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# β4GalT1 promotes inflammation in human osteoarthritic fibroblast-like synoviocytes by enhancing autocrine TNF-α activity

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**Abstract. – OBJECTIVE: Synovial inflammation plays an important role in the pathogenesis of osteoarthritis (OA), and β4GalT1 has been reported to be involved in the inflammatory process. The aim of our study was to investigate the role of β4GalT1 in the progression of inflammation and analyze the association between β4GalT1 and tumor necrosis factor (TNF)-α in human OA fibroblast-like synoviocytes (FLS).**

**PATIENTS AND METHODS: Primary cultured FLS isolated from OA synovial tissues were cultured, and the levels of β4GalT1, TNF-α, MMP-3, p/t-ERK, p/t-JNK, and p/t-P38 were analyzed by Western blotting. An enzyme-linked immunosorbent assay (ELISA) was performed to measure the secretion of TNF-α, interleukin (IL)-1β, and IL-6 in OA-FLS. Immunofluorescence staining was used to examine the co-localization of β4GalT1 and TNF-α or THY1. RT-PCR was used to detect the transfection efficiency of β4GalT1.**

**RESULTS: The expression of β4GalT1 was increased in OA-FLS. β4GalT1 promoted cell invasion, MMP-3 production, and the secretion of TNF-α, IL-1β, and IL-6. si-TNF-α attenuated the β4GalT1-enhanced cell invasion and inflammatory factor secretion in OA-FLS. Furthermore, β4GalT1 increased autocrine TNF-α signaling in OA-FLS. β4GalT1 knockdown successfully decreased autocrine TNF-α activity, while β4GalT1 overexpression increased autocrine TNF-α activity in OA-FLS. Moreover, β4GalT1 enhanced the ERK, JNK, and P38 MAPK signaling pathways through the induction of autocrine TNF-α signaling in OA-FLS.**

**CONCLUSIONS: β4GalT1 may promote the inflammatory progression of OA-FLS by enhancing autocrine TNF-α signaling.**

*Key Words:*
Inflammation Mediators, Osteoarthritis, Synovial Membrane.

### Abbreviations

β4GalT1: β1,4-Galactosyltransferase 1; TNF-α: tumor necrosis factor-α; OA: osteoarthritis; FLS: fibroblast-like synoviocytes; MMP-3: matrix metalloprotease-3; ELISA: Enzyme-linked immunosorbent assay; IL: interleukin; MMPs: matrix metalloproteases; ECM: extracellular matrix; RA: rheumatoid arthritis; IFN-γ: interferon γ; TGF: transforming growth factor; CXCR3: CXC chemokine receptor 3; DMEM: Dulbecco's Modified Eagle's Medium; FBS: fetal calf serum; LPS: lipopolysaccharide; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA: bovine serum albumin; GAPDH: Glyceraldheyde 3-phosphate dehydrogenase; RT-PCR: Reverse transcription polymerase chain reaction; CCK-8: Cell Counting Kit-8; PBS: phosphate-buffered saline; THY1: thymocyte differentiation antigen 1; FITC: fluorescein isothiocyanate; TRITC: tetramethyl rhodamine isothiocyanate; SPSS: Statistical Product and Service Solutions; SD: Standard Deviation; ANOVA: one-way analysis of variance; SCs: Schwann cells; NO: nitric oxid; PGE2: prostaglandin E2.

## Introduction

Osteoarthritis (OA) is a complex degenerative joint disease characterized by articular cartilage degeneration and subchondral bone reactive hyperplasia. Synovial inflammation also plays a key role in the pathological process of OA, even at early stages[1]. Epidemiological studies have shown that damage to the femoral and tibial cartilage is significantly associated with synovial inflammation in OA[2]. Histological changes characterized by synovitis have been found in OA synovial tissues, such as neovascularization, synovial hyperplasia, and macrophage and lymphocyte


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infiltration[3]. Fibroblast-like synoviocytes (FLS) are the most abundant cell type in the synovial membrane, and increasing studies suggest that the activation of OA-FLS may contribute to both cartilage degradation and synovial membrane inflammation through the secretion of various cytokines/chemokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and matrix metalloproteases (MMPs), which may lead to the destruction of bone and cartilage[4-7]. β1,4-Galactosyltransferase 1 (β4GalT1) belongs to the glycosyltransferase protein family; the *β4GalT1* gene encodes a short isoform and a long isoform that differ in their cytoplasmic domains[8]. The short isoform of β4GalT1 is located in the Golgi apparatus, where it transfers galactose from uridine diphosphate-galactose (UDP-Gal) donors to terminal N-acetylglucosamine (GlcNAc) residues of carbohydrate chains through β1,4-linkage to form a β4-N-acetyllactosamine (Gal β1,4GlcNAc) structure[9]. The long isoform of β4GalT1 is located on the cell surface, where it functions as a cell adhesion molecule that binds to N-acetylglucosamine-containing oligosaccharide substrates and ligands in the extracellular matrix (ECM)[10]. Recent findings have shown that β4GalT1 expression was highly correlated with inflammation. A study on β4GalT1-deficient mice suggested that the reduction in acute and chronic inflammatory responses could be attributed to impaired leukocyte infiltration due to the reduced biosynthesis of selectin ligands[11,12]. β4GalT1 up-regulated TNF-α expression by maintaining the mRNA stabilization of TNF-α[13]. Our previous studies found that β4GalT1 expression was markedly changed in the articular cartilage and synovial membranes of patients and arthritis animal models of rheumatoid arthritis (RA) and OA[14-17]. Numerous researches have confirmed that cytokines are involved in the pathogenesis of OA. TNF-α and IL-1β are major proinflammatory cytokines. They are the most frequently studied and detected in OA pathogenesis[18,19]. Likewise, several other pro- and anti-inflammatory cytokines such as interferon gamma (IFN-γ), IL-6, IL-18, IL-8, and transforming growth factor (TGF)-β have been investigated for their roles in OA progression[19]. TNF-α is one of the most important pro-inflammatory cytokines in joint inflammation, and may be involved in the destruction of cartilage and bone in patients with OA[18,20]. TNF-α-induced increases in the ratios of MMPs/TIMPs could partially explain the over-degradation of cartilage ECM in OA[21]. Serum and knee synovial fluid TNF-α levels were shown to be increased in patients with late-stage OA[20]. The levels of serum TNF-α were associated with knee radiographic OA in the elderly[22]. TNF-α also induced IL-1β, IL-6, and IL-8 production in cultured FLS[23]. Our previous studies showed that β4GalT1 could regulate the N-glycan groups of CXCR3, which affected CXCL10/CXCR3 ligand-binding to enhance FLS invasion in RA; additionally, TNF-α was found to up-regulate the expression of *β4GalT1* mRNA in RA-FLS[24,25]. Hence, in this work, we used human synovial membrane tissues and FLS to study the link between β4GalT1 and TNF-α, as well as its role in the progression of inflammation, in OA.

## Patients and Methods

### Patients

Healthy knees were obtained from dead patients within 12 h of death (average age: 59 ± 6.2 years), and the synovial tissues of the healthy knees were examined macroscopically and microscopically to ensure that only healthy tissue was used. Human OA synovial tissues were derived from patients undergoing total knee arthroplasty (average age: 61.2 ± 5.4 years). All patients with OA were evaluated by two certified rheumatologists and were diagnosed with OA based on the criteria of the American College of Rheumatology Diagnostic Subcommittee for OA[26]. These specimens represented moderate-to-severe OA, defined based on macroscopic and microscopic criteria. All patients provided informed consent, and protocols were approved by the Ethical Committee of the Second Affiliated Hospital of Soochow University (Suzhou, China).

### Cell Cultures

The standard operating procedure for synovial fibroblast preparation was performed as described previously[27]. Briefly, synovial tissue was cut into 1- to 2-mm pieces in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA), placed in a Petri dish containing fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C in a humidified 5% $CO_2$ atmosphere for 3 h, and then incubated in DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mg/mL Fungizone) for several days until the cells began to spread. After reaching confluence, cells were suspended by trypsinization. Cells at passage 4-7,

at which point they were a homogeneous population, were used for all experiments. FLS cultures were incubated for the indicated times or until they reached the indicated concentrations with recombinant rat TNF-α (10 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) or lipopolysaccharides (LPS; 10 ng/mL, *Escherichia coli*, 0127: B8; Sigma-Aldrich, St. Louis, MO, USA). For cell signaling experiments, cultured cells at a density of $1.0 \times 10^6$ cells/mL were treated with cell signaling pathway inhibitors including PD98059 (50 μM/mL, Cell Signaling Technology, Danvers, MA, USA), SB202190 (10 μM/mL, Sigma-Aldrich, St. Louis, MO, USA) and SP600125 (20 μM/mL, Sigma-Aldrich, St. Louis, MO, USA) for 30 min before being washed.

### siRNAs and Transfection

Cell transfections were performed as described previously[28]. The target sequence for *β4GalT1* was 5′-AATTGCACACACAAGGAGAC-3′, and the target sequence for TNF-α was 5′-GACAACCAACTAGTGGTGC-3′. OA-FLS was seeded in 24-well plates containing DMEM with 10% fetal bovine serum (FBS) 1 day prior to transfection. For transient transfection, the β4GalT1 expression vector, the β4GalT1 and TNF-α siRNA vectors, and the non-specific vector were transfected into OA-FLS cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions in DMEM with no serum for 37°C. After incubation for 6 h, the medium was replaced with DMEM containing 10% FBS. After 48 h, the cells were used for subsequent experiments.

### Western Blot Assay

Whole cell lysates from OA-FLS were separated by 10% SDS-PAGE. After blocking with 5% bovine serum albumin (BSA) for 2 h, blots were probed with primary antibodies at 4°C for 12 h, including primary antibodies against β4GalT1 (anti-goat, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), TNF-α (anti-mouse, 1:1000, Abcam, Cambridge, MA, USA), MMP-3 (anti-rabbit, 1:500, Sigma-Aldrich, St. Louis, MO, USA), p-ERK, t-ERK, p- JNK, t-JNK, p-P38, t-P38 (anti-rabbit, 1:500, Sigma, St. Louis, MO, USA) and GAPDH (anti-rabbit, 1:1000, Sigma-Aldrich, St. Louis, MO, USA). Membranes were then incubated with appropriate secondary antibodies for 2 h at room temperature. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

### Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed as described previously[28]. The primer sequences used in this study were as follows: β4GalT1-forward: 5'-TACAACTGCTTTGTGTTCAGTGATG-3', β4GalT1-reverse: 5'-GCAGGCTAAACCCGAACTTG-3'; TNF-α-forward: 5'-CGTCGTAGCAAACCACCAAG-3', TNF-α-reverse; 5'-CACAGAGCAATGACTCCAAAG-3'. *GAPDH* was used as an internal control.

### Cell Viability Assay

A Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) was utilized to determine cell viability according the manufacturer's instructions. Briefly, a 100-μL cell suspension (3,000 cells/well) was dispensed in a 96-well microtiter plate, and then pre-incubated in a humidified atmosphere and 5% $CO_2$ at 37°C for 24 h. After transfecting the cells with *β4GalT1* or TNF-α siRNA vectors for 24 h and 48 h, respectively, 10 μL CCK-8 solution was added to each well and then incubated for 2 h. Finally, the absorbance at 450 nm of each well was determined on a microplate reader. Each experiment was performed independently three times.

### Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of TNF-α, IL-6, and IL-1β under various experimental conditions were determined by ELISA (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. When cells were treated with TNF-α, the amount of added TNF-α added was subtracted from the amount measured in the results in order to accurately record TNF-α production in OA-FLS.

### Immunofluorescence Staining

The sections were deparaffinized and rehydrated in a graded alcohol series, and antigen retrieval was performed in citrate buffer (pH 6.0) at 100°C for 15 min. The cells were washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde for 30 min. After blocking, the sections and cells were incubated with antibodies specific for β4GalT1 (anti-goat, 1:100, Santa Cruz, Biotechnology, Santa Cruz, CA, USA), TNF-α (anti-mouse, 1:100, Santa Cruz, Santa Cruz, CA, USA) and thymocyte differentiation antigen 1 (THY1; anti-mouse, 1:100, Biolegend, San Diego, CA, USA) overnight at 4°C. After the sections and cells were rinsed with PBS,

secondary antibodies (fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat; tetramethyl rhodamine isothiocyanate (TRITC)-conjugated donkey anti-mouse, 1:100, Jackson Laboratory, West Grove, PA, USA) were added in a dark room and incubated for 2 h at 4°C. Subsequently, sections and cells were stained with Hoechst (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and washed with PBS. They were then analyzed and imaged by confocal laser scanning microscopy.

### Transwell Assay

A transwell assay was performed as described previously[25]. A cell migration assay was performed with Matrigel invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. The upper chambers were freshly coated with Matrigel, and medium was added to the lower chamber as described above. OA-FLS ($5 \times 10^4$ cells) were suspended in the medium containing 2% fetal bovine serum (FBS) and seeded into Matrigel pre-coated transwell chambers. Cell invasion was allowed to occur for 48 h, and the gel and cells on the top membrane surface were removed with cotton swabs. Cells that had penetrated to the bottom were counted. This experiment was repeated four times.

### Statistical Analysis

Statistical analyses were performed using Statistical Product and Service Solutions (SPSS) software Version 13.0 (SPSS Inc., Chicago, IL, USA). All experiments were repeated at least three times. The data were presented as the mean ± standard deviation (SD). Statistical significance between means was analyzed by one-way analysis of variance (ANOVA) followed by a Least Significant Difference (LSD) post-hoc test. A value of $p < 0.05$ was considered statistically significant.

## Results

### β4GalT1 was Localized in FLS and β4GalT1 and TNF-α Expression Increased in Human OA Synovial Membranes

The results of immunofluorescence showed that β4GalT1 was co-localized with TYH1, a marker of FLS (Figure 1A). We then used Western blotting to analyze the levels of β4GalT1 and TNF-α in OA synovial membrane tissues. Both β4GalT1 and TNF-α expression were increased in OA compared to the control (Figure 1B).

### si-β4GalT1 Attenuated Cell Invasion and the Production of Inflammatory Factors in OA-FLS

β4GalT1 siRNA was used to suppress β4GalT1 expression in OA-FLS; our CCK-8 assay showed that the viability of OA-FLS was not significantly changed after β4GalT1 siRNA vector transfection. (Figure 2A). The levels of MMP-3 were significantly increased after OA-FLS was treated with LPS, while the expression of MMP-3 was reduced by si-β4GalT1 (Figure 2B). Furthermore, the invasiveness of OA-FLS was also decreased by si-β4GalT1 (Figure 2C). Moreover, ELISA results showed that the secretion of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 increased significantly with LPS stimulation, but decreased markedly in β4GalT1-knockdown OA-FLS (Figure 2D-2F).

### Treatment with TNF-α and β4GalT1 Promoted Autocrine TNF-α Signaling in OA-FLS

Our previous work showed that exogenous and endogenous TNF-α promoted the expression of β4GalT1 in RA-FLS[24]. However, in this experiment, we mainly focused on how β4GalT1 affected the expression of TNF-α in FLS. The mRNA and protein levels of TNF-α were increased in OA-FLS after treatment with TNF-α, as determined by RT-PCR and ELISA, respectively (Figure 3A, B). In addition, we found that β4GalT1 was localized with TNF-α in OA-FLS by immunofluorescent staining (Figure 3C). These results encouraged us to investigate the role of β4GalT1 in autocrine TNF-α signaling in OA-FLS. Therefore, we overexpressed β4GalT1 by transfecting the EGFP-β4GalT1 expression vector into OA-FLS. Analysis of EGFP-tagged cells and RT-PCR results indicated that EGFP-β4GalT1 was successfully overexpressed in OA-FLS (Figure 3D). Moreover, ELISA showed that autocrine TNF-α signaling was notably decreased in β4GalT1-knockdown OA-FLS compared to control siRNA-transfected OA-FLS. Conversely, autocrine TNF-α signaling was notably increased in β4GalT1-overexpressed OA-FLS relative to the control (Figure 3E). Similarly, TNF-α expression levels were not affected in either β4GalT1-knockdown or -overexpressed OA-FLS in the absence of TNF-α treatment (Figure 3E).

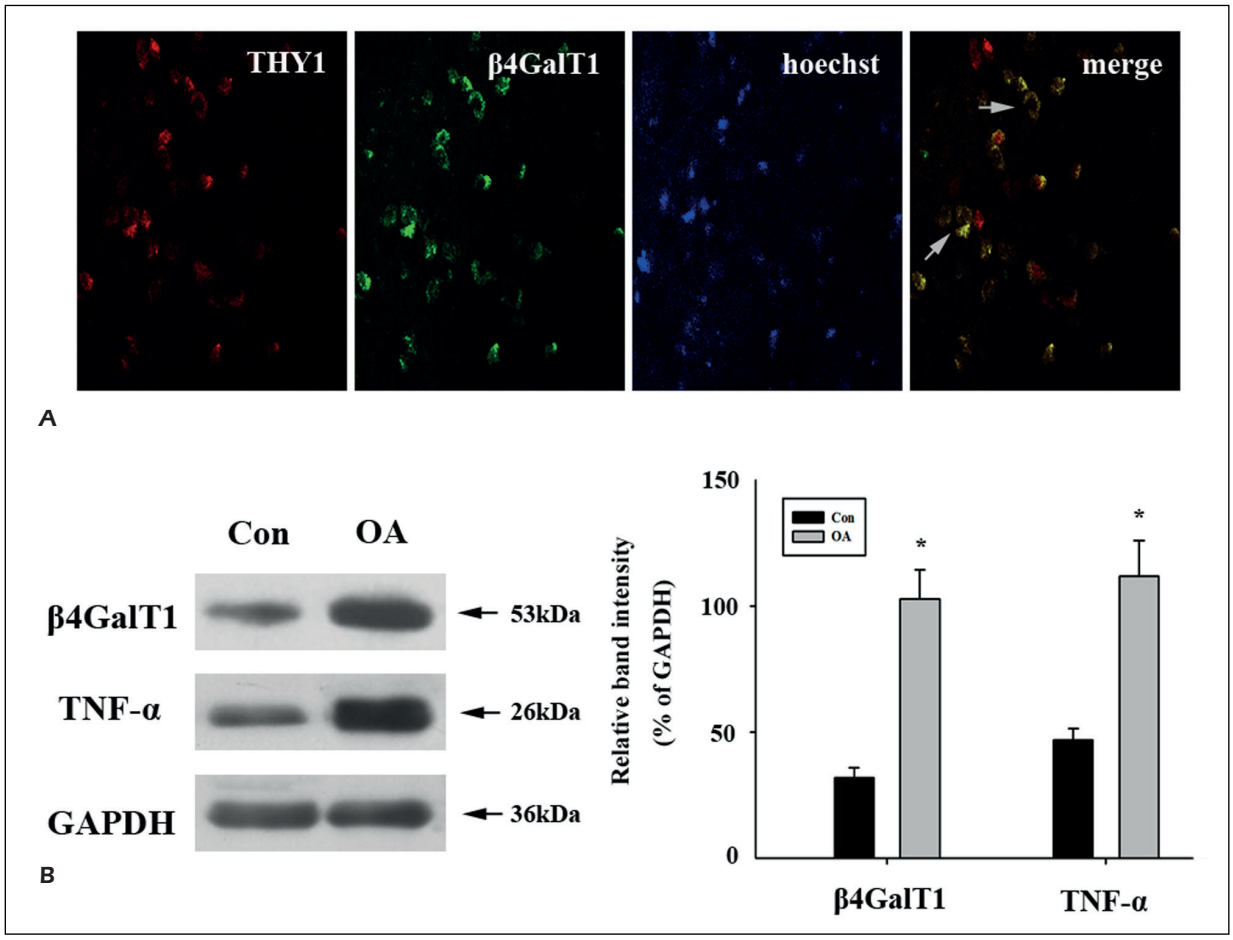


**Figure 1.** The levels of β4GalT1 and TNF-α were increased in human OA synovial membranes. **A**, Immunofluorescence showing that β4GalT1 (green) was co-localized with THY1 (red). **B**, Western blot showing increased levels of β4GalT1 and TNF-α in human OA synovial membranes. GAPDH was used as an internal control. *$p<0.05$.

### *Inhibition of TNF-α Attenuated the Role of β4GalT1 in OA-FLS*

Compared to untreated OA-FLS, both cell invasiveness and the secretion of TNF-α, IL-1β, and IL-6 were significantly increased in β4GalT1-overexpressed cells (Figure 4A-4D). We further assessed the effect of TNF-α on β4GalT1 by transfecting cells with TNF-α siRNA to inhibit the expression of TNF-α; a CCK-8 assay showed that the viability of OA-FLS was not significantly changed after TNF-α siRNA vector transfection (Figure 4A). β4GalT1 was successfully overexpressed in OA-FLS, but cell invasion was not significantly increased in TNF-α-knockdown OA-FLS (Figure 4B). However, β4GalT1 promoted the secretion of TNF-α, IL-1β and IL-6 in OA-FLS, while the inhibition of TNF-α impaired the expression of these factors (Figure 4A, 4C, and 4D).

### *β4GalT1 Promoted MAPK Signaling Pathway Activation and Autocrine TNF-α Signaling in OA-FLS*

Our previous study[28] showed that β4GalT1 promoted autocrine TNF-α signaling through the activation of the extracellular signal-related kinase (ERK), c-jun N-terminal kinase (JNK), and P38 mitogen-activated protein kinase (MAPK) signaling pathways in Schwann cells (SCs). In this experiment, the phosphorylation of the ERK, JNK, and P38 signaling pathways showed no significant changes in β4GalT1 siRNA-transfected OA-FLS compared to control siRNA-transfected OA-FLS without TNF-α stimulation. However, the phosphorylation of the ERK, p38, and JNK signaling pathways was significantly reduced after TNF-α stimulation (Figure 5A). Next, we used the specific inhibitors PD98059, SP600125, and SB202190, which are known to inhibit the phos-

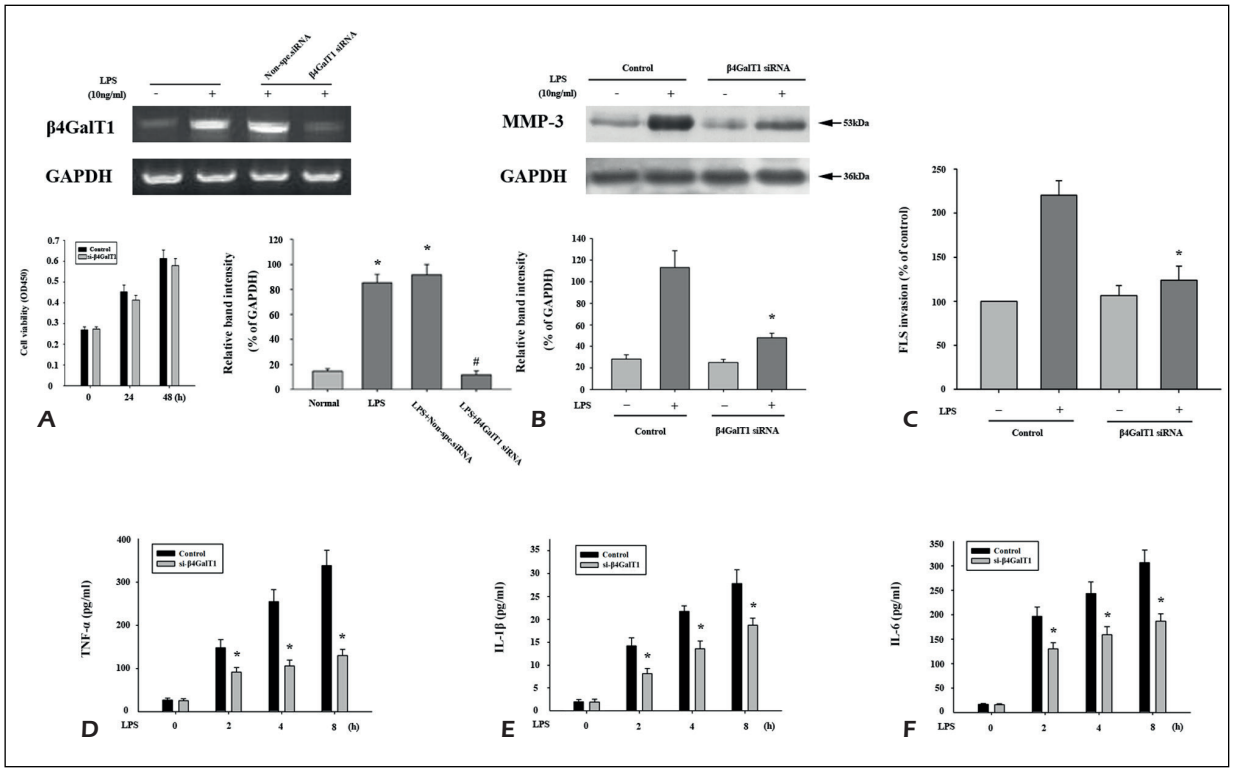


**Figure 2.** The effect of si-β4GalT1 on OA-FLS invasion and cytokine secretion. OA-FLS were transfected with β4GalT1 siRNA and treated with LPS (10 ng/mL) for 8 h. **A**, RT-PCR was used to assess the efficiency of β4GalT1 siRNA, and the viability of OA-FLS after β4GalT1 si-RNA vector transfection was evaluated by a CCK-8 assay. **B**, Western blotting was used to detect the production of MMP-3. **C**, Cell invasiveness was analyzed by a transwell assay and quantified. **D**-**F**, ELISA was used to detect the secretion of TNF-α (**D**), IL-1β (**E**), and IL-6 (**F**). GAPDH was used as an internal control. $^{*,\#}p<0.05$.

phorylation and activation of ERK, JNK, and P38, respectively, to further investigate the role of these pathways in autocrine TNF-α signaling in OA-FLS. We found that autocrine TNF-α signaling was markedly reduced by inhibiting the ERK, JNK, or P38 pathways (Figure 5B). Moreover, autocrine TNF-α signaling was remarkably reduced in β4GalT1-overexpressed OA-FLS pretreated with the ERK, p38, and JNK signaling pathway inhibitors PD98059, SP600125, and SB202190, respectively (Figure 5C).

## Discussion

In the present study, we found that β4GalT1 and TNF-α were highly expressed in OA synovial membranes, and that β4GalT1 was co-localized in OA-FLS. A large number of studies have reported that OA-FLS secretes many inflammatory factors such as TNF-α, IL-1β, IL-6, and MMPs under stimulatory conditions[29]. Therefore, OA-FLS is important for regulating the inflammation of the synovium, which can result in cartilage degradation and disease pathology[29,30]. However, the functions and mechanisms of β4GalT1/TNF-α in OA-FLS remain to be fully elucidated.

Recently, extensive evidence has shown that β4GalT1 plays an important role in the inflammatory process of RA. Previous studies have shown that β4GalT1 promoted synovial inflammation by associating with E-selectin to mediate the influx of inflammatory cells into the synovium[17], and regulated N-glycan groups of CXCR3 to affect CXCL10/CXCR3 ligand binding and enhance FLS invasion in RA[25]. However, little is known of the role of β4GalT1 in OA. When cells were treated with β4GalT1 siRNA, the expression of active MMP-3 in OA-FLS was decreased. The degradation of the ECM plays an important role in the development and progression of OA. Hasegawa et al[31] have reported that FLS produced enzymes to degrade the ECM, such as MMPs, which have been found to be over-activated in OA, including MMP-3 (stromelysin 1). MMP-3 has a critical effect on cartilage destruction in OA[19]. β4GalT1 siRNA also reduced the secretion of TNF-α, IL-1β, and IL-6. In articular cells, TNF-α and IL-1β

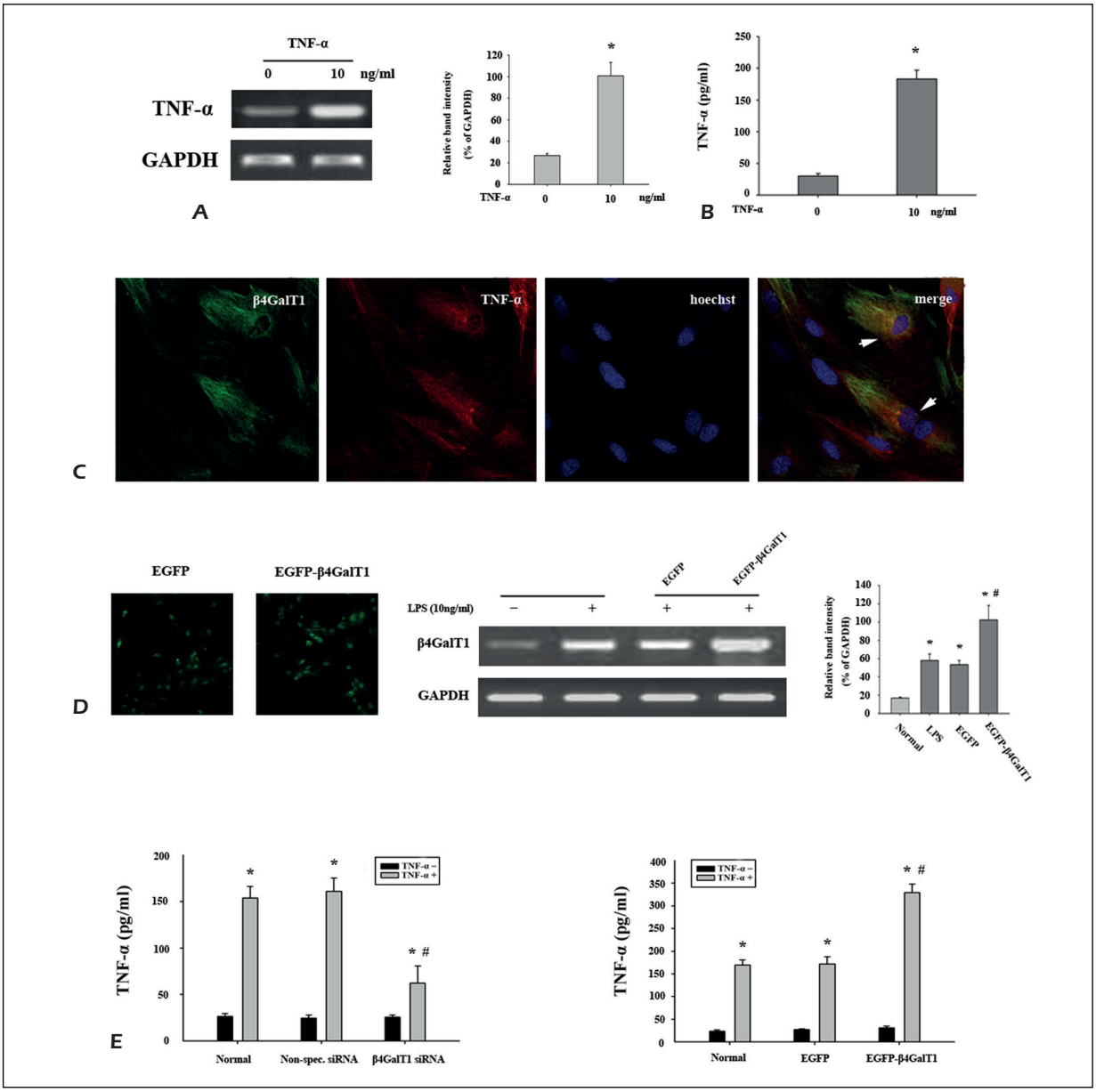


**Figure 3.** Treatment with TNF-α and β4GalT1 affected autocrine TNF-α activity in OA-FLS. OA-FLS were treated with TNF-α (10 ng/mL) for 24 h. **A**, Analysis of the relative mRNA expression of TNF-α by RT-PCR. **B**, Measurement of TNF-α secretion by ELISA. **C**, Immunofluorescence showed strong staining for β4GalT1 (green) and TNF-α (red) in OA-FLS, and co-localization was observed through merged confocal laser scanning microscopy (yellow). **D**, EGFP-β4GalT1 was expressed in OA-FLS as determined by fluorescence imaging and RT-PCR. OA-FLS were overexpressed with β4GalT1 or transfected with β4GalT1 siRNA and then treated with TNF-α (10 ng/ml) for 24 h. **E**, ELISA showed that TNF-α secretion was significantly decreased in β4GalT1-knockdown OA-FLS relative to control siRNA-transfected OA-FLS after treatment with TNF-α; in contrast, autocrine TNF-α activity increased in β4GalT1-overexpressed OA-FLS. The amount of TNF-α added for stimulation was subtracted from the results of TNF-α production by ELISA. GAPDH was used as an internal control. *$p$<0.05.

amplify and perpetuate OA disease progression by inducing the production of proinflammatory cytokines such as IL-6 and other chemokines[32,33]. We found that when LPS stimulated OA-FLS, the knockdown of β4GalT1 significantly reduced cell invasion. The results obtained from β4GalT1 overexpression were directly related to this effect. Our findings indicated that β4GalT1 may play a role in the progression of inflammation in OA; however, the mechanism is still unknown. TNF-α can induce synovial fibroblasts to produce inflammatory cytokines, including IL-1β, IL-6, IL-8 and itself, which further promote the secretion of MMPs, resulting in the destruction

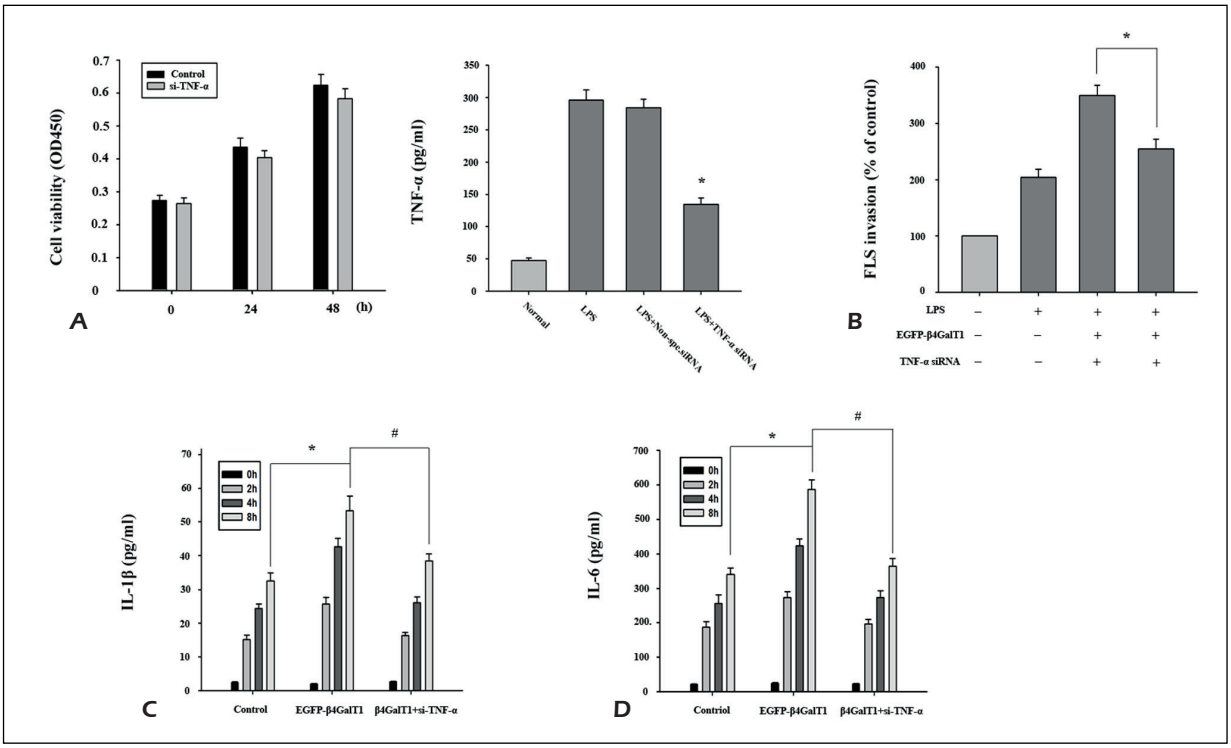


**Figure 4.** The effect of TNF-α on β4GalT1-enhanced OA-FLS inflammation. OA-FLS were overexpressed with β4GalT1 and transfected with or without TNF-α siRNA. Then, cells were treated with LPS (10 ng/mL) for 8 h. **A**, The efficiency of TNF-α siRNA was assessed by ELISA, and the viability of OA-FLS after TNF-α si-RNA vector transfection was evaluated by a CCK-8 assay. **B**, Cell invasiveness was analyzed by a transwell assay and quantified. **C-D**, ELISA was used to analyze the concentrations of IL-1β and IL-6. $^{*,\#}p<0.05$.

of bone and cartilage in RA-FLS[34]. Previously, we demonstrated[24] that exogenous and endogenous TNF-α upregulated β4GalT1 expression in RA-FLS. Exogenous TNF-α successfully induced the expression and release of endogenous TNF-α in RA-FLS[35]. In this study, we found that autocrine TNF-α and β4GalT1 were co-localized with TNF-α in OA-FLS. Furthermore, the silencing of β4GalT1 decreased TNF-α expression, while the overexpression of β4GalT1 increased autocrine TNF-α signaling in TNF-α-treated OA-FLS. TNF-α also stimulated the release of nitric oxide (NO) and prostaglandin $E_2$ ($PGE_2$), which promoted to the inflammation and destruction of articular cartilage by inhibiting the synthesis of anabolic macromolecules such as collagen and proteoglycan, and by enhancing the production of MMPs[18]. Moreover, we also found that si-TNF-α decreased cell invasion, as well as the expression of TNF-α, IL-1β, and IL-6; these results demonstrated that si-TNF-α attenuated the effects of β4GalT1 in OA-FLS progression. Considering these results together, it can be concluded that β4GalT1 plays a potential role in the inflammatory process of OA-FLS by mediating autocrine TNF-α signaling. The ERK, JNK, and P38 MAPK signaling pathways activate the expression of many inflammatory cytokines, chemokines, and MMPs in OA[36,37]. Previous studies[28,38] have shown that the expression of β4GalT1 could be affected by the ERK, JNK, and P38 MAPK signaling pathways, and that β4GalT1 may promote autocrine TNF-α signaling through the activation of MAPK signaling pathways in SCs. Consequently, we further explored whether β4GalT1 expression was related to the activation of the ERK, JNK, and P38 MAPK signaling pathways and the secretion of TNF-α in OA-FLS. Our data indicated that β4GalT1 knockdown repressed the phosphorylation and activation of the ERK, JNK, and p38 MAPK signaling pathways in the presence of TNF-α in OA-FLS. Moreover, autocrine TNF-α signaling was dramatically reduced in β4GalT1-overexpressed OA-FLS treated with ERK, p38, and JNK signaling pathway inhibitors. Our data demonstrated that β4GalT1 played its role through promoting the activation of the ERK, JNK, and P38 MAPK signaling pathways, as well as upregulating autocrine TNF-α signaling.

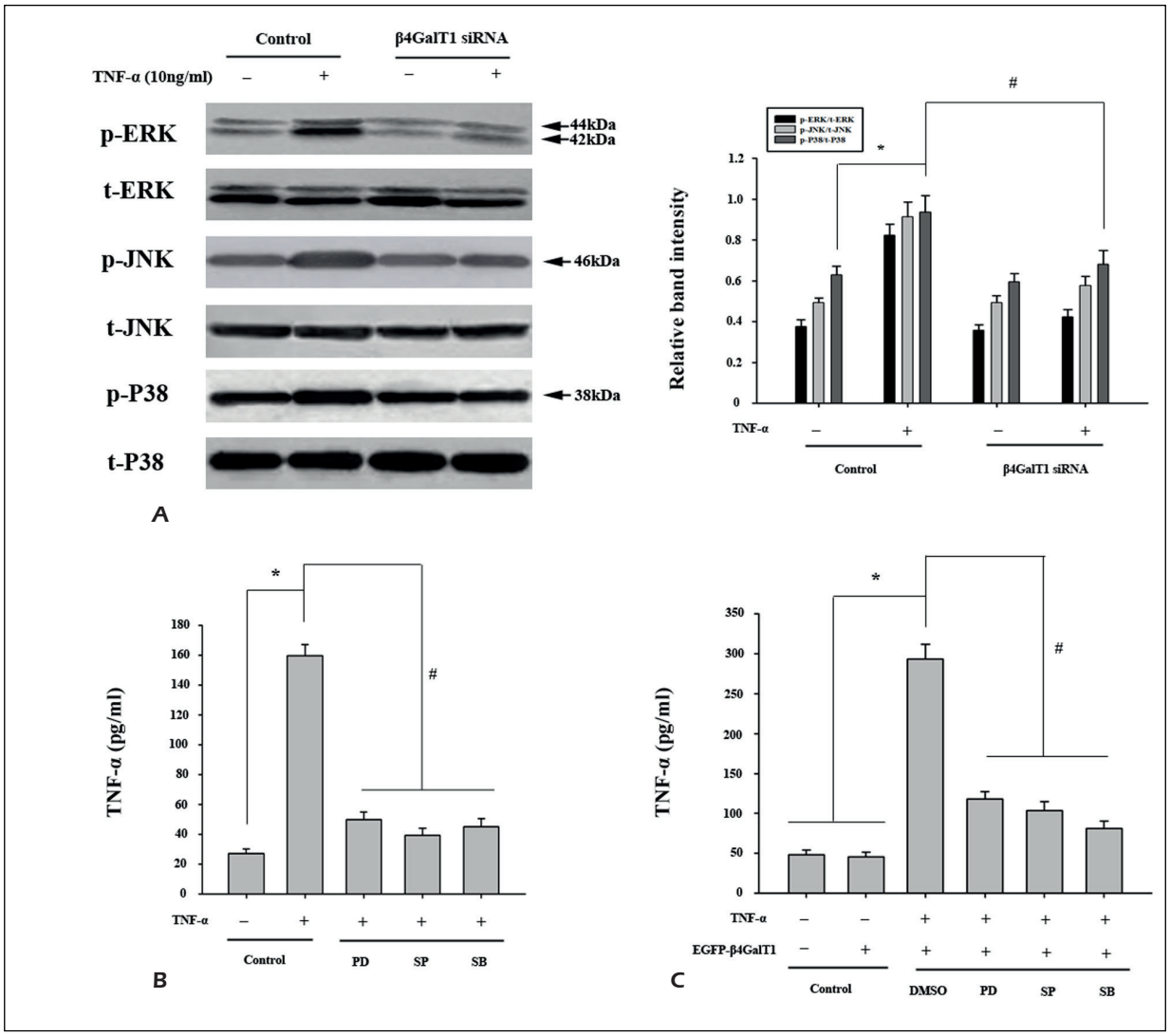


**Figure 5.** β4GalT1 affected MAPK signaling pathway activation and autocrine TNF-α activity in OA-FLS. OA-FLS were transfected with β4GalT1 siRNA and treated with TNF-α (10 ng/mL) for 24 h. **A**, The levels of p-ERK, p-JNK, and p-P38, as well as t-ERK, t-JNK, and t-P38 in OA-FLS were analyzed by Western blotting, and the ratio of their expression was analyzed. TNF-α secretion was analyzed by ELISA. **B**, OA-FLS were pretreated with PD98059 *(PD)*, SP600125 *(SP)*, and SB202190 *(SB)* and treated with TNF-α. **C**, OA-FLS were transfected with EGFP control vector or EGFP-β4GalT1-overexpression vector and pretreated with DMSO, PD98059, SP600125, and SB202190, followed by TNF-α treatment. The amount of TNF-α added for stimulation was subtracted from the results of TNF-α production by ELISA. $*,^{\#}p<0.05$.

## Conclusions

We detected that β4GalT1 was shown to promote autocrine TNF-α signaling and to enhance the activation of the ERK, JNK and p38 MAPK signaling pathways, leading to cell invasion and the production of TNF-α, IL-1β, and IL-6 in OA-FLS. Despite these findings, the molecular mechanisms controlling the activation of TNF-α and other pathogenesis-related cytokines by β4GalT1 in OA need to be further investigated. These findings may provide a novel means to improve the effectiveness of OA treatments.

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## Conflict of Interests

The Authors declare that they have no conflict of interests.

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