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Original Article

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USP14 promotes pyroptosis of human annulus fibrosus cells derived from patients with intervertebral disc degeneration through deubiquitination of NLRP3

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Abstract

Intervertebral disc degeneration (IVDD) is a general disorder that results in low back pain and disability among many affected individuals. However, the current treatments for IVDD are limited to relieving the symptoms but do not solve the fundamental issue. In this study, the role of USP14 in mediating the activation of the NLRP3 inflammasome and the pyroptosis of AF cells from IVDD patients is determined *in vitro*, and gain- and loss-of-function assays of USP14 and the NLRP3 inflammasome are conducted. Pyroptosis of AF cells is detected by flow cytometry. The inflammatory cytokines (IL-1β and IL-18) and protein levels of NLRP3, active Caspase-1, Aggrecan, MMP3 and ADAMTS-5 are determined by ELISA and western blot analysis, respectively. The correlation between USP14 and NLRP3 is measured by coimmunoprecipitation and ubiquitination analysis. Upregulation of USP14 is accompanied by increased level of the NLRP3 inflammasome in AF cells from IVDD patients; furthermore, a positive correlation between them is observed. *USP14* knockdown inhibits pyroptosis in AF cells by inducing ubiquitination of NLRP3, while overexpression of USP14 has the opposite effect, which is inhibited by the NLRP3 inflammasome inhibitor INF39. USP14 exerts its positive regulatory effect on AF cell pyroptosis by modulating the NLRP3/Caspase-1/IL-1β and IL-18 signaling axes.

Key words Intervertebral disc degeneration, USP14, NLRP3 inflammasome, deubiquitination, pyroptosis

Introduction

An intervertebral disc (IVD) is a three-element construct consisting of a nucleus pulposus (NP) in the center and an annulus fibrosus (AF) in the surroundings, which are connected by cartilaginous endplates (CEs) [1]. Alterations in the structural and biomechanical properties of IVD can induce a series of abnormal consequences in spinal alignment, flexibility, motion, or neural anatomy, which in turn lead to dysfunction of IVD, even degenerative disc disease (DDD) [1,2]. Currently, DDD has become a very common disorder worldwide; for instance, over 52 million people suffer from IVD-related pain, with the clinical cost exceeding $40 billion annually [3]. Notably, the present treatments for DDD are confined to relieving the symptoms but not to solving the fundamental issue [1,4]. Furthermore, the molecular mechanism underlying the occurrence of IVD degeneration (IVDD) remains largely unclear [5].

IVDD is frequently characterized by significant degradation of extracellular matrix (ECM), which is regulated by catabolic and anabolic genes [6], and increased loss of the annulus fibrosus (AF) cells [7]. Accordingly, ECM homeostasis and AF cell apoptosis play important roles in preserving the integrity and stability of the AF structure [8], which contributes to the development of disorders in human disc tissue [9,10]. Although the NP and CEP are essential for maintaining the function of the IVD, degeneration of the AF often leads to IVD dysfunction, which subsequently develops into disc herniation [10]. Therefore, the stability of the AF is also critical for maintaining the health of the IVD. Moreover, multiple pathways mediating AF programmed cell death, including classical apoptosis and autophagy, have been identified and described [11,12]. However,

whether other pathways also play a similar role has not been reported.

It has been recognized that almost all diseases occurring in humans and animals are tightly related to inflammation [13]. Growing evidence has demonstrated that inflammation is also associated with degenerative disc disease [14,15]. Moreover, persistent inflammation within the AF plays a crucial role in mediating disc degeneration [16]. The key role of the NLRP3 inflammasome consisting of the NLRP3 protein, Caspase-1, and ASC in modulating inflammation has been well documented [13]. On one hand, elevated levels of NLRP3 inflammasome proteins are often correlated with the deteriorated extent of IVDD [17,18]. On the other hand, activation of the NLRP3 inflammasome is frequently linked to increased inflammation and pyroptotic cell death (i.e., pyroptosis) [19]. Furthermore, NLRP3-dependent pyroptosis of NP cells has been confirmed recently [20]. Despite these findings, it remains undetermined whether the NLRP3 inflammasome plays a similar role in the pyroptosis of AF cells.

Pyroptosis, a unique inflammation-related form of programmed cell death, is different from other types of cell death, such as autophagy, and is mainly characterized by the occurrence of cell swelling and large bubbles originated from the plasma membrane [21]. It is now clear that upregulation or activation of NLRP3 promotes the maturation or activity of Caspase-1, which causes cell death and facilitates the production of proinflammatory cytokines such as IL-1β and IL-18 [22,23]. The central role of the NLRP3/Caspase-1/IL-1β and IL-18 signaling axes in inducing pyroptosis has been proposed and confirmed [24]. Therefore, any factor regulating this signaling pathway should have beneficial or adverse effects on pyroptosis. Indeed, a series of entities have been confirmed to regulate the NLRP3 inflammasome, such as long noncoding RNAs (lncRNAs), proteins, and molecules [20,25-27], thereby altering the development of related disorders, including IVDD. Moreover, a previous report demonstrated that pyroptosis is also involved in AF cells during IVDD progression [28]. Therefore, the pyroptosis process is essential for maintaining the function of AF cells. However, the precise mechanism still needs to be further explored.

In addition, increasing evidence indicates that deubiquitinases (DUBs) can regulate NLRP3 inflammasome activation [25,29]. In particular, the important roles of the members (i.e., ubiquitin-specific proteases, USPs) of the largest family of DUBs in regulating the NLRP3 inflammasome have been well established [30,31]. However, it remains unexplored whether USP14, an important member of this family, plays a similar role and the mechanism through which it modulates the NLRP3 inflammasome.

In this study, we proposed that USP14 mediates the activation of the NLRP3 inflammasome, thereby provoking pyroptosis in AF cells. To this end, the expression of USP14 and its potential relationship with NLRP3 were first examined under IVDD conditions. Next, the consequences of USP14 downregulation on NLRP3 inflammasome signaling and AF cells were determined. Third, the mechanism of action of USP14 on NLRP3 was investigated. Finally, gain-of-function experiments, together with the application of an NLRP3 inflammasome inhibitor, were conducted to further verify the role of USP14 in regulating the NLRP3/Caspase-1/IL-1β and IL-18 signaling axes and inducing pyroptosis in AF cells.

Materials and Methods

Patient tissue samples

The study protocol complied with the Declaration of Helsinki and was approved by the Ethics Committee of Peking University Third Hospital (approval number S2021170). Informed and written consent was obtained from each participant in this study. From March 2015 to April 2018, degenerative AF samples were collected from 25 patients with intervertebral disc degeneration (IVDD) who underwent operations at the Peking University Third Hospital. The degeneration degree of IVD was assessed according to the modified Pfirrmann grading system [32] from preoperative magnetic resonance imaging scans. Normal control AF samples were collected from 10 patients ($n = 5$ women and 5 men; mean age, 39.6 years; range, 23–49 years) with accidental fractures and nondegenerative specimens. None of the patients in the nondegenerative group reported any previous lumbar pain.

Isolation and expansion of human AF cells

To ensure purity of AF samples, AF tissue was collected after removing all the adjacent NP tissue and cartilage EP and cut into 1 mm^3 pieces. The collected AF tissue was placed in Ham's F-12 medium (Gibco-BRL, Gaithersburg, USA) supplemented with 5% fetal bovine serum (FBS; Gibco-BRL) and 1% penicillin/streptomycin (Gibco-BRL). The tissues were washed three times with Hank's balanced salt solution (Gibco-BRL) containing 1% penicillin/streptomycin to remove blood and other contaminants. After that, the chopped tissue was enzymatically digested for 1 h with 0.2% (w/v) pronase (Calbiochem, La Jolla, USA) in Ham's F-12 medium, followed by incubation in medium containing 130 U/mL collagenase type II (Worthington Biochemical Corp., Lakewood, USA) for 14 h at 37°C. The single-cell suspension was filtered through a sterile nylon mesh filter (pore size 70 μm) to remove tissue debris. The suspended cells were centrifuged at 500 *g* for 100 min and resuspended in Ham's F-12 medium containing 10% FBS and 1% penicillin/streptomycin. Isolated human AF cells were seeded at a density of 10,000 cells/cm^2 and expanded with Ham's F-12 medium supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. The culture medium was changed twice a week, and the cells were detached at 80%–90% confluence using a dissociation buffer composed of 0.05% trypsin-EDTA (Gibco, Grand Island, USA) and 0.01% collagenase P (Roche, Auckland, New Zealand) for 5 min at 37°C. Cells were subcultured at a cell density of 3000 cells/cm^2 for expansion in the same medium as above. The second-passage AF cells were used in the subsequent experiments.

Cell transfection

Different shRNAs targeting human USP14 synthesized by Sangon Biotech (Shanghai, China) were cloned into the pLKO.1 lentiviral vector. USP14 shRNAs (shUSP14#1 position 787–805, 5′-GGAAG CAATAGAGGATGAT-3′; shUSP14#2 position 1057–1075, 5′-GCAAAGAAATGCCTTGTAT-3′; shUSP14#3 position 1467–1485, 5′-GGAAACAAGATGAATGGAT-3′) or scramble shRNA (5′-GGAAT GAATGGAAGAAGAT-3′) as a negative control (shNC) were also purchased from RiboBio (Guangzhou, China). To create a USP14 ectopic expression vector, the coding sequence (CDS) of USP14 was inserted into the pLVX-Puro vector. We used the following primers to amplify the USP14 CDS: *USP14*-F 5′-CGGAATTCATGCCGCTC TACTCCGTTAC-3′; *USP14*-R 5′-CGGGATCCTTACTGTT CACTTTCCTCTTCC-3′. For transfection of 293T cells, the cells were cultured in 6-well plates, and then transfected with pLKO.1-USP14-shRNA (shUSP14) or pLVX-Puro-USP14 (oeUSP14) using

Lipofectamine reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Vectors were harvested after 48 h of transfection and were utilized to transduce AF cells. The pLKO.1-scramble shRNA (shNC) and blank pCDNA3.1(+) (Vector) were used as the negative controls. Flag-NLRP3 and HA-USP14 expression plasmids were constructed as previously described [33].

Cell death assay

Pyroptotic cell death was evaluated by active Caspase 1 and propidium iodide (PI) staining. Briefly, AF cells were seeded in 6-well plates (5×10^5 cells/well) and allowed to grow until reaching 50% confluence. Cells were then incubated with 660-YVAD-FMK (FLICA® 660 Caspase-1 Assay Kit; ImmunoChemistry Technologies, Bloomington, USA) according to the manufacturer's instructions and with 10 μL PI (Thermo Fisher Scientific, Waltham, USA) for 15 min and analysed by flow cytometry on a CytoFLEX flow cytometer (Beckman Coulter, Krefeld, Germany).

Enzyme-linked immunosorbent assay (ELISA)

The IL-1β and IL-18 contents in the supernatant of AF cells were determined using IL-1β Human ELISA Kit (R&D Systems, Minneapolis, USA) and IL-18 Human ELISA Kit (Thermo Fisher Scientific) according to the manufacturers' instructions.

Determination of the level of lactic dehydrogenase (LDH)

LDH release was measured using a lactate dehydrogenase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Quantitative RT-PCR

To examine the mRNA expression level of USP14, total RNA was prepared from AF cells using TRIzol reagent (Thermo Fisher Scientific). The extracted total RNA was then reverse transcribed into cDNA using the PrimeScript kit (Takara Biomedical Technology Co., Ltd, Beijing, China) with the protocol provided by the manufacturer. qRT-PCR was performed with SYBR Green Master Mix (Applied Biosystems, Forster City, USA) on an ABI 9700 thermocycler (Applied Biosystems). The following thermocycling conditions were used for qPCR: initial denaturation at 95°C for 300 s; 45 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 30 s. Specific primers for USP14 were *USP14*-F 5′-CGAGAAAGGTGAACAAGGACAG-3′ and *USP14*-R 5′-TGCCGATGCAGATGAGGAG-3′. *GAPDH* was used as the internal control gene, and the specific primers for GAPDH were *GAPDH*-F 5′-AATCCCATCACCATCTTC-3′ and *GAPDH*-R 5′-AGGCTGTTGTCATACTTC-3′. The relative expression of USP14 was calculated based on the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Cells were lysed with RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, St Louis, USA). The protein samples (30 μg) were electrophoresed and electrotransferred onto nitrocellulose membranes (Millipore, Billerica, USA). The membranes were first incubated in 5% skim milk, probed with antibodies against USP14 (ab192618; Abcam, Cambridge, USA), NLRP3 (232401; Abcam), collagen I (138492; Abcam), collagen II (ab34712; Abcam), Aggrecan (13880-1-AP; Proteintech, Rosemont, USA), MMP3 (ab52915; Abcam), ADAMTS-5 (ab41037; Abcam), caspase-1 p20 (bs-10442R; Bioss Antibodies Inc., Woburn, USA), and GAPDH (5174; Cell Signaling Technology, Danvers, USA), and finally incubated with the HRP-conjugated secondary antibody (A0208; Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. An enhanced chemiluminescence system (Bio-Rad Laboratories, Hercules, USA) was applied to visualize signals on the membrane.

Coimmunoprecipitation and ubiquitination analysis

For exogenous coimmunoprecipitation experiments, AF cells were cotransfected with Flag-NLRP3 and/or HA-USP14 expression plasmids. At 24 h posttransfection, the cells were harvested and lysed with 500 μL of RIPA lysis buffer containing protease cocktail. Lysates were centrifuged at 14,000 *g* for 5 min. The supernatant was transferred to a new tube and precipitated with 20 μL of anti-Flag or anti-HA affinity gel (Biotool, Houston, USA) for 2 h at 4°C. For *in vivo* coimmunoprecipitation, the extracted protein from AF cells was incubated with Protein A/G PLUS-Agarose (sc-2003; Santa Cruz Biotechnology, Santa Cruz, USA;) for 1 h and then with anti-USP14 antibody (ab137432; Abcam), anti-NLRP3 antibody (15101; Cell Signaling Technology), or normal IgG (sc-2027; Santa Cruz Biotechnology) overnight at 4°C. Then, protein A-sepharose was used to pull down the immunocomplexes and the immunocomplexes were analysed by western blot analysis using anti-USP14 (ab192618; Abcam), anti-NLRP3 (ab232401; Abcam) or anti-ubiquitin (ab7780; Abcam) antibodies.

Statistical analysis

Data are expressed as the mean ± SD of at least three independent experiments. All statistics were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, USA). Comparisons between different experimental groups were conducted with ANOVA, followed by Tukey's post-multiple test. $P < 0.05$ represents a significant difference.

Results

The expressions of USP14 and NLRP3 are simultaneously upregulated in IVDD patients

The process of IVDD is accompanied by changes in gene expression [34]. Thus, quantitative RT-PCR and western blot analysis were performed to evaluate the changes in type I and type II collagen expression levels in AF cells. As shown in Figure 1A,B, in AF cells isolated from IVDD patients, the expression of type I collagen was significantly increased and that of type II collagen was significantly decreased.

To explore the potential role of USP14 in IVDD, we first analysed USP14 expression in IVDD patients. Compared with the normal controls, the protein level of USP14 was significantly increased in AF cells isolated from IVDD patients, as clearly indicated by western blot analysis (Figure 1A–C). Next, we analysed the clinicopathological features of 25 patients with IVDD and the protein expression of USP14. The patients were categorized by various parameters, such as age at surgery, gender, body mass index (BMI), and degenerative grade. The corresponding expression level of USP14 in these groups was then assessed following examination of individual patients, as summarized in Table 1. Although there were slight differences in terms of USP14 expression in the age, gender, and BMI intergroups, these divergences failed to reach a significant level ($P > 0.05$). Intriguingly, considerable differences were found among the degenerative grade intergroups. Briefly, as the degree of degeneration was increased, the expression level of USP14 displayed a markedly rising trend ($P < 0.001$). These results suggest that USP14

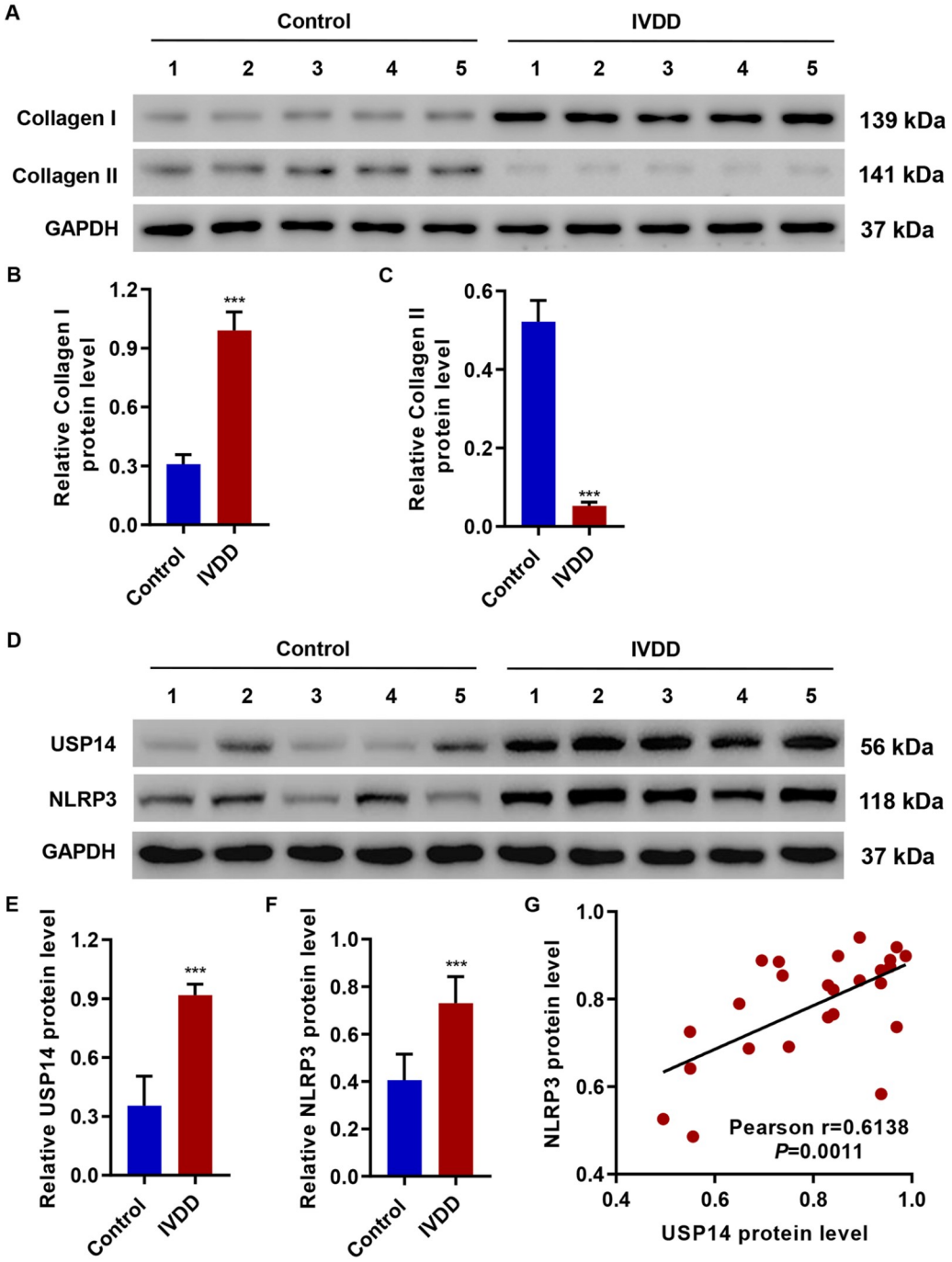

Figure 1. USP14 and NLRP3 expression in AF cells from IVDD patients AF cells were collected from 5 IVDD patients (Pfirrmann grade, patient 1 = II, patient 2 = IV, patient 3 = III, patient 4 = II, patient 5 = V) or normal controls, and the proteins were measured by western blot analysis. (A–C) Type I collagen and type II collagen expressions. (D–F) USP14 and NLRP3 expressions. (G) Pearson correlation scatter plots in patients with IVDD. ***$P < 0.001$ compared with normal controls.

expression is tightly related to the pathological progression of IVDD.

To further characterize the phenomenon observed above and to explore whether there is a potential relationship between USP14 and the NLRP3 inflammasome, their expression levels were intensively examined. Compared with those of the normal controls, the protein levels of USP14 and NLRP3 were significantly increased in IVDD patient samples ($P < 0.001$), as clearly indicated by western blot analysis (Figure 1D–F). Next, the expression levels of USP14 and NLRP3 in individual patients were analysed in depth. Fantastically, a positive correlation was observed between those two ($R = 0.6138$, $P < 0.01$). These results not only demonstrated an increased expression of USP14 and NLRP3 under the condition of IVDD but also manifested a close relationship between them (Figure 1G). Considering the respective roles of USP14 and NLRP3, we speculate

Table 1. Clinicopathological features of 25 patients with intervertebral disc degeneration and the protein expression of USP14

Parameter	Cases	USP14 level	*P* value
Age at surgery			
<45	14	0.834 ± 0.132	0.9151
≥45	11	0.754 ± 0.178	
Gender			
Male	13	0.797 ± 0.177	0.2536
Female	12	0.804 ± 0.133	
BMI			
<24	15	0.789 ± 0.156	0.6535
≥24	10	0.818 ± 0.155	
Degenerative grade			
I	1	0.495 ± 0.045	0.0007
II	4	0.637 ± 0.102	
III	7	0.757 ± 0.081	
IV	5	0.901 ± 0.039	
V	8	0.897 ± 0.145	

BMI indicates body mass index.

that the former may be responsible for the activation of the latter.

USP14 downregulation inhibits pyroptotic cell death in AF cells from IVDD patients, suppresses the activation of NLRP3 and regulates related signaling components

To address the speculation above, shRNA-mediated knockdown of *USP14* was performed, and its expression was examined. Subsequently, the activation of NLRP3 and the levels of its downstream elements were intensively determined. Three shRNAs targeting various regions of USP14 were introduced into AF cells from IVDD patients (hereafter referred to as AF cells), and the mRNA and protein levels of USP14 were examined and quantified (Figure 2A–C). Notably, all three shRNAs significantly downregulated USP14 expression compared with the blank or negative control ($P < 0.001$), while the former two, i.e., Nos. 1 and 2 had a better interfering effect. Therefore, these two shRNAs were utilized for the subsequent experiments. First, pyroptotic cell death of AF cells from IVDD patients was evaluated following the downregulation of USP14. Figure 2D,E demonstrated that knockdown of USP14 led to a considerable decrease in pyroptotic cell death, which was reflected by much less PI- and active Caspase-1-positive cells and LDH release in shUSP14#1- or shUSP14#2-transduced AF cells than in shNC-transduced AF cells ($P < 0.001$). Next, the effect of USP14 downregulation on the expression of NLRP3 and active Caspase-1 was assessed (Figure 2F). Interestingly, the expressions of both NLRP3 and active Caspase-1 were pronouncedly decreased in *USP14*-downregulated AF cells relative to that in control cells. Meanwhile, the levels of IL-1β and IL-18 were determined by ELISA. As indicated in Figure 2G, the contents of both IL-1β and IL-18 in shUSP14-treated AF cells were remarkably lower than those in control cells ($P < 0.001$). Finally, markedly decreased levels of the catabolic genes *MMP3* and *ADAMTS-5* and significantly increased level of the anabolic factor Aggrecan were observed in shUSP14-treated AF cells compared to those in control cells (Figure 2H). Collectively, these results suggest that downregulating USP14 expression impedes the pyroptotic cell death of AF cells from IVDD patients, blocks the activation of NLRP3, and suppresses the production of related signaling components.

USP14 induces deubiquitination of NLRP3

To investigate how USP14 affects the expression of NLRP3, coimmunoprecipitation (co-IP) experiments were conducted to address this issue. When the extracted proteins from AF cells were incubated with anti-USP14 antibody, the interaction of USP14 and NLRP3 was identified. A similar phenomenon was observed when an anti-NLRP3 antibody was used for co-IP (Figure 3A). Moreover, exogenous co-IP was performed to further confirm the interaction between Flag-NLRP3 and HA-USP14 (Figure 3B). As described above, shRNA-mediated *USP14* knockdown resulted in a pronounced decrease in NLRP3 protein level. In contrast, the inhibitory effect of shUSP14 on the NLRP3 level was almost abolished in the presence of MG132 in AF cells when compared to shNC + vehicle control cells (Figure 3C). To further verify the function of USP14 in the deubiquitination of NLRP3, the proteins from shUSP14- or shNC-transduced AF cells were incubated with anti-NLRP3 antibody and then immunoblotted with an anti-ubiquitin antibody. Figure 3D demonstrated that upon reduced expression of USP14, the ubiquitination of NLRP3 was considerably increased, which was revealed by stronger signals in shUSP14-transduced AF cells than in shNC-transduced AF cells following incubation with anti-NLRP3 antibody. Taken together, this evidence suggests that USP14 induces the deubiquitination of NLRP3.

INF39 inhibits the pyroptosis of human AF cells and the activation of the NLRP3 inflammasome induced by USP14 overexpression

To further verify our findings, lentivirus-mediated overexpression of USP14 (named oeUSP14) and INF39, an NLRP3 inflammasome inhibitor that counteracts NLRP3 activation through direct irreversible interaction with NLRP3, was utilized for these purposes. When AF cells from IVDD patients were transduced with oeUSP14 or blank vector, greatly enhanced expression of USP14 at both the mRNA level and protein level was confirmed by quantitative RT-PCR and western blot analysis, respectively, relative to those in control or empty vector-transduced cells (Figure 4A–C). First, pyroptosis in AF cells transduced with oeUSP14 or control vector was assessed by flow cytometry. As indicated in Figure 4D–F, compared with those in the vector-transduced cells, markedly increased pyroptosis and LDH release were found in oeUSP14-transduced AF cells ($P < 0.001$), suggesting that overexpression of USP14 facilitates the occurrence of pyroptosis. In contrast, the addition of INF39 displayed a strong inhibitory effect on pyroptosis in AF cells. Furthermore, the promoting effect of USP14 overexpression could be completely overcome by INF39 treatment.

Next, we sought to determine the levels of NLRP3 and active Caspase-1, as well as related proteins, in the presence of USP14 overexpression or INF39 treatment. As expected, USP14 overexpression greatly enhanced the levels of NLRP3 and active Caspase-1 compared to the control ($P < 0.001$), while this promoting effect of USP14 in AF cells was largely blocked by INF39 (Figure 4G). Similarly, the altered levels of IL-1β and IL-18 in the single or combined presence of USP14 overexpression and INF39 treatment were completely consistent with those observations for NLRP3 and Caspase-1 (Figure 4H).

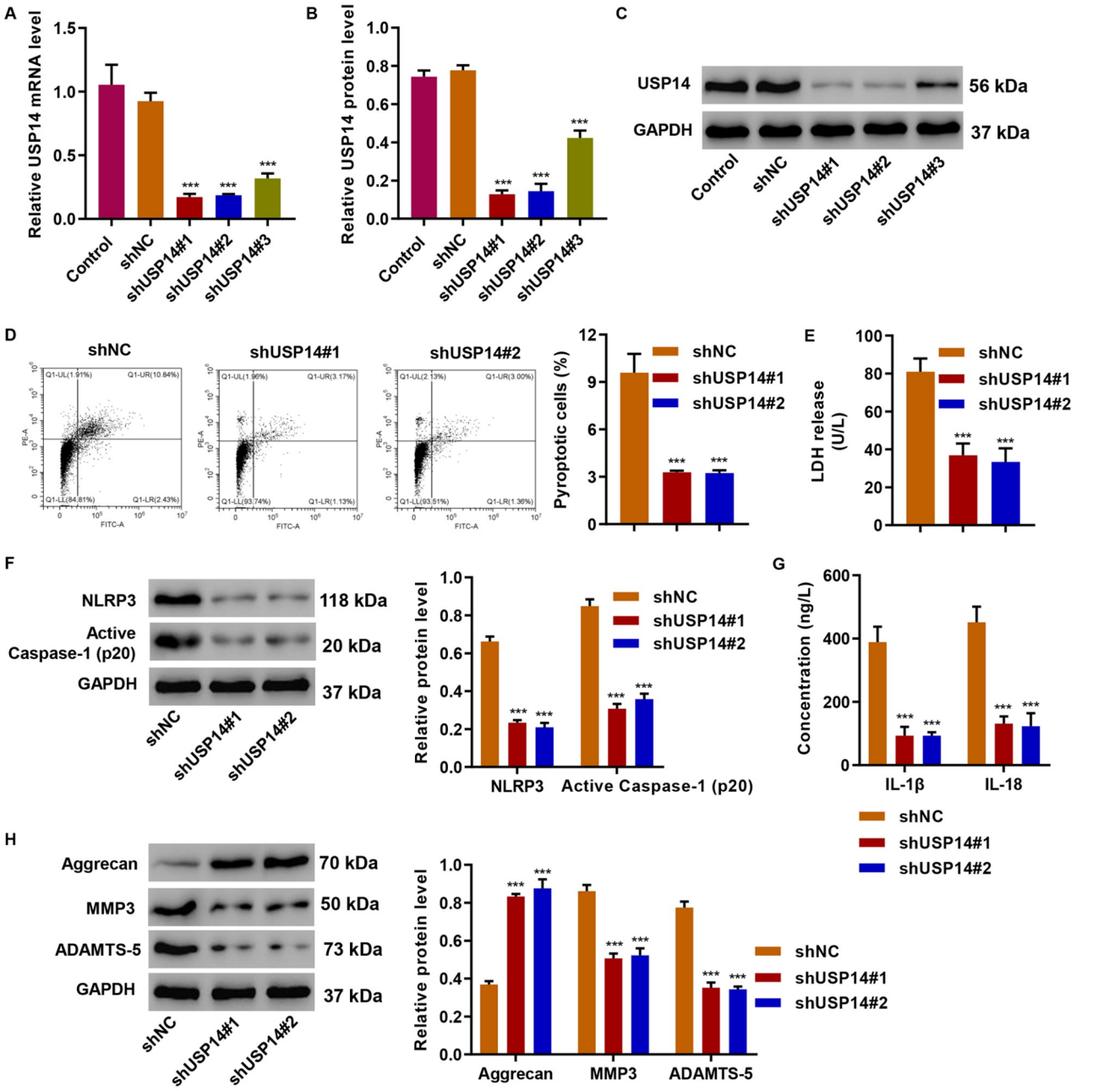

Figure 2. ***USP14*** **knockdown inhibits AF cell pyroptosis** AF cells collected from IVDD patients were transduced with shUSP14 or shNC, and the expression of USP14 was measured by quantitative RT-PCR (A) and western blot analysis (B,C). (D) Cell pyroptosis was assessed by flow cytometry. (E) LDH release. (F,H) The expressions of NLRP3, active Caspase-1 (p20), MMP3, ADAMTS-5 and Aggrecan were examined by western blot analysis. (G) The concentrations of IL-1β and IL-18 were determined by ELISA. ***$P < 0.001$ compared with shNC.

Finally, we determined the levels of several related proteins upon the single or combined overexpression of USP14 and INF39 treatment. Interestingly, much lower expression of Aggrecan and pronouncedly higher levels of MMP3 and ADAMTS5 were found in USP14-overexpressing AF cells than in vector-transduced cells, whereas the impact of USP14 on these proteins was largely reversed by INF39 treatment (Figure 4I). Collectively, these results further confirm that USP14 overexpression facilitates the pyroptosis of AF cells; moreover, this promoting effect is largely mediated by activating the NLRP3 inflammasome.

Discussion

An increasing population suffers from IVDD, and the huge socio-economic burden posed by this disease urgently appeals for a deeper understanding of the underlying mechanism [35]. In this regard, identifying new targets for the development of medicines is of great significance. The present study provided novel insights into the pathogenesis of IVDD from multiple aspects. (1) USP14, a DUB, was confirmed to be involved in the clinical condition of IVDD. (2) The interaction of USP14 with NLRP3 was verified for the first time. (3) USP14 was found to regulate the pyroptosis of AF cells via

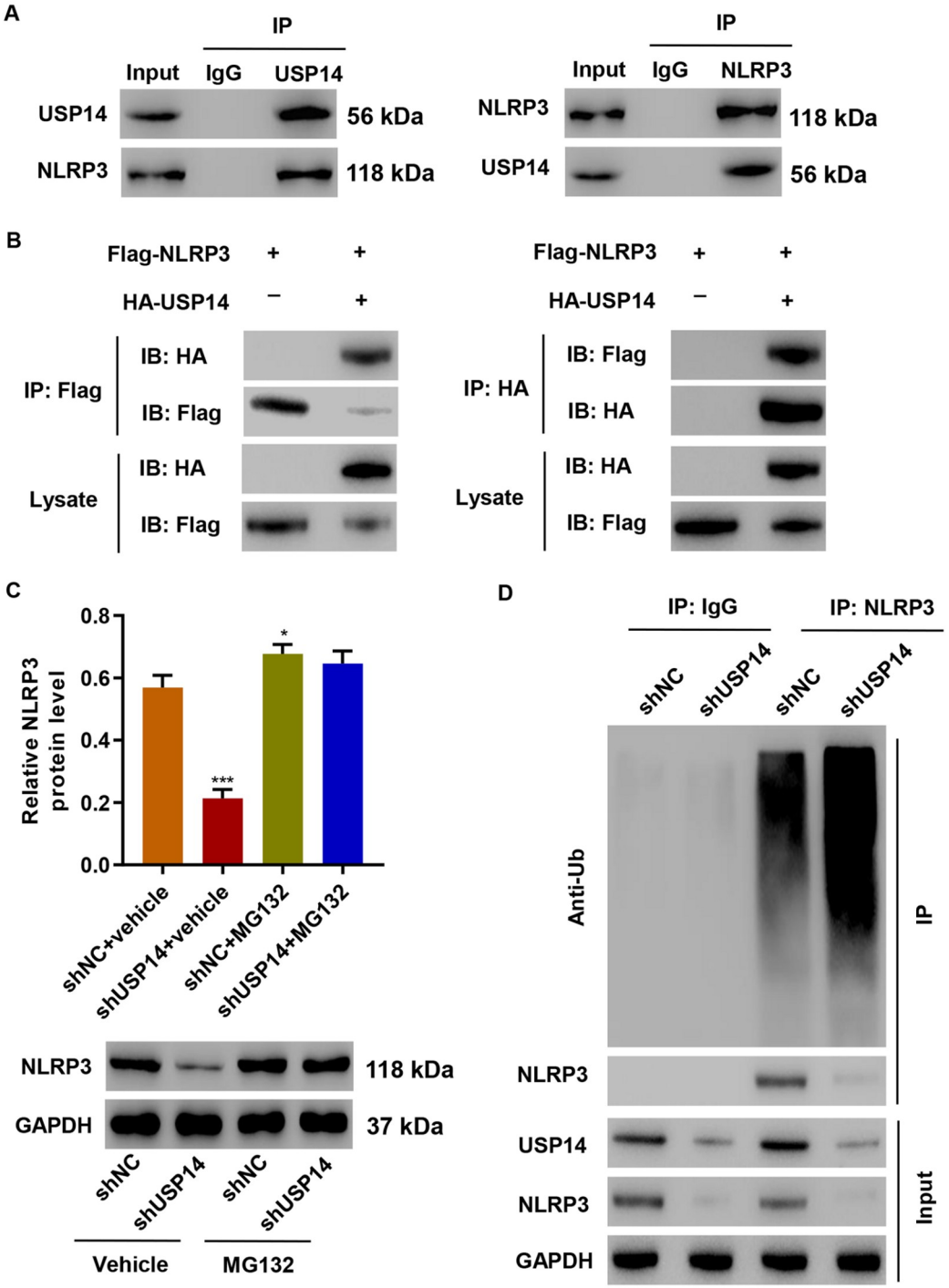

Figure 3. USP14 interacts with NLRP3 and inhibits ubiquitination of NLRP3 (A) The interaction between USP14 and NLRP3 in AF cells was determined by coimmunoprecipitation. (B) The interaction between HA-USP14 and Flag-NLRP3 in transfected AF cells was determined by coimmunoprecipitation. (C) Effects of MG132 (10 μM) or vehicle on shUSP14-mediated NLRP3 expression. (D) AF cells were transduced with shUSP14 or shNC, and NLRP3 was immunoprecipitated and then immunoblotted with the indicated antibodies. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with shNC + vehicle.

NLRP3/Caspase-1/IL-1β and IL-18 signaling.

USP14, as an important member of the USP family, has received great attention for its decisive role in various human disorders [36]. Growing evidence has demonstrated that its high expression is frequently associated with adverse consequences of clinical conditions [37–39]. In this study, we found elevated expression of USP14 in patients with IVDD; furthermore, its level was tightly related to the severity of IVDD, which is in line with the status of

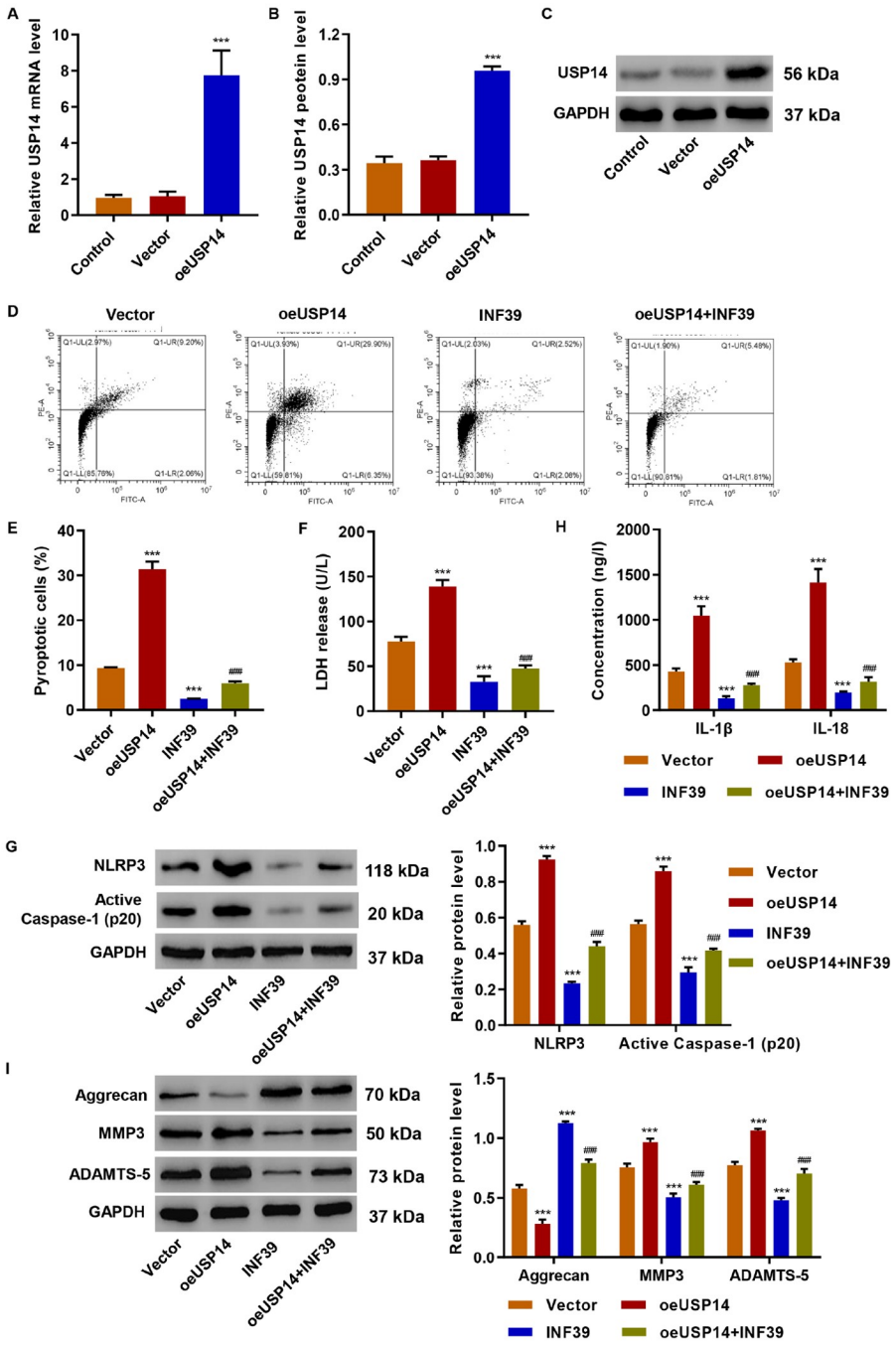

Figure 4. USP14 overexpression promotes AF cell pyroptosis by activating NLRP3 AF cells collected from IVDD patients were transduced with oeUSP14 or blank vector and treated with an NLRP3 inhibitor (INF39; 10 μM), and the expression of USP14 was examined by quantitative RT-PCR (A) and western blot analysis (B,C). (D,E) Cell pyroptosis was assessed by flow cytometry. (F) LDH release. (G,I) The expressions of NLRP3, active Caspase-1 (p20), MMP3, ADAMTS-5 and Aggrecan were measured by western blot analysis. (H) The concentrations of IL-1β and IL-18 were determined by ELISA. ***$P < 0.001$ compared with vector; ###$P < 0.001$ compared with oeUSP14.

USP14 in other diseases [36,40]. Additionally, USP14 was revealed to modulate multiple signaling pathways in response to inflammation [41,42], which is a general feature of numerous disorders, including IVDD, and is characterized by chronic or acute dysfunction of the host response [36]. In terms of inflammation, a pivotal role of the NLRP3 inflammasome in regulating it has been recognized [13]. Considering these findings, we also analysed the level of the NLRP3 protein in patients with IVDD and found pronouncedly increased expression of NLRP3. More importantly, a positive correlation of the expression of USP14 with that of NLRP3 was observed, suggesting a potential relationship between these two proteins.

Although the vital roles of AF cell apoptosis and ECM degradation in the progression of IVDD have been suggested [8,43], information

about how these processes occur remains limited. In this regard, we initially confirmed that downregulation of USP14 led to reduced pyroptosis of AF cells, which was preceded by decreased levels of NLRP3 and Caspase-1, consistent with previous studies [19,44]. This suggested the participation of the NLRP3 inflammasome in mediating AF cell pyroptosis, as further confirmed by the decreased levels of IL-1β and IL-18. Notably, our results revealed for the first time the involvement of pyroptosis in AF cell death and the regulatory role of USP14 in this process. Additionally, USP14 downregulation also facilitated the preservation of the matrix, as verified by the alterations of matrix biomarkers, suggesting that USP14 affects the degradation of ECM in AF cells.

Accumulating studies have demonstrated that many proteins, including DUBs, can regulate the activation of inflammasomes, such as NLRP3, in different ways [31,45,46]. The current study revealed that USP14 modulates the activation of the NLRP3 inflammasome through their interaction, as indicated in the co-IP experiments, which are similar to the results of other studies [47–49]. Additionally, the regulatory effect of USP14 on the protein expression of NLRP3 could be abolished by the proteasome inhibitor MG132 [29,50], suggesting that USP14 regulates NLRP3 level in a proteasome-dependent manner. Indeed, increased ubiquitination of NLRP3 was observed following the downregulation of USP14. Notably, the key domains from those two proteins responsible for the interaction should be further determined.

Finally, we provided gain-of-function evidence to further demonstrate that upregulation of USP14 promotes pyroptosis in AF cells by provoking NLRP3 activation and the productions of IL-1β and IL-18. Aggressive oxidative stress, which induces autophagy and ferroptosis in AF cells, plays an essential role in the initiation and progression of IVDD [51]. Previous evidence supported that NF-κB is activated in intervertebral discs, which correlates with accumulated oxidative stress and increases with age and disc degeneration [52]. In addition, oxidative stress and NF-κB signaling can activate the NLRP3 inflammasome, which is responsible for pyroptosis [53]. Therefore, the reason for the high pyroptosis rate of degenerated AF cells may be due to the increased levels of oxidative stress and NF-κB activation. Additional studies on pyroptosis, which is affected by oxidative stress and NF-κB activation, in IVDD are necessary.

USP14-induced pyroptosis of AF cells almost vanished in the presence of the NLRP3 inflammasome inhibitor INF39 [54,55] and was nearly opposite to that under *USP14* knockdown. Thus, this evidence robustly confirms our initial proposal. As an NLRP3 inflammasome inhibitor, INF39 counteracts NLRP3 activation through direct irreversible interaction with NLRP3. In the present study, NLRP3 expression was also inhibited by INF39, which was consistent with previous studies [56–58]. It has been shown that INF39 can inhibit the activation of the NF-κB transcription factor, which is crucial for NLRP3 expression [58,59]. These results suggest that INF39 may regulate NLRP3 expression via the NF-κB signaling pathway. Moreover, the therapeutic effects of INF39 on IVDD in animal models should be further investigated.

In conclusion, our study provides the first evidence to show that USP14 participates in the pathogenesis of IVDD. Overexpression of USP14 can promote pyroptosis in AF cells, consistent with its increased expression along with the deteriorated extent of IVDD. In contrast, its downregulation has the opposite consequence. The vital role of USP14 in regulating the pyroptosis of AF cells is mediated by the NLRP3/Caspase-1/IL-1β and IL-18 signaling pathways via its interaction with and deubiquitination of NLRP3. Therefore, inhibiting the expression or activity of USP14 is beneficial for treating IVDD; accordingly, it can be a useful target for the development of pharmacological agents.

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