Distinctive roles of tumor necrosis factor receptor type 1 and type 2 in a mouse disc degeneration model

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Abstract

Background: Elevated tumor necrosis factor alpha (TNF-α) expression is correlated with the progression of intervertebral disc degeneration (IVDD). Progranulin binding to tumor necrosis factor receptor (TNFR) and its derivative Atstrin are effective for treating inflammatory arthritis. We hypothesize that Atstrin has a protective effect in IVDD through different roles of TNFR receptor type 1 (TNFR1) and TNFR receptor type 2 (TNFR2) in degenerated discs.

Methods: IVDD models were established in TNFR1−/−, TNFR2−/− mice and their control littermates. Nucleus Pulposus (NP) samples from human patients and IVDD murine models were evaluated by X-ray, micro-MRI, μCT, histological staining and immunofluorescence staining. NP cells isolated from wild-type (WT), TNFR1−/− and TNFR2−/− mice were treated with TNF-α or Atstrin and then assayed by Western blotting, qRT-PCR, and ELISA.

Results: TNFR1 and TNFR2 expression was significantly elevated in the disc tissues of both human patients and IVDD murine models. TNFR1 knockout contributed to reduced disc degeneration. In contrast, TNFR2 knockout was associated with enhanced IVDD severity, including degraded cellular composition, increased cell apoptosis and elevated vertebral destruction. Atstrin protected against IVDD in WT and TNFR1−/− mouse models but had no effect in TNFR2−/− IVDD models. Additionally, in vitro NP cell-based assays demonstrated that TNF-α-stimulated catabolism and Atstrin-activated anabolism depended on TNFR1 and TNFR2, respectively.

Conclusion: TNFR1 is associated with the degenerative progression of IVDD, while TNFR2 contributes to the protective effect on the discs. Atstrin protects against IVDD at least partially by inhibiting the TNFs/TNFR1 inflammatory/catabolic pathway and activating the TNFR2 protective/anabolic pathway.

The translational potential of this article: This study demonstrates that TNFR1 and TNFR2 have disparate roles in disc degeneration and highlights the potential use of Atstrin as a therapeutic agent against IVDD in mice.

1. Introduction

Low back pain (LBP) is one of the most common age-related conditions causing functional disability and activity limitations [1–3]. Intervertebral disc degeneration (IVDD) is considered one of the strongly associated causes of LBP [4,5]. IVDD refers to a cascade that begins with changes to the cell microenvironment and progresses to structural breakdown and functional impairment [6,7]. This degenerative progress has been extensively characterized by molecular, morphological and biomechanical alterations to the discs and surrounding joint tissues [8]. IVDD is often associated with inflammatory cascades [9–12]. In particular, the expression and signaling of proinflammatory tumor necrosis factor alpha (TNF-α) are upregulated in degenerated discs [12–16]. Although TNF-α inhibitors (TNFIs) have been accepted as highly effective anti-inflammatory biologics, risks and limitations result in unsatisfactory therapeutic effects for up to 50% of patients [17–20]. Moreover, the efficacy of clinically available TNFI in LBP treatment remains controversial [21–26].
TNF-α interacts with two receptors, TNF receptor type 1 (TNFR1) and TNF receptor type 2 (TNFR2). TNFR1 is expressed in most cell types and is considered the primary receptor mediating TNF's proinflammatory effects, including activation of the transcription factors NF-κB and MAP kinases, leading to the transcription of proinflammatory cytokines. TNFR2 is primarily expressed in immune and endothelial cells as well as chondrocytes [27]. The roles of TNFR2 signaling in normal and degenerated tissues remain less well characterized, although accumulating evidence supports a protective role of TNFR2 signaling in some inflammatory conditions, particularly inflammatory arthritis [28]. Increasing evidence indicates that these two receptors play distinct roles in various pathophysiological processes, including neurodegeneration and inflammatory arthritis [27–31].

A prior global genetic screen led to the identification of TNFR as the preferential progranulin (PGRN)-associated receptor; PGRN and TNF-α bind to TNFR1 with comparable affinity, and PGRN acts as a naturally occurring antagonist of the TNF-α/TNFR1 interaction [27]. In contrast, PGRN interacts with TNFR2 at approximately 600-fold higher levels than TNF-α [27], and PGRN directly binds to and activates the TNFR2 proinflammatory pathway [32]. PGRN has been evaluated as a promising therapeutic target in various diseases, including common neurological diseases, inflammatory autoimmune diseases, cancer, tissue repair, and rare lysosomal storage diseases [33–37]. Despite the anti-inflammatory effects of PGRN, its multiple functions and oncogenic activity prohibit practical therapeutic implementation [38]. Accordingly, a PGRN derivative composed of half units of granulins A, C, and F plus linkers P3–P5 appears to be the “minimal” engineered molecule that retains binding affinity to TNFR2. This derivative, referred to as Atstrin, lacks the oncogenic activity of PGRN but retains preventative and therapeutic efficacy in murine models of TNF-α-driven inflammatory disease [27]. However, the effect and mechanism of how Atstrin plays a role in IVDD need to be clarified.

This study aimed to investigate the role of TNFR1 and TNFR2 in the course of IVDD and to determine whether Atstrin has therapeutic prospects in the treatment of IVDD.

2. Materials and methods

2.1. Patient samples

Approval for the collection of patient disc samples was obtained from the Ethics Committee of Jiangyin Affiliated Hospital of Nanjing University of Chinese Medicine and performed in accordance with ethical standards. NP samples comprising the control group were collected from vertebral fracture patients without disc degeneration (n = 4). NP samples of the IVDD group were collected from lumbar disc herniation patients (Pfirrmann grade III and IV) (n = 4). Inflammatory cytokine and TNF receptor expression in these disc samples was detected by real-time qRT-PCR analysis. All qRT-PCR amplifications were performed in triplicate, and the experiments were repeated at least three times.

2.2. Murine IVDD model

To further confirm the roles of the TNF receptor, we performed animal experiments. All animal studies were performed in accordance with institutional guidelines and with approval by the Institutional Animal Care and Use Committee of Southeast University. Animals were housed with ad libitum access to food and water under a 12-h light/dark cycle within a rodent barrier facility. The phenotype of mice was confirmed by genotyping when the mice were 4–6 weeks old. IVDD or sham surgery models were established in randomly allocated, experimentally native 12-week-old male C57BL/6 background wild-type (WT), TNFR1-knockout (TNFR1−/−), TNFR2-knockout (TNFR2−/−), and NF-κB reporter mice via needle puncture of caudal intervertebral discs in accordance with previously published reports [39,40]. Each group included 3 mice. Surgeries were performed under ketamine/xylazine-induced general anesthesia (Ketaset 90 mg/kg body weight/xylazine 10 mg/kg body weight, intraperitoneal injection) in a sterile procedure room. In the IVDD surgery model groups, each mouse underwent disc puncture at the tail. A small sagittal skin incision was made from Co2 to Co6 to help locate the disc position for needle insertion in the tail. Subsequently, Co3–Co5 coccygeal discs were punctured using a 31G syringe needle. The syringe needle was inserted into the Co3–Co5 disc along the vertical direction and then rotated in the axial direction by 180° and held for 10 s. The puncture was made parallel to the end plates through the AF into the NP using a 31-G needle, which was inserted 1.5 mm into the disc to depressurize the nucleus. In the sham surgery model group (control group), only a small sagittal skin incision was made in each mouse tail. Macroscopic morphological changes to the intervertebral space were examined at 4 and 8 weeks after model induction by film X-ray and micro-MRI. Mice were sacrificed via CO2 overdose followed by cervical dislocation at 4 or 8 weeks post model induction and processed for histological examination of morphological and molecular changes to intervertebral disc and vertebral tissues using H&E, Safranin O, TRAP and immunohistochemical staining. The expression of relevant anabolic and catabolic proteins in intervertebral discs and in vertebral bone at adjacent degenerative discs was further analyzed by immunofluorescence staining (IF), Western blot, and RT–PCR. Vertebral bone changes in adjacent degenerative discs were further analyzed by micro-CT (Siemens Inveon CT). Six mice from each genotype were employed for each experimental endpoint.

2.3. Hematoxylin-eosin (H&E) and immunohistochemical staining

The tail samples of mice were collected and incubated with 4% PFA in PBS overnight at 4 °C. Then, the tissues were washed with PBS, decalified with 10% EDTA for 2–4 weeks, and embedded in paraffin. Midsagittal sections were obtained and stained with H&E and Safranin O–fast green. The procedures were guided by a well-established protocol. The sections were examined under a light microscope (Axio Scope. A1, Zeiss, Oberkochen, Germany), and the lesion areas were measured by ImageJ (ImageJ 1.52a). H&E– and Safranin O-stained sections were scored across 5 categories of degenerative changes, with scores ranging from 5 points (1 in each category) for a normal disk to 15 points (3 in each category) for a severely degenerated disc. The five categories include the cellularity of the annulus fibrosis, the morphology of the annulus fibrosus, the border between the annulus fibrosus and nucleus pulposus, the cellularity of the nucleus pulposus, and the morphology of the nucleus pulposus.

For immunohistochemistry staining, sections were pretreated with 0.1% trypsin for 30 min at 37 °C. Sections were washed with PBS three times, followed by treatment with 0.25 U/ml chondroitinase ABC (Sigma–Aldrich) for 1 h and then 1 U/ml hyaluronidase (Sigma–Aldrich) for 1 h at 37 °C. To reduce nonspecific staining, sections were blocked at room temperature with 20% normal horse serum diluted in 3% BSA for 1 h. Without washing after blocking, TNFR1 and TNFR2 antibodies (1:200 dilution; DSHB) and aggrecan neopteopeptide antibodies (ab3208, 1:200 dilution; Abcam) were diluted in 20% normal horse serum with 3% BSA at 4 °C overnight. Sections were prepared for detection using the Vectorstain Elite ABC kit following the manufacturer’s guidelines at 25 °C for 1 h. Immunoreactivity was visualized using 0.5 mg/ml 3,3’-diaminobenzidine (DAB) in 50 mM Tris–HCl substrate, pH 7.8. Methyl green (1%) was used for counterstaining.

2.4. Film X-ray and MRI

Mice were euthanized with an intraperitoneal injection of 5 mg of pentobarbital sodium, and all mouse tails were harvested. First, we captured X-ray photos of these mouse tails using an X-ray machine in our lab. Midsagittal MRI images of all tail discs were acquired using micro-MRI (7-T Bruker Biospec 70/30) and qualitatively analyzed the MRI results according to the degenerative changes. The change in disc height
was evaluated by the disc height index (DHI) [40].

2.5. IVIS imaging

An IVIS spectrum imaging core was used according to the protocol (PerkinElmer IVIS Spectrum). The XGI-8 anesthesia system was used in conjunction with the IVIS Spectrum to anesthetize mice undergoing bioluminescent whole-body imaging. Four Nf-x reporter IVDD model mice were established via needle puncture of caudal intervertebral discs. Two IVDD model mice were g 100 μl of vehicle (sterile phosphate buffered saline, PBS) or Atstrin (dosed at 2.5 mg/kg body weight [28]) via intraperitoneal injection once weekly beginning from the day of model induction until sacrifice at 4 weeks post model induction. Another two mice as the control group underwent sham surgery.

2.6. NP cells culture

The mouse disc tissues were isolated from tail discs using a stereotaxic microscope, washed twice with phosphate-buffered saline (PBS), cut off the annulus fibers (AF) at the outside of each disc tissue, and then cut into 2–3 pieces. NP cells were released from these NP tissues by incubation with 0.25 mg/ml type II collagenase (Invitrogen, Carlsbad, CA, USA) for 12 h at 37 °C in Dulbecco’s modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA). NP cells were seeded at 1 x 10⁶ cells in 12-well plates and cultured in F12 basal medium supplemented with 2.5% Matrigel, 10% fetal bovine serum plus 2.5 mg/ml L-ascorbic acid-2-phosphate (Sigma). The medium was changed twice per week for the duration of cell pellet culture.

2.7. qRT-PCR

qRT-PCR was performed to measure mRNA expression. The mice were euthanized, and the tails were cut off. The discs of these tails were separated, snap-frozen into liquid nitrogen, and transferred to –80 °C until use. The cultured cells were also collected. Total RNA was isolated with TRIzol Reagent (Ambion, Life Technologies, Carlsbad, CA, USA) from disc tissues or cultured cells according to the manufacturer’s instructions. RNA quantity was determined using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Quantification of mRNA expression was performed on the StepOnePlus Real-Time PCR System (Life Technologies) using TaqMan Gene Expression Assays (Life Technologies) specific for TNF, TNFR1, TNFR2, MIP-1α, MCP-1, IFN-γ, IL-6, IL-1α, Aggrecan, Collagen II, TRAP, Cathepsin, BAX, Bcl-2, and MMP-13. Data were normalized to the internal control, GAPDH. The primers for specific amplification of murine genes are as follows: 5'-GAGGTCGACCTGGGACAGAGAC-3' and 5'-TTGTTGTTTGTGAGTGTGAG-3' for TNF-α; 5'-CCGGGAGAAGGGATAGCTT-3' and 5'-TCGGACAGTCCTACAACAGT-3' for TNFR1; 5'-ACACCTCATACAAAAGGGGAC-3' and 5'-AGGTTTTCTGATCGTATG-3' for TNFR2; 5'-ACAGGGCTTTAGGCAAGT-3' and 5'-ACTTACAGGTTCCCGG-3' for MIP-1α; 5'-GATAAAGGACACCCCAATCC-3' and 5'-TCTCCTGGGCAACATGGTGTC-3' for MCP-1; 5'-TCCGGTAACTGATGAGTGGTTG-3' and 5'-TCCGGTTTCCCTGTTTATATATG-3' for TNF-α; 5'-GAGGTCGACCTGGGACAGAGAC-3' and 5'-TTGTTGTTTGTGAGTGTGAG-3' for TRAP; 5'-TTGAGGACCTGGGACAGAGAC-3' and 5'-TTTTATCCTGGCCACAC-3' for TNFR1; 5'-GGGCTTCTTCTCTTCTTC-3' and 5'-ATATAAAGGTTTGTGATTATG-3' for TNFR2; 5'-AATCTCACAGGACACATCA-3' and 5'-AAGGTTGCTCATGCTCTCATCT-3' for IL-6; 5'-ACTGGTGGTCTACAAACCC-3' and 5'-CTGGATGGTTTACCAAGGAACC-3' for Aggrecan; 5'-ACAGGGCTTTAGGCAAGT-3' and 5'-TTGTTGTTTGTGAGTGTGAG-3'.

2.8. Immunofluorescence

The frozen sections were first penetrated with 0.1% Triton X-100 for 10 min and then blotted with 5% donkey serum in PBS for 30 min. The sections were then incubated with primary antibodies overnight at 4 °C and subsequently rinsed with PBS and incubated with secondary antibodies at room temperature for 1 h. After rinsing with PBS 3 times, the slides were mounted with Vectashield mounting medium with DAPI (H-1200, Vector Laboratories, Burlingame, CA, USA). The slides were subsequently screened under an immunofluorescence microscope (Axio Scope. A1, Zeiss, Oberkochen, Germany).

2.9. Assays to examine the therapeutic effects of atstrin in IVDD in vivo

To investigate the protective effects of Atstrin on IVDD, IVDD model mice were administered 100 μl of vehicle (sterile phosphate buffered saline, PBS) or Atstrin (dosed at 2.5 mg/kg body weight [28]) via intraperitoneal injection once weekly beginning from the day of model induction until sacrifice at 4 or 8 weeks post model induction. Six mice of each genotype were employed for each experimental endpoint.

2.10. Assays to examine the catabolic and anabolic activity of TNF-α and atstrin in nucleus pulposus cells in vitro

We separated the nucleus pulps from WT, TNFR1+/− and TNFR2+/− mice separately. Then, NP cells were isolated via incubation of intervertebral nucleus pulposus tissues in DMEM for 5 days. Cells were then seeded into a six-well plate and treated with TNF-α (10 ng/ml) or Atstrin (100 ng/ml) for further experiments [21]. At the indicated time points, NP cells were harvested for analysis by WB and RT-PCR. Culture media was collected, and the levels of the secretatory inflammatory cytokines IL-6 and IL-1β were assessed by ELISA.

2.11. Statistical analysis

ImageJ (ImageJ 1.52a) was implemented for image quantification as necessary. For statistical analysis, GraphPad Prism 7 Software (GraphPad Software, San Diego, CA, USA) was used. A significant difference between two experimental groups was identified by multiple t tests, including one per row. To compare the significant differences among multiple groups, we used the mean ± SD and one-way analysis of variance. A p value < 0.05 was considered statistically significant.

3. Results

3.1. TNFR1 and TNFR2 are significantly upregulated in both human and mouse degenerative disc tissues

Transcript levels of various inflammatory cytokines known to be involved in inflammatory/degenerative diseases in nucleus pulposus samples collected from patients with spinal trauma (vertebral fracture patients without disc degeneration, n = 4) and disc degeneration (grade
C IVDD patients, n = 4) were measured by real-time qPCR. Among the cytokines screened, TNF-α, TNFR1 and TNFR2 were significantly increased in biopsies from patients with degenerative nucleus pulposus compared to control tissues (Fig. 1A and B). Similarly, we sought to examine the effect of IVDD on the expression levels of TNFR1 and TNFR2 in IVDD model mice. As shown in Fig. 1C & D, the expression levels of TNFR1 and TNFR2, detected by immunohistochemistry, were also significantly elevated in the nucleus pulposus of the murine IVDD group compared to the control group.

3.2. Deficiency of TNFR1 and TNFR2 is associated with decreased and enhanced IVDD severity, respectively

Histological examination and quantitative analysis were performed on mouse disc samples at four and eight weeks post-IVDD surgery. H&E staining (Fig. 2A&B, S4) showed pathological changes in discs in all IVDD mice with three different genetic backgrounds at 4 and 8 weeks; degenerative changes were particularly pronounced in TNFR2−/− mice at 8 weeks postsurgery. H&E and Safranin O staining showed reduced disc degeneration in TNFR1−/− mice compared to WT and TNFR2−/− mice, with greater maintenance of the distinct nucleus pulposus morphology compared to loss of the boundary with the annulus fibrosus (Fig. 2A-D). Loss of the distinct annulus fibrosus morphology was more severe in TNFR2−/− IVDD discs, where chondrocytes accounted for approximately 75% of the annulus fibrosus cells (Fig. 2C&D). TNFR2−/− IVDD discs showed more bulged, ruptured or serpentine fibers in the annulus fibrosus; a moderate to severe decrease in the number of NP cells; and rounded or irregularly shaped NP morphology (Fig. 2C&D). X-ray images of IVDD mouse tails revealed milder and more severe decreases in the space of the intervertebral disc area and increased ossification in the endplates of TNFR1−/− and TNFR2−/− mice compared to WT mice (Fig. 2E&F, S6). These changes were confirmed by micro-MRI scanning under general anesthesia. MRI results showed decreased disc signals in all groups following needle puncture, with a noticeably greater reduction in signal in TNFR2−/− IVDD mice (Fig. 2G&H). Collectively, these findings showing that loss of TNFR1 protects against while loss of TNFR2 accelerates the progression of IVDD suggest that TNFR1 and TNFR2 may mediate destructive and protective processes in the pathogenesis of IVDD, respectively.

3.3. Loss of TNFR1 and TNFR2 leads to significantly distinctive changes in cell matrix composition in IVDD

We performed a series of histological assays to examine changes in cell matrix composition following IVDD model establishment. MMP13 is one of the main cytokines involved in intervertebral disc degeneration. As revealed by immunofluorescence analysis (Fig. 3A), matrix metalloproteinase MMP13 expression was increased in the degenerated discs. More robust expression of MMP13 was observed in TNFR2−/− IVDD than in TNFR1−/− and WT mice (Fig. 3A&B). In addition, the absence of TNFR1 attenuated the expression of MMP13 in IVDD discs compared with WT mice. Fig. 3C shows immunohistochemistry staining of the catabolic marker Aggrecan degenerative-generated neoepitope in intervertebral disc samples from sham and IVDD groups of different genotypes. The corresponding quantitative analysis (Fig. 3D) demonstrated significantly elevated signaling in the TNFR2−/− group relative to the WT or TNFR1−/− groups. Primary components comprising the disc, Collagen 2 and Aggrecan, were decreased at mRNA expression levels in degenerated discs, especially in TNFR2−/− mice (Fig. 3E,F), and the decrease in these two markers was less pronounced in TNFR1−/− mice than in WT mice. These results illustrate increased catabolic activities and reduced expression of anabolic markers in all degenerative discs. TNFR2

Figure 1. The levels of TNFR1 and TNFR2 are elevated in human and mouse degenerative disc tissues. A. A heat plot representation of real-time PCR mRNA expression of inflammatory cytokines and receptors in human control and IVDD intervertebral discs. TNF-α, TNFR1, TNFR2, and IL-1α mRNA were significantly increased in degenerative disc group (n = 4) relative to control (n = 4). B. Quantitative analysis of Fig. 1A. C. In the IVDD mice models, the degenerated intervertebral disc tissues (n = 5) exhibited higher expressions of TNFR1 and TNFR2 relative to sham controls (n = 5) as detected by immunohistochemistry. D. Quantitative analysis of (*p < 0.05, **p < 0.01).
deficiency led to more severe degenerative changes with regard to these markers of cellular metabolism compared with TNFR1/C0/C0 and WT mice, while TNFR1 deficiency resulted in ameliorated degenerative changes relative to WT mice.

3.4. Loss of TNFR1 and TNFR2 leads to less and more local vertebral bone destruction in IVDD, respectively

In clinical intervertebral disc degeneration cases, not only are the discs affected, but the adjacent vertebrae also exhibit degenerative changes. Accordingly, we investigated local changes in the vertebral bone of IVDD model mice. Interestingly, we found that loss of TNFR1 and TNFR2 led to a significant decrease and increase in osteoclasts in vertebral bodies adjacent to degenerative discs, respectively. Tartrate-resistant acid phosphatase (TRAP) staining revealed an increase in osteoclast number at the endplate adjacent to degenerative discs in all three different genetic background mice with IVDD; however, evidence for enhancement of osteoclastogenesis was more pronounced in the TNFR2/C0/C0 group (Fig. 4A&B, S7), and TNFR1/C0/C0 IVDD discs had fewer TRAP-positive cells than WT IVDD discs. Correspondingly, real-time qPCR results showed that the mRNA expression levels of TRAP and Cathepsin K were significantly downregulated and upregulated in the TNFR1/C0/C0 and TNFR2/C0/C0 IVDD groups, respectively, compared to WT mice with IVDD (Fig. 4C&D).

To further assess vertebral bone quality in our IVDD model mice, we performed micro-CT scanning and quantified the bone mineral density (BMD) of the vertebra adjacent to the degenerated discs. The TNFR2/C0/C0 IVDD group had the lowest vertebral BMD among all three groups (Fig. 4E&F). These results indicate the development of osteoporosis in the vertebra adjacent to the degenerative disc and, more importantly, that TNFR2/C0/C0 IVDD model mice exhibit a more severe loss of BMD than WT and TNFR1/C0/C0 mice. Taken together, these results highlight clear differences in bone quality at degenerative disc-adjacent vertebrae and further indicate that TNFR1 and TNFR2 play opposite roles in disc degeneration.
degeneration by prompting and inhibiting the loss of vertebral bone near degenerated discs, respectively.

3.5. Deficiency of TNFR1 and TNFR2 is associated with decreased and increased apoptosis in degenerated discs, respectively

Cell apoptosis plays an important role in various degenerative diseases, including IVDD. We next sought to examine whether TNFR1 and TNFR2 also affected disc cell apoptosis in IVDD. We first performed an apoptosis assay with an Annexin V approach. Increased apoptosis in all degenerated discs with different genotypes was observed. Additionally, significantly reduced apoptosis was observed in TNFR1 /−/− IVDD mice compared with WT IVDD mice, whereas the most drastic increase in apoptosis was observed in TNFR2 /−/− IVDD mice (Fig. 5A&B). Furthermore, real-time qPCR analysis of intervertebral disc tissue following needle puncture-induced IVDD showed increased pro-apoptotic Bax expression and decreased anti-apoptotic Bcl-2 expression in all IVDD model mice compared to sham groups. Compared to those in the WT IVDD group, deletion of TNFR1 reduced Bax but increased Bcl-2 expression, whereas ablation of TNFR2 increased Bax but decreased Bcl-2 expression in the discs of corresponding IVDD mice (Fig. 5C).

3.6. Atstrin protects against IVDD in WT and TNFR1 /−/− mice but largely loses its protective effects in TNFR2 /−/− mice

Our previous studies demonstrated that Atstrin plays a protective function in inflammatory diseases such as arthritis without any significant side effects [28]. To further determine whether Atstrin also has therapeutic effects on IVDD, we established a needle puncture-induced IVDD model in NF-κB reporter mice and administered Atstrin through weekly intraperitoneal injection for four weeks. IVIS imaging (PerkinElmer IVIS Spectrum) illustrated significantly reduced NF-κB-mediated luciferase signal activation in the Atstrin-treated group relative to control mice with needle puncture-induced IVDD (Fig. 6A&B). To further
determine the dependence of Asttrin therapeutic effects on TNFRs in IVDD, an IVDD model was established in WT, TNFR1/−/− and TNFR2/−/− mice followed by treatment with Asttrin for four weeks. Histological analyses showed significant improvement of disc degeneration scores in Asttrin-treated TNFR1/−/− and WT mice relative to the PBS injected IVDD mice; however, no therapeutic effect of Asttrin was observed in TNFR2/−/− IVDD mice and histological scores of disc degeneration were statistically indistinguishable from those of PBS treated IVDD mice (Fig. 6C&D). MRI scanning also demonstrated that Asttrin attenuated the narrowing of the intervertebral disc space and loss of NP signals in TNFR1/−/− IVDD mice relative to WT and TNFR1/−/− mice (*p < 0.05, **p < 0.01, Bar = 50 μm, n = 6, data were presented as mean ± SD).
WT mice. B. Quantification of A. C. and D. Expressions of Bax and Bcl-2 in the discs of sham and IVDD mice were measured by real time qPCR analysis (*p < 0.05, **p < 0.01, n = 6, data were presented as mean ± SD).

4. Discussion

The intervertebral disc plays an important ‘spacer’ and ‘stabilizer’ role in the optimal anatomical and biomechanical function of the spine. IVDD is a common degenerative disease correlated with aging that is closely related to low back pain, which contributes to disability, decreased quality of life in patients, and increased health care costs [41–43]. As a central inflammatory molecule, TNF-α expression has already been examined in human degenerated intervertebral discs [10, 44–47], and anti-TNF therapies have shown therapeutic promise in in vitro models of disc degeneration [44,45]. Although native disc cells express very few TNF receptors, we demonstrated that the expression of both TNFR1 and TNFR2 is significantly upregulated in degenerated human discs relative to nondegenerated controls. We also established a needle puncture-induced IVDD model in C57BL/6 WT mice and similarly found that both TNFR1 and TNFR2 were increased in these IVDD mice. To distinguish the roles of TNFR1 and TNFR2 in inflammatory signaling, key mediators of anabolic and catabolic cellular responses were monitored following in vitro treatment of disc cells isolated from WT, TNFR1−/− and TNFR2−/− mice with TNF-α or Atstrin, an engineered protein composed of three TNFR-binding fragments of PGRN [28]. From these results, we demonstrated that the activation of phosphorylated p65 (p-p65) by TNF-α largely depends on TNFR1, while Atstrin’s anabolic effect mainly depends on TNFR2 in disc cells.

To further confirm the respective roles of TNFR1 and TNFR2 in degenerative discs in vivo, we established an IVDD model in WT, TNFR1−/− and TNFR2−/− mice. Compared with all three sham conditions, IVDD model establishment was associated with obviously increased catabolic activity and reduced anabolic activity in disc tissues. These changes in catabolic and anabolic markers illustrated an overall protective effect of TNFR1 deficiency and detrimental effects of TNFR2 deficiency on IVDD progression. MMP13 and the intermediate degrada- tion product of aggrecan were increased in each group of IVDD mice, although TNFR2 deficiency was associated with the most dramatic increases. qRT–PCR further confirmed that the expression of collagen 2 and aggrecan, key components of disc composition, was downregulated in IVDD mice, with the most significant downregulation in TNFR2−/− mice.

Apoptosis plays an important role in many degenerative diseases, and the individual contributions of TNFRs to TNF-induced apoptosis may be cell type- and pathology-dependent [48]. In disc degeneration, we found that loss of TNFR2 accelerated cell apoptosis, as assessed through Annexin V staining and qRT–PCR analysis. In patients, mild to severe bone loss is also a common feature of vertebrae adjacent to degenerated discs. Interestingly, we also found enhanced osteoclast activity and reduced bone mineral density, as assessed by TRAP staining and microCT, respectively, in IVDD model mice with three different genetic backgrounds. Compared to WT IVDD mice, TNFR1−/− mice showed less osteoclast staining and less BMD loss, while the opposite trend was observed in TNFR2−/− mice. These results suggest that TNF receptors are also involved in the cellular apoptosis and intervertebral bone loss associated with IVDD and that TNFR1 and TNFR2 may play opposite roles in these pathological processes. These results suggest that the metabolic processes of nucleus pulposus cells and surrounding bone cells change to varying degrees during IVDD. The inflammatory response may promote apoptosis and osteoclast activity to varying degrees.

Importantly, the in vivo limitations of the application of TNF inhibition against IVDD may be attributable to simultaneous nonspecific blockade of protective TNFR2-mediated activity. For instance, the TNF inhibitor etanercept (Enbrel) is a fusion soluble TNFR2 extracellular protein and works by binding to and blocking TNF-α, but it may also bind to and inhibit other TNFR2-binding molecules (e.g., PGRN) [28], resulting in nonspecific blockade of TNF-α proinflammatory signaling,
Figure 6. Atstrin has therapeutic effects on needle-puncture induced IVDD mice. A. IVIS showed that Atstrin can attenuate the inflammatory signal in IVDD model discs in NF-κB reporter mice. Red arrow indicates elevated signal in PBS-treated IVDD mice. B. Quantification of A. C. Safranin O staining on IVDD model discs of indicated genotype with or without Atstrin treatment. D. Histological degeneration scores. E. MRI scanning. Red arrows indicate attenuated IVD space in TNFR2−/− mice. F. Quantification of E (n.s. = not significant, *p < 0.05, **p < 0.01, Bar = 50 μm, n = 6, data were presented as mean ± SD).
which may also be a reason for the controversial effects of TNF-α on LB as well as the lack of efficacious TNF-targeting agents for IVDD. Our findings that loss of TNFR1 can protect against while loss of TNFR2 can accelerate the needle puncture-induced disc degeneration model suggest that a new therapeutic strategy implementing selective interactions with TNF receptors may hold promise in addressing IVDD. In our previous studies, we demonstrated that Atstrin, which binds to TNFR2 with a much higher binding affinity than TNF-α, exerts protective and therapeutic effects in inflammatory disease [28]. To determine whether Atstrin has therapeutic effects in IVDD and the molecular mechanisms involved, we treated WT, TNFR1 fl, and TNFR2 fl IVDD mice with weekly systemic Atstrin administration for four weeks. We found that Atstrin could effectively attenuate disc degeneration in WT and TNFR1 fl IVDD mice. Intriguingly, Atstrin largely lost its protective function against IVDD progression in the absence of TNFR2. Overall, this evidence suggests that TNFR2 mediates the local homeostasis of disc tissues and maintains the capacity for survival and regeneration of disc tissue, but the possible mechanism of TNFR2's beneficial and protective effect on IVDD needs further study.

In summary, our study demonstrates that TNFR1 and TNFR2 have disparate roles in disc degeneration and that TNFR2 plays a beneficial and protective role in the intervertebral disc and vertebral bone. In addition, Atstrin has therapeutic effects against IVDD in mice, and these effects may primarily depend on the presence of TNFR2.

Author contributions
S.Wang and G. Sun, designed and performed experiments, collected and analyzed data, and co-wrote the paper. P.Fan, L.Huang and Y.Chen assisted with experiments, and collected and analyzed data. C.Chen designed and supervised this study, analyzed data, and wrote and edited the manuscript.

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Declaration of competing interest
None declared.

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