Inhibition of Leukotriene A_4 Hydrolase Suppressed Cartilage Degradation and Synovial Inflammation in a Mouse Model of Experimental Osteoarthritis

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Research Article

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Abstract

Chronic inflammation plays an important role in the osteoarthritis (OA) pathology but how this influence OA disease progression is unclear. Leukotrienes (LT) are biologically active lipids that are implicated in various pathological process. Leukotriene B4 (LTB_4) is a potent proinflammatory lipid mediator generated from arachidonic acid through the sequential activities of 5-lipoxygenase, 5-lipoxygenase–activating protein, Leukotriene A4 hydrolase (LTA_4H) and its downstream product leukotriene B4 (LTB_4 or BLT1). The aim of this study is to investigate the involvement of the LTB_4 pathway in OA disease progression. Both clinical human samples and mice experimental OA models were used to determine whether LTA_4H could serve as a potential therapeutic target for OA. We also determined whether the LTA_4H pathway was associated with cartilage degeneration and synovitis inflammation in OA mice models and human articular chondrocytes. We found that both LTA_4H and LTB_4 receptor BLT1 were highly expressed in human and mice OA cartilage. Inhibition of LTA_4H suppressed cartilage degeneration and synovitis in OA mice model. Furthermore, inhibition of LTA_4H promoted cartilage regeneration by upregulating chondrogenic genes expression such as aggrecan (ACAN), collagen 2A1 (COL2A1) and SRY-Box transcription factor 9 (SOX9). Our results indicate that the LTA_4H pathway is a crucial regulator of OA pathogenesis and suggest that LTA_4H could be a therapeutic target in combat OA.

Key Message

- LTA_4H and LTB_4 receptor BLT1 were highly expressed in human and mice OA cartilage.
- Inhibition of LTA_4H could suppress cartilage degeneration and synovitis in OA mice model.
- Inhibition of LTA_4H significantly promoted cartilage regeneration by upregulating chondrogenic genes in human chondrocytes.

Introduction

Osteoarthritis (OA) remains the leading cause of disability, morbidity, and mortality worldwide with no cure [1]. OA is characterized by cartilage degradation, osteophytes, subchondral bone sclerosis and synovitis, causing pain and reduced function of the affected joints. In the past few years, growing evidence has shown that non-resolved persistent synovitis is important in OA radiographic progression [2]. The proper shift from inflammatory to non-inflammatory status is essential to avoid tissue damage and the development of chronic inflammation [3]. Resolution of inflammation is an active process involving a molecular pathway switching from inflammatory eicosanoids and pro-resolving mediators. We demonstrated that promoting inflammation resolution by a pro-resolving lipid mediator, Resolvin D1, attenuated OA-like changes in a mouse model of post-traumatic OA [4]. Although the mechanism of persistent chronic inflammation in OA is incompletely understood, improper/inactivated resolution pathways may play an important role.

Inflammatory cascades in OA cartilage and synovial joints are associated with various cytokines and lipid mediators, mainly the eicosanoids and pro-inflammatory mediators [5]. Eicosanoid molecules produced mediators have been highlighted and linked to inflammatory and cellular metabolic responses in many studies, of which leukotrienes (LTs) are recognized as having a pivotal role [6–10]. Eicosanoids, including prostaglandins and LTs, are biologically active lipids implicated in various pathological processes, such as inflammation and cancer [11]. Leukotriene A4 hydrolase (LTA_4H) is classically recognized as an epoxide hydrolase to generate leukotriene B4 (LTB_4) from leukotriene A4 in response to inflammatory stimulus [12]. This enzyme is expressed mainly in neutrophils and other cell types, including endothelial cells and articular chondrocytes [13–15]. Leukotriene B4 is a pro-inflammatory lipid mediator generated from membrane phospholipid by the 5-lipoxygenase (5-LOX) enzymatic action at arachidonic acid (AA) during the initial phases of inflammation. LTB_4 regulates inflammatory cytokine expression and recruitment of neutrophils primarily through its G protein-coupled receptor BLT1, which is only activated by LTB_4 [16, 17]. The involvement of LTB_4 has been studied in various diseases, such as inflammatory arthritis and metabolic disorders [18, 19]. Given the central role of LTB_4 in regulating inflammatory response mechanisms through recruitment and activation of leukocytes, modulation of the LTB_4 pathway has been considered a new therapeutic approach in treating several diseases.

Previous studies have shown that an elevated level of LTB_4 is found in the synovium, synovial fluid, articular cartilage, and subchondral bone from patients with OA [20, 21]. It was also revealed that increased expression of LTA_4H occurs in human OA synovial fluid [5]. However, the relationship and mechanism of LTA_4H and OA have not been well studied. In the current study, we firstly investigated the expression of LTA_4H and BLT1 in OA cartilage. Based on these observations, we further studied the involvement of leukotriene in disease pathogenesis using an established OA model. We tested whether LTA_4H inhibitor can affect OA development and progression using both *in vivo* and *vitro* models.

Materials And Methods

Study approval

Animal studies were approved by the Institutional Animal Care and Use Committees and Institutional Biosafety Committees of Central South University, China (CSU; 2013-05). Male C57BL/6 mice were purchased from the Animal Centre of Central South University (Changsha, Hunan, China). At the start of the experimental protocols, mice were 6 weeks old. Collection of human OA tissues from discarded knee replacement surgeries were approved by the Queensland University of Technology (QUT) and the St Vincent Private Hospital Ethics Committees (Human ethics number: #1400001024). Informed consent was obtained from all human subjects.

Human cartilage specimen preparation

Human cartilage was obtained from patients with OA (Kellgren-Lawrence grade 4) undergoing total knee replacement. Osteochondral plugs (including cartilage and subchondral bone) were taken from the tibial

plateau region. All samples were prepared for histology and immunohistochemical analysis by washing in Phosphate-Buffered Saline (PBS) and immediately fixed in 4% paraformaldehyde (PFA). The samples were then decalcified in 10% ethylenediaminetetraacetic acid (EDTA, pH = 7.2-7.4). The specimens were embedded in paraffin, and 5 μm-thick sections were obtained as described in our previous studies [22].

Cell culture and bestatin treatment *in vitro*

Human chondrocyte cell line C28I2 was seeded into 6-well plates at a density of 1×10^6 cells/well and cultured in DMEM containing 10% foetal bovine serum (FBS) and 1% of penicillin and streptomycin (PS). At 90% confluence, cells were serum-starved for 24 h and then were incubated in the presence or absence of 10 ng/ml interleukin-1β (IL-1β) or 50 nM bestatin with culture medium for 24 h.

Cell viability test

Human chondrocyte cell line C28I2 was seeded into 96-well plates at a density of 5000 cells/well with culture medium. After 24 h, cells were serum-starved for 24 h and then were incubated in 0, 10, 50, 100, and 200 nM of bestatin separately. Cell viability test and cell count were performed according to our previous publication [23].

Preparation of bestatin

Bestatin was purchased from Cayman Chemical, 2-hydroxypropyl-β-cyclodextrin (HPCD) and polyethylene glycol 400 (PEG-400) was obtained from Sigma Aldrich. The oral formulation for bestatin was prepared as previously described [13]. Briefly, 100 μl PEG-400 and 100 mg of bestatin were gently mixed before dissolve in a 20% (w/v) aqueous solution of HPCD. Then, the solution was mixed at room temperature to obtain a homogeneous mixture at 2 mg/ml concentration.

Induction of OA in mice and bestatin treatment

Mice were group-housed (5 animals/cage) in ventilated cages in a temperature-controlled room on 12-h light/dark cycles with ad libitum access to standard mouse food and water and routine veterinary assessment. C57BL/6 mice (Animal Centre of Central South University, Changsha, Hunan, China) were randomly assigned and underwent destabilization of the medial meniscus (DMM) surgery to induce OA as previously described [22]. Briefly, OA was induced by dissecting the medial meniscus ligament to destabilize the medial meniscus in the knee joint of the hind limb on the right side. Mice in the sham group were surgically treated in which the ligament was visualized but not dissected. One week after experimental OA induction, mice were treated orally with 30 mg/kg bestatin [24] or vehicle (PEG-400 and HPCD). All experimental mice were then received twice-weekly oral bestatin after DMM surgery at a dose of 30 mg/kg for a total of 8 weeks. Mice were sacrificed 8 weeks after surgery for histological analysis.

Histologic evaluation of knee joint in mice

After euthanasia, the knee joints of hind limbs were fixed in 4% PFA, decalcified in 10% EDTA, embedded in paraffin and sectioned for histological and immunohistochemical analysis. Sections were stained with Safranin-O (Saf-O)/Fast green to evaluate cartilage degradation and synovitis. The severity of OA was assessed in the medial compartment of the knee using a modified Mankin scoring system (0-14) as previously described [22]. The degree of synovitis was scored using a 0-6 scoring system that measured the thickness of the synovial lining cell layer on a scale (0=1–2 cells, 1 = 2–4 cells, 2=4–9 cells and 3 = 10 or more cells) and cellular density in the synovial stroma on a scale of 0–3 (0 = normal cellularity, 1 = slightly increased cellularity, 2 = moderately increased cellularity and 3 = greatly increased cellularity). The sum of both parameters was used for analysis. Synovitis scores obtained from all four quadrants (lateral femur, lateral tibia, medial femur, and medial tibia).

Immunohistochemistry

Immunohistochemistry was performed according to our previously published methods [25, 26]. Sections were incubated overnight at 4°C with antibodies against LTA_4H (Abcam, Melbourne, VIC, Australia; dilution 1:250) and leukotriene B_4 receptor (BLT1, LSBio, Sapphire Bioscience, Redfern, NSW, Australia; dilution 1: 50) followed by incubation with anti-rabbit or anti-rat secondary antibody (1:5000 dilution; LI-COR Biosciences, Nebraska, USA) and visualization with 3,3-diaminobenzidine chromogen. The percentage of positive cells was determined by counting the number of immunostained cells and dividing by the total number of cells visualized by a hematoxylin counterstain using ImageJ (National Institute of Health, Bethesda, BA, USA).

Quantitative Real-time PCR

Total RNA was extracted with TRIzol, and cDNA was synthesized using the SensiFAST cDNA Synthesis Kit according to the manufacturer's protocol. SYBR Green detection chemistry was used on the QuantStudio Real-Time PCR system (Applied Biosystems, Thermo Scientific, Scoresby, VIC, Australia). Quantitative measurements of all primers used in this study were determined using ($2^{-\Delta\Delta Ct}$) method, and GAPDH expression were used as the internal control, as described previously by our group [25, 27–29].

Statistical analysis

Statistical difference of the results was tested using the unpaired Student’s t-test comparison of two variables. One-way ANOVA followed by post-hoc test was used for comparison of more than two variables. All analyses were performed using GraphPad Prism 7, and $p < 0.05$ is significant. All data are presented as mean ± SD.

Results

Increased LTA_4H and BLT1 in OA cartilage

To study the leukotriene A4 pathway in the articular cartilage, we first divided the cohort of OA patients into two groups based on a histomorphometry analysis for the semi-quantitative estimation of OA extent on Safranin-O-stained cartilage sections. As exemplarily shown in Fig. 1A for intact smooth cartilage surface (grade 1, G1) and severely degenerated and fibrillated OA regions (grade 4, G4), significant cartilage damage associated with elevations of Mankin score (12.14 ± 1.574, n=7) were observed in G4 OA sites; compared to G1 group (Mankin score = 3.286 ± 0.951; n=7) (Fig. 1B).

We then examined LTA_4H and BLT1 expression in OA cartilage of humans and mice DMM OA model. The percentage of LTA_4H immunostained chondrocytes were markedly elevated in OA-affected G4 cartilage compared with undamaged G1 regions of patient matched OA cartilages (Fig. 1A, C). BLT1 levels were also significantly increased in severe G4 versus mild G1 cartilage (Fig. 1A, D). To further ascertain whether leukotriene plays a role in OA pathogenesis, we examined LTA_4H and BLT1 expression in OA cartilage of mice. Similar results were found in experimental OA mice, in which LTA_4H and BLT1 were significantly increased in knee cartilage from DMM mice comparing to Sham surgery mice (Fig. 1E, F). These results indicated that the expression of LTA_4H and BLT1 is in accordance with the disease severity.

Inhibition of LTA_4H level suppressed OA progression in articular cartilage

The articular cartilage of mice with DMM-induced OA exhibited pathological OA changes characterized by Safranin-O loss and detectable cartilage roughness (Fig. 2A), with a Modified Mankin score of 11.833 ± 1.169 (Fig. 2B). In contrast, the knee joint of normal mice exhibited no visible changes on the articular cartilages with a significant lower Mankin score (0.857 ± 0.690) than that in mice with DMM-induced OA (Fig. 2A, B). Bestatin is a well-characterized inhibitor of LTA_4H and was shown to attenuate LTB_4 synthesis in several previous studies [30, 31]. Inhibition of LTA_4H level in mice with DMM-induced OA suppressed OA progression with a lower Mankin score (8.000 ± 1.095) compared to DMM-induced OA mice with vehicle (Fig. 2A, B). These results suggested that increased leukotriene may be one of the reasons causing OA as inhibition of which could suppress OA progress in DMM mice.

Inhibition of LTA_4H level reduced synovitis in OA mice

Mice with DMM-induced OA also revealed an increased influx of synoviocytes and lining layer thickening in synovium compared to the respective control mice (Fig. 3A). The synovitis scores reiterated these observations with a significant higher score in DMM-induced OA mice (Synovitis score: 4.417 ± 0.540 compared to 1.208 ± 0.485) (Fig. 3B). The role of leukotriene in synovitis in mice was examined by oral administration of LTA_4H inhibitor-bestatin. Bestatin treatment reduced the thickness of the synovial lining cell layer and cellular density in the synovial stroma in mice with DMM (Synovitis score: 2.917 ± 0.585) compared to vehicle-treated mice with DMM (Synovitis score: 1.625 ± 0.345) (Fig. 3A, B). However, oral administration of bestatin had no significant effect on synovitis in mice without DMM (Fig. 3A, B). These results indicated that bestatin treatment also reduced synovitis.

In vitro inhibition of LTA_4H significantly affect gene expression profile in chondrocytes

We further tested the effect of LTA_4H inhibitor on chondrocytes *in vitro*. To test whether bestatin plays a direct role on chondrocytes, we validated the potential effect of bestatin by gene expression profile analysis. We first tested cell viability under different concentrations of bestatin (Fig. 4A, B). Our results indicated that 0-200 nM of bestatin treatment showed no toxic effect on chondrocytes for 24 h. Next, we tested the effects of 50 nM bestatin treatment on chondrocytes phenotype. Bestatin treatment significantly upregulated COL2A1 and inhibited SOX9 expression in chondrocytes in the absence of IL-1β (Fig. 4D, F). No significant effects of bestatin on the expression of aggrecan (ACAN), Runt-related transcription factor 2 (RUNX2), a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) and MMP13 were observed (Fig. 4C, E, G **and H)**. To establish an *in vitro* OA model, the cells were stimulated with IL-1β (10 ng/ml). The expression of RUNX2 gene caused by IL-1β in chondrocytes was significantly inhibited by bestatin (Fig. 4E). The gene expression of ACAN, COL2A1 and SOX9 was robustly upregulated by bestatin, but there were no significant changes in the cartilage degradation marker, ADAMTS5 (Fig. 4G). In response to bestatin treatment MMP-13 showed a decreased gene expression however this effect was not significant (Fig. 4H).

Discussion

Growing evidence shows that the resolution of inflammation represents a critical therapeutic target in OA treatment [32]. The involvement of lipid mediators in inflammatory events has been brought into focus with several studies pointing its role in a multitude of cellular responses, including cell growth and death and inflammation via receptor-mediated pathways. [33]. A constitutive low-grade inflammatory response accompanies the OA, and the leukotriene signaling is now considered a potent chemoattractant agent related to acute inflammatory responses and maintenance of chronic inflammation [34, 35]. In this study, we examined the role of leukotriene pathway in the development of OA. We found that the level of LTA_4H and LTB_4 receptor BLT1 was increased in articular cartilage from OA patients and mice with post-traumatic OA. Moreover, inhibition of LTA_4H decreased cartilage tissue damage and synovial inflammation. These results indicate that upregulation of LTB_4 could be an important event in OA pathogenesis, and its upregulation may be involved in the mechanisms that trigger both cartilage degeneration and synovial inflammation.

Firstly, the production of LTA_4H and LTB_4 in OA has been previously shown. Paredes *et al*. have shown that human OA subchondral osteoblasts constitutively produce LTB_4, whereas He *et al.* and Lascelles *et al.* have demonstrated a high amount of LTB_4 in the synovial tissue from human and animal models of OA, respectively [20, 36, 37]. Overexpression of LTA_4H in human OA synovial fluid was also observed, and treatments with chemical inhibitors of 5-lipoxygenase- activating protein/5-lipoxygenase (FLAP/5-LOX) were shown to inhibit the severity of OA [5, 38, 39]. Our experiment found that human OA cartilage/chondrocytes expressed increased LTA_4H and LTB_4 receptor BLT1. These findings are in corroboration with a study performed by Martel-Pelletier *et al.* that revealed the presence of FLAP and 5-LOX (as determinants of LTB_4 synthetic capacity) in chondrocytes [39]. In the mouse model of post-traumatic OA, we observed significant changes in the cartilage damage and synovitis and correlated with

an increase in the expression of LTA_4H and BLT1. Similarly, Attur *et al.* showed that the level of lipoxygenase-derived products such as LTB_4 was found to be significantly increased in OA cartilage [40]. Although LTB_4 expresses mainly in inflammatory and immune cells, including macrophages, granulocytes, mast cells, T and B lymphocytes, our and other studies all demonstrate that the inflammatory leukotrienes are also produced by human OA chondrocytes [40]. LTB_4 is stimulated by diverse signaling molecules, including pro-inflammatory cytokines, such as Tumor necrosis factor-α (TNF-α) and IL-1β; and itself [12]. The LTB_4 synthesis in OA chondrocytes is increased by growth factors such as Transforming growth factor β1 (TGFβ1) and 1, $25(OH)_2D_3$ in the inflammatory response [39]. Thus, LTA_4H and LTB_4 in OA tissues could be considered an important event to the OA pathogenesis and pathophysiology.

The inflammatory process in diseases is due to an imbalance between pro-inflammatory and anti-inflammatory molecules and a deficiency of bioactive lipid mediators in the resolution phase of inflammation [17]. It has been reported that LTB_4 contributes to persistent inflammation in arthritis by delivering IL-1 into the joint through neutrophils [41]. More recent studies have shown that $BLT1^{-/-}$ mice were protected entirely from collagen-induced arthritis development and systemic insulin resistance in diet-induced obese mice [18, 19]. Our previous study has shown that intra-articular treatment of pro-resolving lipid mediator resolvin D1 diminishes the progression of OA in mice [4]. Leukotriene has also been shown to increase the production of pro-inflammatory cytokines, IL-1β and TNF-α in dose-dependent human OA synovial membrane [36, 42]. Modulating the secretion of lipid mediators may therefore attenuate the severity of the disease. In this study, we considered the LTA_4H as the candidate to investigate the effect of leukotriene activity on OA progression since it is the essential enzyme that catalyzes the distal step in LTB_4 generation. Blocking LTA_4H by bestatin *in vivo* has shown protective and preventive effects in various diseases such as pulmonary hypertension and oesophagal adenocarcinomas [13, 43]. Another advantage of bestatin is the low toxicity [43]. Therefore, we used the LTA_4H inhibitor bestatin and tested if inhibiting LTB_4 production has beneficial effects in mice with post-traumatic OA. In our study, bestatin significantly inhibited the presence of LTA_4H and BLT1 production in articular cartilage of mice with DMM-induced OA. Our results indicate that LTA_4H inhibition decreases cartilage degradation and synovitis in a mouse model of post-traumatic OA. This decrease in synovial inflammation could be because LTB_4 is a critical mediator in the cascade of complement, lipids, cytokines, and chemokines. In BLT1 knock out mice, LTB_4 has shown crucial roles in T helper 2 (Th2)-type cytokine interleukin-13 (IL-13) production from lung T cells and recruitment of antigen-specific effector $CD8^+$ T cells [44]. In inflammatory arthritis, it first initiates and then continues neutrophil inflammation [45]. Therefore, blockade of the LTB_4-BLT1 pathway might be a promising therapeutic strategy in OA patients. However, clinical trials to date have shown that compared with traditional disease-modifying antirheumatic drugs, oral LTB_4 receptor and 5-LOX inhibitors have little clinical benefit [46] however whether or not this is the case with OA patients' needs further investigation.

In conclusion, our study applied oral LTB_4 receptor inhibitor bestatin for feeding mice after OA surgery. Reduced cartilage damage and synovium inflammation were observed in mice joints after 8 weeks, indicating that oral bestatin uptake in early OA might be a potential drug for OA therapy targeting Leukotriene A_4 hydrolase.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Author contribution

Conceptualization: I.P., X.M., R.C.; methodology and formal analysis: X.W., I.P., A.S.; drafting of the article: A.S., X.W., I.P.; review and editing: All authors; supervision: I.P.; Acquisition of funding: I.P., X.M. Y.W.. All authors have read and agreed to the published version of the manuscript.

Ethics approval

Animal studies were approved by the Institutional Animal Care and Use Committees and Institutional Biosafety Committees of Central South University, China (CSU; 2013-05). Collection of OA tissues from discarded knee replacement surgeries were approved by the Queensland University of Technology and the St Vincent Private Hospital Ethics Committees (Human ethics number: #1400001024).

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Figures

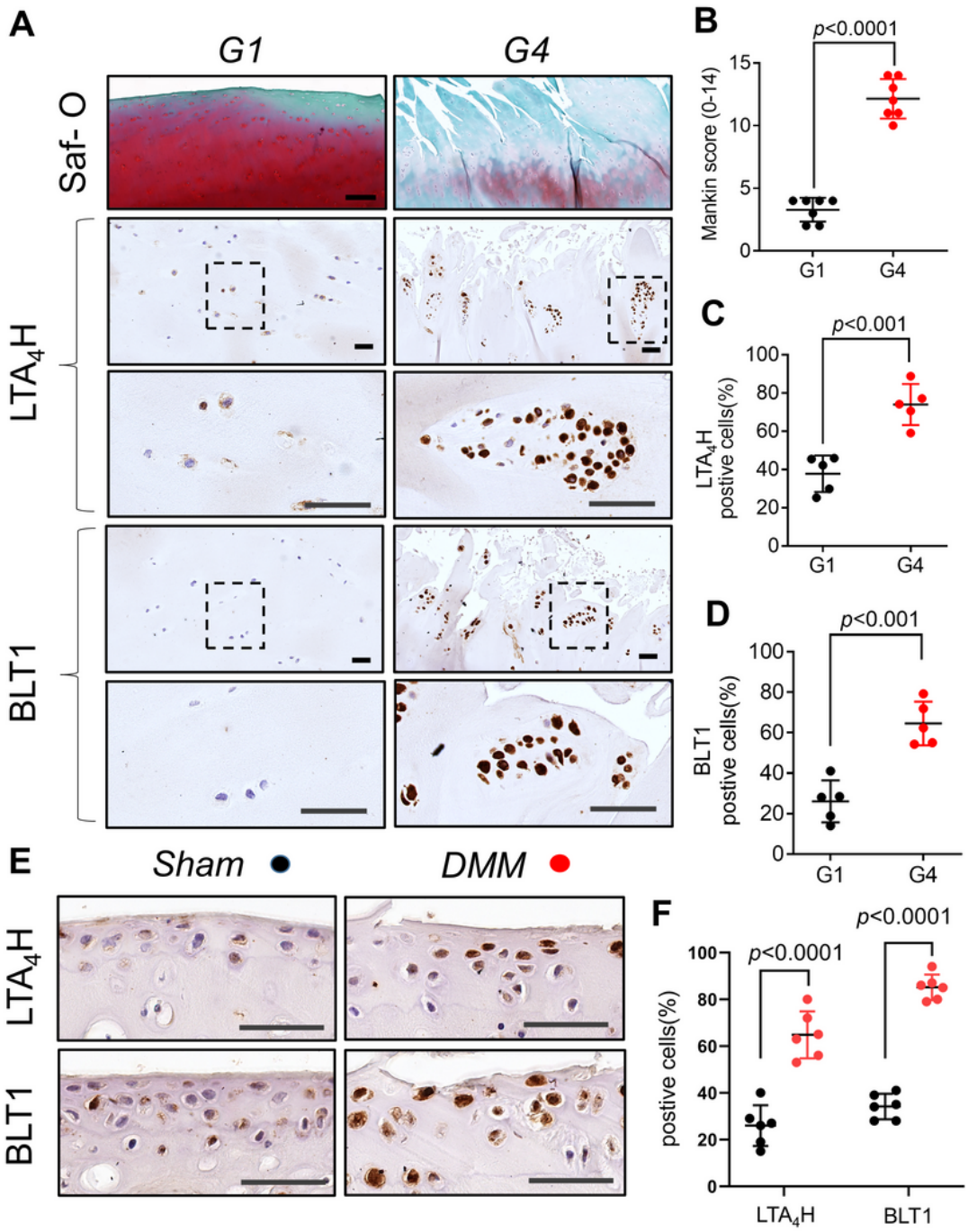

Figure 1

Increased leukotriene A4 hydrolase (LTA_4H) and leukotriene B4 receptor 1 (BLT1) in OA cartilage

(A) Representative Safranin-O (Saf-O) and fast green stained sections of G1 or G4 knee regions. Similar sections were stained with LTA_4H and BLT1. Scale bars, 200 μm. **(B)** Severity of articular cartilage degradation was graded using Mankin scoring system. Graphs represent mean ± SD (n=7). The

percentage of LTA_4H **(C)** and BLT1 **(D)** positive cells per knee section were counted. Graphs represent mean ± SD (n=5). **(E)** Sham and DMM mice knees were stained with LTA_4H and BLT1 (n=6). Positive cells per knee section were counted **(F)**. Scale bars, 20 μm. Graphs represent as mean ± SD. $p < 0.05$ is considered significant. DMM, the surgical destabilization of the medial meniscus.

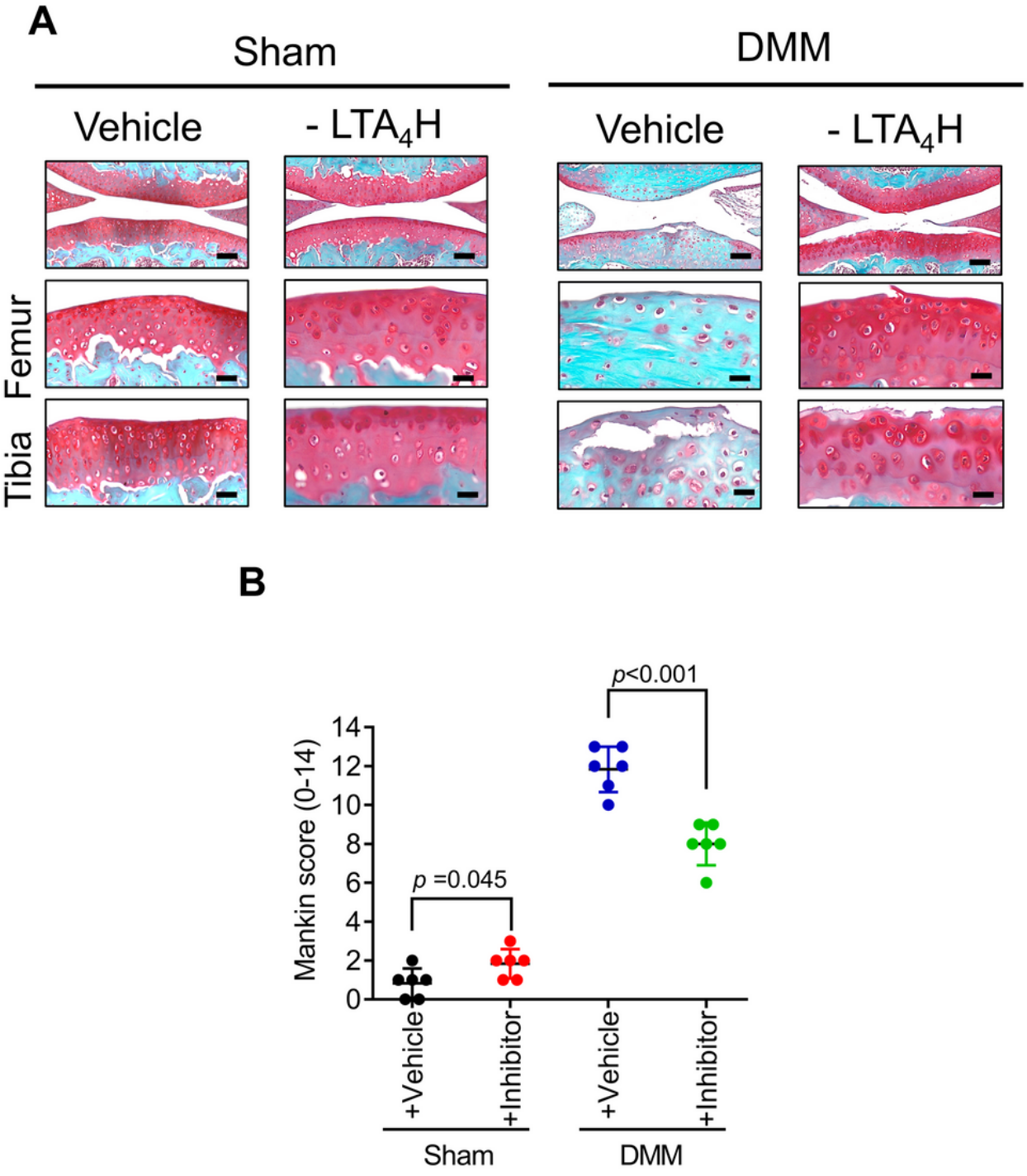

Figure 2

Inhibition of LTA_4H level suppressed OA progress in articular cartilage

(A) Representative Saf-O and fast green stained sagittal sections of sham and DMM mice knee with/without LTA_4H inhibitor. Scale bars in the top panel, 100 μm; bottom panel, 20 μm. (**B)** Severity of articular cartilage degradation was graded using Mankin scoring system. Graphs represent mean ± SD (n=6). $p < 0.05$ is considered significant.

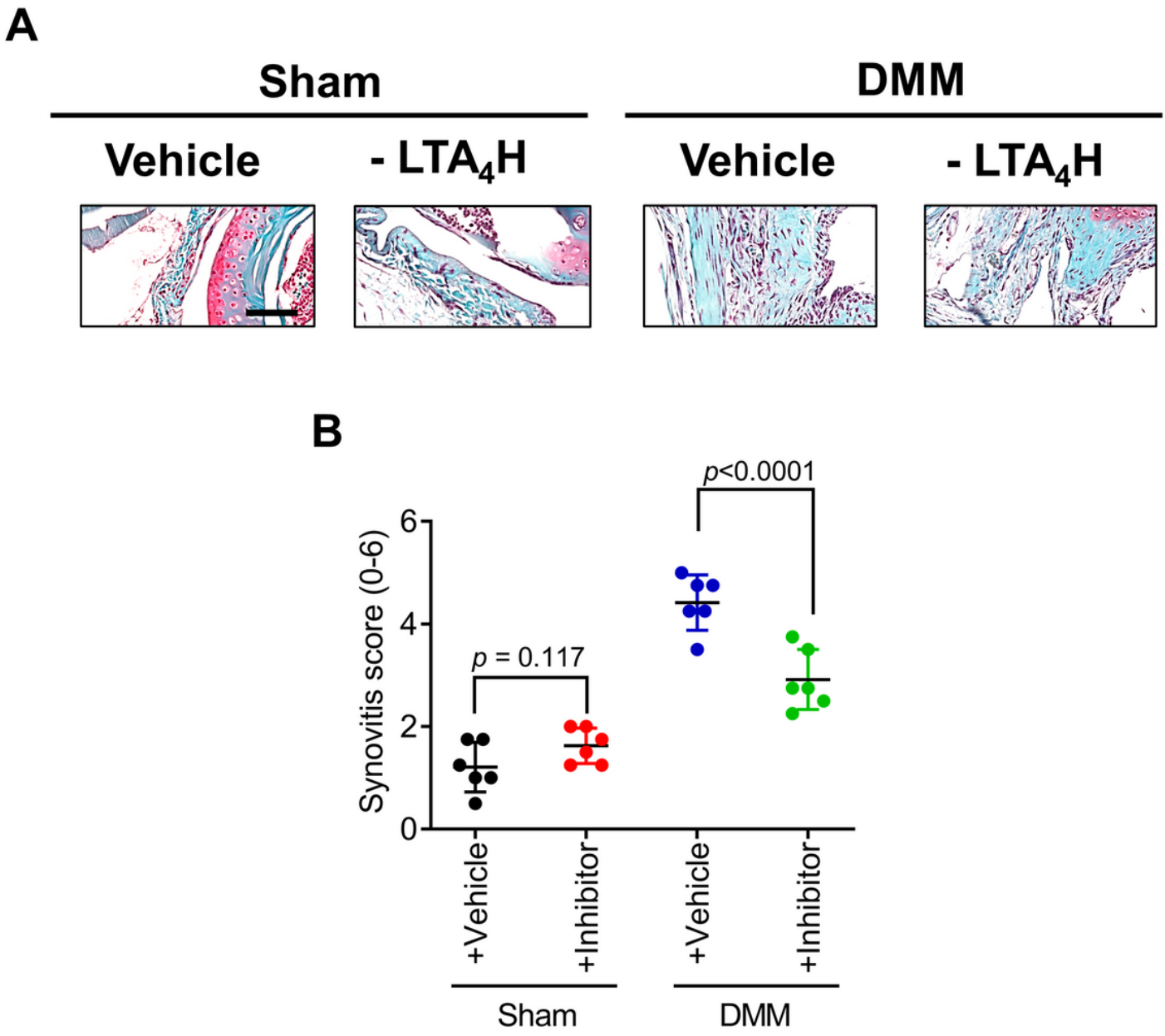

Figure 3

Inhibition of LTA_4H level reduced synovitis in OA mice

(A) Representative Saf-O and fast green stained sagittal sections of sham and DMM mice knee with/without LTA_4H inhibitor. Scale bars, 100 μm. **(B)** Severity of articular synovitis inflammation was graded using the Synovitis scoring system. Graphs represent mean ± SD (n=6). $p < 0.05$ is considered significant.

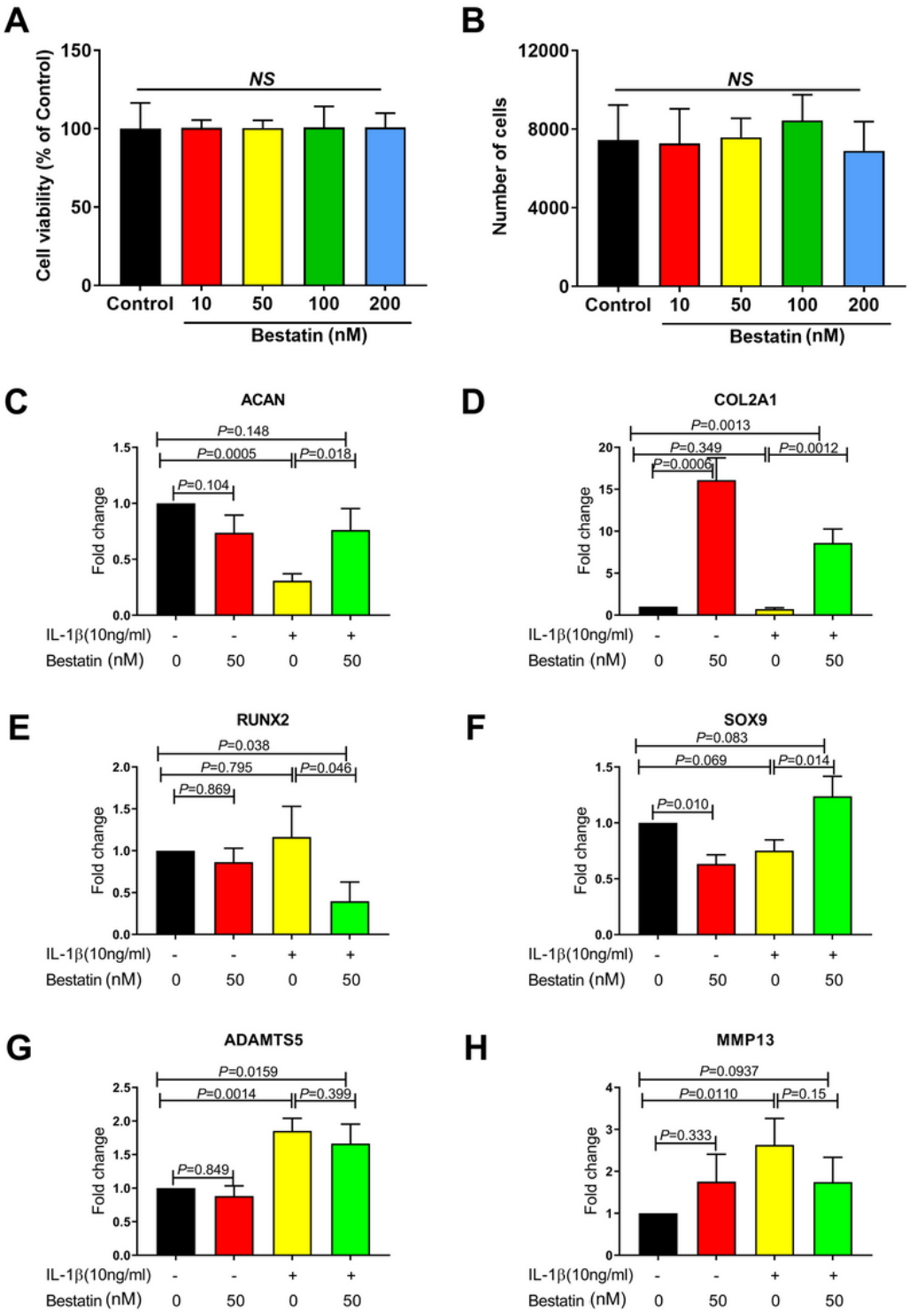

Figure 4

***In vitro* inhibition of LTA_4H significantly affect gene expression profile in chondrocytes.**

Cell viability **(A)**, cell counts **(B)** and qPCR analysis of ACAN **(C)**, COL2A1 **(D)**, RUNX2 **(E)**, SOX9 **(F)**, ADAMTS5 **(G)** and MMP13 **(H)** mRNA in C28I2 chondrocytes cell line with/without LTA_4H inhibitor under normal and inflammation condition (IL-1β treatment). Data represented as mean ± SD (n=6). $p < 0.05$ is considered significant.