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Original Article

β1,4-galactosyltransferase-I protects chondrocytes against TNF-induced apoptosis by blocking the TLR4 signaling pathway

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Abstract: Osteoarthritis (OA) is the most common degenerative disease of the cartilage and is characterized by inflammation of the synovial membrane and subchondral osteosclerosis. β1,4-galactosyltransferase-I (β1,4-GalT-I) is a crucial regulator of inflammation based on its role in the stimulation and sustenance of inflammation in OA. In the present study, we aimed at elucidating the expression pattern and potential biological activity of β1,4-GalT-I in chondrocytes isolated from OA patients. Chondrocytes were isolated from the cartilage and cultured. Western blotting and quantitative real-time polymerase chain reaction (qRT-PCR) were used to analyze β1,4-GalT-I expression. Isolated chondrocytes were stimulated with tumor necrosis factor (TNF). Our results indicate significantly enhanced expression of β1,4-GalT-I in cultivated chondrocytes upon stimulation with TNF. β1,4-GalT-I inhibited the inflammation and cell death triggered by TNF. In addition, β1,4-GalT-Iinhibited the expression of Toll-like receptor 4 (TLR4) and phosphorylation of p65 and IKK. In conclusion, our findings suggest the protective effect of β1,4-GalT-I in chondrocytes against OA induced by TNF based on its ability to block the TLR4 signaling pathway. Our results also indicate significant contribution of β1,4-GalT-I towards the anti-inflammation in the cartilage of patients suffering from OA.

Keywords: β1,4-galactosyltransferase-I, osteoarthritis, chondrocytes, apoptosis, TLR4, inflammation

Introduction

Osteoarthritis (OA) is the most common degenerative disease that affects the human articular cartilage, especially in adults over 65 years of age [1]. In the United States, approximately 37% of adults from OA [2]. OA is characterized by extracellular matrix (ECM) damage and loss of tissue cellularity [3]. Programmed cell death of chondrocytes significantly contributes to the etiology of OA cartilage degeneration [4]. Damaged or injured chondrocytes stimulate the production of reactive oxygen species (ROS) and release of inflammatory factors such as interleukins (ILs) and tumor necrosis factor (TNF), both of which accelerates their cell death [5, 6].

Chondrocytes are the only type of mature cells present in a normal articular cartilage and are associated with the generation of ECM and regulation of ECM turnover [7]. Thus, tissue homeostasis and integrity of the cartilagerelies on the cellular and biochemical activity of chondrocytes [8]. In adults, the articular cartilage tissue is produced by mitosis. Chondrocytes in adults have limited proliferative capacity and are activated only in response to injury, emphasizing the importance of chondrocyte viability in sustaining the activity and structure of the cartilage [9]. Joint diseases including OA cause chondrocyte apoptosis [10], a crucial contributor to the etiology of OA [11]. Progression of OA is determined by chondrocyte survival and theirability to resist cell death. Accordingly, chondrocyte cell death by apoptosis was found to be associated with the typical degeneration of cartilage seen in OA [12]. Thus, inhibition of chondrocyte cell death might prove to be an innovative strategy to avoid cartilage damage and to preserve its integrity.

Recently, several studies have focused on elucidating the molecular mechanism involved in

OA. Accordingly, certain crucial modulators and possible treatment targets for OA have been identified, namely, matrix metalloproteinases (MMPs) [13], nuclear factor kappa light chain enhancer of activated B cells (NFκB), microRNAs (miRs) [14], and possible involvement of mitogen-activated protein kinase (MAPK) pathway [15]. β1,4-galactosyltransferase-I (β1,4-GalT-I) is a member of the big GalT family consisting of seven members, Gal-T1 to Gal-T7 [16]. Residing in the Golgi apparatus, it adds the galactose from uridine diphosphate-galactose (UDP-Gal) donor in β1,4-linkage to the terminal N-acetylglucosamine (GlcNAc) carbohydrate chain to generate β4-N-acetyllactosamine (Galβ1,4GlcNAc). A part of β1,4-GalT-I present on the cell surface serves as a cell adhesion molecule in multiple cellular reactions by binding oligosaccharide substrates or ligands consisting of N-acetylglucosamine in the ECM [17]. β1,4-GalT-I is associated with various biological processes, such as embryonic maturation, reactions between sperm and egg, neurite extension, compaction of embryo to become morula, metastasis of malignant embryonic cells, and migration of mesenchymal cells, wherein, it modulates specific adhesion between cells or between cells and the matrix [18-20]. Under pathological conditions, β1,4-GalT-I acts as a crucial regulator of inflammation asit stimulates and sustains inflammation [21, 22]. However, the expression of β1,4-GalT-I and its biological function during inflammation in chondrocytes in OA patients remains to be elucidated.

Materials and methods

Isolation, culture, and stimulation of chondrocytes

Chondrocytes isolated from the cartilage tissue of OA patients were cultured as previously described. Briefly, cartilage tissues were cut into smaller pieces and the fragments were digested in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand. Island, N.Y., USA) containing 0.2% collagenase (Sigma, St. Louis, MO) for 2 h at 37°C. The isolated cells were cultured overnight in DMEM supplemented with 100 U/mL penicillin-streptomycin and 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD). After incubation, cells were washed to remove non-adherent cells, and the adherent cells were cultured in fresh culture medium. The resultant first-passage cells were used for further investigation. This study has been approved by the Ethics Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University. All study participants had given their written informed consent before participating in the study.

Cells were plated at a density of 1×10^4 cells/mL and cultured at 37°C with 5% CO_2. The culture medium was changed every 48 h. Cells were passaged when they reached 90% confluency. Cells incubated in culture medium containing 100 ng/mL (final concentration) human recombinant TNF (Peprotech, Rocky Hill, NJ) for 24 hwere used for subsequent assays.

Transfection of chondrocytes

Plasmids pcDNA3.1 (Vector) and β1,4-GalT-I-pcDNA3.1 (β1,4-GalT-I) were purchased from Shanghai Gene Pharma Co., Ltd (Shanghai, People's Republic of China). A day prior to transfection, isolated human chondrocytes were seeded at a density of 4×10^5 cells/well in 6-well plates. Transfection admixture was prepared by dissolving 4 μg of plasmid DNA and 3 μL of Turbofect reagent (Fermentas, Glen Burnie, MD, USA) in 500 μL of DMEM/F12 medium without serum by gentle pipetting. The prepared transfection admixture was added to the culture medium 20 min following incubation, and the cells were further incubated for 6 h.

Cell survival assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay was used to analyze cell survival. Chondrocytes were seeded in 96-well platesand incubated for 24 h at 37°C. At the indicated time points, 10 μL of MTT solution (5 mg/mL, Beyotime Institute of Biotechnology, Shanghai, China) was added to each well and incubated for 4 h at 37°C. Following incubation, 150 μL of dimethyl sulfoxide (DMSO, Beyotime Institute of Biotechnology) was added to each well and the plates were subjected to vibration for 10 min for dissolving the formed formazan crystals. The absorbance (A) was measured at 570 nm. All experiments were repeated three times, and average values were used for plotting the proliferation curve.

Flow cytometry (FCM) analyses

Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, China) was used to

analyze cell death. Briefly, cells were seeded at a density of 10^5 cells/well in 6-well plates. Cells were washed twice with cold PBS, resuspended, treated with 5 μL of Annexin V-FITC and 10 μL of PI, and incubated for 1 h at room temperature in dark. Floating and adherent cells were analyzed on a flow cytometer (Beckman Coulter, USA) using the FlowJo software (Tree Star, Inc., USA) to detect apoptotic cells.

RNA Isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using the TRIzol reagent (Life Technologies) and purified using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was carried out using the Superscript III kit (Life Technologies). Complementary DNAs were analyzed by qRT-PCR. Primers used in the present study were as follows: internal control GAPDH, 5'-GCAAGTTCAACGGCACAG-3' (sense), and 5'-GCCAGTAGACTCCACGACATA3' (antisense); TGF-β, 5'-TACAACTGCTTTGTGTTCAGTGATG-3' (sense) and 5'-GCAGGCTAAACCCGAACTTG-3' (antisense). Expression of the relative messenger RNA (mRNA) was quantified by qRT-PCR using SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). For the detection of p38, the PCR cycling included 40 cycles of denaturation at 95°C for 45 sec, annealing at 65°C for 45 sec, and extension at 72°C for 2 min. All experiments were carried out in triplicate and were subjected tothree independent runs. Gene expression was evaluated usingthe Real-Time StatMiner (Integromics, Madrid, Spain). Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA)

Inflammation promoting factors in the serum were analyzed using IL-6, IL-1β, IL-8, and TNF-α ELISA Kits (R&D Systems, USA) according to the manufacturer's instructions.

Western blotting analysis

Cell lysates were homogenized inlysis buffer (Beyotime, China). Protein quantification was carried out using the Bradford assay (Bio-Rad, Hercules, CA, USA). Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Enhanced chemiluminescence (ECL) plus detection reagent (Pierce, Rockford, IL) was used to measure immunoreactive bands and evaluation was done using Omega 16ic Chemiluminescence Imaging System (UltraLum, CA). Primary antibodies used in the study were as follows: rabbit anti-Phospho IKKα/β (2697, Cell Signaling Technology, USA), β-actin (1:1000, Sigma, USA), Bax (1:1000, CST, 2772S, USA), rabbit anti-Phospho-NF-κB p65 (3033, Cell Signaling Technology, USA), and Bcl-2 (1:1000, CST, 2876, USA).

Statistical analysis

Results are represented as mean ± SEM. Results were analyzed using a two-tailed, unequal-variance Student's t-test, or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Nonparametric test was applied to different assessments where necessary. $P < 0.05$ was indicative of a statistically significant difference.

Results

TNF induces enhanced expression of β1,4-GalT-Iin chondrocytes

Expression of β1,4-GalT-I at the transcriptional level in chondrocytes was analyzed by qRT-PCR. Expression of β1,4-GalT-I was significantly enhanced in the TNF group compared to that in the control group (**Figure 1A**). At the translational level, results of western blot analysis revealed significantly enhanced levels of β1,4-GalT-I protein in chondrocytes in the TNF group compared to that in the control group (**Figure 1B**).

β1,4-GalT-I inhibits TNF-triggered chondrocyte cell death

Treatment of chondrocytes with TNF significantly increased the number of dead cells. However, the number of apoptotic cells induced by TNF was significantly lower in the β1,4-GalT-I transfected group compared to that of the vector transfected group (**Figure 2A-C**). In addition, overexpression of β1,4-GalT-I promoted chondrocyte cell survival compared to that of the vector control group (**Figure 2D**). These results indicate inhibition of TNF-induced cell death in chondrocytesby β1,4-GalT-I.

β1,4-GalT-I inhibitsTNF-induced expression of apoptosis-related proteinsinchondrocytes

Expression levels of pro- and anti-apoptotic proteins are crucial for the process of cell

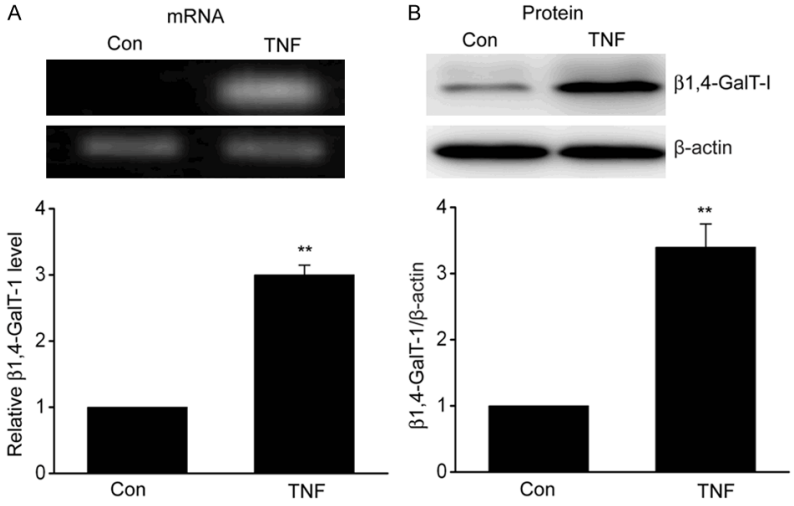

Figure 1. Enhanced expression of β1,4-galactosyltransferase-I (β1,4-GalT-I) in chondrocytes stimulated with tumor necrosis factor alpha (TNF-α). A. Expression of β1,4-GalT-I at the transcriptional level was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) in the TNF and control group. B. Representative immunoblots as well as quantitative analysis of β1,4-GalT-I in the TNF and control group. Results are represented as means ± SEM. $^{**}P < 0.01$ vs. control group (Student's t-test).

death. In the present study, we analyzed the role β1,4-GalT-I on the expression of proteins related to cell death in chondrocytes. In chondrocytes, TNF inhibited the expression of Bcl2, enhanced the expression of Bax, and stimulated the activation of caspase-3 (**Figure 3**). TNF induced expression of Bcl2 was significantly enhanced in the β1,4-GalT-I transfected group compared to that of the vector transfected group. In addition, overexpression of β1,4-GalT-I significantly suppressed the TNF-induced expression of Bax and stimulation of caspase-3 compared to that of the vector control group. These results prove that β1,4-GalT-I could inhibit the TNF-triggered chondrocyte cell death by inhibiting the expression of proteins associated with the cell death pathway.

β1,4-GalT-I inhibits TNF-induced inflammatory cytokines in chondrocytes

Recent research has revealed the role of inflammatory cytokines in accelerating chondrocyte cell death [23]. In the present study, we analyzed the role of β1,4-GalT-I on inflammatory cytokines in chondrocytes. Treatment with TNF enhanced the levels of IL-1β, IL-6, TNF, and IL-8 in chondrocytes. This TNF-induced increase of cytokines was inhibited in chondrocytes transfected with β1,4-GalT-I (**Figure 4A-D**). Thus, our results indicate that β1,4-GalT-I inhibited TNF-induced chondrocyte cell death by suppressing inflammatory cytokines.

β1,4-GalT-I inhibits TNF-induced stimulation of toll-like receptor 4 (TLR4) signaling pathway in chondrocytes

TLR4 signaling pathway plays an important role in inflammation and apoptosis. In the present study, we probed the influence of the overexpression of β1,4-GalT-I on the TLR4 signaling pathway. Results of western blot analysis revealed significantly enhanced expression of TLR4 and phosphorylation of p65 and IκB kinase (IKK) in chondrocytes treated with TNF (**Figure 5**). Overexpression of β1,4-GalT-linhibited the stimulation of TLR4 signaling. Taken together, our results prove the inhibitory role of β1,4-GalT-I onthe TLR4 signaling pathway.

Discussion

OA is the most common paralyzing degenerative joint disease that affects synovial joints causing cartilage injury, inflammation of the synovial membrane, and subchondral bone remodeling [24]. Inflammation of the synovial membrane occurring secondary to articularcartilage (AC) degradation is a primary event in the progression of OA [25, 26]. Earlier studies have reported β1,4-GalT-I to be crucial in promoting inflammation in the synovial tissue, both in patients and in collagen induced murine RA models [27]. Results of our present study indicate enhanced expression of β1,4-GalT-I in chondrocytes stimulated with TNF compared to that of the control. Thus, it can be speculated that β1,4-GalT-I might have a crucial impact on the cartilage in OA patients.

OA is characterized by apoptosis of chondrocytes in the joint cartilage [28]. Chondrocytes residing in the cartilage regulate the anabolic-catabolic balance that is required for matrix maintenance and tissue function [29]. Chondrocytes in OA patients are exposed to both

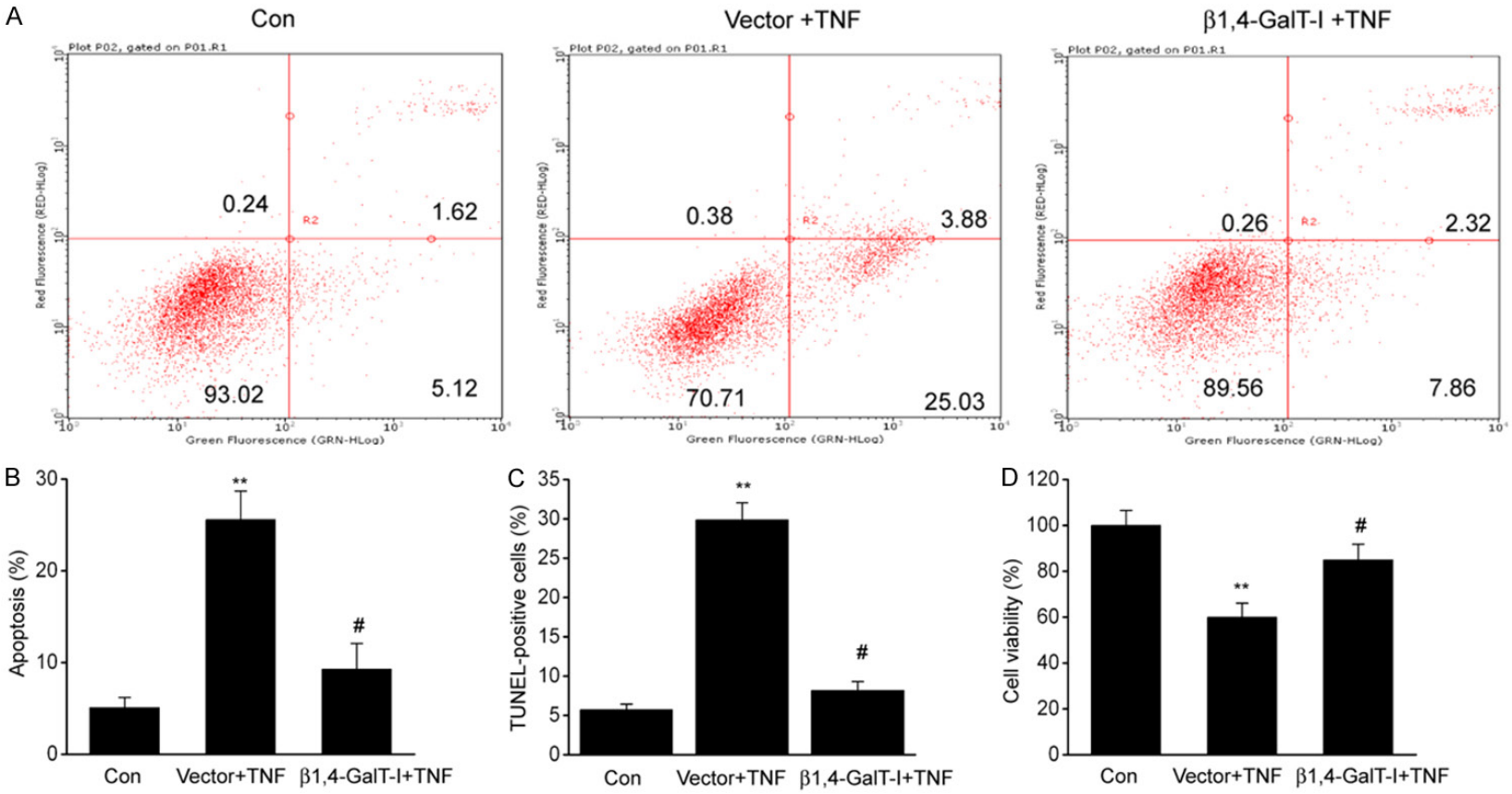

Figure 2. β1,4-galactosyltransferase-I (β1,4-GalT-I) inhibited tumor necrosis factor (TNF)-induced chondrocyte cell death. Chondrocytes were transfected with β1,4-GalT-I plasmid (β1,4-GalT-I) or empty vector (Vector) and stimulated with TNF. A. Representative image of cell apoptosis determined via flow cytometry. B. Quantification data for the apoptotic cells for each group. C. Evaluation of cell apoptosis by TUNEL assay. D. Evaluation of cell survival by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Results are represented as means ± SEM. $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ vs. vector group. One way ANOVA.

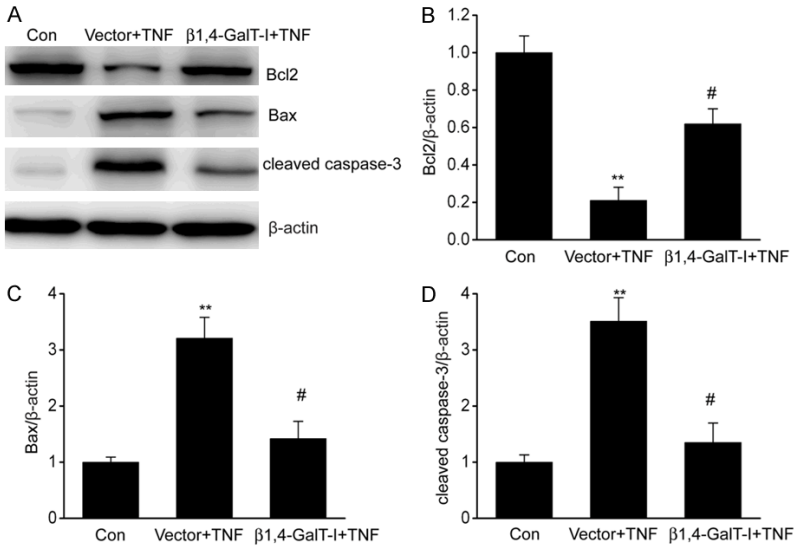

Figure 3. β1,4-galactosyltransferase-I (β1,4-GalT-I) inhibited tumor necrosis factor (TNF)-induced expression of apoptosis related proteins in chondrocytes. Chondrocytes were transfected with β1,4-GalT-I plasmid (β1,4-GalT-I) or empty vector (Vector) and stimulated with TNF. (A-D) Representative immunoblots (A) and quantitative analysis of Bcl2 (B), Bax (C) and caspase-3 (D) in chondrocytes. Results are represented as means ± SEM. $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ vs. vector group. One way ANOVA.

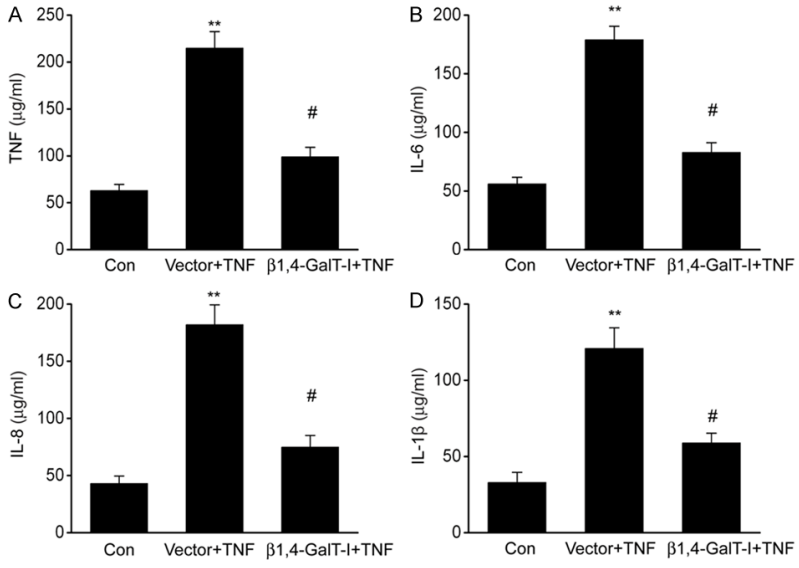

Figure 4. β1,4-galactosyltransferase-I (β1,4-GalT-I) inhibited tumor necrosis factor (TNF)-induced production of inflammatory cytokines in chondrocytes. Chondrocytes were transfected with β1,4-GalT-I plasmid (β1,4-GalT-I) or empty vector (Vector) and stimulated with TNF. (A-D) Analysis of TNF (A), interleukin (IL)-6 (B), IL-8 (C) and IL-1β (D) using enzyme linked immunosorbent assay (ELISA). Results are represented as means ± SEM. $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ vs. vector group. One way ANOVA.

physical and chemical stress such as high temperature, local inflammation, and unphysiological high-weight loading [30]. Till date, number of biological and chemical stress factors involved in the development and progression of OA etiology have been identified [31, 32]. In the present study, our results revealed that overexpression of β1,4-GalT-I could inhibit TNF-induced chondrocyte cell death by inhibiting the expression of cell death promoting proteins including Bax and active caspase-3 and by upregulating the expression of the cell death counteracting protein Bcl2. Our findings shed new insight onthe role of β1,4-GalT-I in the regulation of chondrocyte cell death in OA.

Development of OA is primarily characterized by inflammation. Results from recent research emphasize the impact of innate and acquired immune cells and inflammatory cytokines in the progression of OA [33, 34]. These immune cells responsible for phagocytosis and antigen presentation are crucial for the production of pro-inflammatory cytokines such as IL-6, TNF-α, IL-1β and oncostatin M [35]. In chondrocytes, TNF-α and IL-1β stimulates various signaling pathways resulting in downregulation of proteoglycans and collagen type II, and upregulates cell death and MMP-9, thereby contributing to the etiology of OA [36]. In addition, inflammatory cytokines inhibit the migration of chondrogenic progenitor cells in the OA cartilage and impedes the success of therapeutic strategies involving cell-free scaffolds for endogenous cell recruitment and *in situ* cartilage regeneration [37]. In the present study, β1,4-GalT-I inhibited

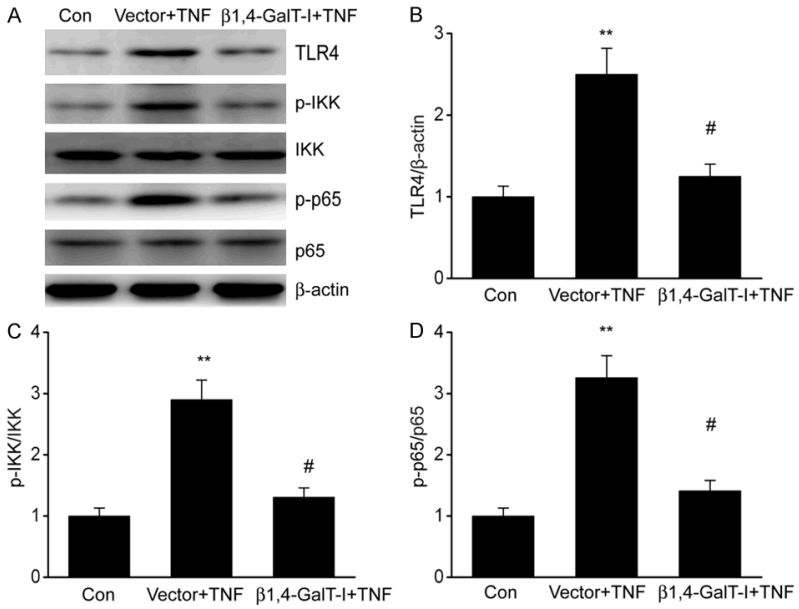

Figure 5. β1,4-galactosyltransferase-I (β1,4-GalT-I) inhibited tumor necrosis factor (TNF)-induced stimulation of toll-like receptor 4 (TLR4) signaling pathway inchondrocytes. Chondrocytes were transfected with β1,4-GalT-I plasmid (β1,4-GalT-I) or empty vector (Vector) and stimulated with TNF. (A-D) Representative immunoblots (A) and quantitative analysis of TLR4 (B), p-IκB kinase (IKK) (C) and p-p65 (D) in chondrocytes. Results are represented as means ± SEM. $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ vs. vector group. One way ANOVA.

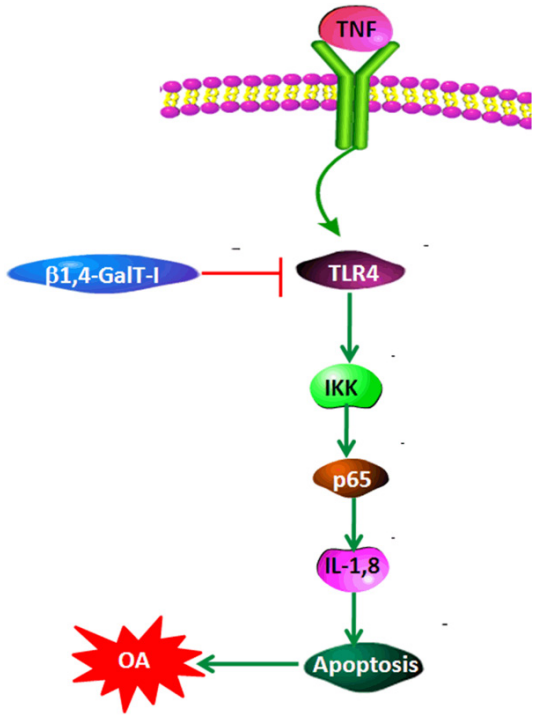

Figure 6. Schematic layout representing the protective effect of β1,4-galactosyltransferase-I (β1,4-GalT-I) in chondrocytes against tumor necrosis factor (TNF)-induced osteoarthritis via inhibition of TLR4 signaling pathway.

the production of IL-8, TNF, IL-1β and IL-6, resulting in the inhibition of chondrocyte cell death. Thus, β1,4-GalT-I has the potential to block the TNF-induced chondrocyte cell death byinhibiting the production of inflammatory cytokines.

TLR4 signaling pathway plays an important role in inflammation and cell death [38]. TLR4-modulated innate immune reactions regulate inflammation and the related catabolic processes [39]. TLR4 bindsto several agonists of which few are released in response to tissue injury. The expression of TLR4 is enhanced in the cartilage during the course of the development of OA [40]. A number of TLR4 agonists isolated from the joints of OA patients are capable of triggering inflammation in *ex vivo* tissue samples of these patients [41]. Certain pathways that regulate TLR4 signaling in cartilage together with inhibitors of TLR4 signaling might serve as potential disease-modifying agents in the treatment OA [42]. In the present study, β1,4-GalT-I could block the stimulation of TLR4 signaling pathway by inhibiting the expression of TLR4 and phosphorylation of p65 and IKK in chondrocytes. Thus, our findings prove that β1,4-GalT-I could inhibit TNF-induced chondrocyte cell death by inhibiting the stimulation of TLR4 signaling pathway (**Figure 6**).

In conclusion, results of our present study prove the protective effect of β1,4-GalT-I against TNF-induced OA by blocking the TLR4 signaling pathway. These results might pave the way for innovative therapeutic strategies aimed at using the β1,4-GalT-I–TLR axis to prevent the development and progression of OA.

Disclosure of conflict of interest

None.

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