# Experimental study of TNF-α receptor gene transfection by ultrasound-targeted microbubble destruction to treat collagen-induced arthritis in rats *in vivo*

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**Abstract.** Ultrasound-targeted microbubble destruction (UTMD) is a novel method for gene transfection. The aim of the present study was to identify the most suitable method of tumor necrosis factor (TNF)-α receptor (TNFR) gene transfection using UTMD for systemically treating a rat model of collagen-induced arthritis (CIA). Plasmids encoding the TNFR and enhanced green fluorescent protein (EGFP) with or without microbubbles were locally injected into the skeletal muscle and synovial membrane of CIA rats. The rats were divided into the following 6 groups: i) Group 1, plasmid + microbubble + ultrasound (muscle group); ii) group 2, plasmid + microbubble + ultrasound (joint group); iii) group 3, plasmid + ultrasound; iv) group 4, plasmid + microbubble; v) group 5, plasmid only and; vi) group 6, untreated controls. Rats were sacrificed at 2, 4 and 8 weeks of treatment. The transfection efficiency of the plasmids in the muscle or synovium was observed by fluorescence microscopy. Arthritis scores were calculated and serum levels of TNF-α were measured prior to and following treatment. Bilateral ankle joints were obtained and stained to observe synovial inflammation and the expression of TNF-α. EGFP expression was detected in all treated groups at each time point, and the fluorescence intensity of groups 1 and 2 was significantly greater than that of the other groups (P<0.05). For groups 1 and 2, the reductions in joint scores and serum levels of TNF-α were significant compared with the other groups (P<0.05). The number of synovial inflammatory cells and the synovial expression of TNF-α presented similar results among all experimental groups and no significant difference was observed between groups 1 and 2. Therefore, the results of the present study suggest that UTMD significantly enhanced the efficiency of TNFR gene transfection in the muscle and inflamed synovium of rats with. Regardless of whether the transfected TNFR gene was injected into the muscle or joint, it was continuously expressed in the rats for at least 8 weeks, which may improve arthritic symptoms and reduce the levels of inflammatory factors in the synovial tissues and peripheral blood.

## Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by persistent synovial inflammation of the joint and invasion of the cartilage and bone. The etiology of RA remains unclear; however, it is thought that dysregulation of the internal environment may induce the pathophysiological process of RA, which is closely associated with immune, genetic and environmental factors (1,2). Tumor necrosis factor-α (TNF-α) serves a key role in RA development and progression, and it is the earliest effective inflammatory factor expressed during the process of inflammation (3). Therefore, suppression of TNF-α activation may inhibit disease progression and further alleviate or cure the disease (4). As a cutting edge treatment strategy, gene therapy facilitates the transfection of genes into tissues, which is different from traditional drug injection, and allows for the continuous and efficient delivery of therapeutic proteins to the target site, thereby achieving the goal of sustained treatment for the disease (5). Generally, gene transfection is performed using biological, chemical and physical methods, and each has its own advantages and disadvantages (6).

Ultrasound-targeted microbubble destruction (UTMD) is a novel method of gene transfection. It utilizes ultrasonic cavitation to increase the permeability of the cell membrane and promote the uptake of plasmids, and is considered to be a highly efficient and safe method (7,8). A previous study confirmed that UTMD effectively delivers insulin like growth factor-1 (IGF-1) plasmid DNA into the injured Achilles tendons of rats, effectively promoting tendon regeneration and inhibiting adhesion (9). An additional study confirmed the successful transfection of the EGFP gene into the inflammatory synovial membrane of rabbits via UTMD (10).

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Since muscle cells are abundant in the body, transfected muscle cells may constantly express and release therapeutic proteins. In addition, intramuscular injection is a simple procedure, therefore, muscle cells are an ideal target for gene therapy. The pathological basis of RA is characterized by the secretion of a variety of inflammatory factors by the synovium, which has a rich blood supply. Therefore, it may also be a useful target for gene therapy. In the present study, UTMD was performed to transfect the TNF-α receptor (TNFR) gene into muscle and synovial cells in the inflamed joints of rats with collagen-induced arthritis (CIA). The transfection efficiency and therapeutic outcomes were then compared to identify an optimal strategy for treating RA using gene therapy. To the best of the author's knowledge, there have only been few studies on this topic.

**Materials and methods**

*Establishment of a rat model of CIA.* A total of 108 healthy female Sprague-Dawley rats (age, 6-7 weeks; weight, ~200 g) were provided by Chengdu Dashuo Experimental Animal Co., Ltd., Chengdu, China, and were housed in a temperature-controlled room (20-26˚C), at 50% humidity, with standard and sufficient food and water and 12/12 h light/dark cycles. All procedures (including rat anesthesia and euthanasia procedures) involving animals were approved by the Sichuan University Research Committee for Animal Research (Sichuan, China; approval no. 2016052A). Bovine type II collagen acetic acid solution (Chondrex, Redmond, WA, USA) was emulsified in an equal volume of incomplete Freund's adjuvant (Chondrex). Each rat was immunized intradermally at the base of the tail with 0.2 ml emulsion (200 $\mu$g collagen/rat). A booster injection was administered (0.1 ml emulsion; 100 $\mu$g collagen/rat) intradermally in the tail at 7 days following the initial immunization. Arthritic progression was monitored daily.

*Amplification, extraction and purification of plasmid DNA.* The present study used the pEGFP plasmid (Roche Diagnostics, Basel, Switzerland) that expresses enhanced green fluorescent protein (EGFP), and a TNFR plasmid (GeneCopoeia, Inc., Rockville, MD, USA) that expresses TNFR. The manufacturer provided these plasmids transfected into *Escherichia coli* and labeled with EGFP. Plasmid extraction and purification were performed according to the instructions provided with the plasmid extraction kit (Qiagen GmbH, Hilden, Germany), and an ultraviolet spectrophotometer was used to determine plasmid concentration by measuring the optical density (OD) at wavelengths of 260 and 280 nm. If the OD 260/280 nm ratio was between 1.8 and 2.1, then the sample was considered to be pure and uncontaminated. The plasmid was diluted to 30 $\mu$g/100 $\mu$l and stored at -20˚C prior to use.

*Preparation of the SonoVue-DNA complexes.* The SonoVue microbubble suspensions (Bracco Imaging S.p.A, Milano, Italy) were prepared at a density of 2-5x10^8/ml and a concentration of 5 mg/ml using 0.9% saline solution. SonoVue (100 $\mu$l) was then mixed with 100 $\mu$l (30 $\mu$g) of the plasmid DNA solution and stored at 4˚C prior to each experiment.

*Experimental grouping.* CIA rats were randomly assigned to the following 6 groups: Group 1, plasmid + microbubble + ultrasound (muscle group); group 2, plasmid + microbubble + ultrasound (joint group); group 3, plasmid + ultrasound; group 4, plasmid + microbubble; group 5, plasmid only and; group 6, untreated controls. Aside from group 2, which was injected at the ankle joint, the other groups were injected in the tibialis anterior muscle. Groups 1, 2 and 4 were injected with the SonoVue/TNFR mixture (300 $\mu$g plasmid/rat), groups 3 and 5 were injected with the same dosage of TNFR plasmid alone, and group 6 received no treatment. An ultrasound therapy device (Sonic Master ES-2; OG Giken, Co., Ltd., Okayama, Japan) associated with a probe frequency of 1 MHz, transducer area of 10 cm$^2$ and a pulse repetition frequency of 100 Hz, was used in this study. The output power (2 W/cm$^2$), duty cycle (20%) and time (5 min) were selected according to the parameters used in our previous study (10).

*Arthritis scoring.* After 2, 4 and 8 weeks of treatment, swelling of the joints in the CIA rats was observed and recorded for arthritis scoring. For each limb, arthritis was graded on a scale of 0 to 4 (Fig. 1): 0 represents a normal joint; 1 represents redness or swelling of one type of joint (the interphalangeal joint, metatarsophalangeal joint or the ankle joint); 2 represents redness or swelling of two types of joints; 3 represents redness or swelling of all types of joints and; 4 represents severe swelling below the knee joint, with loss of anatomical landmarks and the rat failing to bear weight. The scores of all four limbs were added together to give a total score for each rat; the highest possible score was 16.

*Sample collection.* Blood samples were collected in each group prior to treatment, and the serum level of TNF-α was detected by ELISA. At 2, 4 and 8 weeks following treatment, rats were anesthetized by isoflurane inhalation, placed in a supine position and blood samples from both tibialis anterior muscles and ankle joints were obtained. Blood samples were incubated at 4˚C for 24 h, before they were centrifuged at 4˚C at 1,500 x g for 20 min. The serum was subsequently collected and stored at -80˚C. Muscles were fixed with 10% formalin for 48 h at room temperature. Joints were fixed in 10% formalin for 72 h, and then decalcified in 10% EDTA for 60 days at room temperature. Samples were embedded in paraffin and sectioned to 4-$\mu$m thickness for hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining as described below.

*Serological and pathological analysis.* The serum levels of TNF-α were determined using a commercial ELISA kit (cat. no. RTA00; R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's protocol. Differences in TNF-α levels were calculated by subtracting the serum levels of TNF-α at different time points (2, 4, and 8 weeks) from the serum levels prior to treatment.

*H&E staining.* Sections stained with H&E for 5 min and 5% eosin staining for 5 sec at room temperature were observed using a light microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan), and 5 typical fields for each sample were selected to observe joint arthritis. Samples were

Figure 1. Appearance of rat hind paws indicating progressive collagen-induced arthritis. Representative images of rat hind paws with arthritis scores of (A) 1, (B) 2, (C) 3 and (D) 4 are presented.
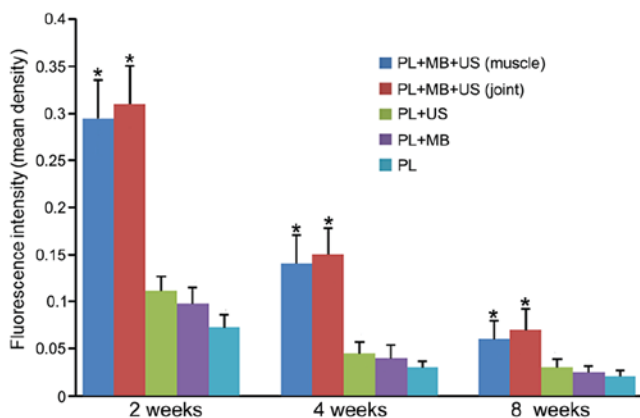


Figure 2. Enhanced green fluorescent protein analysis of groups 1 to 5 at different time points. The fluorescence intensity at 2 weeks was higher than that at 4 and 8 weeks in all groups. The intensity in the PL+MB+US (muscle) and PL+MB+US (joint) was significantly greater than that in the other groups at the corresponding time points. No significant difference was identified between the PL+MB+US (muscle) and PL+MB+US (joint) groups and no significant difference was identified among the remaining groups. *P<0.05 vs. the PL+US, PL+MB or PL groups. PL, plasmid; MB, microbubble; US, ultrasound.

fluorescence intensity. The mean value of three measurements was used for statistical analysis.

*IHC staining.* Tissue sections were deparaffinized and rehydrated prior to IHC analysis. A total of 100 $\mu$l 10% goat serum (cat. no. SP-KIT-B3; Fuzhou Maxim Biotechnology Development Co., Ltd., Fuzhou, China) was added to each section for blocking for 15 min at room temperature. Samples were incubated with anti-TNF-$\alpha$ antibody (1:100; cat. no. ab220210; Abcam, Cambridge, UK) at 4˚C overnight. The horseradish peroxidase conjugated goat secondary antibody (100 $\mu$l Bottle A from the ChemMate™ Envision; cat. no. K5007; Dako Agilent Technologies, Inc., Santa Clara, CA, USA) was added and incubated at 37˚C for 45 min. DAB working liquid was then added and staining was observed under a light microscope (magnification, x100). The sections were then observed using a light microscope (Olympus BX51; Olympus Corporation) and five random fields were selected. The Image-Pro Plus 6.0 system (Media Cybernetics) was used to measure the mean density of TNF-$\alpha$ staining, and the mean values were calculated and used for statistical analysis.

*Western blotting.* The expression of TNF-$\alpha$ in the synovium was examined by western blotting. Total protein was extracted using a EpiQuik Whole Cell Extraction kit (EpiGentek Group, Inc., Farmingdale, NY, USA) according to the manufacturer's protocol. The bicinchoninic acid protein determination method (cat. no. P0012; Beyotime Institute of Biotechnology, Haimen, China) was utilized and 40 $\mu$g protein was subsequently loaded per lane. The samples were separated by 5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. Membranes were subsequently blocked with 50 ml/l non-fat milk for 2 h at room temperature. The rabbit anti-TNF-$\alpha$ polyclonal antibody (1:500; cat. no. ab6671;

scored from 0 to 3 individually depending on the infiltration of inflammatory cells, synovial thickening, cartilage degradation and bone destruction according to a previous study (11).

*Fluorescence staining.* In order to determine the plasmid transfection efficiency, the muscle and synovial samples of rats in each group were stained with DAPI (1 $\mu$g/ml) for 5 min at room temperature and the expression of EGFP was evaluated. A total of ten fields of view for each section were selected at random and observed using a fluorescence microscope (Nikon ECLIPSE TE2000-U; Nikon Corporation, Tokyo, Japan). The Image-Pro Plus 6.0 system (Media Cybernetics, Inc., Rockville, MD, USA) was used to measure the mean
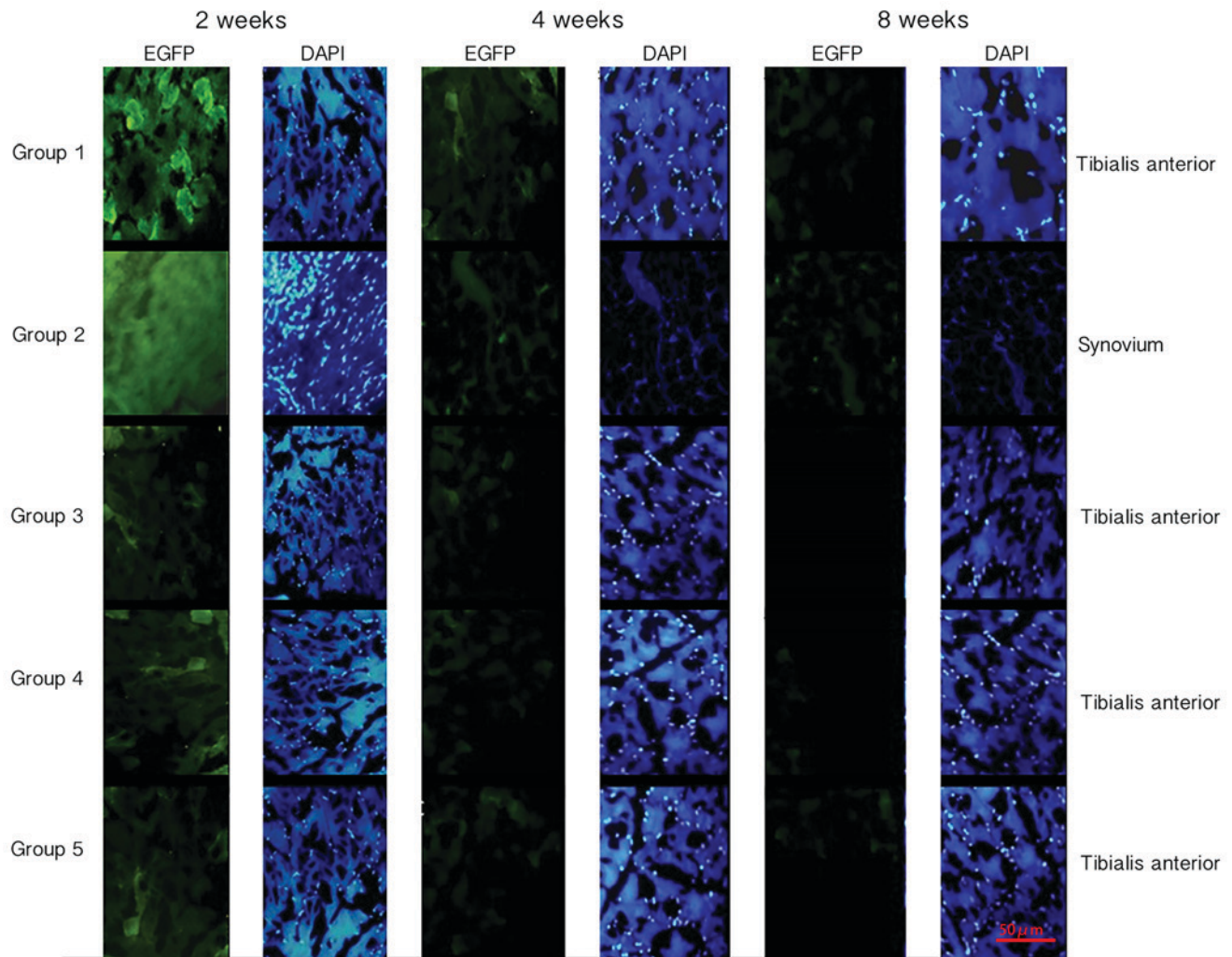
Figure 3. Fluorescence microscopy examination of EGFP expression and DAPI staining of the tibialis anterior and synovium of the rat ankle at 2, 4 and 8 weeks (magnification, x200). Group 1, plasmid + microbubble + ultrasound (muscle group); Group 2, plasmid + microbubble + ultrasound (joint group); Group 3, plasmid + ultrasound; Group 4, plasmid + microbubble; Group 5, plasmid only. EGFP, enhanced green fluorescent protein.

Abcam) was added and incubated for 2 h at room temperature. The membranes were then incubated with horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (1:2,000; cat. no. ab6721; Abcam) for 1 h at 37°C. The protein bands were normalized to GAPDH (1:500; cat. no. ab6671; Abcam) was incubated for 2 h at room temperature. A Pierce™ ECL Western Blotting Substrate (cat. no. 32106; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was utilized as the visualization reagent. A BioSpectrum Imaging System (SmartChemi 500; Sagecreation, Beijing, China) was used to capture the luminescent signals and optical density of the protein bands, which were subsequently quantified using analysis software provided by the manufacturer (Lane 1D™ 2011 edition; Beijing Sage Creation Science Co., Ltd., Beijing, China).

*Statistical analysis.* Statistical analysis was performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). The data are presented as the mean ± standard deviation. Comparisons between two groups were performed using the Wilcoxon rank sum test. Multigroup comparisons were performed using the Kruskal-Wallis H rank sum test. Pairwise comparisons were
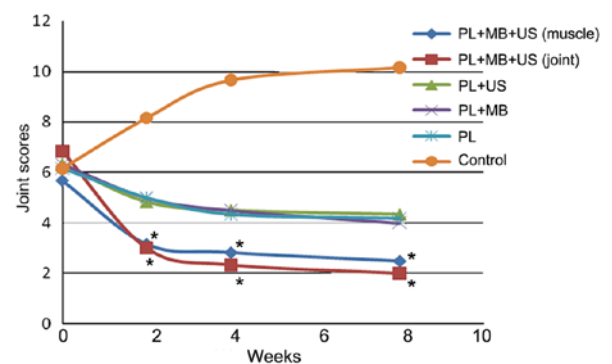


Figure 4. Arthritis scores at different time points for each group. For all treatment groups, the scores decreased with increasing treatment time. A significant reduction occurred at 2 weeks; at which point the scores stabilized. The score reduction in the PL+MB+US (muscle) and PL+MB+US (joint) groups was greater than in other groups (P<0.05); however, the muscle injection group showed no significant difference when compared with the joint injection group (P>0.05). *P<0.05 vs. the PL+US, PL+MB, PL or control groups. PL, plasmid; MB, microbubble; US, ultrasound.

made using the Nemenyi test. P<0.05 was considered to indicate a statistically significant difference.
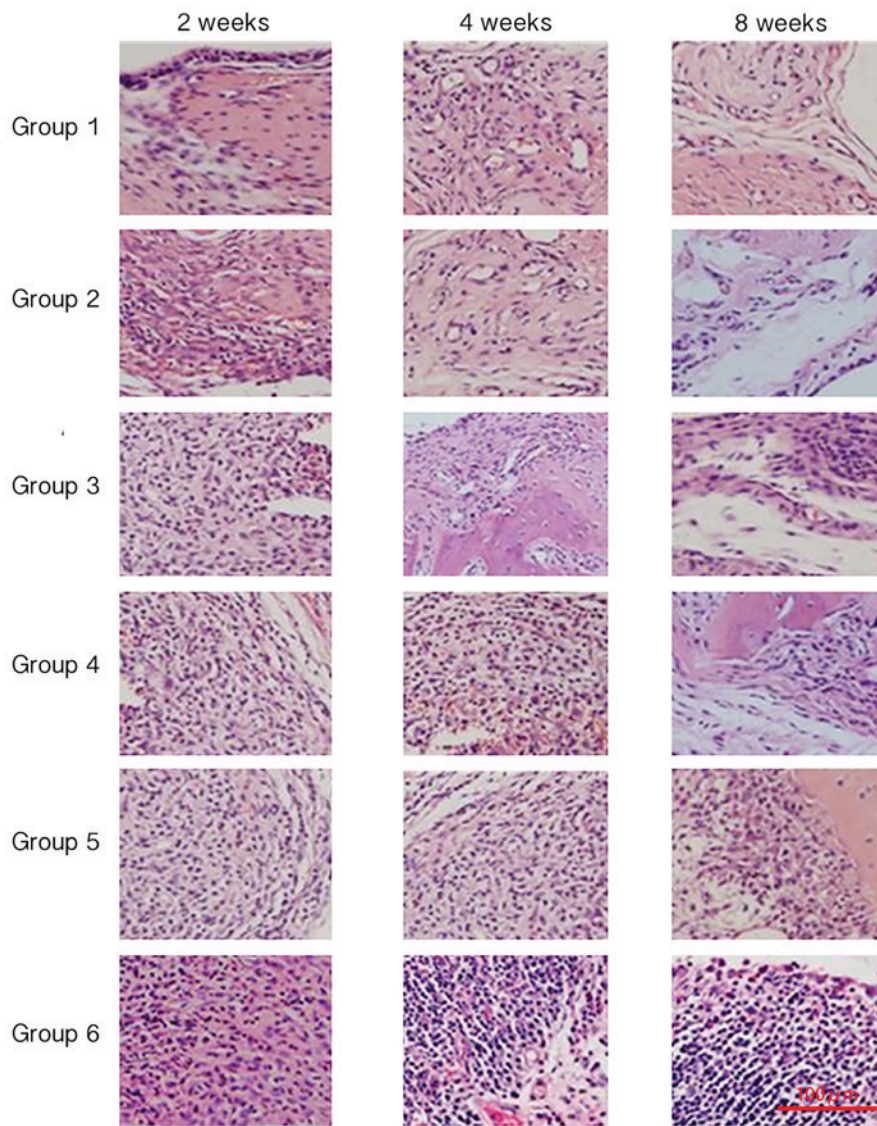
Figure 5. Hematoxylin and eosin staining of the synovium in groups 1 to 6. In groups 1-5, cellular infiltration at 2 weeks was greater than at 4 and 8 weeks. Groups 1 and 2 exhibited milder inflammation compared to the remaining groups at all corresponding time points, and no marked difference between groups 1 and 2 were observed. The control group demonstrated the most severe inflammatory cell infiltration (magnification, x200). Group 1, plasmid + microbubble + ultrasound (muscle group); Group 2, plasmid + microbubble + ultrasound (joint group); Group 3, plasmid + ultrasound; Group 4, plasmid + microbubble; Group 5, plasmid only; Group 6, untreated control.

## Results

*Transfection efficiency at different time points.* The plasmids employed in the present study were labeled with EGFP; therefore, the level of green fluorescence was observed under a fluorescence microscope following transfection. In addition, DAPI was used to label double-stranded DNA in the nucleus and displayed blue fluorescence under the microscope. Quantitative analysis of green fluorescence was performed using Image-Pro Plus software. Green fluorescence was observed in the muscle tissues of groups 1, 3, 4 and 5 at each time point, and the fluorescence intensity at 2 weeks was greater than that at 4 and 8 weeks for all groups (Figs. 2 and 3). The fluorescence intensity in group 1 was greater than that of the other groups at all time points, and the difference was statistically significant (P<0.05; Figs. 2 and 3). In group 2, green fluorescence was observed in the synovial tissue, and the intensity showed no significant difference when compared with group 1 (Figs. 2 and 3). The control group exhibited no fluorescence in the muscle or joint tissues (data not shown).

*Arthritis scoring.* Arthritis was scored at 2, 4 and 8 weeks following treatment. According to the results, the treatment groups exhibited decreasing arthritis scores over time (Fig. 4). The most marked reduction occurred at 4 weeks, after which point, a gradual decline was observed until the rate eventually stabilized. The score reduction in groups 1 and 2 was significantly greater than that in the other groups at 2, 4 and 8 weeks (P<0.05), regardless of whether the injection site was the muscle or joint. The score reductions among groups 3, 4 and 5 were not significantly different (P>0.05; Fig. 4). The scores in the control group exhibited an initial increase and then stabilized over time; however, these scores remained higher than those of the other experimental groups at 2, 4 and 8 weeks following treatment (Fig. 4).

*Pathological analysis using H&E staining.* Inflammatory cell infiltration and synovial hyperplasia were observed to evaluate synovial inflammation at different time points among the different groups. At 2 weeks of treatment, significant cellular infiltration was observed in all groups; most notably in the control group (Fig. 5). At 2, 4 and 8 weeks of treatment, the inflammatory reaction was reduced in all treatment groups. Groups 1 and 2 exhibited the lowest number of inflammatory cells and the least synovial inflammation, and there was no marked difference between groups 1 and 2 (Fig. 5). Groups 3, 4 and 5 exhibited more severe inflammation when compared with groups 1 and 2, and group 6 demonstrated the most severe inflammatory reaction (Fig. 5).

*Pathological analysis by IHC staining.* IHC staining was performed in tissue sections obtained at different time points and from different groups, and the expression of TNF-α was detected. The results demonstrated that the staining intensity in groups 1-5 decreased gradually with increasing treatment time and reached their lowest point at 8 weeks following treatment (Fig. 6). However, group 6 exhibited a gradual increase in staining intensity, reaching a peak at 8 weeks following treatment, and was higher than that of the other groups (P<0.05; Fig. 6). The staining intensity in groups 1 and 2 was significantly lower than the other four groups, and no significant difference between groups 1 and 2 was observed (P>0.05). The staining intensity of groups 3, 4 and 5 groups remained lower than that of group 6 at all the corresponding time points (P<0.05), but no significant difference among these groups was identified (P>0.05; Fig. 6).

*Western blotting analysis.* The western blotting results demonstrated that the expression of TNF-α in groups 1 and 2 were markedly decreased at 4 and 8 weeks post-treatment when compared with group 6 (P<0.05; Fig. 7). In addition, TNF-α levels gradually decreased over time and reached their lowest point at 8 weeks following treatment. In group 1, TNF-α levels were slightly higher than in group 2 at all time points, but no significant difference was identified between them (P>0.05; Fig. 7).

*Serological analysis.* Inflammation was evaluated by measuring the serum levels of TNF-α at different time points among the experimental groups. The results of the ELISA demonstrated that serum levels of TNF-α were negatively correlated with treatment duration (Fig. 8). The concentration was at its highest at the beginning of the treatment, and then gradually decreased to its lowest point at 8 weeks following treatment. At 2, 4 and 8 weeks following treatment, a significant reduction in TNF-α in groups 1 and 2 was observed when compared with the remaining groups (P<0.05; Fig. 8). Group 1 exhibited a greater reduction in serum TNF-α levels when compared with group 2; however, this did not reach statistical significance (P>0.05). When compared with group 6, TNF-α levels in groups 3, 4 and 5 were significantly lower (P<0.05); however, no significant difference was observed among groups 3, 4 and 5 (P>0.05; Fig. 8). The original level of serum TNF- α was ~50 pg/ml (data not shown), as the duration of treatment increased, the serum level of TNF-α in group 6 gradually increased by ~40 pg/ml, which indicated a plateaued at ~90 pg/ml (Fig. 8).
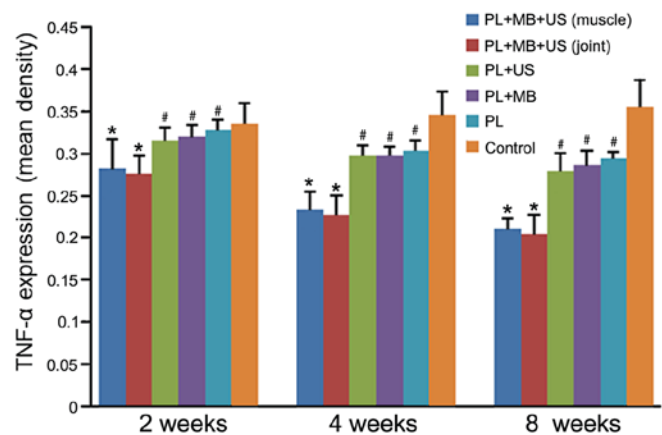


Figure 6. Quantitative analysis of TNF-α expression by immunohistochemical staining. PL+MB+US (muscle) and PL+MB+US (joint) groups exhibited significantly lower staining intensities at all corresponding time points when compared with the other groups (P<0.05). No significant difference between the PL+MB+US (muscle) and PL+MB+US (joint) groups was observed. No significant difference among the PL+US, PL+MB and PL groups was also observed (P>0.05). *P<0.05 vs. the PL+US, PL+MB, PL or control groups; #P<0.05 vs. the control group. TNF-α, tumor necrosis factor-α; PL, plasmid; MB, microbubble; US, ultrasound.

## Discussion

RA is a common inflammatory joint disease. It is an autoimmune disease with a complex pathogenesis and is thought to be associated with abnormal regulation of the internal environment (1). The main clinical symptoms of RA include joint pain and stiffness accompanied by movement disorders. Severe symptoms may affect a patients' quality of life, and may even lead to disability and dysfunction, especially among the young and middle-aged individuals. Therefore, it is of great clinical significance to investigate novel treatment strategies for RA. Although the etiology of RA is currently unclear, immune, genetic, environmental and lifestyle factors (such as smoking) are thought to be closely associated to the development of RA. At present, it is generally considered that RA development involves an imbalance between autoimmune induction and pro-inflammatory/anti-inflammatory factors, which leads to chronic inflammation (3,12). According to their mechanisms of action, clinical medicines for RA treatment are classified into nonsteroidal anti-inflammatory drugs, glucocorticoids, disease-modifying anti-rheumatic drugs and TNF-α inhibitors. However, despite these various treatment options for RA, their curative effects are variable and far from satisfactory.

As a cutting-edge treatment, gene therapy delivers cDNA encoding therapeutic proteins to cells in the target tissue and facilitates continuous and efficient local secretion of gene products. This negates the disadvantages of direct injection and provides sustained treatment for the disease (5). There are currently a number of gene therapy strategies; cDNA transfection can be administered physically, chemically, or biologically. However, these methods differ from each other in terms of efficiency, operation feasibility, as well as therapeutic safety. For instance, although chemical transfection is cheaper, easier to operate and requires no special experimental conditions,
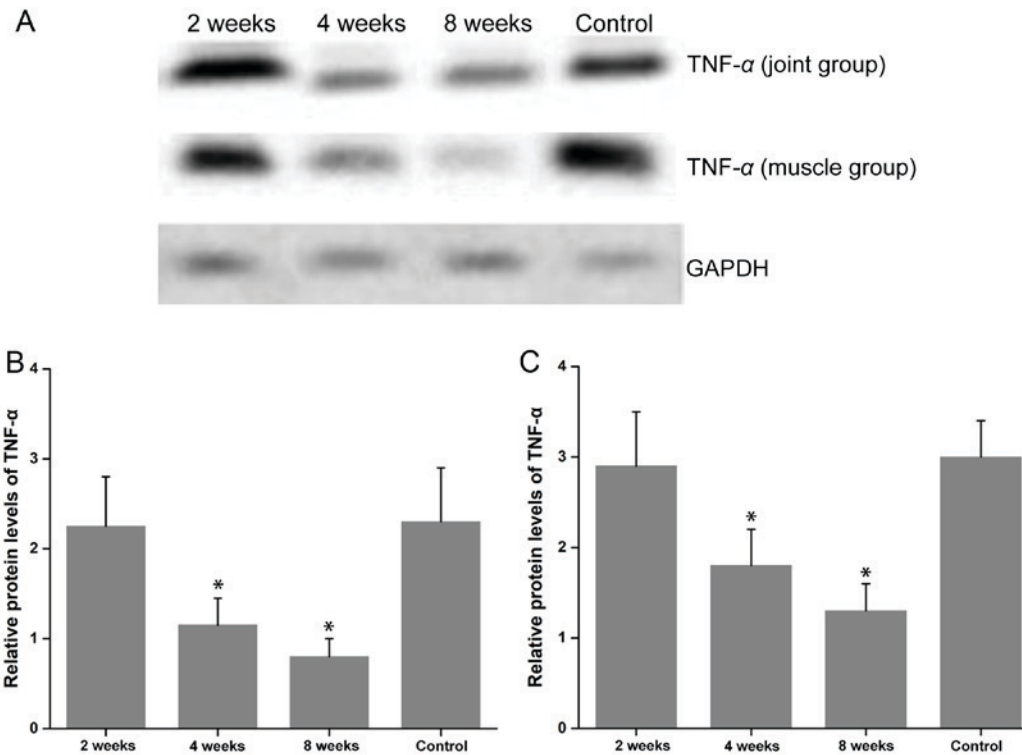
Figure 7. (A) Western blot analysis of TNF-α protein expression in the synovial tissues of the (B) PL+MB+US (muscle) group and the (C) PL+MB+US (joint) group at 2, 4 and 8 weeks. TNF-α expression in both groups decreased at 4 and 8 weeks post-treatment compared with the controls, and TNF-α levels gradually decreased over time and reached their lowest point at 8 weeks following treatment. *P<0.05 vs. Control. TNF-α, tumor necrosis factor-α; PL, plasmid; MB, microbubble; US, ultrasound.
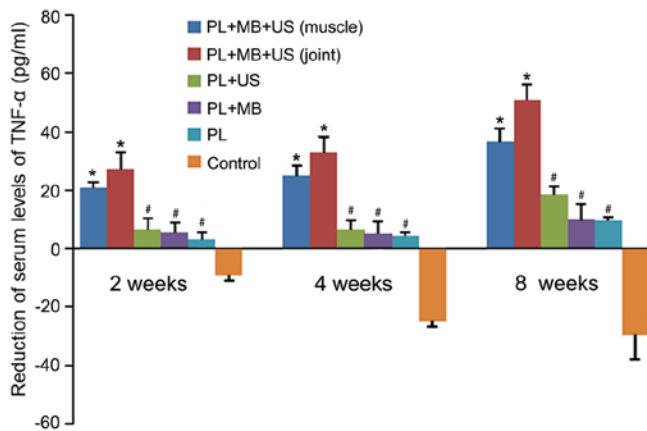


Figure 8. Serum levels of TNF-α at different time points. A reduction in serum TNF-α levels in the PL+MB+US (muscle) and PL+MB+US (joint) groups was greater than that in the other groups at all corresponding time points. *P<0.05 vs. the PL+US, PL+MB, PL or control groups; #P<0.05 vs. the control group. TNF-α, tumor necrosis factor-α; PL, plasmid; MB, microbubble; US, ultrasound.

the efficiency is low and liposomes may be toxic to cells (5). Physical transfection includes electroporation, cell microinjection and gene gun techniques. These techniques provide high transfection efficiencies; however, they also require more advanced equipment and more complex surgical tools. Furthermore, they use high pressure pulses that may cause significant cell death (13,14). Biological methods primarily involve viral vector-mediated transfection. This technique is highly efficient, easy to operate and involves continuous and

stable expression of exogenous genes either *in vitro* or *in vivo*. Since viral vectors only exert their function following integration with the nuclear genome of host cells, this technique introduces a risk of carcinogenesis and teratogenicity (9,10). Therefore, non-viral vectors, such as plasmids, are considered to be safer and have lower immunogenicity when used to carry therapeutic genes; however, their transfection efficiency, duration of action, and expression levels are far lower than those of viral vectors.

UTMD is a new combined transfection method that uses microbubbles as the vector. Following intravenous injection or local administration of plasmids encoding the genes of interest, the cavitation effects of ultrasound are utilized to increase membrane permeability, promote gene uptake and optimize vector delivery, which in turn enhances the efficiency and safety of transfection (7,15,16). In a previous study, IGF-1 cDNA was successfully transfected into rat Achilles tendons using UTMD, which promoted tendon regeneration and provided novel insights into the clinical treatment of tendon injury (9). An additional study also confirmed that UTMD mediated the successful transfection of the EGFP gene into the inflammatory synovium and muscle of antigen-induced arthritis rabbits, which resulted in the continuous production of fluorescent proteins (10). The optimal conditions were determined to be a 1-MHz ultrasound pulse applied for 5 min with a power output of 2 W/cm$^2$ and a 20% duty cycle. Under these conditions, EGFP was expressed at the highest levels and no normal tissue was damaged (10).

As RA is a systemic disease, the introduction of UTMD-based gene therapy for RA may confer a more favorable treatment outcome, and also provide novel insights

for the clinical treatment of RA. In the present study, UTMD was utilized to transfect the TNFR gene into muscle cells or the synovium of the inflammatory joints of CIA rats. The transfection efficiency of different methods at different sites was compared, and inflammatory cytokines and the severity of arthritis were evaluated to identify an optimal strategy for using UTMD in the treatment of RA. The CIA model was established based on the principle of immune inflammation induced by alloantigens, and its clinical symptoms primarily include the development of multi-terminal arthritis. The model was first established by Trentham *et al* in 1977 (17), and it has now become the most commonly used arthritis model (16).

Yovandich *et al* (18) injected plasmids into the knee joints of rabbits and rats. The results revealed that the reporter gene was expressed in the synovial tissues; however, the transfection efficiency was relatively low and expression lasted for only 2-5 days, after which point the protein was absorbed and degraded rapidly by the synovial tissues. Lawrie *et al* (19) investigated ultrasonic irradiation-combined naked DNA transfection and observed that gene expression in vascular endothelial cells was elevated 10-fold. In a subsequent study, Lawrie *et al* (20) combined microbubbles and plasmids together with ultrasonic irradiation and obtained an increased expression level, as well as a plasmid transfection efficiency of 300-fold higher than that of naked DNA transfection. In a previous study performed by our research group, microbubbles and plasmids were injected simultaneously into the knee joints of rabbits to optimize the transfection conditions. Further experiments involving the combined administration of ultrasonic irradiation with microbubbles and plasmids, demonstrated a much higher transfection efficiency of EGFP when compared to ultrasonic irradiation and plasmids, plasmids and microbubbles, or plasmid only methods (3).

The present study used an EGFP-labeled TNFR plasmid. The two genes are simultaneously replicated and expressed, and the transfection efficiency of the TNFR gene was therefore evaluated by fluorescence intensity analysis and DAPI staining. According to the results, fluorescence was detected in the muscles and synovium, indicating successful transfection of the gene. The PL+MB+US muscle group exhibited significantly higher fluorescence and staining intensities when compared with the other groups at the corresponding time points; however, no significant difference with the PL+MB+US joint group was observed. The PL+US, PL+MB and PL groups exhibited lower transfection efficiencies; however, no significant difference among these three groups was observed. Therefore, UTMD significantly improved gene transfection efficiency in the muscles and inflammatory synovium.

Previous studies have evaluated the level of inflammation in CIA rat models using an arthritis scoring system. It is a simple and common method to determine relief or aggravation of joint inflammation, by assessing the degree of joint redness or swelling (21,22). In the present study, the arthritis scores in the two PL+MB+US groups were the lowest at all time points with the most significant reductions compared with other treatment groups and the control. This indicated that UTMD-mediated transfection was associated with the most significant remission of inflammation and the most positive treatment outcome. The PL+US, PL+MB and PL groups were not significantly different in terms of the reduction in arthritis scores. As the control group was untreated, its score increased gradually and plateaued at a certain level.

In the normal synovial tissues of rats, there is a low number of synovial cells and no inflammatory cell infiltration or significant neovascularization is apparent. Synovitis is caused by the activation and infiltration of systemic or local monocytes, macrophages and mast cells, and by the formation of new vessels. In the inflammatory synovium, massive inflammatory cell infiltration and synovial thickening, as well as neovascularization were observed (23). Using H&E staining, the degree of inflammation can be determined by observing the percentage of inflammatory cells and the thickness of the synovium. In the present study, the PL+MB+US groups exhibited the fewest inflammatory cells, no obvious synovial hyperplasia and the mildest inflammation. No significant difference was identified between the two PL+MB+US groups with different injection sites. The control group presented with the most severe pathology with the highest percentage of inflammatory cells, the thickest synovium and the most significant inflammation.

Under inflammatory conditions, synoviocytes secrete factors such as TNF-α, and RA patients show high levels of TNF-α in their serum and synovium of inflammatory joints (24). By stimulating osteoclast formation, TNF-α induces bone invasion and joint injury, which subsequently aggravates inflammation (3). Therefore, TNF-α is considered to be a pathogenic factor and a marker. In clinical practice, inflammatory remission or aggravation could be evaluated by measuring the levels of TNF-α in the synovium or blood. Therefore, Lee *et al* (23) evaluated the effects of TNF-α silencing in RA treatment and observed that the experimental group exhibited a significant reduction in TNF-α levels when compared to the control, and also confirmed that the serum levels of TNF-α were positively associated with the level of inflammation. In the current study, inflammatory remission was evaluated and the treatment efficacies were compared among the different treatment groups by measuring the levels of TNF-α. According to the results, the PL+MB+US groups exhibited the greatest decrease in TNF-α levels when compared with the remaining groups, indicating that UTMD performed better than all other transfection modalities. Although the muscle injection group demonstrated a smaller reduction compared with the joint injection group, the difference was not statistically significant. The authors hypothesize that this be associated with the enriched blood supply in the inflammatory joint synovium, which may promote the absorption of transfected genes. However, this remains to be verified in future studies with larger sample sizes.

Olkkonen *et al* (25) identified the presence of TNF-α in the synovium of RA patients, and the degree of arthritis or synovitis was evaluated by measuring the TNF-α concentration using IHC or additional methods. The results of the present study demonstrated that TNF-α was detected at all time points, and the staining intensity in the treatment groups was decreased with increasing treatment duration, which suggests that treatment resulted in varying degrees of remission. Furthermore, the staining intensity of the PL+MB+US groups was significantly lower than that of the remaining groups, and the joint injection group exhibited a greater reduction when compared with the muscle injection group; however,

the difference was not significant. The staining intensity of TNF-α in the control rats increased gradually with increasing treatment duration, no significant variation was observed following 8 weeks of treatment (and 12 weeks after the model was established). This suggests that, inflammation will progress to a chronic phase at 3 months without treatment. The results of IHC analysis were consistent with those of the serum ELISA analysis, which confirmed that UTMD-mediated transfection improved synovial and systemic inflammation and was associated with an improved disease outcome when compared with other transfection methods for the treatment of arthritis.

However, the present study has some limitations. Namely, evaluation of H&E staining was based on the observation of inflammatory infiltration and synovial hyperplasia, which is relatively subjective, thus objective criteria may be required for future studies. In addition, the observation period was relatively short and will be extended in future studies.

In conclusion, the results of the present study demonstrated that UTMD-mediated gene transfection could successfully introduce the TNFR gene into muscle cells and the inflammatory synovium, and the transfection efficiency was significantly higher when compared with other transfection modalities. The transfected TNFR gene was successfully expressed at a continuous level in CIA rats, and improved the symptoms of arthritis, as well as reduced TNF-α expression in the synovial tissues and peripheral blood. No significant differences in treatment outcome were observed between the two sites of injection (muscle or joint).

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Not applicable.

## Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

LW conducted the animal experiments, analyzed and interpreted the experimental data and was a major contributor in writing the manuscript. XT performed the animal experiments and analyzed the pathological data. XX performed the animal experiments and revised the manuscript. YT supervised the study design and performed the animal experiments. LQ contributed to the conception and design of the study, interpretated the data and critically reviewed the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

All experimental procedures were performed in strict accordance with institutional regulations and the study was approved by the Sichuan University Research Committee for Animal Research.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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