RESEARCH ARTICLE

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Roles of TREM2 in degeneration of human nucleus pulposus cells via NF-κB p65

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Abstract

Nuclear factor (NF)-κB p65 plays a key role in the development of intervertebral disc degeneration (IDD). Herein, we found that messenger RNA levels of human triggering receptor expressed on myeloid cells-2 (TREM2) and NF-κB p65 were upregulated and strongly positive correlated ($r^2 = 0.299$, $P = 0.0126$) in nucleus pulposus (NP) tissues of patients with IDD. To investigate the role of TREM2 in the development of IDD and whether NF-κB p65 was the underlying mechanism, whereby TREM2 played its role, we established TREM2-siRNA–transfected human degenerative NP cells and TREM2-overexpression vector–transfected human normal NP cells. Degeneration of human NP cells was assessed by measuring cell apoptosis, cell proliferation, and the secretion of tumor necrosis factor-α, interleukin (IL)-1β, and IL-6. Protein levels of Bcl2, Bax, total NF-κB p65 in whole cell lysates, cytoplasm NF-κB p65, and nuclear NF-κB p65 were determined to evaluate underlying mechanisms. Our data elucidated that TREM2 silencing was a therapy for human degenerative NP cells through inhibiting cell apoptosis, promoting cell proliferation, suppressing production of tumor necrosis factor-α, IL-1β, and IL-6, and the mechanisms included decreasing Bax while enhancing Bcl2, downregulating total NF-κB p65, and retarding NF-κB p65 nuclear translocation. On the contrary, upregulated TREM2 showed the opposite effects, accelerating the degeneration of human normal NP cells. Downregulating TREM2 and total NF-κB p65 and inhibiting NF-κB p65 nucleus translocation were also confirmed in NP tissue samples of four IDD rats. We concluded that TREM2 functioned as a promoter in the degeneration of human NP cells. Downregulating TREM2 may be a novel therapeutic strategy for human IDD.

KEYWORDS
human degenerative nucleus pulposus (NP) cells, human normal NP cells, nuclear factor (NF)-κB p65, triggering receptor expressed on myeloid cells-2 (TREM2)

1 | INTRODUCTION

Intervertebral disc degeneration (IDD) is identified as successive changes in an intervertebral disc, which result

in the reduction of disc cells and extracellular matrix (ECM) in the disc.[1] IDD is caused by multiple risk factors, such as genetic predisposition, lifestyle, aging, and inadequate metabolite transport,[2] and the disc can be weakened to such an extent that structural failure occurs. Currently, IDD remains an important global problem

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Ming Bai and He-Ping Yin have contributed equally to this study.

with higher incidence, for which treatment is expensive, poorly effective, and limited to alleviating the symptoms rather than etiologies. Besides, IDD is also a major cause of low back/neck/radicular pains,[3] confusing our life, and producing a great financial burden on our whole sociality. Research work based on molecular mechanisms increasingly attracts our attention to see the biology underlying this disease and is very necessary to improve our current IDD therapies.[4]

Inflammation pathways is a major factor leading to ECM degradation and apoptosis of disc cells,[5] contributing to the pathogenesis of IDD. The release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-α/β, and IL-6/7 by disc cells accelerates ECM degradation, enhances chemokine production, and promotes changes in cell phenotype,[3] causing injury of nucleus pulpous (NP) tissues. Upregulated chemokines, in turn, promote infiltration and activate T cells, B cells, macrophages, neutrophils, and mast cells, further amplifying the inflammatory cascade. Inflammatory response is important in maintaining NP tissue homeostasis, and if properly balanced, it may contribute to degenerative NP tissue repair/regeneration.[6]

The nuclear factor (NF)-κB family of protein consists of transcription factors, which modulate cellular responses to inflammation, damage, and stress,[7] and also plays a key role in regulating apoptosis of NP cells.[8] Among its family members, p50/p65 heterodimer is recognized as the most abundant form.[7] In nondegenerative NP cells, NF-κB p65 is inactive and sequestered by IκB proteins, thus mainly found in the cytoplasm; however, in degenerated NP cells, it was found in the nucleus. The severer the degenerative degree of NP cells, the higher the expression of NF-κB p65.[9] NF-κB is activated by the inflammatory factor, oxidation, genetic toxicity, chemical reagents, or other stress and induces the subsequent IκB kinase activation. Activated IκB kinase further phosphorylates IκB proteins, contributing to IκB ubiquitination and degradation, which accelerates the translocation of NF-κB into the nucleus, where it modulates the transcription of targeted genes that are related to cell survival, growth, apoptosis, and generation of inflammatory cytokines. Although, the underlying mechanisms of IDD are still unclear, yet, currently, ameliorating inflammation in the disc and suppressing apoptosis of intervertebral disc cells via the NF-κB pathway represents effective possibilities to block human IDD.

A human triggering receptor expressed on myeloid cells-2 (TREM2), also known as chr6p21, is a cell surface receptor, expressed in myeloid-derived cells.[10] TREM2 and its soluble form inhibit cell apoptosis in microglia[11] and induce inflammatory responses via the NF-κB p65

pathway in many neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease.[12,13] However, fewer reports have elucidated the roles of TREM2 in IDD, and its association with NF-κB p65 in this process. Thus, the current study highlighted a critical role of TREM2 in regulating cell viability, apoptosis, and inflammatory response in both degenerative NP cells and human normal NP cells and revealed the involvement of transcriptional regulation of NF-κB p65 in this process, providing the mechanistic insights into TREM2 in IDD.

2 | MATERIAL AND METHODS

2.1 | NP tissue harvested and primary NP cell isolation

Twenty patients with IDD and 20 corresponding healthy people were recruited, which was approved by the local Human Ethics Committee of The Affiliated Hospital of Inner Mongolia Medical University, and a written informed agreement was achieved from each individual involved in our study. NP samples from three healthy individuals and three patients with IDD were harvested to isolated human normal and degenerative NP cells. Briefly, intervertebral discs from prominent to spinal canal were collected and maintained in phosphate-buffered saline containing 100× streptomycin/penicillin (P1400-100; Solarbio, Beijing, China). NP tissues were separated from the disc, then underwent enzymatic digestion using 0.25% (wt/vol) Trypsin-EDTA solution (T1300-100; Solarbio) and 0.075% (wt/vol) collagenase I (17454.01; SERVA) to isolated NP cells. We cultured isolated cells at 37°C under 5% CO_2 in a medium of Dulbecco's modified Eagle's medium (DMEM)-F12 (SH30023.01B; Hyclone, Logan, UT) with 10% (v/v) fetal bovine serum (16000-044; Gibco, Invitrogen, Carlsbad, CA), 0.1 mg/mL streptomycin, and 100 U/mL penicillin (P1400-100; Solarbio, Beijing, China). We changed the culture medium every other day until adherent cells showed more than 95% of survival rate evaluated by trypan blue staining.

2.2 | Cell transduction

To study the role of downregulated TREM2 in human degenerative NP cells, designed small hairpin RNA targeting TREM2 was synthesized and inserted to Addgene plasmid PLKO.1 expression vector (pLKO.1-TREM2). Then, pLKO.1-TREM2 (1000 ng), Addgen plasmid psPAX2 (900 ng), and Addgen plasmid pMD2.G (100 ng) were coinfected into 293T cells (ATCC, Shanghai, China) via 30 μL Lipofectamine 2000 (Invitrogen), according to a manufacturer's proposal. After transduction for 4-6 hours,

cells were maintained in a complete culture medium. Viral particles with TREM2 silencing were harvested at 48 and 72 hours and subsequently used for transfection of human degenerative NP cells. Three small interfering RNA (siRNA) targeting TMEFF2 (GenBank NM_018965.3) presented in Table 1 were designed with the most effective being observed in the second siRNA-TMEFF2 sequences (Table 1, label 2). Therefore, in our study, we chose siRNA-TMEFF2 sequences (Table 1, label 2) as the optimal sequences to knockdown TREM2.

Meanwhile, to study the role of upregulated TREM2 in human normal NP cells, 293T cells were infected with lentiviral vector encoding TMEFF2 using the method mentioned above. Designed primers were the following: TREM2-Forward: 5′-GCGAATTCATGGAGCCTCTCCGGCTGCTC ATCT (EcoRI); TREM2-Reverse 5′-CGGGATCCTCACGTG TCTCTCAGCCCTGGCAGA (BamHI). After 48 or 72 hours, viral particles with TREM2 overexpression were harvested and subsequently used for infecting human normal NP cells. Lentiviral vector without TMEFF2 encoding was considered as the corresponding empty vector.

2.3 | Cell counting kit-8 and flow cytometry analysis

Normal or human degenerative NP cells in logarithmic growth were respectively digested by Trypsin-EDTA solution (Solarbio), seeded in a 96-well plate (5×10^3/well), and then maintained at 37°C overnight. Collected cells were treated with or without recombinant lentiviral liquid (5×10^9 pfu/mL, 0.1 μL), and continued to culture. At 0, 24, 48, and 72 hours, the absorbance (optical density value) at 450 nm was detected for cell viability using the commercially available cell counting kit-8 (CP002, SAB, Shanghai, China).

Normal or human degenerative NP cells were seeded in a six-well plate (5×10^5/well); they grew adherently until reaching 50% of confluence. Collected cells were treated with

or without recombinant lentiviral liquid (5×10^9 pfu/mL, 0.1 μL) and continued to culture. At 48 hours, cell apoptosis was assessed using the Annexin V-FITC apoptosis detection kit (Beyotime, C1062, Shanghai, China) and flow cytometry (Biosciences, Mountain View, CA). Briefly, cells were maintained with 5 μL fluorescein isothiocyanate–labeled recombinant annexinV (Annexin V-FITC) for 15 minutes in the dark at 4°C followed by 5 μL propidium iodide for another 15 minutes according to the provided instructions. The percentage of apoptotic cells was defined as the ratio of annexin V-positive cells to propidium iodide–negative cells. Fluorescein isothiocyanate–labeled recombinant annexinV staining was measured in the FL1 (green) channel of BD flow cytometry, whereas propidium iodide staining was measured in the FL2 (red) channel of BD flow cytometry.

2.4 | Preparation of nuclear and cytoplasmic fractions

To obtain nuclear and cytoplasmic extracts, NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (#78835; Thermo Scientific) were used according to the manufacturer's instructions. Briefly, cells were washed with phosphate-buffered saline and then resuspended in ice-cold cytoplasmic extraction reagent (CER I) followed by CERll. Cell suspension was incubated on ice for 1 minute and then centrifuged at 4°C at 16 000g (12 000 rpm) for 5 minutes. The supernatants (cytoplasmic protein extracts) were isolated and maintained at −80°C for Western blot analysis. Meanwhile, the precipitates (nuclear protein extracts) were dispersed in an ice-cold nuclear protein extraction reagent and maintained at 0°C for 40 minutes, with vigorous shaking for 15 seconds every 10 minutes. Cell suspension was centrifuged at 16 000g for 10 minutes at 4°C (12 000 rpm), and the supernatants (nuclear protein extracts) were isolated and maintained at −80°C for Western blot analysis.

TABLE 1 siRNA-TMEFF2 sequences

Label 1	TREM2 sequence siRNA sequence	5′-GCCTCTTGGAAGGAGAAAT-3′, start point 589 5′-CCGGGCCTCTTGGAAGGAGAAATCTCGAGATTTCTCCTTCCAAGAGGCTTTTT-3′ (Forward); 5′-AATTAAAAAGCCTCTTGGAAGGAGAAATCTCGAGATTTCTCCTTCCAAGAGGC-3′ (Reverse)
Label 2	TREM2 sequence siRNA sequence	GCCTGCATCTTTCTCATCA, start point 642 5′-CCGGGCCTGCATCTTTCTCATCACTCGAGTGATGAGAAAGATGCAGGCTTTTT-3′ (Forward); 5′-AATTAAAAAGCCTGCATCTTTCTCATCACTCGAGTGATGAGAAAGATGCAGGC-3′ (Reverse)
Label 3	TREM2 sequence siRNA sequence	CCAGGGTATCAGCTCCAAA, start point 753 5′-CCGGCCAGGGTATCAGCTCCAAACTCGAGTTTGGAGCTGATACCCTGGTTTTT-3′ (Forward); 5′-AATTAAAAACCAGGGTATCAGCTCCAAACTCGAGTTTGGAGCTGATACCCTGG-3′ (Reverse)

siRNA, small interfering RNA; TREM2, triggering receptor expressed on myeloid cells-2.

2.5 | Real-time polymerase chain reaction and Western blot analysis

Cell samples were washed with phosphate-buffered saline (BYL40657; JRDUN Biotechnology, Shanghai, China) twice, then lysed at 4°C in a radioimmunoprecipitation assay (RIPA) lysis buffer (R0010; Solarbio) containing protease and phosphatase inhibitors. Cell lysates were heated above 95°C for 10 minutes and centrifuged for 10 minutes at 12 000g (2000 rpm). Cell supernatant was stored at −80°C for further real-time polymerase chain reaction (RT-PCR) and Western blot analysis. As for human or rat intervertebral disk tissue, samples (200 mg) were cut into small pieces, homogenized at 4°C in 1500-2500 μL of RIPA buffer (R0010; Solarbio), and then centrifuged at 4°C at 12 000g (2000 rpm) for 15 minutes, The supernatant was separated and stored at −80°C for further RT-PCR and Western blot analysis.

To assess messenger RNA (mRNA) levels of TMEFF2 and NF-κB p65 in NP tissue samples or cell samples, RT-PCR was conducted by ABI Prism 7300 SDS Software (Applied Biosystem, Foster City, CA) following the manufacturer's proposal. The materials used in this process were the following: Trizol reagent (1596-026; Invitrogen), RevertAid First Strand cDNA Synthesis Kit (#K1622; Fermentas, Ontario, CA), and SYBR Green PCR Kit (Thermo Scientific, Shanghai, China). The primers used in our study are presented in Table 2.

Western blot analysis was conducted to detect protein levels of TMEFF2, total NF-κB p65, Bcl2, and Bax in NP tissue samples or cell samples. Briefly, the total protein level in supernatants was determined using the bicinchoninic acid (BCA) protein assay kit (PICPI23223; Thermo Scientific). Twenty-five micrograms of total protein was separated through 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separated TMEFF2, total NF-κB p65, Bcl2, or Bax were electrophoretically transferred onto nitrocellulose (NC) membranes (HATF00010; Millipore, Bedford, MA). After blocking with 5% nonfat milk, NC membranes were incubated with primary antibodies: TMEFF2 antibody (1:1500 dilution; Ab201621; Abcam, Cambridge, MA), NF-κB p65 antibody (1:1000 dilution; #8242; CST), Bcl2 antibody (1:500 dilution; Sc-492; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Bax antibody (1:500 dilution, Sc-493; Santa), H3 antibody (1:1000 dilution; #4499; CST, Beverly, MA), β-action (1:1000 dilution; #4970; CST), and glyceraldehyde 3-phosphate dehydrogenase antibody (1:2000 dilution; #5174; CST) at 4°C overnight followed by horseradish peroxidase–conjugated antibodies (1:1000 in dilution; Beyotime) for 1 hour at 25°C. Immunoreactive bands were analyzed by the chemoluminescence (ECL) detection system (GE Healthcare/Amersham Biosciences). In our article, glyceraldehyde 3-phosphate dehydrogenase served as an internal control to normalize TMEFF2, total NF-κB p65, Bcl2, and Bax. H3 served as an internal control to normalize nuclear NF-κB p65. β-action served as an internal control to normalize cytosolic NF-κB p65.

2.6 | ELISA for TNF-α, IL-β, IL-6, and level determination

After transfection for 48 hours, supernatants were harvested, and then centrifuged (2000-3000 rpm at 4°C for 20 minutes) to remove cell debris. The concentrations of TNF-α, IL-1β, and IL-6 and in supernatants were assessed by human TNF-α enzyme-linked immunosorbent assay (ELISA) Kit (AE13959HU; Abebio, Beijing, China), human IL-1β ELISA Kit (AE58504HU; Abebio) and human IL-6 ELISA Kit (AE62763HU; Abebio), respectively, following the manufacturer's instructions.

2.7 | Rat IDD model

Eight rats were randomly divided into the healthy and IDD groups (n = 4/group). A rat IDD model was established by needle puncture for tail discs according to a reported literature,[14] and the process of which abided by the ethical use of animals approved by local Animal Ethics Committee of The Affiliated Hospital of Inner Mongolia Medical University. NP tissues were harvested at 4 weeks after surgery. Healthy rats

TABLE 2 Primers used in RT-PCR analysis

Name	GenBank	Primer (5′-3′)
TREM2	NM_001271821.1, at 374-620 position	Forward: TGGCACTCTCACCATTACG; Reverse: CCTCCCATCATCTTCCTTCAC; 247 bp, 59% GC.
NF-κB p65	NM_001145138.1, at 72-303 position	Forward: GAATGGCTCGTCTGTAGTG; Reverse: TGGTATCTGTGCTCCTCTC; 232 bp, 68% GC.
GAPDH	NM_001256799.1, at 1065-1174 position	Forward: CACCCACTCCTCCACCTTTG; Reverse: CCACCACCCTGTTGCTGTAG; 110 bp; 54% GC.

bp, base pairs; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NF-κB p65, nuclear factor-κB p65; TREM2, triggering receptor expressed on myeloid cells-2.

without any surgery were considered as the corresponding control.

2.8 | Statistical analysis

GraphPad Prism 7 was used for graphing. Data are displayed as mean ± SEM (replicates used to calculate the SEM were added in all figure legends). The Student t test combined with one-way analysis of variance was conducted for statistical comparisons, and a difference with P value < 0.05 was considered significant. Pearson's correlation coefficients (r) and linear regression were carried out to analyze correlation.

3 | RESULTS

3.1 | TREM2 and NF-κB p65 were highly expressed and positively correlated in IDD

To investigate the expressions of TREM2 and NF-κB p65 and their possible correlations in the development of IDD, mRNA and protein levels of TREM2 and NF-κB p65 in NP tissues from 20 patients with IDD and 4 healthy people were measured. Our data suggested that TREM2 and NF-κB p65 were remarkably higher in patients with IDD compared with

the healthy people ($P < 0.01$; Figure 1A,B). Meanwhile, the correlation between mRNA levels of TREM2 and NF-κB p65 was analyzed by Pearson r analysis and linear regression, and it was significantly positive ($r = 0.5794$, $P = 0.0126$; Figure 1C). Besides, we also assessed the expressions of TREM2 and NF-κB p65 in cell samples from three patients with IDD and three healthy people. Our data suggested that mRNA and protein levels of TREM2 and NF-κB p65 were significantly higher in degenerative NP cells compared with normal NP cells (Figure 1D,E), substantiating the involvement of TREM2 in the degeneration of human NP cells.

3.2 | siRNA-TREM2 prevented cell apoptosis whereas promoted proliferation of human degenerative NP cells

To verify the role of TREM2 silencing in proliferation and apoptosis of human degenerative NP cells, cells were transfected with siRNA-NC or siRNA-TREM2, and then cell apoptosis and cell proliferation were assessed by flow cytometry and cell counting kit-8, respectively. Significantly reduced mRNA and protein levels of TREM2 demonstrated a successful TREM2 knockdown within human degenerative NP cells (Figure 2C,D). As shown in Figure 2A, the apoptotic rate of human degenerative cells
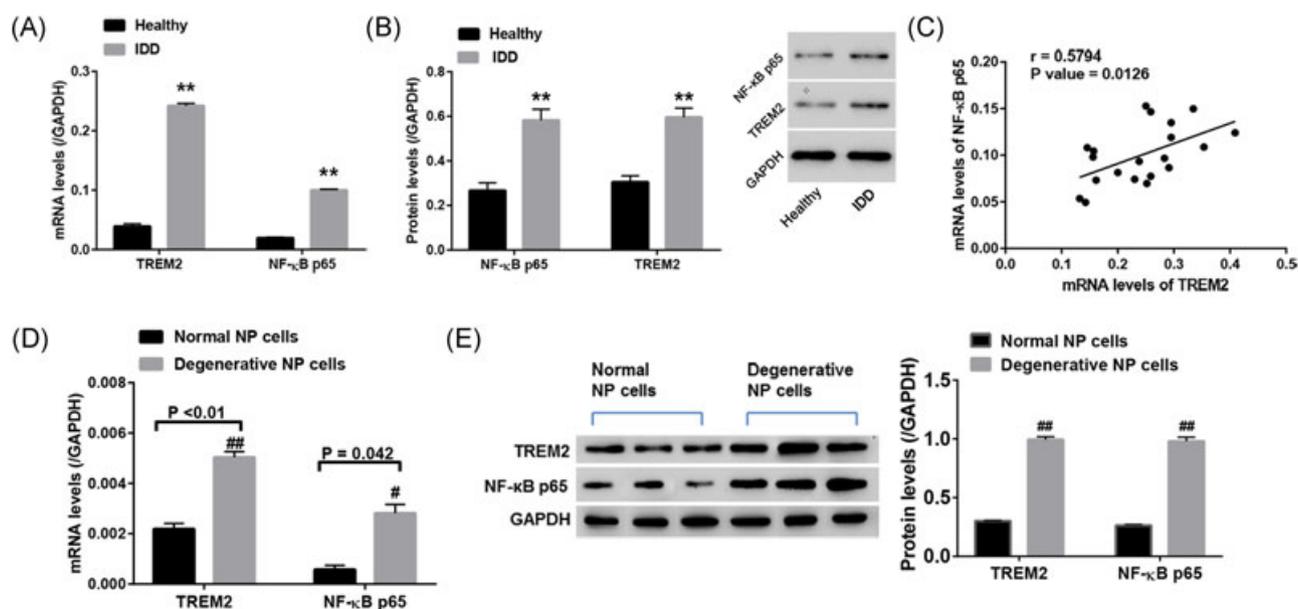

FIGURE 1 TREM2 and NF-κB p65 were upregulated in nucleus pulposus (NP) tissues of patients with intervertebral disc degeneration (IDD). A, mRNA levels of TREM2 and NF-κB p65 assessed by RT-PCR in NP samples from 4 healthy people and 20 patients with IDD. B, Protein levels of TREM2 and NF-κB p65 assessed by the Western blot analysis in NP samples from four healthy people and four patients with IDD. C, Pearson's r analysis demonstrating the positive correlation between mRNA levels of TREM2 and NF-κB p65 ($r = 0.5794$, $P = 0.0126$, n = 20). D, mRNA and E, protein levels of TREM2 and NF-κB p65 in NP cell samples from three healthy people and three patients with IDD. GAPDH was the control for the levels of TREM2 and NF-κB p65. Expressions refer to total gene and protein levels in tissue extracts. There were three parallels for each sample to perform the statistical test. **$P < 0.01$ versus the healthy group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA; NF-κB p65, nuclear factor-κB p65; RT-PCR, real-time polymerase chain reaction; TREM2, triggering receptor expressed on myeloid cells-2

was significantly increased compared with human normal cells ($P < 0.01$). The apoptotic rate in the siRNA-NC group was similar to that of degenerative NP cells without viral transfection and was significantly decreased (by approximate one-half) in the siRNA-TREM2 group compared with siRNA-NC. Meanwhile, Figure 2B shows that there was no difference in cell proliferation between degenerative NP without viral transfection and the siRNA-NC group; however, siRNA-TREM2 remarkably increased cell proliferation at 24, 48, and 72 hours with cell viability being promoted at 11.7%, 28.6%, and 35.7%, respectively.

3.3 | Effect of siRNA-TREM2 on Bax and Bcl2 expressions in human degenerative NP cells

To investigate whether TREM2 silencing influenced the expressions of Bcl2 and Bax in human degenerative NP cells, cells were transfected with siRNA-NC or siRNA-TREM2, and then protein levels of Bcl2 and Bax were determined. As shown in Figure 3D, siRNA-NC had almost no effects on

Bcl2 and Bax expressions. However, siRNA-TREM2 remarkably upregulated Bcl2 whereas downregulated Bax compared with the siRNA-NC group ($P < 0.01$).

3.4 | Effect of siRNA-TREM2 on the proinflammatory response of human degenerative NP cells

To investigate the effect of TREM2 on inflammatory response of human degenerative NP cells, cells were transfected with siRNA-NC or siRNA-TREM2, and then the concentrations of proinflammatory cytokines TNF-α, L-1β, and IL-6 in the cell culture supernatant was detected using ELISA. Figure 3A-C shows that the contents of TNF-α, IL-1β, and IL-6 were significantly enhanced in degenerative cells compared with normal NP cells ($P < 0.01$). There was almost no difference in TNF-α, IL-1β, and IL-6 concentrations between degenerative NP cells and siRNA-NC–transfected degenerative NP cells ($P = 0.6550$ in Figure 3A, 0.7829 in Figure 3B, and 0.9392 in Figure 3C). However, siRNA-TREM2 remarkably reduced the levels of TNF-α, IL-1β, and IL-6 compared with siRNA-NC (all $P < 0.01$).
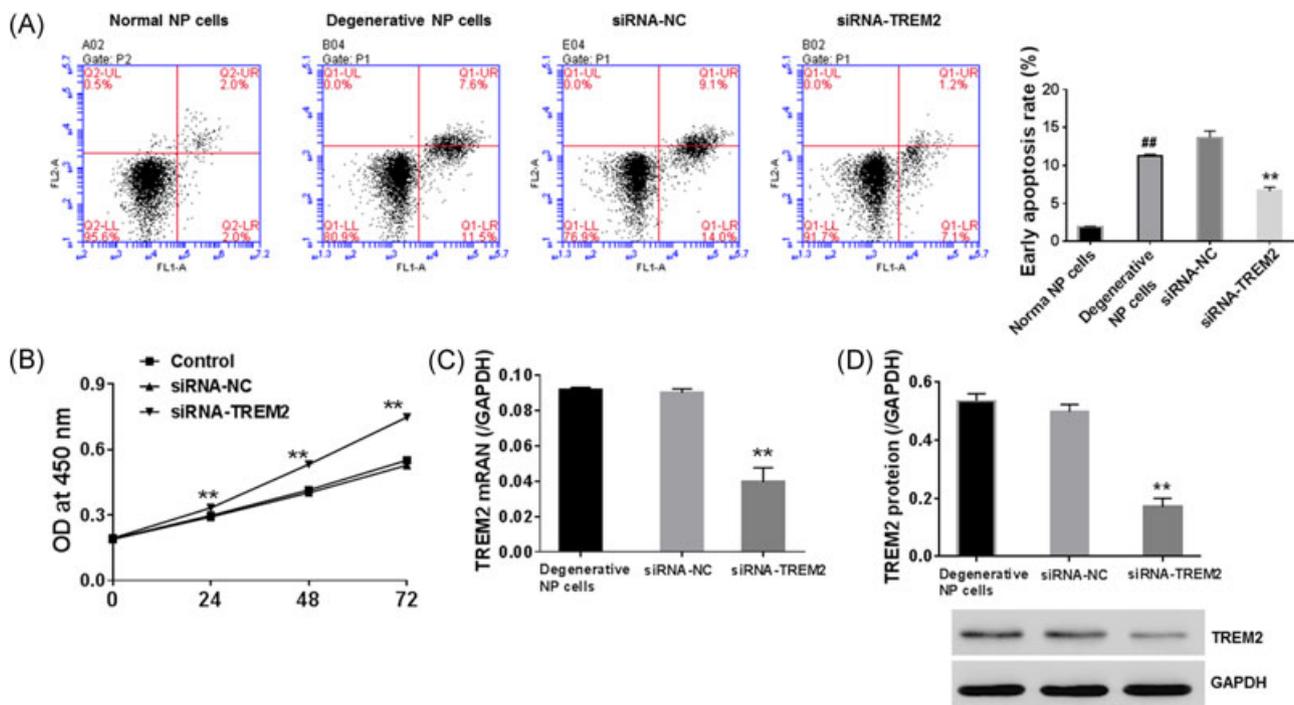

FIGURE 2 Knockdown of TREM2 prevented apoptosis whereas promoted proliferation of human degenerative NP cells. A, Human normal NP cells or degenerative NP cells transfected with or without siRNA-TREM2 were double stained with Annexin V-FITC and PI, and analyzed by BD flow cytometry. The percentage of cells undergoing early apoptosis was presented in the lower right quadrant; B, cell proliferation assessed by CCK8 at 0, 24, 48, and 72 hours. C,D, Obvious reduced TREM2 mRNA and protein, demonstrating a successful construction of TREM2 silencing within human degenerative NP cells. There were three parallels for each experiment to perform the statistical test. ##$P < 0.01$ versus human normal NP cells; **$P < 0.01$ versus siRNA-NC. Annexin V-FITC, fluorescein isothiocyanate–labeled recombinant annexinV; CCK8, cell counting kit-8; mRNA, messenger RNA; NC, nitrocellulose; NP, nucleus pulposus; PI, propidium iodide; siRNA, small interfering RNA; TREM2, triggering receptor expressed on myeloid cells-2
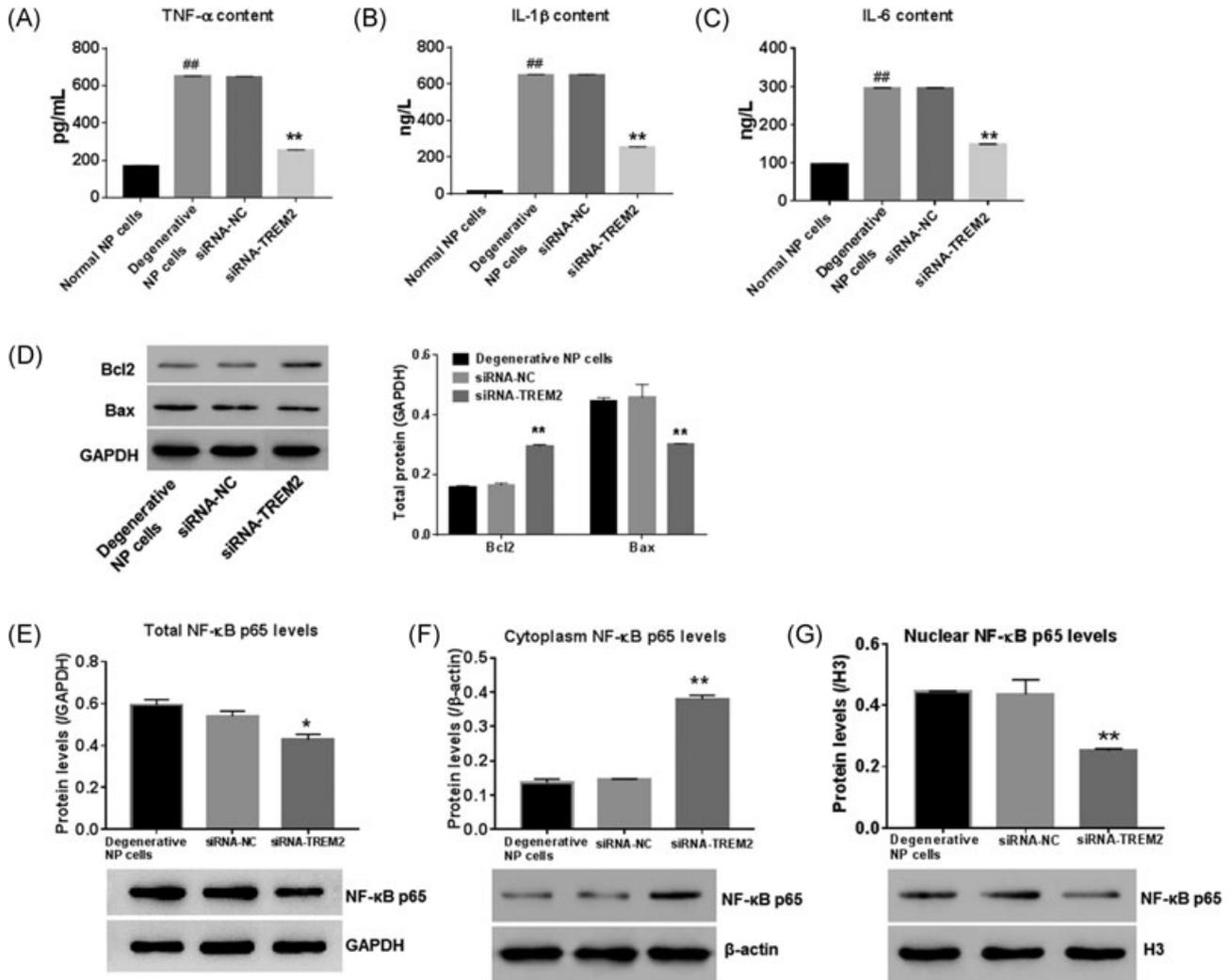
FIGURE 3 Roles of TREM2 silencing in the secretion of (A) TNF-α, (B) IL-1β, and (C) IL-6 in cultural supernatants, and protein expressions of (D) Bcl2 and Bax, (E) total NF-κB p65 levels in whole cell lysates, (F) cytoplasm NF-κB p65 as well as (G) nuclear NF-κB p65 in human degenerative NP cells. GAPDH served as the control for the levels of Bcl2, Bax, and total NF-κB p65; H3 served as the loading control for nuclear NF-κB p65, and β-action served as the loading control for cytosolic NF-κB p65. There were three parallels for each experiment. $*P < 0.05$; $**P < 0.01$ versus siRNA-NC. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; NC, nitrocellulose; NF, nuclear factor; NP, nucleus pulposus; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; TREM2, triggering receptor expressed on myeloid cells-2

3.5 | Role of siRNA-TREM2 in NF-κB p65 expressions in human degenerative NP cells

To investigate the association between TREM2 and NF-κB p65, protein levels of total NF-κB p65, plasma NF-κB p65 and nuclear levels of NF-κB p65 were measured in human degenerative NP cells transfected with siRAN-TREM2. Figure 3E-G shows that siRNA-NC had no distinct effect on protein levels of total NF-κB p65, cytoplasm NF-κB p65, and nucleus NF-κB p65. However, siRNA-TREM2 significantly decreased total NF-κB p65 and meanwhile increased cytoplasm NF-κB p65 whereas decreased nuclear NF-κB p65 (all

$P < 0.01$) compared with the siRNA-NC group, demonstrating that siRNA-TREM2 downregulated total NF-κB p65 and inhibited nuclear translocation of NF-κB p65.

3.6 | TREM2 overexpression promoted apoptosis and inhibited proliferation of human normal NP cells

To study whether upregulated TREM2 regulated cell apoptosis and proliferation of human normal NP cells, cells were transfected with an empty vector or vector encoding TREM2 (TREM2 overexpression group), and

then cell apoptosis and proliferation were assessed. Significantly enhanced mRNA and protein levels of TREM2 demonstrated a successful establishment of lentivirus-mediated TREM2 overexpression within human normal NP cells (Figure 4C,D). As shown in Figure 4A, the apoptotic rate of human normal NP cells with TREM2 overexpression was significantly higher than the normal NP cells transfected with the empty vector. Figure 4B shows that TREM2 overexpression decreased cell proliferation at 24, 48, and 72 hours with cell viability being inhibited at 15.1%, 26.9%, and 33.3% compared with the empty vector. Meanwhile, the empty vector had almost no effect on both cell apoptosis ($P = 0.6838$ in Figure 4A) and proliferation (Figure 4B).

3.7 | Effect of TREM2 overexpression on Bax and Bcl2 expressions in human normal NP cells

To study the proapoptotic effect of TREM2 overexpression at a molecular level, protein levels of Bcl2 and Bax within human normal NP cells were also measured. As shown in Figure 5D, the empty vector exerted almost no effect on Bcl2 and Bax expressions. However, lentivirus-mediated TREM2 overexpression evidently decreased Bcl2 expression whereas increased Bax expression, compared with the empty vector group ($P < 0.01$).

3.8 | Effect of TREM2 overexpression on proinflammatory response of human normal NP cells

To study the effect of TREM2 overexpression on inflammatory response of human normal NP cells, the production of TNF-α, L-1β, and IL-6 in TREM2 overexpression–transfected human normal NP cells was assessed using ELSK. Figure 5A-C shows that there was no difference between normal NP cells and empty vector–transfected cells on the levels of TNF-α, IL-1β, and IL-6 (P value was 0.7676, 0.1399, and 0.9359 in Figure 5A-C, respectively). However, lentivirus-mediated TREM2 overexpression significantly increased the production of TNF-α, IL-1β, and IL-6 ($P < 0.01$), indicating that the upregulation of TREM2 remarkably promoted the productions of proinflammatory cytokines of human normal NP cells.
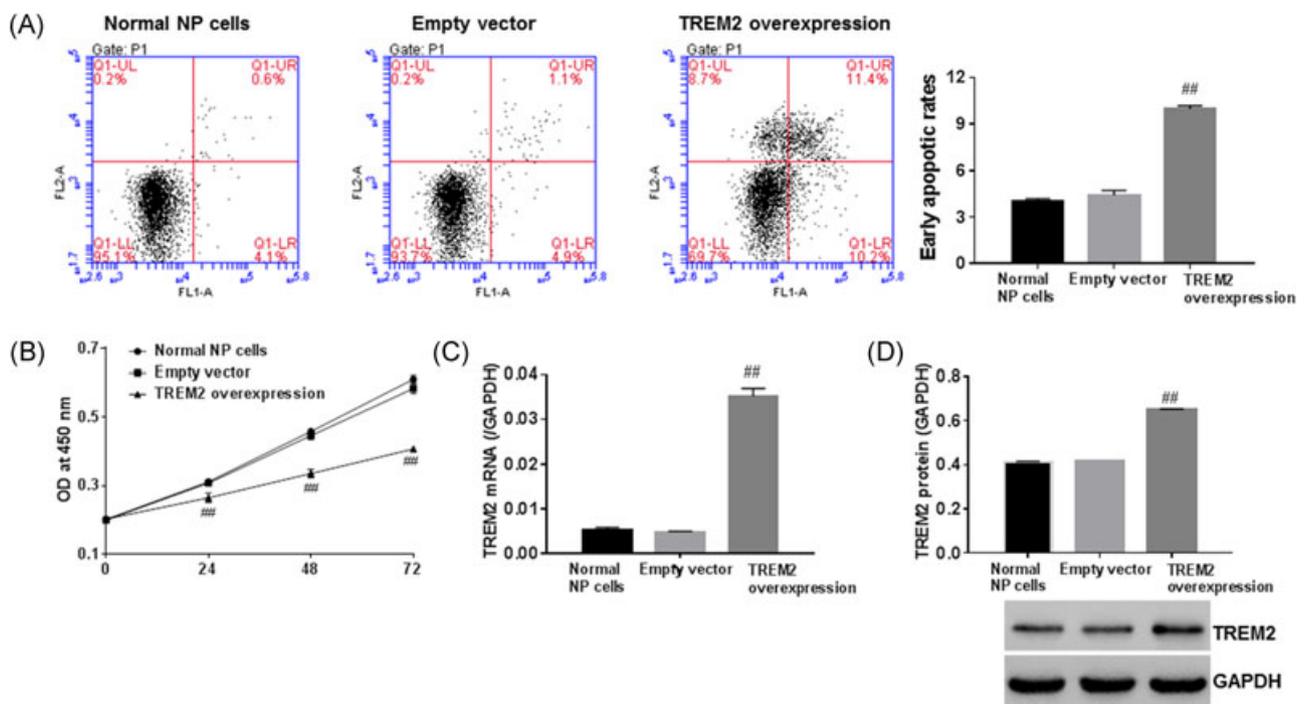

FIGURE 4 TREM2 overexpression promoted apoptosis whereas inhibited proliferation of human normal NP cells. Cells were transfected with empty vector or vector encoding TREM2 expression. After transduction, A, cell apoptosis was assessed by flow cytometry at 48 hours. B, Cell proliferation was determined by CCK8 at 0, 24, 48, and 72 hours. C,D, Obvious increased TREM2 mRNA and protein expressions, demonstrating a successful construction of lentivirus-mediated TREM2 overexpression within human normal NP cells. There were three parallels for each experiment. **$P < 0.01$ versus the empty vector. CCK8, cell counting kit-8; mRNA, messenger RNA; NP, nucleus pulposus; TREM2, triggering receptor expressed on myeloid cells-2
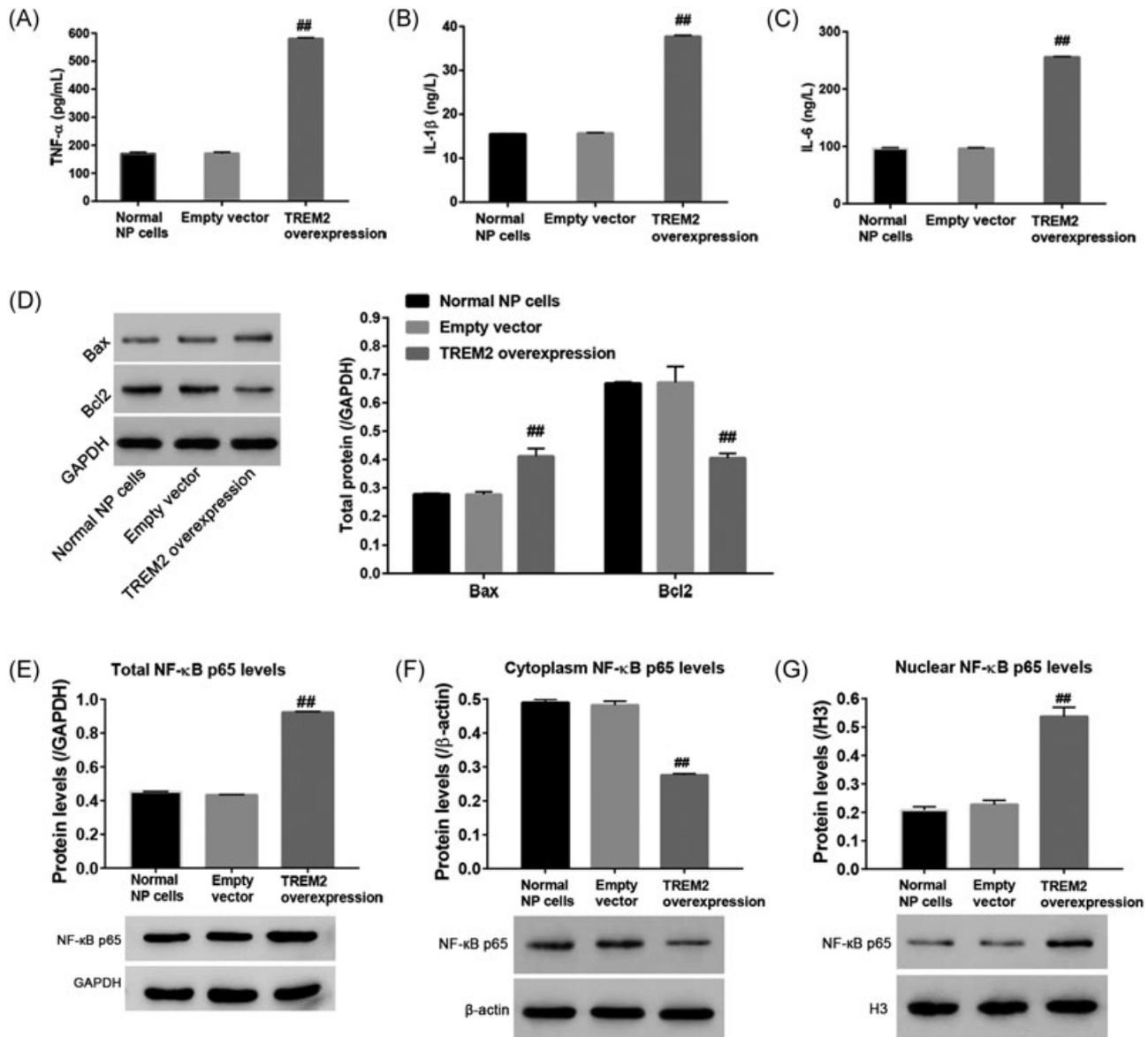
FIGURE 5 Roles of TREM2 overexpression in the secretion of (A) TNF-α, (B) IL-1β, (C) IL-6, and protein expressions of (D) Bcl2, Bax, (E) total NF-κB p65 in whole cell lysates, (F) cytoplasm NF-κB p65 as well as (G) nuclear NF-κB p65 in human normal NP cells. GAPDH served as the control for the levels of Bcl2, Bax, and total NF-κB p65; H3 served as the control for nuclear NF-κB p65 level, and β-action served as the control for cytosolic NF-κB p65. There were three parallels for each experiment to perform the statistical test. $^{##}P < 0.01$ versus empty vector. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; NF, nuclear factor; TNF-α, tumor necrosis factor-α; TREM2, triggering receptor expressed on myeloid cells-2

3.9 | Role of TREM2 overexpression in NF-κB p65 expressions in human normal NP cells

To investigate the association between TREM2 and NF-κB p65, protein levels of total NF-κB p65, plasma NF-κB p65, and nuclear levels of NF-κB p65 were measured in human normal NP cells transfected with TREM2 overexpression. Figure 5E-G shows that lentivirus-mediated TREM2 overexpression obviously enhanced the expression of total NF-κB p65, and meanwhile decreased plasma NF-κB p65 expression, whereas increased the nuclear NF-κB p65

expression ($P < 0.01$) compared with the empty vector group, suggesting that TREM2 overexpression upregulated the total NF-κB p65 expression and promoted NF-κB p65 nuclear translocation.

3.10 | NF-κB p65 inhibitor PDTC alleviated TREM2 overexpression–induced degeneration of human NP cells

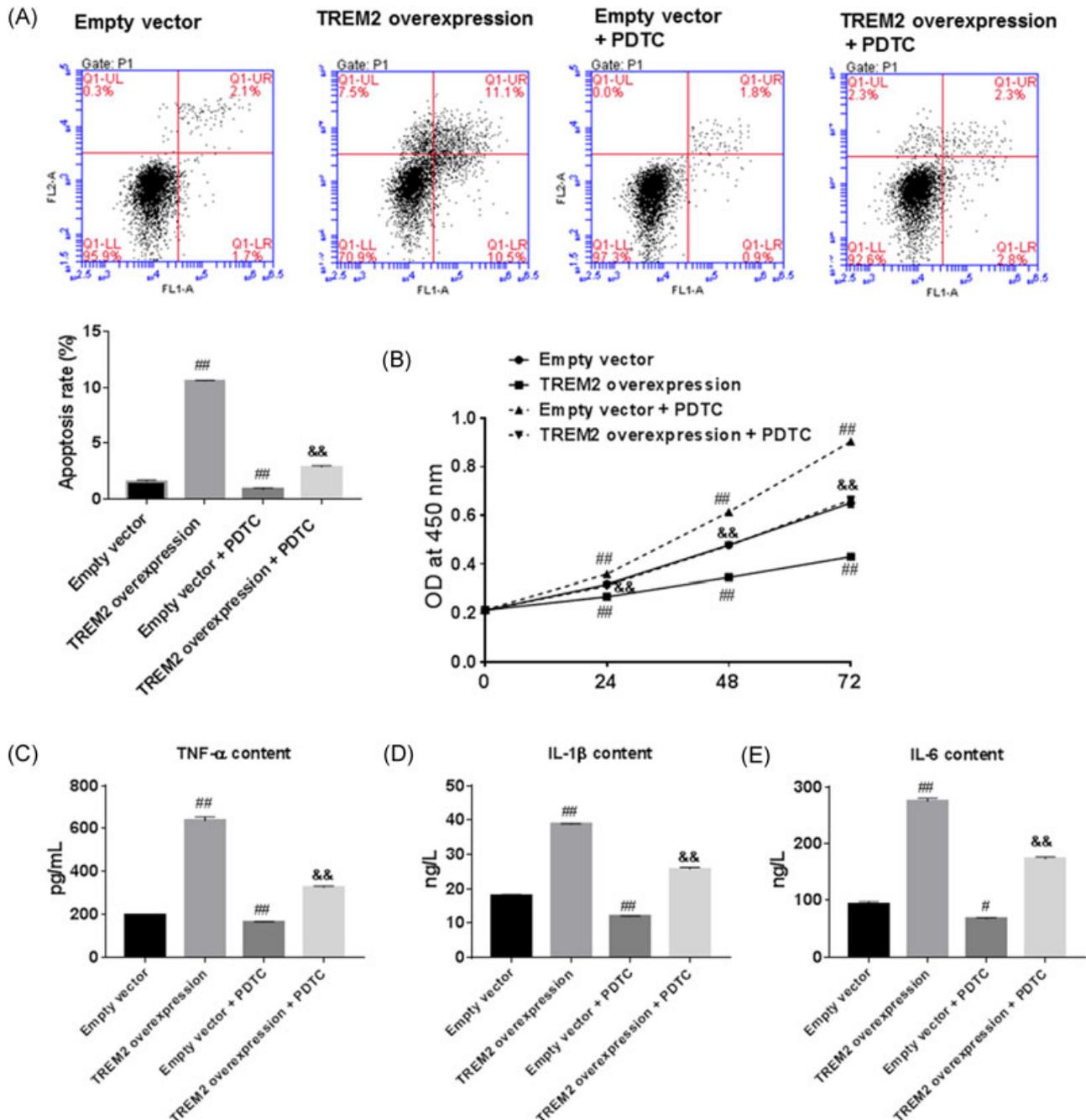To study whether TREM2 exerted its effect via NF-kB p65, human normal or TREM2 overexpression–transfected cells

FIGURE 6 Roles of the NF-kB p65 inhibitor PDTC in degeneration of normal NP cells induced by TREM2 overexpression. Human normal cells transfected with empty or TREM2 overexpression vector were treated with PDTC, and then (A) cell apoptosis, (B) proliferation, and (C) concentration of TNF-α, IL-1β, and IL-6 were assessed. There were three parallels for each experiment. **$P < 0.01$ versus empty vector; &&$P < 0.01$ versus TREM2 overexpression. IL, interleukin; NF, nuclear factor; NP, nucleus pulposus; PDTC, pyrrodlidine dthiocarbamate; TNF-α, tumor necrosis factor-α; TREM2, triggering receptor expressed on myeloid cells-2

were treated with pyrrodlidine dthiocarbamate (PDTC, an NF-κB p65 inhibitor), and then cell apoptosis, proliferation, and production of TNF-α, IL-1β, and IL-6 were assessed, respectively. Figure 6 shows that PDTC significantly reduced cell apoptosis and the production of TNF-α, IL-1β, and IL-6 while enhancing proliferation in the empty vector group, confirming that inhibiting NF-κB p65 was a therapeutic target for the degeneration of human normal NP cells. On

the contrary, TREM2 overexpression exerted the opposite effect on the events mentioned above, promoting degeneration of human NP cells; however, with additional PDTC treatment, TREM2 overexpression induced the changes in cell apoptosis and cell proliferation, and the production of TNF-α, IL-1β, and IL-6 was significantly reversed to almost normal level, suggesting the involvement of NF-kB p65 in degenerative regulation of TREM2 in human NP cells.
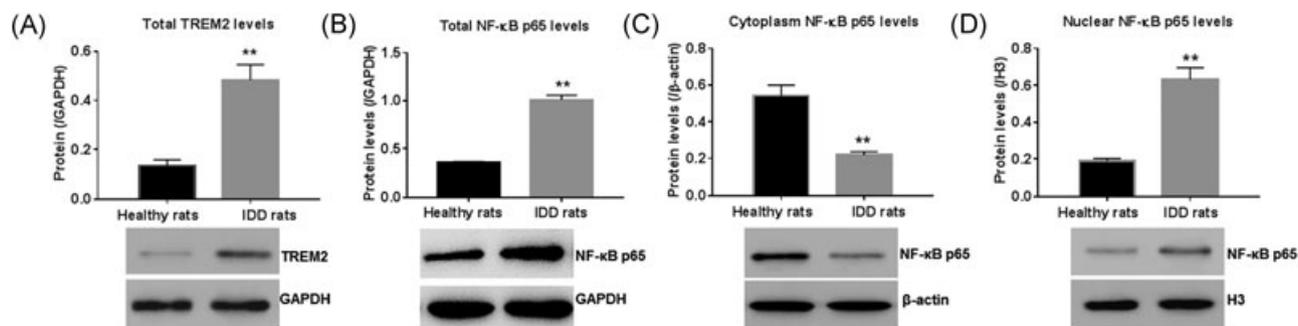
FIGURE 7 Protein expressions of TREM2, total NF-κB p65, cytoplasm NF-κB p65, and nuclear NF-κB p65 in NP tissue from four healthy rats and and four IDD rats. GAPDH served as the control for total NF-κB p65, H3 served as the loading control for nuclear NF-κB p65, and β-action served as the loading control for cytosolic NF-κB p65. There were three parallels for each sample to perform the statistical test. **$P < 0.01$ versus healthy rats. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IDD, intervertebral disc degeneration; NF, nuclear factor; NP, nucleus pulposus; TREM2, triggering receptor expressed on myeloid cells-2

3.11 | TREM2, total NF-κB p65, plasma NF-κB p65, and nuclear NF-κB p65 assessment in NP tissue of rat with IDD

We further assess protein expressions of TREM2, total NF-κB p65, plasma NF-κB p65, and nuclear NF-κB p65 in NP tissue from four healthy and four IDD rats. Figure 7 shows that the levels of TREM2 and total NF-κB p65 were significantly upregulated in IDD groups compared with healthy rats (Figure 7A,B) Meanwhile, nuclear NF-κB p65 was remarkably enhanced, whereas plasma NF-κB p65 was obviously downregulated in NP tissue obtained from IDD rats (Figure 7C,D), suggesting the involvement and the potential association of TREM2 with NF-κB p65 in IDD rats.

4 | DISCUSSION

Abnormal cell apoptosis, viability, and inflammatory response are inextricable in the process of IDD. TREM2 has shown important roles in regulating inflammation and cell apoptosis.[11,15] However, the roles of TREM2 in IDD have not been revealed currently. Total protein and gene expressions of NF-κB p65 were usually upregulated in the process of IDD, and restraining the NF-κB pathway activation is considered as an effective method to prevent this disease.[16,17] NF-κB was involved in the neuroinflammatory regulation of TREM2 in age-related macular degeneration[13] and Alzheimer's disease.[12] Besides, transcription regulation of p65 and its correlation to monocyte chemoattractant protein, and regulated upon activation, normal T cell expressed and secreted was informative in the severity of Type 2 diabetic nephropathy.[18] However, whether transcriptional regulation of NF-κB p65 was correlated with TREM2 in degeneration of human NP cells

remained extremely poor. Our data indicated that expressions of TREM2 and total NF-κB p65 were dramatically upregulated in NP tissues and cell samples from patients with IDD (Figure 1A, B, D, and E), as well as in NP tissues samples from rats with IDD (Figure 7A,B), Besides, we also found that TREM2 was significantly positive correlated with NF-κB p65 expression ($r = 0.5794$, $P = 0.0126$; Figure 1C) in human degenerative NP tissues, suggesting the involvement of TREM2 and its association with NF-κB p65 in IDD disease. We isolated human normal and degenerative NP cells from three healthy individuals and three patients with IDD to study the roles of TREM2 and its association with NF-κB p65, further investigating whether TREM2 would serve as a cellular therapy for preventing human IDD.

Reduction of NP cells is involved in the pathogenesis of IDD, and their enhancement may be a vital mechanism to improve the function of NP tissue. In our current study, we confirmed the apoptosis of degenerative cells compared with normal NP cells (Figure 2A). TREM2 regulates neuronal cell proliferation and apoptosis; however, the roles of TREM2 in proliferation and apoptosis of NP cells remain extremely poor. Our data suggested that siRNA-TREM2 inhibited cell apoptosis while increased the proliferation of human degenerative NP cells (Figure 2A,B), demonstrating that the knockdown of TREM2 ameliorated the degeneration of NP cells by accumulating NP cell number. Bax (apoptosis-promoting protein) and Bcl2 (apoptosis-inhibiting protein) are two classical apoptotic regulatory proteins and are also two known targets of NF-κB; therefore, we measured Bax and Bcl2 protein levels to assess the roles of TREM2 in cell apoptotic regulation at molecular levels. Our data indicated that upregulating the Bcl2 while downregulating Bax was involved in this process (Figure 3D). Interestingly, antiproliferative and proapoptotic effects of siRAN-TREM2 have been addressed in human liver cancer cells, and

downregulating Bcl2 while upregulating Bax was involved,[19] suggesting that TREM silencing may cause different effects on cell proliferation and apoptosis in some cancer cells and NP cells. Even though Figure 2A shows that the antiapoptotic effect of siRNA-TREM2 was rather moderate, and the apoptotic rate of human degenerative cells transfected with siRNA-TREM2 appeared to be still higher than that in healthy cells, suggesting that maybe there were other TREM2-independent proapoptotic pathways in this process. Besides, we also thought that the proproliferative results of siRAN-TREM2 may be partly dependent on its antiapoptotic effect on human degenerative NP cells. On the contrary, TREM2 overexpression showed an opposite effect, accelerating the degeneration of normal NP cells by reducing NP cell numbers (Figure 4A,B). TREM2 overexpression decreased Bcl2 expression whereas increased Bax expression in human normal NP cells, further substantiating the direct involvement of TREM2 in cell apoptotic regulation at molecular levels (Figure 5D).

Excessive release of proinflammatory cytokines TNF-α, IL-1β, and IL-6 by disc cells accelerates ECM degradation and promotes changes in cell phenotype,[3] resulting in NP tissues injury. A properly balanced inflammatory response contributes to the repair or regeneration of degenerative NP tissue.[6] In our current study, we confirmed severe inflammatory response of degenerative cells, as evidenced by significantly increased expression of TNF-α, IL-1β, and IL-6 compared with normal NP cells (Figure 3A-C). Downregulating TREM2 is most likely to ameliorate neuroinflammation and attenuate neurodegeneration.[20] In our study, we looked at the roles of TREM2 in the inflammatory response of human NP cells. Our data showed that siRNA-TREM2 prevented the production of TNF-α, IL-1β, and IL-6 in human degenerative NP cells, demonstrating that the knockdown of TREM2 may serve as a minimal-inflammatory therapy to alleviate cell degeneration. On the contrary, lentivirus-mediated TREM2 overexpression within human normal NP cells enhanced the production of TNF-α, IL-1β, and IL-6 in cell culture supernatants (Figure 5A-C), and TREM2 may be considered as a proinflammatory factor to accelerate cell degeneration.

siRNA-TREM2 decreased the total expression of NF-κB p65 in human degenerative NP cells ($P < 0.01$; Figure 3E), whereas TREM2 overexpression increased the total expression of NF-κB p65 in human degenerative NP cells ($P < 0.01$; Figure 5E). To study whether NF-κB p65 was the mechanism, whereby TREM2 exerted its effect on the degeneration of human NP cells, normal NP cells overexpressing TREM2 were treated with the NF-κB p65 inhibitor PDTC. We found that PDTC prevented the degeneration of empty vector–transfected normal NP cells

through significantly reducing cell apoptosis, promoting cell proliferation, and inhibiting the production of TNF-α, IL-1β, and IL-6 (Figure 6), substantiating that downregulating NF-κB p65 is effective for IDD intervention. However, with additional PDTC treatment, TREM2 overexpression-induced degeneration of human normal NP cells was significantly reversed, indicating the involvement of NF-kB p65 in this process. Even so, the levels of cell apoptosis and cell proliferation and the section of TNF-α, IL-1β, and IL-6 observed in normal NP cells with TREM2 overexpressing were not the reverse of those observed in healthy cells, suggesting maybe other TREM2-depended pathways in this process.

When NF-κB p65 was inactive, it was found in the cytoplasm of normal NP cell. When NF-κB p65 was activated, it would transfer to the nucleus to regulate the degenerative process of NP cells.[9] In the current study, we further assessed whether nucleus translocation of NF-κB p65 was involved in the degenerative regulation of siRNA-TREM2 in human NP cells. Our data suggested that siRNA-TREM2 increased cytoplasm NF-κB p65 expression whereas decreased nucleus NF-κB p65 expression (Figure 3F,G), suggesting that inhibiting NF-κB p65 nucleus translocation was involved in antidegenerative functions of siRNA-TREM2 in human degenerative NP cells. On the contrary, TREM2 overexpression decreased cytoplasm NF-κB p65 whereas increased nucleus NF-κB p65 (Figure 5F,G), suggesting that promoting NF-κB p65 nucleus translocation was involved in prodegenerative functions of TREM2 overexpression in human normal NP cells. Moreover, the involvement and the potential association of TREM2 with NF-κB p65 were also confirmed in a rat IDD model (Figure 7).

In conclusion, TREM2 and NF-κB p65 were upregulated and positively correlated in human degenerative NP tissue. Downregulating TREM2 is a potential method to prevent the degeneration of NP cells via suppressing cell apoptosis, promoting cell proliferation, and preventing inflammation in human degenerative NP cells. However, abnormal upregulation of TREM2 within normal NP cells contributed to the degeneration of human normal NP cell. TREM2 completed its function through regulating total NF-κB p65 expressions and NF-κB p65 nucleus translocating. Thus, our data underscored the possibility of TREM2 as a therapeutic target for the treatment of human IDD.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interests.

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