Paper I

Human articular chondrocytes express functional leukotriene B$_4$ receptors

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Abstract

Leukotriene B$_4$ (LTB$_4$) is a potent chemoattractant associated with the development of osteoarthritis (OA), while its receptors BLT1 and BLT2 have been found in synovium and subchondral bone. In this study, we have investigated whether these receptors are also expressed by human cartilage cells and their potential effects on cartilage cells. The expression of LTB$_4$ receptors in native tissue and cultured cells was assessed by immunohistochemistry, immunocytochemistry, polymerase chain reaction (PCR) and electron microscopy. The functional significance of the LTB$_4$ receptor expression was studied by Western blotting, using phospho-specific antibodies in the presence or absence of receptor antagonists. In further studies, the secretion of pro-inflammatory cytokines, growth factors and metalloproteinases by LTB$_4$-stimulated chondrocytes was measured by multiplex protein assays. The effects of LTB$_4$ in cartilage signature gene expression in cultured cells were assessed by quantitative PCR, whereas the LTB$_4$-promoted matrix synthesis was determined using 3D pellet cultures. Both receptors were present in cultured chondrocytes, as was confirmed by immunolabelling and PCR. The relative quantification by PCR demonstrated a higher expression of the receptors in cells from healthy joints compared with OA cases. The stimulation of cultured chondrocytes with LTB$_4$ resulted in a phosphorylation of downstream transcription factor Erk 1/2, which was reduced after blocking BLT1 signalling. No alteration in the secretion of cytokine and metalloproteinases was recorded after challenging cultured cells with LTB$_4$; likewise, cartilage matrix gene expression and 3D tissue synthesis were unaffected. Chondrocytes express BLT1 and BLT2 receptors, and LTB$_4$ activates the downstream Erk 1/2 pathway by engaging the high-affinity receptor BLT1. However, any putative role in cartilage biology could not be revealed, and remains to be clarified.

Key words: BLT1; BLT2; cartilage; chondrocytes; inflammation; leukotriene B$_4$; osteoarthritis.

Introduction

The metabolism of arachidonic acid by the cyclooxygenase (COX) or 5-lipoxygenase (5-LO) pathways generates eicosanoids, which have been implicated in the pathophysiology of a variety of human diseases, including arthritis of the joints (Molloy & McCarthy, 2005; Korotkova & Jakobsson, 2014).

Arachidonic acid released from cell membranes by events such as inflammation or trauma can be converted to unstable precursor leukotriene A$_4$ (LTA$_4$) via the 5-LO and 5-LO-activating protein, and further into leukotriene B$_4$ (LTB$_4$) by LTA$_4$ hydrolase (Peters-Golden & Henderson, 2007). In the presence of COX-1 or COX-2, arachidonic acid is converted into prostaglandin H$_2$, and subsequently into prostaglandin E$_2$ by prostaglandin E synthase (Li et al. 2009). Leukotriene B$_4$ (LTB$_4$) is a powerful inflammatory mediator implicated in diseases such as asthma, atherosclerosis and rheumatoid arthritis (RA) by the recruitment and activation of leukocytes (Tager & Luster, 2003; Peters-Golden & Henderson, 2007; Di Gennaro & Haeggstrom, 2014). Importantly, LTB$_4$ levels are enhanced in synovial tissue and fluid, cartilage and subchondral bone during both osteoarthritis (OA) and RA (Ahmadzadeh et al. 1991;

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Wittenberg et al. 1993; Paredes et al. 2002; Attur et al. 2012).

Leukotriene B$_4$ binds two G-coupled receptors: the high-affinity LTB$_4$ receptor 1 (BLT1) and the low-affinity LTB$_4$ receptor 2 (BLT2; Yokomizo et al. 2001a). The BLT1 receptor was initially described in humans in myeloid cells (Yokomizo et al. 1997), though later studies have reported its presence in non-myeloid tissues as well, including sub-chondral osteoblasts and synovial cells (Paredes et al. 2002; Hashimoto et al. 2003). BLT1 activation in non-myeloid cells promotes the cell differentiation, migration, proliferation and chemokine production needed to recruit neutrophils (Kim & Luster, 2007). BLT2 is ubiquitously expressed in humans (Yokomizo et al. 2000), while in synovial tissue the expression of BLT2 surpasses that of BLT1 (Hashimoto et al. 2003).

Prostaglandins are also involved in OA development (Amin et al. 1997; Li et al. 2009), with the current first-line treatment of OA including COX inhibitors (McAlindon et al. 2014). However, blocking COX-regulated arachidonic acid pathways leads to a shunting of the system to the 5-LO pathway, as well as an undesired elevation of LTB$_4$ levels (Marcouiller et al. 2005; Maxis et al. 2006; Attur et al. 2012).

Despite studies arguing for the importance of LTB$_4$ and its receptors on RA and OA disease development, the expression and function of LTB$_4$ receptors in chondrocytes has not yet been reported. The aim of this study was to assess the expression of LTB$_4$ receptors BLT1 and BLT2 in cartilage, and the biological effects of LTB$_4$ on cultured chondrocytes.

Materials and methods

Human material and cell culturing

Macroscopically healthy looking cartilage was harvested from the lateral femoral condyle of 15 patients (age 55–82 years) undergoing total knee replacement (TKR), and the overall grading of cartilage according to the ICRS/Outerbridge score (Kleemann et al. 2005) was 2–4. In addition, we obtained surplus cells from the autologous chondrocyte implantation (ACI) of 10 patients (aged 31–65 years); these cells were harvested and expanded as previously described (Brittberg et al. 1994; Peterson et al. 2002), with the procedure also demanding a normal joint on X-ray and arthroscopy (Knutsen et al. 2004). The patients participated by informed consent, and the Regional Ethical Committee of North Norway approved the study.

Cartilage from TKR was kept in a sterile saline and processed within 2 h from harvest. Specimens were processed directly as described for immunohistochemistry and electron microscopy, enzymatically digested for the determination of BLT receptors and signature genes by quantitative polymerase chain reaction (qPCR) or processed for expansion in culture by mincing into ~1-mm^3 pieces,

followed by digestion in 0.8 mg mL^{-1} of collagenase XI solution (cat. no. C9407; Sigma-Aldrich) for 4 h at 37 °C during gentle agitation. Chondrocytes were plated in 25-cm^2 culture flasks containing Dulbecco's Modified Eagle's Medium/Ham's F-12 Mixture, and supplemented with ascorbic acid (62 mg L^{-1}), penicillin (2500 U L^{-1}), streptomycin (2.5 mg L^{-1}) and 20% foetal bovine serum (FBS; cat. no. S0115; Biochrome). After cell attachment, cultures were further propagated in a medium supplemented with 10% serum and the cell number was serially expanded in appropriate vessels, and subsequently used for experimentation at passages 3–4.

Immunohistochemistry and immunocytochemistry

The immunolabelling of BLT1 and BLT2 receptors in native cartilage and cultured chondrocytes was performed using polyclonal rabbit anti-human BLT1 antibody (cat. no. 120114; Cayman Chemicals), and/or polyclonal rabbit anti-human BLT2 antibody (cat. no. 120124; Cayman Chemicals) or no primary antibody for control. For immunohistochemistry, biopsies from five TKR donors were fixed in paraformaldehyde and prepared as previously described (Berg et al. 2010). Sections were incubated overnight at 4 °C in BLT1 or BLT2 antibody diluted at 1 : 100. Slides were stained using an anti-rabbit horseradish peroxidase (HRP) SuperPicTure™ Polymer detection kit (cat. no. 87-9263; Invitrogen), and counterstained with haematoxylin. Isotype control was used to assess non-specific binding. For immunocytochemistry, an appropriate number of expanded chondrocytes from four TKR donors were seeded on fibronectin-coated slides as previously described (Berg et al. 2010). Slides were incubated overnight at 4 °C in BLT1 or BLT2 antibody diluted at 1 : 250. After washing in phosphate-buffered saline (PBS), the slides were incubated with secondary antibody Alexa Fluor® goat anti-rabbit IgG (cat. no. A11008; Invitrogen) at 4 °C for 14 h until nuclear staining and mounting using DAPI-Fluoromount-G™ (cat. no. 0100-20; Southern Biotech). The slides were examined in a Zeiss Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany).

Reverse transcriptase (RT)-PCR of BLT1 and BLT2 mRNAs

As previously described, a RT-PCR was performed (Berg et al. 2010). In brief, total RNA was extracted from two different TKR cell cultures using an RNeasy® Mini Kit (cat. no. 74106; Qiagen), and converted to cDNA using a Superscript® III Reverse Transcriptase (cat. no. 18080-093; Invitrogen). The PCR reaction volume of 50 μL included 5 μL cDNA, 150 nM of each specific primer, ultrapure water and MasterMix (cat. no PRME2200100; 5 Prime). Sequences for the specific primers for human BLT1 (sense 5′-TATGTCTGCGGAGT CAGCATGTACGC-3′, antisense 5′-CCTGTAGCCGACGCCCTAT GTCCG-3′) yield a 345-bp product, while human BLT2 (sense

5′-AGCCTGGAGACTCTGACCGCTTTCG-3′, antisense 5′-GAC GTAGAGCACCGGGTTGACGCTA-3′) yields a 320-bp product. Adenine phosphoribosyltransferase (APRT; sense 5′-CCCGA GGCTTCCTCTTTG GC-3′, antisense 5′-CTCCCTGCCCTTAAGC GAGG-3′), which yields a 300-bp product, was included for RNA quality control; in case of contaminating DNA, a 800-bp product would occur. The PCR was run on a GeneAmp® PCR System 9700 (Applied Biosystems), with an amplification profile of: 7 min at 94 °C (denaturation), 30 s at 94 °C (denaturation), 30 s at 55 °C (annealing), 1 min at 72 °C (extension) and 30 cycles completed with a 10-min extension at 72 °C. RT-PCR products were separated on Novex® 6% TBE gel (cat. no. EC6265BOX; Invitrogen) and stained with SYBR® Safe DNA gel stain (cat. no. S33102; Invitrogen), while images were developed using a G-Box (Syngene). To help confirm that BLT1 and BLT2 transcripts matched those reported in GeneBank®, the PCR products were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (cat. no. 4337455; Applied Biosystems) and GeneAmp® PCR Systems 9700 (Applied Biosystems).

Immunoelectron microscopy

Cartilage specimens from two different TKR donors were freshly collected, washed in ice-cold PBS and fixed in 4% formaldehyde in a 200 mM HEPES buffer, pH 7.5, for 24 h. Cryosections were prepared, and immunolabelling was performed as described elsewhere (Tokuyasu, 1986). Antibodies against BLT1 (diluted 1 : 20) were detected by protein A–gold complexes, and the dried sections were then examined in a JEOL JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV. Additionally, negative controls were routinely included in parallel by the omission of primary antibodies.

Relative quantification of BLT1 and BLT2 receptor by qPCR

Extraction of RNA was from three sources: cartilage biopsies from TKR biopsies ($N = 3$), cultured cells from TKR biopsies at passage 4 ($N = 5$) and surplus cells from ACI at passage 4 ($N = 5$). For RNA extraction from cartilage, the biopsies were minced to ~1-mm^3 pieces, and chondrocytes were then rapidly released by digestion in a 0.25% trypsin solution (cat. no. T3924; Sigma-Aldrich) for 60 min and then in a 0.8 mg mL^{-1} collagenase XI solution for 6 h at 37 °C during gentle agitation. The freshly isolated cells were lysed using Qiazol, followed by DNase I treatment and clean-up on RNeasy mini-spin columns (cat. no. 74804; Qiagen) according to the manufacturer's instructions. Cultured cells were then extracted using a PerfectPure RNA Cultured Cell Kit (cat. no. 2302340; 5 prime), including DNase I treatment according to the manufacturer's instructions. RNA was quantified by spectrophotometry (Nano Drop ND-1000), and diluted at 1 : 5 to a final concentration of 9.82 ng μL^{-1}

before RT using a High-Capacity RNA to cDNA kit (cat. no. 4387406; Invitrogen) according to the manufacturer's instructions. The relative quantification experiment was run with hydrolysis probes targeting BLT1 (LTB4R, Hs01 938704_s1) and BLT2 (LTB4R2, Hs01885851_s1). Based on previous validation experiments, ribosomal protein L13A (RPL13A, Hs04194366_g, all probes from Life Technologies) was used as the reference gene. The reaction volume of 10 μL included 5 μL TaqMan® Fast Universal PCR Mastermix No AmpErase® UNG (cat. no. 4366072; Applied Biosystems), 2.5 μL water, 2 μL cDNA (50 ng μL^{-1}) and 0.5 μL of the respective assays. Three technical replicates of each sample were applied to a MicroAmp® Fast Optical 96-well reaction plate (cat. no. 4346906; Applied Biosystems), and water and no-RT samples were added for the negative control. The plates were run on StepOnePlus, using the following cycling conditions: hold at 95 °C for 20 s, 40 cycles at 95 °C for 1 s and then at 60 °C for 20 s. Data were analysed in Microsoft Excel, a t-test was used to test significance, and the differences were considered significant at $P < 0.05$.

Western blotting of phosphorylated protein kinases

Cultured chondrocytes from three ACI donors were starved for 24 h before LTB$_4$ (cat. no. 20110; Cayman Chemicals) was added at a final concentration of 10^{-8} M for 3 and 5 min each. For inhibition studies, cultured chondrocytes were challenged with BLT1 receptor antagonist U-75302 at 3 × 10^{-7} M (cat. no. 70705; Cayman Chemicals) or BLT2 receptor antagonist LY2552833 at 10^{-6} M (cat. no. 70715; Cayman Chemicals) for 1 h before being stimulated with LTB$_4$ for 5 min (Yokomizo et al. 2001b; Lundeen et al. 2006). Cells were lysed in a 150 μL radioimmunoprecipitation buffer (cat. no. 20-188; Millipore), including protease- and phosphatase inhibitor, and an equal amount of protein was separated with gel electrophoresis and blotted to polyvinylidene fluoride membranes. Membranes were incubated overnight at 4 °C with phospho-p44/42 mitogen-activated protein kinase (MAPK) (Erk 1/2) rabbit mAb (cat. no. 4370; Cell Signaling) and loading control β-actin (13E5) rabbit mAb (cat. no. 4970; Cell Signaling) at a final dilution of 1 : 2000. Next, the membranes were incubated for 1 h with anti-rabbit IgG HRP-linked antibody (cat. no. 7074; Cell Signaling), and bands were detected using chemiluminescence.

Multiplex protein analyses

The protein concentration in culture supernatants was analysed by antibody-based multiplex protein arrays. Chondrocytes from three different TKR cultures were seeded on 24-well plates at a concentration of $1.0 × 10^5$ cells well^{-1}, and incubated overnight in a medium with a 10% FBS. Thereafter, the cells were starved overnight in a medium containing 0.1% FBS before challenging with LTB$_4$ at a concentration of 10^{-12} M, 10^{-10} M, 10^{-8} M or vehicle only at 37 °C

for 18 h. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), along with inflammatory cytokines interleukin (IL)-1β, tumour necrosis factor (TNF)-α, IL-6, IL-8 and monocyte chemoattractant protein (MCP)-1, were analysed in an 8-plex assay (cat. no. Z50007ZORG; Bio Rad). Matrix metalloproteinases (MMP) of -1, -2, -3, -8, -9 and -13 were measured using a multiplex MMP assay (cat. no. LMP000; R&D Systems). Samples were diluted at 1 : 4 and analysed in duplicate using the Bio-Plex 200 System (Bio-Rad). The results are the average of two independent experiments, and were analysed in Excel using a one-way ANOVA.

qPCR of cartilage signature genes

Surplus cultured chondrocytes from four ACI procedures were grown in parallel, in which one culture remained untreated for the control, and the other stimulated with LTB$_4$ at a final concentration of 10^{-8} M for 18 h. Chondrocytes were harvested in a lysis solution and RNA extracted using the PerfectPure RNA Cultured Cell Kit (cat. no. 2302340; 5 prime) according to the manufacturer's instructions, including DNase I treatment. RNA was quantified using Nano Drop ND-1000, and converted to cDNA with a High-Capacity RNA to cDNA kit (cat. no. 4387406; Invitrogen) according to the manufacturer's instructions. For the qPCR, the following pre-optimized hydrolysis probes from Applied Biosystems were used: collagen 1A1 (COL1A1, Assay ID: Hs00164004_m1), collagen 2A1 (COL2A1, Assay ID: Hs00264051_m1), aggrecan (ACAN, Assay ID: Hs00153936_m1) and SOX9 (SOX9, Assay ID: Hs00165814_m1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Assay ID: Hs99999905_m1) was included as a reference gene after validating a stable expression in monolayer cultures. A reaction volume of 20 μL contained 10 μL of TaqMan® Fast Universal PCR Mastermix No AmpErase® UNG (cat. no. 4366072; Applied Biosystems), 7 μL of water, 2 μL of cDNA (50 ng μL^{-1}) and 1 μL of the respective assays. Samples in three technical replicates were applied to a MicroAmp® Fast Optical 96-well reaction plate (cat. no. 4346906; Applied Biosystems); water for a negative control and cDNA from freshly isolated cartilage cells were added in triplicate for positive control and inter-plate calibration. Plates were analysed on an Applied Biosystems 7900HT Fast Real-Time PCR System with the following cycling conditions: hold at 95 °C for 20 s, 40 cycles at 95 °C for 1 s and then 60 °C for 20 s.

3D pellet cultures

Surplus ACI chondrocytes expanded in monolayers were detached by an enzyme-free detachment solution (cat. no. S-004-B; Millipore) and a short (30 s) trypsinization. Cells were then resuspended in a complete growth medium, and 150 μL aliquots (50 000 cells) were added to Nunc™ 96-well polystyrene conical bottom MicroWell™ plates

(cat. no. 249935; Thermo Scientific) and centrifuged at 400 **g** for 10 min to make pellets. After 2 days, rounded shaped and consistent spheroids were transferred to a low attachment 24-well plate in a serum-free medium containing insulin-transferrin-selenite (cat. no. 354352; BD Biosciences). For the treated group, 10^{-8} M LTB$_4$ was added to the medium. Spheroids were further incubated for 6 days, and the medium with or without LTB$_4$ was changed every second day. Lastly, spheroids were collected and prepared for histology as described under immunohistochemistry. To evaluate the matrix synthesis, slides were stained with Alcian blue to detect glycosaminoglycans or a collagen type II antibody.

Results

Initial experiments were performed to assess the expression of LTB$_4$ receptors in cartilage tissue and cells, and native cartilage sections from five different donors were immunolabelled using BLT1 or BLT2 antibodies. A positive brown staining confirmed the presence of BLT1 receptor (Fig. 1A) and BLT2 receptor (Fig. 1B), while immunocytochemistry using respective antibodies showed that cultured chondrocytes at passages 3–4 also expressed BLT1 (Fig. 2A) and BLT2 receptors (Fig. 2B). The expression of mRNA encoding these receptors was confirmed by RT-PCR using specific BLT1 and BLT2 primers, as shown in Fig. 3A,B, respectively. The sequencing of the products revealed an identical sequence to the GeneBank sequence for BLT1 and BLT2 (data not shown). The subcellular distribution of the BLT1 receptor was assessed by immunoelectron microscopy, and the specific detection of BLT1 by immune-gold labelling exhibited a predominant expression of the receptor in the plasma membrane of chondrocytes from native tissue, and to a much lesser extent in cytoplasm (Fig. 4A–D). The relative qPCR revealed that the expression of BLT1 was significantly higher in cultured chondrocytes from non-OA joints (ACI) than cartilage biopsies from OA cases (TKR; Fig. 5A). Likewise, the gene expression of BLT1 was significantly higher in cartilage compared with cultured cells from TKR (Fig. 5C). The expression of BLT2 was higher in cultured ACI cells compared with cultured TKR cells, but equally expressed in expanded TKR cells and cartilage (Fig. 5B,D).

In order to assess the BLT1 and BLT2 downstream signalling, cells were stimulated with 10^{-8} M LTB$_4$, followed by the Western blotting of phospho-Erk 1/2 (p44/42 MAPK). Figure 6 shows a modest background phosphorylation of Erk 1/2 in untreated chondrocytes (Lane 1); however, an LTB$_4$ challenge for 5 min markedly increased phosphorylation (Lane 3). The blocking of the BLT1 receptor with the specific receptor antagonist U-75302 before the LTB$_4$ challenge reduced the signal to background phosphorylation (Lane 4). In contrast, a specific blockage of the BLT2 receptor with the antagonist LY2552833 had no major impact (Lane 5),
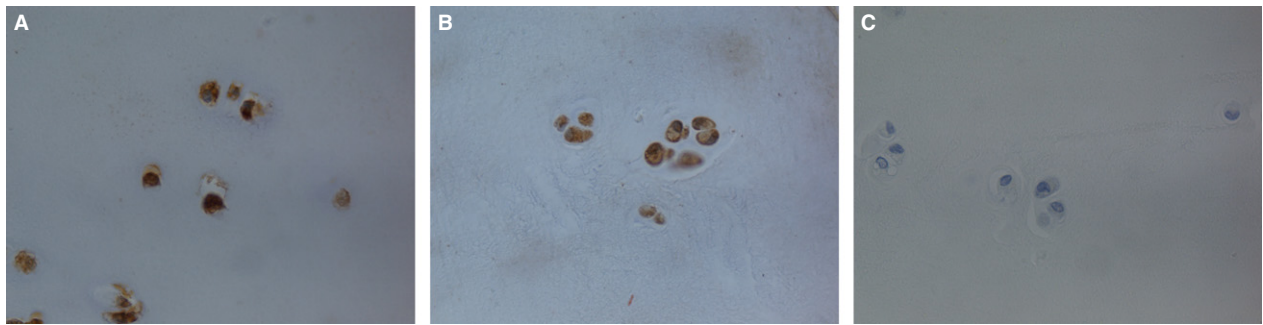
Fig. 1 Expression of BLT1 and BLT2 in cartilage tissue (60 × micrograph). Cartilage tissue sections stained with BLT1 antibody (A), BLT2 antibody (B) and isotype control (C).
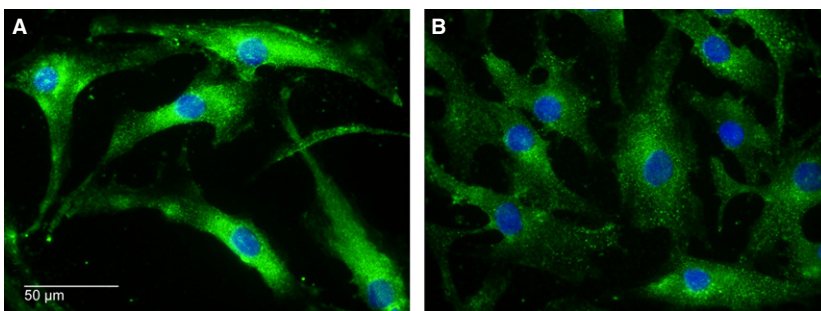

Fig. 2 Expression of BLT1 and BLT2 in cultured chondrocytes. Immunofluorescence microscopy of cultured chondrocytes stained with BLT1 antibody (A), BLT2 antibody (B). Secondary antibody conjugated with Alexa Fluor488 and DAPI-stained nuclei. Isotype controls had no staining (not shown). Scale bar: 50 μm.
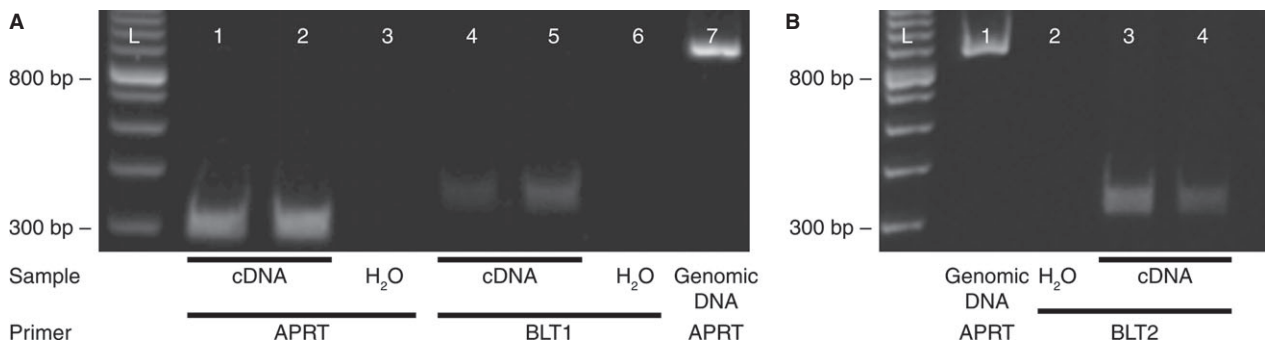

Fig. 3 Expression of BLT1 and BLT2 mRNA in cultured chondrocytes by RT-PCR. (A) Lane L: DNA ladder. Lanes 1 and 2: mRNA control, APRT primer yields 300-bp bands and no trace of the 800-bp bands that appears when APRT is primed to genomic DNA (Lane 7). Lanes 4 and 5: cDNA from two donors and specific BLT1 primers yield 345-bp bands. Lanes 3 and 6: negative control. (B) Lane L: DNA ladder. Lane 1: mRNA control. Lane 2: negative control. Lanes 3 and 4: cDNA from two donors and specific BLT2 primers yield 320-bp bands.
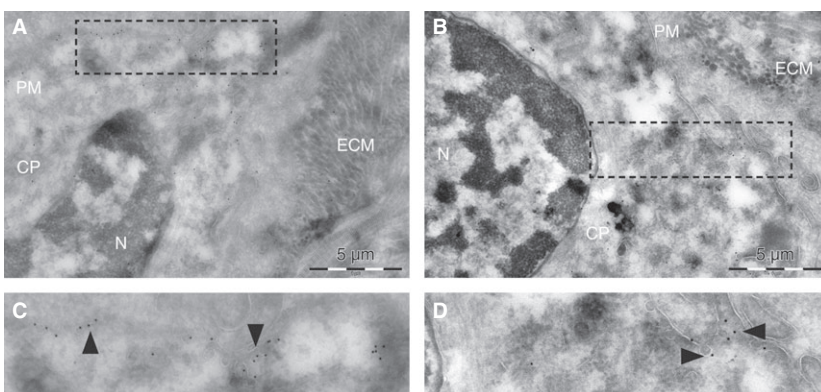

Fig. 4 Subcellular localization of BLT1 receptors in cartilage cells by immune-cryoelectron microscopy. (A and B) Two different cartilage sections stained with gold-conjugated anti-BLT1 antibody binding to BLT1 receptor in plasma membrane. (C and D) Enlarged windows of (A) and (B), respectively. Gold particles are marked with arrow heads. CP, cytoplasm; ECM, extracellular matrix; N, nucleus; PM, plasma membrane. Scale bars: 1 μm.

A Healthy vs. disease — **BLT1** — *P*-value < 0.001 — Non-OA / OA

B **BLT2** — *P*-value < 0.001 — Non-OA / OA

C Tissue vs. culture — **BLT1** — *P*-value = 0.018 — Cartilage / Culture

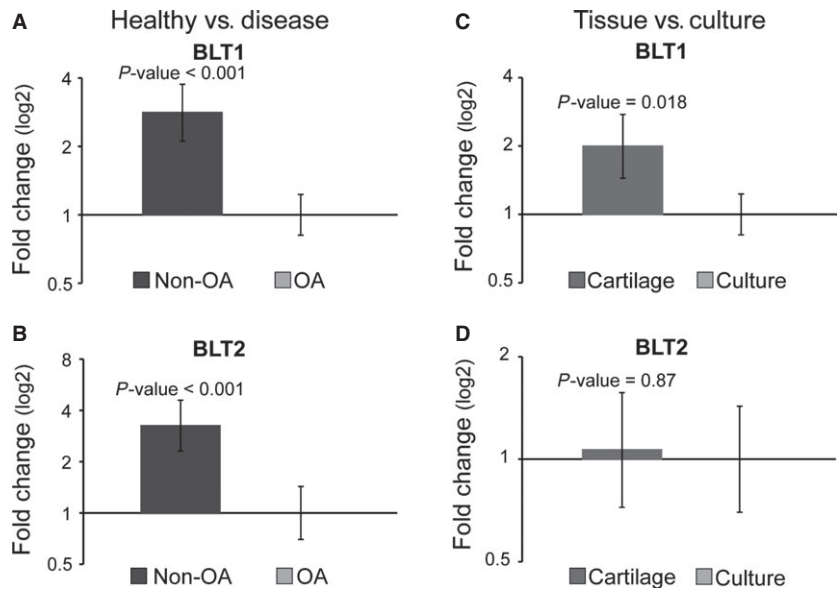**D** **BLT2** — *P*-value = 0.87 — Cartilage / Culture

Fig. 5 Differential expression of BLT1 and BLT2 mRNA in cartilage cells from osteoarthritis (OA) vs. non-OA. Expression of BLT1 (A) and BLT2 (B) in cultured cells from five healthy ACI donors (Non-OA) compared with cultured cells from five TKR (OA) donors. (C and D) A comparison of cartilage from TKR to cultured cells from TKR for BLT1 and BLT2, respectively.
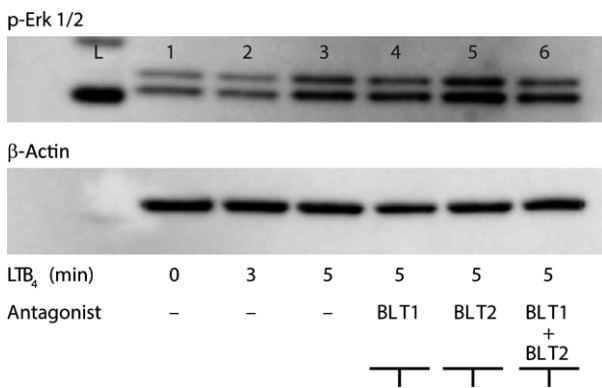

Fig. 6 Functional assessment of BLT receptors by Western blot. p-Erk 1/2 (44/42 kD). Lane L: biotinylated ladder. Lanes 1–3: Erk 1/2 phosphorylation at 0, 3 and 5 min exposure to leukotriene B$_4$ (LTB$_4$). Lane 4: phosphorylation of Erk 1/2 in the presence of BLT1 receptor antagonist (U-75302 at 3×10^{-7} M). Lane 5: phosphorylation of Erk 1/2 in the presence of BLT2 receptor antagonist (LY2552833 at 10^{-6} M). Lane 6: phosphorylation of Erk 1/2 in the presence of both receptor antagonists. Beta-actin (45 kD) was used as loading control.
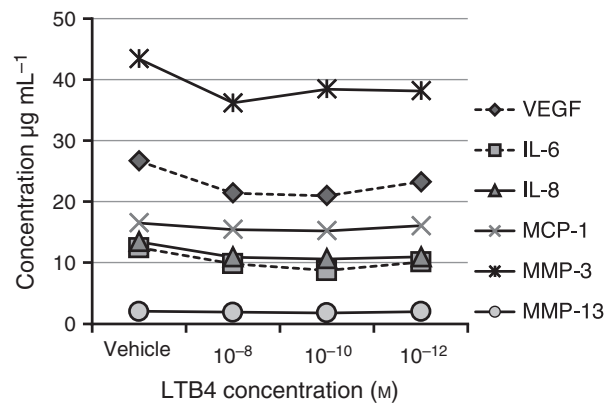

Fig. 7 Secretion of growth factors, matrix metalloproteinases (MMPs) and inflammatory mediators after stimulation of chondrocytes by leukotriene B$_4$ (LTB$_4$). Levels measured in supernatants from cultured chondrocytes after 24 h of stimulation with LTB$_4$ at concentrations of 10^{-12} M, 10^{-10} M, 10^{-8} M and vehicle. IL, interleukin; MCP, monocyte chemoattractant protein; VEGF, vascular endothelial growth factor.

whereas concurrent blocking of the receptors conferred the blocking of BLT1 alone (Lane 6).

Having confirmed the expression of functional BLT1 in chondrocytes, we assessed the potential effects of LTB$_4$ on major biological parameters in subsequent experiments. A multiplex analysis of supernatants of chondrocytes cultured in the presence of LTB$_4$ at increasing doses revealed no significant change in the secretion of growth factors, pro-inflammatory cytokines or MMPs. Levels of bFGF, PDGF, IL-1β, TNF-α, MMPs 1, 2, 8 and 9 were undetectable, with values falling under the detection limit of the assay in both the treated and untreated groups. VEGF, IL-6, IL-8, MCP-1 and MMPs 3 and 13 were detected, although LTB$_4$ did not

significantly alter the secretion of these molecules when analysed using ANOVA (Fig. 7).

To assess the potential effect of LTB$_4$ on chondrocytes differentiation, the expression of some cartilage signature genes, including aggrecan, Sox-9, collagen 1A1 and collagen 2A1, were analysed using qPCR. Four biological replicates were included, and data were normalized against GAPDH. Relative gene expression in chondrocytes stimulated with 10^{-8} M LTB$_4$ normalized to untreated controls is illustrated in Fig. 8. Furthermore, stimulating cultured chondrocytes with LTB$_4$ did not alter the expression of specific cartilage signature matrix genes.

Lastly, the cartilage-forming capability of cultured chondrocytes was assessed in the presence or absence of 10^{-8} M LTB$_4$. After 6 days, tissue with the characteristics of 'imma-
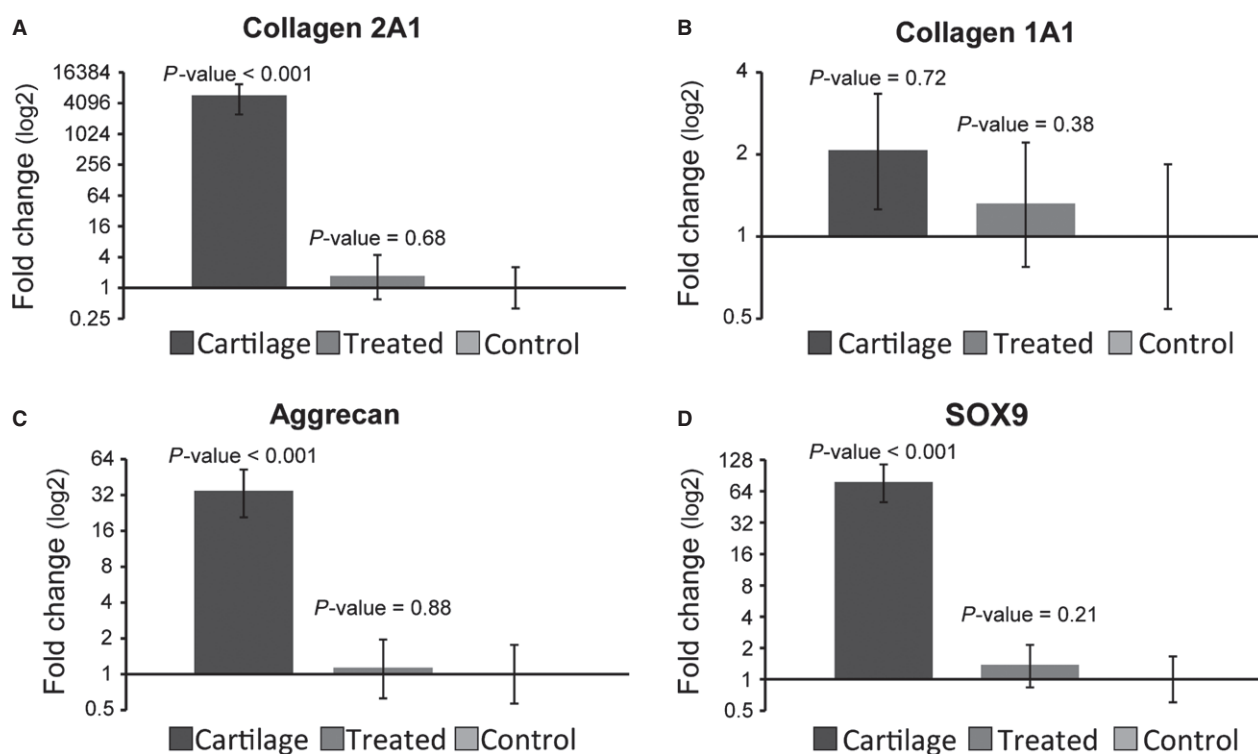
Fig. 8 Cartilage signature gene expression by qPCR. Relative expression of cartilage signature genes in cultured chondrocytes challenged with LTB$_4$ for 18 h. The results are normalized to untreated chondrocytes. A cartilage sample was included to visualize expression in cartilage vs. cultured cells. *P*-values are a result of *t*-testing cartilage vs. control and treated vs. control, respectively.

ture' cartilage was formed in both settings, revealing similar features regarding size, shape, the amount of extracellular matrix and cell density. Specific staining with Alcian blue for the detection of glycosaminoglycans, and with anti-collagen type II antibodies to evaluate the LTB$_4$ effect on matrix synthesis, demonstrated no observable differences (Fig. 9).

Discussion

A potential role of LTB$_4$ in RA and OA has been suggested based on cell culture and tissue models, exhibiting an increased production of LTB$_4$ in RA and OA joints, along with reports demonstrating the presence of BLT1 and BLT2 receptors in synovial tissue and subchondral bone (Paredes et al. 2002; Hashimoto et al. 2003; Attur et al. 2012). Moreover, LTB$_4$ receptor knockout mice were protected from RA development (Kim et al. 2006; Mathis et al. 2010). In the present study, we show for the first time that the LTB$_4$ receptors BLT1 and BLT2 are expressed by human chondrocytes, both *in vivo* and *in vitro*. Additionally, subcellular localization studies of cartilage tissue using cryo-immuno-electron microscopy unveiled a predominant expression of BLT1 at the plasma membrane in chondrocytes from native tissue.

One of the important findings of our study was the differential expression of BLT1 and BLT2 in chondrocytes from OA vs. healthy patients. A qPCR analysis revealed that both BLT1 and BLT2 expression was higher in cartilage cells from non-diseased joints. This downregulation of receptor expression in diseased tissue could represent a protective mechanism to help ameliorate tissue damage caused by chronic inflammation. Likewise, the expression of the BLT1 receptor diminished during cell expansion in monolayers (Fig. 5), with this outcome possibly being part of the overall cellular dedifferentiation process undergone by chondrocytes during expansion in monolayer cultures.

Previous studies with non-myeloid cells have reported that LTB$_4$ stimulation results in the downstream phosphorylation of signal transduction factors such as Akt and Erk 1/2, as well as the subsequent enhancement of migration and proliferation (Lundeen et al. 2006; Watanabe et al. 2009). In the present study, we could see evidence of an increased downstream phosphorylation of the BLT1 receptor when chondrocytes were stimulated with LTB$_4$. The signal appears to be mediated via the BLT1 receptor, as blocking with the BLT1 receptor antagonist U-75302 led to less phosphorylation of Erk 1/2, while blocking BLT2 did not (Fig. 6).

Earlier studies have shown that chondrocytes secrete a wide range of growth factors, cytokines and MMPs (Berg et al. 2010; Polacek et al. 2010, 2011). A critical phenomenon associated with the onset and progression of OA is the enhanced expression of MMPs within the joint tissue (Goldring & Marcu, 2009), with pro-inflammatory cytokines
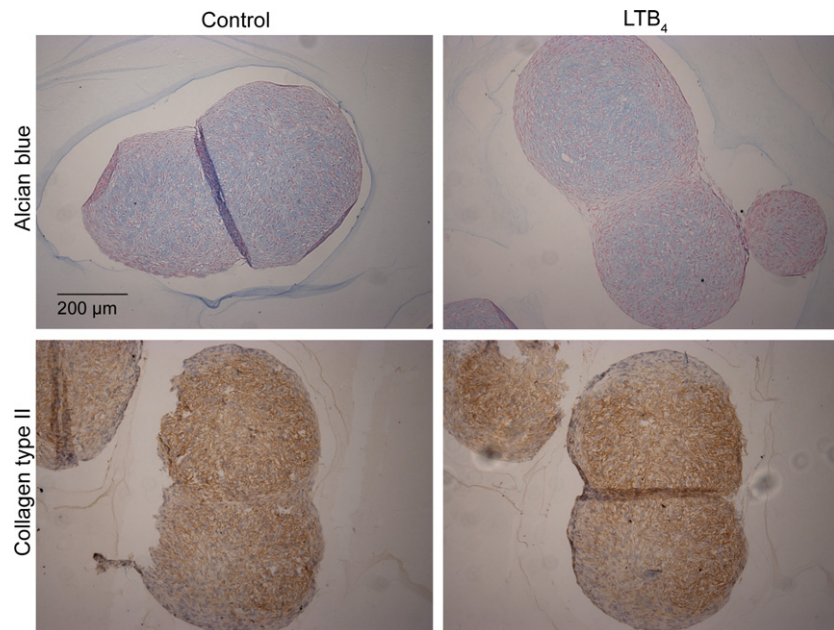
Fig. 9 Histology and immunohistochemistry of scaffold-free 3D chondrocyte spheroids. Chondrocyte pellets incubated for 6 days with leukotriene B$_4$ (LTB$_4$) at 10^{-7} M vs. unchallenged controls. The brown colour corresponds to expression of collagen type II, whereas the blue colour is indicative of matrix proteoglycans. The folds in the upper left and lower right image are artefacts of preparation. Scale bar: 200 μm.

such as IL-1β and TNF-α being strong inducers of MMP production and driving forces in cartilage deterioration (Martel-Pelletier, 2004). The endogenous synthesis of LTB$_4$ by synovium is a crucial element involved in the upregulation of IL-1β and TNF-α in OA (He et al. 2002). We therefore hypothesized that the secretion of MMPs, cytokines and/or growth factors would be affected by LTB$_4$ stimulation. In our analyses, MMP-3, MMP-13, VEGF, IL-6, IL-8 and MCP-1 were readily detected in culture supernatants, though their synthesis was not altered by LTB$_4$ at any dose tested (Fig. 7). The secretion of other mediators, including TNF-α and IL-1β, was below the detectable range. *In vivo*, the production of MMPs is promoted by IL-1β and TNF-α (Martel-Pelletier et al. 2004). Thus, a reasonable assumption is that the levels of these pro-inflammatory cytokines were insufficient in our assays, or that other synergistic factors required were not present in our *in vitro* systems.

The most prominent physiological role of chondrocytes is the homeostasis of the extracellular matrix and the maintenance of cartilage integrity (Goldring & Marcu, 2009). In this regard, the gene expression of specific cartilage matrix molecules is used as a functional marker of chondrocyte phenotype and differentiation status. It has previously been reported that in cultured chondrocytes stimulated with PGE$_2$, aggrecan gene expression was significantly downregulated, whereas the gene encoding collagen type II remained unchanged (Attur et al. 2008). Our data revealed an unaltered gene expression of collagen type 1A1, collagen type 2A1, aggrecan or Sox9 after 18 h of incubation with LTB$_4$ (Fig. 8). The decreased expression of BLT1 receptors observed after monolayer expansion might be the cause of the lack of effect upon LTB$_4$ challenge.

When chondrocytes are cultured in monolayers, they gradually dedifferentiate into a more fibroblast-like cell, a transformation characterized by an upregulation of collagen I and a downregulation of collagen II. In pellet cultures, the cells partially regain their original phenotypic traits in a process involving the downregulation of mitotic activity and the upregulation of matrix synthesis (Martinez et al. 2008). Intriguingly, the treatment of chondrocytes in 3D pellets with the PGE$_2$ has been shown to reduce collagen types I and II content (Jakob et al. 2004). We hypothesized that adding LTB$_4$ during 3D cultures could affect chondrocyte redifferentiation and matrix synthesis, but in our hands there was no marked difference in the morphological or immunohistochemical characteristics of the 3D constructs (Fig. 9).

Concluding remarks

We have demonstrated that human articular chondrocytes express LTB$_4$ receptors BLT1 and BLT2, and that at least the BLT1 receptor is functional, as shown by its capability to activate intracellular signalling pathways upon ligand binding. The physiological role of these receptors in human chondrocytes, particularly during joint inflammation, requires further investigation.

Conflict of interests

The authors have no competing interest to declare.

Authors contributions

AKH carried out the quantitative PCR, Western blotting, interpretation of data and the drafting of the manuscript. JTI performed the PCR, the immunoassays and the interpretation of the data. IM carried out the electron microscopy

and 3D culture assay, interpreted the data and drafted the manuscript. YF participated in the study design and coordination, helped to draft the manuscript, interpret the results and revise the manuscript. BS participated in the design and coordination, helped to draft the manuscript, performed the immunohistochemistry and revised the manuscript. All authors read and approved the final manuscript.

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