Inhibition of transient receptor potential canonical 6 attenuates fibroblast-like synoviocytes mediated synovial inflammation and joint destruction in rheumatoid arthritis

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Abstract

Objective

We aimed to define the importance of transient receptor potential canonical 6 (TRPC6) expression and function in fibroblast-like synoviocytes (FLSs) and to investigate the contribution of TRPC6 in the model of rheumatoid arthritis (RA).

Methods

We compared TRPC6 expression levels in FLSs from RA patients (RA-FLSs), and in FLSs from osteoarthritis (OA) patients (OA-FLSs). By using vitro functional assays which united with small interfering RNA-induced knockdown and functional modulation of TRPC6 in RA-FLSs. Finally, we confirmed the effectiveness of regulating TRPC6 in a collagen induced arthritis (CIA) mice model.

Results

We found that FLSs expressed the TRPC6 as their major Transient receptor potential canonical channel. Both mRNA and protein expression of TRPC6 were found somewhat higher levels in RA-FLSs than in OA-FLSs. Moreover, inhibiting expression of TRPC6 in vitro reduced proliferation of, as well as inflammatory mediator and protease production by, RA-FLSs, whereas opening native TRPC6 enhanced both proliferation and inflammatory mediator of RA-FLSs. Additionally, a TRPC6 deficiency in mice blunted the development of experimental RA, CIA models, reduced joint and bone damage, and inhibited FLS invasiveness and proliferation.

Conclusion

Our results demonstrated a critical role of TRPC6 in regulating FLSs mediated inflammation. Therefore, TRPC6 represents potential therapeutic targets in RA.

Key words

rheumatoid arthritis, transient receptor potential canonical 6, fibroblast-like synoviocytes

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Introduction

The main pathological features of rheumatoid arthritis (RA) are proliferation of synovial lining cells, infiltration of interstitial inflammatory cells, microvascular proliferation, angiogenesis, destruction of cartilage and bone tissue, and joint dysfunction (1). Activated fibroblast-like synoviocytes (FLSs) are thought to play a pivotal role in occurrence and progression of RA (2). More and more research demonstrates that the activation of synovial cells, for instance FLSs, can secrete a large number of inflammatory cytokines, chemokines which can induce the accumulation and activation of inflammatory cells (1). In return, the cytokines from inflammatory cells were able to accelerate the synovial cells activation (3). The cascade of reactions resulted in the rapid activation of synovial cells, and the outburst of synovial hyperplasia, the prominent formation of RA pannus was able to invade the adjacent cartilage and bones. Therefore, understanding the mechanism of FLSs activation and reducing the pathogenic characteristics of RA-FLSs is of great importance to establish a new target in treatment of RA.

Calcium (Ca^{2+}) acted as a second messenger in many cell types. Previous studies have shown that intracellular calcium concentration ($[Ca^{2+}]i$) played an important role in the proliferation and apoptosis of RA-FLSs (4). The extracellular calcium influx channel can be divided into three main categories, voltage-dependent calcium channel (VOC), receptor operated calcium channel (ROC) and store operated calcium channel (SOC). In particular, SOC was involved in immune responses and inflammatory diseases. The difference of expression and function of SOC channel-related proteins in inflammatory cells such as T, B lymphocytes could affect $[Ca2^{+}]i$, which consequently resulting in cellular functions change. These effects could regulate proliferation of inflammatory cells, secretion of inflammatory factors, apoptosis, and migration. However, the role of SOC in progress of RA remained unclear.

Previous studies have found that pretreatment with SOC inhibitor, YM-58483, could prevent and relieve collagen induced arthritis (CIA); when CIA was successfully induced, treatment with YM-58483 could decreased production of IL-1β, IL-6 and TNF-α, improved degree of arthritis disease, attenuated the extent of cartilage and bone erosion damage, and in a dose-dependent manner (5). A similar study observed that in $CD4^{+}$ T cells derived from RA patients, SOC structural protein expression and function were upregulated (6). These results suggested that SOC was a potential drug targets for the treatment of RA.

RA is believed to be a chronic inflammatory autoimmune disease, influenced by both genetic and environmental factors (7). It is currently believed that the SOC formation based Transient receptor potential canonical (TRPC) channel is involved in the regulation of cell proliferation, gene transcription, and other physiological effects through the mediation of Ca^{2+} mediated calcium influx (SOCE). Alawi *et al*. reported TRPC 5 was involved in the joint inflammation (8). Furthermore, a genome-wide association study (GWAS) on RA found that mutations in the TRPC6 gene in patients with RA and was associated with RA disease progression (9). However, the contribution of TRPC6 in the pathogenesis of RA remains poorly revealed. In this study, we adopted a potent and selective TRPC6 agonist, Hyp9 (10, 11), to study the role of TRPC6 in synovial inflammation and joint destruction in RA disease model.

Materials and methods

Animals and cells

This study was approved by the ethics committee of Sun Yat-sen Memorial Hospital (Guangzhou, China). Thirteen patients with OA (Supplementary Table S1) and 18 patients with RA (defined according to the criteria of the American College of Rheumatology [12, 13]), provided their written informed consent. FLSs were isolated from synovial tissues and maintained in culture as previously described (14). FLSs were used between passages 3 and 8 for all experiments.

Experiments involving mice were executed by the Institutional Animal Care and Ethics Committee of Sun Yat-sen Memorial Hospital and complied with the Guide for the Care and Use of Laboratory Animals, which was published

by the US National Institutes of Health. Animal studies were reported in compliance with the ARRIVE guidelines. Age-matched 129Sv/C57BL/6J TRPC6 WT and TRPC6-/- mice were obtained from Dr S.E. Tilotta (National Institute for Environmental Health Sciences, USA). All mice were bred from established breeding pairs and were used at 8–12 weeks of age.

Arthritis induction and assessment of arthritis

Collagen-induced arthritis CIA was induced in C57BL/6 mice according to common methods shown in an earlier publication (14). Experiments were performed in accordance with the Principles of Laboratory Animal Care which directed by the Sun yat-sen university. Chicken type II collagen (4 mg/mL final concentration) (Chondrex, USA) was dissolved in 0.1 M acetic acid at 4°C overnight and then emulsified with an equal volume of complete Freund's adjuvant containing 4 mg/mL Mycobacterium tuberculosis. 8-week-old male mice were immunised subcutaneously at the base of the tail with 100 μL of the emulsion. At day 21 from primary induction, mice were boosted intraperitoneally with 100 μL of bovine type II collagen emulsion prepared with incomplete Freund's adjuvant. Arthritis was assessed blindly by using four paws from each mouse according to previous described scores criteria (15). Paw swelling was assessed by measuring the thickness of affected paws with calipers. Serum cytokines were measured by using a Luminex suspension array technology (BD Biosciences, San Jose, CA).

Operation of ion channel expression and function

We used Hyp9 (Sigma-Aldrich USA) as a TRPC6 agonist (10, 11). TRPC6 small-interfering (si) RNA, non-silencing control siRNA and GAPDH siRNA were obtained from GenePharma Biotech and Engineering Company (Shanghai, China). The sequences of TRPC6 siRNA oligonucleotides were present as follows: plus-sense: CCGCUAUGAACUCCUUGAATT and anti-sense: UUCAAGGAGUUCAUAGCGG TT. When the FLSs were grown to about 60% confluence, transient transfections were applied by using Lipofectamine RNAiMAX (Invitrogen USA). The FLSs were incubated with 100nM siRNA for 6 h in serum-free medium. And then the cell medium containing 10% serum was added and incubated for 42 h before experiments. At the end of the culture, the effect of the siRNA on TRPC6 expression was analysed using quantitative real-time PCR and Western blot analysis.

Immunofluorescence

RA-FLS was grown overnight on confocal dishes (NEST China). They were fixed in instantly prepared ice-cold 0.01 M PBS-4% formaldehyde for 15 min and rinsed 3 times with 0.01 M PBS. After washing and blocking by BSA-TBS-T (3% bovine serum albumin in 10 mM Tris, pH 8.0/0.15M NaCl+0.3% TritonX-100) with soft rocking at room temperature for 30 min, cells were incubated with the primary antibodies against TRPC6 (ACC-017, Alomone Labs, Israel) (1:500 dilution in 3%BSA) overnight at 4°C. The secondary antibody (goat anti- Rabbit IgG conjugated to Alexa Fluor 488, 1:1000 dilution in3%BSA, Invitrogen, USA) was incubated for 1 h in the dark at room temperature, followed by washes. Negative controls, in which the primary antibody was neglected, were similarly treated as described above. The nuclei were stained with the fluorescent DNA-binding dye 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI, Roche, Switzerland) for 15 min at room temperature. Staining was detected with a confocal laser scanning microscopy (Leica TCS SP8, Germany).

Calcium imaging

Cells were grown on confocal dishes and loaded in Hanks' balanced salt solution (HBSS) containing Fluo-4/AM (5 μM, Molecular Probes Eugene, OR, USA) and Pluronic F-127 (0.03%, Sigma, St. Louis, MO, USA) at 37°C for 30 min. The fluorescence intensity of Fluo-4 in FLSs was recorded using confocal laser scanning microscopy (Leica TCS SP8, Germany). The HBSS used in following experiments contained 1.8 mM CaCl2. Hyp9, LPS (Sigma-Aldrich, USA) and IL-1β (Peprotech, USA) were used to stimulate FLSs when the baseline is stable. The $[Ca^{2+}]$ i was expressed as a relative-ratio value (F/F0) of the actual fluorescence intensity (F) divided by the average baseline fluorescence intensity (F0). Data from 15 to 20 cells were compiled from a single run, and at least three independent experiments were conducted.

Quantitative real-time PCR (qPCR)

Total RNA was isolated from the RA-FLSs and then transfer to cDNA by using Takara PrimeScript® RT reagent kit (Takara, Dalian, China) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the StepOnePlus™ Real-Time PCR system. The primers applied for real-time PCR are listed in Supplementary Table S2. 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 (ΔΔCt). All experiments were performed in triplicate.

Western blot analysis

Western blot analysis was made as described previously (11). The protein concentrations were measured using a BCA protein assay (Pierce, Rockford, IL, USA). Primary antibodies were diluted 1:1000 for TRPC6, p38 mitogen-activated protein kinase (MAPK), p-p38MAPK, extracellular signal regulated kinase (ERK), phospho -ERK, Jun N-terminal kinase (JNK), phospho -JNK, protein kinase B (AKT), phospho -AKT, nuclear factor-kappaB (NF-κB) p65, phospho -NF-κB p65 and GAPDH , and 1:500 for IKKα/β, phospho-IKKα/β, IκBα, and phospho-IκBα. Densitometry was performed using a ChemiDoc™ MP-Bio-Rad system.

Histopathologic analysis

Mice were sacrificed by cervical dislocation at day 50 after first immunisation. Knee tissues were randomly collected, then fixed with 10% paraformaldehyde, decalcified in 5% formic acid and embedded in paraffin. Next, 7 μm sections were stained with haematoxylin and eosin (H&E) and evaluated by light microscopy. Estimations of the histological pathologies were quantified by cellular infiltration, synoviocyte

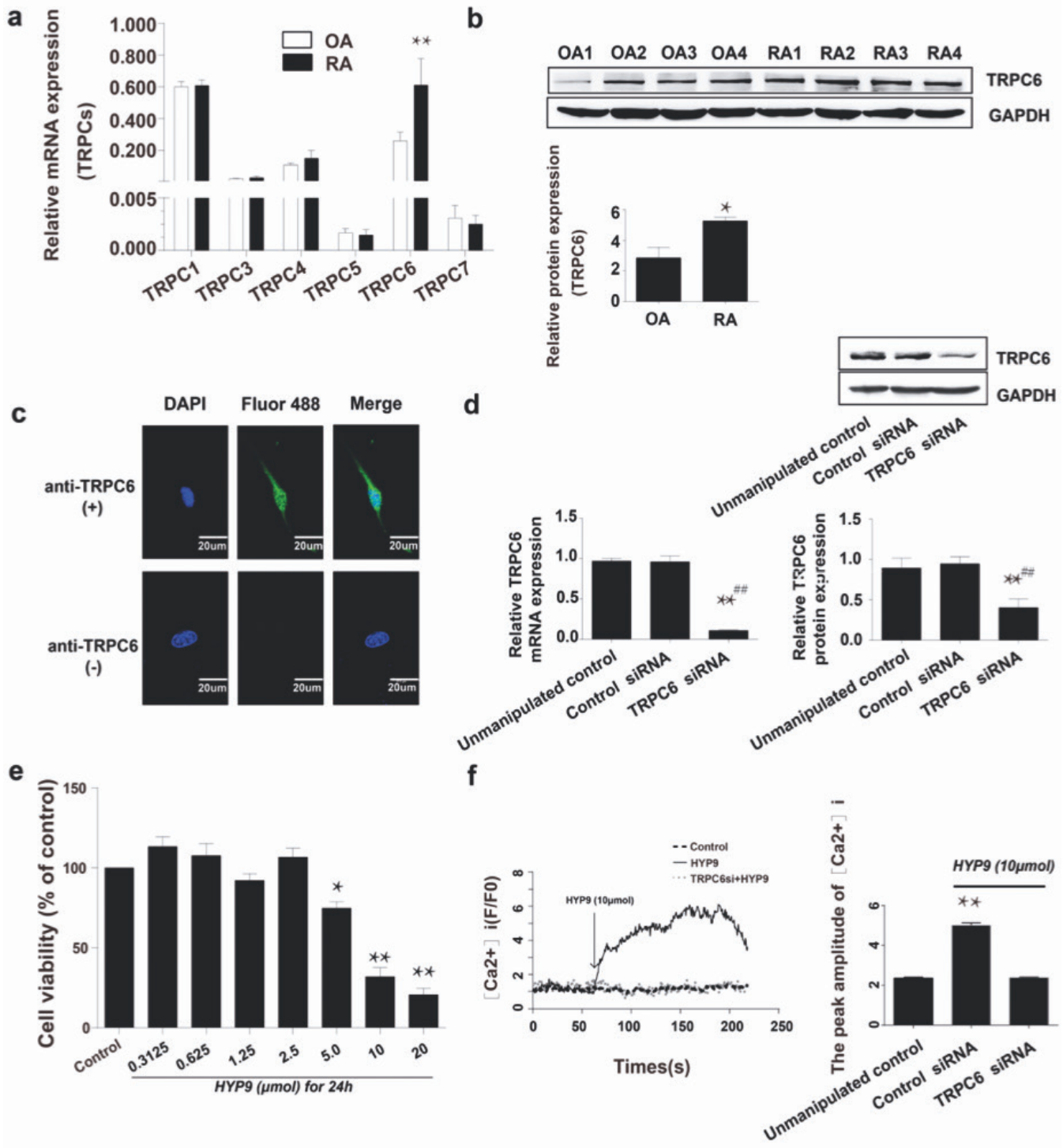

Fig. 1. Expression forms of TRPC6 in FLSs from patients with RA.
a: TRPC subfamily mRNA expression in FLSs. TRPC mRNA expression was determined by qPCR analysis. The data show the means ± SEM from FLSs obtained from the synovial tissues of patients with RA (n=6) and OA (n=6) (**p<0.01 *vs*. OA). **b**: Typical images of Western blot analyses showing the expression of TRPC6 in RA-FLSs (n=5) and OA-FLSs (n=5). The data represent the means ± SEM of the densitometric scanning (*p<0.05 *vs*. OA).
c: The localisation of TRPC6 in RA-FLSs. Immunofluorescence staining was used to determine the expression of TRPC6. The nuclei were stained with DAPI, Representative laser confocal microscopy images show the staining of TRPC6 from patients with RA (n=4) and OA (n=4).
d: TRPC6 siRNA significantly impeded the mRNA and protein expressions of TRPC6.
e: Cell viability by CCK-8 assay was significantly attenuated in a concentration-dependent manner in RA-FLSs were treated with hyp9 (0-20μM) for 24 h in serum-free medium (n=4) (*p<0.05, **p<0.01 *vs*. control).
f: Hyp9 activated TRPC6 resulting in Ca2+ influx. Intracellular calcium concentration ([Ca2+]i) dynamics were monitored using fura-4 fluorescence methods; (n=4), (**p<0.01 *vs*. unmanipulated control).
FLSs: Fibroblast-like synoviocytes; DAPI: 4'-6-diamidono-2-phenylindole-dihydrochloride; TRPC: Transient receptor potential canonical. siRNA: Small-interfering RNA; CCK: Cell Counting Kit. F/F0, Actual fluorescence intensity over the average baseline fluorescence intensity.

proliferation, pannus formation and cartilage damage, which were scored using the following parameters: inflammation - 0 (normal) to 4 (severe inflammation with necrosis and oedema), and joint destruction - 0 (normal) to 4 (severe extensive areas of cartilage ulcerations).

Luminex suspension array technology

Homogenate was treated with 0.9% normal saline for 10 min, centrifuged at 1000 g for 10 min and supernatant was used for further analysis. Plasma levels of IFN-γ, IL-1β, IL-6, IL-10, IL-12, IL- 17 and TNF-α were measured by Luminex suspension array using a specific kit, according to manufacturer's instructions (Merck Millipore, Germany). In brief, 25uL of plasma sample was mixed with 25uL of Assay Buffer and 25uL of beads and the mixture was loaded on 96-well plate and incubated at 4°C in dark with shaking overnight. The next day, the liquids in the 96-well plate were removed, and the plate was washed twice with 200uL Wash Buffer. Subsequently, 25uL of antibodies were added into each well and were incubated with 25uL of PE conjugated Streptavidin at room temperature for 30 minutes with shaking. The liquid was removed and the plate was washed twice with 200uL Wash Buffer. Then, 100uL of driving liquid was added into the plate and samples were analysed with MagPlex instrument (Merck Millipore, Germany).

Statistical analysis

Statistical analyses were performed with SPSS 22.0 statistical software (SPSS, IBM, Armonk, NY, USA). Data are expressed as the means ± SEM. All experimental results were replicated 3 to 4 times. The data were normalised as the fold over the mean of the control. 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 TRPC6 in RA-FLSs was related to OA-FLSs

First, we determined the expression types of TRPCs in FLSs. Analysis of FLSs by qPCR revealed that TRPC1-7 were transcribed in FLSs from RA and OA patients. However, we further observed that only TRPC6 expression was increased in RA-FLS when comparing to the expression levels in OA-FLSs (Fig. 1a). Next, we evaluated the protein expression of TRPC6 in FLSs was increased in RA compared with that in OA patients (Fig. 1b). Confocal microscopy revealed that TRPC6 protein was localised predominantly in the plasma membrane (Fig. 1c). In addition, as shown in Fig.1d, the mRNA and protein expressions of TRPC6 were significantly reduced by TRPC6 siRNA, which indicated that the transfection was efficient. To determine whether HYP9 is toxic to RA-FLSs, we incubated RA-FLSs for 24h with varying amounts of HYP9. A HYP9 concentration of 5μM induced approximately 20% cell death, and a concentration of 10 or 20μM induced about 70% or 80% cell death (Fig. 1e). These results showed that HYP9 is not toxic to RA-FLSs in the proper range.

TRPC6 is a considerable regulator of $[Ca^{2+}]i$. Therefore, we further confirmed the role of TRPC6 in calcium homeostasis of RA-FLSs. As shown in Fig. 1f, the HYP9 induced an influx of calcium in RA-FLSs, while this calcium influx was not induced by the inhibition of TRPC6 with its siRNA.

LPS-induced inflammatory response was mediated by TRPC6 in RA-FLSs

In the present study, we treated RA-

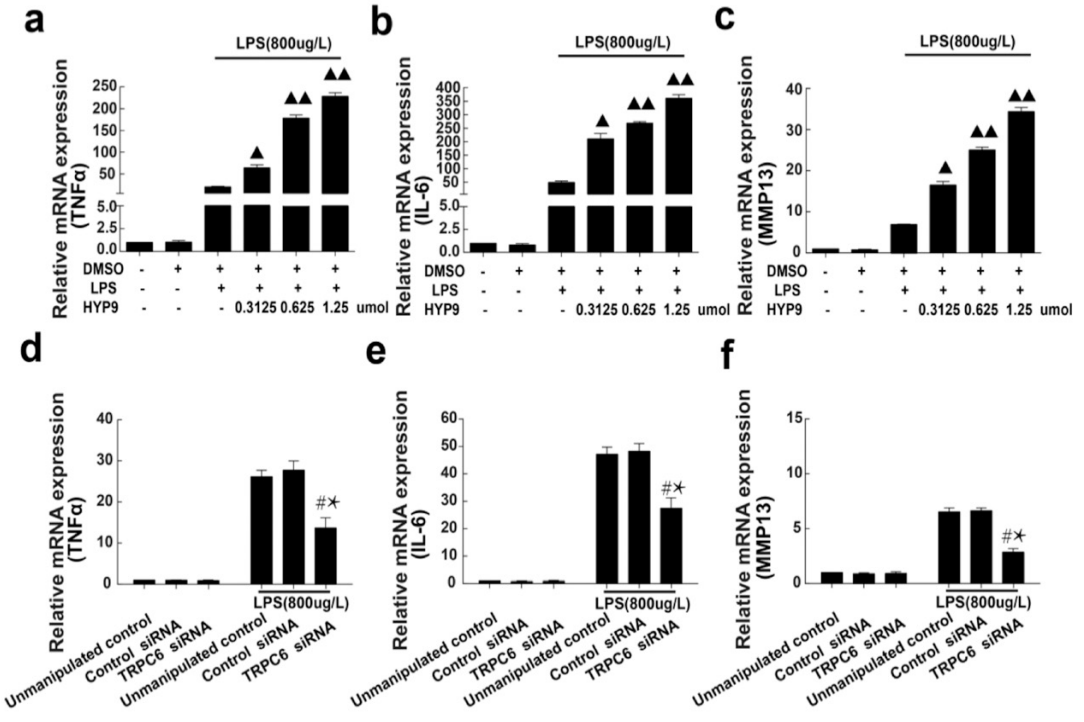

Fig. 2. Effects of TRPC6 on the expression of proinflammatory cytokines and matrix metalloproteinase in RA-FLSs of LPS-induced inflammatory response. RA-FLSs were pretreated with DMSO or various concentrations of a selective TRPC6 agonist, hyp9, for 0.5 h (**a**, **b**, **c**) or were transfected with specific TRPC6 siRNA or control siRNA (**d**, **e**, **f**) and then stimulated with or without LPS (800μg/L) for 6 h. The expression of TNF-α, IL-6, and MMP-13 was measured by qPCR. The data are representative of independent experiments (means ± SEM) from 5 RA patients. ▲ $p<0.05$, ▲▲ $p<0.01$, significantly different from treatment with LPS alone, *$p<0.05$, #$p<0.05$, significantly different from unmanlpulated control or control siRNA.

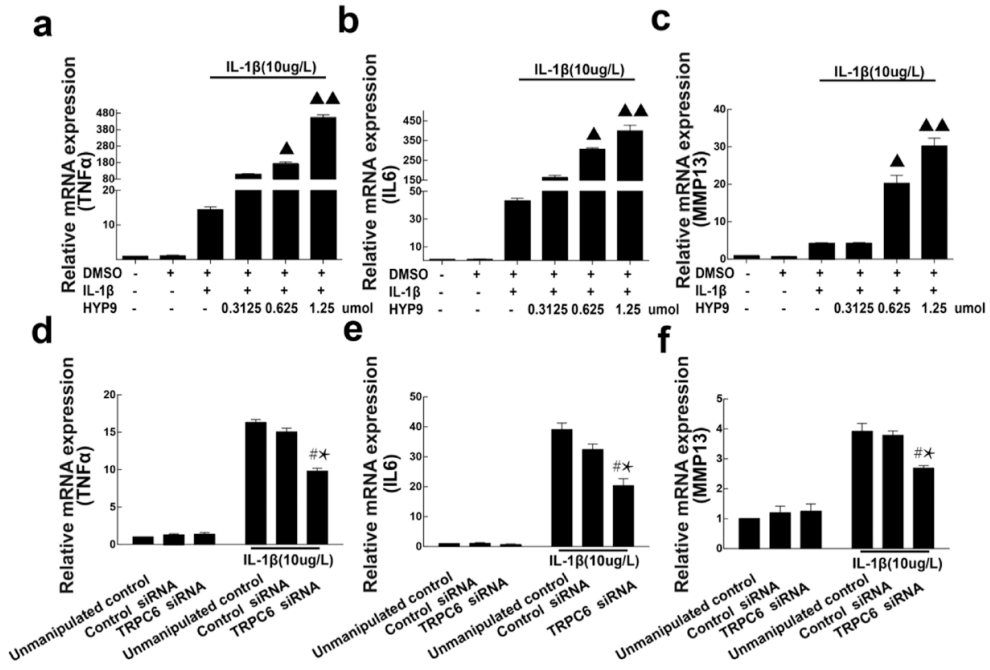

Fig. 3. Roles of TRPC6 on the transcription of proinflammatory cytokines and matrix metalloproteinase in RA-FLSs of IL-1β-induced inflammatory response. RA-FLSs were pretreated with DMSO or different concentrations of TRPC6 agonist, hyp9, for 0.5 h (**a**, **b**, **c**) or were transfected with TRPC6 siRNA or control siRNA (**d**, **e**, **f**) and then irritated with or without IL-1β (10μg /L) for 6h, expression of TNF-α, IL-6, and MMP-13 was measured by qPCR. The data are representative of independent experiments (means ± SEM) from 5 RA patients. ▲ $p<0.05$, ▲▲ $p<0.01$, significantly different from treatment with IL-1β alone, *$p<0.05$, #$p<0.05$, significantly different from un-manipulated control or control siRNA.

FLSs with LPS to establish an inflammation model. We investigated the role of TRPC6 in LPS-induced inflammatory response in RA-FLSs by stimulating with TRPC6 activator and siRNA. RA-FLSs were treated with varying amounts of HYP9 or 800 μg/L LPS for 6 h, the mRNA of TNF-α, IL-6 and Matrix metallopeptidases (MMP) -13 were measured. As shown in Fig. 2a-c, LPS increased the mRNA levels of TNF-α, IL-6 and MMP-13 in RA-FLSs, and the levels further upregulated when RA-FLSs was stimulated together with LPS and HYP9. These results demonstrated that HYP9 could exacerbate LPS-induced inflammatory response. Furthermore, as expected, knockdown of TRPC6 could significantly inhibit LPS-induced expression of TNF-α, IL-6 and MMP-13 (Fig. 2d-f).

TRPC6 increased the inflammatory response to RA-FLSs induced by IL-1β

IL-1β is a major component of the RA inflammatory micro-environment (16). We therefore investigated the role of TRPC6 in the inflammation of RA-FLSs mediated by IL-1β. Related experimental steps for qPCR analysis were performed as previously described. RA-FLSs were pretreated with different concentrations of HYP9 for 30 min and IL-β with and without treated for 6 h. The results of qPCR showed that the mRNA expression of TNF-α, IL-6 and MMP-13 in the HYP9 pretreatment group was significantly higher than that in the IL-1β treatment group, and it was in a dose-dependent manner (Fig. 3a-c). Next, we found that the mRNA expression of TNF-α, IL-6, and MMP-13 in three groups (TRPC6-siRNA group, control siRNA group, and unmanipulated control group), in the absence of IL-1β stimulation, was no significant difference. But, after IL-1β incubated, the mRNA expression levels of TNF-α, IL-6, and MMP-13 in all groups were significantly increased. Compared with the control siRNA group and the unmanipulated control group, the TRPC6-siRNA group presented a significantly lower increase (Fig. 3d-f).

These results demonstrated that HYP9 enhanced IL-1β-mediated inflammatory response. Furthermore, as predicted, knockdown of TRPC6 could significantly inhibited IL-1β induced expression of TNFα, IL-6 and MMP-13 (Fig. 3).

TRPC6 was required for the activation of NF-κB pathway in RA-FLSs induced by LPS

The nuclear factor-kappa B (NF-κB) pathway was well known to play a key role in joint inflammation. Thus, we tested whether NF-κB was the downstream signal pathways of TRPC6. As shown in Fig. 4a, 400μg/L LPS induced rapid phosphorylation of ERK1/2 and NF-κB p65 at 15 min, and it also activated NF-κB p65 at 30, 60, 120min. Knockdown of TRPC6 suppressed LPS-stimulated NF-κB p65 phosphorylation, but ERK1/2, (Fig. 4b). As predicted, HYP9 enhanced LPS-mediated NF-κB p65 phosphorylation (Fig. 4c). These data suggested that TRPC6 mediated LPS-induced activation of NF-κB. Furthermore, we observed a exaltation in phosphorylated IKKα/β following treatment with TRPC6 siRNA in LPS-stimulated RA-FLS (Fig. 4d). Inconsistent with the exaltation IKK activity, knockdown of TRPC6 suppressed the LPS-induced phosphorylation and degradation of IκBα (Fig. 4d). In return, HYP9 enhances the LPS-induced phosphorylation and degradation of IκBα (Fig. 4e). In addition, we also evaluated the role of TRPC6 in regulating the MAPK activation pathways, which was an important pathway in regulating of synovial inflammation and joint destruction in RA. We observed that phosphorylation of p38, JNK and AKT was not significant different induced after treatment with LPS, (Fig. 4a).

TRPC6 enhanced the activation of the NF-κB pathway in RA-FLSs mediated by IL-1β

As shown in Fig. 4a, 5μg/L IL-1β induced speedy phosphorylation of p38 and NF-

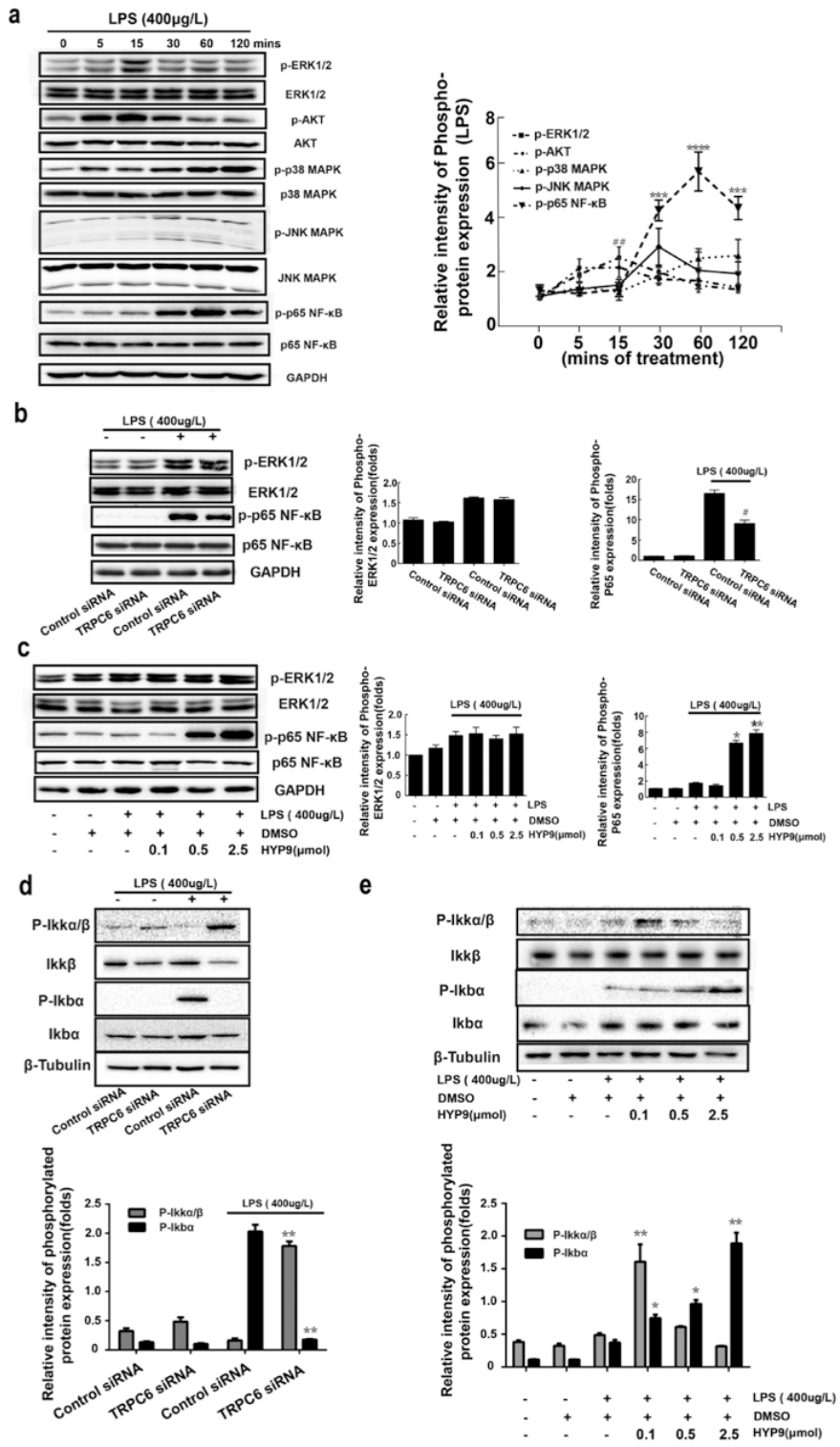

Fig. 4. Involvement of TRPC6 in the LPS-mediated activation of NF-κb pathways by RA-FLSs. RA-FLSs were stimulated by LPS (400μg/L) for 0, 5, 15, 30, 60 and 120 mins; n=4, $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* 0 min p-ERK1/2 group, $^{*}p<0.05$, $^{**}p<0.05$ *vs.* 0 min p-p65NF-κB group (**a**).
RA-FLSs were pretreated with DMSO or various concentrations of a selective TRPC6 agonist, hyp9, for 0.5h, or were transfected with specific TRPC6 siRNA or control siRNA and then stimulated with or without LPS (400 mg/L) for 0.25 or 1.0h. Effects of TRPC6 on the phosphorylation of ERK1/2 and p65NF-κB were induced by LPS (**b**, **c**).
Effect of TRPC6 on IKK and IκBαphosphorylation mediated by LPS. The right or down panel shows a densitometric analysis of immunoblot analyses from four independent experiments.
$^{*}p<0.05$, $^{**}p<0.01$, significantly different from treatment with LPS alone, $^{\#}p<0.05$, significantly different from control siRNA. p: phosphorylation.

κB p65 at 5 min, and it also activated NF-κB p65 at 15, 30, 60 min. Knockdown of TRPC6 suppressed the IL-1β-stimulated phosphorylation of NF-κB p65, also p38 (Fig. 5a). As expected, HYP9 enhanced IL-1β-mediated NF-κB p65 phosphorylation (Fig. 5b). These data suggested that TRPC6 mediated IL-1β-induced activation of NF-κB. Furthermore, we observed a exaltation in phosphorylated IKKα/β following treatment with knockdown TRPC6 in IL-1β-stimulated RA-FLS (Fig. 5c). Inconsistent with the exaltation IKK activity, treatment with TRPC6 siRNA suppressed the IL-1β-induced phosphorylation and degradation of IκBα (Fig. 5d). In return, HYP9 enhanced the IL-1β-induced phosphorylation and degradation of IκBα (Fig. 5e). We also observed that phosphorylation of ERK1/2, JNK and AKT was not significant difference induced after treatment with IL-1β, (Fig. 5a).

Deletion of TRPC6 reduced CIA joint damage

As previously described, CIA mice presented with highly abnormal joint histology including pronounced synovial hyperplasia and pannus formation with immune infiltrates in the synovium and periosteum, angiogenesis, fibrosis, and cartilage erosions (Fig. 6a-b). TRPC6 knockout (KO) group presented low rate of induction and milder disease progression such as invasiveness and proliferation of FLS in mice with CII-induced arthritis.

We next assessed histopathologic changes (Fig. 6d). In normal joints, the synovial membrane usually contains a monolayer of synoviocytes, whereas in CIA mice, synoviocytes over-proliferated and grew into multiple layers that were infiltrated with various inflammatory cells. Inflammation induced by CIA was associated with cellular infiltration, synoviocyte proliferation, pannus formation and cartilage damage. In TRPC6 KO group, arthritis cartilage destruction and inflammatory cell infiltration were both significantly suppressed.

The inflammation cytokine level in blood sample also shown significant changes between CIA group and TRPC6 KO group (Fig. 6f). IL-6, TNF-α and IL-12 in WTCIA group were signifi-

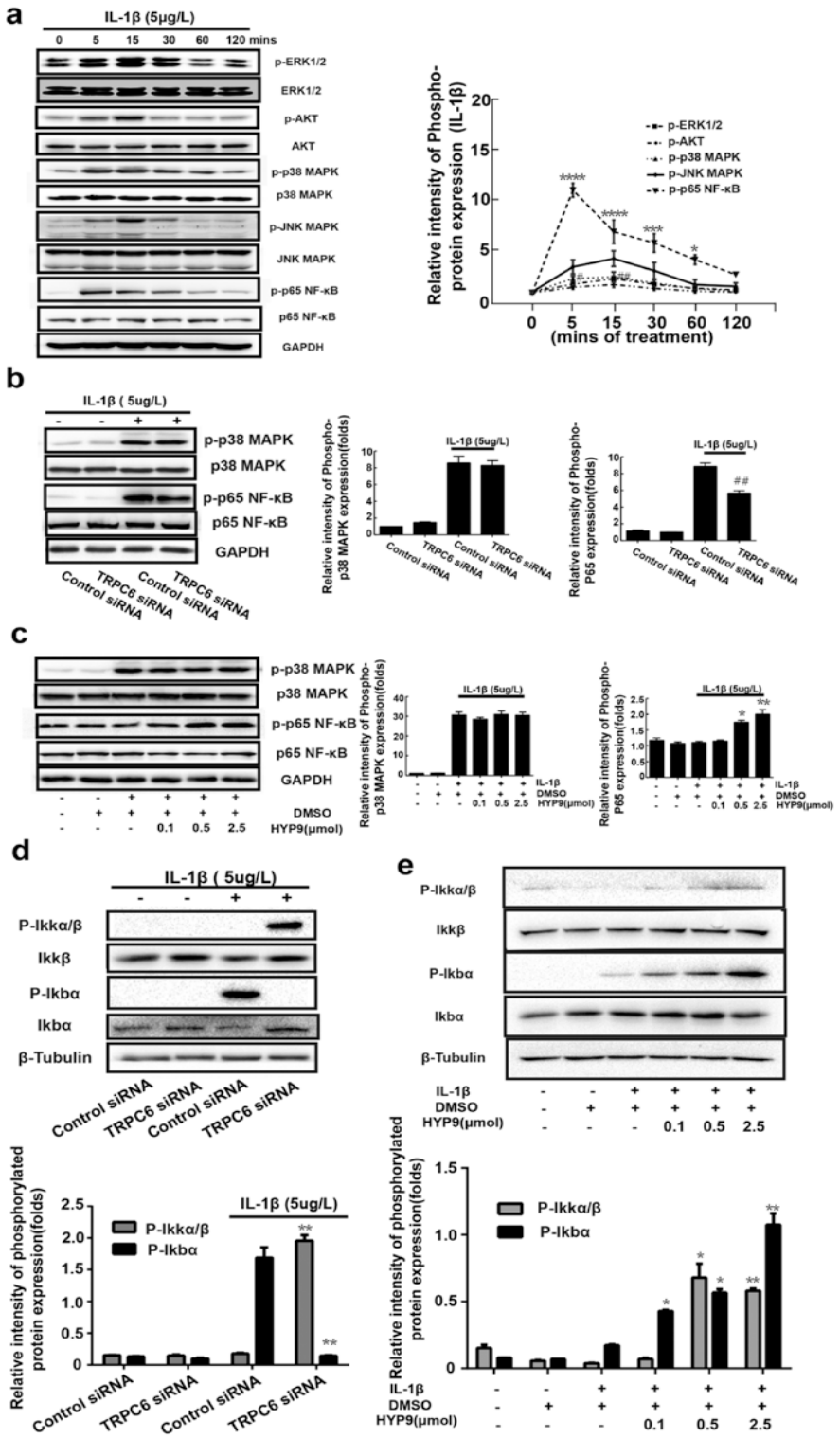

Fig. 5. Participation of TRPC6 in the IL-1β-mediated activation of NF-κb pathways by RA-FLSs. RA-FLSs were stimulated by IL-1β (5μg/L) for 0, 5, 15, 30, 60 and 120 mins; n=4, #p<0.05, ##p<0.01 *vs*. 0 min p-p38MAPK group, *p<0.05, *p<0.05 *vs*. 0 min p-p65NF-κB group (**a**).
RA-FLSs were pretreated with DMSO or various concentrations of a selective TRPC6 agonist, hyp9, for 0.5h, or were transfected with specific TRPC6 siRNA or control siRNA and then stimulated with or without IL-1β (5μg/L) for 0.25 or 1.0h. Effect of TRPC6 on the phosphorylation of p38MAPK and p65NF-κB induced by IL-1β (**b**, **c**).
Effect of TRPC6 on IKK and IκBαphosphorylation mediated by IL-1β. The right or down panel shows a densitometric analysis of Western-blot analysis from four independent experiments.
*p<0.05, **p<0.01, significantly different from treatment with LPS alone, #p<0.05, significantly different from control siRNA. p: phosphorylation.

cantly higher than TRPC6 KO group. The IL-10 level in WT CIA group was lower than the TRPC6KO group, however this difference was not significant.

Discussion

The activated RA-FLSs were thought to play a key role in the occurrence and sustained progression of destructive arthritis. As an important component of the inflammatory environment, pro-inflammatory cytokines were thought to interplay in the pathogenesis of RA. In this study, we demonstrated high TRPC6 expression in RA patients, and find its pro-inflammatory role in FLSs. By blocking TRPC6 expression both *in vitro* and *in vivo*, we further highlighted TRPC6 may become a therapeutic target in RA treatment.

The Ca^{2+} signal was currently thought to regulate the activation of RA-FLSs. Previous studies had found that $[Ca^{2+}]$ i played an important role in the proliferation, apoptosis and gene transcription of RA-FLSs (17, 18). Our study firstly shows that TRPC6 expression was increased in FLSs derived from RA patients, and verified that TRPC6 mediates $[Ca^{2+}]$i in RA-FLSs (Fig 1). These results were indicating that TRPC6 could be related to the progression of RA. We also showed that TRPC6 mediates IL-1β-induced cell proliferation (the data is not shown) in RA-FLSs and mediates IL-1β/LPS-induced cellular inflammatory responses through the NF-κB pathway. These results explained the mechanism of a pro-inflammation effect through TRPC6.

Studies *in vitro* and *in vivo* have shown that Ca^{2+} signalling played an important role in cell growth (18, 19). By using the CCK8 and Edu labelling method, we found that TRPC6 could upregulate the proliferation of IL-1β stimulated RA-FLSs (the data was not shown), which was consistent to these studies. In addition, the results of flow cytometry also showed that TRPC6 could accelerate the transition from RA-FLS to G2/M phase under IL-1β stimulation (the data was not shown), while other frontal studies have shown that TRPC6 was involved in cell proliferation by activating the calcineurin/activated T-cell nuclear factor (CaN/NFAT) pathway in non-

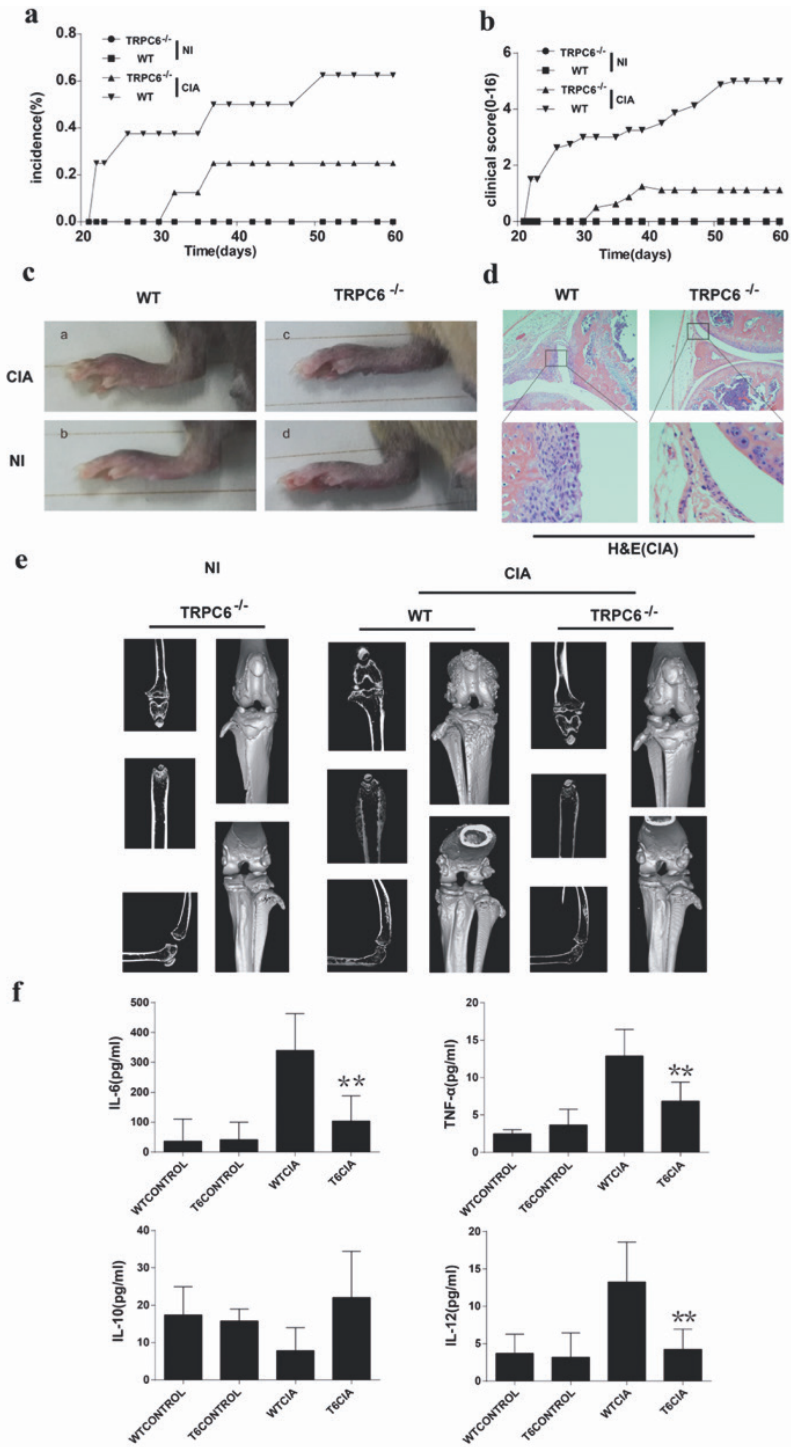

Fig. 6. Delayed severity CIA progression and reduced invasiveness and proliferation of fibroblast-like synoviocytes (FLSs) in CIA in TRPC6 knock out mice.
a: Line graph present the incidence of arthritis onset after the first induction. TRPC6-/- group, the onset of arthritis is day 20 while the WT group is day30. Incidence of arthritis in TRPC6 group is significantly lower than WT group. *p<0.05, **p<0.01.
b: Average clinical score from 4 group during 60 days.
c: TRPC6-/-group paw thickness and ankle swelling were observed.
d: Representative images of joints from TRPC6-/- mice and wildtype mice stained with haematoxylin and eosin (original magnification x 10). The partial enlargement were also shown to present the thickness of synovial cells.
e: Micro-CT reconstruction image show reduction of knee joint damage in mice with CIA.
f: Blood sample were used to measure IL-6, TNF-α, L-10 level and IL-12 level, values are the mean ± SEM (n=6 mice per group), *p<0.05, **p<0.01, TRPC6-/- compare to WT group. WT: wild type; T6:TRPC6; NI: without; CIA: collagen-induced arthritis; H&E: haematoxylin and eosin.

tumour (20) and tumour cells (21-25). Whether TRPC6 could mediate CaN/NFAT pathway conduction through Ca^{2+} signalling, promoting the proliferation of RA-FLSs is what we are going to verify in the follow-up research.

Ca^{2+} signalling was also associated to inflammatory responses. TRPC6 knockout mice present a longer survival time in severe multiple sepsis model and resistant to lethal dose LPS injection. Our previous studies showed that inflammation of lung were significantly reduced in TRPC6 knockout mice (26). In this study, we found that TRPC6 could upregulate the transcription levels of RA-FLSs, TNF-α and IL-6 with the IL-1β and LPS induction. We also found *in vivo* that TRPC6 knockout mice developed a milder arthritis than normal mice, and the levels of TNF-α, IL-6 and IL-12 were lower in blood sample. Therefore, our experiments showed that TRPC6 may regulate inflammatory response in RA disease.

Transcription factors were thought to play a vital role in the destruction of RA cartilage and bone (25). The NF-κB signalling pathway was considered as one of the major signalling pathways of joint inflammation in RA, and regulated expression of a variety of pro-inflammatory mediators, including cytokines such as TNF-α, IL-1β, and IL-6, chemokines, and cell adhesion molecules (22, 23, 27). NF-κB activation in FLSs could promote the transcription of MMPs to enhance the invasion and destruction of FLSs and accelerate the progression of RA disease (28-30). In addition, we observed that TRPC6 inhibition suppressed IL-1β and LPS induction of phosphorylation of IKK and IκBα, as well as the translocation of nuclear NF-κB, suggesting that TRPC6 could regulate the NF-κB pathway by interfering with early cytoplasmic IKK signalling. It has recently been reported that TRPC6-mediated calcium signalling activated NF-κB pathway in lung endothelial cells and upregulated inflammation levels (31). In this study, we also showed that in RA-FLSs, TRPC6 promoted the activation of NF-κB induced by IL-1β and LPS. Several studies explained the activation of NF-κB was a multi-step process. TRPC6-mediated Ca^{2+} signal-

ling activated Myosin light chain kinase, which subsequently promoted the interaction between Myeloid differentiation primary response 88 and interleukin-1 receptor-associated kinase 4. This process finally mediated the NF-κB activation and aggravated the inflammatory response (31). Interestingly, studies have found that TRPC6-mediated calcium signalling activated NF-κB, NF-κB could also reverse-regulated TRPC6, which was consistent to our study. However, research showed that in kidney cells, ROS/PKC signalling inhibited the expression of TRPC6 by activating NF-κB (32). This difference might be related to the difference in the cell type.

The strength of this study was introducing TRPC6-mediated calcium signalling as an entry point, providing a new idea for the activation mechanism of RA-FLS. A newly discovered TRPC6 selective inhibitor which was known as Larixyl Acetate (33) potentially provides the possibility to establish a clinical treatment strategy for RA. Despite the interesting results, this study did not use the TRPC6 inhibitor to discuss the prevention or treatment function in vivo. More important, whether this treatment should be targeted on local synovium or globally should be determined in further study.

Conclusion

TRPC6-mediated calcium signalling was found participate in the activation of RA-FLS by regulating NF-κB. Inhibition of TRPC6 expression can attenuate the progression of induced joint inflammation in CIA model.

Key messages

- TRPC6 contributed to fibroblast-like synoviocyte-mediated synovial inflammation in RA patients.
- TRPC6 mediated the inflammatory pathways in RA fibroblast-like synoviocytes.
- TRPC6 represents a potential therapeutic target in RA.

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