for surgery) under experimental conditions beforedesigned study initiation. They were distributed into the study group before induction based on their body weight. Animals were housed by two in ventilated cages in compliance with the new European Directive ETS 123 in a full specific pathogen-free (SPF) facilities. Animals were fed with commercially availablerodent food (Special Diets Service, Essex, England). Food and sterilized water were available ad libitum. The 3Rs (Replacement, Reduction and Refinement) rule was respected. Cotton, nestlets and tunnels were placed in each cage. Coll2-1 peptide was injected and compared to bovine type II collagen (CIA) or streptococcal cell wall (SCW) or saline solution in female Lewis rats ($n = 8$/group except naïve controls $n = 3$). Coll2-1 peptide was injected subcutaneously (SC) at the base of the tail, at concentrations of 20 and 200 μg/100 μL/animal or intra-articularly (IA) at concentrations of 0.5 and 5 μg/50 μL/animal. Bovine type II collagen was injected SC at the concentration of 200 μg/100 μL (Chondrex, Redmond, USA)[24], SCW extract PeptidoGlycan-PolySaccharide (PG-PS) 100P was injected IA at the concentration of 5 μg/50 μL (BD Bioscience, Erembodegem, Belgium)[25]. Volume of saline solution injected was 100 μL SC and 50 μL IA. The animals

were injected on day 0 under aseptic conditions and monitored for 28 days.

Euthanasia

Animals were euthanized 28 days after injection (D0). They were anesthetized with a subcutaneous injection of a Ketamin/Medetomidine mix and further euthanized by exsanguination.

Histological evaluation

The knees were processed for histological analysis. They were decalcified in HCl/EDTA mix (DC2, Qpath, VWR Belgium) for the appropriate time before inclusion in paraffin. Three frontal sections (5μM) of each compartment were done 200μM apart in the weight bearing zone using a microtome. Slides were stained according to a standard Safranin-O/Fast green protocol. Slides were evaluated under a light microscope by two trained experts blinded for the treatment groups. Each slide was scored for infiltration of inflammatory cells in synovial tissue (0–3), inflammatory cells in the joint cavity (exudate) (0–3), cartilage proteoglycan depletion (0–3), loss of articular cartilage (0–3), bone erosion (0–3)[26]. Infiltration of inflammatory cells in synovial tissue and inflammatory cells in the joint cavity were considered together (sum of the scores) as inflammatory parameters for the analysis of the detailed criteria. Right and left knees were considered together for the histological analysis after SC injection whereas data were considered separately for right (injected knee) and left knees in the case of IA injection.

Statistical analysis

Data from *in vitro* experiments were converted to neperian logarithm, analysed and compared using a repeated-measures one-way ANOVA test followed, if positive, bymultiple comparisons post-test. *P*-values were considered significant when $P < 0.05$. All samples collected from animal experiment were analyzed. Statistical significance of histological scores was evaluated with a parametric one-way ANOVA, followed if positive, by a Tukey's multiple comparison post-test. Graphs were presented as scatter dotplot. Exact *P* values were provided and asterisks representations were also performed. All data were analysed using GraphPad Prism software V5.0.

Results

Effects of Coll2-1 on human synoviocytes in monolayer culture

Addition of Coll2-1 significantly increased IL-8 chemokine gene expression [Fig. 1(A)]. The values in each condition were respectively 2.71 ± 5.36 (95%CI: −1.78 to 7.19) in the control, 5.66 ± 13.1 (95%CI: −5.28 to 16.6) with Coll2-1 0.45 nmol and 7.38 ± 16.0 (95% CI: −5.99 to 20.8) with Coll2-1 4.5 nmol. Globally, values differed between conditions ($P = 0.0042$) and, specifically, between control and Coll2-1 4.5 nmol ($P = 0.0043$). Coll2-1 also tended to increase

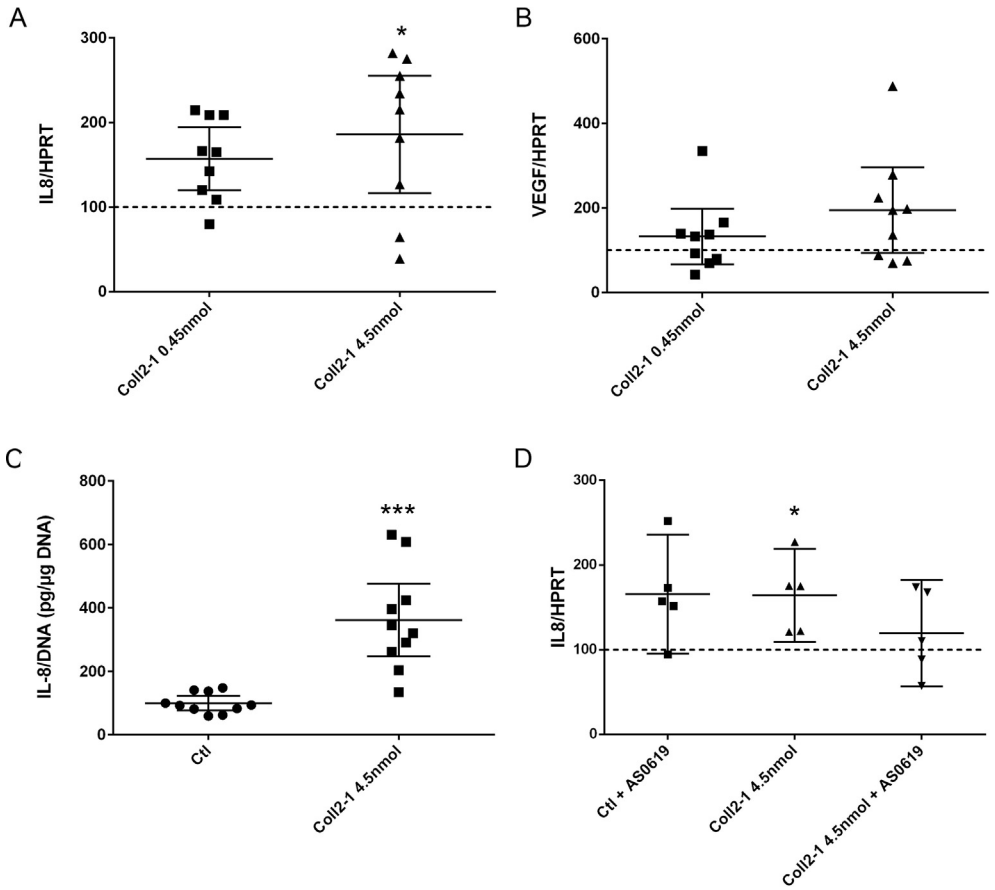

Fig. 1. Effect of Coll2-1 peptide on human synoviocytes in monolayer culture. (A) IL-8 mRNA expression ($n = 9$) **(B)** VEGF mRNA expression ($n = 9$) **(C)** IL-8 production ($n = 10$) **(D)** Competitive inhibition of Coll2-1 by purified IgG and effect on IL-8 gene expression ($n = 5$). Results are expressed as percent control and represented by mean with 95%CI. $^{*}P < 0.05$ and $^{***}P = 0.0001$.

VEGF gene expression [Fig. 1(B)] but had no significant effect on IL-6, MMP-2 and ADAMTS-5 gene expression (data not shown). Consistent with IL-8 gene expression data, a significant increase of IL-8 production ($P = 0.0001$) was observed in the presence of Coll2-1 peptide (4.5 nmol) [Fig. 1(C)]. Pre-incubation of Coll2-1 with anti-Coll2-1 IgG decreased its stimulating effect on IL-8 gene expression [Fig. 1(D)], demonstrating that IL-8 increase is the consequence of Coll2-1 treatment.

Coll2-1 peptide activates the nuclear factor NF-κB

Coll2-1 (4.5 nmol) increased the phosphorylation of p65 NF-κB subunit and its translocation to nucleus ($P = 0.0008$; Fig. 2(A) and (B)). This effect was inhibited by apocynin (0.2mM) and diphenyleneiodonium (6.35 10^{-2} mM) ($P < 0.0001$; Fig. 2(B)) suggesting the involvement of ROS in Coll2-1-induced NF-kB signaling pathway activation. Furthermore, Coll2-1 induced IκBα phosphorylation and degradation ($P = 0.0003$; Fig. 2(A) and (C)). Again, this effect was also reduced using ROS inhibitors ($P = 0.0001$; Fig. 2(A) and (C)). Coll2-1 significantly increased the intracellular production of H_2O_2 by synovial fibroblast cells (Ctl vs Coll2-1 4.5 nmol: −0.13 ± 0.04; 95%CI: −0.24 to −0.02; Fig. 2(D)). Conversely, Coll2-1 decreased the reduced form of glutathione (GSH) (Ctl vs Coll2-1 0.45 nmol: 0.13 ± 0.03; 95%CI: 0.05 to 0.20; Ctl vs Coll2-1 4.5 nmol: 0.15 ± 0.05; 95%CI: 0.004 to 0.29; Fig. 2(E)).

Coll2-1 peptide effects are mediated through TLR-4 receptor

Coll2-1 4.5 nM significantly stimulated IL-8 gene expression. This effect was fully blocked by CLI-095, an inhibitor of TLR-4, at the

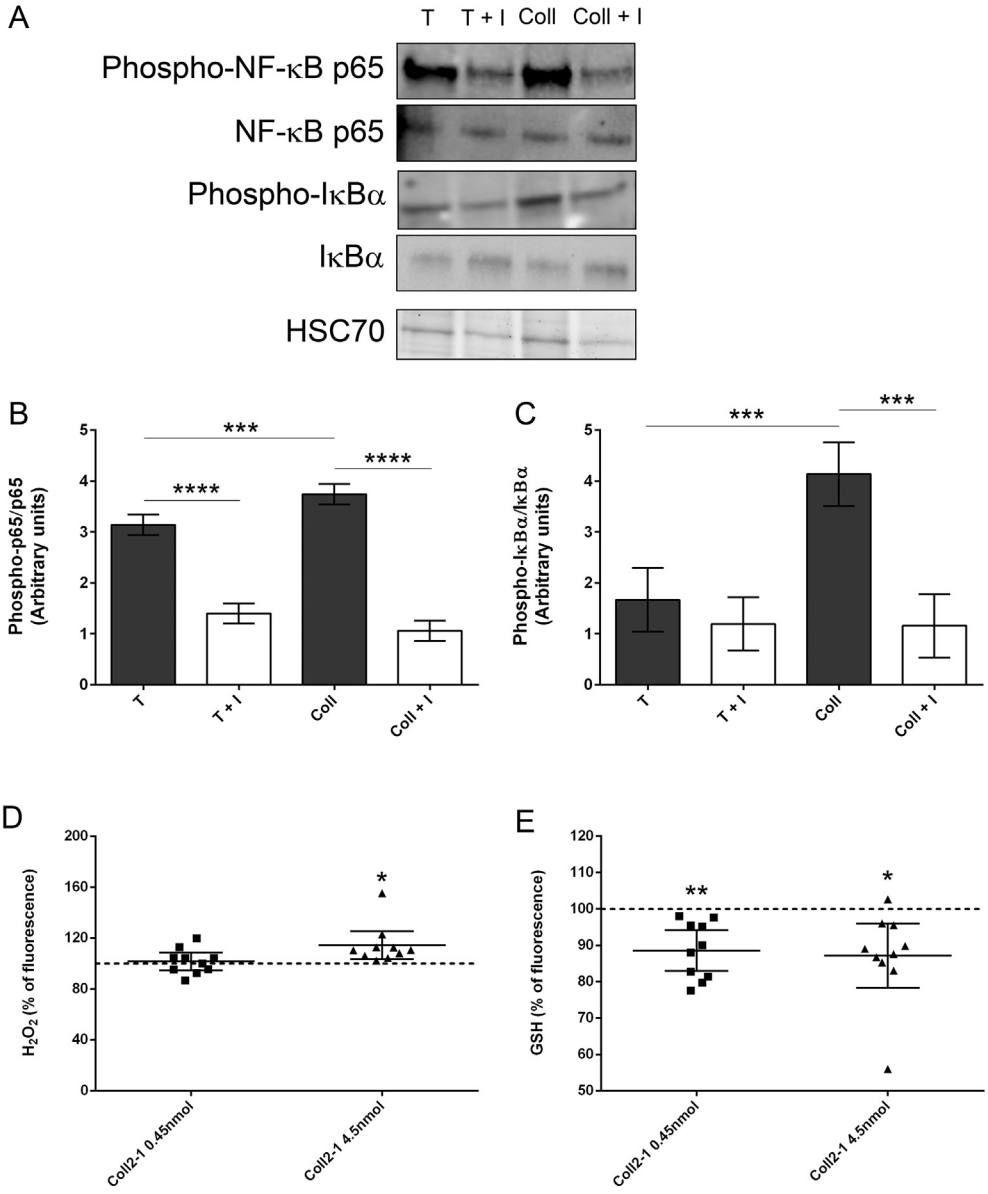

Fig. 2. Coll2-1 peptide activates the nuclear factor NF-kB. (A) Oxidative stress inhibitors effects on Coll2-1 induced phosphorylations of NF-κB p65 and IκBα subunits. Western blot analysis with Coll2-1 treated OA synovial fibroblast cells total protein extracts. Synovial fibroblast cells were pre-treated 1 h with oxidative stress inhibitors (I) before a 24 h treatment with Coll2-1 (4.5 nmol). The protein extracts were probed for phospho NF-κB p65, p65, phospho IκBα, IkBα and HSC70 (control) by Western blot analysis using specific antibodies. T: Control**,** Coll: Coll2-1 and I: apocynin; 0.2mM and diphenyleneiodonium; 6.35 10^{-2} mM; **(B–C)** The bar graphs were generated by quantifying blots from three independent experiments using ImageJ and normalizing the intensity of the bands to the loading control; **(D–E)** Effect of Coll2-1 peptide on H_2O_2 and GSH production in OA synovial fibroblast cells. Synovial fibroblast cells were treated without (Ctl) or with Coll2-1 peptide (0.45 and 4.5 nmol) during 24 h. Results are expressed as percent control (Ctl) and represented by mean with 95%CI ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

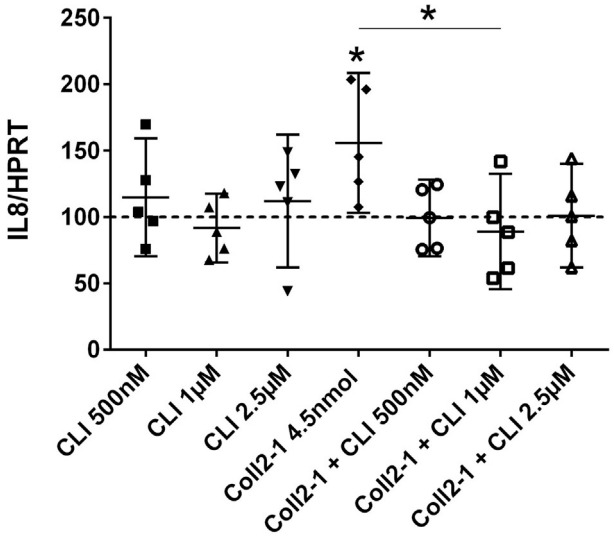

Fig. 3. Coll2-1 peptide effects are mediated through TLR-4 receptor. Synovial fibroblast cells were pre-treated 1 h with CLI-095 (500 nM, 1 and 2.5µM) before a 24 h treatment with Coll2-1 peptide at 4.5 nmol. Total RNA was isolated in cellular extract and IL-8 mRNA expression was analysed by Real Time PCR Technology. Results are expressed as percent control (Ctl) and represented by mean with 95%CI(n = 5). *P < 0.05.

concentrations of 500 nM, 1µM (Coll2-1 4.5 nmol vs Coll2-1 4.5 nmol + CLI-095 1 µM: −4.17 ± 0.92; 95%CI: −5.33 to −3.03) and 2.5µM indicating that this Coll2-1 effect is mediated by binding to TLR-4 (Fig. 3).

Effects of Coll2-1 on human chondrocytes in monolayer culture

In primary human chondrocytes culture, Coll2-1 significantly increased MMP-3 and VEGF gene expression (P = 0.0313 and P = 0.0313, respectively; Fig. 4(A)–(C)), tended to increase ADAMTS-5 gene expression [Fig. 4(D)]. IL-8 gene expression was not detectable (data not shown). Interestingly, MMP-3 expression was reduced by CLI-095 indicating that this one is also mediated by binding to TLR-4 in human chondrocytes [Fig. 4(B)].

Effects of Coll2-1 injected in lewis rats

None of the involved animals reached the ethical endpoints determined in the study. Native bovine Type II collagen and Coll2-1 injected SC significantly increased the global arthritic histological score compared to saline solution (CIA vs Saline: 9.24 ± 0.72; 95%CI: 7.34 to 11.15; Coll2-1,200 vs Saline: 7.48 ± 0.72; 95%CI: 5.58 to 9.39; Coll2-1 20 vs Saline: 8.48 ± 0.72; 95%CI: 6.58 to 10.39) [Fig. 5(A)]. The severity of arthritic histological lesion was similar in both groups while no arthritis was observed in saline group (Fig. 6(A)-B-C-D-E-F). The analysis of the sub-score showed that both native

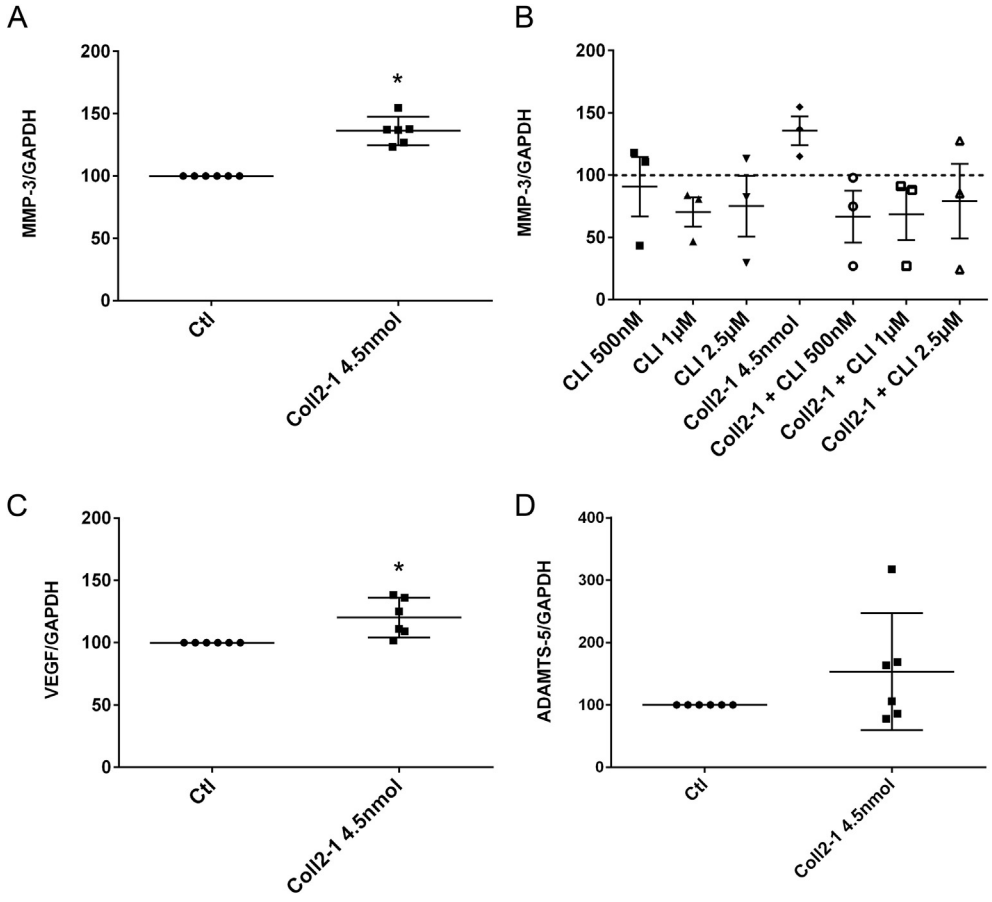

Fig. 4. Effect of Coll2-1 peptide on human chondrocytes in monolayer culture. Chondrocytes were treated without (Ctl) or with Coll2-1 peptide (4.5 nmol) during 24 h. Total RNA was isolated in cellular extract and MMP-3 **(A–B)**, VEGF (**C**) and ADAMTS-5 **(D)** mRNA expression was analysed by Real Time PCR. Results are expressed as percent control (Ctl) and represented by mean with 95%CI (n = six except for Fig. 4(B), (n) = 3). *P < 0.05.

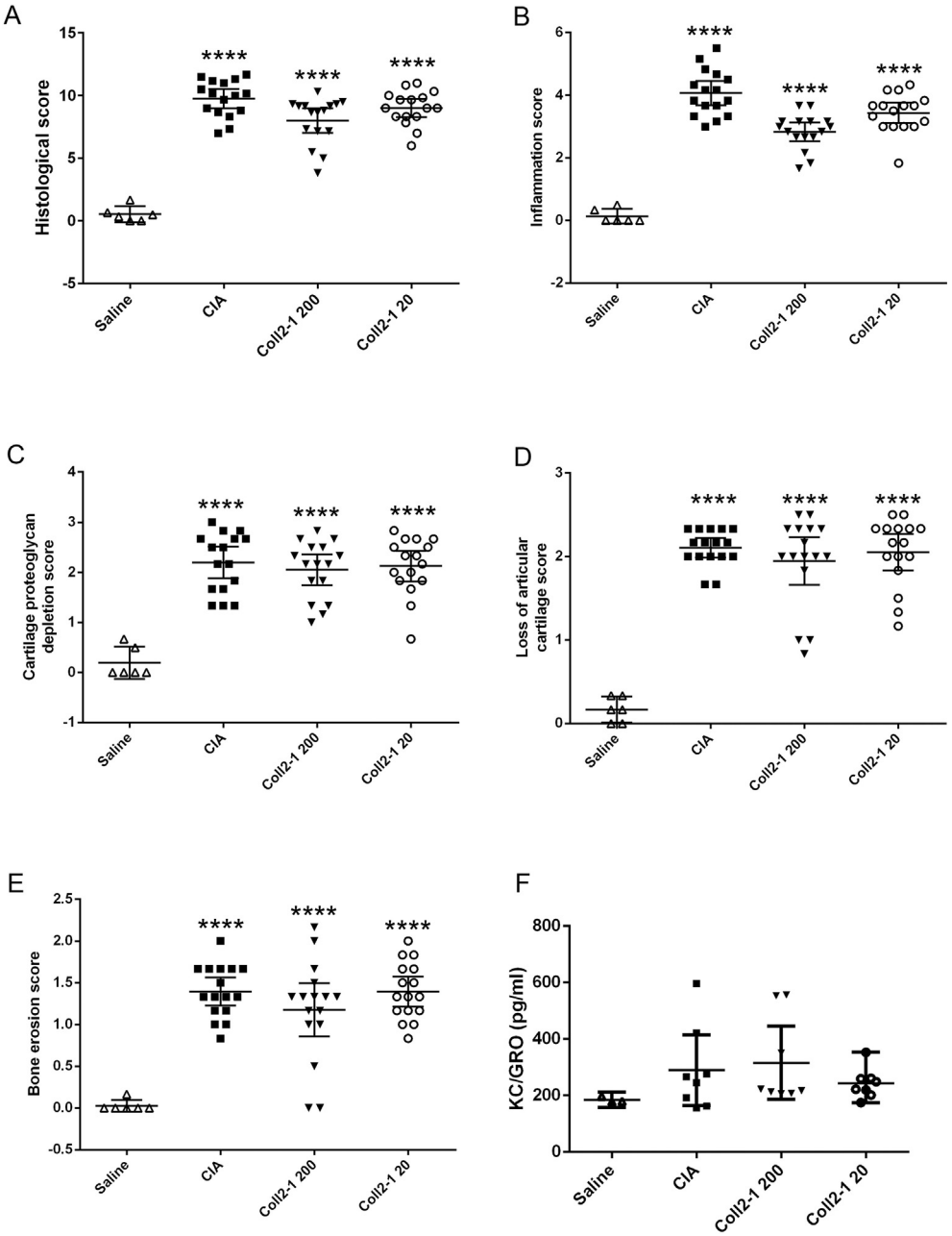

Fig. 5. Arthritis induced by subcutaneous injection of bovine Type II collagen. **(A)** Sum of the global histological score of the right and left knees after SC injection. **(B–E)** Detailled criteria of histological score after SC injection. **(B)** Inflammation (0–6); **(C)** Cartilage matrix proteoglycan loss (0–3); **(D)** Cartilage degradation (0–3); **(E)** Subchondral bone modification (0–3) ($n = 8$ animals/group except naïve controls $n = 3$) and **(F)** Serum KC/GRO levels in Lewis rats after SC injection. Results are represented by mean with 95%CI ($n = 8$/group except naïve control $n = 3$). ****$P < 0.0001$.

bovine type II collagen and Coll2-1 (20 and 200 μg) increased inflammation (CIA vs Saline: 3.93 ± 0.29; 95%CI: 3.16 to 4.71; Coll2-1,200 vs Saline: 2.69 ± 0.29; 95%CI: 1.92 to 3.47 and Coll2-1 20 vs Saline: 3.30 ± 0.29; 95%CI: 2.52 to 4.08; Figs. 5(B) and 6(H)-(J)-(L)), subchondral bone erosion evaluated by the ratio calcified tissue/presence of lacunae (CIA vs Saline: 1.37 ± 0.20; 95%CI: 0.84 to 1.90; Coll2-1,200 vs Saline: 1.15 ± 0.20; 95%CI: 0.62 to 1.68; Coll2-1 20 vs Saline: 1.37 ± 0.20; 95%CI: 0.84 to 1.90; Fig 5(E) and Fig 6(B)-D-F), cartilage degradation visible as a reduction of the thickness (CIA vs Saline: 1.94 ± 0.19; 95%CI: 1.44 to 2.43; Coll2-1,200 vs Saline: 1.78 ± 0.19; 95%CI: 1.28 to 2.28; Coll2-1 20 vs Saline: 1.89 ± 0.19; 95%CI: 1.39 to 2.39; Fig. 5(D)) and reduced cartilage proteoglycans content (CIA vs Saline: 2 ± 0.27; 95%CI: 1.29 to 2.71; Coll2-1,200 vs Saline: 1.86 ± 0.27; 95%CI: 1.15 to 2.57; Coll2-1 20 vs Saline: 1.93 ± 0.27; 95%CI: 1.22 to 2.64; Fig 5(C) and Fig 6(B)-D-F) compared to saline solution.

Both intra-articular injection of SCW and Coll2-1 increased the global arthritic histological score (SCW vs Saline: 10.76 ± 0.80; 95% CI: 8.53 to 12.18; Coll2-1 5 vs Saline: 8.93 ± 0.80; 95%CI: 6.71 to 11.16; Coll2-1 0.5 vs Saline: 8.92 ± 0.80; 95%CI: 6.70 to 11.15; Fig. 7(A)) while saline solution was without effect. No difference between SCW and Coll2-1 was observed on this parameter. When histological sub-scores were analyzed separately, Coll2-1 peptide (0.5 and 5 μg) induced an inflammatory reaction (SCW vs Saline: 5.22 ± 0.42; 95%CI: 4.06 to 6.38; Coll2-1 5 vs Saline: 3.40 ± 0.42; 95%CI: 2.243 to 4.563 and Coll2-1 0.5 vs Saline: 3.07 ± 0.42; 95%CI:

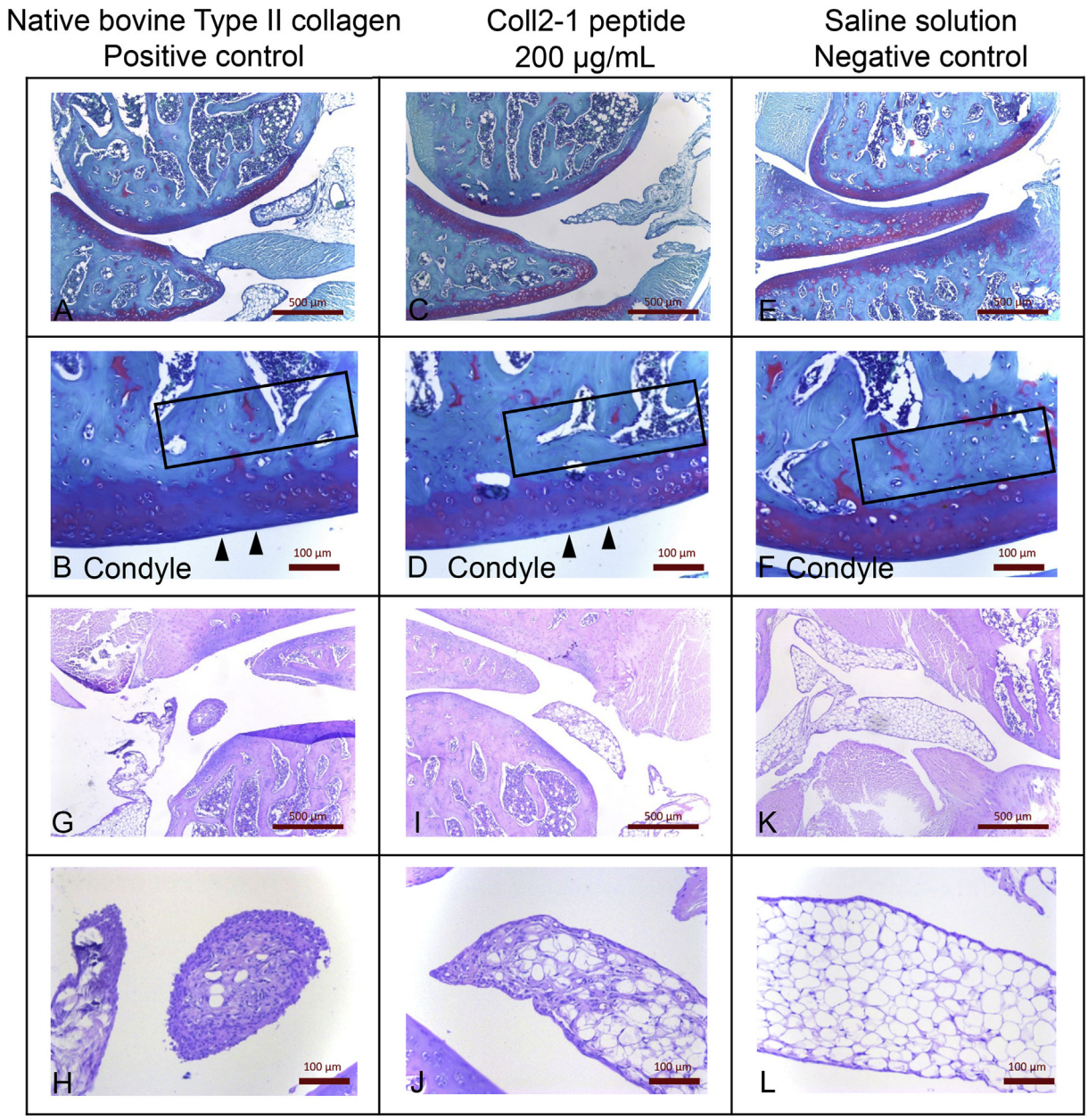

Fig. 6. Arthritis induced by subcutaneous injection of bovine Type II collagen. Representative histological sections of rat joint (Condyle) under three conditions: Native bovine Type II collagen (Positive control), Coll2-1 peptide (200 µg/mL) and Saline solution (Negative control). Cartilage with Safranin-O/Fast green staining **(A–F)** and synovial membrane with H&E **(G–L)**. 5× magnification **(A, C and E; G, I and K)**. 20× magnification **(B, D and F; H, J and L)**. Cartilage proteoglycan depletion was indicated by **arrows**. Loss of cartilage is defined as a decrease of **cartilage thickness**. A tissue example considered for the bone erosion score was highlighted by a **rectangle**.

1.909 to 4.230; Figs. 7(B) and 8(H)-(J)-(L)), the loss of proteoglycans (SCW vs Saline: 2.03 ± 0.27; 95%CI: 1.28 to 2.77; Coll2-1 5 vs Saline: 2.21 ± 0.27; 95%CI: 1.46 to 2.96; Coll2-1 0.5 vs Saline: 2.19 ± 0.27; 95%CI: 1.45 to 2.94; Figs. 7(C) and 8(B)-(D)-(F)), the loss of articular cartilage visible as a reduction of thickness (SCW vs Saline: 2.03 ± 0.25; 95%CI: 1.34 to 2.72; Coll2-1 5 vs Saline: 2.04 ± 0.25; 95% CI: 1.35 to 2.73; Coll2-1 0.5 vs Saline: 2.26 ± 0.25; 95%CI: 1.57 to 2.95; Figs. 7(D) and 8(C)) and modifications of the subchondral bone characterized by the ratio calcified tissue/presence of lacunae (SCW vs Saline: 1.44 ± 0.25; 95%CI: 0.75 to 2.14; Coll2-1 5 vs Saline: 1.29 ± 0.25; 95%CI: 0.60 to 1.98; Coll2-1 0.5 vs Saline: 1.40 ± 0.25; 95%CI: 0.71 to 2.09; Fig 7(E) and Fig 8(B)-D-F) comparable to SCW while saline solution did not significantly affected these parameters. No difference between SCW and Coll2-1 was observed.

Consistent with the increase of IL-8 observed *in vitro* in synoviocytes, the serum KC/GRO production was also increased whatever the model. Native bovine type II collagen and Coll2-1 SC injected, increased KC/GRO production in Lewis rat sera compared to saline solution [Fig. 5(F)]. Both IA of SCW and Coll2-1 also tends to increase the KC/GRO levels while saline solution was without effect [Fig. 7(F)].

Discussion

This *in vitro* study demonstrated that Coll2-1, a peptide located in the triple helical part of the type II collagen molecule and currently used as biomarker of cartilage degradation[20,27,21] stimulated IL-8 production by synoviocytes while IL-6, MMP-2 or

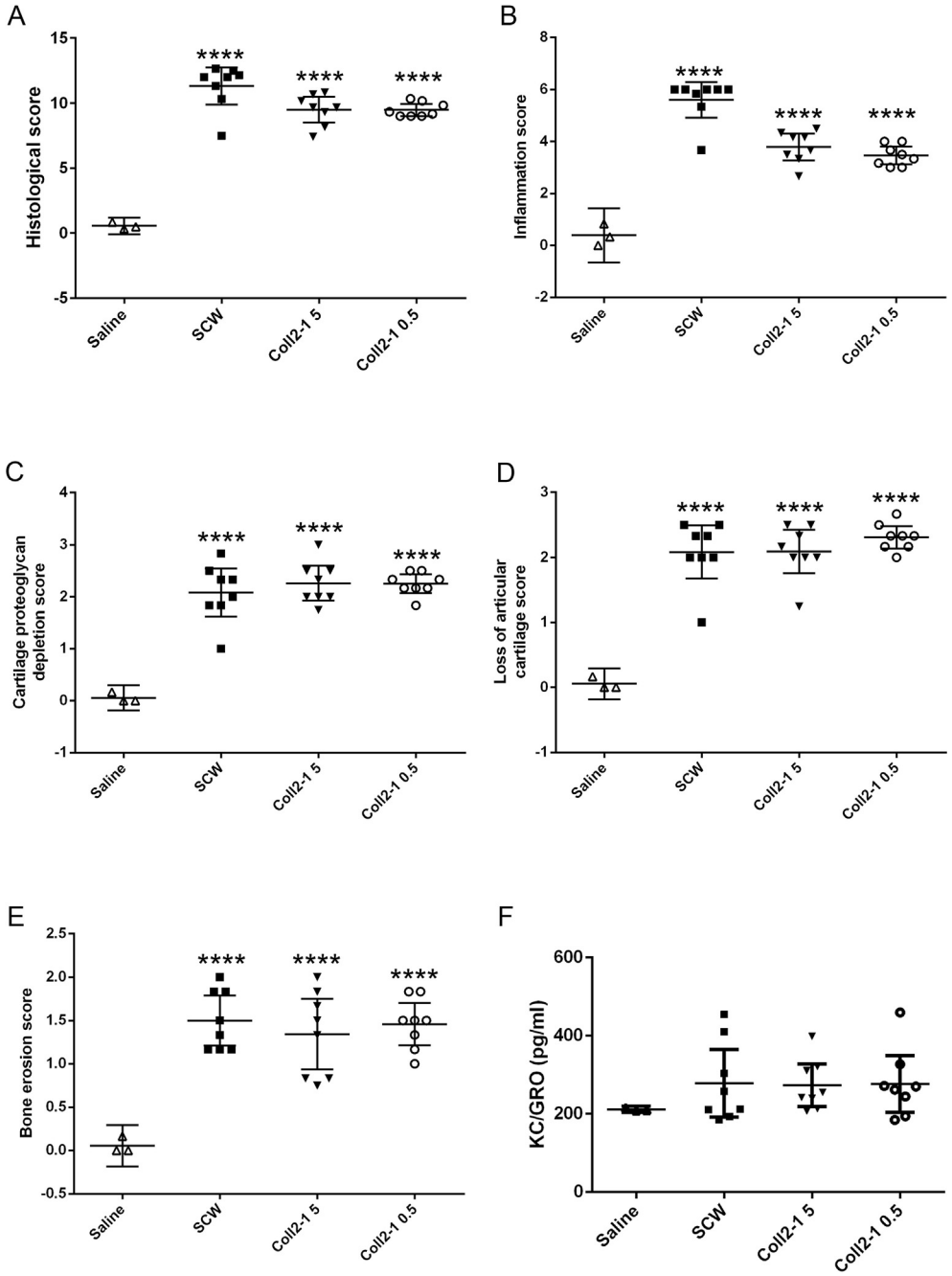

Fig. 7. Arthritis induced by intra-articular injection of Streptococcal cell wall. (**A**) Sum of the global histological score of the right knee after IA injection. **(B–E)** Detailled criteria of histological score after SC injection. **(B)** Inflammation (0–6); **(C)** Cartilage matrix proteoglycan loss (0–3); **(D)** Cartilage degradation (0–3); **(E)** Subchondral bone modification (0–3) ($n = 8$ animals/group except naïve controls $n = 3$) and **(F)** Serum KC/GRO levels in Lewis rats after IA injection. Results are represented by mean with 95%CI ($n = 8$/group except naïve control $n = 3$). ****$P < 0.0001$.

ADAMTS-5 productions were not altered. Moreover, in human chondrocyte culture, Coll2-1 significantly increased MMP-3 and VEGF gene expression. This is the first study describing the significant effects of a type II collagen peptide used at concentrations commonly found in OA patients serum. This supports the extrapolation of the *in vitro* conclusions to *in vivo* situation. The most remarkable effect of Coll2-1 was shown on IL-8 production which was found to be elevated in both serum and synovial fluid of OA patients[28]. This chemokine activates neutrophils at inflammatory site inducing chemotaxis, exocytosis and respiratory burst. This pro-inflammatory effect of type II collagen peptide has been previously suggested through a series of *in vitro* studies performed on chondrocytes[12–16]. In our culture condition, Coll2-1 stimulated MMP-3 production, confirming previous study, but failed to stimulate IL-8 production.

Importantly, our results demonstrated that Coll2-1 effects are mediated through TRL-4, both in synoviocytes and chondrocytes and NF-κB signaling pathway activation. TLR-4, one the most highly expressed TLRs in OA synovial fibroblasts, is involved in OA physiopathology mainly through the activation of MyD88-dependent pathway which promotes innate immune responses including the induction of inflammatory mediators (IL-1β, MMPs, NO, or PGE_2)[29].

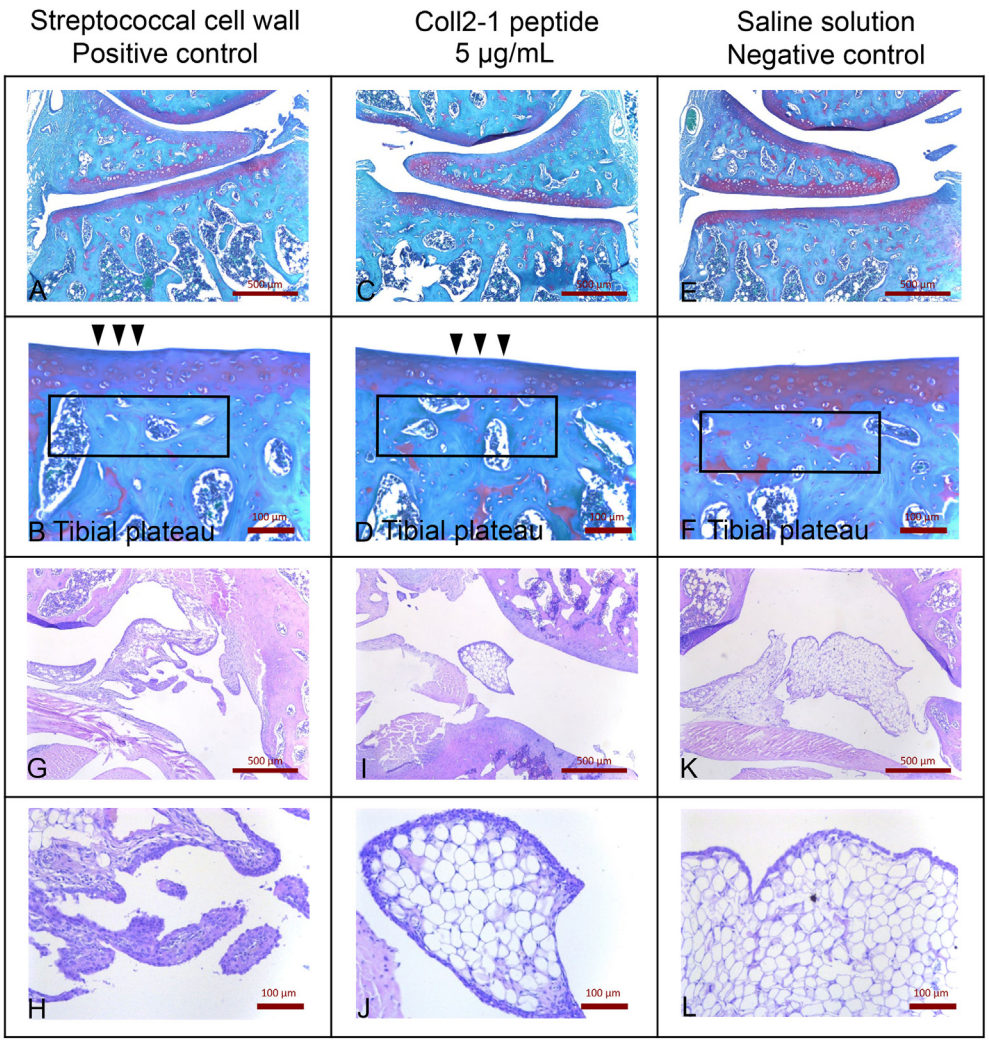

Fig. 8. Arthritis induced by intra-articular injection of Streptococcal cell wall. Representative histological sections of rat joint (Tibial plateau) under three conditions: Streptococcal cell wall (Positive control), Coll2-1 peptide (5 µg/mL) and Saline solution (Negative control). Cartilage with Safranin-O/Fast green staining **(A–F)** and synovial membrane with H&E **(G–L)**. 5× magnification **(A, C and E; G, I and K)**. 20× magnification **(B, D and F; H, J and L)**. Cartilage proteoglycan depletion was indicated by **arrows**. Loss of cartilage is defined as a decrease of **cartilage thickness**. A tissue example considered for the bone erosion score was highlighted by a **rectangle**.

In addition to synoviocytes, TLR-4 is also expressed by numerous joint cells types including chondrocytes, osteoblasts and immune cells (macrophages, monocytes or dendritic cells). Furthermore, its expression in joint tissues is increased with increasing severity of OA and with aging[30]. Finally, TLR-4 binds a number of different agonists, some of which (such as fibronectin or hyaluronan) are released when tissues are damaged.

We established that Coll2-1-exposed synovial cells produced H_2O_2, promoting a state of cellular oxidation. Since we have observed that both apocynin and diphenyleneiodonium decreased phosphorylation of p65 and IkBα, we suggest that e NADPH oxidase system at least is involved in ROS production. ROS are able to stimulate expression of NF-κB-dependent pro-inflammatory cytokine and to promote the formation of an amplification loop that feeds back to further elevation of additional ROS, suggesting that activation of NF-κB may be regulated through an oxidant/antioxidant balance[31]. On the other hand, generated ROS serve as a stimulus for NF-κB activation, probably through effects on upstream kinases[32]. This includes effects either on NF-κB dissociation from its inhibitor IκB, requiring oxidation, regulation of IκB degradation and NF–κB binding to DNA[33]. The contribution of this nuclear factor has been demonstrated to be involved in OA onset and development[34,35]. NF-κB is crucial in the signalling pathway activated by TLRs. resulting in the release of various factors involved in OA pathogenesis such as catabolic factors, cytokines, chemokines and complement initiating a repetitive cascade leading to chronic inflammation and cartilage degradation.

We have also demonstrated that effects induced by Coll2-1 were comparable to those induced by native Type II collagen injected subcutaneously (CIA) or SCW injected into the joint. These models are considered as reference models for the study of arthritis[9,36,37]. Based on inflammation score, Coll2-1 induced infiltration of inflammatory cells in joint, indicating that Coll2-1 may initiate innate immunity. Further, Coll2-1 peptide induced cartilage and bone lesions indicating that *in vivo* Coll2-1 activates joint cells to generate inflammatory and catabolic conditions. Interestingly, in Lewis rat serum, Coll2-1 stimulated the KC/GRO production, a CXC chemokine known as CXCL1 but also as IL-8 related protein in rodents. These data are consistent with the pro-inflammatory effect of type II collagen peptide demonstrated in synoviocytes. This opens perspective of biotherapy development to treat OA.

Of course, our study suffers of some limitations. Proof-of-concept regarding the ability of an antibody or other binding agents to neutralize peptide Coll2-1 still has to be performed.

Nevertheless, the present study gives important information both on the model feasibility and efficacy end-point.

In conclusion, Coll2-1, an end-product of type II collagen degradation known to be a biomarker of cartilage degradation, activates synoviocytes to produce IL-8, chondrocytes to produce MMP-3, and induces arthritis in rat. These findings reinforce the interest of Coll2-1 as a biomarker because it seems to be involved in OA physiopathology. Neutralize Coll2-1 may represent an attractive target for the management of OA progression. The main differentiation of this approach may be the blocking of factors triggering the inflammation rather than the inflammation itself, which represents an innovative way among current therapeutic approaches.

Contributions

Lambert, Cécile: Conception and design of the study, acquisition data, analysis and interpretation of data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, obtaining of funding.

Borderie, Didier: Conception and design of the study, acquisition data, analysis and interpretation of data, critical revision of the article for important intellectual content, final approval of the article, obtaining of funding.

Dubuc, Jean-Emile: Conception and design of the study, acquisition data, critical revision of the article for important intellectual content, final approval of the article.

Rannou, François: Conception and design of the study, analysis and interpretation of data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article.

Henrotin, Yves: Conception and design of the study, analysis and interpretation of data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article.

Lambert, Cécile and Borderie, Didier take responsibility for the integrity of the data analysis.

Competing interest statement

None of the authors have any conflicts of interests in relation to this work.

Role of the funding source

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