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PPAR-γ agonist pioglitazone protects against IL-17 induced intervertebral disc inflammation and degeneration *via* suppression of NF-κB signaling pathway

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ABSTRACT

Interleukin-17 (IL-17) is the production of T helper type 17 (Th17) cells and has been reported to play a pro-inflammatory role in the immunopathogenesis of intervertebral disc degeneration. Peroxisome proliferator-activated receptor γ (PPAR-γ) activators display anti-inflammatory and anti-degeneration roles in osteoarthritis and rheumatoid arthritis. However, the expression level of PPAR-γ and related regulatory mechanisms in the nucleus pulposus tissues are not clear. Herein we report that PPAR-γ was down-regulated both in the nucleus pulposus tissue of intervertebral disc degeneration patient and in the cultured nucleus pulposus cells stimulated with IL-17. This study was undertaken to investigate the potential therapeutic effect of pioglitazone, as a PPAR-γ ligand, and its underlying molecular mechanism in IL-17-induced human intervertebral disc degeneration model *in vitro*. Our results indicate that pioglitazone administration suppressed the production of pro-inflammatory cytokines and down-regulated the mRNA expression levels of inflammatory mediators in the cultured human nucleus pulposus cells and tissue. Consistently, pioglitazone decreased the levels of metalloproteinase and maintained the expression of critical matrix components, such as aggrecan and type II collagen. Moreover, the activation of NF-κB signaling in the nucleus pulposus tissue during the intervertebral disc degeneration development was antagonized by pioglitazone administration. In conclusion, our current findings provide scientific evidence for the assessment of pioglitazone as a potential therapeutic approach to treat the intervertebral disc degeneration.

1. Introduction

Accumulating evidence had demonstrated that intervertebral disc degeneration (IVDD) is an age-related disease, which is thought to be the primary cause of chronic lower back pain [1]. The degenerative process of IVDD is the outcome of unbalance between the degradation and regeneration of intervertebral disc tissue, accompanied by abnormal inflammation activation, nucleus pulposus (NP) cells apoptosis, and degradation of type II collagen (collagen 2) and aggrecan, the main components of extracellular matrix [2]. Multiple pro-inflammatory cytokines and chemokine, such as interleukin-17 (IL-17), IL-1β, tumor necrosis factor-α (TNF-α), CC chemokine ligand-20 (CCL-20), and cyclooxygenase-2 (COX-2) have been found overexpressed in the degenerated NP tissues [3]. The increasing production of inflammatory cytokines up-regulated the expression of the catabolic enzymes, including matrix metalloproteinase-13 (MMP-13) and a disintegrin and metalloproteinase with thrombospondin motifs-7 (ADAMTS-7), which induce severe structural destruction of the intervertebral disc [4,5].

Our previous studies have shown that IL-17, the eponymous cytokine produced by T helper type 17 (Th17) cells, plays a central role in the IVDD through the regulation of pro-inflammatory gene expression [6]. Administration of IL-17 alone in the NP cell culture model can promote the inflammatory reaction [7]. Additionally, a number of studies have shown that IL-17 cooperated with other cytokines to induce a potent synergistic effect, particularly with TNF-α [8,9]. Peroxisome proliferator-activated receptors γ (PPAR-γ) has been shown to have an extensive and powerful anti-inflammatory impact through inhibiting the nuclear factor-κB (NF-κB) signal pathway [10], and the expression level of PPAR-γ is down-regulated in many diseases, such as rheumatoid arthritis [11], atherosclerosis [12], and colitis [13]. However, the role of PPAR-γ in the process of IVDD remains unclear. In this study, we found the low expression level of PPAR-γ in the degenerated

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intervertebral disc tissue and NP cells degeneration model. Then we argue whether the increase of PPAR-γ expression level by administration of pioglitazone, a PPAR-γ agonist, could block the intervertebral disc inflammatory reaction induced disc degeneration.

2. Materials and methods

2.1. Ethics statement

The experiments were approved by the Shandong University Human Subjects Institutional Review Board. A written informed consent document was obtained from each enrolled patient.

2.2. Patients and specimen source

Human pathological specimens were obtained from the patients who accept discectomy procedures from March 2017 to August 2017 in Qilu Hospital of Shandong University, Jinan, China. Eight patients (5 male and 3 female, age range: 25–65 years) diagnosed with 3–4 grade lumbar disc degeneration were enrolled as the IVDD group. In addition, five disc tissue specimens from the patients (3 male and 2 female, age range: 20–31 years) diagnosed with scoliosis were enrolled as the control group. The criteria for screening patients were as described in the previous articles [7,14]. The Pfirrmann grading system was used to determinate the disc degeneration grade based on the magnetic resonance imaging (MRI) and computed tomography (CT) scan analysis.

2.3. NP cells isolation, culture, and treatment

NP tissue samples used for NP cells isolation were collected from the 6 patients who were diagnosed with adolescent idiopathic scoliosis and had undergone spinal fusion surgery. Consent forms were signed by these patients, and this study was approved by the Shandong University Human Subjects Institutional Review Board specifically. The NP cells were collected and cultured, as described in the previous studies [14,15]. In short, to remove the residual blood, the samples were washed three times with cold, aseptic phosphate-buffered saline (PBS). Next, the NP samples were carefully isolated and cut into small pieces of 1 mm^3. Then, the cells were isolated after digestion of the NP fragments by type II collagenase (Sigma, USA) and trypsin. The isolated NP cells were seeded as monolayer and cultured in DMEM/F12 media (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA), 50 mg/ml of ascorbic acid, 1% penicillin and streptomycin (HyClone, USA), at 37 °C, 5% CO_2, 95% air, pH 7.2 for 3 weeks. After culture for 5 days, primary cells grow adherently, but the cells grow slowly; the culture medium was changed for the first time. After that, the medium was changed once every three days. After two weeks of culture, the primary cells grew densely and gradually merged, achieving 80%–90% confluence at 3 weeks. After three passage, primary cells were used for further study.

After pre-culture, the NP cells ($1 * 10^6$/ml) were seeded in 6-well plates and treated with cytokines (IL-17 and TNF-α; PeproTech, USA) and pioglitazone (Sigma, USA). To detect the effect of IL-17 and TNF-α on the expression of PPAR-γ, human NP cells were stimulated by PBS, IL-17 (100 ng/ml), TNF-α (20 ng/ml) or combination of IL-17 (100 ng/ml) and TNF-α (20 ng/ml) for 24 or 48 h, then the cells were collected and detected by real-time RT-PCR and western blot. To detect the effect of pioglitazone on NP cell inflammation and degeneration, human NP cells were cultured and stimulated by PBS, 100 ng/ml IL-17 + 20 ng/ml TNF-α, 100 ng/ml IL-17 + 20 ng/ml TNF-α + 10 μM pioglitazone, 100 ng/ml IL-17 + 20 ng/ml TNF-α + 50 μM pioglitazone for 24 h or 48 h, then the cells and supernatant samples were collected and detected in further study. At the appointed time intervals after stimulation, the supernatants were collected for enzyme-linked immunosorbent assay (ELISA), and the NP cells were collected for protein detection and mRNA quantification.

2.4. Isolation and culture of disc specimens

Intervertebral discs from the patients, diagnosed with adolescent idiopathic scoliosis and undergoing spinal fusion surgery, were isolated under sterile conditions. The specimens were seeded in 6-well plates and maintained with PBS or 100 ng/ml IL-17 + 20 ng/ml TNF-α or 100 ng/ml IL-17 + 20 ng/ml TNF-α + 50 μM pioglitazone for 7 days. The medium of every group was changed every 48 h. After culture for 7 days, the specimens were harvested for western blot and RT-PCR.

2.5. Immunohistochemical staining

Intervertebral disc tissues from each patient of the two groups were fixed in 10% formalin for 72 h. After decalcification, dehydration and clearance with dimethylbenzene, the intervertebral disc tissues were embedded in paraffin for the preparation of 5 μm thick sections. Then, the serial sections were incubated with primary antibodies (rabbit anti-PPAR-γ, 1:150 dilution, Santa Cruz Biotechnology, USA; rabbit anti-IL-17, 1:200 dilution, Abcam, UK; rabbit anti-NF-κB phosphorylated p65 (NF-κB p-p65), 1:200 dilution, Cell Signaling Technology, USA; mouse anti-aggrecan, 1:200 dilution, Abcam, UK) at 4 °C overnight. Then, the sections were incubated with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase HRP (1:500, ZSGB-Bio, China) for 30 min at 37 °C. The Images were captured by IX71-SIF type microscope (Olympus, Japan) at 200 magnification and analyzed using Image-Pro Plus 5.0 software (Media Cybernetics, USA).

2.6. Western blot analysis

Total proteins were extracted and collected from the cultured NP tissues and NP cells from each treatment group, as previously described. Protein of each group was resolved on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and then transferred by electroblotting to polyvinylidene difluoride (PVDF) membranes. After being blocked by 5% milk in Tris-buffered saline-Tween 20 (TBST, 10 mM Tris-HCl, pH 8.0; 150 mM NaCl; and 0.5% Tween 20) and washed by TBST, the blots were respectively incubated with rabbit anti-PPAR-γ (1:500 dilution, Santa Cruz Biotechnology, USA), rabbit anti-COX-2 (1:1000 dilution, Abcam, UK), rabbit anti-ATAMTS-7 (1:1000 dilution, Abcam, UK), rabbit anti-MMP-13 (1:1000 dilution, Abcam, UK), mouse anti-aggrecan (1:500 dilution, Santa Cruz Biotechnology, USA), rabbit anti-collagen 2 (1:1000 dilution, Abcam, UK), rabbit anti-p65 (1:1000 dilution, Cell Signaling Technology, USA), rabbit anti-NF-κB p-p65 (1:1000 dilution, Cell Signaling Technology, USA) and mouse anti-β-actin (1:1000 dilution, Santa Cruz Biotechnology, USA) antibodies overnight at 4 °C. After washing with TBST, all the immunolabeled bands were then incubated with the secondary antibody (1:2000 dilution, Cell Signaling Technology, USA) for 2 h and detected using an enhanced FluorChem imaging system (Amersham Imager 600, General Electric Company, USA). Data were analyzed by ImageJ software (National Institutes of Health, USA). The relative level of the protein (Rel. Protein level) was calculated as the grey value ratio of target protein/β-actin.

2.7. Real-time RT-PCR analysis

Total RNA was collected from the intervertebral disc tissues of the cultured NP tissues and NP cells. In each indicated experimental group, the total RNA was extracted by an RNeasy kit (Qiagen, USA) in accordance with the instructions of the manufacturer. RT-Polymerase Chain Reaction (PCR) kits (Toyobo, Japan) were used to establish the first-strand cDNA. RT-PCR was performed with SYBR Green I dye to detect the DNA synthesis, as previously described. Data indicated in the experimental groups were analyzed by Light-Cycler analysis software 4.0.0.23 (Roche, Switzerland) after normalization to GAPDH. The nucleotide sequences of PPAR-γ, ADAMTS-7, COX-2, MMP-13, IL-1β, CCL-

Table 1
Real-time PCR primers.

Target	Forward Primer, 5′-3′	Reverse Primer, 5′-3′
ADAMTS-7	GCAGGTTGAGAGCTATGTGCT	GCATGGTGCGTGATCTTTAGG
COX-2	GGAACTTTCTGGTCCCTTCAG	TGTGTTTGGAGTGGGTTTCA
CCL-20	GCAAGCAACTTTGACTGCTG	CAAGTCCAGTGAGGCACAAA
IL-1β	TTTGAAGCTGATGGCCCTAAACA	TGTAGTGGTGGTCGGAGATTCGT
MMP-13	TGCTGCATTCTCCTTCAGGA	ATGCATCCAGGGGTCCTGGC
NF-κB2	CAGTGAGAAGGGCCGAAAGAC	CAGGGGCAGGGAGAAGGAG
PPAR-γ	GTGGCCGCAGAAATGACC	CCACGGAGCTGATCCCAA
GAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG

20, NF-κB2, and GADPH primers used for RT-PCR were synthesized, as shown in Table 1. The specific PCR products for each gene were confirmed by melting-curve analysis. All gene relative expression levels in different groups were calculated according to a formula: $2 - \Delta\Delta Ct$.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The supernatant of the intervertebral disc from control and IVDD patients was extracted and the expression of IL-17 was detected by ELISA kit (Abcam, UK).The NP cells were cultured with PBS, 100 ng/ml IL-17 + 20 ng/ml TNF-α, 100 ng/ml IL-17 + 20 ng/ml TNF-α + 10 μM pioglitazone, 100 ng/ml IL-17 + 20 ng/ml TNF-α + 50 μM pioglitazone for 48 h. The levels of CCL-20, prostaglandin E2 (PGE-2), IL-1β, IL-10 and TGF-β in the cellular supernatant of each group was measured through ELISA kit (Abcam, UK) following the manufacturer's instructions.

2.9. Immunofluorescence staining

The NP cells were on coverslips and cultured with PBS, 100 ng/ml IL-17, 20 ng/ml TNF-α or 100 ng/ml IL-17 + 20 ng/ml TNF-α for 48 h. The cells were immunostained for PPAR-γ captured by fluorescence microscope system. The NP cells were on coverslips and cultured with PBS, 100 ng/ml IL-17 + 20 ng/ml TNF-α or 100 ng/ml IL-17 + 20 ng/ml TNF-α + 50 μM pioglitazone for 1 h. The cells were immunostained for NF-κB p65 and captured by fluorescence microscope system.

2.10. Statistical analysis

All data were shown as mean values ± standard deviation (SD). Comparison of various treatments was performed as a paired Student's *t*-test or one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant. GraphPad Prism v.5.0 (GraphPad Software, USA) was used to perform the statistical analysis.

3. Results

3.1. The altered level of PPAR-γ and IL-17 in degenerated intervertebral disc tissue

To evaluate the clinical relevance of IL-17 and PPAR-γ in human NP tissues, surgical intervertebral disc tissues were obtained from the patients and measured by IHC staining. Consistent with the previous studies [7,16–18], a high expression level of IL-17 was observed in the IVDD patients ($n = 8$) compared with the scoliosis control group ($n = 5$) (Fig. 1a, c, e). The current report indicated that the PPAR-γ level was down-regulated in many bones and joint diseases, such as rheumatoid arthritis, osteoarthritis [19]. Western blot was applied to further examine the changes in PPAR-γ expression. Western blot and IHC results indicated lower expression of PPAR-γ in the IVDD compared with the control group (Fig. 1b, d, f). IL-17 was recognized to play a crucial pro-inflammatory role in the IVDD [20], while PPAR-γ had an anti-inflammatory effect in arthritis-related diseases [19]. There may be a relationship between the alternation of IL-17 and PPAR-γ. Then, a primary NP cell culture model was established to investigate the effect of IL-17 on PPAR-γ expression.

3.2. Administration of IL-17 and TNF-α down-regulated the level of PPAR-γ in the NP cells

Previous studies have reported the robust synergistic effect of IL-17 and TNF-α which induces the production of pro-inflammatory cytokines [7,9]. Here, the human primary NP cells were stimulated with PBS, IL-17 (100 ng/ml), TNF-α (20 ng/ml), IL-17 (100 ng/ml) plus TNF-α (20 ng/ml) and then the mRNA expression and protein level were detected in each group. As illustrated in Fig. 2a and b, IL-17 or TNF-α alone can decrease the PPAR-γ expression both at the mRNA and protein level. Significantly lower expression of PPAR-γ was observed in IL-17 plus TNF-α group compared to the group treated with IL-17 or TNF-α alone. The combination of TNF-α and IL-17 had a significant synergistic effect in inhibiting PPAR-γ expression which was verified by the immunofluorescence (Fig. 2c).

3.3. Pioglitazone inhibited the production of pro-inflammatory cytokine levels induced by IL-17 in combination with TNF-α

To investigate the effects of the pioglitazone, a PPAR-γ agonist, at different concentrations, the levels of several pro-inflammatory cytokines derived from PBS-, IL-17 + TNF-α-, IL-17 + TNF-α + 10 μM pioglitazone-, and IL-17 + TNF-α + 50 μM pioglitazone-treated NP cells were measured by real-time RT-PCR, western blot or specific ELISA. Our previous study showed that the CCL-20 mediated IL-17 induced an inflammatory immune response in the IVDD. In addition to CCL-20, the pro-inflammatory mediators IL-1β, COX-2, and PGE-2 also contribute to the pathogenesis of IVDD. As shown in Fig. 3a–c, CCL-20, IL-1β, and COX-2 mRNA levels were significantly increased in the IL-17 + TNF-α treatment group, and pioglitazone at both 10 μM and 50 μM inhibited the pro-inflammatory effect of IL-17 in combination with TNF-α in a dose-dependent manner. Consistent with its effects on mRNA expression, the increased levels of CCL-20, IL-1β, and PGE-2 in the NP cells supernatant induced by IL-17 combined with TNF-α were significantly inhibited by pioglitazone at both 10 μM and 50 μM, as assayed by ELISA (Fig. 3e–g). Western blot was used to test the secreted level in the NP cells, both 10 μM and 50 μM pioglitazone showed a decrease in the appearance of COX-2 at the protein level (Fig. 3d). IL-10 and TGF-β exert anti-inflammatory effects in intervertebral disc degeneration. As shown in Fig. 3h, IL-10 expression in control NP cells is very low, and IL-10 expression in IL-17 and TNF-α -stimulated groups is increased. In addition, low concentration of pioglitazone (10 μM) has a weak effect on IL-10 expression, and high concentration of pioglitazone (50 μM) can significantly increase IL-10 expression in cultured NP cells. Compared with the control group, IL-17 and TNF-α could reduce the expression of TGF-β. Compared with IL-17 and TNF-α group, there is no significant change in the expression of TGF-β after the addition of pioglitazone (Fig. 3i).

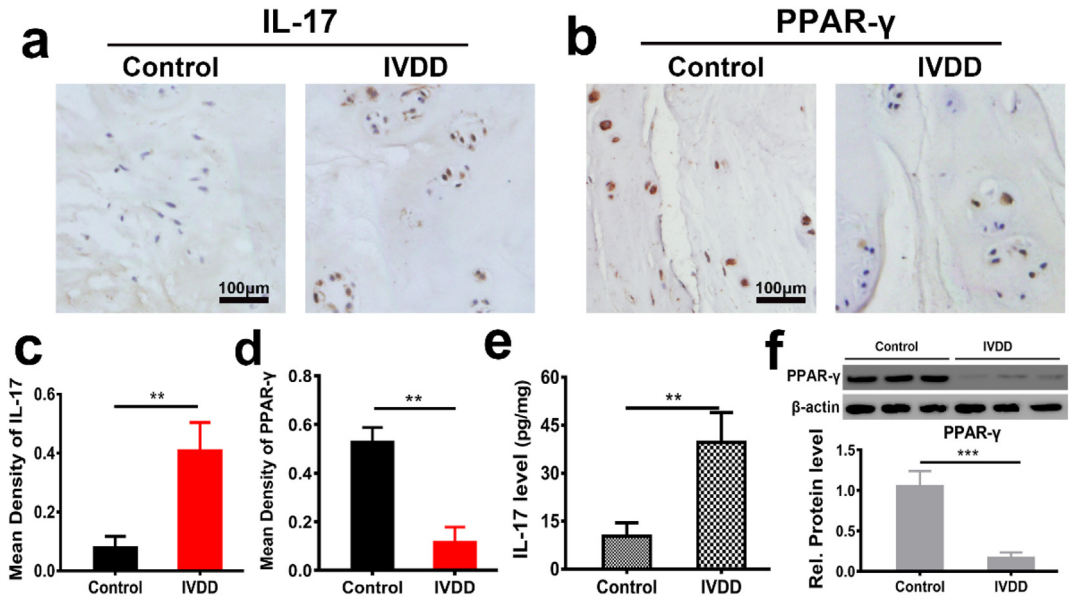


Fig. 1. Expression pattern of PPAR-γ and IL-17 in the human intervertebral disc tissues.
(a–d) Detection of IL-17 (a, c) and PPAR-γ (b, d) expression in the human intervertebral disc tissues of the patients with scoliosis and IVDD, measured by IHC. (e) The IL-17 levels of nucleus pulposus tissue in scoliosis (control group) and IVDD patients were detected. The protein is extracted from the nucleus pulposus tissues and detected by ELISA. (f) Detection of PPAR-γ level in the human intervertebral disc tissues of the control and IVDD patients as assayed by western blot. Scale bar, 100 μm. Significant differences are indicated in the above assays. $^{**}P < 0.01$, $^{***}P < 0.001$.

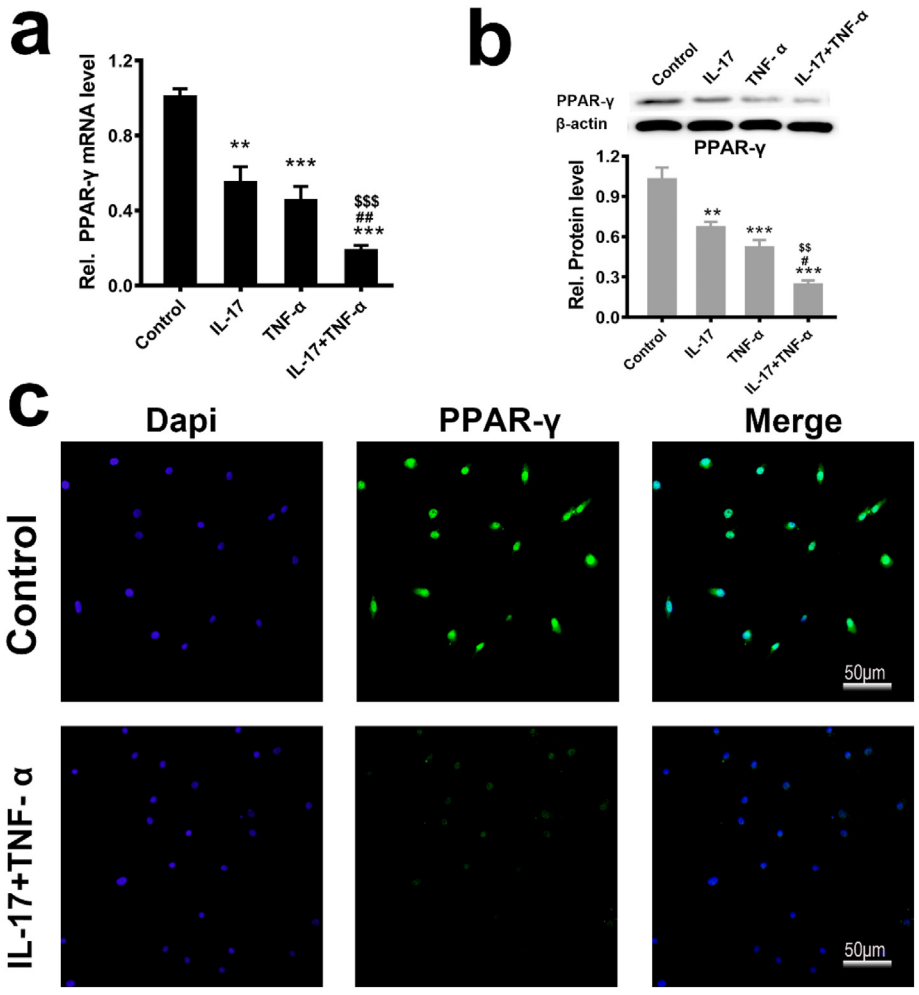


Fig. 2. IL-17 with or without TNF-α inhibited the PPAR-γ expression in the human NP cells.
(a) IL-17 combined with TNF-α had an intensive inhibition on the expression of PPAR-γ compared with that of IL-17 or TNF-α alone. Human NP cells were stimulated by PBS, IL-17 (100 ng/ml), TNF-α (20 ng/ml) or combination of IL-17 (100 ng/ml) and TNF-α (20 ng/ml) for 24 h, then the total mRNA was collected and detected by real-time RT-PCR. (b) Human NP cells were stimulated as indicated above for 48 h, total protein was isolated, and the expression of PPAR-γ was detected by western blot. (c) IL-17 combined with TNF-α could inhibit the expression of PPAR-γ in the human NP cells, tested by immunofluorescence staining. Scale bar, 50 μm. Significant differences are indicated in the above assays. * compared with control group, $^{**}P < 0.01$, $^{***}P < 0.001$; $^{\$}$ compared with IL-17 group, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$; $^{\#}$ compared with TNF-α group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

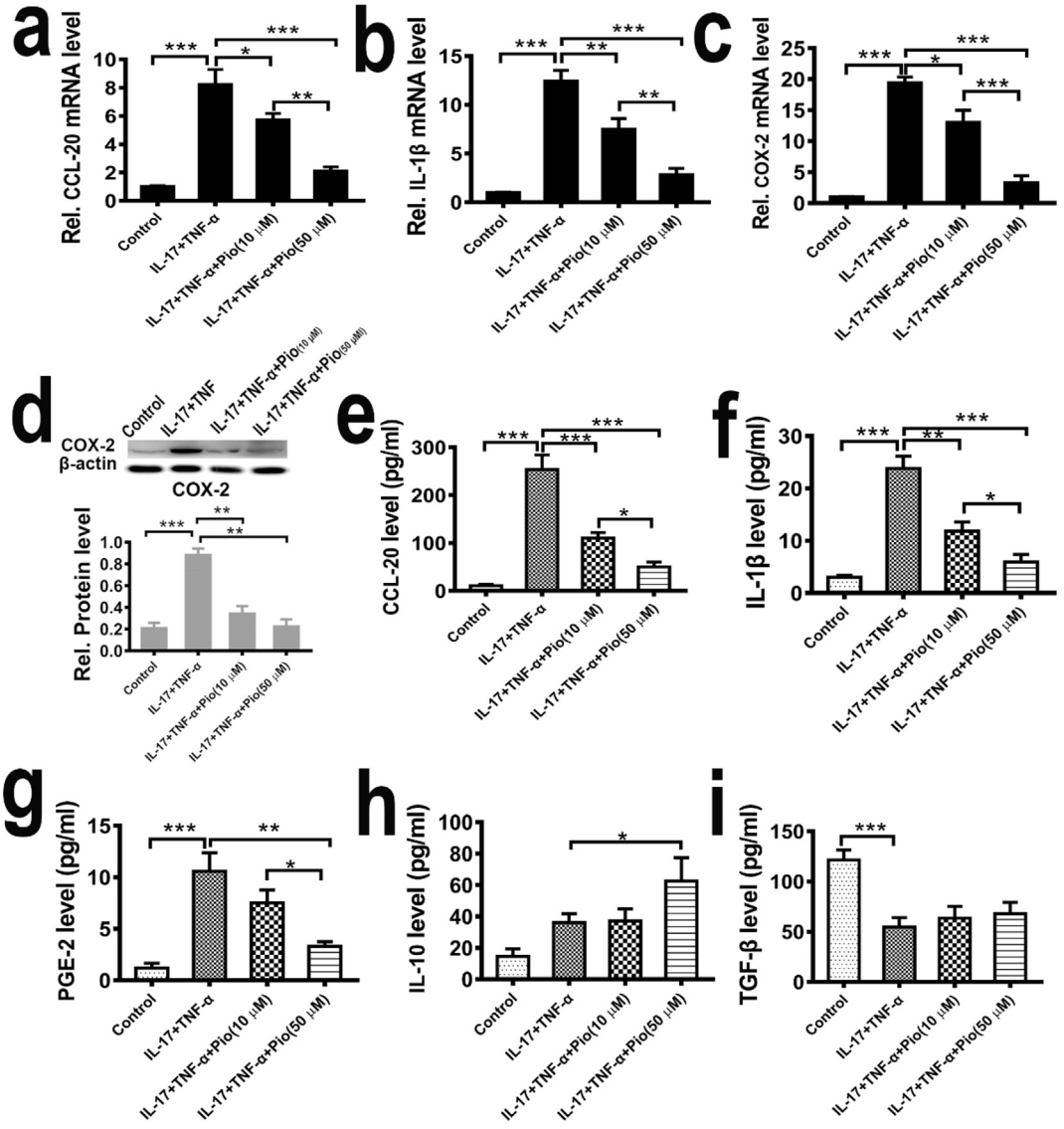


Fig. 3. Elevated levels of inflammatory markers induced by IL-17 combined with TNF-α were inhibited by different concentrations of pioglitazone in the human NP cells.

(a–c) Different concentrations of pioglitazone (10 μM and 50 μM) could significantly inhibit the elevated expression of CCL-20 (a), IL-1β (b), COX-2 (c) induced by IL-17 combined with TNF-α in the human NP cells. Human NP cells were cultured and stimulated by PBS, 100 ng/ml IL-17 + 20 ng/ml TNF-α, 100 ng/ml IL-17 + 20 ng/ml TNF-α + 10 μM pioglitazone, 100 ng/ml IL-17 + 20 ng/ml TNF-α + 50 μM pioglitazone for 24 h. Total mRNA was collected from each group and detected by real-time RT-PCR. (d) Western blot was used to reconfirm the inhibition of pioglitazone on the elevated expression of COX-2 in NP cells induced by IL-17 combined with TNF-α. The NP cells were incubated with indicated stimulations for 48 h, and total protein was collected. (e–g) Elevated levels of CCL-20 (e), IL-1β (f), and PGE-2 (g) induced by IL-17 combined with TNF-α were decreased by different concentrations of pioglitazone. The levels of IL-10 (h) and TGF-β (i) were tested by ELISA. The human NP cells were treated as indicated above for 48 h and cell supernatant of each group was collected for ELISA. Significant differences are indicated. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.4. Pioglitazone reversed the increased metalloproteinase expression and matrix degradation induced by IL-17 with TNF-α

Alternation of enzymes, such as ADAMTS-7 and MMP-13, has been shown to be involved in the degradation of intervertebral discs [21,22]. The cultured NP cells were incubated for 24 h with IL-17 (100 ng/ml) plus TNF-α (20 ng/ml) in the presence of pioglitazone (10 μM and 50 μM), and the levels of ADAMTS-7 and MMP-13 were determined by real-time RT-PCR and western blot analysis. As illustrated in Fig. 4a–c, IL-17 in combination with TNF-α can obviously promote the expression of ADAMTS-7 and MMP-13 both at the mRNA and protein levels compared to the control group. To further assay the degeneration of matrix components, collagen-2 and aggrecan were evaluated by western blot analysis. With the increased levels of pro-inflammation and enzymes, we observed a significant down-regulation of collagen-2 and aggrecan (Fig. 4c–g). Administration of both doses of pioglitazone can inhibit the production of ADAMTS-7 and MMP-13 and enhance the up-regulation of matrix components - collagen-2 and aggrecan in the NP cell cultured model. Application of pioglitazone can reduce the production of nucleus pulposus degrading enzymes and increase the production of aggrecan and collagen2 in nucleus pulposus cells. Compared with the low-dose group (10 μM), the high-dose group (50 μM), showed a more obvious protective effect on nucleus pulposus cells.

3.5. Pioglitazone inhibited IL-17 and TNF-α induced NF-κB pathway activation

As shown in Fig. 5a, the expression of NF-κB p-p65 was increased in

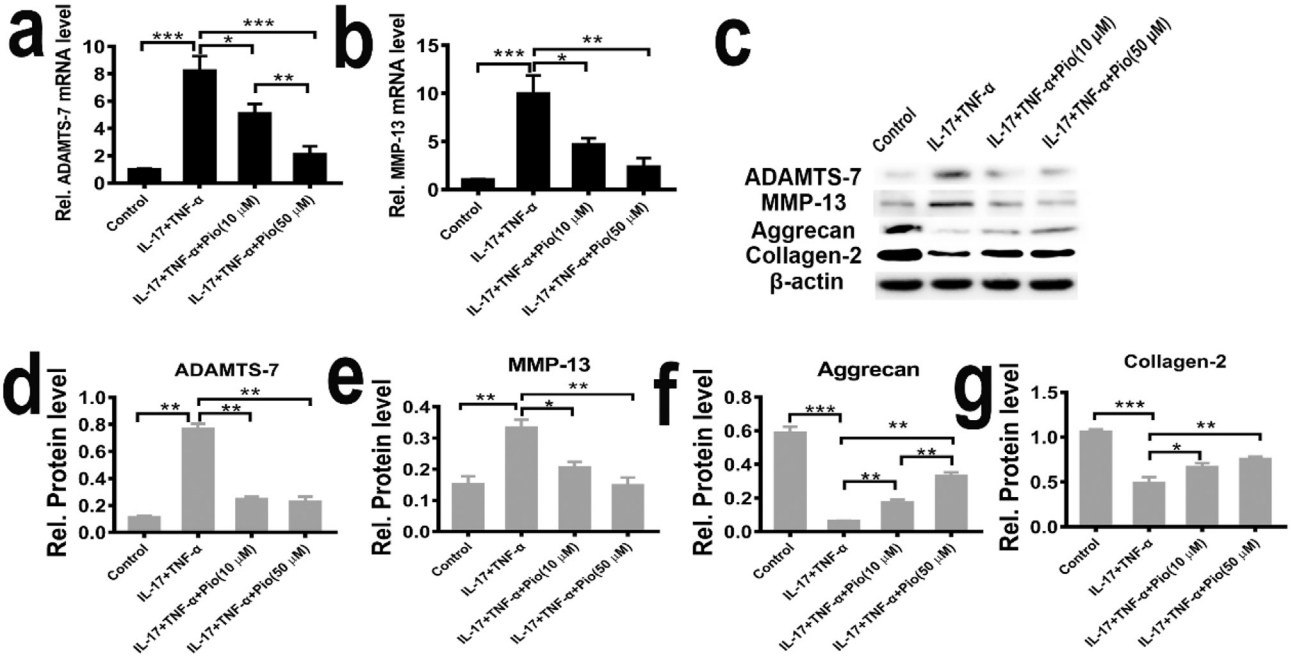


Fig. 4. Pioglitazone inhibited the expression of catabolic enzymes participated in disc degeneration and reduced the degeneration of the main components of the human intervertebral disc.

(a–b) Pioglitazone inhibited the increased expression of ADAMTS-7 (a) and MMP-13 (b) stimulated by IL-17 combined with TNF-α, the human NP cells were treated as indicated in Fig. 3 for 24 h and total mRNA was collected for RT-PCR. (c–g) Treatment of IL-17 in combination with TNF-α stimulated the expression of ADAMTS-7 (d), MMP-13 (e), and inhibited the expression of aggrecan (f), collagen-2 (g), while the effects were inhibited by pioglitazone, as detected by western blot in the human NP cells. Significant differences are indicated. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{**}P < 0.001$.

the IVDD tissues compared with the control group. Since both IL-17 and TNF-α activate NFκB pathway, to enhance the production of pro-inflammatory cytokine and related metabolic enzyme, the primary NP cells were treated with 100 ng/ml IL-17 or 100 ng/ml IL-17 + 20 ng/ml TNF-α in the presence or absence of pioglitazone (50 μM). As shown in Fig. 5b–d, we examined the content of NF-κB p-p65 in total protein of different stimulation groups. The results showed that the expression of NF-κB p-p65 was increased stimulated by IL-17 and IL-17 combined with TNF-α, indicating that NF-κB pathway was activated. Decreased expression of NF-κB p-p65 after pioglitazone showed that pioglitazone can inhibit the activation of NF-κB pathway activated by IL-17 and/or TNF-α to exert anti-inflammatory effects. Fig. 5e showed that the pioglitazone treated group down-regulated the expression of NF-κB p65 level induced by IL-17 or by IL-17 + TNF-α. As shown in Fig. 5f, we found that both IL-17 and TNF can cause activation of the NF-κB pathway and increase the mRNA level of NF-κB2, but IL-17 combined with TNF-α can significantly activate the NF-κB pathway compared to IL-17 or TNF-α alone, consistent with the previous results [9]. To further confirm the effect of pioglitazone, NF-κB p65 was detected through immunofluorescence staining. Fig. 5g showed that pioglitazone abolished the activation of the NF-κB pathway. These results indicated that the NF-κB signaling pathway might be involved in the protective role of pioglitazone in the IVDD.

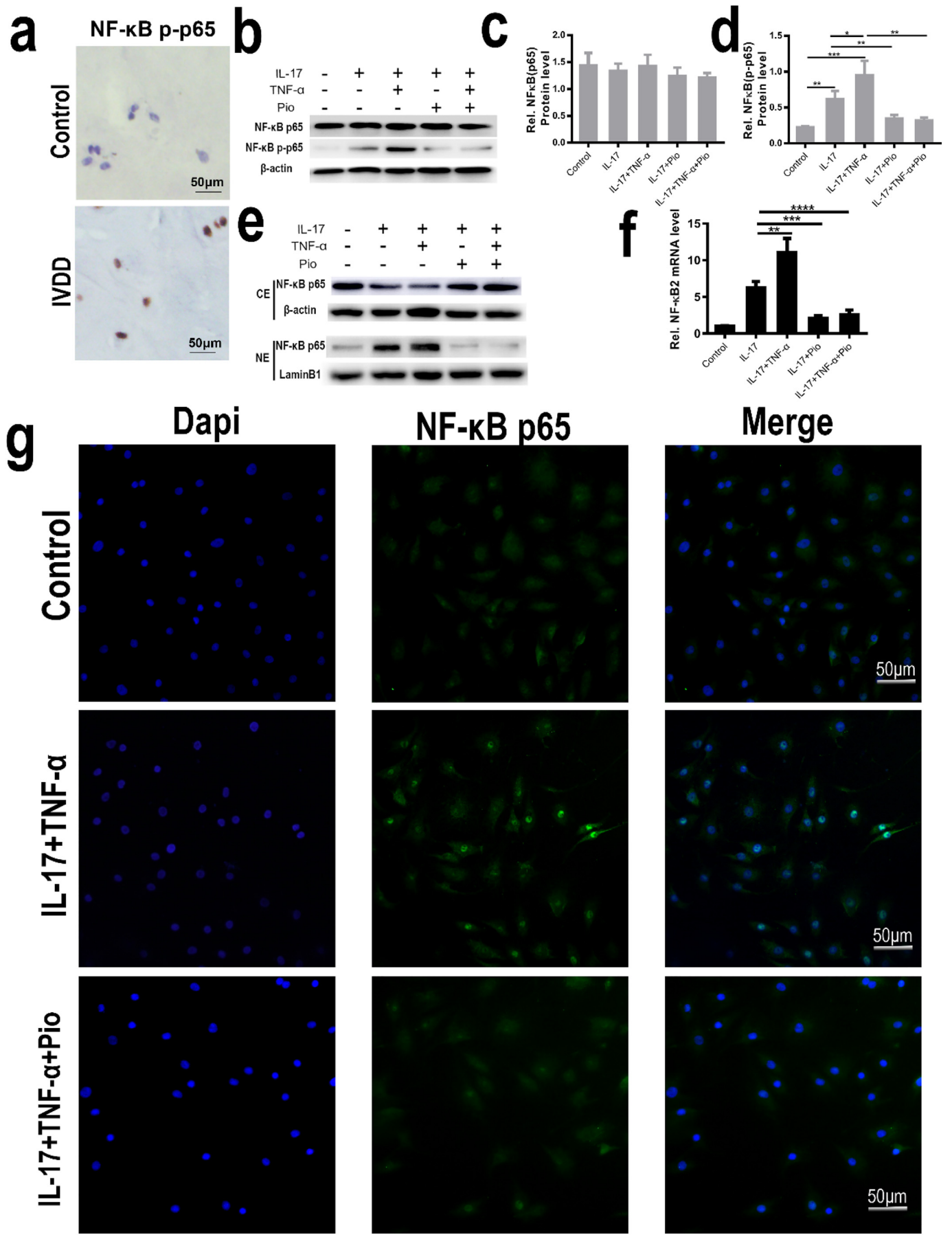
3.6. Pioglitazone inhibited IL-17 and TNF-α induced NP tissues' inflammatory reaction and degeneration in vitro

To further validate the effect of pioglitazone on the NP tissues' inflammatory reaction and degeneration, we used PBS, IL-17 + TNF-α, IL-17 + TNF-α + pioglitazone (50 μM) to stimulate the cultured NP tissues *in vitro*. Pioglitazone inhibited the mRNA expression levels of CCL-20 and IL-1β (Fig. 6a and b). Moreover, pioglitazone reduced the protein expression of COX-2, ADAMTS-7, MMP-13, and increased the expression of collagen-2 (Fig. 6c–g), induced by IL-17 combined with TNF-α. Aggrecan is an essential component of the intervertebral disc matrix. It degrades significantly when the disc degenerates. Here, immunohistochemistry was performed to detect the expression of aggrecan degradation products in the NP tissue stimulated by IL-17 + TNF-α with or without pioglitazone. As shown in Fig. 6h–i, the administration of IL-17 + TNF-α led to the increased expression of aggrecan degradation products, while the additional use of pioglitazone antagonized this trend.

4. Discussion

IVDD is an inflammatory process triggered by the autoimmune system [23]. There are a large number of inflammatory factors and enzymes in the degenerated intervertebral disc tissue, such as IL-1β, TNF-α, PGE-2, ADAMTS-7, MMP-13, and COX-2 [18]. In addition, it was found that CCL-20 is abundantly expressed in NP cells in the degenerated intervertebral disc tissue, which attracts Th17 cells to the inflammatory region mainly by chemotactic [7]. NP, as a major component of the intervertebral disc tissue, has an immune evasion effect. When the disc degeneration occurs, the structure of the intervertebral disc tissue is destroyed, and the contact of the NP with the immune system triggers a series of inflammatory reactions [24,25]. With the development of degenerative severity and accumulation of inflammatory factors and metabolically related enzymes in the intervertebral disc, macrophages and Th cells are drawn to the degenerated intervertebral disc tissue by chemotactic influences of inflammatory factors and the main components of the extracellular matrix of the intervertebral disc, such as collagen-2 and aggrecan, are gradually reduced.

Previous studies have found that Th17 cells are CD4+ helper T lymphocytes subgroup and play an essential role in a series of immune-mediated inflammatory diseases, such as rheumatoid arthritis [26], psoriasis [27], asthma [28], inflammatory bowel disease [29], *etc.* Our previous study found that Th17 cells are also involved in the inflammatory process of intervertebral disc degeneration, and Th17 cells are significantly increased in the peripheral blood and degenerated



(caption on next page)

Fig. 5. Pioglitazone inhibited the activation of the NF-κB signal pathway induced by IL-17 with or without TNF-α.
(a) Levels of NF-κB p-p65 in the normal and degenerated disc tissues were detected by IHC. (b–d) The expression levels of NF-κB p65 and NF-κB p-p65 in total protein of NP cells treated by PBS, 100 ng/ml IL-17, 100 ng/ml IL-17 + 20 ng/ml TNF-α, 100 ng/ml IL-17 + 50 μM pioglitazone and 100 ng/ml IL-17 + 20 ng/ml TNF-α + 50 μM pioglitazone for 1 h were detected. Total protein of each group was extracted and assayed by western blot. (e) Expression of NF-κB p65 in the nucleus and outside the nucleus in the NP cells was detected by western blot, the NP cells were treated as indicated above for 1 h and total protein of each group was collected. (f) The relative mRNA levels of NF-κB2 in the NP cells stimulated by PBS, IL-17, IL-17 + TNF-α, IL-17 + 50 μM Pioglitazone, IL-17 + TNF-α + 50 μM Pioglitazone for 12 h, respectively were assayed by real-time RT-PCR. (d) Expression of NF-κB p65 in the NP cells induced by IL-17 + TNF-α and IL-17 + TNF-α + 50 μM Pioglitazone for 1 h were detected by the immunofluorescence staining. Significant differences are indicated. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ compared to IL-17 treated group. Scale bar, 50 μm.

intervertebral disc tissue in the patients with disc herniation [6,17]. In addition, IL-17, as a characteristic factor of Th17 cells, plays an important pro-inflammatory role in the development of many chronic inflammatory diseases, such as psoriasis vulgaris [30], rheumatoid arthritis [31], and Crohn's disease by promoting the secretion of other inflammatory factors [32]. IL-17 was also found to play an important role in the inflammatory response by stimulating the secretion of many other inflammatory cytokines in degenerated intervertebral disc tissue. Our research group also conducted related experimental studies and found that the levels of IL-17 in the blood and degenerated intervertebral disc tissue of the patients with degenerated intervertebral disc were significantly higher than those of the control group. After degenerative discectomy, the level of IL-17 in the blood of the patients was significantly reduced. And IL-17 can dramatically stimulate the expression of IL-6, CCL-20, and PGE-2 in the cultured NP cells *in vitro* [7,9]. In this study, the IL-17 level was significantly increased in the degenerated intervertebral disc tissue compared with the control group, which was verified again.

IL-17 could up-regulate the levels of a variety of pro-inflammatory chemokines and cytokines by activating the NF-κB, MAPKs, and CCAAT/enhancer binding proteins (C/EBPs) signal pathways [33]. IL-17 signals activation is in part through the NF-κB pathway, and the

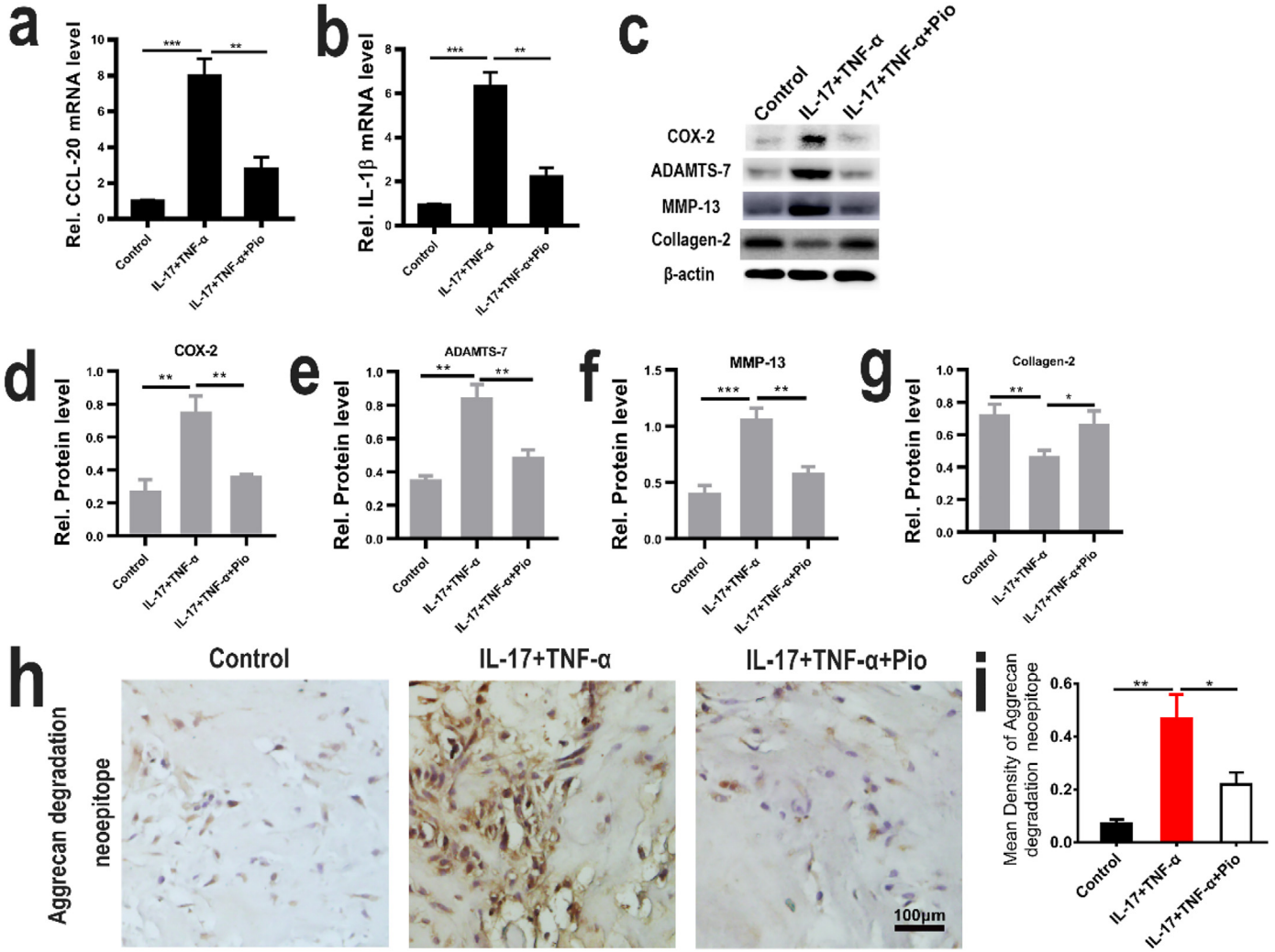


Fig. 6. Pioglitazone inhibited the inflammation and degeneration induced by IL-17 combined with TNF-α in cultured human intervertebral disc tissue.
(a–b) Levels of CCL-20 and IL-1β in the cultured human disc tissues treated with PBS, IL-17 + TNF-α or IL-17 + TNF-α + Pioglitazone for 7 days were detected by RT-PCR. (c) Expression of COX-2 (d), ADAMTS-7 (e), MMP-13 (f), and collagen-2 (g) in the cultured human intervertebral disc tissues treated as indicated above for 7 days was detected by western blot. (h–i) IL-17 combined with TNF-α could markedly increase the level of aggrecan degradation neoepitope, while this effect was inhibited by pioglitazone, as detected by IHC. IL-17 at 100 ng/ml, TNF-α at 20 ng/ml, pioglitazone at 50 μM. Significant differences are indicated. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to IL-17 in combination with TNF-α group.

major signaling pathway of IL-17 is through C/EBP transcription factors. Therefore, IL-17 alone is a weak NF-κB activator. But it can synergize with other cytokines like TNF-α to aggravate inflammatory responses to be a robust pathogenic cytokine. TNF-α increases the expression of the pro-inflammatory factor mainly through the activation of the NF-κB signal pathway and is a robust NF-κB activator [8,34]. IL-17 can promote the expression of TNF-α in degenerated intervertebral disc tissue. And the increased expression of TNF-α can enhance the pro-inflammatory effect of IL-17 to form a positive feedback loop. The synergistic pro-inflammatory effects of IL-17 and TNF-α have been previously confirmed in many cells *in vitro*, such as osteoblasts, fibroblasts, and bone marrow stromal cells [8,35,36]. Our previous study also found that IL-17 or TNF-α alone could stimulate the expression of CCL-20 in NP cells, and this effect was enhanced by IL-17 combined with TNF-α [7]. In this study, we also found that the combination of IL-17 and TNF-α significantly increased the expression levels of IL-1β, CCL-20, COX-2, PGE-2, MMP-13, and ADAMTS-7 in the NP cells and NP tissues cultured *in vitro*. At the same time, the combination of IL-17 and TNF-α could significantly reduce the expression of the main components of the NP, such as collagen-2 and aggrecan. More importantly, IL-17 combined with TNF-α could dramatically activate the NF-κB pathway compared with IL-17 alone. The NF-κB pathway is pivotal in the process of intervertebral disc degeneration. Therefore, finding a drug that inhibits the NF-κB pathway may reduce or reverse the degeneration of the intervertebral disc.

In recent years, the PPAR, as a ligand-activated transcription factor, consists of three members: PPAR-α, PPAR-δ, and PPAR-γ and has been found to be involved in many inflammatory diseases except its effects on adipocyte differentiation and glucose homeostasis. Yasu-Taka Azuma et al. found that PPAR-α contributes to inhibiting the colitis of mice model [37] and PPAR-γ ligand inhibited the inflammatory functions of macrophages [38]. In addition, previous researches have shown that the high expression of PPAR-γ has protective effects on the inhibition of osteoarthritis and atherosclerosis [12,19]. These results suggest that PPAR-γ may be an endogenous anti-inflammatory factor. According to the published investigations, PPAR-γ can inhibit the inflammatory response mainly through competitive inhibition of the NF-κB signaling pathway to terminate NF-κB pathway-elicited inflammation. Therefore, we speculated that PPAR-γ might be involved in the inflammatory process of IVDD. The results showed that the expression of PPAR-γ was significantly lower in the degenerated intervertebral disc tissue than that in the control group. And IL-17 or TNF-α monotherapy can dramatically reduce the appearance of PPAR-γ in NP cells. However, IL-17 combined with TNF-α significantly decreased the expression of PPAR-γ compared with the single group of IL-17 or TNF-α. Next, we applied pioglitazone to increase the expression of PPAR-γ in the NP cells and NP tissues and found that pioglitazone can significantly inhibit the production of pro-inflammatory factors and degrading enzymes induced by IL-17 combined with TNF-α. Here, we demonstrated that pioglitazone application can also increase the production of anti-inflammatory factors (IL-10), but not the expression of TGF-β. At the same time, pioglitazone can inhibit the reduced levels of collagen-2 and aggrecan induced by IL-17 combined with TNF-α. In addition, we also found that pioglitazone significantly inhibited the activation of NF-κB pathway induced by IL-17 or IL-17 in combination with TNF-α. These findings unravel that pioglitazone, as a PPAR-γ activator, has a novel function in inhibiting the inflammation of IVDD and inhibits disc degeneration through NF-κB inhibition.

Lower back pain caused by the degeneration of the intervertebral disc is miserable as well as an economic burden to many patients and is a common social problem. This study demonstrates for the first time that pioglitazone, an activator of PPAR-γ, inhibits intervertebral disc inflammation and reduces degeneration by inhibiting the NF-κB signal pathway. In the future, more animal experiments and large-scale clinical trials are needed to explore detailed mechanism.

Conflict of interest

The authors declare no conflict of interest.

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