Supplementary Material

Scavenger Receptor-Mediated Targeted Treatment of Collagen-Induced Arthritis by Dextran Sulfate−Methotrexate Prodrug

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Experimental Section

Materials

Dextran sulfate (DS) and dextran (Dex) with both number-average molecular weight (M_n) of 5000 g/mol were purchased from J&K Scientific Ltd. (Beijing, P. R. China). MTX was bought from Beijing HuaFeng United Technology Co., Ltd. (Beijing, P. R. China). 1-Ethyl-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC·HCl) and 4-*N*,*N*-dimethylaminopyridine (DMAP) were acquired from GL Biochem Co., Ltd. (Shanghai, P. R. China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (NY, Grand Island, USA). Penicillin and streptomycin were obtained from Huabei Pharmaceutical Co., Ltd. (Shijiazhuang, P. R. China). Lipopolysaccharide (LPS) was bought from Biosharp Co. (Hefei, P. R. China). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich (Shanghai, P. R. China). Bovine type II collagen (BCol II) (2.0 mg/mL), complete Freund's Adjuvant (CFA) (2.0 mg/mL), and incomplete Freund's Adjuvant (IFA) were purchased from Chondrex (Washington, USA). Anti-TNF-α, anti-IL-1β, and anti-IL-6 antibodies were obtained from Abcam (Cambridge, MA, USA). The primers of TNF-α, IL-1β, and IL-6, and housekeeping gene of GADPH were designed by Sangon Biotech (Shanghai, P. R. China). The reverse transcription kit and SYBR Premix Ex Taq^{TM} kit were bought from Takara Biomedical Technology (Beijing, P. R. China). TNF-α, IL-1β, and IL-6 ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA).

Preparations and Characterizations of DS-*g*-MTX and Dex-*g*-MTX

DS-*g*-MTX was prepared by a one-step condensation reaction. Condensing agent was EDC·HCl, and catalyst was DMAP, as shown in Scheme 1. Firstly, DS (2.50 g, 15.4 mmol glucose units) and MTX (308.64 mg, 0.77 mmol) were dissolved in 50.0 mL of MilliQ water and dimethyl sulfoxide (DMSO), respectively. Then, the solution of MTX was dropped into DS solution. The reaction between DS and MTX was conducted at room temperature. After 72 h, dialysis of the mixed solution was carried out in Milli-Q water (molecular weight cut-off (MWCO) = 3500 Da). The flocculent solid matter was obtained by lyophilization. Dex-*g*-MTX was synthesized using the same procedure. FITC was conjugated with various MTXs at a molar ratio of 1:1. FITC (4.28 mg in three copies), MTX (5.0 mg), DS-*g*-MTX (128.21 mg), and Dex-*g*-MTX (119.05 mg) were sufficiently dissolved. Then, FITC was slowly added into three solutions of MTX formulations before ceaseless stir for 48 h. Subsequently, the mixture was dialyzed in Milli-Q water (MWCO = 500 Da for MTX, and MWCO = 3500 Da for MTX prodrug). A final volume of 10.0 mL FITC-labeled solution was obtained in each group. The equivalent concentration of MTX was 0.5 mg/mL.

Proton nuclear magnetic resonance (^{1}H NMR) spectra of DS-*g*-MTX, Dex-*g*-MTX, DS, and Dex in deuterated water (D_2O), and MTX in deuterated dimethyl sulfoxide (DMSO-d_6) were tested on a Bruker AV 600 NMR spectrometer. Fourier-transform infrared (FT-IR) spectra were analyzed on a Bio-Rad Win-IR instrument using potassium bromide (KBr) method. Transmission electron microscopy (TEM) measurements were performed on a JEOL JEM-1011 transmission electron microscope with an accelerating voltage of 100 kV. The sample was prepared by being dissolved in 0.001 M phosphate-buffered saline (PBS), and then dropped on a carbon-coated copper grid and dried at room temperature in the air. The

hydrodynamic diameters (D_hs) of conjugates were tested by dynamic laser scattering (DLS) measurements with a vertically polarized He-Ne laser (DAWN EOS, Wyatt Technology Co., Santa Barbara, CA, USA). The critical micelle concentrations (CMCs) of DS-*g*-MTX and Dex-*g*-MTX were detected by Nile Red as a probe. The MTX contents in DS-*g*-MTX and Dex-*g*-MTX were determined by an ultraviolet-visible (UV-Vis) spectrophotometry with λ_{abs} = 303 nm (UV-1800, Shimadzu, Kyoto, Japan).

***In Vitro* MTX Release**

In vitro release of MTX from DS-*g*-MTX or Dex-*g*-MTX was assessed in PBS at pH 7.4. DS-*g*-MTX or Dex-*g*-MTX was dissolved in 10.0 mL of PBS before being transferred into a dialysis bag (MWCO = 3500 Da). Then the dialysis bag was immersed in 100.0 mL of PBS containing 5% (*V*/*V*) Tween-80 at 37 ℃ under continuous shaking of 70 rpm. At predetermined time intervals, 2.0 mL of release medium was taken for test, and an equal volume of PBS (pH 7.4) containing 5% (*V*/*V*) Tween-80 was added to the system. The amount of MTX was determined by UV-Vis spectrophotometer at λ_{abs} = 303 nm.

***In Vitro* Targeting Ability Analyses and Cytotoxicity Assay**

Both confocal laser scanning microscopy (CLSM) and flow cytometry (FCM) were used to detect the targeting ability of DS-*g*-MTX and Dex-*g*-MTX toward the activated and unactivated macrophages (*i.e.*, RAW 264.7 cells). RAW 264.7 cells were seeded in 6-well plates with a density of 2.0×10^5 cells per well in DMEM containing 10% (*V*/*V*) FBS and 1% (*W*/*V*) penicillin-streptomycin at 37 ℃ in humidified 5% carbon dioxide (CO_2) atmosphere for 12 h.

Cells were activated with 20.0 ng/mL LPS for 48 h. After cell activation, the original medium was replaced with the FITC-labeled DS-*g*-MTX or Dex-*g*-MTX solution (green) in DMEM at a final MTX concentration of 20.0 μg/mL, followed by incubation for 2 h.

CLSM. The activated and unactivated cells on glass coverslips cocultured with the two labeled conjugates were washed three times with PBS and fixed with 4% (*W/V*) PBS-buffered formaldehyde for 30 min at room temperature. Subsequently, cell nucleus was stained with DAPI (blue) for 3 min. The microscopy image of the cellular uptake was taken by CLSM using a LSM 780 CLSM (Carl Zeiss, Jena, Germany) with 10 × eyepiece and 60 × objective.

FCM. Moreover, the activated and unactivated cells cocultured with the two stained conjugates were also washed three times with PBS, followed by digestion with trypsin. The cells were harvested by centrifugation for 5 min at 15000 rpm, 4 ℃. Then the cells were suspended and washed with PBS for two additional times prior to the final suspension with 500.0 μL of PBS. The fluorescence intensity of cellular uptake was detected by FCM (Beckman, California, USA)

Cytotoxicity. The cytotoxicity of DS, Dex, DS-*g*-MTX, Dex-*g*-MTX, and free MTX was measured by a MTT assay toward RAW 264.7 cells. Cells were seeded in 96-well plates with a density of 8000 cells per well for 12 h. Then, the cell culture medium was refreshed with 200.0 μL of medium containing DS, Dex, DS-*g*-MTX, Dex-*g*-MTX, or free MTX at various MTX equivalent concentrations from 4.9 to 20.0 μg/mL. The cells were incubated for another 72 h. After that, 100.0 μL of MTT solution containing 0.05 mg of MTT was added and incubated for a further 4 h. Subsequently, the medium was replaced with 100.0 μL of DMSO. Finally, the absorbance of the solution in each well was measured on a Bio-Rad 680

microplate reader (Hercules, CA, USA) at 490 nm. The cell viability was calculated in accordance with Equation S1.

$$\text{Cell Viability (\%)} = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

Hemolysis Assays

The hemocompatibility properties of DS-*g*-MTX, Dex-*g*-MTX, and free MTX were determined by a spectrophotometry technique. The fresh blood was obtained from the hearts of rabbits in the Laboratory Animal Center of Jilin University. Subsequently, red blood cells (RBCs) were separated by centrifugation at 2500 rpm for 15 min. After careful washing, DS-*g*-MTX, Dex-*g*-MTX, and free MTX solutions of various concentrations were added to the 2% (*V*/*V*) RBCs. After mixing using vortex, the three MTX formulations were thoroughly mixed *via* vortex, and incubated at 37 ℃ in a thermostatic water bath for 3 h. NS and Triton X-100 (1×10^4 mg/mL, a surfactant known to lyse RBCs) were used as negative and positive controls, respectively. RBCs were then isolated by centrifugation at 3000 rpm for 10 min, and 100.0 μL of the supernatant of each sample was transferred to a 96-well plate. The free hemoglobin released from RBCs was measured on a Bio-Rad 680 microplate reader at 540 nm. The hemolysis ratio of RBCs was calculated using Equation S2.

$$\text{Hemolytic Ratio (\%)} = \frac{A_{\text{sample}} - A_{\text{negative control}}}{A_{\text{positive control}} - A_{\text{negative control}}} \times 100 \quad (2)$$

In Equation S2, A_{sample}, $A_{\text{negitive control}}$, and $A_{\text{positive control}}$ were presented as the absorbances of sample, and negative and positive controls, respectively.

***Ex Vivo* Biodistribution of Conjugates**

In this work, all animals were handled according to the protocol approved by the Institutional Animal Care and Use Committee of Jilin University. DBA/1J mice were

maintained in 12 h light/12 h dark cycles with continuous access to food and water. Collagen-induced arthritis (CIA) was induced in male DBA/1J mice at 8 − 10 weeks of age according to the previously published approach [1]. To induce CIA, mice were intradermally injected with BCol II (2.0 mg/mL) emulsified in equal volume of CFA (2.0 mg/mL) at the end of tail. It was followed by a booster immunization at 21 days after the primary immunization with BCol II equally emulsified in IFA. At 48 h after primary immunization, the FITC-labeled DS-*g*-MTX, Dex-*g*-MTX, and free MTX were intravenously injected with a MTX equivalent dose of 5.0 mg per kg body weight (mg/(kg BW)). NS was injected as a control. The mice were sacrificed at 12 h post-injection. The inflamed joints were excised immediately and subsequently washed with NS three times for *ex vivo* imaging of FITC fluorescence on a Maestro 500FL *in vivo* Imaging System (Cambridge Research & Instrumentation, Inc., USA). And the signals were also quantitatively analyzed using Maestro™ 2.4 software (CRi, Woburn, MA, USA).

***In Vivo* Therapeutic Efficacies against CIA Mice**

The CIA mice were induced by the protocol above. In practice, the intravenous administration of 200.0 μL of DS-*g*-MTX, Dex-*g*-MTX, or free MTX at a MTX equivalent dose of 5.0 mg/(kg BW), and NS as a control was carried out on CIA mice at 30, 33, 36, 39, 42, and 45 days after primary immunization (n = 6 for each group). The mice were examined every three days after administration for signs of joint inflammation and were scored as follows: 0 = normal, 1 = mild swelling, and erythema confined to the tarsus or ankle joint, 2 = mild swelling, and erythema extending from the ankle to tarsus, 3 = moderate swelling, and erythema extending from the ankle to tarsus, 4 = severe swelling, and erythema

encompassing the foot, ankle, and digits, or ankylosis of the limb. These paw scores were summed for each limbs of each mouse, giving a maximum possible score of 16 per mouse. Paw thickness (mm) was measured using a caliper for the right and left hind paws of mice.

Histopathological and Immunofluorescence Analyses: The CIA mice were sacrificed at 3 days after the last administration. For histopathological analyses, the knee joints were collected and fixed in 4% (*W*/*V*) PBS-buffered paraformaldehyde overnight, and decalcified using 10% (*W*/*V*) EDTA solution. Then, the joints were embedded in paraffin and cut into slices of 5 mm thickness for hematoxylin and eosin (H&E) staining and immunofluorescence analyses (TNF-α, IL-1β, and IL-6). The histopathological microimages were visualized by a microscope (Nikon Eclipse *Ti*, Optical Apparatus Co., Ardmore, PA, USA) with 10 × eyepiece, 4 × objective for full view of knee joints and 10 × objective for analyses of the histopathological synovitis score (HSS; Table S1) to measure synovitis status and modified Osteoarthritis Research Society Internationalscore (OARSI; Table S2) and evaluate the articular cartilage status [2,3]. Immunofluorescence was detected by CLSM using a LSM 780 (Carl Zeiss, Jena, Germany) with a 10 × eyepiece and 10 × objective. In addition, the relative positive areas of pro-inflammatory cytokines were calculated by ImageJ software (National Institutes of Health, Bethesda, Maryland) for semiquantitative analyses.

RT-PCR Analyses

The total RNA of affected joins isolated from sacrificed mice of all groups was extracted based on the following procedure. The affected joints were ground into powder in liquid nitrogen. Then TRIzol reagent (Thermo Fisher Scientific Inc., Shanghai, P. R. China) was added followed by extraction, purification, and dying of RNA. The RNA concentration of

each sample was detected. The reverse transcription kit was used for conversion to complimentary DNA (cDNA) according to manufacturer's instructions. SYBR Premix Ex Taq^{TM} kit was utilized according to the protocol, and RT-PCR was finally performed on a Stratagene Mx3005P system (Stratagene, La Jolla, CA, USA). As shown in Table S3, the specific primers of TNF-α, IL-1β, and IL-6, and the housekeeping gene GADPH were listed. $\Delta\Delta C_t$ method was used for the analyses of results.

ELISA Assays

Serum samples were collected at 48 days after the primary immunization. The concentrations of TNF-α, IL-1β, and IL-6 in serum were detected by ELISA kits, according to the manufacturer's instructions.

Statistical Analyses

All experiments in this work were successfully conducted in triplicate. Experiment data were expressed as mean ± standard deviation (SD). Statistical significance of the data was calculated using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant, and $P < 0.01$ and $P < 0.001$ were considered highly significant.

References

1. Brand DD, Latham KA, Rosloniec EF. Collagen-induced arthritis. Nat Protoc. 2007; 2: 1269-75.
2. Liu H, Ding J, Wang J, Wang Y, Yang M, Zhang Y, et al. Remission of Collagen-Induced Arthritis through Combination Therapy of Microfracture and Transplantation of Thermogel-Encapsulated Bone Marrow Mesenchymal Stem Cells. PLoS ONE. 2015; 10: e0120596.
3. Liu H, Ding J, Wang C, Wang J, Wang Y, Yang M, et al. Intra-Articular Transplantation of Allogeneic BMMSCs Rehabilitates Cartilage Injury of Antigen-Induced Arthritis. Tissue Eng Part A. 2015; 21: 2733-43.

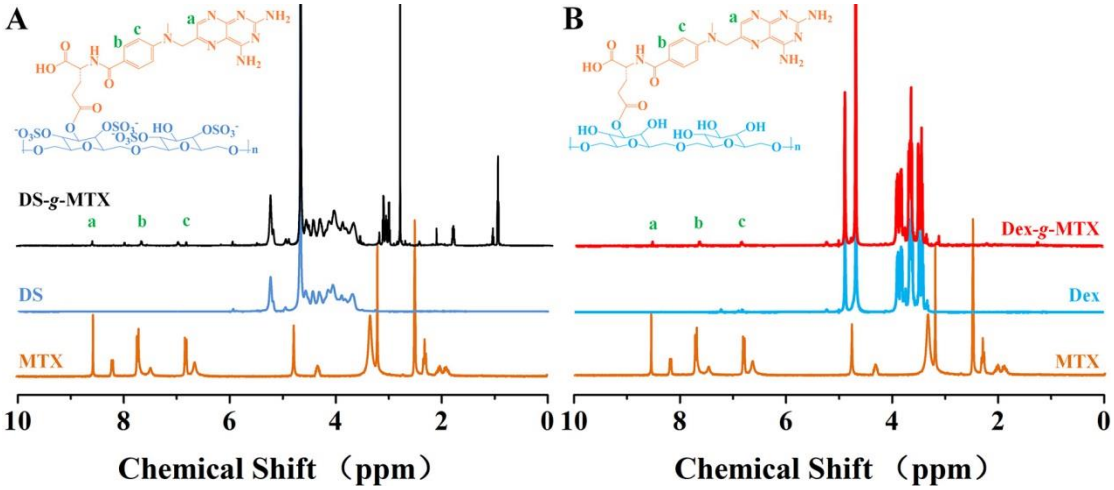

Figure S1. ^{1}HNMR spectra of DS-*g*-MTX(A) and Dex-*g*-MTX (B).

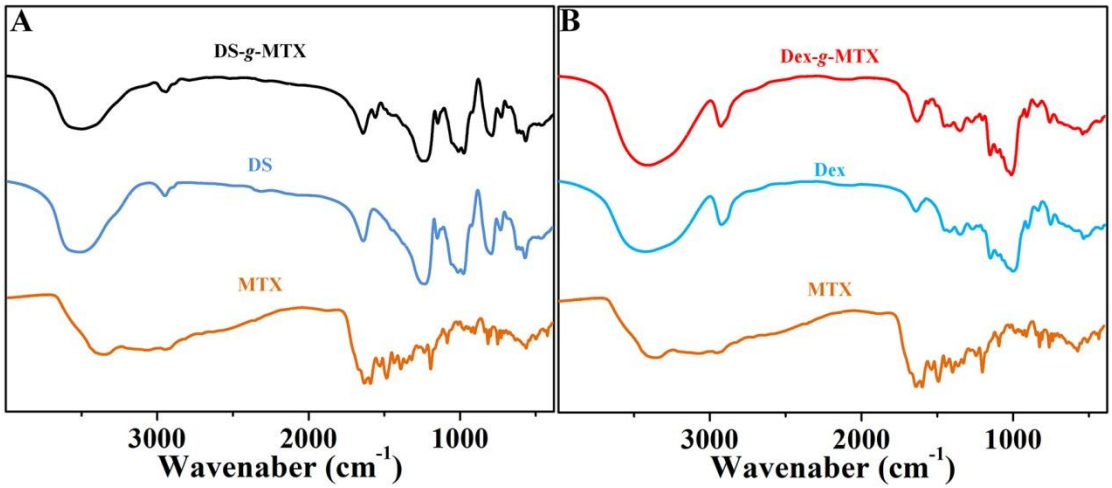

Figure S2. FT-IR spectra of DS-*g*-MTX, Dex-*g*-MTX, DS, Dex, and free MTX.

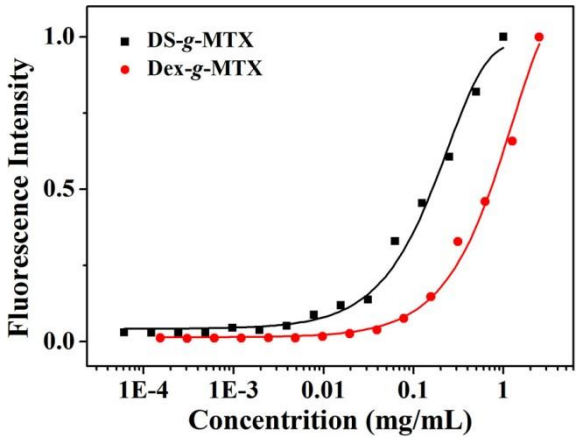

Figure S3. CMCs of DS-*g*-MTX and Dex-*g*-MTX.

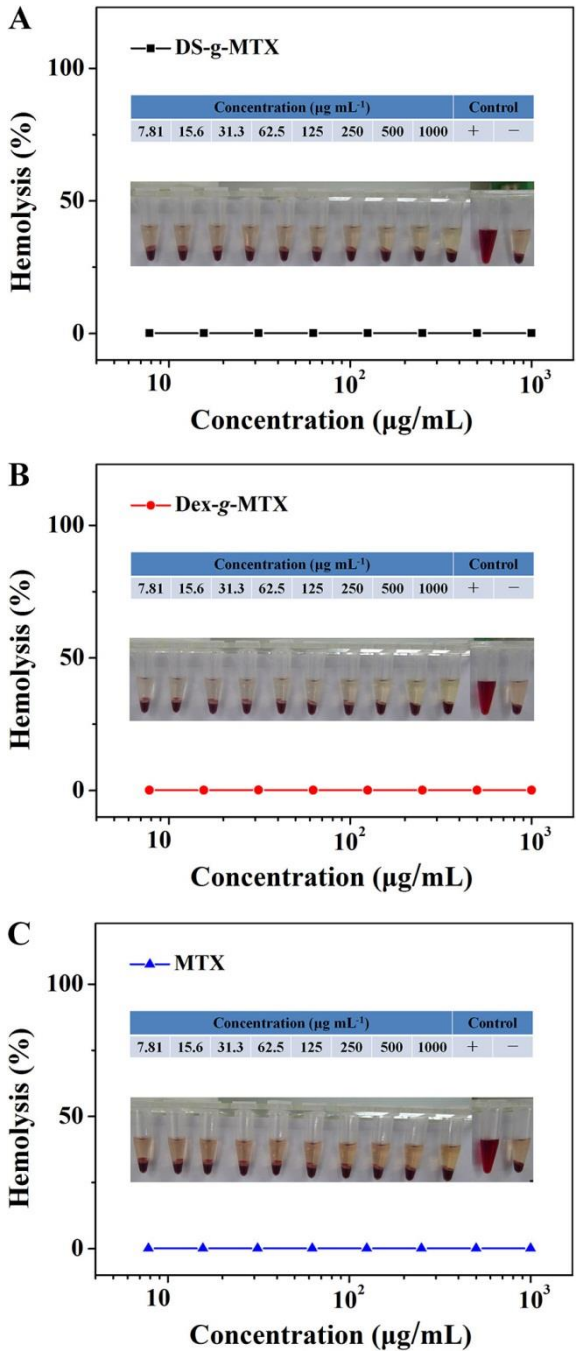

Figure S4. Hemolysis of DS-*g*-MTX (A), Dex-*g*-MTX (B), and free MTX (C). Data were presented as mean ± SD (n = 3).

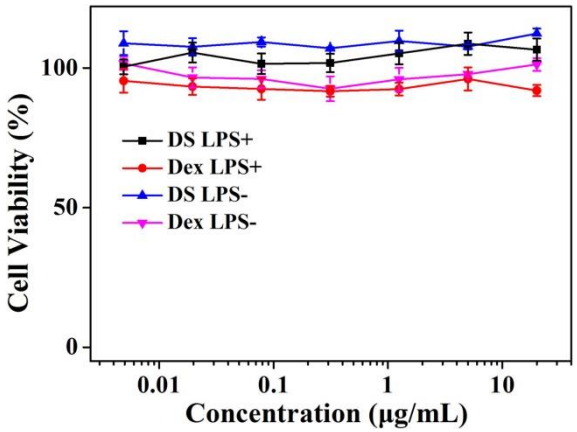

Figure S5. Cytotoxicity of DS and Dex after coincubation for 72 h toward RAW 264.7 cells activated by LPS (+) or not (−) *in vitro*. Data were presented as mean ± SD (n = 3).

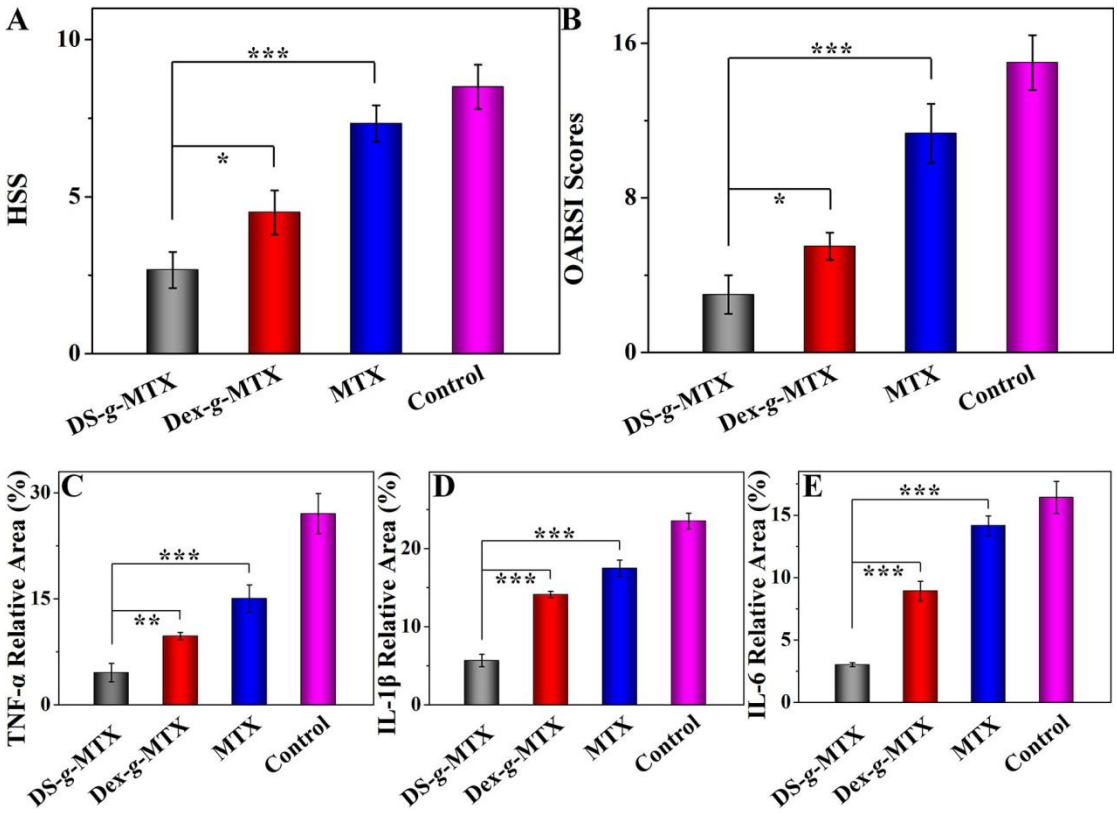

Figure S6. The HSS (A) and modified OARSI scores (B) of DS-*g*-MTX, Dex-*g*-MTX, free MTX, and NS as control groups were determined by the microimages of H&E staining. Relative positive areas of pro-inflammatory cytokines, *i.e.*, TNF-α (C), IL-1β (D), and IL-6 (E), calculated by ImageJ software. Data were presented as mean ± SD (n = 3; *****P < 0.05, ******P < 0.01, *******P < 0.001).

Table S1. Morphological features of synovium.

Feature	Score
A. Hyperplasia or enlargement of synovial lining cell layer	
1. Absent	0
2. Slight enlargement (two to three cell layers). Giant cells are very rare	1
3. Moderate enlargement (four to five cell layers). Some giant cells or lymphocytes	2
4. Strong enlargement (more than six cell layers). Giant cells and lymphocytes are frequent	3
B. Inflammatory infiltration	
1. Absent	0
2. Slight inflammatory infiltration (diffusely located single cells and small perivascular aggregates of lymphocytes and/or plasma cells)	1
3. Moderate inflammatory infiltration (perivascular and/or superficial lymphatic aggregates, and small sized lymphatic follicles without germinal center may be observed)	2
4. Strong inflammatory infiltration (lymphatic follicles with germinal center and/or confluent subsynovial lymphatic infiltration)	3
C. Activation of synovial stroma/pannus formation	
1. Absent	0
2. Slight synovial stroma activation (low cellularity with slight edema, slight fibrosis with some fibroblast, no giant cells)	1
3. Moderate synovial stroma activation (moderate cellularity with a moderate density of fibroblasts, endothelial cells, and giant cells may be detected)	2
4. Strong synovial stroma activation (high cellularity with dense distribution of fibroblasts and endothelial cells, and giant cells are abundant)	3

Table S2. Modified OARSI scores to evaluate the cartilage status microscopically.

Feature	Score
A. Structure	
0. Normal	0
1. Slight surface irregularities	1
2. Moderate surface irregularities	2
3. Severe surface irregularities	3
4. Clefts/fissures into transitional zone (one-third depth)	4
5. Clefts/fissures into radial zone (two-thirds depth)	5
6. Clefts/fissures into calcified zone (full depth)	6
7. Fibrillation and/or erosion to transitional zone (one-third depth)	7
8. Fibrillation and/or erosion to radial zone (two-thirds depth)	8
9. Fibrillation and/or erosion to calcified zone (full depth)	9
10. Fibrillation and/or erosion to subchondral bone	10
B. Cellularity	
0. Normal	0
1. Increase or slight decrease	1
2. Moderate decrease	2
3. Severe decrease	3
4. No cells present	4
C. Chondrocyte cloning	
0. Normal	0
1. Several doublets	1
2. Many doublets	2
3. Doublets and triplets	3
4. Multiple cell nests	4

Table S3. Primer sequences for RT-PCR.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
TNF-α	TATGGCTCAGGGTCCAACTC	GGAAAGCCCATTTGAGTCCT
IL-1β	CTCACAAGCAGAGCACAAGC	CAGTCCAGCCCATACTTTAGG
IL-6	CGGAGAGGAGACTTCACAGAG	CATTTCCACGATTTCCCAGA
GADPH	AGAGAGGGAGGAGGGGAAATG	AACAGGGAGGAGCAGAGAGCAC