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**Hyaluronic acid-methotrexate conjugate
for targeted therapy of rheumatoid arthritis**

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Materials and methods

1. Materials

Sodium hyaluronate (MW = 2.34×10^5 Da), purchased from Lifecore Biomedical LLC (Chaska, USA), was purified by dialysis against distilled water, followed by lyophilization. Methotrexate (MTX) ($\geq 98\%$), *N,N'*-dicyclohexylcarbodiimide (DCC) (99%), and 4-dimethylaminopyridine (DMAP) ($\geq 98\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The NIR dye, Cy5.5, was purchased from Amersham Biosciences (Piscataway, NJ, USA). The water used in all experiments was prepared by using AquaMax-ultra water purification system (Anyang, Korea). RAW 264.7 cells (mouse leukemic macrophage cell line) were purchased from the American Type Culture Collection (Rockville, MD, USA). All other chemicals were obtained from commercial sources, and used as received, without further purification.

2. Synthesis of the HA-MTX conjugate

MTX was chemically conjugated to the backbone of hyaluronic acid (HA), by the esterification reaction in the presence of DCC and DMAP (Fig. S1). In brief, HA (100 mg, 0.264 mmol) was dissolved in 30 ml of formamide at a concentration of 3 mg/ml under vigorous stirring. Then, the solution was further diluted with 25 ml of dimethyl sulfoxide (DMSO) and allowed for stirring at room temperature. MTX (84 mg, 0.184 mmol), dissolved in 5 ml of DMSO, was mixed with DCC (152 mg, 0.737 mmol) and DMAP (90 mg, 0.737 mmol), followed by mild stirring for 30 min to activate the carboxyl group of MTX. The resulting solution was mixed with the HA

solution for the esterification reaction. The reaction was allowed to proceed at room temperature for 24 h, in the dark condition. Thereafter, the products were purified by centrifugation at 3000 rpm for 5 min to remove the dicyclohexylurea residue, followed by dialysis against deionized water for 2 days, using a membrane tube (MW cutoff = 12,000 ~14,000), and lyophilization. As shown in Fig. S2, the chemical structures of HA, MTX, and the HA-MTX conjugate were characterized by using ^1H NMR (JNM-AL300, JEOL, Tokyo, Japan). In order to determine the degree of substitution (DS), defined as the number of MTX per 100 repeating units of HA, the concentration of MTX in the conjugate was observed using a UV-vis spectrophotometer (Optizen 3220UV, Mecasys Co., Ltd, Daejeon, South Korea) by measuring an absorbance at 303 nm appearing from MTX. For this experiment, HA-MTX was dissolved in DMSO/water (1v/1v) mixture, and the calibration curve was obtained using DMSO/water (1v/1v) mixture with different MTX concentrations in the range of 1 μg /ml to 100 μg /ml. The amount of MTX in the conjugate was calculated based on the calibration curve. As a consequence, the DS value of the conjugate was found to be 6.37.

3. *In vitro* drug release behavior

HA-MTX conjugates were dispersed in phosphate (pH 7.4 or 6.8), or acetate buffer (pH 5.0). Each solution was transferred to the cellulose membrane tube (MWCO = 1,000 Da, Millipore). The tube was then immersed in the corresponding buffer medium at 37 °C. The sample was gently shaken in a water bath at 100 rpm. Each medium was refreshed at predetermined time intervals, to preserve the sink condition; and the amount of MTX released was measured, using a UV-Vis spectrophotometer at 303 nm.

4. *In vitro* cellular uptake of the HA-MTX conjugate

RAW 264.7 cells were cultured in DMEM medium (Invitrogen-Gibco, Carlsba, California, USA) containing 10% FBS and 1 % penicillin-streptomycin, at 37 °C in humidified 5 % CO₂ atmosphere. Cells were activated with 10 ng/ml lipopolysaccharide (LPS) for 24 h. For sterilization of the HA-MTX conjugate, the sample was passed through a 0.45 μm syringe filter, followed by exposure to the germicidal lamp for 2 h. After cell activation, the medium was replaced with 2 ml of serum-free DMEM medium containing the Cy5.5-labeled HA-MTX conjugate (50 μg/ml), followed by an incubation for 3 h. The cells were washed twice with PBS (pH 7.4), and fixed, with a 4 % paraformaldehyde solution. The intracellular localization of the HA-MTX conjugate was observed by using confocal microscope system (OLYMPUS F1000, Japan). For the competitive study of cellular uptake of the HA-MTX conjugate, the medium was replaced with 2 ml of serum free DMEM medium containing free HA polymer (5 mg/ml), followed by 1 h incubation. Thereafter, the medium was replaced with 2 ml of the Cy5.5-labeled HA-MTX conjugate, followed by incubation for 3 h.

5. *In vivo* biodistribution of the HA-MTX conjugate

DBA1/J 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 DBA1/J mice (6-8 weeks in age), as previously described [9, 10]. To induce CIA, mice were injected intradermally at the tail, with 200 μg of chicken type II collagen (CII) (2 mg/ml, Chondrex, Redmond, WA, USA), emulsified in 100 μl of Complete Freund's Adjuvant (4 mg/ml). This was followed by a booster

immunization 21 days later, with CII emulsified in Incomplete Freund's Adjuvant. 40 days after primary immunization, Cy5.5-labeled HA-MTX was injected into the tail vein of wild-type (WT) or CIA mice at a dose of 1 mg/kg, to image their *in vivo* biodistribution by using the Optix MS3 system (ART Advanced Research Technologies Inc., Montreal, Canada). The laser power and count time settings were optimized at 3 μ W and 0.3 s per point, respectively. Excitation and emission spots were raster-scanned in 1.5 mm steps over the selected region of interest. A 670 nm-pulsed laser diode was used to excite the Cy5.5 molecules. The NIR fluorescence emission at 700 nm was collected and detected through a fast photomultiplier tube (PMT, Hamamatsu Photonics, Japan), and a time-correlated single photon counting system (TCSPC, Becker and Hickl GmbH, Berlin, Germany), respectively. To estimate the time-dependent profile, the average NIR fluorescence intensity per selected region was calculated as a function of time. All the data were calculated using the region of interest (ROI) function of Optiview Analysis software (ART Advanced Research Technologies Inc.), and values are presented as the means \pm SE, for groups of three animals.

6. *Ex vivo* tissue distribution of the HA-MTX conjugate

Major organs and knees were dissected from WT and CIA mice, at 48 h after the intravenous injection of Cy5.5-labeled HA-MTX (1 mg/kg). NIR fluorescence images of dissected organs and knees were obtained with the Optix MS3 system (ART Advanced Research Technologies Inc.). The tissue distribution of the HA-MTX was quantified, by measuring the average of NIR fluorescence intensity at the ROI. All values are expressed as the means \pm SE of three animals. For histological analysis, the dissected knee joints were fixed in 10 % (v/v) buffered formalin

solution, decalcified using Decalcified Solution-Lite (Sigma-Aldrich Co., MO, USA) for 6 h, embedded in paraffin, and sectioned. The paraffin tissue was sliced to 6-mm thickness, stained with H&E, and examined using a fluorescent microscope (BX51, Olympus, Optical Co. Ltd., Tokyo, Japan). For immunohistochemistry, paraffin-embedded sections were fixed to MAS-GP microscope slides (Matsunami Glass Ind., Ltd., Tokyo, Japan). The tissue sections retrieved were immunostained with monoclonal anti-CD44 antibody (Abcam, Cambridge, UK) and fluorescence-conjugated secondary antibodies (Alexa-488: Molecular probe®, Eugene, OR, USA), according to the manufacturer's instructions. The fluorescence of CD44-Alexa488 and HA-MTX-Cy5.5 was analyzed by using a confocal microscope (LSM 510 MetaDuoScan, Carl Zeiss Micro Imaging GmbH, Germany).

7. Therapeutic efficacy of the HA-MTX conjugate

The CIA mice were treated through the intravenous injection of MTX (0.5 or 2.4 mg/kg), HA-MTX (0.5 mg/kg MTX or 2.5 mg/kg MTX), or vehicle (PBS), once every three days, starting on the day of the booster injection. The mice were examined every other day for signs of joint inflammation, scored as follows: 0 = normal, 1 = mild swelling, and erythema confined to the midfoot and ankle joint, 2 = mild swelling, and erythema extending to the midfoot and ankle joint, 3 = moderate swelling, and erythema extending from the metatarsal joints to the ankle, 4 = severe swelling, and erythema encompassing the foot, ankle and digits. These paw scores were summed for each mouse, giving a maximum possible score of 16/mouse. Paw thickness (mm) was determined using calipers for the right and left paws of mice. Five separate animals were tested for each sample and the results were expressed as the mean \pm SE.

8. Histologic examination of joints

For histological analysis, the dissected knee joints were fixed in 10 % (v/v) buffered formalin solution, decalcified using Decalcified Solution-Lite (Sigma-Aldrich Co., MO, USA) for 6 h, embedded in paraffin, and sectioned. The paraffin tissue was sliced to 6-mm thickness, stained with H&E, and examined using a fluorescent microscope (BX51, Olympus, Optical Co. Ltd., Tokyo, Japan). The extents of synovitis, and bone and cartilage destruction were evaluated, using a five-point scale: grade 0 (no sign of inflammation), grade 1 (mild inflammation with minimal hyperplasia of the synovial lining layer without cartilage destruction), and grades 2 through 4 (increasing degrees of inflammatory cell infiltration or cartilage and bone destruction).

9. Evaluation of cytokine and antibody

Serum samples were obtained 40 days after immunization. Serum levels of TNF- α , IL-6, INF- γ , and IL-12 were determined using a Milliplex Mouse Cytokine panel 1 (Millipore) and the Luminex 100 system (Luminex), according to the manufacturer's instructions. To determine collagen-specific autoantibody levels *in vivo*, serum samples were analyzed using ELISA kits (Chondrex, USA), for CII-specific IgG1 and IgG2a antibody levels.

10. Statistical analysis

The statistical significance of differences among the groups tested was determined, using one-way ANOVA. A *p*-value of less than 0.05 was considered significant.

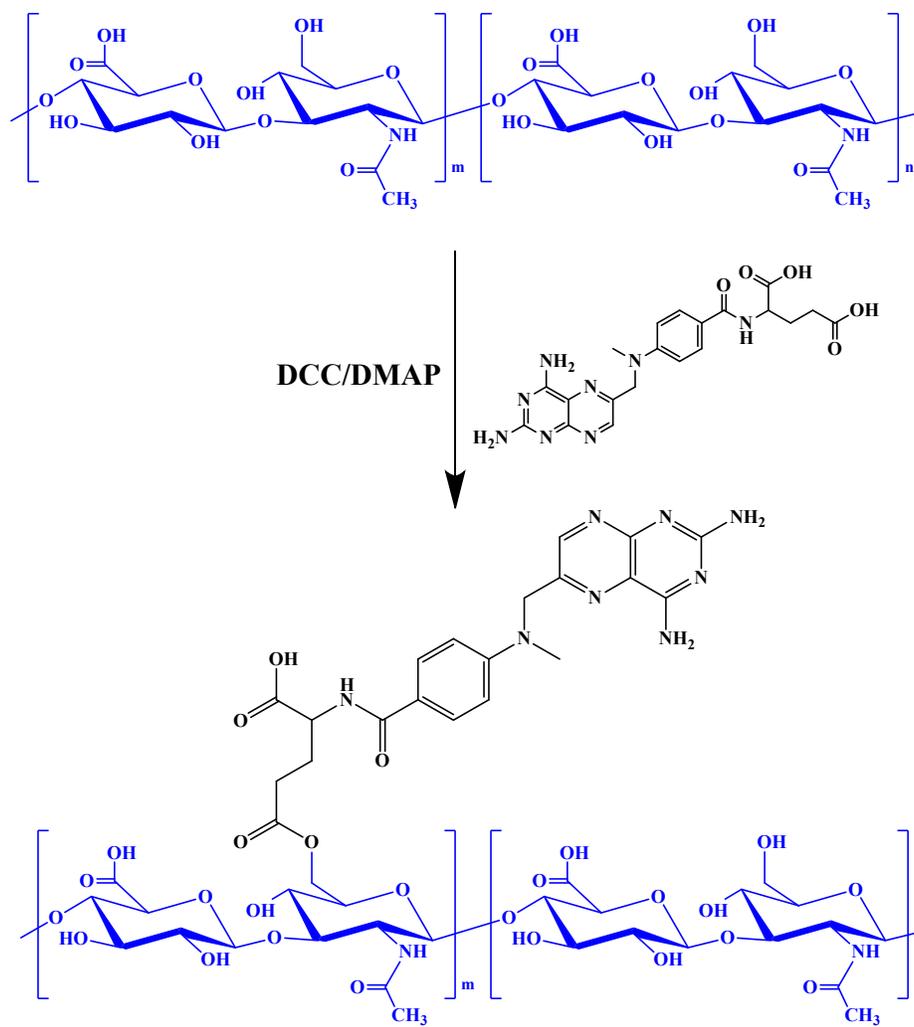


Fig. S1. Synthetic route of HA-MTX conjugate.

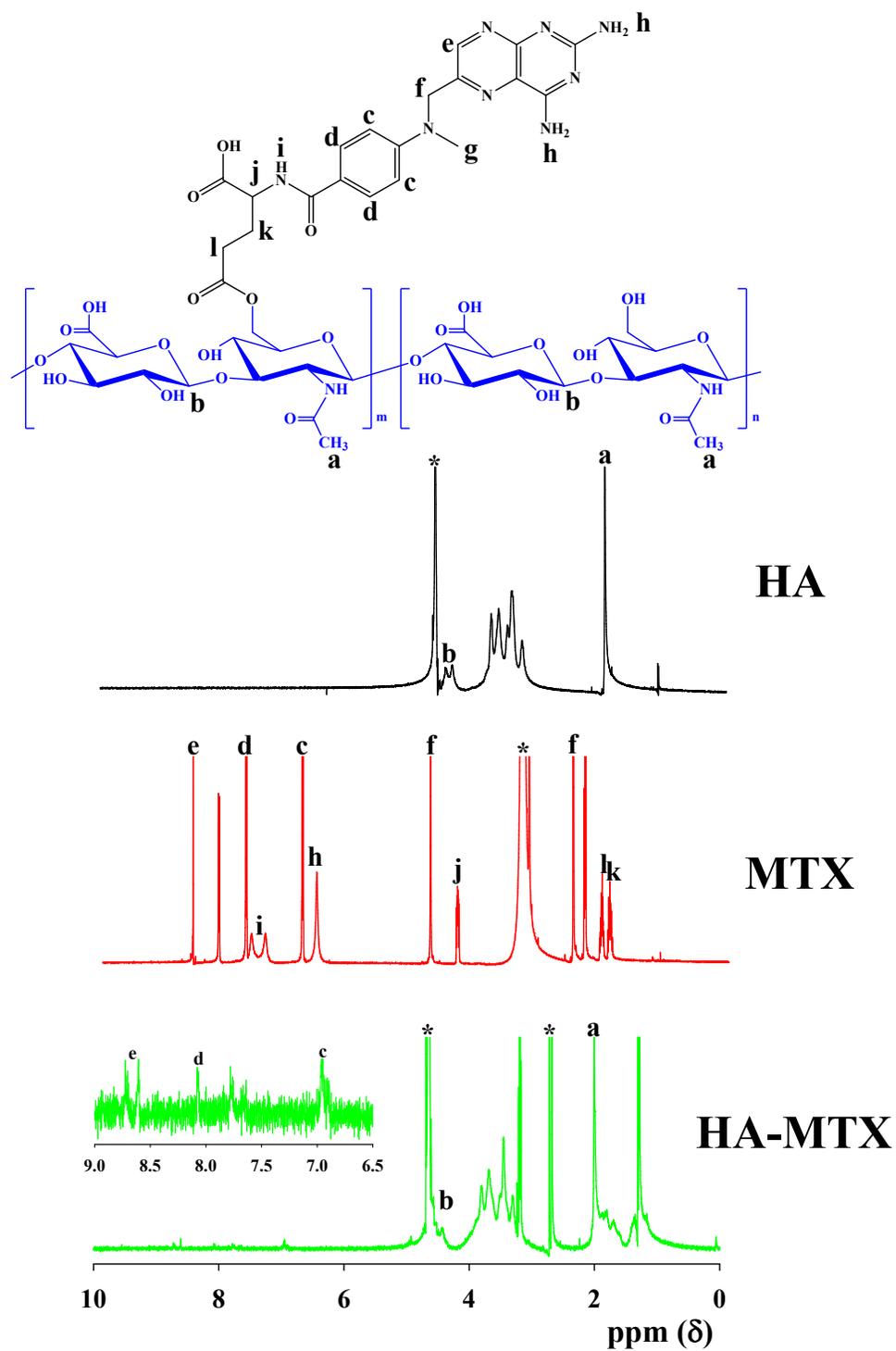


Fig. S2. ¹H NMR spectra of HA, MTX, and HA-MTX conjugate.

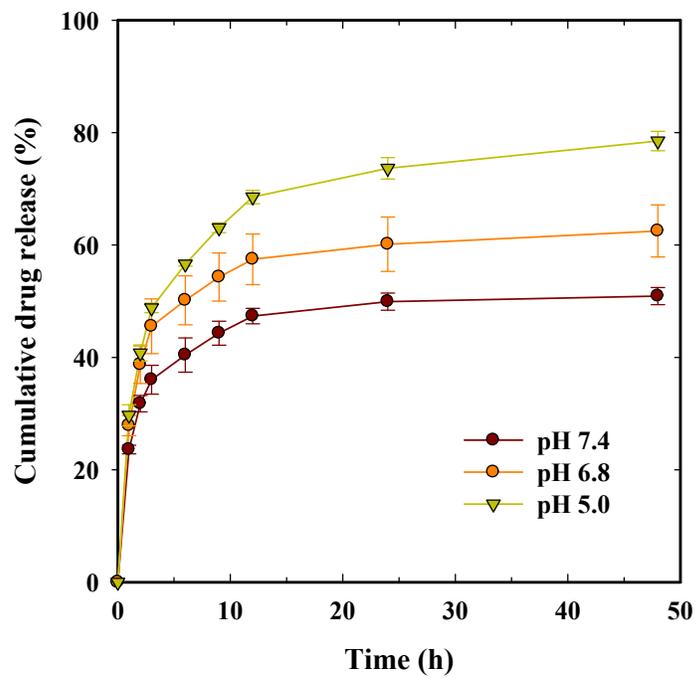


Fig. S3. *In vitro* drug release profile of HA-MTX conjugate in various pH conditions.

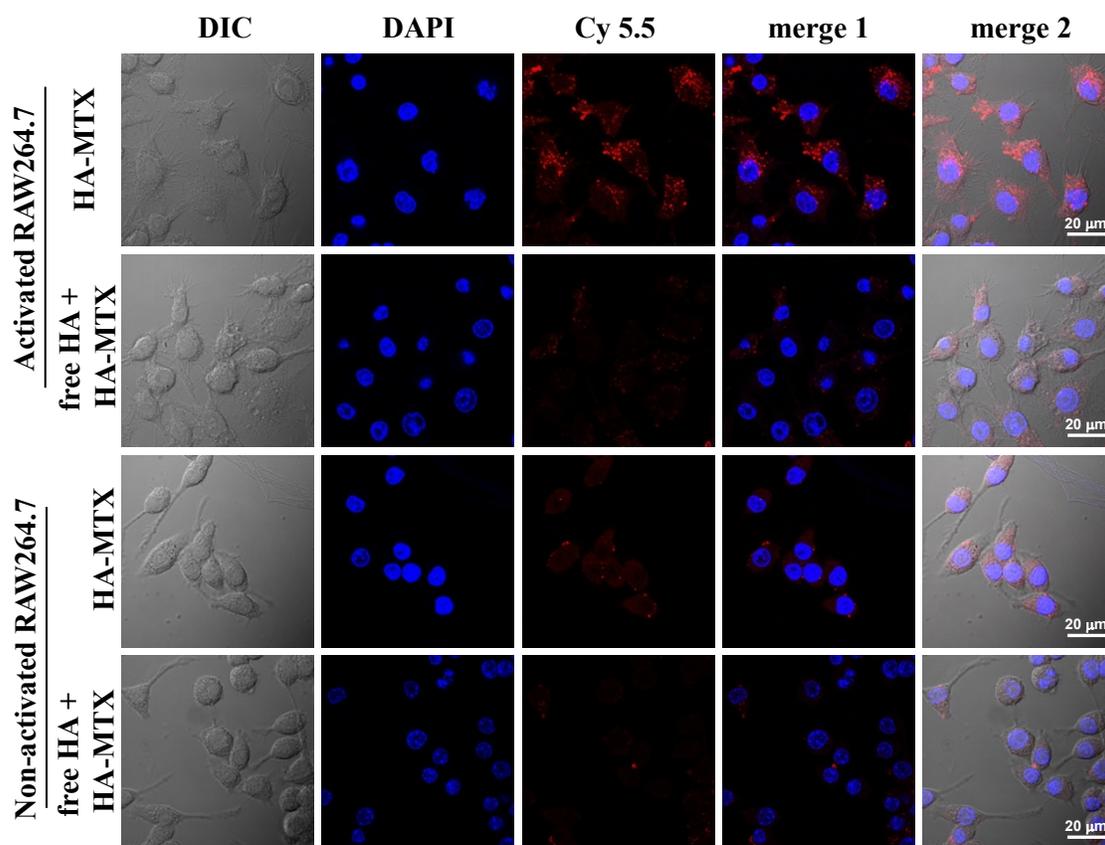


Fig. S4. *In vitro* cellular uptake of the HA-MTX conjugate by activated and non-activated macrophages. The cells were treated with the Cy5.5-labeled HA-MTX conjugate for 3h.

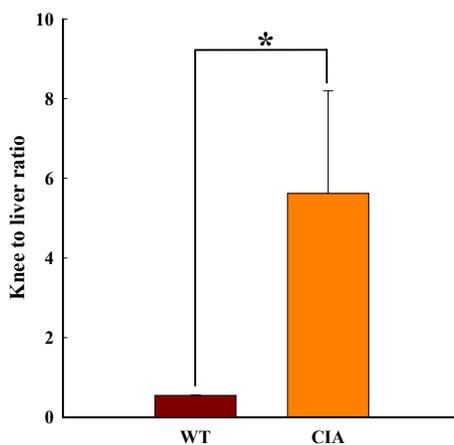


Fig. S5. Fluorescence intensity ratios of knee to liver. * indicates significantly different, compared with the vehicle group ($p < 0.05$). Error bars represent standard errors ($n = 5$).

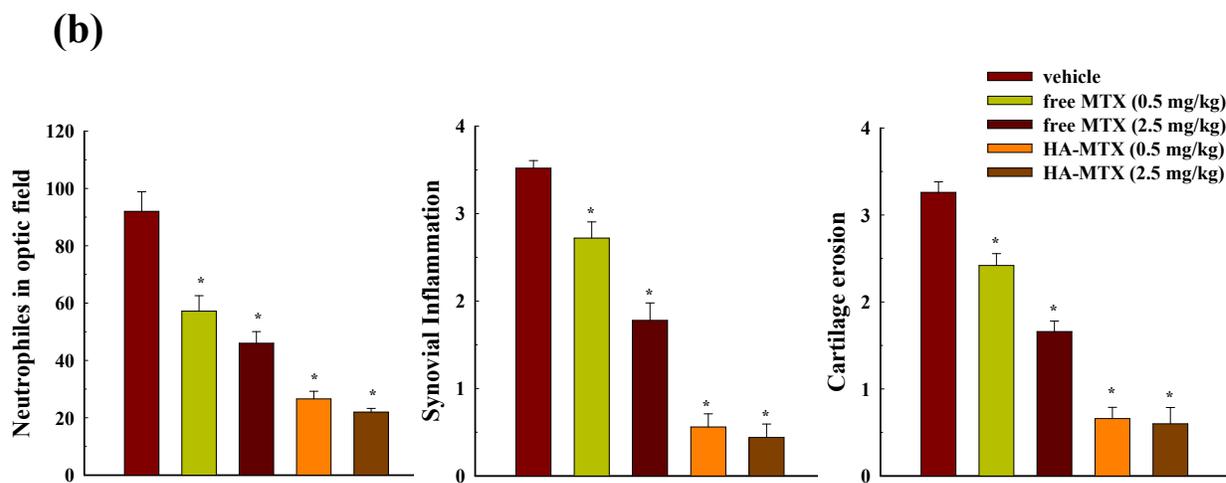
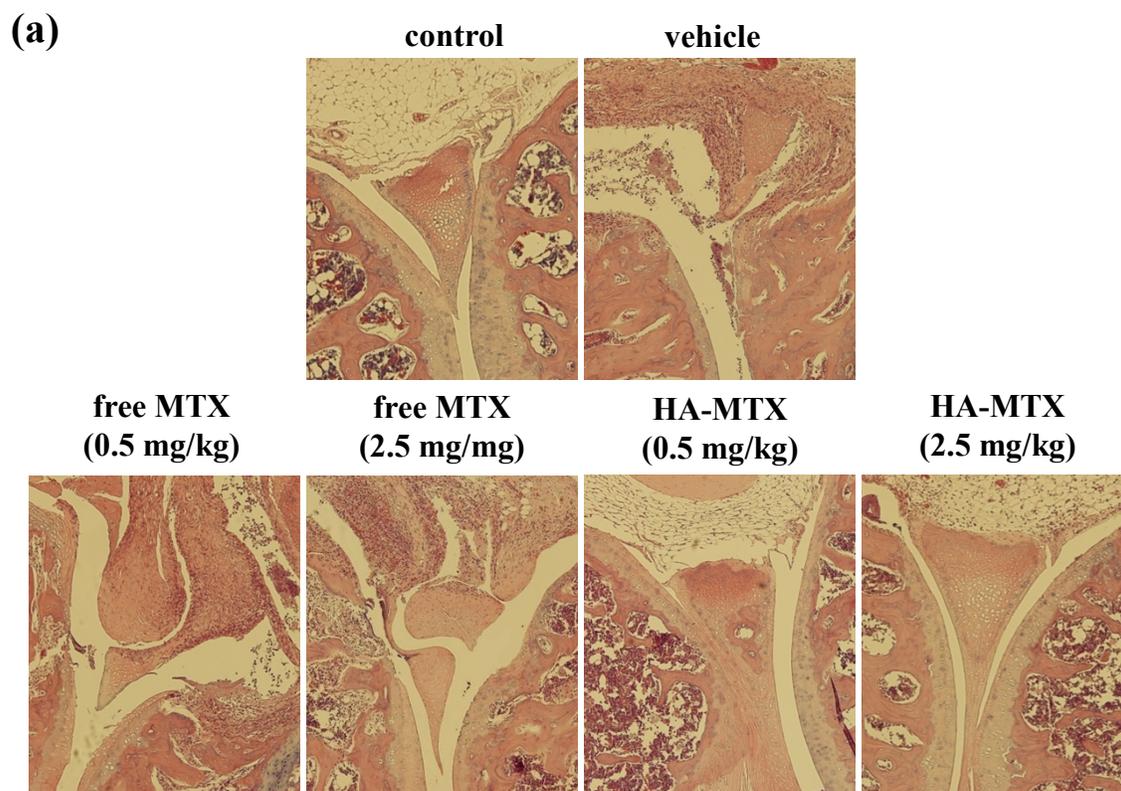


Fig. S6. (a) Representative sections of the knee joints of CIA mice. (b) Histological scores of synovial inflammation, cartilage erosion, and neutrophil infiltration. * indicates significantly different, compared with the vehicle group ($p < 0.05$). Error bars represent standard errors ($n = 5$).

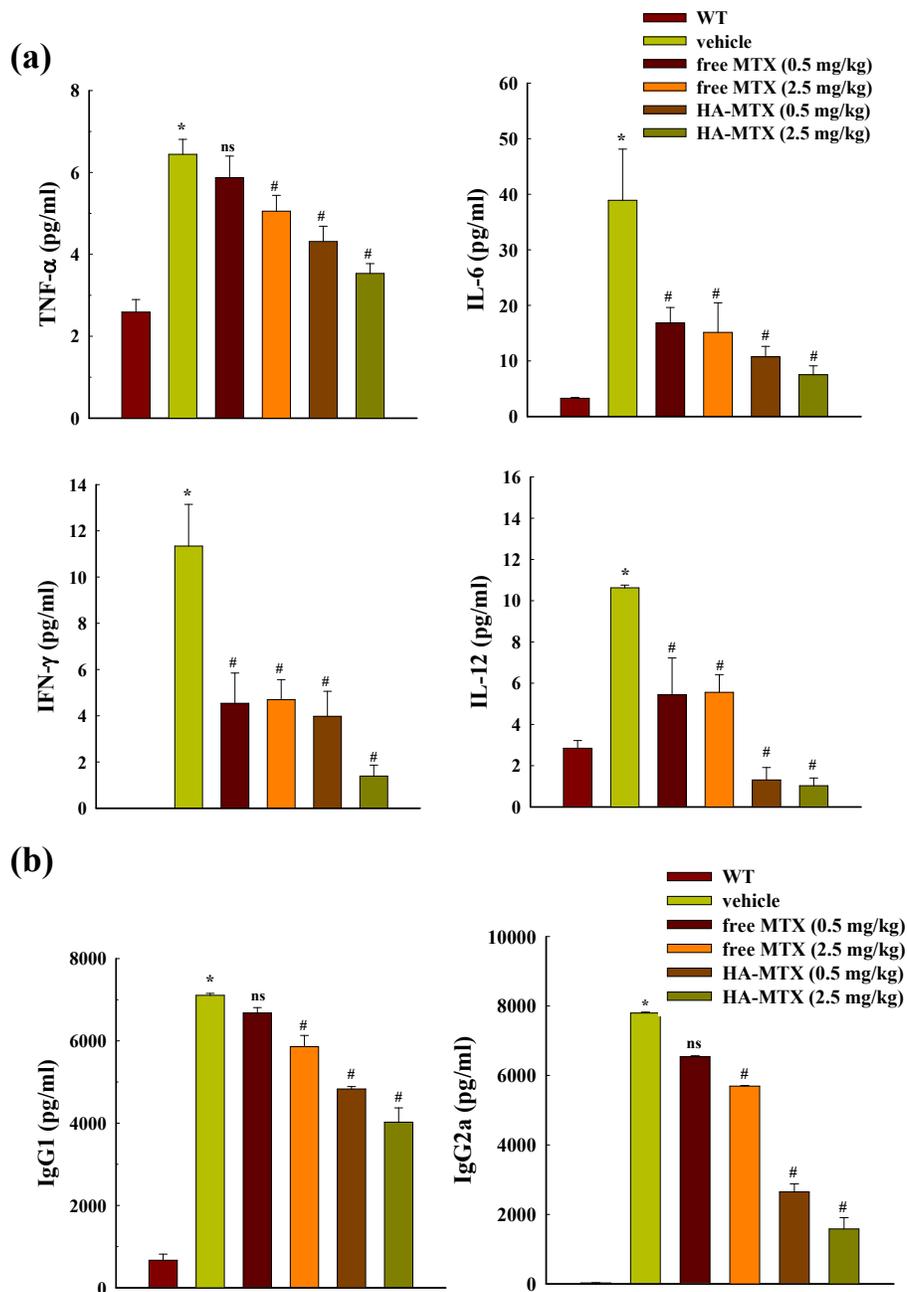


Fig. S7. (a) Bead-based cytokine analysis of serum of the CIA mice using the Luminex 100 system. (b) Quantitative analysis of anti-CII IgG1 and IgG2a in the serum of CIA mice. * indicates significantly different, compared with the control group ($p < 0.05$). # indicates significantly different, compared with the vehicle group ($p < 0.05$). Error bars represent standard errors ($n = 5$).