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Original Article

Propionibacterium acnes induces intervertebral discs degeneration by increasing MMP-1 and inhibiting TIMP-1 expression via the NF-κB pathway

Yuehuan Zheng[1*], Yazhou Lin[2,3*], Zhe Chen[2,3], Yucheng Jiao[2,3], Ye Yuan[2,3], Changwei Li[3], Xiangyang Xu[2], Peng Cao[2,3]

[1]Department of Orthopedics, Ruijin Hospital North, Shanghai Jiaotong University School of Medicine, Shanghai, China; [2]Department of Orthopedics, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China; [3]Shanghai Key Laboratory for Prevention and Treatment of Bone and Joint Diseases with Integrated Chinese-Western Medicine, Shanghai Institute of Traumatology and Orthopedics, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China. [*]Equal contributors.

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Abstract: Emerging evidence suggests that *Propionibacterium acnes* (*P. acnes*) may be a new pathogen implicated in intervertebral disc degeneration (IVDD), although the underlying mechanisms are unclear. Since the most significant biochemical change of IVDD is inability of the extracellular matrix (ECM) synthesis by nucleus pulposus cells, we first analyzed the expression of aggrecan and collagen II in nucleus pulposus tissues of IVDD patients with or without *P. acnes* infection. Compared with the *P. acnes*-negative controls, the expression levels of aggrecan and collagen II were significantly dampened in nucleus pulposus tissues with *P. acnes* infection. Interestingly, we found that *P. acnes* infection strongly increased matrix-degrading metalloproteinase-1 (MMP-1) expression but decreased that of tissue inhibitor of metalloproteinase-1 (TIMP1). Furthermore, the dampened aggrecan and collagen II synthesis concomitant with the increased MMP-1 and decreased TIMP-1 expression seen in *P. acnes*-induced IVDD were confirmed by a rat model. Mechanistically, *P. acnes* infection increased MMP-1 levels, while decreasing TIMP-1 expression via the NF-κB pathway. Overall, these findings reveal that *P. acnes* infection dampens aggrecan and collagen II synthesis in nucleus pulposus cells by increasing MMP-1 and inhibiting TIMP-1 expression via the NF-κB pathway, which may ultimately lead to IVDD.

Keywords: *Propionibacterium acnes*, MMP-1, TIMP-1, NF-κB, Disc degeneration

Introduction

Intervertebral discs (IVDs) are complex anatomical structures with a specific load-bearing organization that gives the spine flexibility [1]. As the inner core of IVDs, the nucleus pulposus (NP) is known to resist mechanical loading by synthesizing the extracellular matrix (ECM) containing proteoglycan and collagen II, thus maintaining the stability of IVDs [2]. The hallmark of progressive IVD degeneration (IVDD) is the incapacity of NP cells (NPCs) to maintain normal homeostatic tissue remodeling, thereby leading to a series of severe sequelae, such as disc herniation, Modic changes, sciatica, and lower back pain, which greatly worsen quality of life and increase the economic and health care burdens of society [3].

Traditionally, excessive mechanical loading, nutritional disorders, traumatic injury, or genetic predisposition are considered the main etiological causes for IVDD [3]. However, recent studies suggested that *Propionibacterium acnes*, a low-virulence anaerobic bacterium, which was found to latently reside inside non-pyogenic IVDs, could induce IVDD. Epidemiological studies and systemic analyses revealed that the prevalence of *P. acnes* in IVDD ranged from 13 to 44% [4-8]. In our previous study, *P. acnes* colonies were identified in non-pyogenic degenerated IVD by anaerobic culture and histological observation [9]. More interestingly, inoculation of *P. acnes* into normal rabbit IVDs induced severe disc degeneration and Modic changes [10]. Although our previous study uncovered that *P. acnes* infection could initiate IVDD by

promoting NPC apoptosis [11], the pathological mechanisms of *P. acnes*-induced IVDD remain unclear and need further investigation.

In the present study, we set out to investigate the mechanisms by which *P. acnes* induces IVDD. Our findings uncover that *P. acnes* infection dampens aggrecan and collagen II synthesis in nucleus pulposus cells by increasing MMP-1 and inhibiting TIMP-1 expression via the NF-κB pathway, which may ultimately lead to IVDD.

Materials and methods

Patients and tissue harvesting

A total of 20 patients were included in this study. The patients underwent discectomy at the single-level lumbar spine due to disc degeneration associated with low back pain and/or sciatica. Patients who received antibiotics within the month preceding surgery were not included in this study. Following the methodology of previous research [9], all tissues were cultured in tryptone soy broth (TSB) for 14 days under anaerobic conditions, then the presence of bacteria in the culture was identified by amplifying the 16S rDNA gene by PCR according to our previous protocol [9].

After culture of the specimen and bacterial identification, the patients who had *P. acnes* only in intervertebral discs were classified as the positive group (n=23). A case-controlled method was used for the quantitative analysis following a previous study [12]. 10 patients who were identified as completely bacteria-free in their intervertebral discs were selected to match each of the positive patients based on the following criteria: 1. same gender; 2. same surgery segment; 3. same symptoms of low back pain only, sciatica only or both; 4. similar ages ± 5 years; 5. similar duration of symptoms ± 3 months.

The study was approved by the Institutional Review Board of Shanghai Ruijin Hospital and informed consent forms were signed by all patients.

Preparation of P. acnes inoculum

P. acnes preparation was performed as described in our previous report [11]. A standard strain of P. acnes (ATCC: 6919, GIM: 1.243, Guangdong Microbiology Culture Center, Guangdong, China) was cultured on Gifu Anaerobic (GAM) broth (Nissui, Tokyo, Japan) for 3 d at 37°C under anaerobic conditions.

Inoculation of P. acnes into rat caudal intervertebral discs

The inoculation of P. acnes into caudal rat IVDs was performed as described in our previous study [11]. The target vertebrae (Ca) 6/7 to (Ca) 8/9 (n=3 per animal) of eight-week-old male Sprague-Dawley rats were identified and marked by palpation and X-ray before surgery. A volume of 2.5 μl *P. acnes* (OD_{600}=3.0), *P. acnes* with BAY11-7082 (NF-κB inhibitor, 10 μM) or saline was inoculated vertically into the nucleus pulposus using a microsyringe with a 28-gauge needle (Hamilton, Nevada, USA). All animal experiments were performed in accordance with the protocol approved by the Shanghai Jiao Tong University (SJTU) Animal Care and Use Committee [IACUC protocol number: SYXK (Shanghai) 2011-0113] and in accordance with the Ministry of Science and Technology of the People's Republic of China animal care guidelines. All surgeries were performed under anesthesia, and all efforts were made to minimize suffering.

Cell culture and stimulation

Primary nucleus pulposus cells were isolated and cultured as described in our previous report [11]. The bacteria were harvested from 3-day cultures in a stationary phase and washed twice with phosphate-buffered saline (PBS). The bacterial density was adjusted to optical density (OD_{600}=2). Then, *P. acnes* were added to the cell culture (5×10^5 cells/well) in a 6-well culture plate at a 100:1 multiplicity of infection (MOI) without antibiotics.

Immunoblotting and immunofluorescent staining

Nucleus pulposus tissues isolated from patients and rats IVDs or nucleus pulposus cells stimulated as described were lysed using a RIPA buffer (pH 7.4) containing a protease inhibitor cocktail (Roche). 10 mg of total protein was used for the Western blots. MMP-1, TIMP-1, aggrecan, collagen II and β-actin were detected by immunoblot with their specific antibodies, respectively. The protein bands were

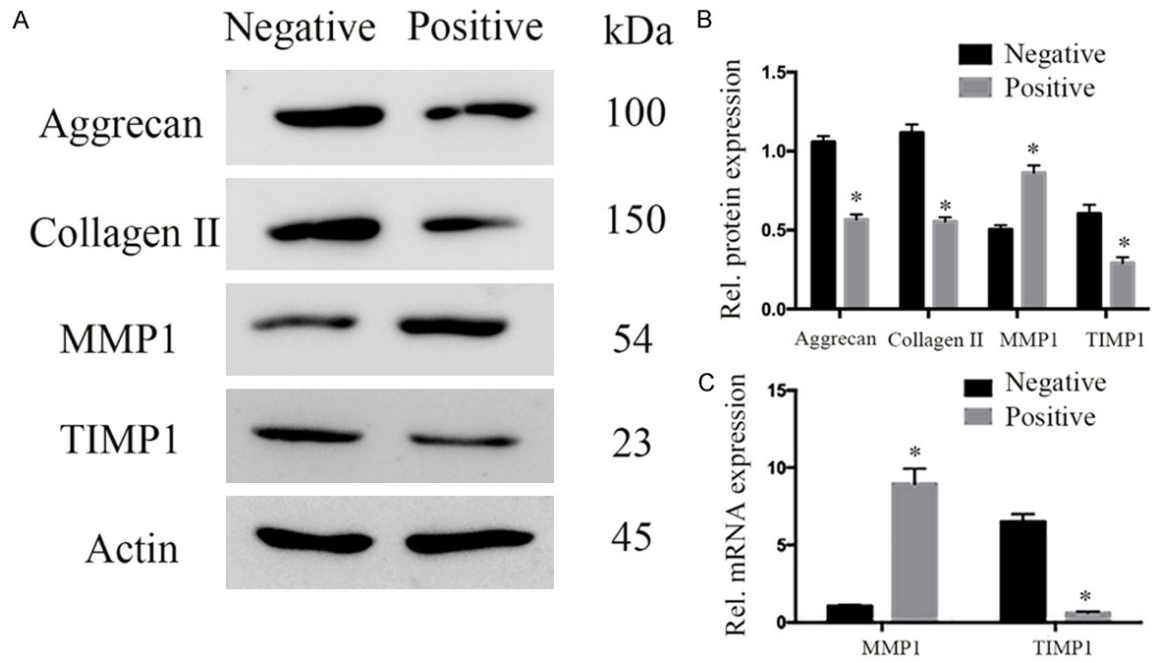

Figure 1. *P. acnes* infection dampened aggrecan and collagen II expression by increasing MMP-1 and decreasing TIMP-1 expression in patients. A, B. Western blot analysis of aggrecan, collagen II, MMP-1 and TIMP-1 expression in nucleus pulposus tissues of patients with or without *P. acnes* IVDs infection. Samples from 10 patients with *P. acnes* IVDs infection were pooled together. C. Quantification of MMP-1 and TIMP-1 mRNA expression in nucleus pulposus tissues of 10 patients with or without *P. acnes* IVDs infection. **P < 0.05*, *P* values were analyzed using Student's *t* test. Data were presented as mean ± SD from three independent experiments.

quantitatively analyzed using an image analysis system (Quantity One software, Bio-Rad, Hercules, CA, USA).

For MMP-1 and TIMP-1 immunofluorescent staining, nucleus pulposus cells were cultured on glass slides as described and then fixed for 30 min in 4% para-formaldehyde. The NPCs were incubated for 16 h at 4°C with MMP-1 and TIMP-1 antibodies respectively. All images were observed using a fluorescence microscope (Axio, Carl Zeiss, Oberkochen, Germany).

Real-time quantitative RT-PCR

Real-time RT-PCR specific primers were used to evaluate gene expression. Rat GAPDH: 5'-TCTACCCACGGCAAGTCC-3' (forward) and 5'-GATGTTAGCGGGATCTCG-3' (reverse); rat MMP-1: 5'-TTTGATGGACCTCAATAT-3' (forward) and 5'-CATTAGTGCTCCTACA-3' (reverse); rat TIMP-1: 5'-TTTGCATCTCTGGCCTCTG-3' (forward) and 5'-CATCTTGATCTCATAACGC-3' (reverse); human GAPDH 5'-CTTAGCACCCCTGGCCAAG-3' (forward) and 5'-TGGTCATGAGTCCTTCCACG-3' (reverse); human MMP-1: 5'-GATGTGGAGTGCCTGATGTG-3' (forward) and 5'-TCTCAATGGCATGGTCCAC-3' (reverse); rat TIMP-1: 5'-ACATCCGGTTCGTCTACACC-3' and 5'-TGATGTGCAAGAGTCCATCC-3' (reverse); RNA analysis was done as previously reported [13].

Statistical analysis

All data are representative of three independent experiments and are as mean ± S.D. We used two-tailed t tests to determine significances between the two groups. We did analyses of multiple groups by one-way ANOVA with a Bonferroni post test of GraphPad prism version 5. For all statistical tests, we considered *P* value < 0.05 to be statistically significant.

Results

P. acnes induces IVDD by increasing MMP-1 and decreasing TIMP-1 expression in patients

To detect the pathological mechanisms of *P. acnes*-induced IVDD, ten *P. acnes*-positive degenerated IVDs were collected from patients; meanwhile, ten *P. acnes*-negative degenerated

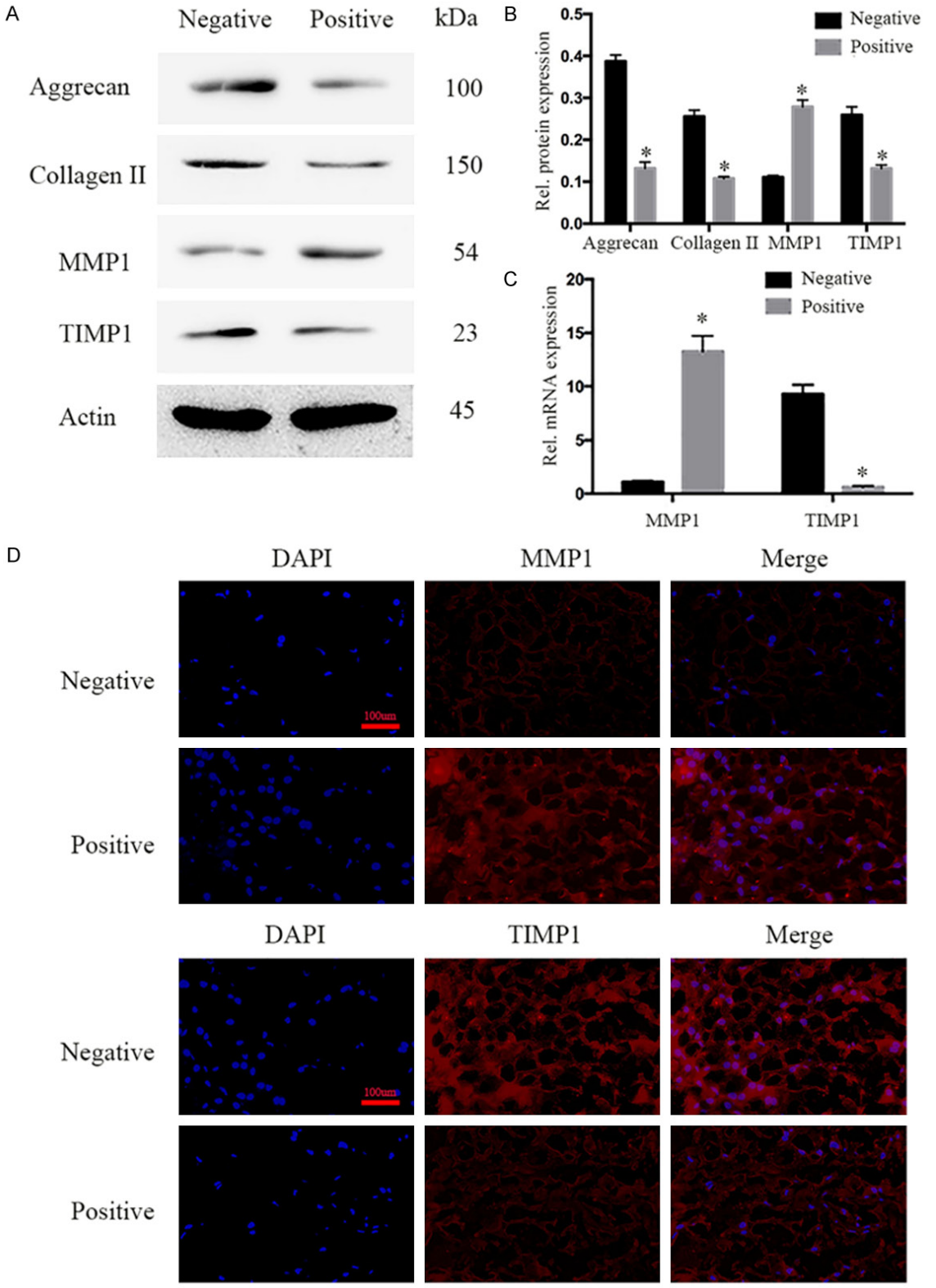

Figure 2. *P. acnes* infection dampened aggrecan and collagen II expression by increasing MMP-1 and decreasing TIMP-1 expression in rats. A, B. Western blot analysis of aggrecan, collagen II, MMP-1 and TIMP-1 expression in nucleus pulposus tissues of rats with or without *P. acnes* caudal IVDs infection. Samples from 6 rats with *P. acnes* caudal IVDs infection were pooled together. C. Quantification analysis of MMP-1 and TIMP-1 expression in nucleus

pulposus tissues of 6 rats with or without *P. acnes* caudal IVDs infection. D. Immunofluorescent staining analysis of MMP-1 and TIMP-1 expression in nucleus pulposus tissues of rats with or without *P. acnes* caudal IVDs infection. Scale bar represents 100 μm. *$P < 0.05$, *P* values were analyzed using Student's t test. Data were presented as mean ± SD from three independent experiments.

IVDs were chosen as controls with a case-controlled matched method used previously [9, 14]. Consistent with our previous report [11], aggrecan and collagen II expressions were significantly decreased in nucleus pulposus tissues from *P. acnes*-positive degenerated IVDs compared to those from *P. acnes*-negative degenerated IVDs (**Figure 1A**, **1B**); from this we predicted that *P. acnes* infection worsened IVDD. Since the concerted activity of matrix-degrading metalloproteinases (MMPs) and aggrecan-degrading enzymes causes a progressive loss of collagens and proteoglycans, and MMP-1 is one of the most important MMPs in ECM degradation [15-18], we then examined whether *P. acnes* dampened aggrecan and collagen II expression by inducing MMP-1 expression. The results showed that *P. acnes* infection strongly increased MMP-1 but decreased the tissue inhibitor of metalloproteinase-1 (TIMP1) expression (**Figure 1A-C**). Taken together, these data demonstrated that *P. acnes* IVDs infection dampened aggrecan and collagen II expression by increasing MMP-1 and decreasing TIMP-1 expression in patients.

P. acnes induces IVDD by increasing MMP-1 and decreasing TIMP-1 expression in rats

To further confirm the dampened aggrecan and collagen II expression resulting from the upregulation of MMP-1 and downregulation of TIMP-1 in *P. acnes*-induced IVDD, *P. acnes* was inoculated into the caudal IVD of rats. We found that *P. acnes* infection significantly decreased aggrecan and collagen II expression in rat nucleus pulposus tissues (**Figure 2A**, **2B**). Interestingly, a Western blot (**Figure 2A**, **2B**), mRNA quantification (**Figure 2C**), and immunofluorescence analyses (**Figure 2D**) further confirmed the increase in MMP-1 and decrease in TIMP-1 expression in nucleus pulposus tissues in response to *P. acnes* IVDs infection. Collectively, these results demonstrated that *P. acnes* IVDs infection dampened aggrecan and collagen II expression by increasing MMP-1 and decreasing TIMP-1 expression in rats.

P. acnes incubation dampens aggrecan and collagen II expression by increasing MMP-1 and decreasing TIMP-1 expression in NPCs

Next, we investigated whether *P. acnes* infection could dampen aggrecan and collagen II expression by increasing MMP-1 and decreasing TIMP-1 expression *in vitro*. Upon co-culturing NPCs with *P. acnes* (MOI=100) for different time periods (12, 24 h), a time-dependent decrease in aggrecan and collagen II was observed (**Figure 3A**, **3B**). Simultaneously, we detected a time-dependent increase in MMP-1 and a decrease in TIMP-1 expression using western blot and real time PCR (**Figure 3A-D**). Consistently, the increased MMP-1 and decreased TIMP-1 expression was further confirmed by immunofluorescence (**Figure 3E**). Taken together, these data revealed that *P. acnes* incubation dampened aggrecan and collagen II expression by increasing MMP-1 and decreasing TIMP-1 expression in NPCs.

P. acnes infection increases MMP-1 and decreased TIMP-1 expression via NF-κB pathway

Having observed that *P. acnes* infection dampened aggrecan and collagen II expression by increasing MMP-1 and decreasing TIMP-1 expression, we next sought to detect the mechanisms by which *P. acnes* infection increases MMP-1 and decreases TIMP-1 expression. Since our previous study demonstrated that the NF-κB signaling pathway mediated *P. acnes*-induced IVDD by regulating aggrecan and collagen II expression, we speculated that NF-κB activation was essential for *P. acnes*-mediated MMP-1 and TIMP-1 expression. To this end, the NF-κB activation was analyzed in response to *P. acnes* infection. The results showed that *P. acnes* incubation increased the phosphorylation of p65 (a subunit of NF-κB) in a time-dependent manner (**Figure 4A**, **4B**). Next, we inhibited NF-κB activation with the NF-κB inhibitor-BAY11-7082 and revealed that in line with the dampened p65 phosphorylation, BAY11 pretreatment significantly decreased MMP-1, but it increased TIMP-1 expression in NPCs (**Figure 4C**, **4D**). Collectively,

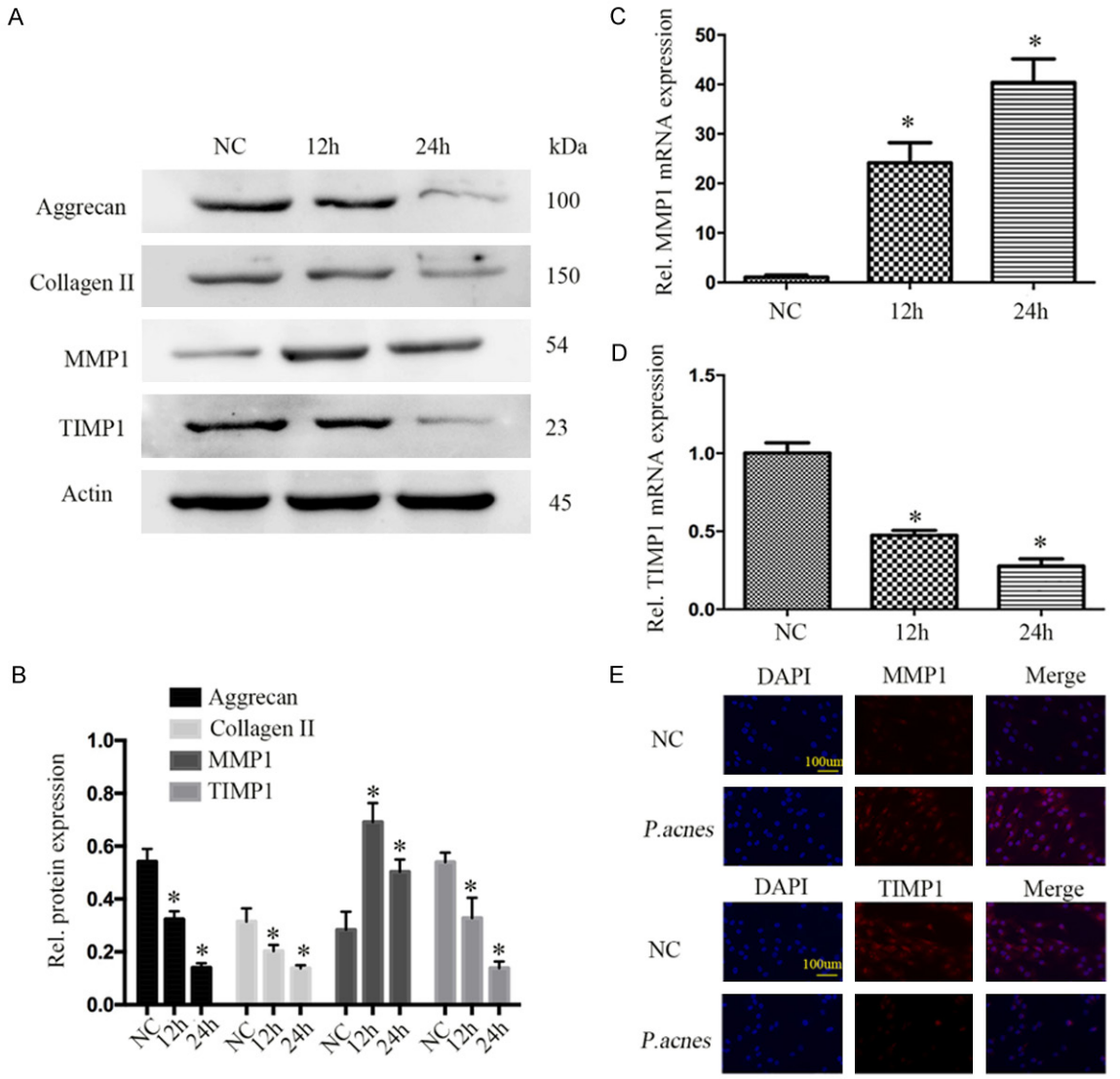

Figure 3. *P. acnes* incubation dampened aggrecan and collagen II expression by increasing MMP-1 and decreasing TIMP-1 expression in nucleus pulposus cells. (A, B) Western blot analysis of aggrecan, collagen II, MMP-1 and TIMP-1 expression in nucleus pulposus cells inoculated with or without *P. acnes* (MOI=100) for different times. (C, D) Quantification analysis of MMP-1 (C) and TIMP-1 (D) mRNA expression in nucleus pulposus cells inoculated with or without *P. acnes* (MOI=100) for different times. (E) Immunofluorescent staining analysis of MMP-1 and TIMP-1 expression in nucleus pulposus cells inoculated with or without *P. acnes* (MOI=100) for 24 hours. Scale bar represents 100 μm. *$P < 0.05$, P values were analyzed using one-way ANOVA. Data were presented as mean ± SD from three independent experiments.

these data demonstrated that *P. acnes* infection increased MMP-1 and decreased TIMP-1 expression via the NF-κB pathway.

Discussion

Since the first isolation of low virulence anaerobic bacteria from non-pyogenic degenerated intervertebral discs [5], the pathological roles of these bacteria in non-pyogenic intervertebral discs have drawn an increasing amount of attention. Accumulating evidence suggests that *P. acnes* is a novel pathogenic factor contributing to intervertebral disc degeneration (IVDD), but the mechanisms by which *P. acnes* induces IVDD are unclear. Here, we found that *P. acnes* infection dampened aggrecan and collagen II synthesis in NPCs by inducing an

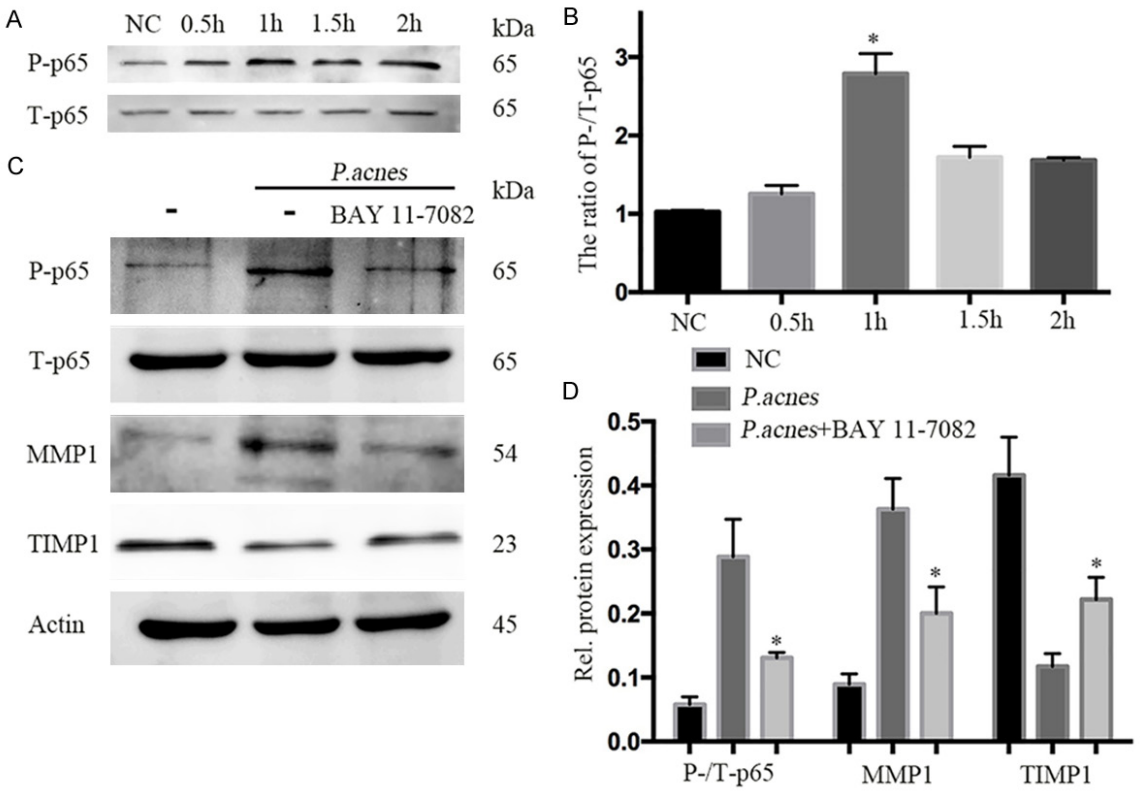

Figure 4. *P. acnes* infection increased MMP-1 and decreased TIMP-1 expression via NF-κB pathway. A, B. *P. acnes* inoculation (MOI=100) induces p65 phosphorylation in a time-dependent manner. C, D. Western blot analysis of P-p65, MMP-1 and TIMP-1 expression in nucleus pulposus cells induced by *P. acnes* (MOI=100) with or without BAY11-7082 pretreatment. *$P < 0.05$, *P* values were analyzed using one-way ANOVA. Data were presented as mean ± SD from three independent experiments.

increase in MMP-1 and a decrease in TIMP-1 expression via the NF-κB pathway, which might lead to IVDD.

The most significant biochemical change in IVDD is the loss of the ECM, which mainly contains proteoglycan and collagen II [19, 20]. The matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitor of metalloproteinases (TIMPs), are important regulators of ECM turnover [21, 22]. Although our previous study demonstrated that *P. acnes* infection decreased aggrecan and collagen II expression [11], whether MMPs and TIMPs were involved in this system was unknown. In this study, we found that *P. acnes* IVDs infection increased MMP-1 expression, while decreasing TIMP-1 expression, in NPCs both *in vivo* and *in vitro*. Furthermore, our results revealed that NF-κB activation was critical for *P. acnes*-mediated MMP-1 and TIMP-1 expression changes. Although MMP-1 is reported as one of the most important MMPs in ECM degradation [15-18], MMP-3, MMP-13, ADAMTS4, and ADAM5 have also been implicated in this process; therefore, the possible regulation of the expression of these MMPs and ADAMTSs by *P. acnes* in disc degeneration is a path for further investigation.

The NF-κB pathway is an important catabolic pathway in the pathophysiology of IVDD [23, 24]. In addition, NF-κB has been reported to play a crucial role in the production of catabolic gene expression, such as MMP-1, MMP-13, ADAMTS4, and ADAMTS5 in chondrocytes [25, 26]. However, whether NF-κB was involved in *P. acnes*-mediated MMP-1 expression was unknown. Here, we found that *P. acnes* increased MMP-1 expression and decreased TIMP-1 expression via the NF-κB pathway, to mediate aggrecan and collagen II expression. In addition, our previous study has demonstrated an NF-κB-dependent inflammatory response

in NPCs in response to *P. acnes* infection [11]. Since inflammatory cytokines, such as IL-1β, TNF-α, and IL-6, are involved in mediating MMP-1 and TIMP-1 expression during disc degeneration [27], we speculate that *P. acnes* regulates MMP-1 and TIMP-1 expression through mediating the expression of inflammatory cytokines, but this still needs further examination.

In conclusion, our study provides the first evidence that *P. acnes* IVDs infection dampens aggrecan and collagen II expression by increasing MMP-1 and decreasing TIMP-1 expression via the NF-κB pathway, which may ultimately lead to IVDD. The confirmation of *P. acnes* as a pathogenic factor for IVDD and elucidation of the underlying mechanisms provides new insights into IVDD and may ultimately lead to the development of novel treatment regimens for IVDD.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Changwei Li, Shanghai Key Laboratory for Prevention and Treatment of Bone and Joint Diseases with Integrated Chinese-Western Medicine, Shanghai Institute of Traumatology and Orthopedics, Shanghai Jiaotong University School of Medicine, 197 Ruijin Er Road, Shanghai 200025, China. Tel: +86 21 64313534; Fax: +86 21 64335742; E-mail: changwei393331@163.com; Drs. Xiangyang Xu and Peng Cao, Department of Orthopedics, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Er Road, Shanghai 200025, China. Tel: +86 21 64313534; Fax: +86 21 64335742; E-mail: xu664531@hotmail.com (XYX); dr_caopeng8@163.com (PC)

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