Expression of TREM-2 and its inhibitory effects on TNF-α induced inflammation in fibroblast-like synoviocytes via inhibiting p38 pathway activation

S.H. Huang[1,2], G.W. Liu[1,3], J.H. Li[4], J.H. Xu[5], D.W. Xu[1], W.Q. Zhang[5], J.R. Huang[1,5]

[1]Department of Orthopaedics, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, P.R. China; [2]Department of Orthopaedics, the Seventh Affiliated Hospital, Sun Yat-Sen University, Shenzhen, P.R. China; [3]Department of Orthopaedics and Traumatology, Qingyuan People's Hospital, The Sixth Affiliated Hospital of Guangzhou Medical University, Guangdong, P.R. China; [4]Key Laboratory of Protein Modification and Degradation, School of Basic Medical Sciences, Guangzhou Medical University, Guangdong, P.R. China; [5]Department of Orthopaedics, Zengcheng District People's Hospital, Guangzhou, P.R. China.

Abstract

Objective

It is not clear whether TREM-2 (the "triggering receptor expressed on myeloid cells 2") is expressed in fibroblast-like synovial cells (FLSs). In this study, we aimed to determine the expression of TREM-2 in rheumatoid arthritis (RA)-FLSs and explore whether and how TREM-2 modulates the function of RA-FLSs.

Methods

Western blot and RT-PCR were used to detect the expression of TREM-2 in RA-FLSs, siRNA and lentivirus were used to down-regulate and up-regulate the expression of TREM-2 in RA-FLSs. Then mRNA expression of IL-1β, IL-6, and MMP-13 was determined by RT-qPCR. Protein secretion of IL-1β, IL-6, and MMP-13 in the supernatant was determined by ELISA assay; expression of cell signal transduction molecules was determined by western blot.

Results

A: Relative to OA-FLSs, mRNA and protein expression levels of TREM-2 in RA-FLSs are significantly elevated. TREM-2 protein is mainly expressed in the cytoplasm of RA-FLSs;

***B**: In RA, the expression of TREM-2 was reduced at first and then up-regulated after stimulation by TNF-α. TREM-2 also inhibited the activation of TNF-α induced of inflammation in RA-FLSs by the p38 pathway, which regulates the production of cytokines and matrix metalloproteinases.*

Conclusion

TREM-2 expressed in RA-FLSs and TNF-α mediated reduction of inflammatory reactions. These phenomena indicated that TREM-2 may be a potential target in the treatment of RA.

Key words

rheumatoid arthritis, p38, triggering receptor expressed on myeloid cells 2, fibroblast-like synovial cell, signal transduction

*ShengHui Huang**
*GuiWang Liu**
JianHua Li, PhD
JinHuang Xu
DaWei Xu
WeiQiong Zhang
JianRong Huang, MD

**These authors contributed equally to this study.*

Please address correspondence to:
Dr JianRong Huang,
107 Yanjiangxi Rd,
Guangzhou 510120, P.R. China.
or
1st Guangming East Road,
Zengcheng Urban,
Guangzhou 511300, P.R. China.
E-mail: guke16@163.com

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Authors' contributions:
HJR conceived and designed the experiments.
HSH and LGW performed the experiments.
XJH and LJH analysed the data.
ZPZ, ZWQ and XDW contributed regents/materials/analysis tools.
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Introduction

TREM is an immune model receptor family discovered in 2000. TREM-2 (triggering receptor expressed on myeloid cells 2), as an immune model receptor, is mainly expressed in macrophages, dendritic cells, microglia cells, fibroblasts, some tumour cells, and other cells (1). TREM-2 inhibits the release of inflammatory mediators, and acts as an important anti-inflammatory receptor *in vivo*. Turnbull *et al*. (2) found that if mouse macrophages were stimulated by lipopolysaccharide (LPS) after TREM-2 gene knockout, the secretion of inflammatory factors increased significantly. Zhu *et al*. (3) found that the expression of TREM-2 was increased for 3–5 days after corneal infection in mice. They also found that activating TREM-2 promoted host resistance to Pseudomonas aeruginosa and accelerated the healing of the keratitis. Recent studies of TREM-2 address a variety of autoimmune diseases, including multiple sclerosis and rheumatoid arthritis. Ohrfelt *et al*. (4) confirmed that the expression of soluble TREM-2 (sTREM-2) in cerebrospinal fluid of patients with multiple sclerosis, which is closely related with the disease status; Tania *et al*. (5) confirmed that the expression of TREM-2 was significantly increased in synovial tissue of patients with acute rheumatoid arthritis.

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease characterised by facet joint pain. It has complex pathogenesis, with loss of immune tolerance as a key mechanism. Fibroblast-like synovial cell (FLS) dysfunction plays a key role in RA inflammation and tissue destruction (6). FLSs are present in synovial joints and are the major effector cells in RA, involved in pannus formation and the erosion of articular cartilage and bone (7).

Previously, we found that TREM-2 is expressed in human bone marrow mesenchymal stem cells (MSCs), and that the adipogenic, osteogenic, and chondrogenic activity of those MSCs are suppressed in the absence of TREM-2. The stimulation of MSCs by LPS could significantly increase the secretion of inflammatory factors in the presence of TREM-2 (8). Li *et al*. (9) reported that the expression of TREM-2 in FLSs was significantly increased in a rat model of rheumatoid arthritis. However, it is not clear whether this increase in TREM-2 was occurring in the RA-FLSs that were the main effector cells of the RA inflammatory factors.

Therefore, in the present study, we investigated expression of TREM-2 in FLSs isolated from patients with RA by different methods, using RNA interference and gene transfection, in order to determine whether TREM-2 expression was down-regulated or up-regulated in the RA-FLSs. We then studied the expression of inflammatory factors and signal transduction molecules in the RA-FLSs, in order to elucidate the regulation and mechanisms of TREM-2 in RA.

Methods

Culture of human FLSs

To compare the protein and mRNA level of TREM-2 between RA-FLSs and OA-FLSs, eleven patients from the Department of Rheumatology of the Third Affiliated Hospital of Sun Yat-sen University who fulfilled the 1987 revised criteria of the American College of Rheumatology (ACR) or 2010 ACR/European League against Rheumatism (EULAR) criteria for RA were recruited as study subjects. All patients had significant inflammation, with a 28-joint disease activity score (DAS28) ≥2.6. Ten patients with osteoarthritis (OA) from the Orthopaedic Department were included as controls with less-inflamed disease. Clinical features of the RA and OA groups are shown in Table I. The protocol was approved by Medical Ethics Committee of the Third Affiliated Hospital of Sun Yat-Sen University, and all patients gave written informed consent.

Synovial tissues were obtained under sterile conditions, and fat, blood vessels, and fibrous tissue were removed. The trimmed synovial tissue samples were washed with phosphate buffered saline (PBS) buffer 2 to 3 times, and the synovial samples were then cut down to small blocks, each about 1 mm^3. The tissue blocks were placed in culture flasks, appropriate amounts of 15% fetal bovine serum (FBS, Gibco division of Thermo Fisher Scientific, USA) con-

taining high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) were added, and the samples were cultured in a constant temperature incubator at 37º C with 5% CO2. The culture medium was replaced twice a week. After the cells divided and the coverage rate of the culture was at least 80%, the synovial cells were dispersed by trypsinisation and transferred to new plastic dishes in a split ratio of 1:3, and after 3 to 5 passages the FLSs were utilised in subsequent experiments. Human RA-FLSs derived from the inflamed synovial tissue obtained from patients with active RA (Cell Applications Inc., California, USA) were studied after 3 to 7 passages for the balance of the experiments. The culture methods were as described above.

The expression of TREM-2 protein as detected by immunofluorescence

The cells were suspended at 2×10^5/ml and inoculated into 24-well plates. When the FLSs density in the cultures was 50–60%, the medium was replaced by serum free medium for 24 h. The cells were fixed with 4% paraformaldehyde or iced methanol for 10 min, permeabilised by 0.3% Triton X-100 for 10 min, and blocked with goat serum for half an hour. The FLSs then were incubated with a primary antibody to TREM-2 (Santa Cruz, California, USA) diluted by goat serum and were stained with Alexa Fluor 488-conjugated goat anti-mouse IgG diluted to 1:1,000 (ZSGB-BIO, Beijing, China). The FLSs were then co-stained with DAPI (ThermoFisher Scientific, Waltham, MA, USA) to visualise nuclei. Finally, the location of TREM-2 was analysed by fluorescence microscopy (Carl Zeiss GmbH, Jena, Germany).

Transient siRNA transfection in RA-FLSs

RA-FLSs were cultured in DMEM medium supplemented with 15% FBS with 1% penicillin-streptomycin and 1% L-glutamine (Invitrogen, Carlsbad, CA, USA) at the permissive temperature of 37°C, as described above. According to the manufacturer's instructions, RA-FLSs were transiently transfected with specific FAM-siRNA for TREM-2 (siTREM-2 sense sequence was 5′-GCCUCUU GGAAG GAGAAAUTT-3′ and the antisense sequence was 5′-AUUUCUCCUUCCAAGAGG CTT-3′) *versus* nonsense control FAM-siRNA (siNC, sense sequence was 5-′UUCU CCGAACGUGUCAGGUTT-3′, and antisense sequence was 5′-ACGUGACACGUU CGGAGAATT-3′) (GenePharma, Shanghai, China), mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After transfection for 6 h, the medium was replaced, and the transfected cells were studied by fluorescence microscopy, with interference efficiency detected by RT-PCR and Western blot.

Table I. Baseline demographic and clinical features of RA and OA.

Characteristic	RA (n=11)	OA (n=10)
Demographic		
Age, median (IQR)	59 (51 to 66)	61 (53 to 65)
Famale (n%)	8 (72.7)	6 (60.0)
Disease status		
Disease duration, median (IQR)	38 (12 to 120)	72 (24 to 120)
CRP (mg/dl)	6.37 (2.13 to 9.05)	0.29 (0.18 to 0.87)
ESR (mm/h)	78 (53 to 107)	16 (11 to 29)
RF (n%)	10 (90.9)	NA
Anti-CCP-positive, n (%)	9 (81.8)	NA
DAS28, median (IQR)	5.79 (4.83 to 6.39)	NA
Previous medications		
Corticosteroids (n%)	4 (36.36)	NA
Methotrexate (n%)	4 (36.36)	NA
Leflunomide (n%)	2 (18.18)	NA
Sulfasalazine (n%)	1 (9.09)	NA
Hydroxychloroquine (n%)	2 (18.18)	NA
Etanercept (n%)	2 (18.18)	NA

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor; anti-CCP: anti-cyclic citrullinated peptide antibodies; DAS28: disease activity score 28-joint assessment; NA: not applicable.

Lentivirus infection in RA-FLS

TREM-2 overexpression was determined by lentivirus infection (GenePharma Shanghai, China). The human TREM-2 mRNA sequence was selected from GenBank (NCBI Reference Sequence: NM_018965.3). Supernatants containing lentivirus were added to the RA-FLS cultures in the presence of hexadimethrine bromide (Polybrene©, Abbott Laboratories, Chicago, IL, USA). The cells overexpressing TREM-2 were selected by puromycin (1 μg/ml), and after 24h stable cell lines were verified by reverse transcription polymerase chain reaction (RT-PCR) and Western blot.

RT-PCR and quantitative real-time PCR (qPCR)

Total RNA was isolated from cell pellets using Trizol (Invitrogen, USA) according to the manufacturer's instructions. RNA purity and quantity was evaluated by spectrophotometer. After reverse transcription, complementary DNA was used as the PCR template. Amplification of cDNA was conducted with Taq polymerase (Takara, Dalian, China) according to the manufacturer's instructions. PCR amplification was performed using specific primers (Table II). The housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was used to normalise tested genes. The PCR products were analysed by electrophoresis in 2% agarose gels stained with ethidium bromide (EB), and bands were visualised and photographed under ultraviolet excitation. The qPCR was performed using QuantiTeck™ SYBR Green PCR kit (Takara, Dalian, China). The constitutively expressed gene encoding GAPDH was used as an internal control and quantification of the mRNA level was performed by the comparative threshold method ($\Delta\Delta$Ct).

Western blot analysis

Cultured human FLSs were lysed with RIPA (Beyotime, Shanghai, China) lysis buffer (1%PMSF). The protein con-

Table II. Primers for RT-PCR and quantitative real-time PCR.

Molecule	Sense primer (5´-3´)	Antisense primer (5´-3´)
TREM-2	GTCTTGCCCCTATGACTCCA	CTGGTAGAGACCCGCATCAT
IL-1β	CCAGCTACGAATCTCCGACC	CATGGCCACAACAACTGACG
IL-6	CCAGGAGAAGATTCCAAAGATG	GGAAGGTTCAGGTTGTTTTCTG
MMP-13	GCTGCCTTCCTCTTCTTGA	TGCTGCATTCTCCTTCAGGA
GAPDH	GAAGGTCGGAGTCAACGG	GGAAGATGGTGATGGGATT

TREM-2: Triggering receptor expressed on myeloid cells-2; MMP: matrix metalloproteinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

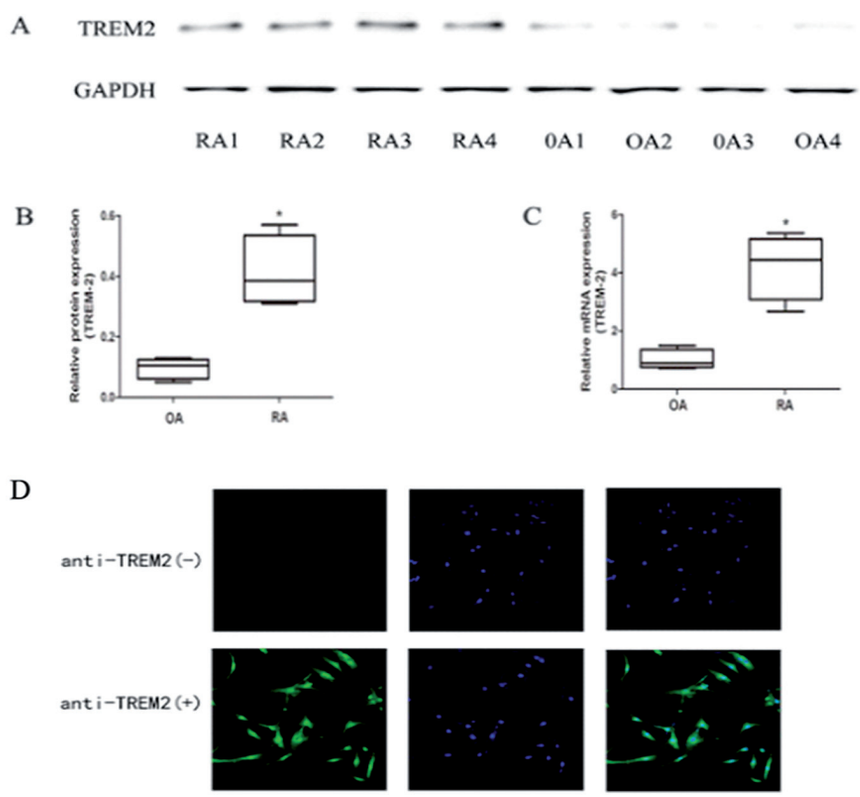

Fig. 1. Expression of TREM-2 in RA-FLSs.
A-B: The expression of TREM-2 protein in RA-FLSs and OA-FLSs was detected by western blotting. **C**: The expression of TREM-2 mRNA in RA-FLSs and OA-FLSs was detected by qPCR. **D**: The expression of TREM-2 in RA-FLSs was detected by immunofluorescence assay (× 200). *$p<0.01$, compared with the OA group (n=3, $\bar{x} \pm s$).

centration was determined using BCA protein assay kit (keyGEN, Nanjing, China). An identical amount of proteins (20 μg) were subjected to SDS-PAGE and target proteins were detected with primary anti-bodies recognising TREM-2 (Santa Cruz, CA, USA), p38 mitogen-activated protein kinase (MAPK), p-p38MAPK, extracellular signal regulated kinase (ERK), p-ERK, Jun N-terminal kinase (JNK), p-JNK, nuclear factor-kappaB (NF-κB) p65, p-NF-B p65 (Cell Signaling Technology, Boston, MA, USA) and GAPDH (Bioworld Technology, St. Louis Park, MN, USA). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (EarthOx Life Sciences, Millbrae, CA, USA), protein bands were visualised using enhanced chemiluminescence (Millipore, Boston, MA, USA) plus Western blot detection reagents followed by exposure to a scanning imager (Bio-Rad, Hercules, CA, USA).

ELISA

Levels of human matrixmetallopreteinases-13 (MMP-13) and interleukin-1β (IL-1β), interleukin-6 (IL-6) were determined with ELISA (enzyme-linked immunosorbent assay) kits in cell-free supernatant (Neobioscience, Shenzhen, China). The cytokines were analysed by standard curves. The OD values were evaluated at a wavelength of 450 nm.

Statistical analysis

Statistical analyses were performed with SPSS 22.0 statistical software (SPSS, IBM, Armonk, NY, USA). All experimental results were replicated 3 to 4 times. The data were consistent with a normal distribution were expressed as mean ± SD; otherwise, logarithmic transformations were performed to make the data consistent with a normal distribution when comparing the OA and RA groups. Data were analysed by the Student's *t*-test or by one-way analysis of variance with Tukey's post-hoc test for multiple comparisons to determine the statistical significance of comparisons. *P*-values <0.05 were considered statistically significant.

Results

High expression of TREM-2 in RA-FLSs relative to OA-FLSs

qPCR and Western blot results showed mRNA and protein expression levels of TREM-2 were significantly increased in RA-FLSs compared to levels in OA-FLSs. By immunofluorescence, the TREM-2 in RA-FLSs was mainly located in the cytoplasm (Fig. 1).

Expression of TREM-2 in the inflammatory response of RA-FLSs mediated by TNF-α

In order to evaluate the effect of TNF-α on the expression of TREM-2 in RA-FLSs, we used TNF-α (20ng/ml) to stimulate RA-FLSs and measure mRNA or protein levels of TREM-2, IL-6, IL-1β, and MMP-13 at different time points of incubation (2, 4, 6, 12, and 24 h).

qPCR results suggested that the mRNA level of TREM-2 was significantly decreased after 2 h of stimulation by TNF-α and then slowly increased. Western blot analyses revealed that the TREM-2 protein levels changed in a similar pattern over time. However, our qPCR results suggested that the secretion of IL-1β, IL-6, and MMP-

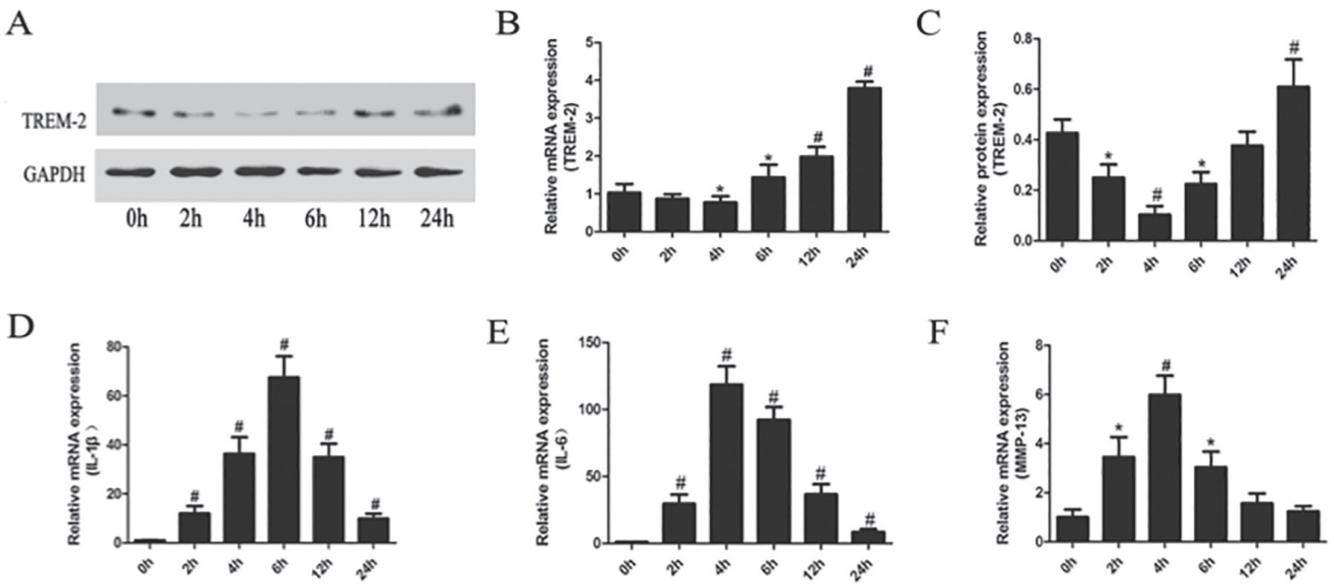

Fig. 2. Expression changes of TREM-2 and inflammatory factors after stimulation with TNF-α in RA-FLSs.
A-B: Western blotting analysis of TREM-2 protein in 0, 2, 4, 6, 12, and 24 h after TNF-α stimulation RA-FLSs. **C**: qPCR analysis of TREM-2 mRNA in 0, 2, 4, 6, 12, and 24 h after TNF-α stimulation RA-FLSs. **D-E-F**: qPCR analysis of IL-1β,IL-6, MMP-13 mRNA in 0, 2, 4, 6, 12, and 24 h after TNF-α stimulation RA-FLSs. *p<0.01, compared with the 0h group (n=4, $\bar{x} \pm s$).

13 significantly increased after 2 h of stimulation by TNF-α and then slowly decreased, These results suggested that TNF-α may be a negative regulator of TREM-2 synthesis, and a positive regulator of IL-1β, IL-6, and MMP-13 secretion (Fig. 2).

Silencing TREM-2 enhances TNF-α mediated inflammation of RA-FLSs

After interference by siTREM-2, mRNA and protein expression levels of TREM-2 were significantly reduced compared with NC (negative control) group and siNC (RA-FLSs interfered by nonsense mRNA) group. There was no effect on the proliferation of RA-FLSs. In order to investigate the role of TREM-2 in the inflammation of RA-FLSs mediated by TNF-α, the cells were incubated with TNF-α (20ng/ml) for 4 or 24 h. The interference group (TNF-α + siTREM-2) and the control groups (TNF-α + siNC and TNF-α + NC) were compared, mRNA and protein expression of IL-6, IL-1β, and MMP-13 were significantly enhanced in the interference group, while there was no significant difference between the siNC group and NC group. We also found no significant difference between the interference group and control group when no TNF-α was present (Fig. 3-4).

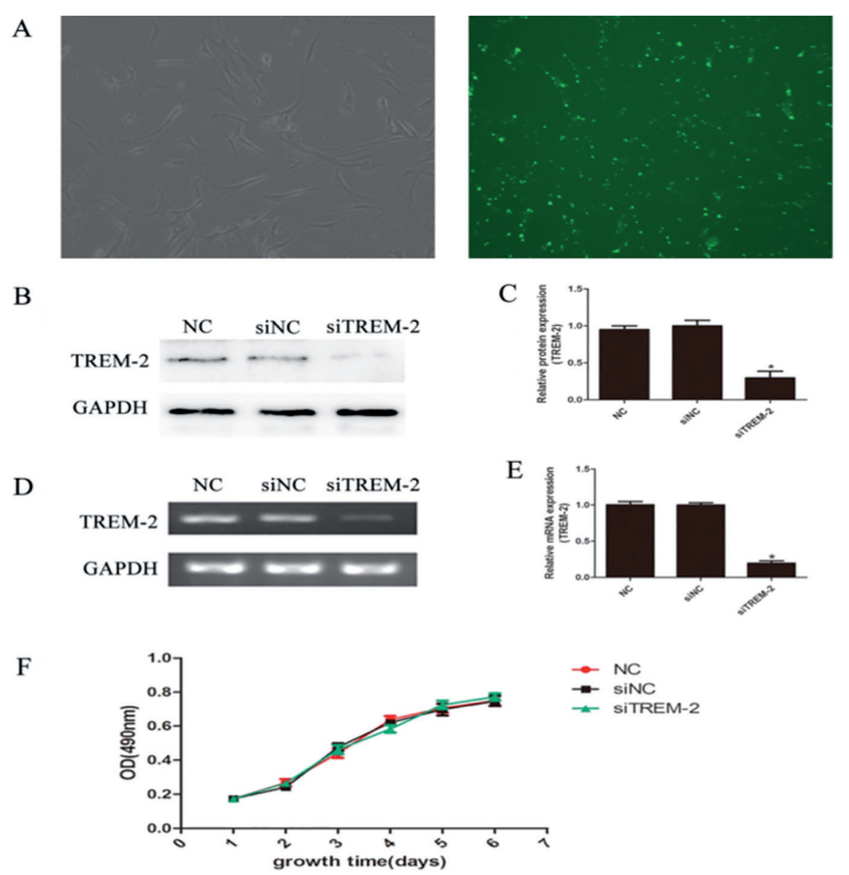

Fig. 3. siRNA silencing expression of TREM-2 in RA-FLSs.
A: RA-FLSs were transfected with FAM-siRNA for 6 h; points of FAM fluorescence were scattered in the cells (x 200); B-C: The protein expression of TREM-2 was detected after siRNA interference by Western blot. *p<0.05, compared with the NC, siNC group (n=3, $\bar{x} \pm s$). D-E: mRNA expression of TREM-2 was detected after siRNA interference by RT-PCR; *p<0.05, compared with the NC, siNC group (n=3, $\bar{x} \pm s$). F: CCK8 method was used to detect the growth curve of RA-FLSs after siRNA interference. *p >0.05, compared with the NC, siNC group (n=3, $\bar{x} \pm s$).

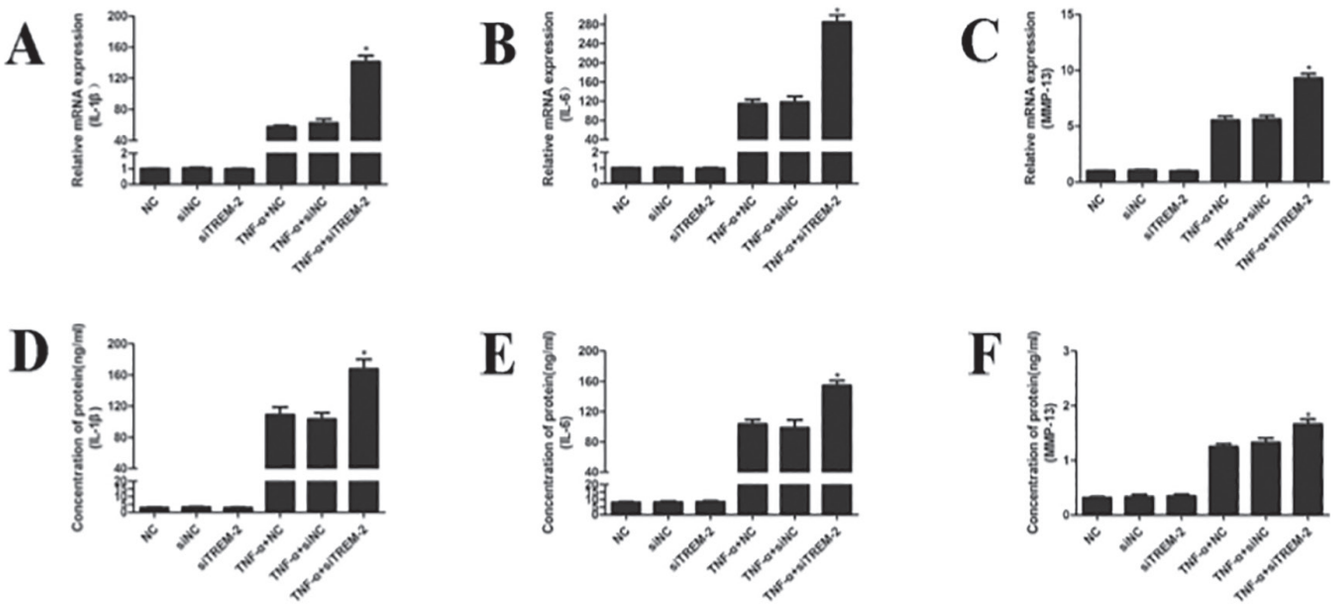

Fig. 4. Silencing TREM-2 enhanced TNF-α mediated inflammation in RA-FLSs.
A-B-C: mRNA expression of the pro-inflammatory cytokines IL-1β, IL-6, and MMP-13 in FLSs was evaluated by qPCR after cultured RA-FLSs were pre-treated with TNF-α (20ng/ml) for 4 h. **D-E-F**: The secretion level of pro-inflammatory cytokines IL-1β, IL-6, and MMP-13 in FLS was evaluated by ELISA after cultured RA-FLSs were pre-treated with TNF-α (20 ng/ml) for 24 h. *$p<0.05$, compared with the TNF-α +NC, TNF-α +siNC group (n=3, $\bar{x} \pm s$).

Overexpression of TREM-2 inhibits the inflammatory response mediated by TNF-α in RA-FLSs

After RA-FLSs were transfected with the lentivirus vector carrying a green fluorescent protein and the TREM-2 gene (LvTREM-2) or lentivirus vector only carrying green fluorescent protein (LvGFP), the results of RT-PCR and Western blot showed that the mRNA and protein expression levels of TREM-2 in FLSs were both significantly increased, while there was no effect on cell proliferation. After TNF-α stimulation for 6 or 24 h, compared with the control groups (TNF-α +LvGFP and TNF-α +NC), IL-6, IL-1β, and MMP-13 mRNA and protein expression were significantly decreased in the transfected group (TNF-α + LvTREM-2). There was no significant difference in mRNA and protein expression of IL-6, IL-1β, and MMP-13 between the transfected group and the control groups without the stimulation of TNF-α (Fig. 5-6).

TREM-2 inhibits the activation of the p38 pathway in RA-FLSs

In TNF-α (20 ng/ml) stimulated RA-FLSs exposed for 0, 15, 30, 60, or 120 min, the Western blot results showed that p-JNK, p-p38MAPK, p-ERK, and p-NF-κB p65 all increased significantly after incubation for 30 and 60 min compared with baseline (0 min) values, indicating that these cellular pathway proteins were phosphorylated.

After TNF-α (20 ng/ml) stimulation of FLSs in the different groups, expression levels of phosphorylated proteins in the cell signaling pathways were detected. TNF-α induced p38 phosphorylation was significantly enhanced in the interference group (TNF-α + siTREM-2), and the phosphorylation level of p38 was significantly decreased in the transfected group (TNF-α + LvTREM-2). After the addition of the p38 specific inhibitor SB203580 (20 μM), secretion levels of IL-1β, IL-6, and MMP-13 decreased significantly in the silent cell groups (SB203580+TNF-α + siTREM-2) compared to the control group (TNF-α + siTREM-2) under the stimulation of TNF-α. After the addition of the p38-specific agonist anisomycin (10 mg/ml), the secretion level of IL-1, IL-6, and MMP-13 in the transfected group (anisomycin + TNF-α + LvTREM-2) was significantly higher than that in the control group (LvTREM-2 + TNF-α).

Discussion

Rheumatoid arthritis (RA) is a systemic disease characterised by autoimmune synovial inflammation that results in progressive joint destruction. Activated RA-FLSs play an important role in maintaining local inflammatory microenvironments and promoting the destruction of cartilage and bone by secreting a large number of inflammatory mediators and MMPs. Therefore, inhibiting the secretion of these agents and enzymes might control RA progression (10, 11).

Previous research found that TNF-α secreted by macrophages and other cells could activate RA-FLSs that then proliferated rapidly and produced copious amounts of cytokines, proteases, adhesion molecules, and growth factors. TNF-α played a crucial role in this RA-FLS-induced pathology (12, 13). The clinical use of TNF-α inhibitors has remarkable curative effects on RA (11). IL-1β, IL- 6, and other inflammatory mediators secreted by RA-FLSs have important roles in the pathophysiology of rheumatoid arthritis (14). Although IL-6 and IL-1β production can lead to secretion of TNF-α by RA-FLSs and macrophages, that TNF-α in turn can produce cascade effects and progression of inflammation leading to chronic bony changes (15). The MMP family includes the main effector agents for producing the bone and car-

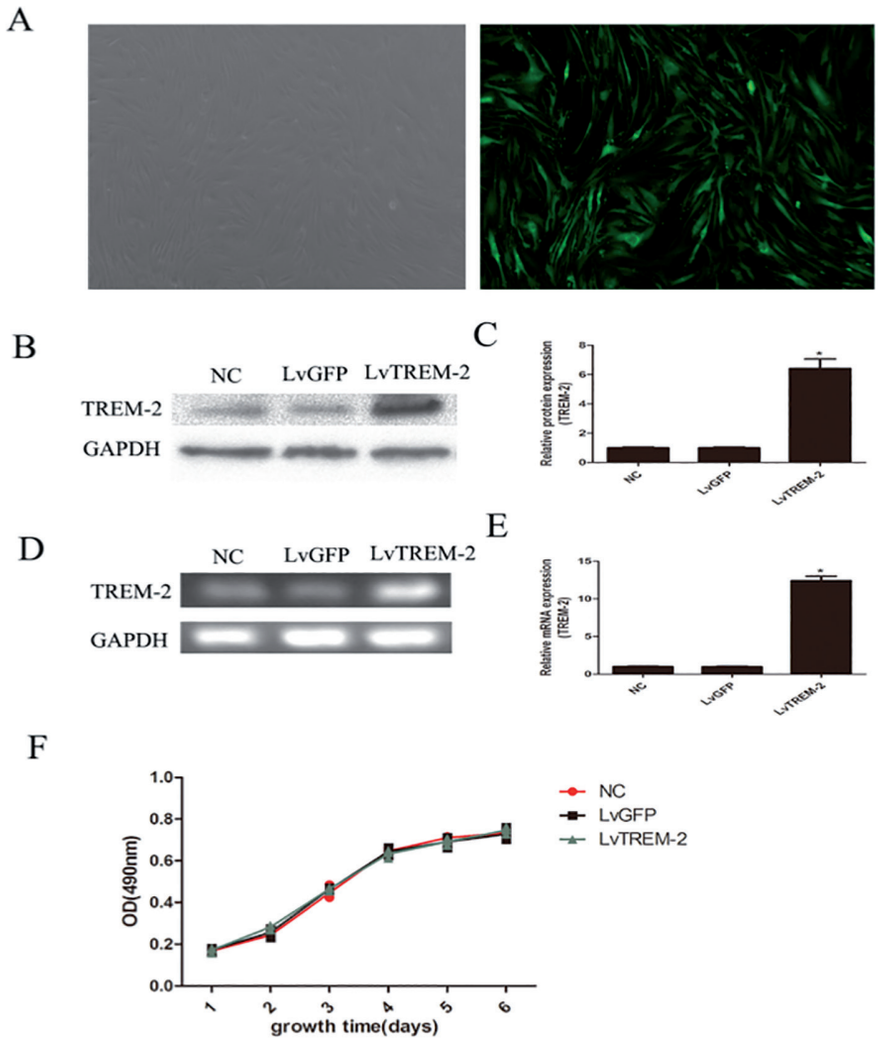

Fig. 5. Overexpression of TREM-2 in RA-FLSs by lentivirus infection.
A: RA-FLS were transfected with GFP-Lentivirus for 6 h; GFP fluorescence was scattered in the cells (×200). **B-C**: Protein expression of TREM-2 was detected after siRNA interference by Western blot, compared with NC, LvGFP groups (n=3, $\bar{x} \pm s$). *p<0.05 compared with the NC, LvGFP group (n=3, $\bar{x} \pm s$). **D-E**: mRNA expression of TREM-2 was detected after siRNA interference by RT-PCR, compared with the NC, LvGFP group (n=3, $\bar{x} \pm s$). *p<0.05, compared with the NC, LvGFP group (n=3, $\bar{x} \pm s$). **F**: CCK8 method was used to detect the growth curve of RA-FLSs after GFP-Lentivirus transfection. *p>0.05, compared with NC, LvGFP groups (n=3, $\bar{x} \pm s$).

tilage erosions induced by TNF-α (16). MMP-13 is one of these collagenases, with significant ability to erode articular cartilage by lysis of type II and IV collagen. It is far more effective for this than other MMPs and is thus regarded as the limiting enzyme for articular cartilage destruction (17, 18).

TREM-2 is expressed in myeloid cells, fibroblasts, and stem cells, as well as other cell types, and its expression changes with the cell environment. The expression of TREM-2 has a close relationship with many diseases. But there were no reports concerning the expression and function of TREM-2 in RA-FLSs (19). The present research confirmed that TREM-2 mRNA and protein (by qPCR and Western blot) is expressed in both RA-FLSs and OA-FLSs, and that the expression of TREM-2 in RA-FLS was significantly higher than that in OA-FLSs. This result suggests that TREM-2 plays a role in RA. Although TREM-2 is considered to be a membrane protein in myeloid cells, our immunofluorescence results suggest TREM-2 is mainly located in the cytoplasm of RA-FLSs. We thus suspect that there is little expression of TREM-2 in non-activated RA-FLSs, and that more TREM-2 is accumulated in the cytoplasm where proteins are synthesised and stored. Our results are consistent with the report of Sessa *et al*. (20), who suggested that TREM-2 in a variety of inactive cells is mainly associated with the Golgi apparatus and is transferred to the cell membrane when the LPS ligand was activated (20).

Previous studies have shown that the expression of TREM-2 in cells such as RAW 264.7 cells, J774.2 macrophages and microglia decreases after stimulation by LPS (21, 22). Although IL-4 and IL-13 can induce expression of TREM-2 in various cells (23), different inflammatory factors affect TREM-2 expression differently. We stimulated RA-FLSs with the pro-inflammatory mediator TNF-α and found TREM-2 expression first decreased and then increased. We speculated that although TNF-α reduces TREM-2 expression directly, it simultaneously stimulates RA-FLSs to produce IL-4, IL-13 and other mediators that then increase generation of TREM-2. This may explain why expression of TREM-2 by activated RA-FLSs was significantly higher than that of OA-FLSs. However, if RA inflammation is correlated with TREM-2 expression in RA-FLSs, it is not yet fully clear, and we would also want to know if there is any correlation between the degree of severity of RA and the concentration or activity of TREM-2 in joint fluid and blood.

The TREM family of immune receptors plays an important role in innate immunity and the inflammatory response. TREM-1 is an inflammatory response amplifier and is also highly expressed in autoimmune diseases such as inflammatory bowel disease (IBD) and RA. In contrast, TREM-2 suppresses the inflammatory response, and some investigators think TREM-2 ligands exist on the surface of abdominal and bone marrow macrophages. Others argue that the TREM-2 ligand is an ionic type, but at this time the natural ligand of TREM-2 has not been clearly identified and remains controversial (24). To verify the function of TREM-2 in RA-FLSs, we reduced TREM-2 expression using a specific siRNA interference fragment in one protocol and increased the TREM-2 expression by a lentivirus car-

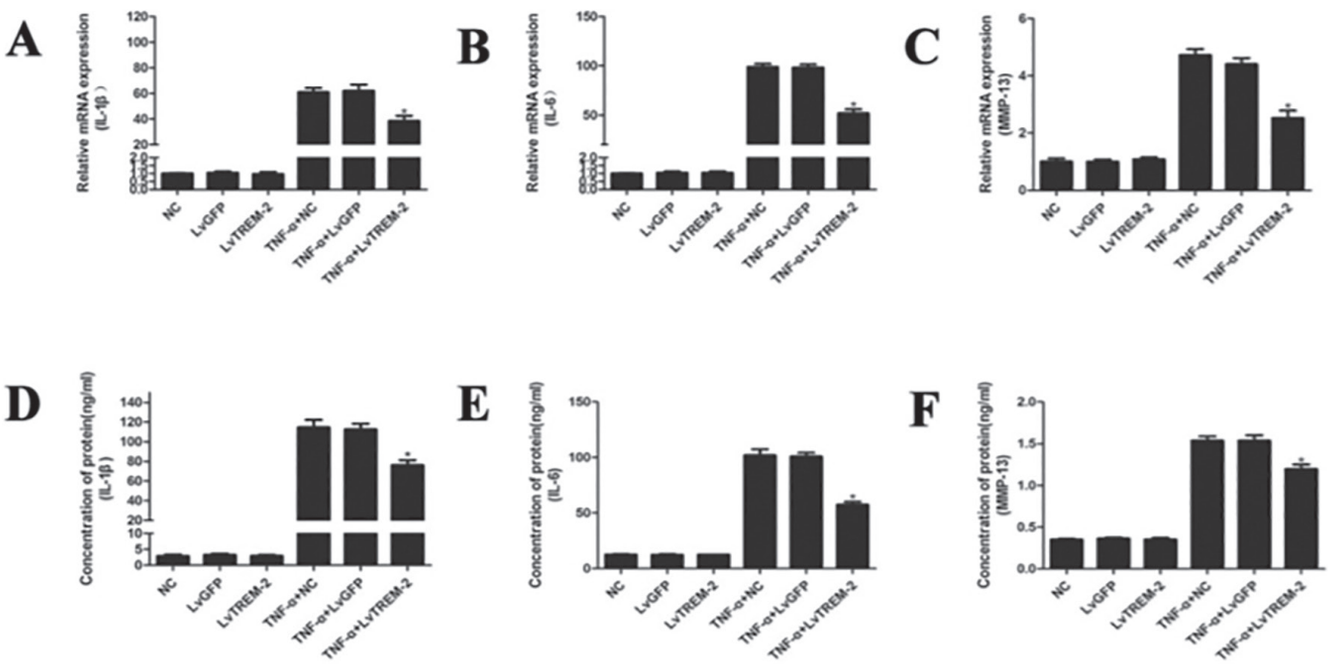

Fig. 6. TREM-2 overexpression attenuates TNF-α mediated inflammation in RA-FLSs.
A-B-C: Cultured RA-FLSs were pre-treated with TNF-α (20 ng/ml) for 4 h. mRNA expression of the pro-inflammatory cytokines IL-1β, IL-6, and MMP-13 in FLSs was evaluated by qPCR. **D-E-F**: Cultured RA-FLSs were pre-treated with TNF-α (20 ng/ml) for 24 h, The secretion level of pro-inflammatory cytokines IL-1β, IL-6, and MMP-13 in RA-FLSs was evaluated by ELISA. *p<0.05, compared with the TNF-α+LvGFP, TNF-α+NC group (n=3, $\bar{x} \pm s$).

rying a TREM-2 fragment in a second protocol. We found that overexpression of TREM-2 did not affect RA-FLSs proliferation. The secretion of IL-1β, IL-6, and MMP-13 increased significantly after suppressing expression of TREM-2 in RA-FLSs, although the secretion of IL-1β, IL-6, and MMP-13 decreased after overexpression of TREM-2. This suggested that TREM-2 may play a role in preventing the destruction of articular cartilage and decreasing progression of RA by suppressing production of these mediators.

TREM-2-mediated TNF-α inflammation, as well as the NF-κB and MAPKs pathways, have a confirmed role in RA flares-ups (25, 26). The activated NF-κB and MAPKs pathways can lead to release of inflammatory mediators and MMPs (27). Paunovic (28) found the MAPKs pathway is a key route for TNF-α-induced inflammation and includes the p38, ERK, and JNK systems. Muller-Ladner (29) reported that TNF-α may play a role in the activation of the NF-κB pathway, which is important in regulating the inflammatory response in joints. Inhibition of the ERK, p38, and NF-κB pathways significantly reduces secretion of IL-1β, IL-6, MMP-3, MMP-13, and RANKL secretion in RA-FLSs (26, 28).

We speculated that the biological effects of TREM-2 involved the NF-κB and MAPKs pathways and control of phosphorylation levels. We reduced the expression of TREM-2 in RA-FLSs and studied the activation of the p38 pathway without activation of the ERK, JNK, and/or NF-κB pathways. When the specific p38 inhibitor SB203580 was applied, the effect of silencing TREM-2 on enhancing the inflammatory response was diminished. Overexpression of TREM-2 was associated with the inactivation of the p38 pathway, without affecting the ERK, JNK, and NF-κB pathways. The effect of over-expression of TREM-2 on the inhibition of the inflammatory response was diminished when the p38 agonist anisomycin was used. We hypothesised that the p38 pathway plays an important role in RA-FLSs, by mediating TREM-2 inflammatory responses. The ERK pathway significantly affects TREM-2 activity during the differentiation of macrophages into osteoclasts (30, 31). In dendritic cells, as well as other cell types, the role of TREM-2 depends on the activation of the ERK pathway, but does not depend on the p38 or JNK pathways (32). In the present study, TREM-2 affected the p38 pathway but did not affect the expression of ERK, indicating that the regulation of TREM-2 in the different cells was variable. In the present study, in the absence of TNF-α, IL-1β, IL-6, TREM-2, and MMP-13 secretion was not significantly changed. This indicated that TREM-2 affected the secretion of inflammatory factors that depended on the participation of other pro-inflammatory mediators. We speculated that this phenomenon was related to low level activation of the p38 pathway when RA-FLSs had not been activated by TNF-α.

Conclusions

In conclusion, in the present study we first confirmed that the expression of TREM-2 occurred in RA synovial cells. We also found that its expression was regulated by TNF-α, and that activation of TREM-2 reduced inflammation by inhibiting the p38 pathway. If the regulatory mechanisms of TREM-2 on the inflammatory response can be further defined, it may prove to be useful as a target for control of inflammation in rheumatoid arthritis.

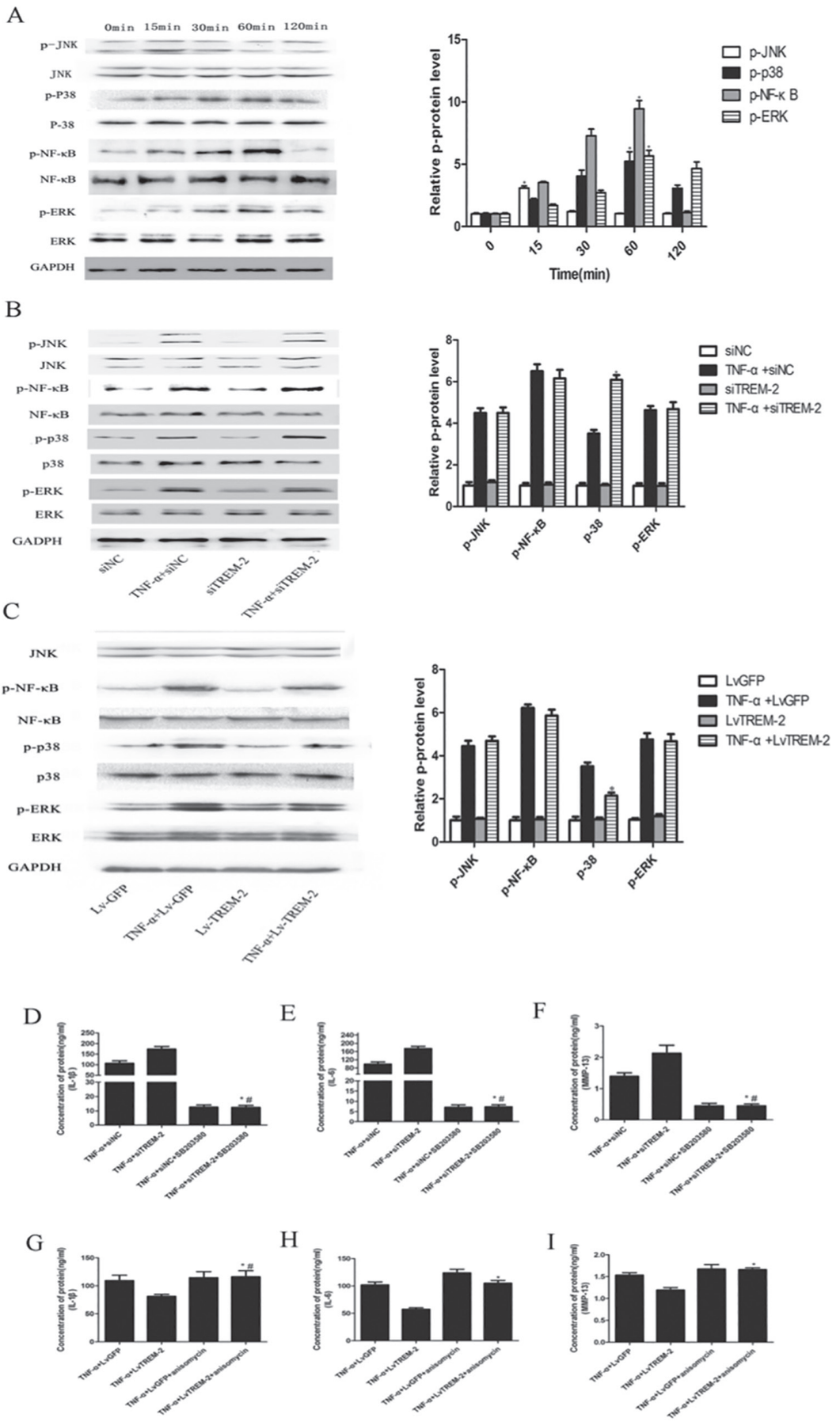

Fig. 7. TREM-2 mediated TNF-α induced inflammation in RA-FLSs via p38 pathway.
A: After RA-FLSs were incubated with TNF-α (20ng/ml) for 0, 15, 30, 60, 120 min, the intracellular levels of ERK1/2 and p-ERK1/2, p38 and p-p38, JNK and p-JNK as well as the levels of NF-κB p65 and p- NF-κB p65 were analysed by Western blot.
**p*<0.05, compared with groups stimulated by TNF-α at other time.
B: Western blot analysis of the intracellular levels of ERK1/2 and p-ERK1/2 (60 min), p38 and p-p38 (30 min), JNK and p-JNK (15 min)as well as the levels of NF-κB p65 and p-NF-κB p65 (60 min) in siNC, TNF-α (20ng/ml)+siNC, siTREM-2, TNF-α (20ng/ml)+siTREM-2 group.
*p<0.05, compared with TNF-α (20ng/ml) + siNC group.
C: Western blot analysis of the intracellular levels of ERK1/2 and p-ERK1/2 (60 min), p38 and p-p38 (30 min), JNK and p-JNK (15 min) as well as the levels of NF-κB p65 and p-NF-κB p65 (60 min) in LvGFP, TNF-α (20ng/ml)+LvGFP, LvTREM-2, TNF-α (20ng/ml)+LvTREM-2 group.
**p*<0.05, compared with the TNF-α (20ng/ml)+LvGFP group.
D-E-F: Cultured RA-FLSs in different group were incubated with SB203580 (inhibitor of p38, 20 μM), and the concentrations of IL-1β, IL-6, and MMP-13 in culture supernatants were determined by ELISA 24h after TNF-α stimulation.
**p*<0.05, compared with TNF-α+siTREM-2 group. #*p*>0.05, compared with TNF-α+siNC+ SB203580 group (n=3, $\bar{x} \pm s$).
G-H-I: Cultured RA-FLSs in different groups were incubated with anisomycin (agonist of p38, 10 mg/ml), and cell concentrations of IL-1β, IL-6, and MMP-13 in culture supernatants were determined by ELISA 24 h after TNF-α stimulation.
**p*<0.05, compared with TNF-α + LvTREM-2 group. #*p*>0.05, compared with TNF-α + LvGFP + anisomycin group (n=3, $\bar{x} \pm s$).

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