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RESEARCH ARTICLE

HUCMSC-derived Exosomes Suppress the Titanium Particles-induced Osteolysis in Mice through Inhibiting CCL2 and CCL3

Shixun Li, PhD[1], Chuangran Wu, MD[1], Sipeng Lin, PhD[1], Zhenkang Wen, PhD[1], Wenqiang Luo, MD[1], Changchuan Li, MD[1], Xiaoyan Wang, PhD[2], Xuejia Li, PhD[2], Liangbin Gao, MD[1], Yue Ding, MD[1]

[1]*Department of Orthopaedic Surgery, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University and* [2]*Guangzhou Saliai Stem Cell Science and Technology Co., LTD, Guangzhou, China*

Objective: Wear particles induce inflammation and the further osteolysis around the prosthesis, has been proven to be the main cause of aseptic hip joint loosening. In this research, we aimed to clarify whether human umbilical cord mesenchymal stem cells (HUCMSCs) could inhibit the titanium particles-induced osteolysis and shed light upon its mechanism.

Methods: The expression of chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 3 (CCL3) and chemokine (C-C motif) ligand 5 (CCL5) were examinjed in clinical specimens of aseptic hip prosthesis loosening patients. Local injection of lentivirus that knocked down CCL2 or CCL3 in a cranial osteolysis mice model were used to exam the effect of CCL2 and CCL3 on titanium particles-induced osteolysis *in vivo*. Transwell assay was used to examine the effect of CCL2 and CCL3 on titanium particles-induced activation of macrophage *in vitro*. Furthermore, the therapeutic effect of HUCMSCs, and exosomes from HUCMSCs were also examed *in vivo and vitro*. Immunohistochemical and real-time PCR were used to examine the expression of relative pathways. Analysis of variance (ANOVA) and Student–Newman–Keuls *post hoc t* test were used to analyze the results and determine the statistical significance of the differences.

Results: Results showed that titanium particles caused the osteolysis at the mice cranial *in vivo* and a large number of macrophages that migrated, while local injection of HUCMSCs and exosomes did inhibit the cranial osteolysis and migration. An exosome inhibitor GW4869 significantly increased the osteolysis area in the mice cranium osteolysis model, and increased the number of migrated macrophages. Immunohistochemical results suggested that the expression of CCL2, CCL3 and CD68 in the cranial in Titanium particles mice increased significantly, but was significantly reduced by HUCMSCs or exosomes. HUCMSC and exosomes down-regulate the expression of CCL3 *in vitro* and *in vivo*.

Conclusion: HUCMSCs and HUCMSC-derived exosomes could suppress the titanium particles-induced osteolysis in mice through inhibiting chemokine (C-C motif) ligand 2, chemokine (C-C motif) ligand 3.

Key words: Aseptic loosening; CCL2; CCL3; Exosome; HUCMSC; Macrophage

Introduction

Total hip arthroplasty (THA) has become one of the most revolutionary advances in orthopedics, for its reconstruction of joint function and durable pain relief. However, the operational life span of hip prosthesis was limited by many complications. It was reported that from 1999 to 2016, the 10-year implant survival rate in New Zealand was 93.6%.[1] Aseptic loosening was reported as the most common

Address for correspondence *Yue Ding and Liangbin Gao, Department of Orthopaedic Surgery Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University Guangzhou, China. Email: dingyue@mail.sysu.edu.cn and gaolb@mail.sysu.edu.cn
Shixun Li and Chuangran Wu are these authors contributed equally to this work.
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long-term cause for the failure of the hip prosthesis, the incidence of which is as high as 1.44/1000 patients per year within 10 years after primary surgery.[2] Revision hip replacement surgery for aseptic loosening patients not only requires excellent surgical techniques, but also brings life-threatening risks to patients. Therefore, the study of the prevention and treatment of hip aseptic loosening is of vital importance for expanding the life span of hip prosthesis and could be helpful for new prosthesis design.

Previous studies have shown that high hydraulic pressure, stress shielding and other effects can lead to the occurrence of aseptic hip joint loosening. However, the wear particles produced by the hip prosthesis stimulating macrophages and then further mediating osteolysis around the prosthesis, has been proved to be the main cause of aseptic loosening.[3] Research has shown the amount of macrophages in the periprosthetic tissue of patients increased significantly.[4] In recent years, the concept of macrophage polarization has been established. After being induced by inflammation signaling, macrophage turned into M1 polarization and responded with effective antigen presentation and production of pro-inflammatory cytokines and chemokines, such as tumor necrosis factor-α (TNF-α), Interleukin-6 (IL-6), Interleukin-1β (IL-1β) and receptor activator of nuclear factor-κB ligand (RANKL).[5] M2 macrophage activation is achieved by suppression of pro-inflammatory cytokine production, and at last produces anti-inflammatory mediators. It was mainly caused by cytokines IL-4 or IL-13.[6] First, in the process of aseptic loosening, after the wear particles were recognized, macrophages polarized from M0 to M1 and secreted a large amount of inflammatory factors, such as TNFα, IL-1β, and then initiated the inflammatory response in the surrounding micro-environment and recruited more inflammatory cells, which also enhanced the osteoclast activity.[7,8] Second, macrophages could act as precursor cells and differentiated into osteoclasts after inflammatory stimulation, which eventually leads to the osteolysis around the prosthesis.[8]

The macrophages were recruited by chemokines. Chemokines were signaling proteins secreted by cells, which have the ability to induce directional chemotaxis of nearby reactive cells. Studies have shown that macrophage chemotaxis is mainly induced by chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 3 (CCL3) and chemokine (C-C motif) ligand 5 (CCL5) in CC subgroups.[9] It has been reported that CCL2 was mainly involved in knee osteoarthritis,[10] CCL2/3 was mainly involved in aseptic loosening.[11] Inhibition of these chemokines might be a potential strategy to the treatment of wear particles induced aseptic hip joint loosening.

Stem cell is a kind of undifferentiated cell that exists in embryonic or adult tissues and have the capacities of self-renewal replication and multi-differentiation.[12] Studies have proven that mesenchymal stem cells (MSCs) own the ability of homing to the injured tissue *in vivo* and repair the damaged tissue through its multi-differentiation potential.[12] MSCs can also improve the local microenvironment, playing an anti-inflammatory response and regulate immune reaction through its paracrine mechanism.[13] Among all kinds of MSCs, HUCMSCs have been proved to have better differentiation and secretion abilities, with a wider range of sources and no ethical controversy.[14] Exosomes that containing complex RNA and proteins, mainly showed the ability to modulate the micro-environment after being released into the extracellular matrix.[15] Thus, exosome might become a potential treatment for wear particles induced aseptic loosening.

In this study, we aim to clarify: (i) which chemokine was involved in the recruitment of macrophage in wear particles induced aseptic loosening; (ii) can HUCMSCs and exosome from HUCMSCs inhibit wear particles induced aseptic loosening; and (iii) did HUCMSCs and exosome from HUCMSCs inhibit wear particles induced aseptic loosening through inhibiting CCL2 or CCL3.

Materials and Methods

Collection of Clinical Specimens

Clinical specimens were obtained from the Synovial membrane around the prosthesis from seven patients (15.1 ± 3.0 years post primary THA) undertaking hip revision in order to treat aseptic loosening in hip prosthesis, and the hip synovial membrane from 13 patients taking primary THA to deal with their femoral head necrosis (FHN). Patients with infection at the affected hip or any tissue all over the body would be excluded. Under the guidance of the Ethics Committee at our hospital, all procedures were approved ([2017] Ethic Record No.26). All patients agreed with this study.

Immunohistochemistry Assay of Clinical Specimens

Tissues were fixed in 4% paraformaldehyde fixation. Serial sectioning every 3.0-μm of the specimens was obtained after being embedded in paraffin blocks. The specimens were then deparaffinaged and dehydrated by xylene and ethanol washes of decreasing concentrations retrospectively. Hydrogen peroxide (3%) was then used for blockage of endogenous biotin or enzymes, sodium citrate was used for antigen retrieval. CCL2 antibody (1:100 diluted, ABclonal Technology, Wuhan, China), CCL3 antibody (1:100 diluted, ABclonal Technology, China) and CCL5antibody (1:100 diluted, ImmunoWay Biotechnology, Su Zhou Shi, China) were used as primary antibodies. The tissues were then incubated with primary antibodies respectively for 2 h at 37°C and washed with Phosphate Buffer Solution (PBS) three times. Afterwards, tissues were incubated with horseradish peroxidaseconjugated (Zhongshanjinqiao Biotechnology, Suzhou, China) as secondary antibody for 0.5 h at 37°C and washed. Then, DAB and hematoxylin was used as chromogenic agent and second stain respectively. At last, all specimens were washed, dried and mounted, then observed using a biomicroscope (DM2000, Leica, Wetzlar, Germany).

A semiquantitative scoring system (Bresalier's scoring system) was used to evaluate the staining intensity. For a

single cell, no stain: 0; weak stain: (1) moderate stain: (2) strong stain: (3) the percentages of stained cells were then counted and recorded. A total of 10 views of each immunostained sections were evaluated by three researchers separately. Scores and the percentage of the positive cells were then averaged and multiplied. Immunoreactivity score (IS) for each case: Σ ($0 \times F0 + 1 \times F1 + 2 \times F2 + 3 \times F3$).[16]

Particles

Titanium particles (Ti particles, Alfa Aesar Ward Hill, MA, USA) ranging from 0.2 to 1.2 μm (on average 0.82 ± 0.12μm) were filtered (Millipore, Burlington, MA, USA) in pure water in a filter holder.[17] After dried and weighted, these particles were sterilized (Ethylene oxide) and then suspended to the concentration of 4×10^8 mm^3/mL in PBS. At last, the level of endotoxin was tested (QCl-1000; Bio Whittaker, Walkersville, MD, USA) to make sure that these particle suspensions were endotoxin free (<0.01 EU/ml).

Cell Culture and Stimulation

The macrophage cell line RAW264.7 (ATCC, Manassas, VA, USA) were used in this study. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco™ DMEM, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) (Gibco, USA). Before further analysis, these macrophages were cultured in six-well plates (Costar, Cambridge, MA, USA) with the quantity of 1×10^6 cells each well overnight. The cell count and viability were assessed by a cell counter (Countstar, BioTech, Urbana, IL, USA) (Exceeding 98% in all trials). All cells were cultured at the temperature of 37°C with 5% CO_2.

The stem cells were obtain from Guangzhou Saliai Stem cell Science and Technology. DMEM with 10% FBS, 1% NEAA, 1% Glutamine were used for the cell culture of HUCMSCs, at the temperature of 37°C with 5% CO_2. The stem cell suspension (2×10^5 cells in 10 μl PBS) was administered.

For the particles or drugs used in this study, particles used in this study was at the the concentration of 100:1 (particle volume [μm^3]: quantity of cells). PBS was chosen as negative control. An inhibitor of exosome, GW4869 (MedChemExpress, China) 2.5 μg/ml, was used to block the release of exosome.[18]

Lentivirial Transfection

One shRNA sequence down-regulating CCL2, one shRNA sequence down-regulating CCL3 and a negative control sequence were used to construct the recombinant lentivirus by Cyagen (Cyagen, Suzhou, China). The titer of the lentivirus was 1×10^8TU/mL. A quantity of 4×10^5 RAW264.7 cells per well were co-cultured with 200 μl Lentivirus in six-well plate and then incubated overnight in DMED medium and 2 μg/ml ploybrene for 24 h. Down-regulation of CCL3 were confirmed by real-time PCR.

Sequences of CCL2-shRNA:

TCGGACTGTGATGCCTTAATTCTCGAGAATTAAGGCATCACAGTCCGA.

Sequences of CCL3-shRNA:

AAGTCTTCTCAGCGCCATATCTCGAGATATGGCGCTGAGAAGACTTG.

Total RNA Extraction and Real-time PCR

Total RNA was extracted from the RAW264.7 cells using RNAiso Plus (Takara, Dalian, China). The concentration of total RNA was measured with a NanoDrop instrument (ND-2000, ThermoFisher Scientific, Waltham, MA, USA). Total RNA was then reversely transcribed into cDNA using Primescript RT MasterMix (Takara, Dalian, China) following the manufacturer's instructions. GAPDH, the housekeeping gene, was used to normalize the relative expression. The CFX Connect system (Bio-Rad, USA) was used then to measure the mRNA levels. Data were calculated by the $2^{-\triangle\triangle}$CT method. The primers of target genes used in this study are shown in Table 1.

TABLE 1 Primers of target genes

GAPDH	F:TGTGTCCGTCGTGGATCTGA	R:TTGCTGTTGAAGTCGCAGGAG
CCL2	F:TAAAAACCTGGATCGGAACCAAA	R:GCATTAGCTTCAGATTTACGGGT
CCL3	F:CAGCTTATAGGAGATGGAGCTATG	R:TCACTGACCTGGAACTGAATG
CCL5	F:GCTGCTTTGCCTACCTCTCC	R:TCGAGTGACAAACACGACTGC
CCR1	F:ATCCTGTTGACGATTGACAGAT	R:TGATGCCAAAAGTAACAGTTCG
CCR5	F:GACATCCGTTCCCCTACAAG	R:AGATGAACACCAGTGAGTAGAG
CCR9	F:CTGGTATTGCACAAGAGTGAAGA	R:CCACACTGATGCACATGATGA

Exosome Extraction and Identification

Exosomes were extracted using multiple rounds of centrifugation from HUSMSCs. First, these cells were centrifuged for 10 min at 300 g. Then the collected supernatant was centrifuged for 10 min at 2000 g. Repeatedly, the collected supernatant was centrifuged for 30 min at 10,000 g, and then for 70 min at 100,000 g. Afterwards, the precipitate was suspended with PBS and centrifuged for 70 min at 100,000 g. Finally, the precipitate was suspended in PBS and stored at −80°C. Before used for experiments, exosomes were examined by electron microscopy. The size distribution of the exosomes was then tested by NanoSight NS300 system (Malvern Panalytical, Malvern, UK).

Transwell Migration Assay

Twenty-four Transwell inserts (BD Biosciences) were used for migration assays. 5×10^4 wild type RAW264.7 cells were planted in the top chamber, 3×10^5 RAW264.7 cells treated by particles, LPS, GW4869, exosomes or co-cultured with HUSMSCs (3×10^5) was planted in the lower chamber as attractant. After 48 h, the cells were stained with 0.5% crystal violet and counted.

Animal Surgery

Seventy male C57BL/6J mice at the age of 8-week obtained from Animal Laboratory (Guangzhou, Guangdong, China) were raised in Laboratory Animal Center in our University (Guangzhou, Guangdong, China) in a specific pathogen free (SPF) condition. All mice took the surgery at the age of 10 weeks. Animal experiments were approved by the Institutional Animal Care and Use Committee at our University (L102022018060E), all animal experiments were performed in accordance with the principles of laboratory animal care.

A total of 10% chloral hydrate was injected intraperitoneal in mice to be anesthetized. On the head of the mice, a midline sagittal incision at the length of 15 mm was cut in order to expose the calvaria. After stripping the periosteum, the calvaria area was covered by a $0.5 \times 0.5 \text{ cm}^2$ gelatin sponge. Titanium particles, lentiviral vector, HUSMSCs, exosomes or GW4869 were injected respectively under the gelatin sponge at mid-line sagittal of the calvaria before suture. Making an incision, placing the gelatin sponge over the calvaria and suturing without injection comprised the Sham group. No deaths or complications were observed. A total of 7 days after operation, all calvaria were harvested and then examined by micro-CT imaging.

Subgroup of the Animals

The mice were divided in to 14 groups, each containing five mice. 30 μl PBS containing 0.3 mg Ti particles was injected in all groups except for the Sham, exosomes, HUCMSCs, CCL2-LV, CCL3-LV and NC-LV groups. 1.5×10^7 HUCMSCs in 30 μl PBS was locally injected in Ti + HUCMSCs, Ti + HUCMSCs+GW4869 and HUCMSCs groups. 2×10^9 exosomes in 30 μl PBS was locally injected in Ti + exosomes, and exosomes groups. 20 μl GW4869 in 30 μl PBS was locally injected in Ti + HUCMSCs+GW4869 and Ti + GW4869 groups. 70 μl of the shRNA lentiviral vector down-regulating CCL2, CCL3 or lentiviral vector containing negative control, at the titer of 1×10^8TU/mL was injected in the Ti + CCL2-LV, CCL2-LV, Ti + CCL3-LV, Ti + NC-LV, CCL3-LV and NC-LV groups. The injection volume was adjusted to 100 μl by PBS in all groups. 100 μl PBS was used as blank control.

CT Scan Analysis

After harvest, the calvaria specimens took a 24-h paraformaldehyde fixation. Afterwards, the level of osteolysis in the calvaria was evaluated by a micro-CT Imaging System (ZKKS-MCT-Sharp, Japan). The radiographic projections were acquired at 60 kV and 667 μA within 240 ms. The associated reconstruction software was used to reconstruct 3D images. The bone volume/ total volume (BV/TV) and bone mineral density (BMD) were measured from a $1 \times 3 \times 3$ mm region of interest in the cross-section slices around the sagittal suture. All projection frames were recorded at least five times and averaged.

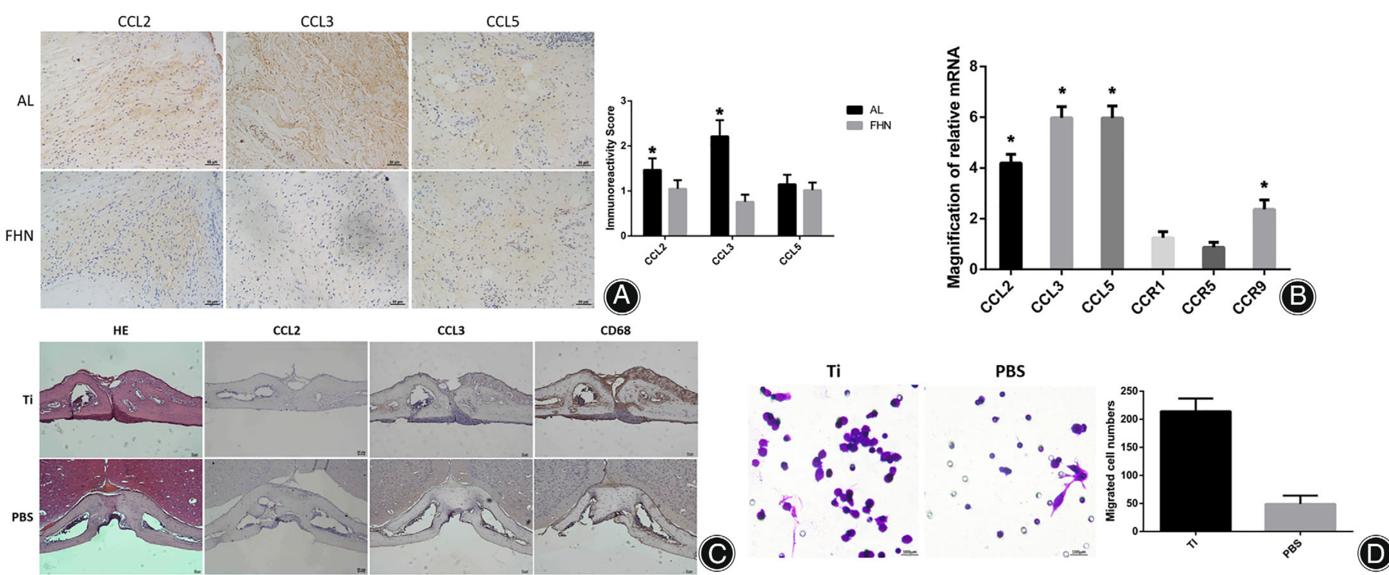

Fig. 1 (A) The immunohistochemical staining of CCL2, CCL3 and CCL5 of hip Synovial membrane from AL and FHN patients, and the immunoreactivity score. CCL2 and CCL3 were significantly higher in AL patients. *: Significant difference comparing with the IS of FHN patients: $P < 0.01$. (B) The mRNA expressions of CCL2, CCL3, CCL5, CCR9 in macrophages were significantly higher after the stimulation of Ti particles (*: Significant difference comparing with negative control, $P < 0.01$). (C) The immunohistochemical staining of CCL2, CCL3 and CD68 of mice calvaria sutura. CCL2, CCL3 and CD68 were highly expressed in the calvaria in Ti group. (D) Transwell Migration Assay showed significant migration of macrophages when the macrophages in lower chamber were stimulated by Titanium particles

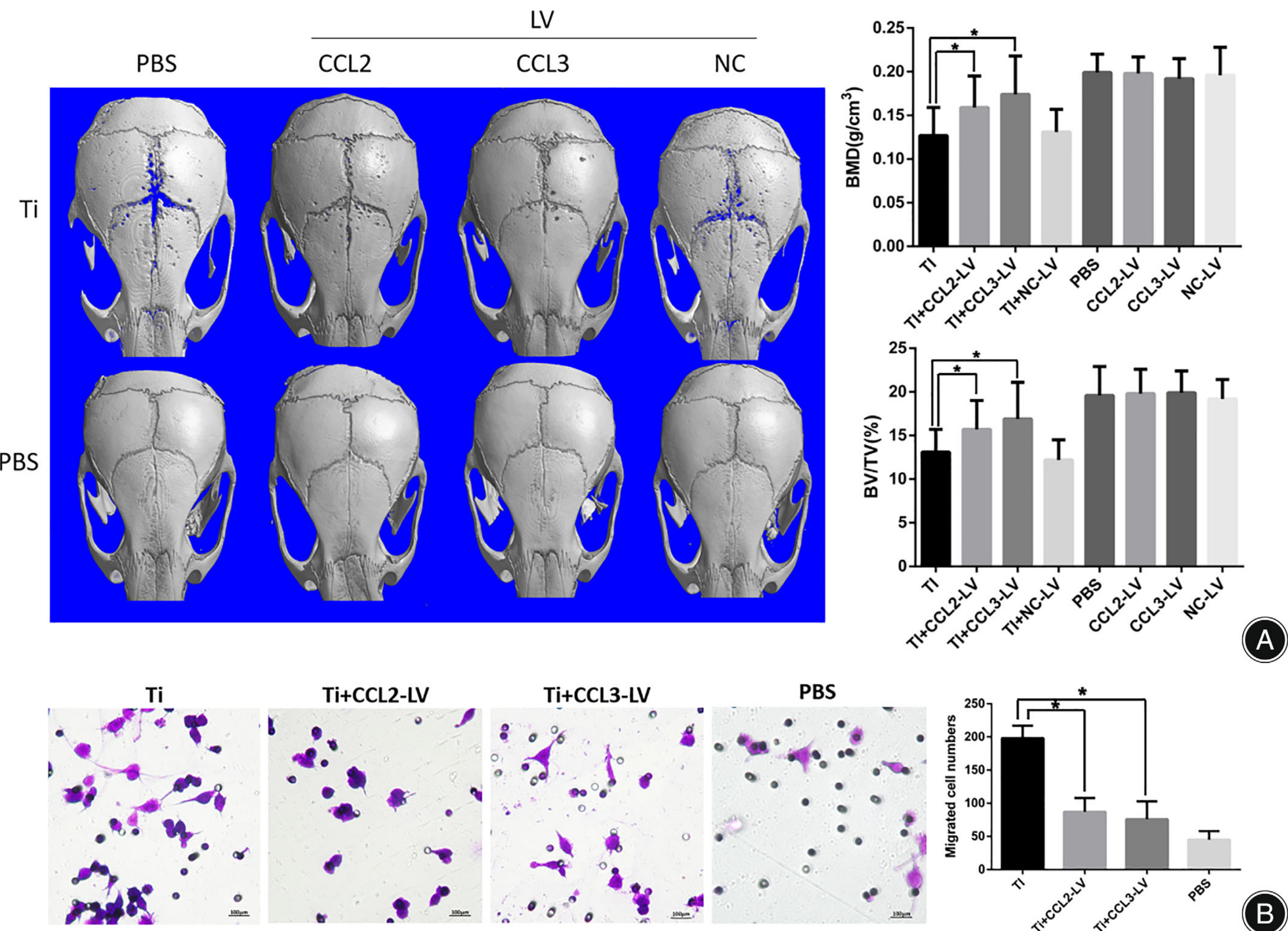

Fig. 2 (A) 3-dimension reconstruction of the mice calvaria in different groups; The BV/TV and BMD of an 1 × 3 × 3 mm region of interest at the cranial suture was significantly higher in Ti + CCL2-LV group and Ti + CCL3-LV group than in Ti group. (*$P < 0.01$). (B) Transwell Migration Assay showed the migration was significantly inhibited by CCL2-LV and CCL3-LV, comparing Ti group and Ti group (*$P < 0.01$)

Immunohistochemistry Assay of Mice Calvaria

After micro-CT scan, mice calvaria were decalcified in 10% ethylene diamine tetra acetic acid (EDTA) for 21 days. After which, the samples were fixed in 4% paraformaldehyde fixation. 4.0-μm serial sectioning of the specimens was obtained after embedded in paraffin blocks. The sections were then deparaffinaged by xylene and dehydrated by ethanol washes of decreasing concentrations. A total of 3% hydrogen peroxide was then used for blockage of endogenous biotin or enzymes, sodium citrate was used for antigen retrieval. CCL2 antibody (1:100 diluted, ABclonal Technology, China), CCL3 antibody (1:100 diluted, ABclonal Technology, China), CD68 antibody (1:100 diluted, ABclonal Technology, China) were used as primary antibodies. The tissues were then incubated with primary antibodies respectively for 2 h at 37°C and washed with phosphate buffer solution (PBS) three times. Afterwards, tissues were incubated with horseradish peroxidase conjugated (Zhongshanjinqiao Biotechnology, China) as secondary antibody for 0.5 h at 37°C and washed. Then, DAB and hematoxylin was used as chromogenic agent and second stain respectively. At last, all specimens were washed, dried and mounted, then observed using a biomicroscope (DM2000, Leica, Wetzlar, Germany).

Statistics

All data were analyzed with SPSS 22.0 software (Chicago, IL, USA). Analysis of variance (ANOVA) and Student–Newman–Keuls *post hoc t* test were used to analyze the results and determine the statistical significance of the differences. All data were expressed as mean ± SD. The value of $P < 0.05$

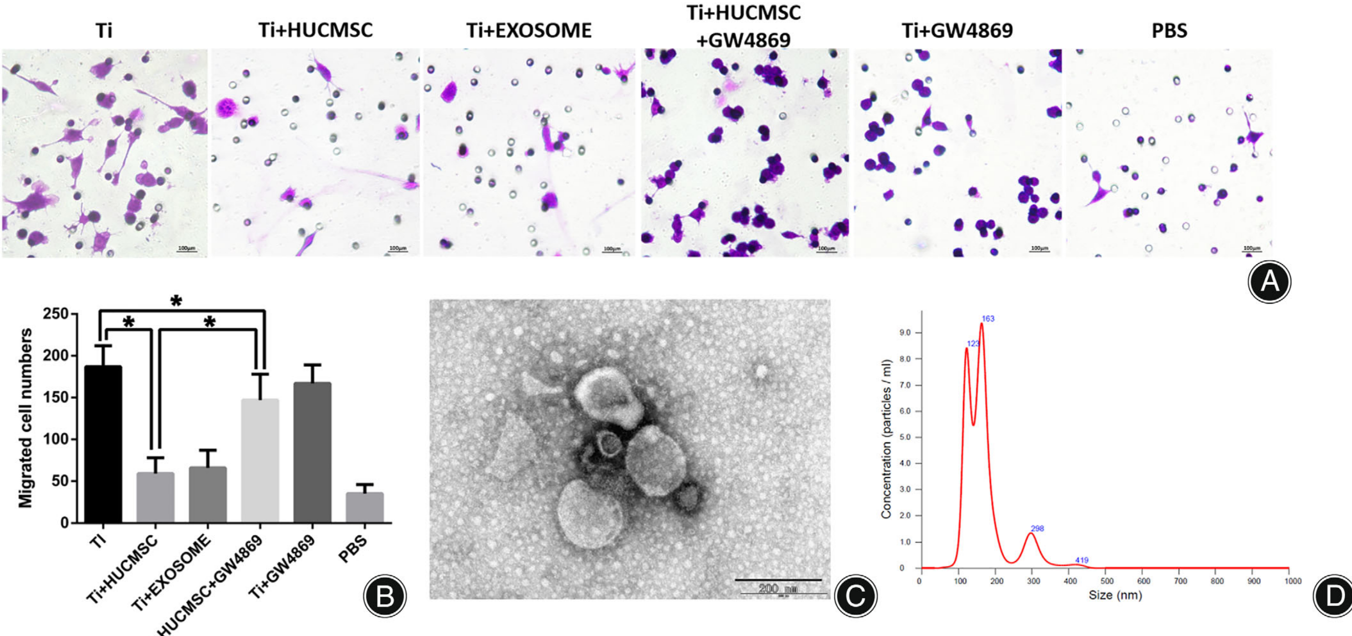

Fig. 3 (A, B) Transwell migration assay showed the migration was significantly inhibited by HUCMSC and exosome, but still large number of migrated macrophages was observed in Ti + HUCMSC + GW4869 group (*$P < 0.01$) (C) Pictures of the exosome through TEM. (D) The size distribution of the exosomes: the diameter of the exosomes was 170.1 nm on average

was considered as statistically significant. Every result *in vitro* came at least three independent experiments.

Results

CCL2 and CCL3 Was Highly Expressed in Aseptic Hip Joint Loosening Clinical Specimens and Aseptic Loosening Animal Model

In clinical specimens, CCL2 and CCL3 were more highly expressed in AL patients than in FHN patients. However, CCL5 show no obvious difference in staining intensity between two groups (Fig. 1A). The immune-reactivity score of CCL3 in AL patients was 2.21 ± 0.12, higher than the IS in FHN patients (0.76 ± 0.16, $P < 0.01$). The IS of CCL2 in AL patients was also significantly higher than the IS in FHN patients (1.47 ± 0.25 *vs* 1.05 ± 0.19, $P < 0.01$). The The IS of CCL5 showed no difference between these two groups (1.15 ± 0.21 *vs* 1.02 ± 0.17, $P > 0.05$) (Fig. 1A).

The mRNA expressions of CCL2, CCL3, CCL5, CCR9 in macrophages were all increased after the stimulated by Ti particles ($P < 0.01$). No significant increase of mRNA expression was observed in CCR1 and CCR5 (1.15 ± 0.21 *vs* 1.02 ± 0.17, $P > 0.05$) (Fig. 1B). To further investigate the expression of CCL2, CCL3 and CD68 in Ti particle induced osteolysis *in vivo*, the calvaria osteolysis mouse model was conducted. CCL2, CCL3 and CD68 were all found higher expressed in the Ti group than in the PBS group through immunohistochemistry assay (Fig. 1C). Transwell migration assay was used to further investigate the migration of macrophages. Results showed that when the macrophages in lower chamber were stimulated by titanium particles, a large quantity of macrophages in upper chamber were migrated (214.1 ± 23.2 *vs* 49.0 ± 15.7 cells, $P < 0.01$) (Fig. 1D).

The Knockdown of CCL2 and CCL3 Through Lentivirus Inhibited the Osteolysis of Mice Cranial in vivo and the Macrophages' Migration in vitro

Transwell migration assay was used to further investigate the effect on the macrophage migration when macrophages in the lower chamber was transfected with CCL2-LV or CCL3-LV. Results showed that the number of migrated macrophage was lower than that of wild type macrophages (198.3 ± 19.4 *vs* 87.9 ± 21.3 *vs* 76.5 ± 27.7 cells, $P < 0.01$) (Fig. 2B). When the calvaria osteolysis mouse model was used, CCL2-LV or CCL3-LV were injected respectively along with the titanium particles in order to evaluate the effect of CCL2 or CCL3 *in vivo*. After harvesting, the calvaria took Micro-CT to quantify BV/TV and BMD. Three-dimension reconstruction of the mice calvaria showed an obvious osteolysis area around the cranial sutures in Ti particles-treated mice. The injection of CCL2-LV or CCL3-LV reduced the osteolysis area around the cranial sutures. But the injection of NC-LV did not decrease the osteolysis (Fig. 2A). The other groups showed no significat osteolysis. Qualitative analysis showed that the BMD and the BV/TV were all increased in Ti particles + CCL2-LV group and Ti

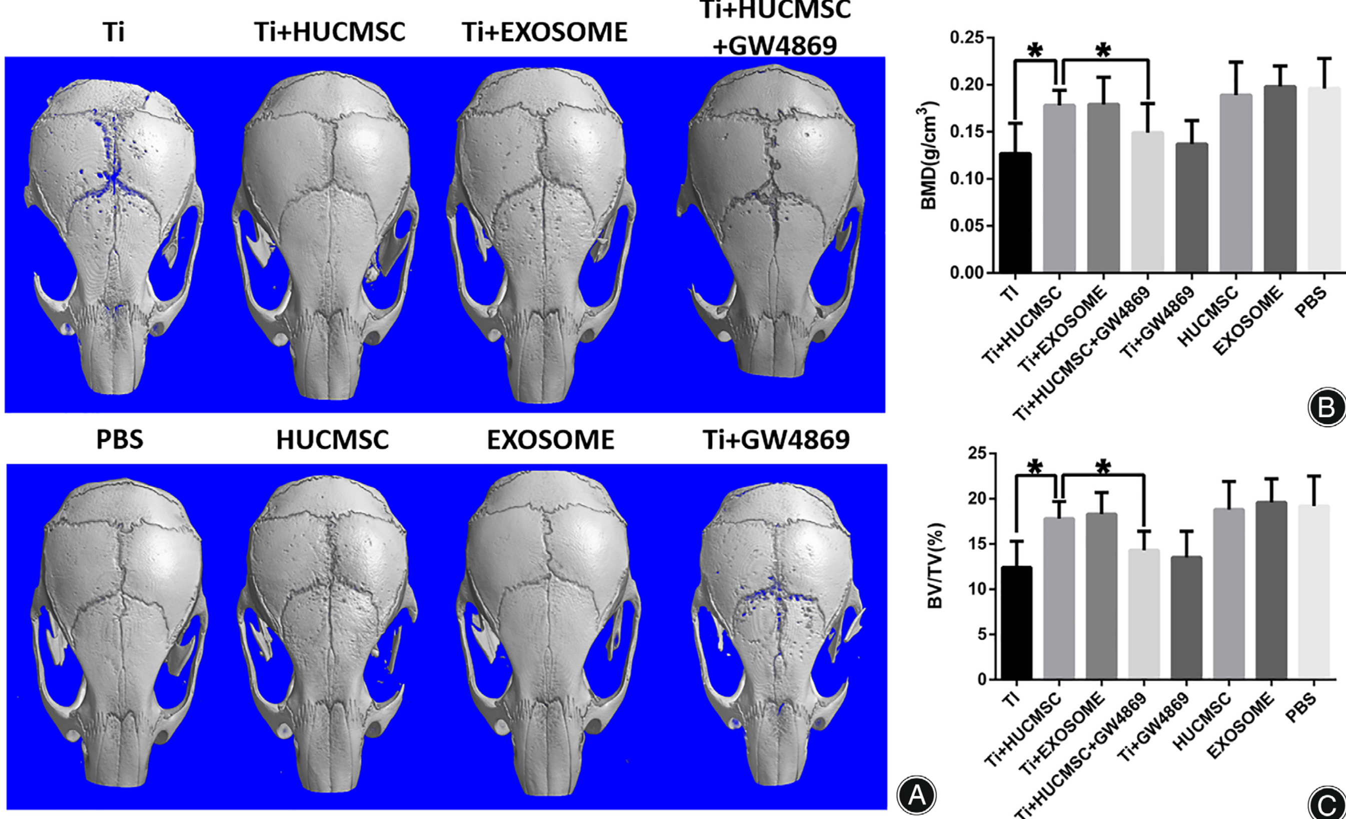

Fig. 4 (A) 3-dimension reconstruction of the mice calvaria in the eight different treatment groups; (B) The BMD of the ROI increased in Ti + HUCMSC and Ti + exosome group compared with Ti group, but reduced again after the usage of GW4869 (*P < 0.01). (C) The BV/TV of the ROI around the cranial suture increased in Ti + HUCMSC and Ti + exosome group compared with Ti group, but reduced again after the usage of GW4869 (*P < 0.01)

particles + CCL3-LV group, compared with the Ti group (BMD: 0.127 ± 0.032 *vs* 0.159 ± 0.036 *vs* 0.174 ± 0.044 g/cm^3, $P < 0.01$. BV/TV: 13.1 ± 2.6 *vs* 15.7 ± 3.3 *vs* $16.9 \pm 4.2\%$, $P < 0.01$), yet no difference was observed between Ti + NC-LV and Ti groups in BMD ($P > 0.05$) or BV/TV ($P > 0.05$) (Fig. 2A). The four non-titanium groups were all significantly higher in BV/TV and BMD than Ti group, no difference was found among these groups.

HUCMSC and Exosomes Derived from HUCMSCs Inhibited the Osteolysis of Mice Calvaria in vivo and the Macrophages' Migration in vitro

Exosomes were examined by electron microscopy (Fig. 3C). NTA showed that the diameter of the exosomes was 170.1nm on average (Fig. 3D). Transwell migration assay was used to further investigate the effect on the macrophage migration when macrophages in the lower chamber was co-cultured with HUCMSC or exosomes. GW4869 was used to block the release of exosomes. Results showed that the number of migrated macrophage was 68.5% and 65.7% lower than that of wild type macrophages in the Ti + HUCMSC and Ti + exosome groups retrospectively (Ti *vs* Ti + HUCMSC *vs* Ti + exosome: 187.2 ± 25.3 *vs* 59.6 ± 19.4 *vs* 66.9 ± 21.1 cells, $P < 0.01$ retrospectively; Fig. 3A,B), yet no difference was observed between the Ti + HUCMSC and Ti + exosome groups. The number of migrated macrophages became larger after the use of GW4869 (59.6 ± 19.4 *vs* 147.8 ± 31.2 cells, $P < 0.01$), but still significantly lower than Ti group. What is interesting, comparing the Ti and Ti + GW4869 groups, we found that the use of GW4869 could also slightly decrease the number of migrated cells, yet no significant difference was found (Fig. 3A,B).

The mice calvaria osteolysis model was generated to investigate the effect of HUCMSC and exosomes *in vivo*. MircoCT examination showed the same tendency as transwell migration assay. Three-dimension reconstruction of the mice calvaria showed the osteolysis area was significantly reduced in HUCMSC or exosomes treated groups around the cranial sutures. The use of GW4869 inhibited the HUCMSC's protection on mice calvaria, the osteolysis area was increased again (Fig. 4A). The other non-Ti groups showed no significat osteolysis. Qualitative analysis showed that the BV/TV and BMD were both increased in the Ti + HUCMSC and Ti + exosome groups compared with Ti

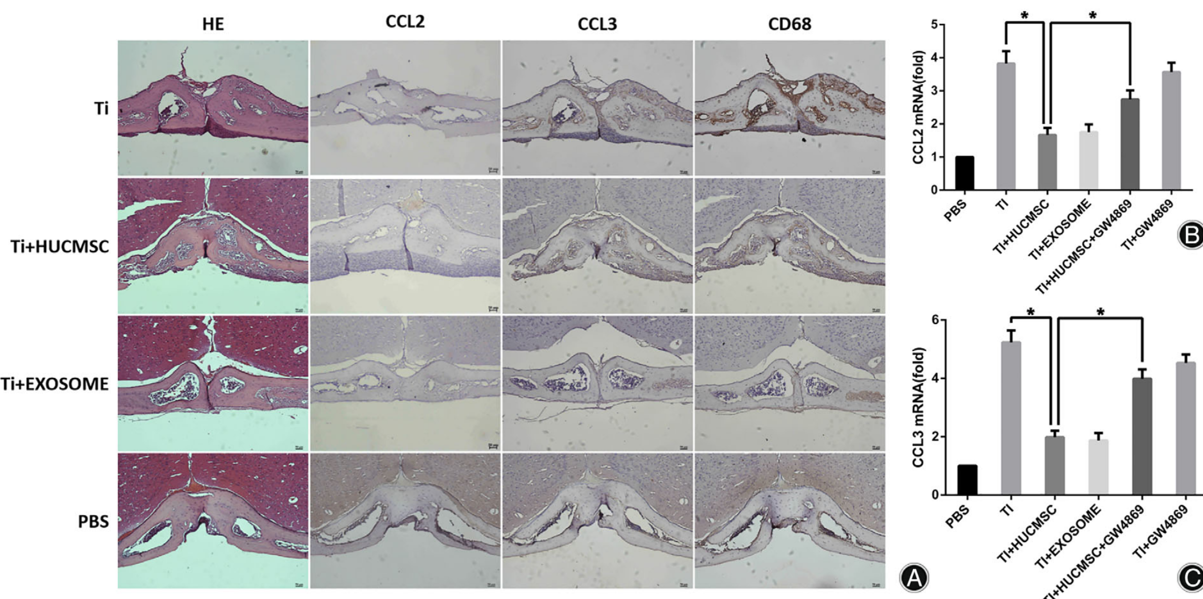

Fig. 5 (A) The HE and immunohistochemical staining of CCL3, CCL3 and CD68 of mice calvaria sutura in different groups. CCL2, CCL3 and CD68 were highly expressed in the calvaria in the Ti group, HUCMSC and exosome down-regulated the expression of CCL2, CCL3 and CD68. (B) The mRNA expression of CCL2 in macrophages was significantly higher after the stimulation of Ti particles. The expression of CCL2 was down-regulated by HUCMSC and exosome, but increased again after the usage of GW4869 (*$P < 0.01$). (C) The mRNA expression of CCL3 in macrophages was significantly higher after the stimulation of Ti particles. The expression of CCL3 was down-regulated by HUCMSC and exosome, but increased again after the usage of GW4869 (*$P < 0.01$)

group (BV/TV: 17.8 ± 1.9 *vs* 18.3 ± 2.4 *vs* 12.4 ± 2.9%, $P < 0.01$ retrospectively. BMD: 0.178 ± 0.016 *vs* 0.179 ± 0.029 *vs* 0.127 ± 0.032 g/cm^3, $P < 0.01$ retrospectively). The BV/TV and BMD were both reduced again after the usage of GW4869 compared with the Ti + HUCMSC group (BV/TV: 17.8 ± 1.9 *vs* 14.3 ± 2.1%, $p < 0.01$. BMD: 0.178 ± 0.016 *vs* 0.149 ± 0.031 g/cm^3, $P < 0.01$; Fig. 4B,C). The three non-titanium groups were all significantly higher than the Ti group in BV/TV and BMD, no difference was found among these three groups.

CCL2 and CCL3 Expression Was Reduced in HUCMSC or Exosome Treated Mice Cranial Osteolysis Model and in HUCMSC or Exosome Treated Macrophage RAW264.7 after Titanium Stimulation

To further investigate whether CCL2 and CCL3 were affected by HUCMSCs or exosomes, immunohistochemistry assay was carried out to evaluated the CCL2 and CCL3 expressions in mice calvaria. Results showed that CCL2, CCL3 and CD68 were highly expressed in the calvaria in the Ti group. But in the Ti + HUCMSC and Ti + exosome groups, the expression was much lower (Fig. 5A). Realtime PCR showed the same results. After stimulation by Ti particles, the mRNA expression of CCL2 and CCL3 were up-regulated ($P < 0.01$). But under the intervention of HUCMSCs or exosomes, the mRNA expression of CCL2 and CCL3 were lower than the Ti group significantly. In Ti + HUCMSCs + GW4869 group, the mRNA expression of CCL2 and CCL3 was increased again, significantly higher than that of the Ti + HUCMSC group ($P < 0.01$; Fig. 5B,C).

Discussion

In this study, our results showed that CCL2 and CCL3 was highly expressed in aseptic hip joint loosening clinical specimens and aseptic loosening animal model, the knockdown of CCL2 and CCL3 through lentivirus did inhibit the osteolysis of mice cranial *in vivo* and the macrophages' migration *in vitro*, indicating that CCL2 and CCL3 might promote the macrophage' migration and further aggravated the inflammation in aseptic loosening. However, HUCMSC and exosomes derived from HUCMSCs inhibited the osteolysis of mice calvaria *in vivo* and the macrophages' migration *in vitro*. What is more, the expression of CCL2 and CCL3 were found to be reduced when treated with HUCMSC or exosomes derived from HUCMSCs *in vivo and vitro*, indicating that HUCMSCs and exosome from HUCMSCs inhibit wear particles induced aseptic loosening through inhibiting CCL2 and CCL3.

As the most common cause for revision hip replacement surgery, aseptic loosening had long been studied. Complex mechanisms had been proven to induce aseptic loosening, such as micro motion, high-pressure in joint fluid and stress shielding.[19] Still, the byproduct of the prosthesis, wear particles, were considered as the major cause of the

aseptic hip joint loosening. It' has been proved that almost all kinds of materials could produce wear particles at every site of the prosthesis, which will activate the macrophage and finally lead to aseptic loosening.[20] Another study had found out that histological examination of per prosthetic tissues from aseptically loosened implants showed that there was around 60 μm^3 of wear debris per macrophage. Particles within the phagocytosable size range (0.1–10 μm) were the most biologically active.[21] Other studies had proved that the concentration of 100:1 (particle volume [μm^3]: quantity of cells) could provide the maximum inflammation response.[22] Thus, in this study, we chose the titanium particles ranging from 0.2 to 1.2 μm (on average 0.82 ± 0.12 μm) at the concentration of 100 μm^3 per cell, to get the maximum inflammation response. However, there were studies showed that very few titanium particles could be phagocytosed.[23] The phagocytosis rate of the titanium particles and its affect on the inflammatory requires more studies.

CCL2 and CCL3 Was Involved in the Recruitment of Macrophage in Wear Particles Induced Aseptic Loosening

After the interaction with wear particles, macrophage would polarize into M1 macrophage and initiate a complex inflammation procedure, plenty of signal pathways was involved, including TLRs, NLRs, NF-κB, NLRP3 inflammasome, caused the secretion of inflammation cytokines.[7] These cytokines could not only activate the osteoclasts and induce the osteolysis, but also activate the tissue around the prosthesis. It has been reported that TNF-α could cause the release of CCL3 in human monocytic cells.[24] In another study, the expression of TNF-α was up-regulated in clinical specimen from the tissues around the prosthesis.[25] In this study, the expression of CCL2and CCL3 were both up-regulated in clinical specimens in most of the cells, mainly fibroblasts. Showing that not only immunocytes, but the cells around the prosthesis all took part in the expression of chemokines. What is more, we tested the mRNA expression for three CCRs:CCR1, CCR5 and CCR9, and found only CCR9 was up-regulated in macrophage when stimulated by titanium particles. But this was only a preliminary result so we could not reach the conclusion that CCR9 was the downstream of the CCLs in aseptic loosening. More work is required to further explore the exact downstream mechanisms. When the large number of macrophages were recruited, many of them would differentiate into osteoclasts and aggravate the osteolysis. Thus, inhibiting the recruitment of macrophages could be a potential method to prevent aseptic loosening.

Studies had showed that CCL2 and CCL3 was involved in the recruitment of macrophages.[11] The inhibition of the MCP-1-CCR2 (CCL2-CCR2) axis reduced the macrophage's response when stimulated by the UHMWPE particles.[26] Other recent studies found out that CCL3 might participated in some other migration regulations such as tumors. By promoting cell migration and invasion, CCL3-CCR5 axis could contribute to the progression of esophageal squamous cell carcinoma.[27] What is more, CCL3 was reported to contribute to the secondary damage after spinal cord injury.[28] In our study, CCL2 and CCL3 were proved to promote the migration of macrophages under the stimulation of titanium particles. When the expression of CCL2 or CCL3 were inhibited, the macrophage's migration was significantly depressed. The mice calvaria osteolysis were also relieved when lentivirus containing CCL2 or CCL3 inhibiting sequences were injected at the meanwhile. Thus, both CCL2 and CCL3 might mediated the migration of macrophages in titanium induced aseptic loosening.

The inhibition of chemokines provided a new treating method for many diseases that have chemokines involved. Studies have found that Curcumin could inhibit the CCL3 production and then further inhibit the migration of the precursors of osteoclast and its osteoclastogenesis.[29] Other studies found that Octominin inhibited LPS-induced CCL3 secretion by blocking TLRs/NF-κB signal transduction, making Octominin a potential treatment in treating inflammatory diseases.[30]

HUCMSCs and Exosome from HUCMSCs Inhibit Wear Particles Induced Aseptic Loosening in vivo and vitro

MSC provide a ideal option to inhibit the expression of CCL3 safely and effectively. It has been reported that HUCMSC could alleviate IBD and polarize neutrophils toward the "N2" phenotype and inhibit the expression of CCL3 by inhibiting activation of ERK signaling pathways.[31] Other research has found that MSCs could selectively block immune cell migration and down-regulate chemokines such as CCL1, CCL3 and chemokine receptors, and further relieve the cutaneous Scl-GVHD.[32] Similarly in our study, HUCMSC inhibited the expression of CCL3 *in vivo* and *in vitro*, and further inhibited the osteolysis around the cranium and macrophage migration.

HUCMSC showed a very promising future in treating aseptic loosening. HUCMSC could be locally injected around the prosthesis regularly to prevent the occurrence of aseptic loosening. Although the immunogenicity of HUCMSC was quite low, there is still a large percentage of patients who suffer the side-effects. In a clinical study, researchers injected HUCMSCs into the patients' knee joints to treat osteoarthritis, more than 50% of the patients suffered adverse events, such as swelling or pain of the knee joint.[33] Thus, exosome might become a better option.

Exosomes containing complex RNA and proteins was an important way for MSC to achieve therapeutic effects on inflammatory diseases.[34] Research has found that chondrocytes-derived exosomes could promote BMSCs' chondrogenic differentiation *via* regulating the Wnt/beta-catenin pathway.[35] BMSCs-derived exosomes could inhibit apoptosis and inflammatory response in spinal cord injury.[36,37] In this study, we used a exosomes inhibitor, GW4869, to block the secretion of exosomes. When exosomes were suppressed, the osteolysis area was increased again in the mice cranium osteolysis model, the number of migrated macrophages was also increased. Which means that

the exosomes from HUCMSCs might participate in the protective effect on titanium induced osteolysis.

However, GW4869 was not a highly selective inhibitor of HUCMSC-derived exosomes. Studies had showed that it could inhibit the TNFα secretion of macrophages.[38] Other studies had reported that GW4869 could attenuate the Lipopolysaccharide activated macrophages induced BMSCs' osteogenic differentiation through inhibiting macrophages' exosomes.[39] In this study, 3-dimension reconstruction of the mice calvaria showed the osteolysis area around the cranial sutures was smaller in the Ti + GW4869 group than in the Ti group. The usage of GW4869 also inhibited the macrophages' migration. As mentioned before, after the interaction with wear particles, macrophage would initiate a complex inflammation procedure and cause the secretion of plenty kinds of inflammation cytokines.[5] GW4869 itself might provide a protecting effect by inhibiting the macrophage-derived exosomes' secretion.

To further investigate the effect of HUCMSC-derived exosomes, we extracted the exosomes from HUCMSC and then investigated whether exosomes alone could achieve the same effect as HUCMSC on aseptic loosening or not. Results showed that exosomes showed the same protecting ability comparing with HUCMSCs.

HUCMSCs and Exosome from HUCMSCs Inhibit Wear Particles Induced Aseptic Loosening through Inhibiting CCL2 or CCL3

At last, we investigated the expression of CCL2 and CCL3 in these HUCMSCs or exosomes treated macrophages. Results showed that HUCMSCs and exosomes could down-regulate the expression of CCL2 and CCL3 *in vivo* and *in vitro*. The decrease on the expression CD68 means the macrophages migration were also inhibited. There might be two possibilities for the down-regulation of CCL2 and CCL3 expression. First, there might be some miRNA in exosomes that could directly inhibited the expression of CCL2 and CCL3. Second, HUCMSCs and exosomes suppressed the expression of inflammation factors, the down-regulation of CCL2 and CCL3 expression showed as a result. The mechanism of the down-regulation of CCL2 and CCL3 requires further research.

There were limitations to this study. RAW264.7 cells were used as the reporter cells to verify the recruitment of macrophages in transwell assay. However, RAW264.7 cells are long-lived macrophages which may cause unpredictable effects on their function of inflammation and migration. Further, it seems that the effect of the local injection of lentivirus has been proved, but the trace of those lentivirus requires further study.

Conclusions

In conclusion, we demonstrated that CCL2 and CCL3 might promote the macrophage migration and further aggravated the inflammation in aseptic loosening. However, HUCMSC and exosomes derived from HUCMSCs inhibited the osteolysis of mice calvaria *in vivo* and the macrophages' migration *in vitro*. HUCMSCs and exosome from HUCMSCs inhibit wear particles-induced aseptic loosening through inhibiting CCL2 and CCL3.

Author Contributions

Shixun Li and Chuangran Wu performed qRT-PCR experiments, Transwell assay, the data analysis and drafted the manuscript. Sipeng Lin and Zhenkang Wen performed the animal surgery and the micro-CT of the specimens. Wenqiang Luo and Changchuan Li collected the clinical specimens and performed the further IHC examinations. Dr. Xiaoyan Wang and Dr. Xuejia Li provided the HUCMSCs that used in this study. Liangbin Gao and Yue Ding designed the study. All authors read and approved the final manuscript.

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