Supplementary information

Ultrasound-augmented anti-inflammatory exosomes for targeted therapy in rheumatoid arthritis

Yuanjiao Tang,[#,a] Zihe Wu,[#,a] Ruiqian Guo,[a] Jianbo Huang,[a] Xiao Rong,[a] Bihui Zhu,[a] Liyun Wang,[a] Lang Ma,*[a] Chong Cheng,[b] and Li Qiu*[a]

[a] *Department of Ultrasound, National Clinical Research Center for Geriatrics, West China Hospital, College of Polymer Science and Engineering, Med-X Center for Materials, Sichuan University, Chengdu 610041, China*
E-mail: malang1989@scu.edu.cn (L. Ma) and qiulihx@scu.edu.cn (L. Qiu)

[b] *State Key Laboratory of Polymer Materials Engineering, Sichuan University, Chengdu 610065, China*

[#] *These two authors contributed equally to this article.*
[*] *Corresponding authors*

Materials

The interleukin-10 (IL-10) was purchased from Genscript (Nanjing, China). The ExoQuick-TC®ULTRA EV Isolation Kit was purchased from SBI (San Francisco, USA). The CD63, TSG101, IL-10 antibodies, and IL-10 ELISA Kit were obtained from Abcam (London, UK). The CD86 and CD206 antibodies were obtained from Proteintech (Chicago, USA). The LinKine™ AbFluor™ 488 was provided by Abbkine (Wuhan, China), and the 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was provided by AAT Bioquest (California, USA). The IL-6 and TNF-α ELISA Kits were acquired from Neobioscience (Shenzhen, China). The bovine type II collagen, complete Freund's adjuvant, and incomplete Freund's adjuvant were acquired by Chondrex(Washington, USA). And the BCA protein assay kit, CCK-8 Kit, and FDA/PI-stained Kit were supplied by Beyotime (Shanghai, China)

Cell proliferation and viability.

To test the safety of exosomes and AI-Exo, cell proliferation and viability were tested. Briefly, human umbilical vein endothelial cells (HUVECs) and RAW264.7 cells were seeded into 96-well plates and incubated overnight, then treated for 24 h with exosomes and AI-Exo at 1 μg mL^{-1} of IL-10 concentration. The cell viability (%) was measured using the CCK-8 kit (Beyotime, Shanghai, China), as described in the manufacturer's guide at 450 nm using a microplate reader. Besides, the living/dead double staining by fluorescein diacetate/propidium iodide FDA/PI was used. The samples were washed with PBS and incubated in staining solution for 5 min and observed under a fluorescence microscope. The living cells were marked with green fluorescence and dead cells were marked with red fluorescence.

Establishment of CIA mice model.

Male DBA/1 mice aged 7~8 weeks were housed in a temperature-controlled room at 20~26 °C, at 50% humidity, with standard food and water and 12/12 h light/dark cycles. All animal experiments were approved by the Institutional Animal

Care and Ethics Committee of Sichuan University (Approval No. 20220606005). The CIA mice model was established according to the manufacturing instruction of Chondrex (Washington, USA). And the immune emulsion was prepared first. Freund 's complete adjuvant (CFA) was used for primary immunization and Freund 's incomplete adjuvant (IFA) was used for enhanced immunization. The 2 mg/mL bovine collagen II/ acetic acid solution was mixed and emulsified with the same volume of adjuvant in the ice water bath by a high-speed shear homogenizer (Kinematica POLYTRON® PT2500E, Switzerland). The mixture was continuously stirred at 20000 r/ min for 2 min, cooled at 0 °C for 5 min, and repeated 2~3 times. When the emulsion was dropped into the water with complete morphology and no blooming, the emulsion was successfully prepared. On the 0th day, 0.15 mL CFA emulsion was injected into the skin of mice at two or more points about 1-2 cm near the tail root for primary immunization. On the 21st day, 0.10 mL IFA emulsion was injected into the same position. Then the arthritic progression was monitored daily. When the joints of mice were significantly red and swollen, the symptoms of lameness and loss of appetite were seen as successful modeling.

Joint score measurement.

Arthritis scores are established by the following criteria where 0 = no edema or arthritis, 1 = swelling in one type of joint, 2 = swelling in two types of joint, 3 = swelling in three types of joint, and 4 = swelling of the entire paw. The scores of all four limbs were added together to give a total score for each mouse, so the highest possible score was 16. The animals were scored every time before treatment and euthanasia.

Imaging evaluation of ankles.

The high-frequency ultrasound was used to evaluate the synovitis and the Micro-CT was used to evaluate the bony changes of the ankles. Ultrasound examinations were performed before the animals were sacrificed with an ultrasonic device (IU22, Philips, Amsterdam, Netherlands). The probe frequency was 7~15 MHz,

and the superficial condition was selected. The probe surface was covered with an ultrasound gel pad to enable total contact on the skin. And the synovial thicknesses of the ankles were measured. As for the Micro-CT scanning, the Quantum GX Micro-CT Imaging System (PerkinElmer; MA, USA) was used, and the scanning parameters were voltage, 80 kV; current, 100 μA; pixel size, 50 μm. Analyze 12.0 software (PerkinElmer; MA, USA) was used to reconstruct 3D images to produce a visual representation of the results.

Histological evaluation of ankles.

Ankles of the mice were dissected and fixed in 10% formalin and decalcified in 10% neutral ethylene diamine tetraacetic acid (EDTA) solution. Decalcified tissue was embedded in paraffin. Moreover, the sections (2.5 mm) were stained with HE, Safranin O-fast green, and TRAP and later subjected to light microscopic examination in a blinded manner. Histopathological changes of the ankles were scored from 0 to 3 individually for inflammatory cell infiltration, pannus formation, synovial hyperplasia, articular cartilage damage, and bone destruction according to the previous study[20] based on H&E staining. The grading scheme was based on the following scales: inflammatory scores (0 = no inflammatory cell infiltrate; 1 = a few inflammatory cell infiltrate; 2 = a part of the joint cavity filled with inflammatory cells; 3 = all of the joint cavity filled with inflammatory cells), synovitis scores (0 = healthy; 1 = mild thickening of the synovium; 2 = substantial thickening of the synovium; 3 = severe thickening of the synovium), cartilage damage scores (0 = normal; 1 = minor destruction of the cartilage surface; 2 = clear loss of cartilage; 3 = cartilage almost absent in the whole joint), and bone destruction scores (0 = normal; 1 = minor signs of destruction; 2 = up to 30 % destruction; 3 = more than 30 % destruction). A total score was obtained by adding those four scores together, so the highest possible score was 12. Cartilage destruction was scored using the modified Mankin method as previously[21] based on Safranin O-fast green staining. The grading scheme was based on the following aspects: Structure (0 = normal; 1 = irregular surface; 2 = pannus; 3 = superficial cartilage layers absent; 4 = slight disorganization; 5 = fissures into

calcified cartilage layer; 6 = disorganization), cellular abnormalities (0 = normal; 1 = hypercellularity; 2 = clusters; 3 = hypocellularity), matrix staining (0 = normal/slight reduction; 1= staining reduced in radial layer; 2 = reduced in interterritorial matrix; 3 = only present in pericellular matrix; 4 = absent). A total score was obtained by adding those three aspects together, so the highest possible score was 13. The total numbers of osteoclasts around the taluses were calculated based on TRAP staining.

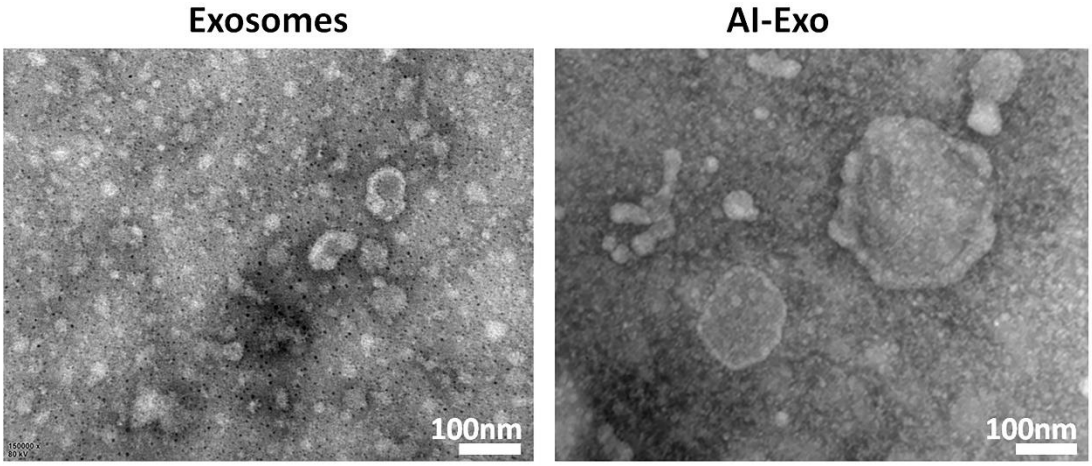

Fig. S1 TEM images (scale bar = 100 nm) of exosomes and AI-Exo.

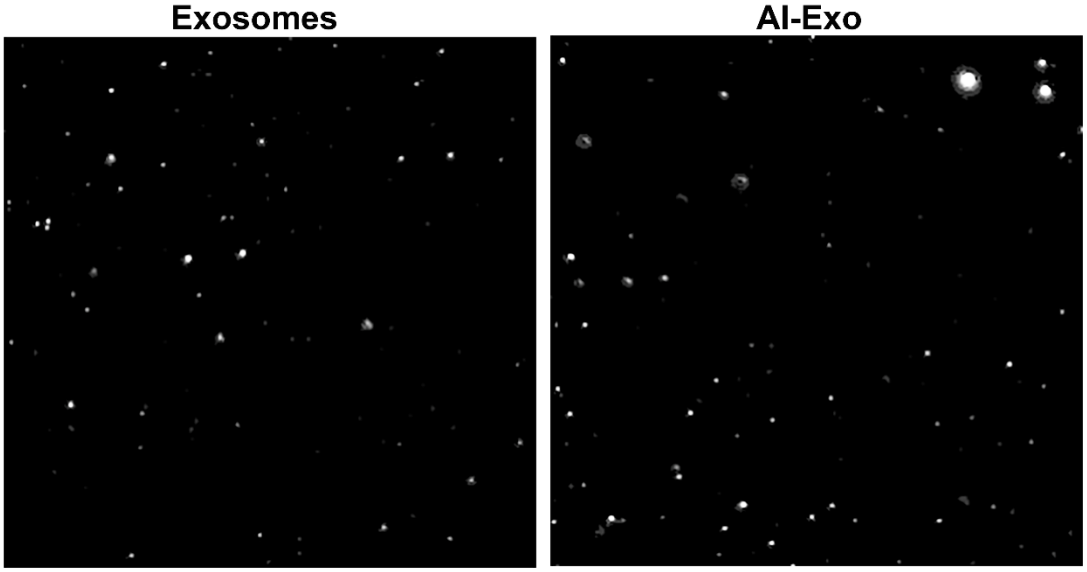

Fig. S2 The nanoparticle tracking analysis of exosomes and AI-Exo.

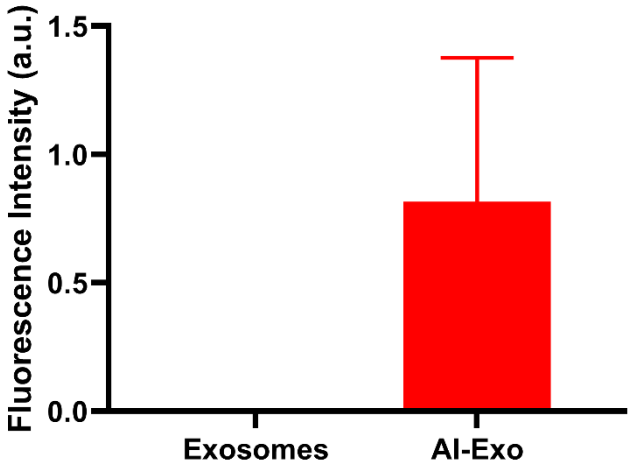

Fig. S3 Green fluorescence intensities of IL-10 between exosomes and AI-Exo. ****P* < 0.001.

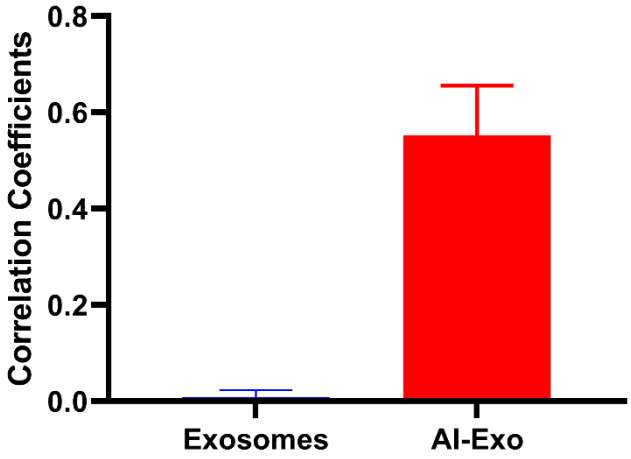

Fig. S4 Correlation coefficients between the green channel and red channel in the exosomes group and the AI-Exo group.

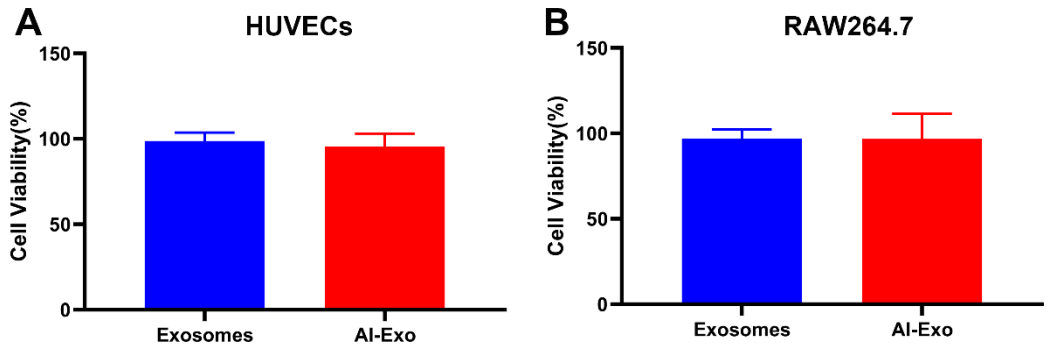

Fig. S5 Cell viabilities of (A) human umbilical vein endothelial cells (HUVECs) and (B) RAW264.7 cells after incubated with exosomes and AI-Exo for 24 h.

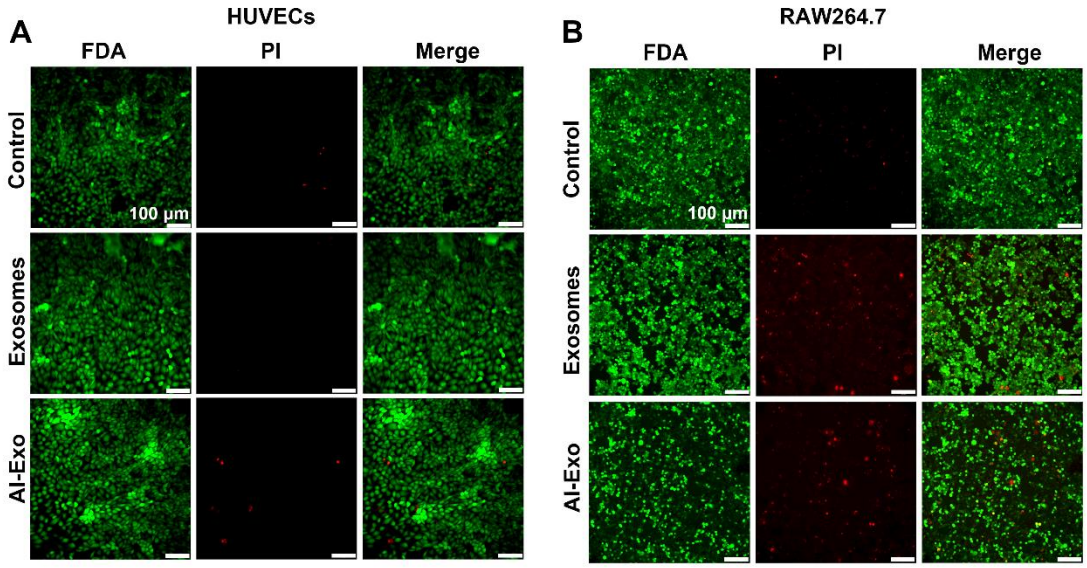

Fig. S6 Fluorescence microscope images of FDA/PI-stained (A) HUVECs and (B) RAW264.7 cells incubated with exosomes and AI-Exo for 24 h.