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Exploring the role of TNF/TNFR superfamily and NF-κB pathway in regulating B7/CD28 expression in rheumatoid arthritis: An *in-vitro* study

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Abstract: **Background**: The TNF/TNFR superfamily and B7/CD28 costimulatory molecules are critical regulators of immune responses; however, their interplay in rheumatoid arthritis (RA) remains unclear. **Objectives**: To investigate whether the TNF/TNFR superfamily regulates B7/CD28 expression via the NF-κB pathway in RA and to evaluate its effects on synovial cell inflammation and apoptosis. **Methods**: Peripheral blood mononuclear cells (PBMCs) and fibroblast-like synoviocytes (FLS) were obtained from 40 patients with active rheumatoid arthritis (RA) (13 males, 27 females; mean age 42.3 ± 8.1 years, range 26-55; DAS28-ESR > 3.2) and 40 age- and sex-matched healthy controls (15 males, 25 females; mean age 41.8 ± 7.9 years, range 26-54) with no history of arthritis or autoimmune diseases; all participants provided written informed consent. A co-culture system of PBMCs and FLS was established and divided into three groups: control (healthy PBMCs + FLS), model (RA PBMCs + FLS) and inhibition (RA PBMCs + FLS treated with PDTC, an NF-κB inhibitor). After 48 hours, cell viability, apoptosis, inflammatory cytokines (TNF-α, IL-1β), COX-2, Bax, Bcl-2, TNF-R1/TNF-R2 mRNA and the expression of CD28 and CD80 on $CD4^+$ and $CD8^+$ T cells were assessed. **Results**: Compared to controls, the model group exhibited increased FLS viability, reduced apoptosis, elevated levels of TNF-α, IL-1β, COX-2, Bcl-2 and TNF-R1/R2 mRNA, along with decreased Bax expression and lower CD28/CD80 expression on $CD4^+$ T cells (all $P < 0.05$). NF-κB inhibition with PDTC significantly reduced FLS viability and increased apoptosis, downregulated IL-1β and COX-2 and restored CD28/CD80 expression on $CD4^+$ T cells ($P < 0.05$), but did not alter TNF-α or TNFR mRNA levels. **Conclusion**: Inhibition of the NF-κB pathway attenuates RA-FLS inflammation and restores B7/CD28 expression on T cells; however, these effects were not directly mediated by changes in TNF/TNFR expression.

Keywords: Apoptosis; Inflammatory response; NF-κB signaling pathway; Rheumatoid arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic autoimmune disease whose pathological process involves the abnormal activation of various immune cells. Among these, T cell dysfunction is considered one of the core drivers of disease onset and progression (Ding *et al*., 2023; Yan *et al*., 2024). The complete activation of T cells requires two signals: the first signal is provided by the T cell receptor recognizing the antigen peptide-major histocompatibility complex, while the second signal, known as the co-stimulatory signal, is provided by the interaction between co-stimulatory molecule receptors on the T cell surface and their corresponding ligands on antigen-presenting cells. Among the numerous co-stimulatory molecules, the B7/CD28 family is one of the most extensively studied and critically important members. CD28, as the primary co-stimulatory receptor on T cells, binds to its ligands B7-1 (CD80) and B7-2 (CD86), providing essential signals for T cell proliferation, differentiation and survival. This interaction plays a crucial role in the initiation and effector phases of the immune response (Stai *et al*., 2024). Studies have shown that abnormalities in the co-stimulatory signals mediated by B7/CD28 are closely associated with the pathogenesis of various autoimmune diseases (Zhao and Yu., 2024). However, the upstream signaling mechanisms regulating the abnormal expression of B7/CD28 in rheumatoid arthritis remain incompletely understood.

The tumor necrosis factor (TNF) and its receptor superfamily constitute another critical component of the immune regulatory network. Members of this family, such as TNF-α and its receptors TNFR1 and TNFR2, are widely involved in various biological processes including cell proliferation, differentiation, apoptosis and inflammatory responses. TNF-α, as a pleiotropic cytokine, plays a complex and even paradoxical role in the pathogenesis of rheumatoid arthritis. On one hand, TNF-α is a well-recognized pro-inflammatory factor that can activate signaling pathways such as NF-κB, promoting the production of downstream inflammatory factors like IL-1β and COX-2, thereby driving synovial inflammation and joint destruction. This forms the basis for TNF-α inhibitors as a cornerstone therapy for RA (Zhou *et al*., 2024). On the

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other hand, TNF/TNFR signaling has also been shown to participate in regulating the homeostasis and apoptosis of immune cells and its signaling dysregulation may be associated with the abnormal survival of autoreactive T cells (Zhang *et al*., 2024). Recent studies have found that the regulation of the NF-κB pathway by TNF/TNFR signaling is not limited to activation. Under specific cell types or pathological backgrounds, TNF signaling can also recruit negative regulatory molecules or induce inhibitory signaling pathways, thereby achieving inhibition of NF-κB activity (Veerasubramanian *et al*., 2024; Boyce *et al*., 2023). This bidirectional regulatory characteristic suggests that the role of TNF/TNFR in RA may be far more complex than its simple pro-inflammatory effect.

Given the central role of T cell abnormalities in RA and the extensive involvement of B7/CD28 co-stimulatory signals and the TNF/TNFR family in immune regulation, exploring the interaction between these two major signaling systems is particularly important. Currently, whether and how the TNF/TNFR family influences the expression of B7/CD28, thereby participating in the immunopathological processes of RA, especially its relationship with the NF-κB pathway, remains inadequately explored. Previous high-throughput sequencing analysis suggested that there were abnormalities in multiple signaling pathways including NF-κB in RA (Zhang *et al*., 2022), providing clues for this study to explore the association among the three.

Based on the above background, this study proposes the following scientific hypothesis: In the pathological microenvironment of RA, the TNF/TNFR superfamily may regulate the expression of downstream B7/CD28 costimulatory molecules by modulating the NF-κB signaling pathway, thereby influencing the activation state of T cells and the apoptotic process of synovial cells. To verify this hypothesis, this study conducted *in-vitro* cell co-culture experiments to observe the changes in TNF/ TNFR-related molecules, B7/CD28 and their expressions on T cell subsets, as well as downstream inflammatory and apoptosis-related indicators in the RA pathological environment and after blocking the NF-κB signal. This study aims to preliminarily clarify the possible regulatory network of the TNF/TNFR superfamily, NF-κB pathway and B7/CD28 co-stimulatory signal in RA, providing a new perspective for understanding the complex immune mechanism of this disease.

MATERIALS AND METHODS

Selection of research subjects

This study strictly adhered to the principles of the Declaration of Helsinki (Dal-Re., 2023) and was approved by the Ethics Committee of Sichuan Orthopedic Hospital (Approval No.: KY2025-065-01). All patients with rheumatoid arthritis and healthy volunteers participating in this study were informed of the purpose and procedures of the research and provided written informed consent.

A total of 40 patients diagnosed with rheumatoid arthritis were consecutively recruited. The cohort included 13 males and 27 females, aged 26-55 years. Inclusion criteria were: 1) Fulfillment of the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria for RA (Aletaha *et al*., 2010); 2) Age between 18 and 70 years; 3) Disease Activity Score (DAS28-ESR) (Prevoo *et al*., 1995) > 3.2, indicating at least moderate disease activity. Exclusion criteria were: 1) Concurrent diagnosis of other autoimmune diseases (e.g., systemic lupus erythematosus, Sjogren's syndrome); 2) Treatment with biologic agents (e.g., TNF-α inhibitors) or JAK inhibitors within the preceding 3 months; 3) History of severe infection or use of glucocorticoids (at doses > 10 mg/d prednisone or equivalent) within the preceding month; 4) Concurrent malignancy, severe hepatic or renal dysfunction.

Simultaneously, 40 healthy volunteers who underwent physical examinations at the Medical Examination Center of Sichuan Orthopedic Hospital during the same period were recruited as the control group. This group comprised 15 males and 25 females, aged 26-54 years. Inclusion criteria for the healthy control group were: 1) Age and sex matched to the RA group; 2) No history of any arthritis or autoimmune disease; 3) No history of infection or medication use within the preceding 3 months. All healthy subjects had no history of arthritis.

Isolation and culture of synovial fibroblasts

Synovial tissue source: Synovial tissues from RA patients were obtained from joints undergoing knee replacement surgery or synovectomy. Control synovial tissues were obtained from normal joint areas of individuals undergoing amputation due to trauma or from arthroscopy. The obtained tissues were immediately placed in ice-cold DMEM medium containing 2% penicillin-streptomycin (Gibco, USA) and transported to the laboratory (Zhao *et al.,* 2016).

Isolation and culture: In a laminar flow hood, the tissues were rinsed three times with PBS (phosphate-buffered saline, pH 7.4) containing 5% penicillin-streptomycin and adipose and fibrous components were removed. The tissues were minced into approximately 1 mm^3 fragments and placed in DMEM medium containing 1 mg/mL Collagenase Type II (Sigma-Aldrich, USA) and 0.15 mg/mL DNase I (Roche, Switzerland). Digestion was performed for 2-3 hours in a 37°C, 5% CO_2 incubator with agitation every 30 minutes. Following digestion, the cell suspension was filtered through a 70 μm cell strainer (BD Falcon, USA). The filtrate was collected and centrifuged at 1500 rpm for 10 minutes and the supernatant was discarded. The cell pellet was resuspended in complete DMEM medium containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin, seeded into 25 cm^2 culture flasks and cultured in a 37°C, 5% CO_2 incubator. The medium was replaced after 24 hours to remove non-

adherent cells. Subsequently, the medium was changed every 3 days. When the cells reached 80-90% confluence, they were subcultured using 0.25% trypsin-EDTA (Gibco, USA) for digestion.

Cell purity identification: To ensure cell purity, fibroblast-like synoviocytes (FLS) from passages 3 to 6 were used for all subsequent experiments in this study. Passage 3 FLS were harvested and surface markers were identified using flow cytometry. After digestion, the cells were stained with FITC-conjugated anti-human CD90 antibody, PE-conjugated anti-human CD14 antibody and APC-conjugated anti-human CD45 antibody (all antibodies purchased from BD Pharmingen, USA). The results showed that the proportion of $CD90^+$, $CD14^-$, $CD45^-$ cells was > 95%, confirming that the cultured cells were highly purified FLS.

Isolation of peripheral blood lymphocytes

Fasting peripheral venous blood samples (10 mL each) were collected from RA patients and healthy controls into EDTA anticoagulant tubes. After diluting the whole blood with an equal volume of PBS, the mixture was carefully layered over Ficoll-Paque PLUS (GE Healthcare, Sweden) separation medium (blood to separation medium volume ratio of 2:1) and centrifuged at 2000 rpm for 20 minutes. The buffy coat layer (PBMC layer) was carefully aspirated and washed twice with PBS (1500 rpm, 10 minutes) to obtain PBMCs. The cells were resuspended in RPMI 1640 medium (Gibco, USA) containing 10% FBS and the cell density was adjusted to 2×10^6 cells/mL for further use.

Lymphocyte-synovial fibroblast co-culture system

FLS (passages 3-6) in the logarithmic growth phase were digested with trypsin and seeded into 24-well plates at a density of 2×10^4 cells per well. They were routinely cultured overnight to allow for adherence. The next day, the supernatant was discarded and the co-culture system was established with the following groups: Control Group: RPMI 1640 complete medium (containing 10% FBS) with PBMCs from healthy controls was added. Model Group: RPMI 1640 complete medium with PBMCs from RA patients was added. Inhibition Group: RPMI 1640 medium with PBMCs from RA patients was added, along with the NF-κB signaling pathway inhibitor PDTC (pyrrolidine dithiocarbamate, Sigma-Aldrich, USA) at a final concentration of 100 μM. Blockade group: Based on the inhibition group, the CD28 costimulatory signal blocker CTLA-4 Ig (Abatacept, BD Pharmingen, USA) was added at a final concentration of 100 mg/mL to further investigate the role of B7/CD28 signaling in the downstream effects regulated by NF-κB. The concentration of PDTC was selected based on preliminary experimental results and relevant literature (Patil *et al*., 2021), confirming its effective inhibition of NF-κB activity with minimal impact on cell viability under the experimental conditions.

In all the above groups, the co-culture ratio of PBMCs to FLS was 10:1 (i.e., 2×10^5 PBMCs were added per well). The co-culture system was established through direct contact, with a total volume of 500 μL per well. The cells were co-cultured for 48 hours in a 37 °C, 5% CO_2 incubator. After co-culture, the non-adherent lymphocytes and adherent FLS were collected separately for subsequent analyses.

Cell viability assay (MTT Method)

Referring to previous literature (Ghasemi *et al*., 2023), FLS were seeded into 96-well plates at a density of 5×10^3 cells per well. After overnight adherence, corresponding PBMCs and treatments were added according to the above-mentioned groups for co-culture. At 24 h, 48 h and 72 h of culture, 20 μL of MTT solution (5 mg/mL, Sigma-Aldrich) was added to each well and incubation was continued for 4 hours. The supernatant was carefully aspirated and 150 μL of DMSO was added to each well. The plates were shaken for 10 minutes to fully dissolve the formazan crystals. The absorbance value of each well was measured at a wavelength of 490 nm using a microplate reader. Five replicate wells were set for each group and the experiment was independently repeated three times.

Apoptosis assay (Annexin V-FITC/PI double staining method)

Apoptosis was detected using the Annexin V-FITC/PI Apoptosis Detection Kit (BD Pharmingen, USA, Cat# 556547). Following co-culture, FLS (digested with trypsin without EDTA) and suspended lymphocytes were collected and washed twice with pre-cooled PBS. According to the manufacturer's instructions, cells were resuspended in 1× Binding Buffer at a density of 1×10^6 cells/mL. Subsequently, 100 μL of the cell suspension (containing 1×10^5 cells) was transferred to a flow cytometry tube, followed by the addition of 5 μL Annexin V-FITC and 5 μL PI. The mixture was gently vortexed and incubated in the dark for 15 minutes at room temperature (25°C). After incubation, 400 μL of 1× Binding Buffer was added to each tube and the samples were immediately analyzed using a BD FACSCanto II flow cytometer. A minimum of 10,000 cells were collected per sample. Data were analyzed using FlowJo software. Annexin V-FITC single-positive cells were identified as early apoptotic cells, PI single-positive cells as necrotic cells and Annexin V-FITC and PI double-positive cells as late apoptotic cells. The total apoptosis rate was calculated as the sum of the early apoptosis rate and the late apoptosis rate. The procedure was performed with reference to the relevant report by Manohar *et al*., (2025).

Flow cytometric analysis of cell surface markers

Based on and modified from the method of Manohar *et al.,* (2025), lymphocytes collected after 48 hours of co-culture were washed with PBS and subjected to surface staining. A total of 1×10^6 cells were resuspended in 100 μL of PBS and the following fluorochrome-conjugated antibodies were added: PerCP-Cy™5.5-conjugated anti-human CD4 antibody (BD Pharmingen, Clone: RPA-T4), APC-

conjugated anti-human CD8a antibody (BD Pharmingen, Clone: HIT8a), PE-conjugated anti-human CD28 antibody (BD Pharmingen, Clone: CD28.2) and FITC-conjugated anti-human CD80 antibody (BD Pharmingen, Clone: L307.4). All antibodies were diluted according to the manufacturer's recommendations. Corresponding isotype control antibodies were also set up to exclude non-specific staining. After incubation at 4ºC in the dark for 30 minutes, the cells were washed twice with PBS, resuspended in 500 μL of PBS and analyzed by flow cytometry.

Flow cytometry gating strategy: Data were acquired using a BD FACSCanto II flow cytometer. First, the lymphocyte population was gated (P1) on an FSC-A/SSC-A scatter plot. Within the P1 gate, doublets were excluded by FSC-A/FSC-H to obtain a single-cell population (P2). Within the P2 gate, the expression of CD4 and CD8 was analyzed to gate $CD4^+$ T cells and $CD8^+$ T cells, respectively. Finally, the percentages of CD28 and CD80 expression were analyzed within the $CD4^+$ and $CD8^+$ T cell populations, respectively. Fluorescence Minus One (FMO) controls were used for all analyses to establish precise gates.

Cytokine detection (ELISA)

Cell culture supernatants were collected after 48 hours of co-culture and centrifuged to remove cell debris. The concentrations of TNF-α and IL-1β in the supernatants were measured according to the instructions of the human TNF-α ELISA kit (R&D Systems, USA, Cat# DTA00D) and the human IL-1β ELISA kit (R&D Systems, USA, Cat# DLB50), following procedures described in previous literature (Axelsson., 2022). Each sample was tested in triplicate and cytokine concentrations were calculated based on standard curves.

Western blot analysis

FLS were collected after 48 hours of co-culture, washed with pre-cooled PBS and lysed in RIPA lysis buffer containing protease inhibitors (Beyotime, China, Cat# P0013B) on ice for 30 minutes. Following the method described in relevant literature (Begum *et al*., 2022), the lysates were centrifuged at 12,000 rpm for 15 minutes at 4°C and the supernatants were collected. Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific, USA, Cat# 23225). A total of 30 μg of protein was subjected to SDS-PAGE gel electrophoresis and then transferred to PVDF membranes (Millipore, USA, Cat# IPVH00010). After blocking with 5% non-fat milk for 1 hour at room temperature, the membranes were incubated with primary antibodies: rabbit anti-human Bax monoclonal antibody (dilution 1:1000, Cell Signaling Technology, USA, Cat# 14796S), rabbit anti-human Bcl-2 monoclonal antibody (dilution 1:1000, Cell Signaling Technology, USA, Cat# 15071S), rabbit anti-human COX-2 monoclonal antibody (dilution 1:1000, Abcam, UK, Cat# ab179800) and mouse anti-human GAPDH monoclonal antibody (dilution 1:5000, Proteintech, China, Cat# 60004-1-Ig), overnight at 4°C. Following TBST washes, the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (dilution 1:5000, Cell Signaling Technology, USA, Cat# 7074S) for 1 hour at room temperature. Protein bands were visualized using an ECL chemiluminescence kit (Millipore, USA, Cat# WBKLS0500). The grayscale values of the bands were analyzed using ImageJ software and the relative expression levels of the target proteins were calculated using GAPDH as an internal control.

Real-time quantitative PCR (RT-PCR)

According to the method described in previous literature (Harshitha and Arunraj., 2021), total RNA was extracted from FLS after 48 hours of co-culture using TRIzol reagent (Invitrogen, USA). A total of 1 μg of RNA was reverse transcribed into cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan). Using cDNA as a template, amplification was performed on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) with TB Green® Premix Ex Taq™ II (TaKaRa, Japan). The reaction conditions were as follows: initial denaturation at 95ºC for 30 seconds, followed by 40 cycles of denaturation at 95ºC for 5 seconds and annealing/extension at 60ºC for 34 seconds. All reactions were performed in triplicate. The relative expression levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with GAPDH serving as the internal reference gene. The primer sequences were validated and synthesized by Sangon Biotech (Shanghai) Co., Ltd., with specific sequences listed in table 1.

Statistical analysis

All data were statistically analyzed using SPSS 26.0 software. Measurement data were expressed as mean ± standard deviation (mean ± SD). Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA). If homogeneity of variance was met, pairwise comparisons between groups were conducted using the LSD-t test; if homogeneity of variance was not assumed, Welch's test was used followed by Games-Howell multiple comparisons. A P value < 0.05 was considered statistically significant.

RESULTS

Inhibition of the NF-κB pathway reduces FLS viability and promotes apoptosis

As shown in table 2, compared with the control group, the viability of FLS in the model group was significantly increased at 24 h, 48 h and 72 h of co-culture ($P < 0.001$). Compared with the model group, the FLS viability in the inhibition group, treated with the NF-κB inhibitor PDTC, was significantly reduced at all three time points ($P < 0.001$), suggesting that under RA pathological conditions, inhibition of NF-κB signaling can reverse the abnormal proliferation of FLS.

Table 1: Sequence of each primer.

Gene		Sequences	Length (bp)
GAPDH	Forward	5 ' -GAAGGTGAAGGTCGGAGTC-3 '	226
	Reverse	5 ' -GAAGATGGTGATGGGATTTC-3 '	
TNF-R1	Forward	5 ' -ACCAAGTGCCACAAAGGAAC-3 '	192
	Reverse	5 ' -CTGCAATTGAAGCACTGGAA-3 '	
TNF-R2	Forward	5 ' -TTCGCTCTTCCAGTTGGACT-3 '	167
	Reverse	5 ' -CACGAAGGTCTGGTTGTCCT-3 '	

Table 2: Comparison of cell viability in each group.

Group	N	24 h	48 h	72 h
Control	5	1.01±0.02	1.01±0.03	1.01±0.01
Model	5	3.78±0.34	10.52±2.55	10.09±1.16
Inhibition	5	1.12±0.03	3.22±0.21	5.43±0.31
Blockade	5	1.20±0.05	3.64±0.23	6.12±0.36
F	—	303.410	51.342	177.317
P	—	< 0.001	< 0.001	< 0.001
①vs②	P	< 0.001	< 0.001	< 0.001
①vs③	P	0.005	< 0.001	< 0.001
②vs③	P	< 0.001	< 0.001	< 0.001
④vs③	P	0.015	0.017	0.012

Note: ① is the control group; ② is the model group; ③ is the inhibition group; ④ is the Blockade group.

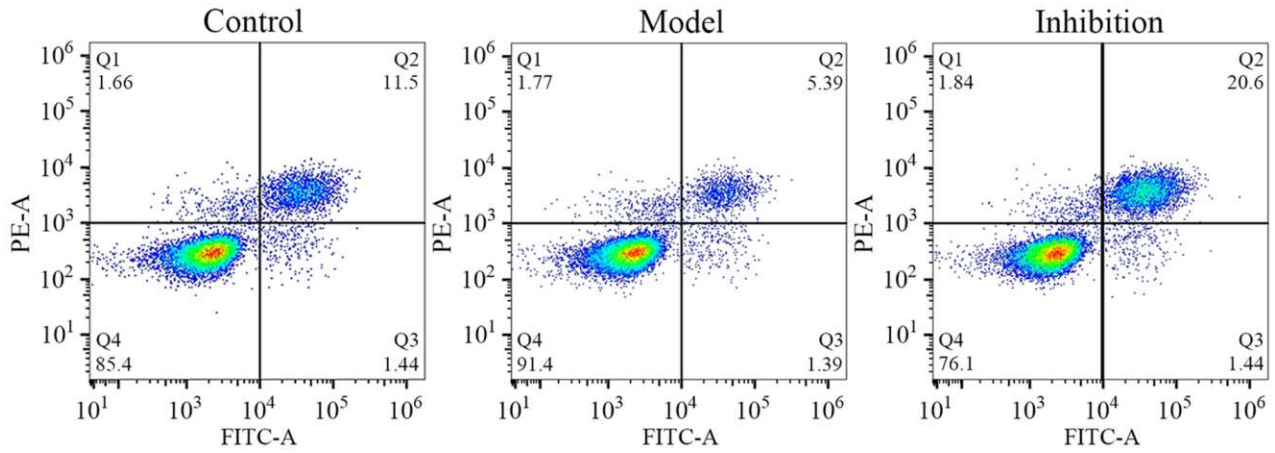

Fig. 1: Cell apoptosis in each group.

Further apoptosis detection (Fig. 1) revealed that the total apoptosis rate of FLS in the model group was significantly lower than that in the control group ($P < 0.001$), suggesting that there is apoptosis resistance in FLS in the pathological microenvironment of RA. In contrast, the inhibition group exhibited the highest total apoptosis rate among all groups, significantly exceeding both the model and control groups ($P < 0.001$). The detection of apoptosis-related proteins (Table 3) showed that in the FLS of the model group, the expression of the anti-apoptotic protein Bcl-2 was the highest and the expression of the pro-apoptotic protein Bax was the lowest. The expression of Bcl-2 in the inhibition group was significantly lower than that in the model group ($P < 0.001$) and the expression of Bax was significantly increased ($P < 0.001$). These results indicate that the activation of the NF-κB signaling pathway is associated with the apoptotic resistance of RA-FLS.

To further observe whether the B7/CD28 signal is related to the apoptotic changes of FLS induced by PDTC, the CD28 blocking antibody CTLA-4 Ig was added to the inhibition group (blocking group). The results showed that compared with the inhibition group, the apoptosis rate of FLS in the Blockade group was significantly reduced ($P < 0.05$), while the expression of Bcl-2 increased and the expression of Bax decreased ($P < 0.01$), suggesting that the CD28 signal may be associated with the apoptotic changes of FLS induced by PDTC.

Table 3: Comparison of Bax and Bcl-2 in each group.

Group	N	Bcl-2	Bax
Control	5	0.377±0.041	0.426±0.036
Model	5	0.987±0.147	0.144±0.067
Inhibition	5	0.398±0.076	0.244±0.065
Blockade	5	0.575±0.102	0.153±0.024
F	—	41.580	35.585
P	—	< 0.001	< 0.001
①vs②	P	< 0.001	< 0.001
①vs③	P	< 0.001	< 0.001
②vs③	P	< 0.001	< 0.001
④vs③	P	0.014	0.019

Note: ① is the control group; ② is the model group; ③ is the inhibition group; ④ is the Blockade group.

Table 4: Comparison of TNF-α, IL-1β, COX-2, TNF-R1 mRNA and TNF-R2 mRNA in each group.

Group	N	TNF-α(ng/L)	IL-1β(ng/L)	COX-2(%)	TNF-R1 mRNA	TNF-R2 mRNA
Control	5	2.44±0.13	7.34±1.24	0.17±0.03	1.47±0.16	1.33±0.17
Model	5	8.87±1.23	18.12±1.33	0.78±0.21	2.56±0.21	2.53±0.11
Inhibition	5	8.03±0.24	10.68±1.25	0.22±0.08	2.87±0.22	2.55±0.12
Blockade	5	8.06±0.23	12.74±1.03	0.36±0.05	2.85±0.24	2.58±0.17
F	—	107.284	68.683	28.901	49.986	91.430
P	—	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
①vs②	P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
①vs③	P	< 0.001	0.003	0.227	< 0.001	< 0.001
②vs③	P	0.172	< 0.001	< 0.001	0.052	0.791
④vs③	P	0.845	0.022	0.011	0.894	0.755

Note: ① is the control group; ② is the model group; ③ is the inhibition group; ④ is the Blockade group.

Changes in the expression profiles of TNF/TNFR-related molecules and inflammatory factors

As shown in table 4, compared with the control group, the levels of TNF-α, IL-1β, TNF-α R1/R2 mRNA expression and COX-2 protein level in the model group and the inhibition group were significantly increased ($P < 0.001$), confirming the successful construction of the RA *in-vitro* inflammatory model.

Compared with the model group, the expressions of IL-1β and COX-2 in the inhibition group were significantly decreased ($P < 0.001$), while there were no significant differences in the level of TNF-α ($P = 0.812$) and the mRNA expression of TNF-R1/R2 ($P = 0.086$). This result indicates that PDTC mainly affects the expression of downstream inflammatory signaling molecules, but has no significant correlation with the expression level of TNF/TNFR itself.

In the blocking group, the levels of IL-1β and COX-2 were slightly higher than those in the inhibition group ($P < 0.05$), but still lower than those in the model group ($P < 0.01$), suggesting that the CD28 signal may be associated to some extent with the maintenance of the downstream inflammatory response of NF-κB. After CD28 blockade, no significant changes were observed in the expression of TNF-α and its receptor ($P > 0.05$), indicating that the action site of B7/CD28 is located downstream of TNF/TNFR.

Changes in the expression of co-stimulatory molecules B7/CD28 on T cell subsets

As shown in Table 5, compared with the control group, the positivity rates of CD28 and CD80 on $CD4^+$ T cells in the model group were significantly decreased ($P < 0.01$), suggesting that under the inflammatory stimulation of RA, the T-cell co-stimulation signal may be in an abnormal state. Compared with the model group, the positive rates of CD28 and CD80 on $CD4^+$ T cells in the inhibition group were significantly increased ($P < 0.001$). On $CD8^+$ T cells, the expression trend of CD80 was similar to that of $CD4^+$ T cells (the lowest in the model group and increased in the inhibition group, $P < 0.001$).

In the blocking group, due to the addition of CD28 blocking antibody, the positive rate of CD28 detectable on $CD4^+$ T cells was significantly lower than that in the inhibition group ($P < 0.001$), confirming the success of the blocking. It is worth noting that the expression of CD80 in the blocking group remained at a relatively high level and there was no statistically significant difference compared with the inhibition group ($P > 0.05$), suggesting that the upregulation of CD80 by PDTC may not be mediated by CD28 feedback signaling.

Table 5: Expression of CD28 and CD80 on CD4+ and CD8+ T cells in each group.

Group	N	CD28+onCD4+	CD80+onCD4+	CD28+onCD8+	CD80+onCD8+
Control	5	19.26±5.12	10.04±0.04	15.26±8.12	11.03±0.03
Model	5	10.56±5.43	2.12±0.13	8.56±1.43	2.13±0.15
Inhibition	5	14.13±3.17	8.45±0.24	14.13±3.16	9.43±0.25
Blockade	5	9.61±2.02	8.48±0.21	9.13±2.05	9.49±0.23
F	—	5.476	2035.119	2.843	2311.807
P	—	0.009	< 0.001	0.071	< 0.001
①vs②	P	0.031	< 0.001	0.107	< 0.001
①vs③	P	0.093	< 0.001	0.779	< 0.001
②vs③	P	< 0.001	< 0.001	0.007	< 0.001
④vs③	P	0.028	0.835	0.018	0.703

Note: ① is the control group; ② is the model group; ③ is the inhibition group; ④ is the Blockade group.

However, there was no statistically significant difference in the expression of CD28 on $CD8^+$ T cells among the three groups (F = 2.843, P = 0.071), suggesting that the regulation of co-stimulatory molecules by NF-κB signaling may have T cell subpopulation specificity.

DISCUSSION

Rheumatoid arthritis is an autoimmune disease characterized by chronic synovial inflammation and joint destruction, in which the abnormal activation of T cells plays a central role in its pathogenesis (Zheng *et al*., 2024; Jahid *et al*., 2023). In this study, by establishing a co-culture system of lymphocytes from RA patients and fibroblast-like synoviocytes, we investigated whether the TNF/TNFR superfamily regulates the expression of the co-stimulatory molecules B7/CD28 through the NF-κB pathway, thereby influencing the inflammatory response and cell apoptosis in RA.

This study found that inhibiting the NF-κB signaling pathway could reduce the activity of FLS and increase their apoptosis, accompanied by down-regulation of Bcl-2 expression and up-regulation of Bax expression. This finding is consistent with the classical anti-apoptotic function of NF-κB (Liu *et al*., 2025). It suggests that under pathological conditions of RA, activation of the NF-κB pathway may confer resistance to apoptosis in FLS by upregulating anti-apoptotic factors such as Bcl-2, thereby promoting synovial hyperplasia.

To further investigate whether B7/CD28 signaling is associated with NF-κB-regulated apoptosis changes in FLS, this study added the CD28 blocking antibody CTLA-4 Ig on the basis of the inhibition group. The results showed that blocking CD28 signaling attenuated the pro-apoptotic effect induced by PDTC, as evidenced by a decreased apoptosis rate, increased Bcl-2 expression and decreased Bax expression. This observation suggests that B7/CD28 costimulatory signaling may be associated with the regulation of RA-FLS apoptosis by the NF-κB pathway. However, it should be emphasized that the above data only indicate phenotypic changes following CD28 blockade and are insufficient to determine whether B7/CD28 serves as a direct mediator of NF-κB-regulated FLS apoptosis. The interaction between T cells and FLS may involve multiple signaling pathways and soluble factors and the underlying mechanisms remain to be further elucidated.

In terms of the expression of inflammatory factors, the levels of TNF-α, TNF-R1 mRNA and TNF-R2 mRNA in both the model group and the inhibition group were significantly higher than those in the control group, but there was no statistically significant difference between the two groups. This result indicates that the NF-κB inhibitor PDTC did not significantly affect the expression levels of TNF-α and its receptors. Combined with the phenomenon that PDTC can effectively reduce the expressions of IL-1β and COX-2, it can be inferred that PDTC mainly acts on the downstream inflammatory signal transduction link of the NF-κB pathway, rather than affecting the expression of TNF/TNFR itself. It should be particularly noted that the data from this study do not support the inference that "TNF/TNFR functions by inhibiting the NF-κB pathway". Firstly, after PDTC intervention, the expression of TNF-α and its receptor did not change significantly, precluding a definitive inference regarding the regulatory direction between TNF/TNFR and NF-κB. Secondly, the existing literature generally holds that TNF-α is a classic activator of the NF-κB pathway (Kim *et al*., 2024) and the data of this study do not contradict this. Therefore, the specific regulatory relationship between TNF/TNFR and NF-κB in RA still needs to be further clarified through the experimental design of direct intervention in TNF/TNFR signaling.

Regarding the expression of T cell costimulatory molecules, this study found that after inhibiting the NF-κB pathway, the expression of CD28 and CD80 on $CD4^+$ T cells was significantly restored compared with the model group, approaching levels observed in the control group. This observation appears not entirely consistent with the classic understanding that NF-κB promotes T cell activation. Possible reasons include: first, NF-κB inhibition may alter

the differentiation status or activation characteristics of T cell subsets, thereby indirectly affecting the expression of costimulatory molecules (Kuo *et al*., 2025); second, as an antioxidant, PDTC may influence the expression of cell surface molecules through NF-κB-independent pathways (Nan *et al*., 2023). Therefore, when interpreting experimental results obtained with PDTC, careful distinction between its specific effects and non-specific effects is warranted. Notably, the expression of CD28 on $CD8^+$ T cells showed no significant differences among the groups, suggesting that the regulation of costimulatory molecules by NF-κB signaling may be T cell subset-specific. In the blockade group, although CD28 signaling was effectively blocked, CD80 expression remained at relatively high levels, with no statistically significant difference compared with the inhibition group. This observation suggests that the upregulation of CD80 by PDTC may occur independently of the CD28 feedback signaling pathway.

Regarding the regulation of apoptosis, this study found that in the inhibition group, Bax expression was increased and Bcl-2 expression was decreased, further supporting the inference that the NF-κB pathway may influence FLS survival through the regulation of Bcl-2 family proteins. Although the role of TNF/TNFR signaling in apoptosis has been extensively studied (Javaid *et al*., 2024; Qin *et al*., 2024), TNFR1 and TNFR2 may exert distinct or even opposing functions in RA. TNFR1 is typically associated with pro-inflammatory and pro-apoptotic signaling (Messaoud-Nacer *et al*., 2022), whereas TNFR2 is more linked to cell survival and immunomodulation (Zhang *et al*., 2022). This study did not differentiate between the functions of the two receptors and future investigations employing specific blockade or gene knockout approaches are warranted to further elucidate their respective roles in RA.

Several factors should be considered when evaluating the reliability and generalizability of the conclusions of this study. First, the relatively small sample size may limit statistical power and the broad applicability of the findings. Second, while PDTC is a widely used NF-κB inhibitor, its antioxidant and metal-chelating properties may affect the activity of other transcription factors, such as AP-1, or indirectly modulate cellular functions through reactive oxygen species depletion. As such, the conclusions of this study should be interpreted as preliminary correlative evidence and more specific intervention strategies are warranted in future studies. Third, the observation that PDTC treatment did not alter the expression levels of TNF-α or its receptors suggests that TNF/TNFR may function upstream of or in parallel to the NF-κB pathway; however, this inference is based on correlative data and lacks direct experimental evidence from TNFR-targeted interventions. Fourth, the absence of functional T cell assays limits the ability to evaluate the actual impact of altered CD28/CD80 expression on T cell function. Fifth, the PBMC-FLS co-culture system cannot fully replicate the *in-vivo* joint microenvironment, which includes factors such as hypoxia, mechanical stress and contributions from other immune cell populations. Therefore, the precise role of the TNF/TNFR-NF-κB-B7/CD28 regulatory axis in the pathogenesis of RA requires further validation using animal models, such as collagen-induced arthritis.

CONCLUSION

In summary, this study found that inhibition of the NF-κB signaling pathway alleviated the inflammatory response in RA-FLS and restored the expression levels of B7/CD28 on T cells, whereas the expression of TNF/TNFR itself was not affected by PDTC. These observations suggest that the NF-κB pathway plays an important role in the regulation of inflammation and T cell costimulatory signals in RA, although the specific regulatory relationship between TNF/TNFR and this pathway remains unclear. Future studies integrating multi-omics analysis with functional validation are needed to further elucidate the immunoregulatory mechanisms of TNF/TNFR signaling in RA, thereby providing new targets for precision therapy.

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Authors' contributions
Jingjing Liu: Funding acquisition, supervision, writing – original draft; Dan Pan: Methodology, data curation, formal analysis, writing – original draft; Weiguang Hou: Investigation, methodology, writing – review and editing; Huiwu Zhang: Supervision, resources, software, Project administration; Runan Pan: validation, visualization, writing – original draft; Qing Pan: Data curation, formal analysis, writing – original draft; Chengjie Tang: Conceptualization, methodology design, writing – review and editing. All authors read and approved the final manuscript.

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Data availability statement
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval
This study strictly adhered to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the Sichuan Orthopedic Hospital (Approval No.: KY2025-065-01). All patients with rheumatoid arthritis and healthy volunteers participating in this study

were informed of the purpose and procedures of the research and provided written informed consent. This study was performed in adherence with the STROBE guidelines. See supplementary file for the STROBE checklist.

Conflict of interest

The authors declare that they have no conflicts of interest.

Supplementary data

https://www.pjps.pk/uploads/2026/07/SUP1785321666.pdf

REFERENCES

Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO 3rd, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B, Costenbader KH, Dougados M, Emery P, Ferraccioli G, Hazes JMW, Hobbs K, Huizinga TWJ, Kavanaugh A, Kay J, Kvien TK, Laing T, Mease P, Menard HA, Moreland LW, Naden RL, Pincus T, Smolen JS, Stanislawska-Biernat E, Symmons D, Tak PP, Upchurch KS, Vencovsky J, Wolfe F and Hawker G (2010). 2010 Rheumatoid arthritis classification criteria: An American college of rheumatology/European league against rheumatism collaborative initiative. *Arthritis Rheum.*, **62**(9): 2569-2581.

Axelsson B (2022). Detection of cytokine-secreting cells by enzyme-linked immunospot (ELISpot). *Methods Mol Biol.*, **2386**: 61-79.

Begum H, Murugesan P and Tangutur AD (2022). Western blotting: A powerful staple in scientific and biomedical research. *Biotechniques.*, **73**(1): 58-69.

Boyce BF, Li J, Yao Z and Xing L (2023). Nuclear factor-kappa B regulation of osteoclastogenesis and osteoblastogenesis. *Endocrinol Metab (Seoul)*., **38**(5): 504–521.

Dal-Re R (2023). Waivers of informed consent in research with competent participants and the Declaration of Helsinki. *Eur J Clin Pharmacol*., **79**(4): 575-578.

Ding Q, Hu W, Wang R, Yang Q, Zhu M, Li M, Cai J, Rose P, Mao J and Zhu YZ (2023). Signaling pathways in rheumatoid arthritis: Implications for targeted therapy. *Signal Transduct Target Ther.*, **8**(1): 68.

Ghasemi M, Liang S, Luu QM and Kempson I (2023). The MTT assay: A method for error minimization and interpretation in measuring cytotoxicity and estimating cell viability. *Methods Mol Biol*., **2644**: 15-33.

Harshitha R and Arunraj DR (2021). Real-time quantitative PCR: A tool for absolute and relative quantification. *Biochem Mol Biol Educ.*, **49**(5): 800-812.

Jahid M, Khan KU, Rehan-Ul-Haq and Ahmed RS (2023). Overview of rheumatoid arthritis and scientific understanding of the disease. *Mediterr J Rheumatol.*, **34**(3): 284-291.

Javaid N, Ahmad B, Patra MC and Choi S (2024). Decoy peptides that inhibit TNF signaling by disrupting the TNF homotrimeric oligomer. *FEBS J.*, **291**(19): 4372-4391.

Kim TW, Koo SY, Riessland M, Chaudhry F, Kolisnyk B, Cho HS, Russo MV, Saurat N, Mehta S, Garippa R, Betel D and Studer L (2024). TNF-NF-κB-p53 axis restricts *in-vivo* survival of hPSC-derived dopamine neurons. *Cell.*, **187**(14): 3671-3689.e23.

Kuo CY, Huang CY, Chen HM, Chen LC and Kuo ML (2025). Antagonism of CD28 blocks allergic responses in the ovalbumin-induced asthmatic model mice. *Int Immunopharmacol.*, **148**: 114071.

Liu H, Lu H, Fan X, Chen S, Chen X and Gao W (2025). Probing the molecular mechanism of kaempferol in relieving rheumatoid arthritis based on network pharmacology. *Sci Rep.*, **15**(1): 12645.

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**(4): 402-408.

Manohar SM, Chaubal S, Mukherjee S, Arfuso F, Dharmarajan A and Warrier S (2025). Flow cytometry: A versatile tool for stem cell research. *Biochem Biophys Res Commun.*, **793**: 153033.

Messaoud-Nacer Y, Culerier E, Rose S, Maillet I, Rouxel N, Briault S, Ryffel B, Quesniaux VFJ and Togbe D (2022). STING agonist diABZI induces PANoptosis and DNA mediated acute respiratory distress syndrome (ARDS). *Cell Death Dis.*, **13**(3): 269.

Nan L, Zhang Y, Song H, Ye Y, Jiang Z and Zhao S (2023). Influence of light-emitting-diode-derived blue light overexposure on rat ocular surface. *J Ophthalmol.*, **2023**: 1097704.

Patil S, Reda R, Boreak N, Taher HA, Melha AA, Albrakati A, Vinothkumar TS, Mustafa M, Robaian A, Alroomy R, Kharaf RJA, Kameli TS, Alkahtani A, Baeshen HA, Patil VR and Testarelli L (2021). Adipogenic stimulation and pyrrolidine dithiocarbamate induced osteogenic inhibition of dental pulp stem cells is countered by cordycepin. *J Pers Med.*, **11**(9): 915.

Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LBA and van Riel PLCM (1995). Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum*., **38**(1): 44-48.

Qin X, Xu W, Hu J, Dong Y, Ding R, Huang S, Zhao Z, Chang H, Wang X and Dong S (2024). Structure-activity relationship study of Pseudellone C as anti-glioma agents by targeting TNF/TNFR signaling pathway. *Eur J Med Chem.*, **278**: 116799.

Stai S, Lioulios G, Xochelli A, Papadopoulou A, Yannaki E, Kasimatis E, Christodoulou M, Moysidou E, Samali M, Testa T, Iosifidou AM, Iosifidou MA, Tsoulfas G, Stangou M and Fylaktou A (2024). Vaccination with tozimameran induces T-cell activation, but not senescent or exhaustive alterations, in kidney transplant recipients. *Vaccines*, **12**(8): 877.

Veerasubramanian PK, Wynn TA, Quan J and Karlsson FJ

(2024). Targeting TNF/TNFR superfamilies in immune-mediated inflammatory diseases. *J Exp Med*., **221**(11): e20240806.

Wallberg F, Tenev T and Meier P (2016). Time-lapse imaging of cell death. *Cold Spring Harb Protoc*., **2016**(3): pdb.prot087395.

Yan G, Song R, Zhang J, Li Z, Lu Z, Liu Z, Zeng X and Yao J (2024). MIF promotes Th17 cell differentiation in rheumatoid arthritis through ATF6 signal pathway. *Mol Med*., **30**(1): 237.

Zhang C, Weng Y, Wang H, Zhan S, Li C, Zheng D and Lin Q (2024). A synergistic effect of triptolide and curcumin on rheumatoid arthritis by improving cell proliferation and inducing cell apoptosis via inhibition of the IL-17/NF-κB signaling pathway. *Int Immunopharmacol*., **142**(Pt A): 112953.

Zhang R, Chang C, Jin Y, Xu L, Jiang P, Wei K, Xu L, Guo S, Sun S and He D (2022). Identification of DNA methylation-regulated differentially expressed genes in RA by integrated analysis of DNA methylation and RNA-Seq data. *J Transl Med.*, **20**(1): 481.

Zhang X, Lao M, Xu J, Duan Y, Yang H, Li M, Ying H, He L, Sun K, Guo C, Chen W, Jiang H, Zhang X, Bai X and Liang T (2022). Combination cancer immunotherapy targeting TNFR2 and PD-1/PD-L1 signaling reduces immunosuppressive effects in the microenvironment of pancreatic tumors. *J Immunother Cancer.*, **10**(3): e003982.

Zhao F and Yu JS (2024). Overview of dendritic cells and related pathways in autoimmune uveitis. *Open Life Sci.*, **19**(1): 20220887.

Zhao J, Ouyang Q, Hu Z, Huang Q, Wu J, Wang R and Yang M (2016). A protocol for the culture and isolation of murine synovial fibroblasts. Biomed Rep, 5(2): 171-175.

Zheng Y, Wei K, Jiang P, Zhao J, Shan Y, Shi Y, Zhao F, Chang C, Li Y, Zhou M, Lv X, Guo S and He D (2024). Macrophage polarization in rheumatoid arthritis: Signaling pathways, metabolic reprogramming and crosstalk with synovial fibroblasts. *Front Immunol.*, **15**: 1394108.

Zhou S, Xue W and Tan J (2024). Design, synthesis and antirheumatoid arthritis mechanism of TLR4 inhibitors. *ACS Omega.*, **9**(34): 36232-36241.