

Lactoferrin from *Camelus dromedarius* Inhibits Nuclear Transcription Factor-kappa B Activation, Cyclooxygenase-2 Expression and Prostaglandin E2 Production in Stimulated Human Chondrocytes

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ABSTRACT

Background: Osteoarthritis (OA) is a progressive joint disorder, which remains the leading cause of chronic disability in aged people. Nuclear factor-kappa B (NF-κB) is a major cellular event in OA and its activation by interleukin-1β (IL-1β) plays a critical role in cartilage breakdown in these patients. **Objective:** In this study, we examined the effect of lactoferrin on NF-κB activation, cyclooxygenase-2 (COX-2) expression and prostaglandin E2 (PGE₂) production in stimulated human articular chondrocytes. **Materials and Methods:** Human chondrocytes were derived from OA articular cartilage and treated with camel lactoferrin and then stimulated with IL-1β. Gene expression was determined by TaqMan assays and protein expression was studied by Western immunoblotting. NF-κB activity and PGE₂ levels were determined by ELISA based assays. NF-κB activity was also determined by treatment of chondrocytes with NF-κB specific inhibitor Bay 11-7082. **Results:** Lactoferrin inhibited IL-1β-induced activation and nuclear translocation of NF-κB p65 in human OA chondrocytes. Lactoferrin also inhibited mRNA/protein expression of COX-2 and production of PGE₂. Moreover, Bay 11-7082 also inhibited IL-1β-induced expression of COX-2 and production of PGE₂. The inhibitory effect of lactoferrin on the IL-1β induced expression of COX-2 or production of PGE₂ was mediated at least in part via suppression of NF-κB activation. **Conclusions:** Our data determine camel lactoferrin as a novel inhibitor of IL-1β-induced activation of NF-κB signaling events and production of cartilage-degrading molecule PGE₂ via inhibition of COX-2 expressions. These results may have important implications for the development of novel therapeutic strategies

for the prevention/treatment of OA and other degenerative/inflammatory diseases.

Key words: Chondrocytes, cyclooxygenase-2, interleukin-1β, lactoferrin, nuclear factor-kappa B, osteoarthritis, prostaglandin E2

SUMMARY

- Lactoferrin shows anti-arthritis activity in IL-1β stimulated primary human chondrocytes.
- Lactoferrin inhibits IL-1β-induced NF-κB activation.
- Lactoferrin inhibits production of cartilage degrading PGE2 via inhibition of COX-2 expression.

Abbreviations Used: OA: Osteoarthritis; IL-1β: Interleukin-1 beta; NF-κB: Nuclear factor-kappa B; COX-2: cyclooxygenase-2; PGE₂: prostaglandin E₂.

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INTRODUCTION

Osteoarthritis (OA), the most common forms of arthritis, which afflicts millions of individuals across the world resulting in impaired quality of life and increased health costs.^[1] Many risk factors that contribute to disease onset have been identified including both systemic and biomechanical factors, but the direct etiology of this progressive joint disease remains to be identified.^[1,2] There are currently no disease-modifying treatments, and the only effective treatment is a surgical joint replacement.^[3] Chondrocytes are the only cellular components of cartilage. Under normal physiologic conditions, chondrocytes maintain equilibrium between anabolic and catabolic activities that is necessary for the preservation of the structural and functional integrity of the tissue. Chondrocytes express inflammatory mediators such as interleukin (IL)-1β, cyclooxygenase-2 (COX-2), proteolytic enzymes, which under normal conditions mediate a very low matrix turnover required for cartilage remodeling.^[4] However, in OA, production of these inflammatory mediators including COX-2 or prostaglandin E2 (PGE₂) becomes significantly higher than the normal range, resulting in cartilage destruction and pain.^[5,6] In OA, evidence show that excessive production of PGE₂ activates/increases production of metalloproteinases and also proteoglycan, which further enhance the catabolic degradation of cartilage.^[6-8] IL-1 is a key inflammatory and catabolic cytokine in the pathophysiology of OA, represents one

of the possible treatment targets.^[9,10] IL-1β induced overexpression of COX-2 is also well reported in OA, which strongly contributes to the inflammation and cartilage degradation.^[10,11] Now it is well documented that selective inhibition of PGE₂ production via inhibition of COX-2 activity is the most appropriate therapeutic target for anti-inflammatory or anti-arthritis therapy,^[12] therefore the development of selective COX-2 inhibitors is now on the rise and inhibition of COX-2 activity is assumed to be a potential therapeutic target for OA therapy. Another most important therapeutic target of OA is the nuclear transcription factor-kappa B (NF-κB), which is intimately involved in the regulation

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of numerous genes (including COX-2) in the setting of the inflammatory response.^[13] Since inflammatory processes play a fundamental role in the damage of articular tissues, many *in vitro* and *in vivo* studies have examined the contribution of components of the NF- κ B signaling pathways to the pathogenesis of OA.^[13,14] Inflammation, cartilage degradation, cell proliferation, angiogenesis and pannus formation are processes in which the role of NF- κ B is prominent.^[13-15] Therefore, targeting or pharmacologic modulation of NF- κ B pathways also seems to be an effective therapy for OA.

Free iron in the joints is toxic and has proinflammatory effects, many studies show that iron accumulation in the inflamed synovium is well correlated with arthritic activity.^[16,17] Moreover, iron chelators reduce inflammation in patients and animal models.^[18,19] These findings clearly indicate that elimination of toxic iron from the joints has a clinical use for arthritis patients. A candidate iron-binding protein is a lactoferrin, which is released by degranulating neutrophils at sites of inflammation and is increasingly being recognized as a potential anti-inflammatory agent by virtue of its ability to bind iron when transferrin cannot do so.^[17,20-23] Lactoferrin is abundant in milk and in most biological fluids, its protective effects range from anti-inflammatory to anti-cancer activities that have been recently reviewed by García-Montoya *et al.*^[24] This wide range of lactoferrin activity is made possible by mechanisms of action involving not only the capacity of lactoferrin to bind iron but also interactions of lactoferrin with molecular and cellular components of both hosts and pathogens.^[24] Recently, lactoferrin from milk of various sources has also been found to inhibit inflammatory signals in various cell types.^[22-25] However, its role on chondrocytes has never been investigated. In the present study, we addressed the question for the first time of a possible inhibitory effect of lactoferrin from *Camelus dromedarius* milk on IL-1 β -induced expression of COX-2 and production of PGE₂ in human OA chondrocytes. Our results showed that lactoferrin suppressed the IL-1 β -induced COX-2 expression and PGE₂ production in primary human OA chondrocytes, and these effects were concomitant with inhibited activation of the transcription factor NF- κ B. Our results thus identify a unique mechanism of action of a dietary constituent and suggest that use of lactoferrin or compounds derived from it may have cartilage sparing effect in arthritis.

MATERIALS AND METHODS

Specimen selection and articular chondrocytes preparation

The present study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for human samples. Informed consent was obtained for all investigations on human subjects. With Institutional Review Board approval, discarded cartilage samples were obtained from the knee joints of OA patients who underwent joint replacement surgery. The macroscopic cartilage degeneration was determined by staining of femoral head samples with India ink and the cartilage with the smooth articular surface ("unaffected cartilage") was resected and used to prepare chondrocytes by enzymatic digestion as we described previously.^[26,27] Histological analysis of some of the "unaffected cartilage" samples was performed on 5 μ m thick sections stained with haematoxylin and eosin and Safranin O and grade using Mankin score.^[28] Grading of the histology slides (not shown) revealed that all of the cartilage pieces taken from the unaffected area had a Mankin score of <2 for structure and Mankin score of 1 for cellularity. Isolated chondrocytes were plated at a density of 1.2×10^6 /ml in 6 well tissue culture dishes (Millipore, Darmstadt, Germany) in complete Dulbecco's Modified Eagle's medium (DMEM) as we previously described.^[29-31]

Isolation of lactoferrin

Lactoferrin was isolated from *C. dromedarius* (camel) milk as described previously.^[32] Briefly, milk was skimmed by centrifugation at $2500 \times g$ at 4°C for 30 min. Skim milk was then diluted 1:1 with the phosphate-buffered saline-T (0.04 M NaH₂PO₄, 0.8 M NaCl, 0.04% [v/v] Tween 20, pH 7.4) and it was incubated with cation exchanger SP-Sepharose at 4°C overnight. Afterward, the SP-Sepharose was washed with the washing buffer (0.02 M NaH₂PO₄, 0.4 M NaCl, 0.02% [v/v] Tween 20, pH 7.4) to elute the unbound proteins. The gel then packed into a column and lactoferrin was eluted with the elution buffer (0.02 M NaH₂PO₄, 1 M NaCl, pH 7.4). The peak fractions were a pool and filtered by Acrodisc PF filter (catalog # 4658, Pall Corporation, Cornwall, UK). The filtered fractions were then electrophoresed on 10% sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) for further confirmation and were used in cell treatment.

Treatment of primary human osteoarthritis chondrocytes with interleukin-1 β and lactoferrin

We first determined whether IL-1 β and lactoferrin affect the viability of human OA chondrocytes *in vitro*. Human OA chondrocytes (1.2×10^6 /well) were plated in 6 well culture dishes (catalog # PIMWS0650, Millipore, Darmstadt, Germany) in complete DMEM medium (catalog # SLM-120-B, Millipore) and serum-starved for 12 h/overnight. Chondrocytes were treated with various doses of IL-1 β (5–10 ng/ml) and lactoferrin (25–150 μ g/ml) and after 24 h incubation cytotoxicity of IL-1 β and lactoferrin was examined by using CellTiter-Glo Luminescent Cell Viability Assay (catalog # G7573, Promega, WI, USA). Primary chondrocytes were pretreated with different doses of lactoferrin for 2 h prior to stimulation with IL-1 β as we described previously.^[29-31,33] Chondrocytes cultured without IL-1 β or lactoferrin served as controls. All experiments were performed within 4–6 days of the primary culture to avoid dedifferentiation of OA chondrocytes.

Quantitative real-time-polymerase chain reaction

Real-time quantitative polymerase chain reaction (qRT-PCR) was used to quantify the expression of mRNA for COX-2, type 2 collagen (COL2A1), COL10A1, aggrecan (ACAN), and SRY-box containing gene 9 (SOX-9) with expression of endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as we described previously.^[26,31,34] Total RNA was separated from OA chondrocytes by total RNA isolation kit (catalog # AM1560, Ambion, CA, USA). First-strand cDNA was synthesized using 1 μ g total RNA and the SuperScript First-Strand cDNA synthesis kit (catalog # 75780, Affymetrix Inc., OH, USA). Primers used for PCR-assisted amplification are listed in Table 1. PCR amplification was carried out using the core kit for SYBR Green or Taqman reagent (Applied Biosystem, Foster City, CA) and the step one RT-PCR System (Applied Biosystems). Typical profile times used were initial step, 95°C for 10 min, followed by a second step at 95°C for 15 s and 60°C for 60 s for 40 cycles with melting curve analysis. The level of target mRNA was normalized to the level of GAPDH and compared to control (untreated sample). Data were analyzed using $\Delta\Delta$ CT method.^[35]

Western immunoblotting

Expression of proteins in treated or untreated human primary OA chondrocytes were determined by Western blotting as we described previously.^[36] Total cell lysates were prepared using the Pierce RIPA buffer (catalog # 89901, Thermo Scientific, LI, USA). Total cell lysates (35 μ g/lane) were resolved by SDS-PAGE (10% resolving gel with 4% stacking) and transferred to PVDF immobilon-P transfer membranes (catalog # IPVH00010, Millipore Corporation, Bedford, MA). Membranes

were blocked with non-fat dry milk powder in tris-buffered saline and 0.1% Tween-20. Blots were probed with 1:200–1:1000 diluted primary antibodies specific for the target protein. Immunoreactive proteins were visualized by using 1:1000 diluted horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence (GE Healthcare, Milwaukee, WI). Images were captured using G: Box Imaging System (Syngene, MD, USA).

Prostaglandin E2 ELISA

PGE₂ production in the culture medium of treated or untreated human OA chondrocytes was quantified using commercially available ELISA kit (catalog # 514010, Cayman Chemical Company, MI, USA) according to the instructions of the manufacturer. The plate was read using an automatic microplate reader (Anthos Zenyth 3100 Multimode Detectors, Salzburg, Austria).

Nuclear factor-kappa B activity assays

Inhibition of NF-κB p65 activity by lactoferrin in IL-1β stimulated human OA chondrocytes was determined in the nuclear extracts using a highly sensitive Transcription Factor ELISA Kit according to the instructions

of the manufacturer (catalog # ab133128, Abcam, MA, USA). The plate was read using an automatic microplate reader (Anthos Zenyth 3100 Multimode Detectors, Salzburg, Austria). The nuclear cell extract was prepared by our published procedure as we described previously.^[26,31,34]

Densitometric analysis

Measurements of the scanned bands were performed using UN-SCAN-IT software (Silk Scientific Corporation, Orem, UT). Each band was scanned 5 times, and the mean band intensity (pixels per band) was obtained. Data were normalized to suitable loading controls and expressed as mean ± standard deviation followed by appropriate statistical analysis.

Statistical analysis

All measurements were performed in duplicates and repeated at least 3 times using chondrocytes prepared from a different age- and sex-matched OA cartilage samples. Statistical comparisons were performed by one-way ANOVA analysis followed by Tukey's *post-hoc* analysis or two-way ANOVA followed by Bonferroni *post-hoc* tests using GraphPad Prism-5 (San Diego, CA, USA) and *P* < 0.05 was considered significant. Values shown are mean ± standard error of mean unless stated otherwise.

RESULTS

Preparation of lactoferrin

Lactoferrin was prepared from camel milk by cation exchange chromatography using SP-Sepharose column. The isolated lactoferrin was eluted in a single symmetrical peak [Figure 1a] and was shown a single band on SDS-PAGE [Figure 1b].

Human osteoarthritis chondrocytes in monolayer maintain their chondrogenic phenotype

We determined whether primary human OA chondrocytes used in these studies maintained their phenotype by analyzing the expression of COL2A1, ACAN, SOX-9 mRNA, which are considered to be the signature of the chondrogenic phenotype.^[26,31,37] Our results show that primary human OA chondrocytes in monolayer culture maintained their phenotype, when they were plated (1.2×10^6 /ml) in complete DMEM medium with 10% fetal

Table 1: Details of primers used in microRNA expression studies

Gene name	Accession number	Forward primer	Reverse primer
COX-2	NM_000963	5'-CAA ATC CTT GCT GTT CCC ACC CAT-3'	5'-GTG CAC TGT GTT TGG AGT GGG TTT-3'
COL2A1	NM_001844	5'-ACG TGA AAG ACT GCC TCA GC-3'	5'-TTT CAT CAA ATC CTC CAG CC-3'
COL10A1	NM_000493	5'-TGA TCC TGG AGT TGG AGG AC-3'	5'-GAG ATC GAT GAT GGC ACT CC-3'
ACAN	NM_013227	5'-GAC CTG CAA GGA GAC AGA GG-3'	5'-CCA CTG GTA GTC CTT GGG CAT-3'
SOX-9	NM_011448	5'-GAT TTT TCA CGC AGC CCT AA-3'	5'-ATA CAG TCC AGG CAG ACC CA-3'
GAPDH	NM_002046	5'-TCG ACA GTC AGC CGC ATC TTC TTT-3'	5'-ACC AAA TCC GTT GAC TCC GAC CTT-3'

COX-2: Cyclooxygenase-2; COL2A1: Type 2 collagen; COL10A1: Type 10 collagen; ACAN: Aggrecan; SOX-9: SRY-box containing gene 9; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

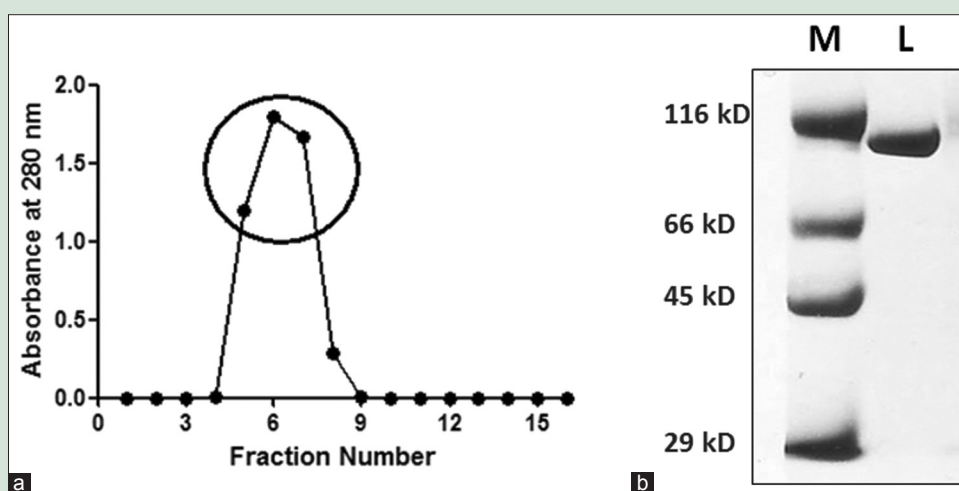


Figure 1: Preparation of lactoferrin from camel milk. (a) Elution profile of lactoferrin from camel milk on cation exchange SP-Sepharose column. The flow rate was 5 ml/min and the volume of the fractions was 3 ml. (b) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of pool of peak fractions (shown in circle in Figure 1a). M stands for molecular weight markers. L stands for isolated lactoferrin

calf serum and allowed to grow at 37°C and 5% CO₂ in a tissue culture incubator, as judged by the continued expression of COL2A1, ACAN and SOX-9 mRNAs, whereas COL10A1 mRNA was not expressed (data not shown). Based on this data, chondrocytes were used within 3–6 days after plating to avoid dedifferentiation of OA chondrocytes.

Lactoferrin inhibits interleukin-1 β -induced expression of cyclooxygenase-2 mRNA and protein and the production of prostaglandin E₂ in primary human osteoarthritis chondrocytes

Primary OA chondrocytes (70–80% confluent) were pretreated with lactoferrin (50–75 μ g/ml) for 2 h, then stimulated with IL-1 β (10 ng/ml) for 24 h. No cytotoxic effect of lactoferrin was noted at the dose used (results not shown). The level of COX-2 mRNA was quantified by a highly sensitive and specific quantitative RT-PCR method, and values were compared with control. Our results showed that OA chondrocytes treated with IL-1 β had a higher level of COX-2 mRNA compared with

unstimulated OA chondrocytes. However, COX-2 mRNA levels showed a marked decline in the samples pretreated with lactoferrin and then stimulated with IL-1 β [Figure 2a]. To determine whether inhibition of gene expression also affected protein level, total cell lysate were assayed for COX-2 protein using Western immunoblotting. As shown in Figure 2b, pretreatment with 50–75 μ g/ml of lactoferrin significantly decreased the IL-1 β -induced COX-2 protein expression of IL-1 β -stimulated primary human OA chondrocytes ($P < 0.01$). Not only these, but we also determined the culture medium of the same treated or untreated primary human OA chondrocytes for PGE₂ production. Our data showed that enhanced PGE₂ production by IL-1 β was significantly inhibited by lactoferrin in a dose-dependent manner [$P < 0.01$; Figure 2c].

Lactoferrin suppresses interleukin-1 β -induced activation and nuclear translocation of factor-kappa B in primary human osteoarthritis chondrocytes

NF- κ B is an important transcriptional regulator of inflammatory cytokines gene expression and plays a crucial role in the immune and

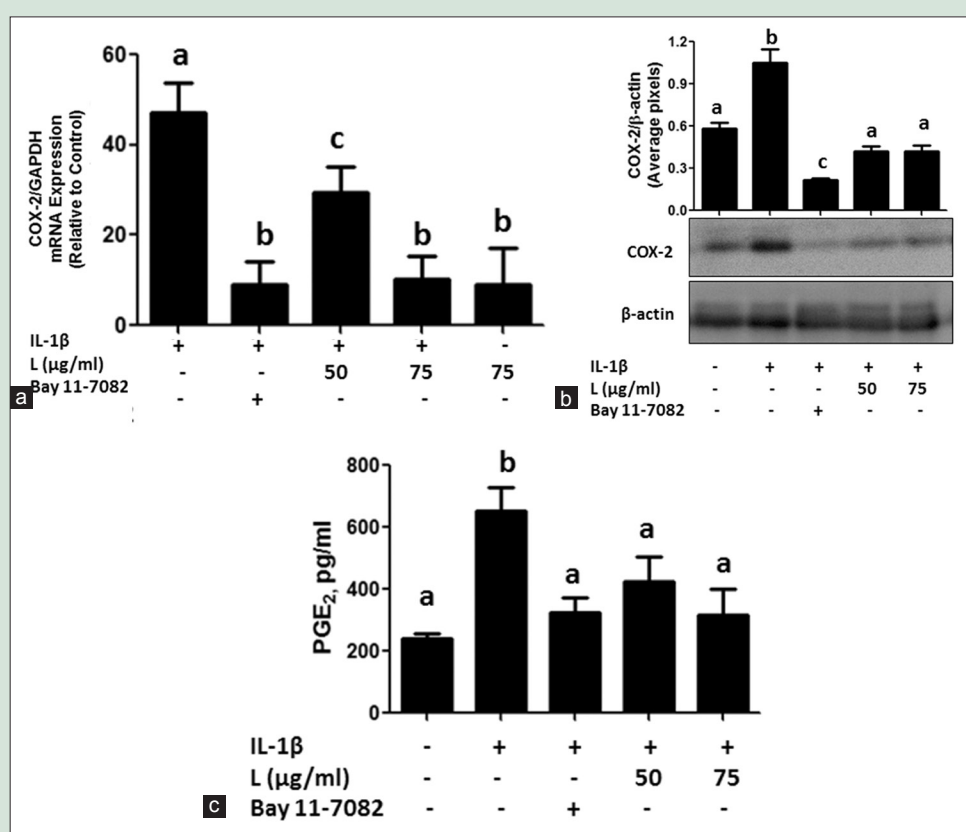


Figure 2: Lactoferrin inhibits cyclooxygenase-2 expression and prostaglandin E₂ production. (a) Effect of lactoferrin (L) and nuclear factor-kappa B inhibitor (Bay 11-7082) on the gene expression of cyclooxygenase-2 in interleukin-1 β -stimulated human osteoarthritis chondrocytes determined by quantitative real time-polymerase chain reaction. Primary human chondrocytes (70–80% confluent) were pretreated with L (50–75 μ g/ml) or Bay 11-7082 (10 μ M) for 2 h and stimulated with interleukin-1 β (10 ng/ml) for 24 h. Expression of cyclooxygenase-2 mRNA was normalized to glyceraldehyde 3-phosphate dehydrogenase and compared to the levels present in control. Results are representative (mean $\pm$ standard error of mean) of duplicate experiments with chondrocytes obtained from osteoarthritis donors and values differ without a common letter $P < 0.01$. (b) Effect of lactoferrin and nuclear factor-kappa B inhibitor on the protein expression of cyclooxygenase-2 in interleukin-1 β -stimulated human osteoarthritis chondrocytes. Primary chondrocytes were pretreated with L (50–75 μ g/ml) for 2 h and stimulated with interleukin-1 β (10 ng/ml) for 24 h and cell lysates were prepared. Cyclooxygenase-2 protein was analyzed by Western immunoblotting. Band images were digitally captured and the band intensities were obtained using the Un-Scan-It software and are expressed in average pixels of five independent scans. (c) Effect of lactoferrin and nuclear factor-kappa B inhibitor on the production of prostaglandin E₂ in interleukin-1 β -stimulated osteoarthritis chondrocytes culture medium. Primary chondrocytes were pretreated with L (50–75 μ g/ml) for 2 h and stimulated with interleukin-1 β (10 ng/ml) for 24 h. Prostaglandin E₂ production was analyzed in cell culture supernatant by ELISA. Data shown are cumulative of three experiments and differ without a common letter $P < 0.01$.

inflammatory response. To investigate the mechanism responsible for the inhibitory effect of lactoferrin on COX-2 expression, we examined the effect of lactoferrin on NF- κ B activation and translocation to the nucleus by estimating the nuclear cell fractions using NF- κ Bp65 transcription factor specific assays kit (Abcam). Exposure of OA chondrocytes to IL-1 β significantly enhanced the DNA binding activity of NF- κ Bp65 compared to controls ($P < 0.001$) and increasing doses of lactoferrin (50–75 μ g/ml) significantly reduced the IL-1 β -induced DNA binding activity of NF- κ B p65 ($P < 0.05$) [Figure 3]. To further strengthen the relation of inhibition of NF- κ B pathway and the expression of COX-2 in our studies, we next investigated the effect of a pharmacological agent, Bay-11, a known inhibitor of NF- κ B, on the expression of COX-2 and production of PGE₂. Treatment of chondrocytes with the Bay-11 (10 μ M), significantly blocked the IL-1 β -induced COX-2 expression both at mRNA and protein levels [$P < 0.05$; Figure 2a and b] as well as PGE₂ production [$P < 0.05$; Figure 2c]. Together these results suggest that lactoferrin exerts its inhibitory effect on COX-2 expression via modulation of the activation and DNA binding activity of NF- κ B [Figure 4].

DISCUSSION

This is the first report that shows lactoferrin, an iron-binding protein inhibits the inflammatory activity in IL-1 β stimulated human OA chondrocytes. This is achieved by blocking of NF- κ B activation in human chondrocytes. As COX-2 gene is NF- κ B dependent, inhibition of NF- κ B also inhibits its mRNA and protein expression and its product, PGE₂ in IL-1 β -stimulated human OA chondrocytes. OA is a chronic disorder of synovial joints characterized pathologically by focal areas of damage to the articular cartilage, centered on load-bearing areas, which is associated with the new bone formation at the joint margins, changes in the subchondral bone, variable degrees of mild synovitis, and thickening of the joint capsule.^[38] The severity of OA differs from patient to patient, but the very common clinical symptoms include pain, reduced range of motion, inflammation, and deformity.^[38] Now it is widely accepted that the onset

of OA is well associated with excessive production of pro-inflammatory cytokines including IL-1 β .^[9,39] It is well documented that IL-1 β has a pivotal role in cartilage matrix destruction via upregulation of the production of proteases and downregulation of the synthesis of proteoglycan and collagen.^[9-11,39] It also upregulates excessive expression of COX-2,^[10] which is a major PGE₂ synthetic enzyme and is well known to involve in the pathogenesis of chronic inflammation.^[5] Although very low levels of PGE₂ may have anabolic functions in cartilage, in OA tissues, it is predominantly performed catabolism, leading to an inhibition of proteoglycan synthesis, increased proteases, and increased COL2A1 degradation.^[6-8,40] As PGE₂ is usually found at high levels in synovial fluid in OA patients and OA animal models, is regarded as a possible therapeutic target in OA.^[40] Although arthritis is present in every population and OA is the most common joint disorder, the treatment is still limited only to a few classes of drugs, primarily nonsteroidal antiinflammatory drugs, corticosteroids, and products from plants.^[41,42] While providing relief from pain, however, none of the agents has been shown to inhibit disease progress or to inhibit cartilage degradation; they also have varying degrees of side effects including gastrointestinal toxicity.^[41]

There are a number of studies documenting the beneficial health effects of iron-binding glycoprotein lactoferrin. Most of these studies place emphasis on the antibacterial, antiviral, antiparasitic, antiallergic, catalytic, antioxidant, and anticancer properties,^[24] which have now been attributed, at least in part, to the ability to inhibit the inflammatory processes.^[20-25] Now it has been accepted that it is a multifunctional protein involves in many physiological functions, including regulation of iron absorption and immune responses.^[17,43] Lactoferrin is found in blood circulation, mucosal surfaces, granules of neutrophils, and in various secretory fluids, such as milk, bile, tears, nasal secretion, pancreatic juice, and saliva.^[44] It is reported that lactoferrin in blood is normally low (5–7 μ g/ml) but at the sites of inflammation its concentration raised remarkably, as in arthritic patients it reaches up to 100 μ g/ml, whereas in arthritic animal model its concentration becomes up to 200 μ g/ml.^[17,24,45] This high release of lactoferrin at the sites of inflammation is well reported

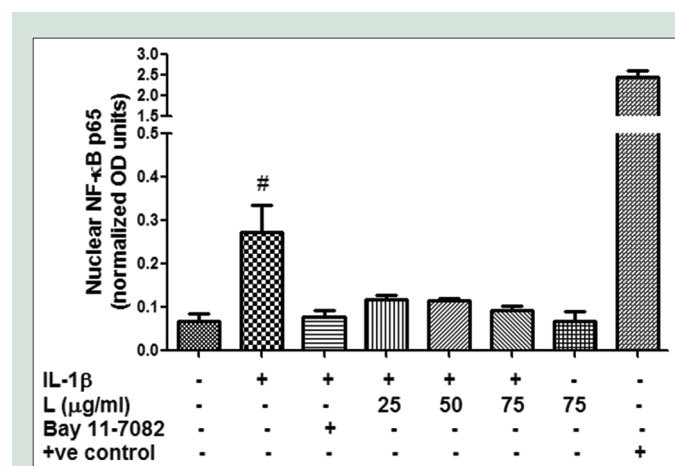


Figure 3: Lactoferrin inhibited the activation of nuclear transcription factor-kappa B in interleukin-1 β -stimulated human osteoarthritis chondrocytes. Primary chondrocytes (70–80% confluent) were pretreated with lactoferrin (L) (25–75 μ g/ml) and Bay 11-7082 (10 μ M) for 2 h and stimulated by interleukin-1 β (10 ng/ml) for 30 min. Activated nuclear factor-kappa B p65 in the nucleus was determined by highly specific transcription factor ELISA kit (Abcam). The positive control nuclear extract supplied with the kit was used. Data are representative of three experiments and is presented as mean $\pm$ standard error of mean $^*P < 0.05$ versus untreated chondrocytes; $^{\#}P < 0.05$ versus L or Bay 11-7082 treated chondrocytes

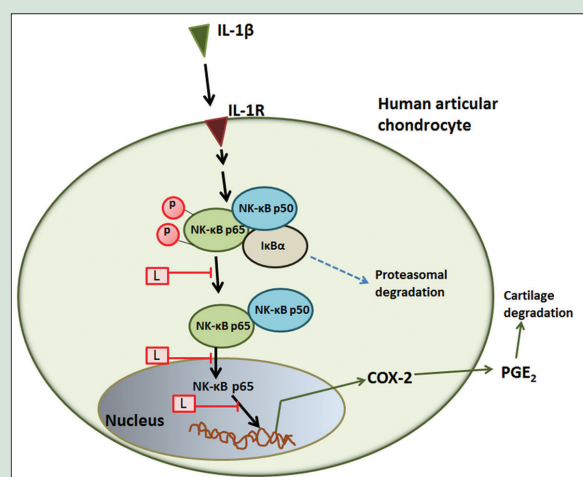


Figure 4: Overview of lactoferrin mediated inhibition of interleukin-1 β -induced signaling events to cyclooxygenase-2 in human osteoarthritis chondrocytes. Interleukin-1 β through interleukin-1 receptor activates interleukin-1 β -induced signaling to cyclooxygenase-2 or prostaglandin E2 via activation of nuclear transcription factor-kappa B. Nuclear factor-kappa B complex was demonstrated by nuclear nuclear factor-kappa Bp65. All possible offered targets of lactoferrin induced inhibition to cyclooxygenase are shown. IL-1 β : interleukin-1 beta; IL-1R: receptor for IL-1; L: lactoferrin; NF- κ B: Nuclear factor-kappa B; p-NF- κ B: phosphorylated NF- κ B; COX-2: cyclooxygenase-2; PGE₂: prostaglandin E2

to play an anti-inflammatory role. As it modules the expression of many inflammatory mediators such as tumor necrosis factor- α , IL-1 β , IL-6, IL-8, nitric oxide, granulocyte-macrophage-colony-stimulating factor, intercellular adhesion molecules in patients, and animal models.^[17,20-25] Despite these important roles of lactoferrin in inflammation, its chondroprotective effect in joints of articular cartilage is not well understood. In this study, we show for the very 1st time that lactoferrin inhibited the IL-1 β -induced gene and protein expression of COX-2 and production of PGE₂ in primary human OA chondrocytes.

Activation of the master transcription factor NF- κ B leads to the coordinated expression of many genes that encode cytokines, chemokines, enzymes, and adhesion molecules involved in mediator synthesis and the further amplification and perpetuation of the inflammatory reaction.^[13-15,45] NF- κ B is present in the cytosol in an inactive state, complexed with the inhibitory I κ B proteins, its activation occurs by the induction of phosphorylation, which mediates proteasomal degradation of I κ B.^[46] It is also well documented that NF- κ B is known to be involved in IL-1 β -mediated effects of IL-1 receptor signaling, and that expression of COX-2 gene is dependent on the activation of transcription factor NF- κ B.^[46] Suppression of NF- κ B activation has been linked with anti-inflammatory activity. Therefore, we postulated that lactoferrin mediates its inhibitory effects on COX-2 expressions at least in part, through the suppression of NF- κ B activity [Figure 4]. In IL-1 β -stimulated human OA chondrocytes, lactoferrin inhibited nuclear translocation of the NF- κ Bp65, as determined by DNA binding activity of nuclear NF- κ Bp65 in OA chondrocytes. These data further confirms that lactoferrin attenuates the inflammatory stimuli-induced activation and DNA binding activity of NF- κ B in human chondrocytes. In order to further confirm that lactoferrin specifically inhibited the IL-1 β -induced activation of NF- κ B and inhibited the expression of COX-2 and production of PGE₂, we treated chondrocytes with NF- κ B specific inhibitor, Bay 11-7082. Treatment of chondrocytes with Bay 11-7082 reduced the IL-1 β -stimulated gene and protein expression of COX-2 or production of PGE₂. To this, based on our results, we can add that lactoferrin is a potent inhibitor of IL-1 β -induced induction of COX-2 and production of PGE₂ at a physiologically achievable concentration (50–75 μ g/ml). We, therefore, conclude that inhibition of arthritis following local administration of lactoferrin in an arthritic animal model^[17] and lactoferrin mediated inhibition of IL-1 β -induced production of cartilage-degrading molecule PGE₂ via inhibition of COX-2 expression provide new insights into the mechanism of its action. Therefore, it is important to suggest that lactoferrin or compounds derived from it have important implications for the development of novel therapeutic strategies for the prevention/treatment of OA.

CONCLUSION

This study provides an important new insight into the molecular basis of the cartilage protective and antiarthritis effects of camel lactoferrin. Our data show for the 1st time that lactoferrin inhibits the inflammatory activity against IL-1 β -induced activation of human OA chondrocytes. This could block the activation of NF- κ B signaling events. Our data also show a potent inhibitory effect of lactoferrin on IL-1 β -induced production of cartilage-degrading molecule PGE₂ via suppression of IL-1 β -induced expression of COX-2 mRNA and protein in human OA chondrocytes. These findings are of important to understand the chondroprotective mechanisms and clinical applications of camel lactoferrin.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

- Glyn-Jones S, Palmer AJ, Agricola R, Price AJ, Vincent TL, Weinans H, *et al.* Osteoarthritis. *Lancet* 2015;386:376-87.
- Lee AS, Eilman MB, Yan D, Kroin JS, Cole BJ, van Wijnen AJ, *et al.* A current review of molecular mechanisms regarding osteoarthritis and pain. *Gene* 2013;527:440-7.
- Health Quality Ontario. Total knee replacement: An evidence-based analysis. *Ont Health Technol Assess Ser* 2005;5:1-51.
- Gao Y, Liu S, Huang J, Guo W, Chen J, Zhang L, *et al.* The ECM-cell interaction of cartilage extracellular matrix on chondrocytes. *Biomed Res Int* 2014;2014:648459.
- Abramson SB. The role of COX-2 produced by cartilage in arthritis. *Osteoarthritis Cartilage* 1999;7:380-1.
- Attur M, Al-Mussawir HE, Patel J, Kitay A, Dave M, Palmer G, *et al.* Prostaglandin E2 exerts catabolic effects in osteoarthritis cartilage: Evidence for signaling via the EP4 receptor. *J Immunol* 2008;181:5082-8.
- Bu X, Zhao C, Dai X. Involvement of COX-2/PGE (2) Pathway in the Upregulation of MMP-9 Expression in Pancreatic Cancer. *Gastroenterol Res Pract* 2011;2011:214269.
- Wu K, Fukuda K, Xing F, Zhang Y, Sharma S, Liu Y, *et al.* Roles of the cyclooxygenase 2 matrix metalloproteinase 1 pathway in brain metastasis of breast cancer. *J Biol Chem* 2015;290:9842-54.
- Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol* 2011;7:33-42.
- Tsuzaki M, Guyton G, Garrett W, Archambault JM, Herzog W, Almekinders L, *et al.* IL-1 beta induces COX2, MMP-1, -3 and -13, ADAMTS-4, IL-1 beta and IL-6 in human tendon cells. *J Orthop Res* 2003;21:256-64.
- Daheshia M, Yao JQ. The interleukin 1beta pathway in the pathogenesis of osteoarthritis. *J Rheumatol* 2008;35:2306-12.
- de Boer TN, Huisman AM, Polak AA, Niehoff AG, van Rinsum AC, Saris D, *et al.* The chondroprotective effect of selective COX-2 inhibition in osteoarthritis: Ex vivo evaluation of human cartilage tissue after *in vivo* treatment. *Osteoarthritis Cartilage* 2009;17:482-8.
- Roman-Blas JA, Jimenez SA. NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. *Osteoarthritis Cartilage* 2006;14:839-48.
- Marcu KB, Otero M, Olivetto E, Borzi RM, Goldring MB. NF-kappaB signaling: Multiple angles to target OA. *Curr Drug Targets* 2010;11:599-613.
- Rigoglou S, Papavassiliou AG. The NF-kB signalling pathway in osteoarthritis. *Int J Biochem Cell Biol* 2013;45:2580-4.
- Dabbagh AJ, Trenam CW, Morris CJ, Blake DR. Iron in joint inflammation. *Ann Rheum Dis* 1993;52:67-73.
- Guillen C, McInnes IB, Vaughan D, Speekenbrink AB, Brock JH. The effects of local administration of lactoferrin on inflammation in murine autoimmune and infectious arthritis. *Arthritis Rheum* 2000;43:2073-80.
- Barton JC. Chelation therapy for iron overload. *Curr Gastroenterol Rep* 2007;9:74-82.
- Kocaoglu B, Akgun U, Erol B, Karahan M, Yalcin S. Preventing blood-induced joint damage with the use of intra-articular iron chelators: An experimental study in rabbits. *Arch Orthop Trauma Surg* 2009;129:1571-5.
- Sanchez L, Calvo M, Brock JH. Biological role of lactoferrin. *Arch Dis Child* 1992;67:657-61.
- Wong SH, Francis N, Chahal H, Raza K, Salmon M, Scheel-Toellner D, *et al.* Lactoferrin is a survival factor for neutrophils in rheumatoid synovial fluid. *Rheumatology (Oxford)* 2009;48:39-44.
- Yeom M, Park J, Lee B, Choi SY, Kim KS, Lee H, *et al.* Lactoferrin inhibits the inflammatory and angiogenic activation of bovine aortic endothelial cells. *Inflamm Res* 2011;60:475-82.
- Inubushi T, Kawazoe A, Miyauchi M, Kudo Y, Ao M, Ishikado A, *et al.* Molecular mechanisms of the inhibitory effects of bovine lactoferrin on lipopolysaccharide-mediated osteoclastogenesis. *J Biol Chem* 2012;287:23527-36.
- García-Montoya IA, Cendón TS, Arévalo-Gallegos S, Rascón-Cruz Q. Lactoferrin a multiple bioactive protein: An overview. *Biochim Biophys Acta* 2012;1820:226-36.
- Valenti P, Catizone A, Pantanella F, Frioni A, Natalizi T, Tendini M, *et al.* Lactoferrin decreases inflammatory response by cystic fibrosis bronchial cells invaded with *Burkholderia cenocepacia* iron-modulated biofilm. *Int J Immunopathol Pharmacol* 2011;24:1057-68.

26. Rasheed Z, Haqqi TM. Endoplasmic reticulum stress induces the expression of COX-2 through activation of $\text{eIF}2\alpha$, p38-MAPK and NF- κ B in advanced glycation end products stimulated human chondrocytes. *Biochim Biophys Acta* 2012;1823:2179-89.
27. Rasheed Z, Akhtar N, Haqqi TM. Advanced glycation end products induce the expression of interleukin-6 and interleukin-8 by receptor for advanced glycation end product-mediated activation of mitogen-activated protein kinases and nuclear factor- κ B in human osteoarthritis chondrocytes. *Rheumatology (Oxford)* 2011;50:838-51.
28. Mankin HJ, Dorfman H, Lippello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. *J Bone Joint Surg Am* 1971;53:523-37.
29. Rasheed Z, Akhtar N, Haqqi TM. Pomegranate extract inhibits the interleukin-1 β -induced activation of MKK-3, p38 α -MAPK and transcription factor RUNX-2 in human osteoarthritis chondrocytes. *Arthritis Res Ther* 2010;12:R195.
30. Akhtar N, Rasheed Z, Ramamurthy S, Anbazhagan AN, Voss FR, Haqqi TM. MicroRNA-27b regulates the expression of matrix metalloproteinase 13 in human osteoarthritis chondrocytes. *Arthritis Rheum* 2010;62:1361-71.
31. Rasheed Z, Anbazhagan AN, Akhtar N, Ramamurthy S, Voss FR, Haqqi TM. Green tea polyphenol epigallocatechin-3-gallate inhibits advanced glycation end product-induced expression of tumor necrosis factor- α and matrix metalloproteinase-13 in human chondrocytes. *Arthritis Res Ther* 2009;11:R71.
32. Conesa C, Sánchez L, Rota C, Pérez MD, Calvo M, Farnaud S, *et al.* Isolation of lactoferrin from milk of different species: Calorimetric and antimicrobial studies. *Comp Biochem Physiol B Biochem Mol Biol* 2008;150:131-9.
33. Shukla M, Gupta K, Rasheed Z, Khan KA, Haqqi TM. Bioavailable constituents/metabolites of pomegranate (*Punica granatum* L) preferentially inhibit COX2 activity *ex vivo* and IL-1 β -induced PGE2 production in human chondrocytes *in vitro*. *J Inflamm (Lond)* 2008;5:9.
34. Rasheed Z, Akhtar N, Khan A, Khan KA, Haqqi TM. Butrin, isobutrin, and butein from medicinal plant *Butea monosperma* selectively inhibit nuclear factor- κ B in activated human mast cells: Suppression of tumor necrosis factor- α , interleukin (IL)-6, and IL-8. *J Pharmacol Exp Ther* 2010;333:354-63.
35. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:e45.
36. Rasheed Z, Akhtar N, Anbazhagan AN, Ramamurthy S, Shukla M, Haqqi TM. Polyphenol-rich pomegranate fruit extract (POMx) suppresses PMACI-induced expression of pro-inflammatory cytokines by inhibiting the activation of MAP Kinases and NF- κ B in human KU812 cells. *J Inflamm (Lond)* 2009;6:1.
37. de Crombrughe B, Lefebvre V, Behringer RR, Bi W, Murakami S, Huang W. Transcriptional mechanisms of chondrocyte differentiation. *Matrix Biol* 2000;19:389-94.
38. Fingleton C, Smart K, Moloney N, Fullen BM, Doody C. Pain sensitization in people with knee osteoarthritis: A systematic review and meta-analysis. *Osteoarthritis Cartilage* 2015;23:1043-56.
39. Jotanovic Z, Mihelic R, Sestan B, Dembic Z. Role of interleukin-1 inhibitors in osteoarthritis: An evidence-based review. *Drugs Aging* 2012;29:343-58.
40. Martel-Pelletier J, Pelletier JP, Fahmi H. Cyclooxygenase-2 and prostaglandins in articular tissues. *Semin Arthritis Rheum* 2003;33:155-67.
41. Fendrick AM, Greenberg BP. A review of the benefits and risks of nonsteroidal anti-inflammatory drugs in the management of mild-to-moderate osteoarthritis. *Osteopath Med Prim Care* 2009;3:1.
42. Beg S, Swain S, Hasan H, Barkat MA, Hussain MS. Systematic review of herbals as potential anti-inflammatory agents: Recent advances, current clinical status and future perspectives. *Pharmacogn Rev* 2011;5:120-37.
43. Guillén C, McInnes IB, Kruger H, Brock JH. Iron, lactoferrin and iron regulatory protein activity in the synovium; relative importance of iron loading and the inflammatory response. *Ann Rheum Dis* 1998;57:309-14.
44. Albar AH, Almeshdar HA, Uversky VN, Redwan EM. Structural heterogeneity and multifunctionality of lactoferrin. *Curr Protein Pept Sci* 2014;15:778-97.
45. Alghasham A, Rasheed Z. Therapeutic targets for rheumatoid arthritis: Progress and promises. *Autoimmunity* 2014;47:77-94.
46. DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive I κ B kinase that activates the transcription factor NF- κ B. *Nature* 1997;388:548-54.

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