Transplantation of IL-1β siRNA-modified bone marrow mesenchymal stem cells ameliorates type II collagen-induced rheumatoid arthritis in rats

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Abstract. Rheumatoid arthritis (RA) is a chronic autoimmune disease that causes erosion of articular cartilage and bone and has adverse effects on both patients and livestock animals. The aim of the present study was to investigate the role of interleukin-1β (IL-1β) in the pathogenesis of RA, and to further determine whether injection of IL-1β small interfering RNA (siRNA) or transplantation of IL-1β siRNA + bone marrow mesenchymal stem cells (BMSCs) can ameliorate RA in rats. A collagen-induced arthritis (CIA) rat model was established by injecting type II collagen for 4 weeks. Next, CIA rats were randomly divided into three groups and injected or transplanted with PBS, IL-1β siRNA and IL-1β siRNA + BMSCs for another 4 weeks. The CIA rat model was successfully established, as demonstrated by the higher toe swelling value, thymus and spleen/body weight, immobility time and serum IL-1β concentration, as well as lower body weight, climbing time and mRNA expression of programmed death-1 (PD-1), transforming growth factor-β1 (TGF-β1) and forkhead box protein 3 (Foxp3) in the spleen, compared with control rats. Furthermore, histopathology results demonstrated that joint swelling and redness were observed in the knee joints of CIA rats. H&E results revealed that CIA rats presented erosive destruction of the bone and ulceration of the articular cartilage. In addition, *in vitro* results demonstrated that IL-1β expression was successfully silenced after IL-1β siRNA transfection in lipopolysaccharide-stimulated BMSCs. When compared with the results of PBS rats, both IL-1β siRNA injection and IL-1β siRNA + BMSC transplantation significantly increased the body weight, climbing time and mRNA expression of PD-1, TGF-β1 and Foxp3 in the spleen, while significantly reduced the immobility time and serum IL-1β concentration. In addition, when compared with that of IL-1β siRNA injection, IL-1β siRNA + BMSC transplantation exhibited markedly higher therapeutic efficacy against CIA. These results demonstrated that higher IL-1β contributed to the pathogenesis of CIA, and that IL-1β siRNA injection ameliorated CIA, while its combination with BMSCs exerted synergistic effects, which may be beneficial against RA.

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Abbreviations: BMSCs, bone marrow mesenchymal stem cells; CIA, collagen-induced arthritis; IL-1β, interleukin-1β; LPS, lipopolysaccharide; RA, rheumatoid arthritis; RNAi, RNA interference; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA; TGF-β1, transforming growth factor-β1

Key words: collagen-induced arthritis, interleukin-1β small interfering RNA, bone marrow mesenchymal stem cells, rheumatoid arthritis, rats

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that is primarily characterized by the destruction of multiple joints, causing substantial pain, swelling, persistent synovitis, systemic inflammation and an increase in proinflammatory cytokines levels (1,2). It affects >1% of the adult population worldwide and leads to joint damage and the loss of physical function (1,2). In addition, RA is common in companion animals, livestock and wild animals, and the joint swelling caused by RA affects movement and productivity, which has an important negative impact on the economic value in the farming industry (3). Although treatment options for RA have gradually expanded, no effective therapeutic approach currently exists for the improvement of joint destruction, since its potential pathogenesis has not been fully elucidated. Furthermore, although various drugs, such as methotrexate, leflunomide and aspirin have already been used for RA therapy, effective drugs with few side effects and low cost are still

lacking (4-6). Gene therapy has gained considerable interest recently, as it can effectively prevent these shortcomings to a great extent. Therefore, identifying the key genes involved in the pathogenic mechanisms of joint destruction is essential for RA gene therapy (3).

Numerous studies have demonstrated that proinflammatory genes, such as interleukin 1 (IL-1) and tumor necrosis factor-α were markedly increased in the pathogenesis of RA. Therefore, the inhibition of certain inflammatory cytokines, especially IL-1, has become the major focus of RA clinical research since the 1990s (7,8). A previous study revealed that higher serum IL-1β concentrations were observed in the synovial fluid of patients with RA (9). An animal study has also demonstrated that IL-1β injections cause severe joint destruction, which is a typical symptom of human RA (10). These findings suggested that IL-1β serves a vital role in the pathogenesis of RA, and that IL-1β inhibition may be considered a good therapeutic option for RA treatment.

RNA interference (RNAi), a post-transcriptional gene silencing method driven by small interfering RNA (siRNA), is a powerful tool for the prevention and cure of various diseases (11-15). Due to its ability to specifically and efficiently silence gene expression in a sequence-specific manner, RNAi serves major roles in biological processes (16-19), and has been considered to be a promising strategy for the therapy of numerous cancers and cardiovascular diseases, among others (20,21). However, the clinical application of siRNA is still in contention due to its inherent instability in biological fluids along with poor or non-specific cellular uptake (22-24). Therefore, the use of IL-1β siRNA alone for RA treatment is limited. Thus, there is an urgent requirement to elucidate a more effective tool for target gene transfer in RA gene therapy.

Mesenchymal stem cells (MSCs) are multipotent stem cells that are considered to be effective cellular delivery vehicles in numerous types of cell and gene therapies. As a type of MSCs, bone marrow MSCs (BMSCs) have been extensively investigated (25-27). A previous study has demonstrated that protein expression in BMSCs appeared to be readily altered after the transfection of exogenous genes (28), suggesting that BMSCs have high potential as target cells for gene therapy. It has also been reported that BMSC transplantation exhibited high efficiency in the treatment of experimental autoimmune encephalomyelitis (29), indicating that BMSC transplantation is feasible in the treatment of inflammatory diseases. Furthermore, it has been demonstrated that BMSCs were able to regulate the proliferation or survival of T lymphocytes and exerted an immunosuppressive effect by releasing soluble factors, such as transforming growth factor β1 (TGF-β1) and hepatocyte growth factor (30). Based on the aforementioned advantages, exploring the potential of IL-1β siRNA-modified BMSCs for RA treatment, and comparing the therapeutic effect between IL-1β siRNA alone and IL-1β siRNA + BMSCs requires further elucidation.

The aim of the present study was to establish an RA rat model by injecting collagen and to further determine the role of IL-1β in RA pathogenesis. The successfully constructed RA rat model was used to evaluate the therapeutic effect of IL-1β siRNA alone or in combination with BMSCs in RA.

Materials and methods

Animals. A total of 40 female Wistar rats (age, 8-10 weeks; weight, 200-250 g) were purchased from the Comparative Medicine Center of Yangzhou University [Yangzhou, China; certificate of quality, SCXK (Su) 2017-0044]. The rats were housed at 23±3˚C with a relative humidity of 50±10% and subjected to a 12-h light/dark cycle with free access to food and water. All animal experimental protocols were approved by the Animal Care and Use Committee of Yangzhou University (Yangzhou, China).

Experimental design. In the present study, two consecutive experiments were performed. First, a collagen-induced arthritis (CIA) rat model was established by injecting animals twice with type II collagen for 4 weeks (cat. no. C9301; Sigma-Aldrich; Merck KGaA). The first injection was performed at the beginning of modeling, and the second injection was administered 1 week after the first injection. After modeling for 1-3 and 4 weeks, the CIA rat model was evaluated, and rats injected with the same volume of PBS were used as controls. After 4 weeks, the successful CIA rats were injected with PBS and IL-1β siRNA or transplanted with IL-1β siRNA + BMSCs for an additional 4 weeks. The rats were injected twice over the 4-week period to strengthen the therapeutic effect, once at the beginning of the 1st week and another time at the beginning of the 2nd week of treatment. The effects were evaluated at 1-3 and 4 weeks post-transplantation.

Induction of CIA model. The CIA rat model was generated as previously described (31) with certain modifications. CIA rats were first subcutaneously injected at the root of tail with 1 ml bovine type II collagen emulsified in Freund's complete adjuvant (1 mg/ml; cat. no. F5881; Sigma-Aldrich; Merck KGaA). After 1 week, the rats were immunized a second time with half the volume of the same substance. Body weight and toe swelling values were measured once a week after the first immunization, totally for 4 weeks, to preliminary assess the CIA rat model. Toe swelling was measured with a vernier caliper at the same joint. In addition to body weight and toe swelling values, other parameters such as the immobility time, climbing time, serum concentrations of IL-1β, histopathology of the knee joint and expression of certain immune-response related marker genes were assessed to determine model success.

Forced swimming test. Behavioral changes were measured via forced swimming tests once a week after the first immunization, totally for 4 weeks. One day before the formal test, rats were placed and trained in transparent glass cylinders with a height of 93 cm, a bottom diameter of 54 cm and a depth of 60 cm. The water temperature was maintained at 22±1˚C. Each cylinder contained 1 rat, and different cylinders were separated from each other with black organic glass. Rats swam in the water tank for 15 min to adapt to the swimming environment prior to the formal experiment, when the rats were allowed to swim for 6 min. At the end of the experiment, both the immobility time and climbing time were recorded and analyzed. Climbing time referred to the time that the rats began having difficulties to float in the water, especially indicated by the

continuous movement of their front claws on the water surface, or when limb movements were used to keep the head above the water and the water surface fluctuated constantly.

ELISA. At 0, 2 and 4 weeks, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg body weight; Sigma-Aldrich; Merck KGaA) (32-35). No signs of peritonitis were observed. A total of 1 ml blood sample was obtained from the orbital venous plexus, then centrifuged at 3,000 x g at 4˚C for 5 min to separate the serum. The serum concentrations of IL-1β were determined via ELISA (IL-1β Rat ELISA Kit; cat. no. BMS630; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The absorbance was measured at 450 nm. Furthermore, BMSCs were lysed on ice for 1 h using 0.5% Triton X-100 and cell lysate was collected. Both the BMSC cell lysate and supernatant were also used for detecting IL-1β via ELISA.

Sample collection. Four weeks after modeling, rats were sacrificed by decapitation after being anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg body weight). The spleen and thymus were subsequently removed and weighed to calculate the organ index (organ index=tissue wet weight/body weight). Furthermore, the spleen was collected and stored at -80˚C to isolate total RNA and detect the mRNA expression levels of certain immune-response related marker genes. mRNA expression of programmed death-1 (PD-1), transforming growth factor-β1 (TGF-β1) and forkhead box protein 3 (Foxp3) in the spleen was determined via reverse transcription-quantitative PCR (RT-qPCR). Symptoms such as pain, weight loss, loss of appetite or weakness were set as humane endpoints for the present study; however, no rats were sacrificed before the completion of the experiment as a result of displaying any of these symptoms.

Histopathology of the knee joint of CIA rats. A total of 4 weeks after modeling, the whole knee joints were dissected, fixed in 4% paraformaldehyde solution for 24 h at room temperature, decalcified in 10% ethylene diamine tetraacetate at room temperature for 30 days (with the solution renewed once a week) and embedded in paraffin. Standard 4-μm-thick frontal sections were prepared and stained with hematoxylin for 10 min and eosin for 1.5 min (H&E) at room temperature. The synovial tissue sections were observed using light microscopy (CX23; Olympus Corporation; magnification, x20, x100, x200 or x400) and evaluated in a blinded manner.

Rat BMSC isolation using direct adherence. A total of 4 healthy 3-4-week-old specific pathogen free-grade male Wistar rats weighing 100-120 g were sacrificed by anesthesia with intraperitoneal injection of pentobarbital sodium (65 mg/kg body weight) followed by decapitation and soaking in 75% ethanol at room temperature for 10 min. Then, the femur and tibia were isolated under sterile conditions. Samples were immersed in DMEM (cat. no. 11965092; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin. Joint capsules at the end of the diaphysis were removed without isolating the epiphysis, and the diaphysis was then divided, and the connective tissue was stripped from the diaphysis to avoid a mixed cell population. A disposable aseptic syringe was used to collect antibiotic-supplemented DMEM and to repeatedly wash the bone marrow cavity to collect cells in a sterile petri dish. The obtained BMSC suspension was centrifuged at 250 x g for 5 min at 26˚C and rinsed once in DMEM containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). Cells were further resuspended in complete medium (90% DMEM, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) and transferred to a 25-cm^2 plastic culture flask for incubation at 37˚C in a 5% CO_2 incubator. Cells isolated from one rat were cultured in one flask.

Cell culture and siRNA transfection. The BMSCs were cultured in DMEM containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator with 5% CO_2. Cells were seeded at a density of $1x10^5$ cells per well in a six-well plate. When the cells reached 60-70% confluency, they were treated with lipopolysaccharide (LPS; cat. no. L8880; Beijing Solarbio Science & Technology Co., Ltd.; 500 ng/ml in complete DMEM) for 24 h at 37˚C to simulate the RA inflammatory process. IL-1β concentrations in cells and culture medium were determined using IL-1β Rat ELISA kit (cat. no. BMS630; Thermo Fisher Scientific, Inc.). IL-1β siRNA (sense, 5'-GGAAGGCAGUGUCACUCAUTT-3' and antisense, 5'-AUGAGUGACACUGCCUUCCTT-3') and a scrambled siRNA negative control (sense, 5'-UUCUCCGAA CGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUU CGGAGAATT-3') were purchased from Invitrogen (Thermo Fisher Scientific, Inc.), and 100 nM siRNA was transfected into BMSCs for 12 h at 37˚C via Transfast Transfection Reagent (Promega Corporation), followed by removal of the transfection medium and addition of the complete medium, and BMSCs were stimulated with LPS for 24 h to simulate the RA pathogenesis. Next, the cells and culture medium were harvested, and IL-1β concentration was determined using IL-1β Rat ELISA kit.

RT-qPCR. Total RNA from spleen tissue or BMSCs was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The integrity of the extracted total RNA was verified via 1.0% agarose gel electrophoresis. The purity of the total RNA was determined according to the A260/A280 nm value using a spectrophotometer. DNase I was then added to remove genomic DNA contamination at 42˚C for 3 min. RNA was reverse transcribed into cDNA, and the reaction components were as follows: Total RNA template (2 μg), M-MLV reverse transcriptase (Takara Biotechnology Co., Ltd.) (40 U/μl), oligo(dT) (100 nM), dNTP mix (1 mM), DTT (0.1 M), RNAse inhibitor (40 U/μl) and deionized water (RNAse-free) to a total volume of 20 μl. The mixture was incubated at 42˚C for 60 min, heated to 70˚C for 10 min, cooled in iced water and stored at -20˚C. qPCR was performed by mixing 10 μl Power SYBR Green PCR Master mix (Thermo Fisher Scientific, Inc.), cDNA (2 μl, diluted 1:20) and forward and reverse primers (0.2 μM each) to a final PCR volume of 20 μl on an ABI Prism 7700 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions of qPCR were as follows: Initial denaturation at 95˚C for 5 min; followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 62˚C for 30 sec and extension at 72˚C for 30 sec; and final

Table I. PCR primers used in the present study.

Name	Gene reference number	Sequence (5'-3')
PD-1	NM_001106927	F: GCCCGCTTCCAGATCGTAC R: AGGGTCTTCCTTGCGTCCAG
TGF-β1	NM_021578.2	F: TGCTGCCGCTTCTGCTCCCACTC R: ATAGATTGCGTTGTTGCGGTCCAC
Foxp3	NM_001108250.1	F: GCTTGTTTGCTGTGCGGAGAC R: GTTTCTGAAGTAGGCGAACAT
IL-1β	NM_031512	F: TTCAAATCTCACAGCAGCAT R: TCCCACGAGTCACAGAGG
β-actin	NM_031144.2	F: CCTCTGAACCCTAAGGCCAA R: GTCTCCGGAGTCCATCACAA

PD-1, programmed death-1; Foxp3, forkhead box protein 3, F, forward; R, reverse.

extension at 60˚C for 60 sec. mRNA expression was normalized to β-actin, and relative gene expression was quantified using the comparative $2^{-\Delta\Delta Cq}$ method (36,37). The primers used are presented in Table I.

Western blotting. BMSC total protein was extracted using RIPA lysis buffer containing 2 mM EDTA, 100 mM NaCl, 5% SDS, 50 mM NaF, 0.1 mM Na_3VO_4, 100 μM 4-benzenesulfonyl fluoride hydrochloride, 1 mM benzamidine, 50 mM HEPES (pH 7.4) and 10 μg/ml aprotinin. Protein concentration was then determined with BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 40 μg protein sample was mixed with loading buffer (187.5 mmol/l Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 150 mmol/l DTT and 1% bromophenol blue) and denatured for 5 min by boiling at 100˚C before being loaded on a 10% SDS-polyacrylamide gel. The proteins were transferred onto a PVDF membrane after electrophoresis, blocked with 3% BSA (cat. no. A8020; Beijing Solarbio Science & Technology Co., Ltd.) for 90 min at room temperature, and then incubated with primary anti-IL-1β antibodies (1:1,000; ab9722; Abcam) overnight at 4˚C. After washing three times for 10 min each with TBS-0.05% Tween-20, the membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated antibodies (1:5,000; cat. no. ab205718; Abcam) for 2 h at room temperature. Finally, via enhanced chemiluminescence using the SuperSignal West Pico kit (Pierce; Thermo Fisher Scientific, Inc.), the bands were visualized and captured with the VersaDoc 4000 MP system (Bio-Rad Laboratories, Inc.). Quantity One 4.6 software (Bio-Rad Laboratories, Inc.) was used to calculate the band density values automatically. β-actin (1:5,000; cat. no. ab8227; Abcam) was used as a reference protein. IL-1β protein levels were expressed as the relative-fold change of the LPS + Transfast + BMSCs or the negative control group. The experiment was performed in triplicate.

In vivo IL-1β siRNA treatment. Successful CIA rats were randomly divided as follows: i) PBS group, which was used as a negative control; ii) IL-1β siRNA group and iii) IL-1β siRNA + BMSC group. Both PBS and 100 nM IL-1β siRNA rats were injected via the caudal vein with Entranster™-*in vivo* (cat. no. 18668-11-2; Engreen Biosystem, Ltd.). BMSCs were plated in 24-wells at a density of $2.4x10^4$ cells/well. When cells reached 60-70% confluency, 100 nM IL-1β siRNA was transfected into BMSCs for 12 h at 37˚C via Transfast Transfection Reagent (Promega Corporation). Then, IL-1β siRNA + BMSCs cells were immediately injected twice via the caudal vein in rats with BMSCs stock solutions (resuspended in PBS) of $1x10^7$ cells/kg, on days 3 and 6 after 4 weeks of CIA modeling. The therapeutic effect was assessed at 1-3 and 4 weeks post-transplantation by means of body weight, toe swelling value, immobility time, climbing time and serum IL-1β concentration. The mRNA expression of immune-response related marker genes in the spleen was determined after therapy for 4 weeks.

Statistical analysis. All data are presented as the mean ± SEM. Statistical analyses were carried out with SPSS software v20.0 for Windows (IBM Corp.). Differences between two groups were evaluated using independent Student's t-test, while differences among three groups were evaluated using one-way ANOVA followed by Tukey-Kramer post-hoc test. All the assays conducted were repeated at least three times. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment and evaluation of a CIA rat model induced by collagen II. Toe swelling value, immobility time and climbing time are three typical indicators to evaluate whether the CIA rat model was successfully established (38). After modeling for 4 weeks, stiffness, deformity and subcutaneous nodules were observed on the surface of the skin area of CIA rat joints (data not shown). In addition, although the growth curves of both CIA and control rats were gradually increased, the body weight of CIA rats was significantly lower than that of the controls after modeling for 2 weeks (Fig. 1A). Compared with control rats, CIA rats demonstrated increased foot swelling values, whose joints could not bear their own body weight, resulting in crawling difficulties; however, no rats were sacrificed before the completion of the experiment, as a result of displaying any of the predetermined following humane endpoints, such as pain, weight loss, loss of appetite or weakness. Furthermore,

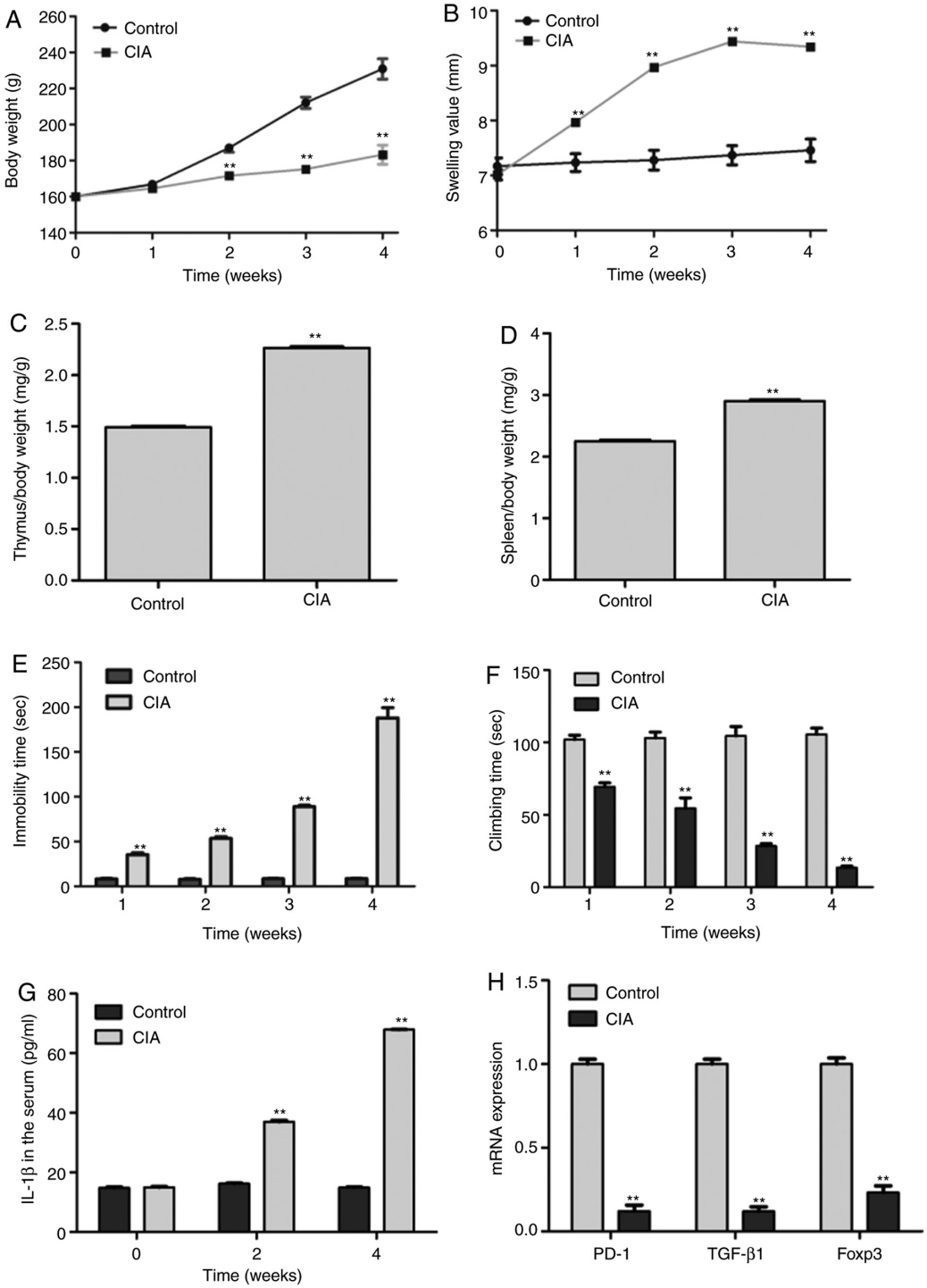

Figure 1. Establishment and evaluation of the CIA rat model. (A) Body weight. (B) Toe swelling value. (C) Thymus index and (D) spleen index. (E) Immobility time. (F) Climbing time. (G) Serum IL-1β level. (H) PD-1, TGF-β1 and Foxp3 mRNA expression in the spleen. Values are presented as the mean ± SEM. CIA rats, n=30; control rats, n=10. **P<0.01 vs. control rats. PD-1, programmed death-1; Foxp3, forkhead box protein 3; CIA, collagen-induced arthritis.

during the 1st week of modeling, the volume of both feet started to swell and reached a peak at 3 weeks that was then stabilized (Fig. 1B), which indicated a progressively increasing foot swelling value of CIA rats relative to controls. Immune organ index analysis demonstrated that both the spleen and thymus index in CIA rats were significantly elevated compared with those of control rats (2.91±0.07 vs. 2.25±0.10 mg/g; and 2.27±0.05 vs. 1.49±0.10 mg/g; respectively), indicating that the pathological changes and immune response in CIA rats were more severe than those in the control rats (Fig. 1C and D). The forced swimming results revealed that the immobility time in CIA rats was significantly higher (Fig. 1E), while the climbing time was lower than that of the control rats after modeling for 1-3 and 4 weeks, indicating that hind limb movement disorders were becoming more severe with the extension of the modeling time (Fig. 1F). The results of ELISA demonstrated that CIA rats exhibited higher serum IL-1β concentrations than that of the control rats after modeling for 2 and 4 weeks (Fig. 1G). In

addition, the RT-qPCR results revealed that the mRNA expression of the immune-related marker genes PD-1, TGF-β1 and Foxp3 were significantly decreased in the spleen compared with those of control rats (Fig. 1H). Overall, the results suggested that CIA model rats were successfully established, and that the pathogenesis of CIA was closely associated with elevated serum IL-1β concentration.

Histopathology of the knee joints of CIA rats. Compared with the knee joints of control rats, joint swelling and redness were observed in CIA model rats (Fig. 2A and B) after modeling for 4 weeks. In addition, the H&E staining results of tissue sections of synovium demonstrated that the articular synovial cells of the control rats were normal in morphology and were neatly arranged (Fig. 2C and D), while the CIA model rats presented much more severe RA symptoms, which were characterized by evident erosive destruction of the bone and ulceration of the articular cartilage (Fig. 2E and F), as well as severe mixed infiltration of inflammatory cells, mainly including neutrophils, lymphocytes and macrophages, in the synovial membrane, articular cartilage and joint cavity. In addition, multinucleated giant cells in the articular cavity, minor bleeding, fibrinous exudation and mild neovascularization were observed in CIA model rats but not in the control rats (Fig. 2G and H).

IL-1β expression in LPS-stimulated BMSCs with or without IL-1β siRNA transfection. In the present study, both RT-qPCR and western blotting were performed to confirm that IL-1β siRNA transfections were successful in BMSCs. The results demonstrated that when compared with scrambled siRNA negative control, IL-1β siRNA transfection significantly reduced IL-1β mRNA and protein expressions (Fig. 3A and B). In the current study, BMSCs were stimulated with LPS (LPS + BMSCs) to simulate the classic inflammatory symptoms of RA, and IL-1β concentrations in the cell supernatant and lysate were analyzed by ELISA. The results revealed that IL-1β concentrations in the cell supernatant (335.29±5.17 vs. 121.76±3.10 ng/ml) and lysate (176.65±6.33 vs. 78.52±3.24 ng/ml) were both significantly elevated in LPS + BMSCs compared with those of BMSCs (Fig. 3C and D).

In addition, IL-1β concentrations in both the cell supernatant (347.84±6.17 vs. 52.66±1.72 pg/ml) and lysate (178.29±2.18 vs. 28.47±1.27 pg/ml) were reduced in the LPS + BMSCs + IL-1β siRNA group compared with those in the LPS + BMSCs + Transfast group (Fig. 3E and F), suggesting that IL-1β siRNA transfection was able to successfully reverse the increased IL-1β concentration in both the supernatant and lysate of LPS-stimulated BMSCs.

Furthermore, the RT-qPCR and western blotting results revealed that IL-1β mRNA (0.28±0.02 vs. 2.74±0.15) and protein expression were significantly reduced in the LPS + BMSCs + IL-1β siRNA group relative to the LPS + BMSCs + Transfast group (Fig. 3G and H), further demonstrating that IL-1β expression was able to be successfully silenced after IL-1β siRNA transfection in LPS-stimulated BMSCs.

Therapeutic effect of IL-1β siRNA combined with BMSCs on CIA model rats. The timeline of modeling and transplantation therapy is presented in Fig. 4A. The results demonstrated that compared with PBS-injected rats, the body weight of both IL-1β siRNA-injected rats and IL-1β siRNA + BMSCs-transplanted rats was significantly increased after therapy for 1-3 and 4 weeks (Fig. 4B). Furthermore, the toe swelling of rats that received IL-1β siRNA and IL-1β siRNA + BMSCs was significantly lower than that of PBS rats after therapy for 2, 3 and 4 weeks (Fig. 4C). The forced swimming results demonstrated that compared with PBS rats, the immobility time in rats treated with IL-1β siRNA and IL-1β siRNA + BMSCs was significantly reduced (187.90±5.4 vs. 156.02±4.58 vs. 106.45±7.97 sec), while the climbing time was significantly increased (13.62±2.17 vs. 43.68±4.74 vs. 62.22±3.36 sec) after therapy for 1 week. Furthermore, with the prolongation of the treatment, the immobility time was reduced gradually in the 3rd and 4th weeks compared with the 1st and 2nd weeks of treatment in both IL-1β siRNA and IL-1β siRNA + BMSCs rats, while the climbing time was increased gradually. Furthermore, compared with IL-1β siRNA rats, IL-1β siRNA + BMSCs rats demonstrated lower immobility time and higher climbing time in the 4th week of treatment (Fig. 4D and E), suggesting that both IL-1β siRNA and IL-1β siRNA + BMSCs resulted in a gradually improved recovery over time, and the combination treatment achieved a more prominent improvement compared with that of IL-1β siRNA treatment alone.

The ELISA results revealed that the serum IL-1β concentrations in both IL-1β siRNA and IL-1β siRNA + BMSCs rats were significantly lower than those of PBS rats after treatment for 2 (44.34±2.19 vs. 31.27±1.87 vs. 69.15±0.55 pg/ml, respectively) and 4 (31.76±1.59 vs. 20.21±0.83 vs. 65.44±1.94 pg/ml, respectively) weeks. Furthermore, IL-1β siRNA + BMSCs rats presented notably lower IL-1β concentration than IL-1β siRNA rats (Fig. 4F). The RT-qPCR results demonstrated that the mRNA expression of PD-1, TGF-β1 and Foxp3 in spleen was significantly increased in both IL-1β siRNA and IL-1β siRNA + BMSCs rats compared with that in PBS rats, and IL-1β siRNA + BMSCs rats exhibited a higher increase than IL-1β siRNA rats (Fig. 4G). Overall, the above behavioral tests and inflammation assessment indicated that both IL-1β siRNA and IL-1β siRNA + BMSCs were able to significantly ameliorate the CIA symptoms, while IL-1β siRNA + BMSCs transplantation exhibited a higher therapeutic efficacy compared with that of IL-1β siRNA injection alone in CIA rats.

Discussion

RA modeling is essential for understanding the pathogenesis of RA and for finding effective treatments. Current methods for establishing RA models include: Adjuvant arthritis (AA), CIA, ovalbumin-induced arthritis and avridine-induced arthritis (AIA), among which the AA and CIA modeling methods are widely used (39,40). In our previous preliminary study, both CIA and AA modeling methods were used, and it was revealed that when compared with the AA method, the CIA rat model was more likely to present the typical pathological symptoms of human RA in several aspects, including synovial inflammation and slow and late onset of chronic persistent inflammation, suggesting that CIA is an ideal model for RA (data not shown). This is in concordance with previous research, which indicated that CIA is widely used as an RA model in antirheumatic drug screening, since it exhibits various similarities to human RA (41).

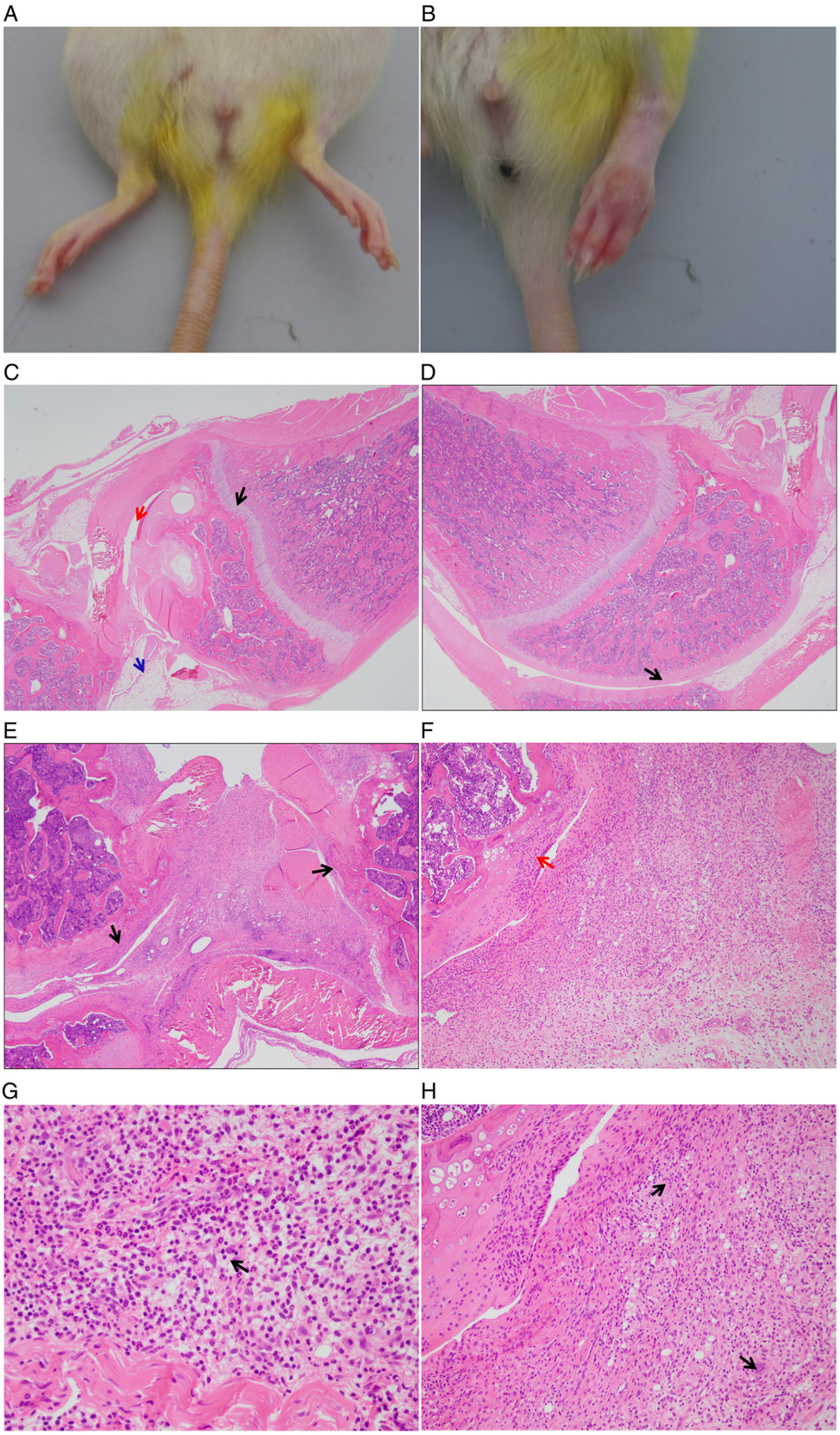

Figure 2. Histopathological analysis of the knee joints of CIA rats after modeling for 4 weeks. (A) Knee joint of control rats. (B) Knee joint of CIA rats with obvious swelling and redness symptoms. (C and D) Hematoxylin and eosin staining of synovial tissue from the knee joints of control rats, with smooth articular cartilage surface and neatly arranged synovial tissue cells. (C) The black arrow indicates the normal growth plate, the red arrow indicates normal articular cavity and the blue arrow indicates normal synovium (magnification, x20). (D) The black arrow indicates normal articular cartilage (magnification, x20). (E) Erosive bone destruction of CIA model rats. Black arrows indicate erosion of the articular cartilage (magnification, x20). (F) Ulceration of the articular cartilage in knee joints. The red arrow indicates erosion of the articular cartilage (magnification, x100). (G) Infiltration of inflammatory cells in knee joints, including neutrophils, lymphocytes and macrophages. The black arrow indicates mixed cell inflammation, which composed of a large number of neutrophils and lymphocytes and macrophages (magnification, x400). (H) Multinuclear giant cells in the articular cavity and deposition of fibrin clots in knee joints. Black arrows indicate multinuclear giant cells (magnification, x200). CIA, collagen-induced arthritis.

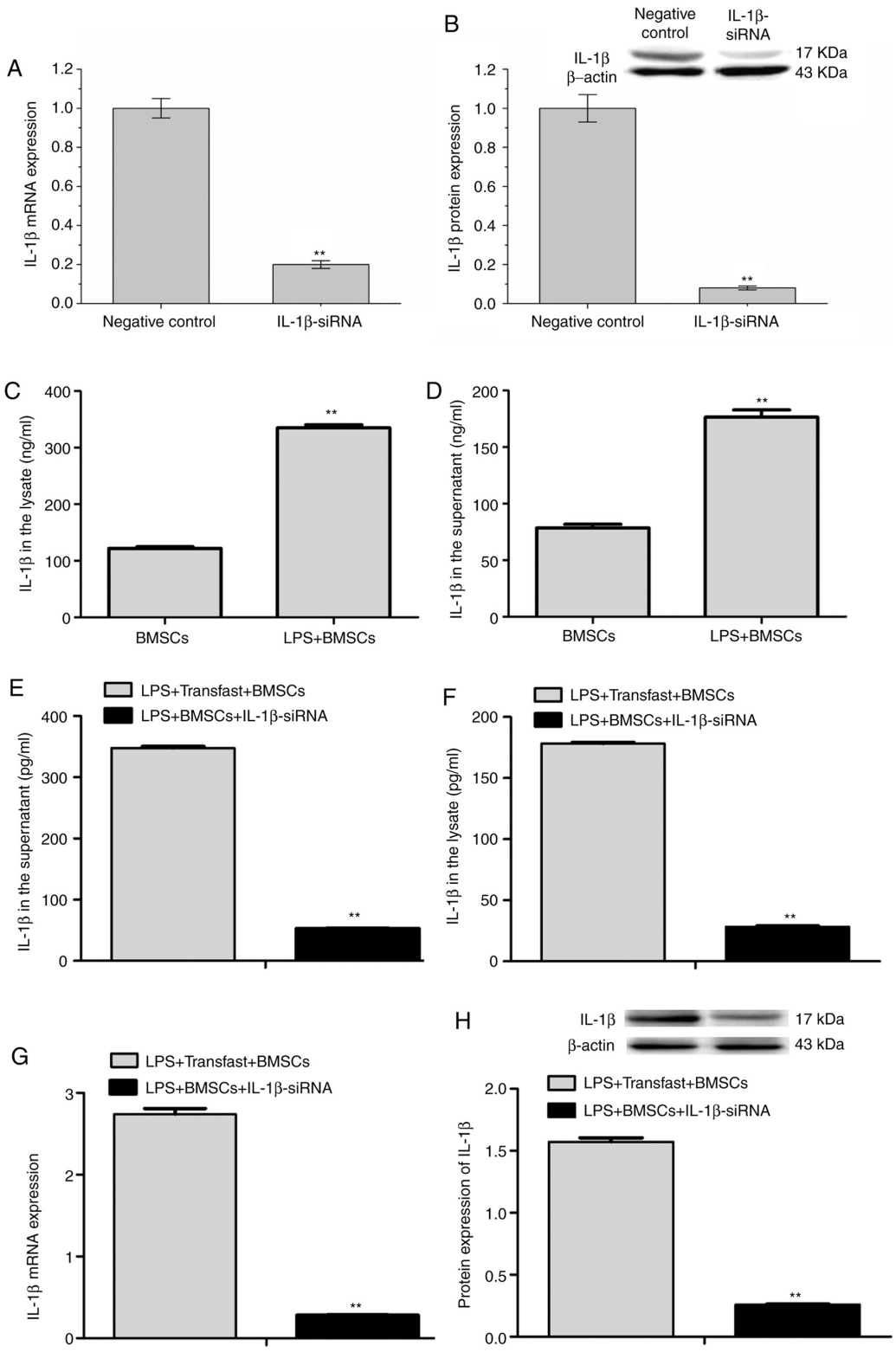

Figure 3. IL-1β expression in LPS-stimulated BMSCs with or without IL-1β siRNA transfection. (A) mRNA and (B) protein expression of IL-1β in BMSCs. IL-1β concentration in the (C) cell lysate and (D) culture supernatant of LPS stimulated BMSCs. IL-1β concentration in the (E) culture supernatant and (F) cell lysate of LPS-stimulated BMSCs with IL-1β siRNA transfection. (G) mRNA and (H) protein expression of IL-1β in LPS-stimulated BMSCs with IL-1β siRNA transfection. Data are presented as the mean ± SEM (n=6/group). **P<0.01 vs. negative control, BMSCs or LPS + Transfast + BMSCs. LPS, lipopolysaccharide; si, small interfering; BMSCs, bone marrow mesenchymal stem cells.

The present study improved the CIA modeling method by first injecting combined emulsions of type II collagen and complete Freund's adjuvant, while a previous study demonstrated that the successful rate of CIA modeling was 40% (38). A total of 7 days after the first immunization, the mixed antigen was injected again, which guaranteed the high success rate (>80%; data not shown) and stability of the CIA model. Furthermore, 200-250 g male adult rats were selected for CIA modeling,

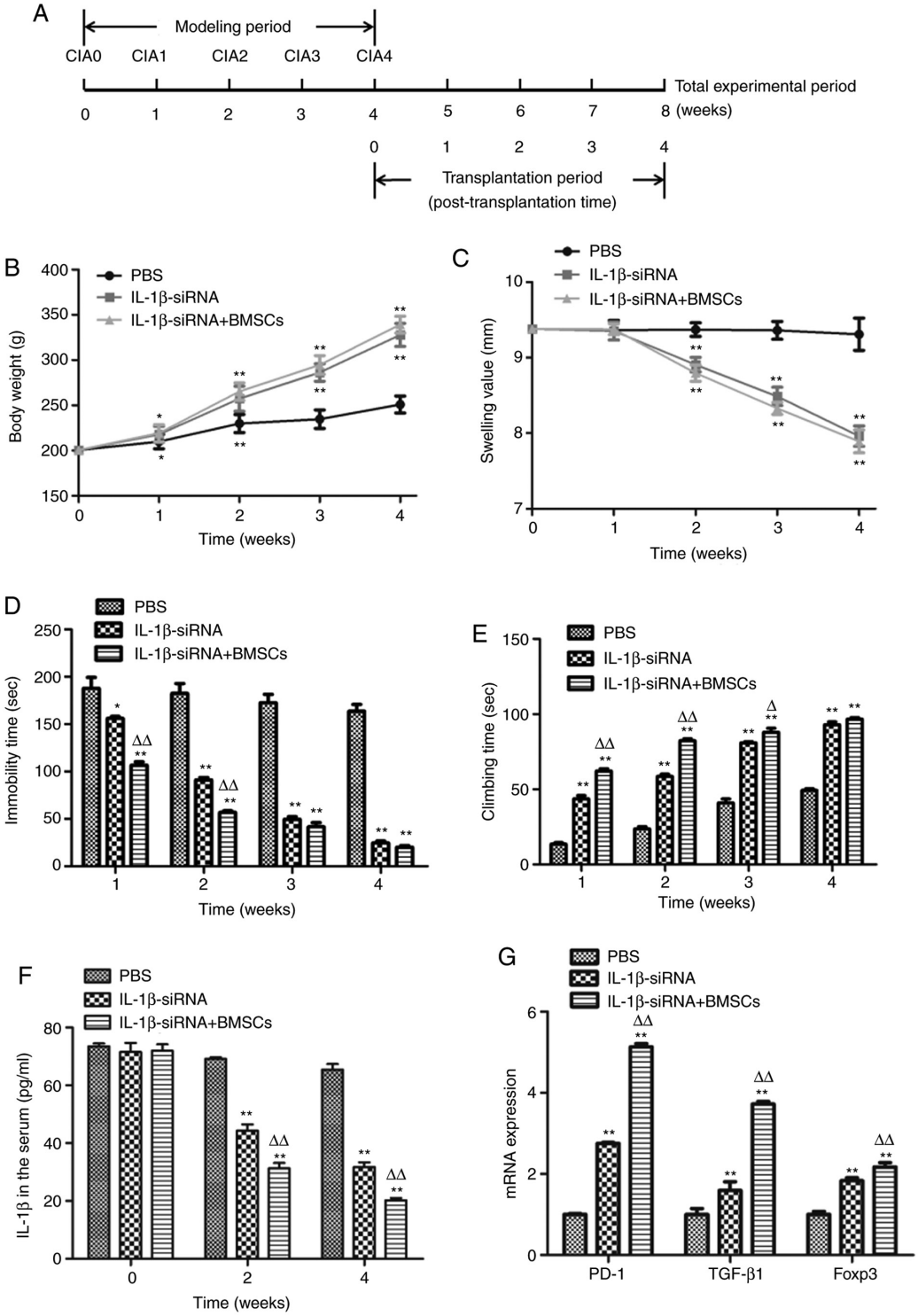

Figure 4. Therapeutic effect of IL-1β siRNA combined with or without BMSCs on the CIA rat model. (A) Timeline of the experimental design. (B) Body weight. (C) Toe swelling value. (D) Immobility time. (E) Climbing time. (F) Serum IL-1β concentration. (G) mRNA expression of PD-1, TGF-β1 and Foxp3 in the spleen. Data are presented as the mean ± SEM (n=8/group). *P<0.05, **P<0.01 vs. PBS; $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 vs. IL-1β siRNA. CIA, collagen-induced arthritis; PD-1, programmed death-1; Foxp3, forkhead box protein 3; si, small interfering; BMSCs, bone marrow mesenchymal stem cells.

mainly based on a previous study, which demonstrated that age serves a key role in the construction of the CIA model and rats <21 days or >9 months of age were not easy to be modeled because of the effect of age and sex (42). After modeling, CIA rats were assessed by testing important indicators associated with RA. The results revealed that the swelling value, immune organ index, immobility time and serum IL-1β concentration were significantly increased, while the body weight, climbing time and PD-1, TGF-β1 and Foxp3 mRNA expression in the spleen were reduced, suggesting that a stable and reliable CIA model was established, which contributed to the follow-up gene and cell therapy experiment. In addition, histopathology analyses also demonstrated severe joint destruction of CIA rats, which also indicated successful RA modeling.

Abnormal inflammatory cytokines are considered to be involved in RA pathogenesis, among which the most important one that is associated with articular cartilage injury is IL-1β. IL-1β mainly exists in the synovial fluid and serum of patients with RA and serves important regulatory roles in synovial inflammation. It is highly expressed in the synovial membrane during inflammation in RA disease and can be used as an indicator to judge RA severity (43,44). A previous study has demonstrated that IL-1β was able to induce endothelial cells to promote the aggregation of lymphocytes, neutrophils and macrophages, and increase the secretion of prostaglandins by neutrophils, thereby increasing the expression of collagenase, chondrocytes and fibroblasts in synovial cells, as well as the secretion of JAK2 and JAK3 kinases, which led to the occurrence of local inflammatory response in joints (45,46). Therefore, the examination of IL-1β concentration is important for the evaluation of RA modeling success. The present study demonstrated that the serum IL-1β concentration of CIA rats was elevated, suggesting that IL-1β served crucial roles in RA development, and may have induced the production of proteinases and osteoclast activation, thus leading to joint destruction. RA is also caused by the imbalance of T cells, which in turn leads to increased proinflammatory and reduced anti-inflammatory cytokines (47,48). A previous study demonstrated that PD-1 null mice developed late-onset inflammatory arthritis and mild glomerulonephritis, indicating that PD-1 was important for *in vivo* peripheral self-tolerance and was involved in the negative regulation of the immune response (49). Furthermore, another study in human and murine arthritis demonstrated that the PD-1 gene was closely associated with RA pathogenesis, while a PD-1 agonist ameliorated RA activity by reducing inflammatory cytokine production (50). TGF-β1 and Foxp3 are also key molecules of the immune system apparatus (51). In the present study, the mRNA expression of PD-1, TGF-β1 and Foxp3 in the spleen was significantly reduced in CIA rats, which confirmed the imbalance of cytokines in the CIA rat model and the successful establishment of the RA model. These results suggested that the blockade of these three genes may have increased T and B cell activation and proliferation, leading to IL-1β production.

RNAi is a relatively novel technology for gene silencing, and RNAi-based therapy is a promising strategy that reduces joint inflammation in experimental arthritis (52,53). BMSCs, as matrix stem cells, undergo chondrogenic differentiation and are capable of promoting cartilage repair and tissue regeneration (54). BMSCs can not only differentiate into multiple tissue cells, but also enhance the repair of tissue damage caused by inflammation by secreting various matrix molecules, which suggests that they may aid in regenerating RA cartilage (54-56). Mature articular cartilage has a limited capacity for self-regeneration after injury due to the lack of blood and nerve supply, resulting in poor articular cartilage recovery after damage (57). RA is mainly caused by cartilage destruction and synovial damage (58). In the present study, in order to simulate the inflammatory process of RA, BMSCs were treated with LPS. The results demonstrated that IL-1β expression was significantly induced, suggesting the important role of IL-1β in the pathogenesis of RA. IL-1β siRNA with high silencing efficiency was subsequently transfected into LPS-stimulated BMSCs. The results revealed that the mRNA and protein expression of IL-1β was significantly inhibited in LPS-stimulated BMSCs, indicating that the exogenous IL-1β siRNA could be successfully transfected into BMSCs and produced a high and stable silencing effect. Previous animal studies have determined that allogeneic BMSC transplantation was able to successfully repair cartilage defects in the local microenvironment (26,27). Another study demonstrated that BMSC co-therapy with TGF-β1 can repair cartilage defects more effectively than treatment with TGF-β1 alone (59), suggesting an enhanced effect of BMSCs in the gene therapy of arthritic diseases.

In the current study, *in vivo* injections of IL-1β siRNA alone and IL-1β siRNA + BMSCs were used to treat RA rats. Based on the unique superiority of BMSCs compared with differentiated cells, it was hypothesized that IL-1β siRNA transfection and BMSC transplantation could produce a better therapeutic effect. Compared with the effects observed in PBS-injected rats, both IL-1β siRNA-injected rats and IL-1β siRNA-BMSCs-transplanted rats exhibited a significant therapeutic effect at the morphological, behavioral, histopathological and immunological level, and IL-1β siRNA-BMSCs transplantation exhibited a better therapeutic effect on the remission of symptoms compared with that of IL-1β siRNA injection alone, indicating that IL-1β siRNA-BMSCs achieved a more prominent improvement of RA. However, in the present study, the therapeutic effects were evaluated by comparisons with PBS-injected CIA rats only. The lack of a control (vehicle treatments only for both modeling and therapy stages) as the baseline is a limitation to be considered.

The present study demonstrated that the blockade of IL-1β was able to ameliorate RA-associated clinical symptoms by increasing body weight and reducing swelling time and immobility time and increasing climbing time, which is consistent with previous results (60). As an effective immunosuppressive factor, TGF-β1 exhibits an anti-inflammatory effect and serves an important role in the pathogenesis of several inflammatory diseases. A previous study demonstrated that inhibiting the expression of TGF-β1 led to autoimmune disorders in RA, while its overexpression resulted in RA improvement (47).

The results of the present study revealed that the mRNA expression of TGF-β1 was significantly lower in the spleen of RA rats than that of control rats, suggesting that decreased expression of TGF-β1 may not be adequate to inhibit the development of autoimmune inflammation, which may also be one of the reasons for RA incidence. In addition, the mRNA expression of Foxp3 was also decreased significantly in the spleen of RA rats, which may have promoted $CD4^+CD25^+$ regulatory T cell populations and contributed to the decreased secretion of TGF-β1 (61). In the therapeutic experiment, both TGF-β1 and Foxp3 mRNA expression levels were significantly increased in the spleen of IL-1β siRNA-injected rats and IL-1β siRNA-BMSCs-transplanted rats, indicating that RA may be potentially improved by the promotion of $CD4^+CD25^+$ regulatory T cell population and function (62,63).

In summary, the current study demonstrated the important role of IL-1β in the pathogenesis of RA and IL-1β siRNA-BMSCs transplantation significantly decreased the

severity of RA by reducing IL-1β concentration and improving biological behaviors. The results suggested that IL-1β was implicated in the pathogenesis of RA, and that IL-1β siRNA was effective in RA therapy, while its combination with BMSCs exerted a synergistic therapeutic effect. The findings of the present study may provide a theoretical basis for the improvement of RA using IL-1β siRNA + BMSC transplantation. The present findings provide the first evidence, to the best of our knowledge, that IL-1β siRNA-BMSCs can repair cartilage defects of RA more effectively and may represent a novel strategy for RA treatment.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HX and SP designed the experiments, supervised the laboratory work and critically revised the manuscript. XD mainly performed the experiments and analyzed the data. YW assisted with data analysis, discussion of results and writing of the manuscript. TZ, YL and AZ provided samples and carried out the detection of the serum parameters. XD and YW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval (approval no. SYXK-SU-2007-0005) was obtained from the Institute of Animal Care and Use Committee of Yangzhou University (Yangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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