Gingival mesenchymal stem cells derived from patients with rheumatoid arthritis treats experimental arthritis

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Keywords: Rheumatoid arthritis, GMSCs, Collagen induced arthritis, Humanized animal model, Therapeutic effects

Posted Date: November 7th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3505508/v1

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Abstract

Background

Therapeutic strategy using mesenchymal stem cells (MSCs) has been accepted as a novel therapy for treating rheumatoid arthritis (RA). Human gingiva derived MSCs (GMSCs) are superior in regulating immune responses. To avoid the potential risks of allogenic MSC, autologous MSCs are the optimal candidate. However, whether autologous GMSCs from RA patients are therapeutic remains unknown.

Methods

In this study, we compared the therapeutic efficacy of GMSCs derived from patients with RA (RA-GMSCs) and that from health donors (H-GMSCs) in vivo and in vitro. Then, we utilized RNA-sequencing, the molecular and cellular assays to determine the immunomodulatory molecules that contribute to therapeutic effect of RA-GMSCs on both collagen-induced arthritis (CIA) and humanized synovitis models.

Results

We demonstrated that GMSCs derived from patients with RA (RA-GMSCs) and that from health donors (H-GMSCs) shared the similar expression of immunomodulatory molecules. Moreover, RA-GMSCs were as effective as H-GMSCs in suppressing T cell proliferation, proinflammatory cytokines secretion, as well as osteoclast differentiation in vitro. In addition, RA-GMSCs had a robust therapeutic effect on collagen-induced arthritis (CIA) model. Specifically, RA-GMSCs decreased the frequency of Th1 and Th17 cells whereas enhanced Treg cells, reducing the joint histopathological scores of lymphocytes, osteoclasts and cartilages. Importantly, RA-GMSCs were also effective in suppressing inflamed synoviocytes (RA-FLSs) proliferation, migration and invasion in vitro, and cartilage invasion in a humanized synovitis model in vivo.

Conclusion

Our study implies that manipulation of RA-GMSCs is therapeutic in CIA mice and humanized synovitis models and may have a therapeutic potential in RA patients using autologous GMSCs in the future.

Graphical abstract

Highlights
1. RA-GMSCs share the similar immunomodulatory phenotype with H-GMSCs, as well as immunosuppressive capacity.
2. RA-GMSCs have a robust therapeutic effect on collagen-induced arthritis (CIA) model by suppressing T cell immune response and protecting bone erosion.
3. RA GMSCs are effective in suppressing RA-FLSs invasion in a humanized synovitis model. Use of autologous GMSCs deserves a clinical trial in the future.

**Background**

Rheumatoid arthritis (RA) is an autoimmune disease caused by disorders of the immune system. Inflammatory cells infiltration in pathological sites and bone/cartilage destruction in joints are the major characteristics of RA[1, 2]. It has been accepted that regulatory T (Treg) cells and T helper cells (Th1 and Th17) exert a curial role in driving the process in the disease[3]. Those effector T (Teff) cells combine with B cells and innate effector cells together promote the pro-inflammatory cytokines production and autoantibodies secretion[4]. In addition, resident fibroblast-like synoviocytes (RA-FLSs) can be activated by the pro-inflammatory cytokines, resulting in cartilage invasion and bone destruction[3, 5, 6]. To date, therapeutic managements of RA include the combination of pharmacological (non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs) and corticosteroids, as well as and non-pharmacological (yoga, acupuncture, massage and physiotherapy) approaches[7]. However, few therapeutic approaches are applied to radically eradicate the complex diseases[8].

Mesenchymal stem cells (MSCs) possess powerful immunomodulatory and tissue regeneration capacities and thus can be a potential candidate for the treatment of RA [9, 10]. So far, bone marrow derived MSCs (BMSCs)[11], umbilical cord-derived MSCs (UC-MSCs)[12] and adipose tissue derived MSCs (AD-MSCs)[13] are somehow effective in treating RA. However, it still remains the problems that hinder their further applications in clinical practice, such as difficulty in harvesting and tumorigenesis[14]. Particularly, RA patient derived BMSCs are inefficient in treating autoimmune diseases, limiting use of autologous MSC in treating RA and other autoimmune diseases[15, 16]. Although allogeneic MSCs are effective in controlling the disease development, the side effects and fate of these cells after cell therapy have been considered as the major constraint[17]. Thus, the development of autologous MSC therapy will be a best option.

Recently, we and others have reported GMSCs, a quite different population of MSCs isolated from human gingiva, showed a superior therapeutic effect on several mice models of autoimmune diseases (AIDs) (including RA) and humanized animal models[18–26]. Besides, compared with other tissue derived MSCs, GMSCs are easy to obtain and proliferate fast at low dose growth factors condition in vitro. Also, they are uniformly homogenous and low carcinogenicity[14]. However, it is unclear whether autologous GMSCs from RA patients have an efficiency in alleviating RA pathologic process.
Herein, we investigated the therapeutic effect of RA patient derived GMSCs (RA-GMSCs) in collagen induced arthritis (CIA) mice model. By comparing the immunophenotype differences in protein and mRNA levels, RA-GMSCs and that derived from health donors (H-GMSCs) showed similar immunomodulatory molecule expressions. Moreover, RA-GMSCs also similarly suppressed T cell proliferation, T cell produced TNF-α and osteoclast formation in vitro. Importantly, RA-GMSCs were equally therapeutic in CIA model by delaying the disease onset, declining the clinic scores, improving the joint pathology, and protecting bone destruction. Additionally, like H-GMSCs, RA-GMSCs also had the capacity in suppressing the proliferation, migration and invasion of RA fibroblast-like synoviocytes (RA-FLSs) in vitro and in a humanized synovitis in vivo model, highlighting its clinical application potential. In conclusion, RA-GMSCs are as therapeutic as H-GMSCs in CIA mice model and humanized synovitis model, and thus may be employed to treat RA patients as an autologous MSC cell therapy.

Materials and methods

Animals

C57BL/6 (JAX:000664), NOD-SCID (JAX:001303) and DBA1/J (JAX:000670) mice were used in this project. All mice were originated from Jackson Laboratory and maintained in specific pathogen-free (SPF) animal facility of Guangdong laboratory animals monitoring institute. Mice aged from 6 to 8 weeks were used in this study and were treated by National Institutes of Health (NIH) guidelines for the use of experimental animals. All protocols were approved by Guangdong laboratory animals monitoring institute. The protocols were approved by the Institution Review Boards of Guangdong laboratory animals monitoring institute (IRB:IACUC2019022).

Gingival-derived mesenchymal stem cells

Human gingiva tissue samples were obtained following the routine dental procedures at the Division of Dentistry in the Third Affiliated Hospital at Sun Yat-sen University and the Division of Dentistry in the Sixth Affiliated Hospital at Sun Yat-sen University. All the donors had agreed the content of informed consents form in this project. The protocols were approved by the Institution Review Boards (IRB:201809). GMSCs were isolated and cultivated as previously described [19, 27]. For in vivo treatments, one donor derived GMSCs were used in individual mouse model, and GMSCs from more than three donors were used in one experiment. Gender and age matched RA-GMSCs and H-GMSCs were used in one experiment to compare their capacities.

Flow Cytometry

For flow cytometry analysis, fluorescence conjugated human mAbs were from BioLegend (San Diego, CA) as follows: CD90, CD39, CD73, CD44, HLA-DR, CD80, HLA-ABC, CD29, CD34, CD45, CD86, and CD105. Cell subsets were incubated with mAbs above individually. Samples were detected using FACS Calibur flow cytometer and analyzed with Cell Quest Software (Becton, Dickinson). The final data were processed with
FlowJo Software (Tree Star, Ashland, OR) and presented in plot or histogram figures. The antibodies’ details were in Table 1.

Table 1
The biomarkers for phenotypic identification of GMSCs

<table>
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<tr>
<th>REAGENT or Resource</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
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<tbody>
<tr>
<td>PE anti-human CD29 (TS2/16)</td>
<td>BioLegend</td>
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<td>PE anti-human CD34 (561)</td>
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<tr>
<td>PerCP/Cy5.5 anti-human CD45 (HI30)</td>
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<td>304208</td>
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<tr>
<td>FITC anti-human CD73 (AD2)</td>
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<td>FITC anti-human CD80 (2D10)</td>
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<td>PE anti-human CD86 (IT2.2)</td>
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<tr>
<td>APC anti-human CD105 (43A3)</td>
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<td>FITC anti-human HLA-ABC (W6/32)</td>
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<tr>
<td>PerCP/Cy5.5 anti-human HLA-DR (L234)</td>
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<td>307628</td>
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RAN sequencing

RA-GMSCs and H-GMSCs were isolated and cultured as described above, and total RNA was extracted using the RNeasy mini kit (Invitrogen). The cDNA libraries were constructed using NEBNext Ultra RNA Library Prep Kit following manufacturer’s recommendations. Then, the products were sequenced using a 125bp/150bp paired-end mode. Illumina sequencing were performed on an Illumina HiSeq platform and single cells were encapsulated into emulsion droplets using Chromium Controller (10x Genomics). All the procedure were executed in Beijing Novogene Bioinformatics Technology Co., Ltd.

Quantitative real-time PCR

After isolated and cultured RA-GMSCs and H-GMSCs, total RNA was extracted using RNA kit (Tiangen Biotech) according to manufacturer’s instructions. cDNA was synthesized using RT-Master Mix (TaKaRa) and amplified with qRT-PCR in ABI prism 7700 sequence-detection system (Applied Biosystem, Foster City, CA) using the specific primers for human. in In each sample, the data were proceeded by analyzing the relative gene expression and normalized with GAPDH expression. The primers in this study were synthesized by Applied Biosystems and the sequences were in Table 2.
### Table 2
The primers and sequences of RT-qPCR

<table>
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<th>Gene</th>
<th>Forward primer</th>
<th>Reversed primer</th>
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<td>hNT5E</td>
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<tr>
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<td>GTGCATCAACACAGGGCGCTCTTTC</td>
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<tr>
<td>hCOX2</td>
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<td>hB7H1</td>
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<td>hADORA2B</td>
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<td>hGAPDH</td>
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**In vitro T cell assays**

To evaluate suppressive functions of RA-GMSCs and H-GMSCs on T cells *in vitro*, DBA1 mouse splenic T cells were enriched using a nylon wood dropping method. The enriched T cells were then labeled with CFSE (1 µM, Biolegend). The lymphocytes attached on nylon wood were flushed and used as antigen presenting cells (APCs) and were devitalized by mitomycin (50 µg/ml, Sigma). Then the CFSE labeled T cells (0.3× 10^6 cells/well) were co-cultured with devitalized APCs (the ratio of T cells to APCs was 1:1) in the presence of anti-mouse CD3ε (0.05 µg/ml, Biolegend) for T cell proliferation. The graded ratios of RA-GMSCs or H-GMSCs to T cells were 1:1, 1:50 and 1:100. Cells were maintained in RPMI culture medium for 72 hours with the presence of 10% FBS and 1% penicillin/streptomycin.

For the TNF-α production assay, enriched T cells (0.3× 10^6 cells/well) were co-cultured with devitalized APCs (the ratio was 1:1) in the presence of anti-mouse CD3ε (0.05 µg/ml, Biolegend) and anti-mouse CD28 (1 µg/ml, Biolegend). The graded ratios of RA-GMSCs or H-GMSCs to T cells were 1:1, 1:50 and 1:100. Cells were maintained in RPMI culture medium for 72 hours with the presence of 10% FBS and 1% penicillin/streptomycin.

**Osteoclastogenesis assay in vitro**

For osteoclast formation assay, bone marrow cells from C57BL/6 mice were collected and CD11b + cells were isolated using autoMACS by incubating biotin anti-mouse CD11b antibody (BioLegend) and anti-
biotin microBeads (Miltenyi Biotec). The cell population purity more than 95% were used for the next experiment. The obtained cells were then seeded in 48-well plates (0.5 × 10^6/well) in α-MEM culture medium with 10% FBS and 1% penicillin/streptomycin. Osteoclast was generated with mouse M-CSF (50 ng/mL) for 3 days, and then induced with mouse RANKL (50 ng/mL) and mouse M-CSF (50 ng/mL) (R&D systems, Minneapolis, MN, USA) for another 6 days[25]. RA-GMSCs or H-GMSCs were seeded in the system at day 0. For osteoclast formation assay, the induced cells were detected using tartrate-resistant acid phosphatase (TRAP) kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions, and TRAP + cells were counted with microscopy.

**RA FLSs proliferation, migration and invasion in vitro**

RA synovial biopsies were finely minced into small pieces and cultivated in Dulbecco’s modified Eagle's medium (DMEM) (Hyclone Laboratories, LOGAN, UT, USA) with the presence of 10% FBS and 1% penicillin/streptomycin in a humidified incubator at 37 °C and 5% CO₂[28]. RA FLSs were identified by morphology and surface marker expression, < 1% CD11b+, < 1% CD68+, < 1% FcgRII and FcgRIII receptor + and > 96% CD90+. Cells from passages 3–5 were used for the subsequent experiments.

For RA FLSs proliferation in vitro, RA FLSs were cultivated in 96-well plates with the cell number of 4000 per well. The graded ratios of RA-GMSCs or H-GMSCs to RA FLSs were 1:1, 1:50 and 1:100 for 3 days. Cell proliferation was detected using the cell counting kit-8 (CCK-8, Dojindo, Tokyo, Japan) according to the manufacturer's instructions. RA FLSs migration or invasion assays were performed in a trans-well culture system. For RA FLSs migration, RA FLSs cells (8000/well) were seeded in the trans-well chambers (8.0 μm pore size, Corning Inc., Corning, NY, USA) with serum-free DMEM. DMEM containing 10% FBS was added in the lower wells as a chemoattractant added. For RA FLSs migration, trans-well chambers were firstly coated with matrigel basement membrane matrix (Corning Inc., Corning, NY, USA) according to the manufacturer's instructions. RA-GMSCs or H-GMSCs were seeded in the lower wells at the ratio of 1:1, 1:50 and 1:100 for 3 days. To evaluate the RA FLSs migration or invasion, unmigrated cells were removed and the migrated cells were fixed with 4% paraformaldehyde after the remove of unmigrated cells. Then, stained the fixed cells with crystal violet (Sigma) and washed 3 times. The results were assessed using microscopy.

**RA-GMSCs proliferation, migration and differentiation in vitro**

For RA-GMSCs proliferation in vitro, RA-GMSCs were seeded in 96-well plates with the cell number of 4000 per well for 3 days. Cell proliferation was detected using the cell counting kit-8. For RA-GMSCs migration assay, RA-GMSCs (8000 cells/well) were seeded in the trans-well chambers with serum-free α-MEM. α-MEM containing 10% FBS was used as a chemoattractant added in the lower wells. Proliferation and migration capacities were detected daily for 3 days.

For RA-GMSCs osteogenic differentiation, RA-GMSCs were incubated in 24-well plates with a number of 3× 10^4 per well for 24 h. Then, osteo-inductive condition was established as follows[29]: α-MEM containing 10% FBS, dexamethasone (0.1 mM, Sigma), β-glycerophosphate (10 mM, Alfa Aesar),
ascorbate-2-phosphate (50 mM, Sinopharm Chemical Reagent Co.) and 1% penicillin/streptomycin (Gibco). Cells were induced for 3 weeks, and the culture medium was refreshed at every 3 days. Finally, the induced cells were fixed with 4% paraformaldehyde for 15 min and stained with Alizarin Red S (pH 4; Sigmae Aldrich). The results were assessed using microscopy.

For adipogenic differentiation, RA-GMSCs were incubated in 24-well plates with a number of $3 \times 10^4$ per well for 24 h. Then, adipogenic-inductive condition was established as follows[29]: α-MEM containing 10% FBS, dexamethasone (0.1 mM, Sigma), indomethacin (200 mM, Sigma), insulin (10 mM, Sigma), isobutyl methylxanthine (0.5 mM, Sigma), and 1% penicillin/streptomycin (Gibco). Cells were induced for 3 weeks, and the culture medium was refreshed at every 3 days. Finally, the induced cells were fixed with 4% paraformaldehyde for 15 min and stained with Oil Red O (Sigma). Adipogenic differentiation was observed with microscope.

**Induction of collagen-induced arthritis (CIA)**

CIA mice model was developed as previously reported[25]. First, type II bovine collagen (CII, 2 mg/mL) was emulsified with Complete Freund's adjuvant (CFA) with a ratio of 1:1. Then, the mixture was intradermally injected into the DBA1/J mice tail. Each group mice were randomly divided with or without a single intravenous injection of RA-GMSC or H-GMSCs ($2 \times 10^6$ per mice) at 14 days post immunization. Data were collected at day 60 after mice sacrificed.

**Evaluation of clinical scores**

Clinic scores of the mice were evaluated every other day. Paws' swelling score was assessed using a well established 0 to 4 scoring system. Each paw score was judged as previous documented[30]: 0, no erythema/swelling; 1, mild swelling confined to the tarsal bones or ankle joint; 2, mild swelling including ankle and tarsal bones; 3, moderate swelling ranged from the ankle to the metatarsal joints; 4, severe swelling encompassing the ankle, foot and digits, or ankylosis of the limb.

**Micro-computed tomography (Micro CT) imaging**

Mice joints were fixed with 4% paraformaldehyde. Micro CT was performed as previous reported[25]. The *in vivo* imaging of the three-dimensional bone was performed in high-resolution micro-CT system (Viva CT 40, Scanco, Switzerland). A entire single mouse foot was scanned using the parameters below: 211 image slices, 17.5 µm voxel size at 55 kV, 145 µA, 200 ms integration time. The micro-CT images were converted to 8-bit and imported into Mimics software (Materialise, Belgium), then filtered using discrete Gaussian filtering (variance = 1; max kernel width = 1). Bone erosion was quantified on the interest metatarsophalangeal joint volumes. Consistent image intensity threshold was used to segment second through fourth metatarsal and phalangeal bones from surrounding soft tissue. Three interested volumes were selected from each metatarsophalangeal joint and were oriented consistently based on the 3D longitudinal axis of the third metatarsal.

**Humanized synovitis model**
For the evaluation of RA FLSs invasion *in vivo*, a humanized synovitis model was developed as previously documented[31, 32]. In the first operation, a cartilage-gelatin sponge sandwich was implanted subcutaneously into the left side of NOD SCID mouse. Then, post two weeks, another cartilage-sponge complex containing RA FLSs (5 × 10^5) was implanted under the right flank of the same mouse. Obtained the tissues at day 60, and stained with hematoxylin and eosin (H&E) to assess RA FLSs invade into cartilage.

**Criterion for clinical score of humanized synovitis model**

Invasion[31]: 0, no or minimal invasion; 1, visible invasion; 2, invasion; 3, deep invasion. Perichondrocytic cartilage degradation[17]: 0, chondrons intact halo compared with the cartilage before implantation; 1, one diameter of the chondron; 2, between one and two diameters of a chondron; 3, more than two diameters of a chondron.

**Statistical analysis**

One-way ANOVA analysis was used to statistic the treatment groups. All the results data were presented as Means ± S.E.M. GraphPad Prism Software (version 8.01) was used to perform statistical analyses. P < 0.05 was considered as statistically significant.

**Results**

**RA-GMSCs and H-GMSCs are similar in immunophenotypes**

To determine the differences between RA-GMSCs and H-GMSCs in immunomodulatory phenotypes, RA-GMSCs and H-GMSCs cell surface markers were examined using flow cytometry. The results revealed that RA-GMSCs expressed all the markers such as CD90, CD73, CD39, CD44, CD29, CD105, HLA-DR, HLA-ABC, CD34, CD86, CD45 and CD31 that were similar to H-GMSCs (Fig. 1A). It has been previously reported that CD39-CD73 signaling is a key pathway for MSCs to execute their immune modulate capacity[20, 33]. Indeed, we have documented that H-GMSCs can suppress xeno-graft- versus-host disease[20], atherosclerosis[21], autoimmune arthritis[24], and lupus nephritis[26] via CD39-CD73 signaling pathway. We noted that RA-GMSCs also expressed high levels of CD39 and CD73 that were comparable to H-GMSCs (Fig. 1A).

In addition to CD39-CD73 signaling pathway, IDO, PD-1, COX-1, COX-2, ROR2, IL-10 and TGF-β pathways may also involve in immunosuppressive functions of MSCs[34, 35]. Therefore, we detected these molecule expressions in RA-GMSCs and H-GMSCs using RAN-seq. The results showed that both RA-GMSCs and H-GMSCs highly and similarly expressed IDO, PD-1, COX-1, COX-2, ROR2, IL-10 and TGF-β, hinting that RA-GMSCs may be capable of suppressing inflammatory responses and related diseases (Fig. 1B).

However, when the proliferation and migration capacities of RA-GMSCs and H-GMSCs were compared, we observed that RA-GMSCs had evidently lower proliferation rates and migration capacities than H-GMSCs.
(Fig. S1). Therefore, it is of vital to determine whether RA-GMSCs have immune regulatory functions.

**RA-GMSCs suppress T cell inflammatory responses and osteoclastogenesis in vitro**

Teff is important in initiating and maintaining RA progression[28, 36]. Also, Teff exacerbrates immune dysfunction and damage by secreting pro-inflammatory cytokines[37, 38]. Thus, we need to determine whether RA-GMSCs can suppress Teff proliferation *in vitro* using a GMSCs and murine T cell co-culture system. In brief, mouse splenic T cells were enriched using a nylon wood dropping method. The enriched T cells were labeled with CFSE and co-cultured with mitomycin devitalized antigen presenting cells (APCs) with anti-mouse CD3ε (0.05 µg/ml). T cells proliferation suppression was detected by adding RA-GMSCs or H-GMSCs into the established system. CFSE dilution rates were used to determine the results after 3 days of culture. As expected, RA-GMSCs strongly suppressed T cell proliferation with a dose-dependent effect that was highly similar to H-GMSCs (Fig. 2A).

In addition to Teff proliferation, TNF-α function profile is central to RA pathophysiology[39–41]. TNF-α promotes leukocyte activation, adhesion and migration, angiogenesis, and chemokine expression[42]. More importantly, TNF-α combines with RANKL (receptor activator of NFκB ligand) that contributes to the activation and effector function of osteoclasts[42]. We sought to investigate whether RA-GMSCs can suppress T cell TNF-α production *in vitro*. Similar to GMSCs and T cell co-culture system as described above, splenic enriched T cells were co-cultured with devitalized APCs plus anti-mouse CD3ε (1 µg/ml). RA-GMSCs or H-GMSCs were individually co-cultured in the system for 3 days. TNF-α level on CD4+T cells was detected using flow cytometry. Like H-GMSCs, RA-GMSCs effectively dampened TNF-α production from CD4+T cells with a dose-dependent effect (Fig. 2B).

During the pathogenesis of RA, osteoclastogenesis is another important pathologic factor which results in bone destruction and imbalance[43, 44]. To explore the possibility that RA-GMSCs suppress the osteoclastogenesis, CD11b+ cells were sorted from mice bone marrow and stimulated with M-CSF (50 ng/ml) for 3 days. Osteoclasts were induced in the presence of M-CSF (50 ng/ml) and RANKL (50 ng/ml) for another 6 days. RA-GMSCs or H-GMSCs were co-cultured in the system for the last 6 days, TRAP+ multinucleated cells were counted and significantly lower in both RA-GMSCs and H-GMSCs than that in the baseline group without GMSCs (Fig. 2C), suggesting that RA-GMSCs possess the similar capacity to H-GMSCs in dampening osteoclastogenesis. Collectively, these data document that RA-GMSCs are as effective as H-GMSCs in regulating Teff cell inflammatory responses and osteoclastogenesis.

**RA-GMSCs can alleviate pathologic severity and T cell immune responses in CIA model**

RA-GMSCs display their ability to inhibit Teff cell response and osteoclastogenesis *in vitro*, hinting they may have a therapeutic potential in experimental arthritis. To address this possibility, a collagen-induced arthritis (CIA) mice model, one of best models to mimic RA pathologic process was employed. Bovine type II collagen (CII) was emulsified with complete Freund’s adjuvant (CFA) and intracutaneously injected into DBA1/J mice. RA-GMSCs or H-GMSCs were intravenously transferred into CIA model at day 14 post
immunization. Mice were sacrificed and data were collected at day 60 (Fig. 3A). In fact, we and others have previously reported that GMSCs lack MHC-II and human GMSCs can be successfully transplanted into mice[21]. Primary Dermal Fibroblast (PDF) was utilized as control cells to exclude the non-specific role of GMSCs, due to their similar morphological characteristics but lack of immune regulatory function. As expected, both the infusion of RA-GMSCs and H-GMSCs significantly delayed the incidence of CIA, compared to PDF cells (Fig. 3B). Also, the clinic scores of RA-GMSCs and H-GMSCs treated mice were obviously decreased (Fig. 3C). The H&E, Toluidine Blue, and TRAP staining also revealed that both joint pathologic lesions, cartilage destruction and osteoclastogenesis were significantly reduced in the CIA mice that received RA-GMSCs or H-GMSCs, compared to mice received PDF cells (P < 0.01**) (Fig. 3, D). Thus, RA-GMSCs are therapeutic in CIA model by attenuating lymphocytes infiltration and protecting cartilage erosion.

The immunopathogenesis of RA is characterized by deficiency in the immune regulation and alteration of the peripheral immune tolerance of CD4 + T cells[12, 45]. Therefore, we also tried to understand whether RA-GMSCs can regulate CD4 + T cell immune response in CIA mice. Indeed, the frequency of CD4 + Foxp3 + regulatory T (Treg) cells were higher in CIA mice received RA-GMSCs than that in CIA mice, although the H-GMSCs therapy had a greater frequency of CD4 + Foxp3 + T regulatory cells (Fig. 4A). Interestingly, the frequency of TNF- + Treg cells in RA-GMSCs or H-GMSCs treatment CIA mice were significantly lower than that in CIA model mice (Fig. 4B). As TNF- expressed in Treg cells represents an unstable population of Treg cells in inflammatory milieu[36], thus, GMSCs may promote Treg stability to contribute to their therapeutic effect on experimental arthritis. We also examined frequency alterations of TNF- + subset and Th17/Th1 cells in RA-GMSCs or H-GMSCs treatment mice. We noted, compared to model group, the frequencies of TNF-+ T cells and Th17/Th1 cells in both RA-GMSCs and H-GMSCs treatment groups were significantly lower than that in the CIA mice (Fig. 4, C and D). Thus, RA-GMSCs display the potent ability to modulate T cell immune responses in CIA inflammatory milieu.

Bone loss caused by pro-inflammatory cytokines is a hallmark of RA [46, 47]. We next investigated bone changes in RA-GMSCs or H-GMSCs treatment CIA mice using micro-CT imaging as we previously reported[25, 48]. Interestingly, bone volumes in both CIA mice received RA-GMSCs or H-GMSCs were markedly higher than that in CIA mice received no cells (Fig. 4E). The long-term chronic inflammation may result in a permanent alternation to immune system and MSCs. We have noted that compared to H-GMSCs, the osteogenesis capacity of RA-GMSCs was obviously declined although the adipogenesis function of RA-GMSCs was no significantly changed (Fig. S2, A and B). We further confirmed the osteogenesis difference of RA-GMSCs and H-GMSCs using RAN-seq technology. Consistent with the osteogenesis results, the expression of bone morphogenetic protein 6 (BMP6) in RA-GMSCs was predominantly decreased compared to H-GMSCs (Fig. S2C). BMP6 is a member of the transforming growth factor β (TGFβ) superfamily, which is responsible for the in vivo bone-induction[49, 50]. Besides, BMP6 is responsible for cell proliferation and apoptosis[50]. This may explain the phenomena that RA-GMSCs had a slower proliferation compared with H-GMSCs (Fig. S1). However, RA-GMSCs retained osteoclast suppressive capacity during the long-term chronic inflammation environment in patients with RA. Taken together, RA-GMSCs are still effective in protecting bone from destruction in CIA mice.
RA-GMSCs suppress RA-FLSs tumor-like characteristics in vitro

A number of evidence indicated that inflammatory milieu activated RA-FLSs display many biological features as tumor cells, including high proliferation, migration and tissue invasion[51]. The increased RA-FLSs causes an invasive hyperplastic lining tissue mass known as a pannus[52]. Targeted on RA-FLSs may have a therapeutic role in RA and also a limited added effect on host defense[52]. We first determined whether RA-GMSCs can modulate RA-FLSs proliferation, migration and invasion. Experiments were conducted in vitro using cck8 kit and Transwell chamber system individually. Unlike PDF control cells, both RA-GMSCs and H-GMSCs similarly suppressed inflamed FLSs proliferation when co-cultured for 3 days (Fig. 5A). Moreover, both GMSCs markedly dampened inflamed FLSs migration with a dose-dependent effect (Fig. 5B). Moreover, both GMSCs were equally effective in hindering inflamed RA-FLSs invasion in a Matrigel incubated Transwell chamber system with a dose-dependent effect (Fig. 5C). Collectively, these data indicate that GMSCs from patients with RA maintain their capacity in suppressing RA inflamed FLSs tumor-like characteristic in vitro.

RA-GMSCs suppress RA-FLSs tumor-like characteristics in a humanized synovitis model

To further explore the functional activity of GMSCs on RA-FLSs in vivo, we then established a humanized synovitis model. The model was developed as we previously reported[31, 53, 54]. Fresh normal human cartilage was transplanted with absorbable gelatin sponge subcutaneously into the back of NOD SCID mice. After the insertion, RA-FLSs (5×10^5 cells) were injected into the sponge. RA-GMSCs or H-GMSCs (2×10^6 cells) were injected into the RA-FLSs transferred sponges. Mice were euthanatized at day 60, the implants were removed and fixed in 4% buffered formalin for H&E staining. Consistent with the in vitro results, both RA-GMSCs and H-GMSCs transplanted cartilages had a significant less RA-FLSs infiltration (Fig. 6A), lower invasion scores and cartilage degradation scores when compared to control mice (P < 0.01***) (Fig. 6B). Together, RA-GMSCs are also effective in attenuating RA-FLSs invasion in humanized synovitis model. Thus, we provide proofs that GMSCs derived from patients with RA can exert a therapeutic role in experimental arthritis by suppressing T cell immune response and inflamed FLSs tumor-like characteristic, and importantly, by protecting CIA mice from bone destruction.

Discussion

RA is a long-term refractory chronic autoimmune disease characterized by bone and cartilage destruction[1]. MSCs possess stem cell multipotency and robust immunomodulatory properties[26]. Therefore, MSCs had been considered as a potential approach in treating autoimmune, including RA[55–57]. We and others have previously reported that GMSCs showed excellent capacities in treating streptozotocin-induced T1DM[18], collagen-induced arthritis[19], xeno-graft-versus-host disease[20], atherosclerosis[21], experiential colitis[22], lupus nephritis[26], contact hypersensitivity[58], and allergic conjunctivitis[59]. As for the clinical application, safety has been considered as the major constraint[60]. To avoid the unpredictable safety risk, autologous MSCs from patients may be a better option. GMSCs
have been attested effective in treating CIA[19]. However, it is unclear whether autologous GMSCs from RA patients have an efficiency in alleviating RA pathologic process.

Here, we attested that administration of RA GMSCs is able to attenuates CIA process, a severe manifestation in patients with RA. To perform a systemic study, we employed a battery of in vitro and in vivo experiments to assess the potential capacities of RA-GMSCs in curing CIA model. We compared the immunophenotypes of RA-GMSCs and H-GMSCs using flow cytometry and qPCR technologies to initially evaluate the possible difference of them. Next, we used T cells and GMSCs co-culture system to further investigate the immunsuppressive capacities of RA-GMSCs. Osteoclast induction system was applied to assess the osteoclastogenesis suppressive function of RA-GMSCs in vitro. To ensure RA-GMSCs can exert suppressive function in vivo, we developed a CIA model using DBA1/J mice. Indicators, such as incidence, clinic scores, pathologic staining (H&E, Toluidine Blue, and TRAP), immune cell frequency change, and bone erosion using micro-CT, were used to evaluate disease treating capacity of RA-GMSCs. Moreover, we used a humanized synovitis model to assess whether RA-GMSCs can modulate RA FLSs tumor-like behaver in vivo. By combining this battery of experiments, we have established a scientific system to investigate the treatment ability of RA-GMSCs. Importantly, the established system can be applied to evaluate the capacities of other candidates in treating RA.

MSCs treatment have been accepted as a promising approach in treating autoimmune diseases because of their immunomodulatory properties. It is documented that BMSCs, UC-MSCs and AD-MSCs are therapeutic in RA patients by decreasing inflammatory markers and DAS28 scores[11–13]. However, not all of them are safe. For example, some BMSCs might have more problems than the solution in inflammatory rheumatic diseases [11]. UC-MSCs and AD-MSCs are difficult with harvest and slow growth, although they are safer than BMSCs, hindering their clinical applications[18]. Differing from other tissue derived MSCs, GMSCs can be obtained easily, proliferate faster, and low carcinogenicity[14], making them superior to other tissues-derived MSCs in treating the autoimmune diseases[61]. Moreover, we have recently reported that GMSCs are highly safe in various animals including mouse, rat, dog and monkeys[62]. Thus, GMSCs could be a potential candidate for treating incurable AIDs.

Autologous tissue derived MSCs are safe and well tolerated in treating AIDs, but not all of them are effective in attenuating the disease in RA patients. For instance, autologous BMSCs failed to alleviate the disease development in RA patients[63]. In the current study, we reported that RA-GMSCs reserved immunomodulatory capacity in vitro, and importantly in CIA and humanized synovitis models, indicating that RA-GMSCs may be applicable in treating RA patients. Notably, RA-GMSCs showed impaired osteogenesis function caused by the declined expression of BMP6. The phenomenon may be caused by the long-term exposure to chronic inflammatory milieu of RA-GMSCs. Thus, understanding the molecular mechanisms will further to explore the underlying targets to protect RA patients from bone destruction.

Conclusion
In summary, we provided a battery of scientific proofs that RA-GMSCs shared the similar immunophenotypes to H-GMSCs. Furthermore, RA-GMSCs can suppress T cell immune responses and osteoclastogenesis in vitro. Importantly, RA-MGSCs can exert a therapeutic role in treating CIA and humanized synovitis models. Taken together, these data indicate that the administration of autologous GMSCs may have a promising therapeutic role in patients with RA.

**Abbreviations**

MSCs: mesenchymal stem cells; GMSCs: gingiva derived MSCs; RA-GMSCs: GMSCs derived from patients with RA; H-GMSCs: GMSCs derived from health donors; RA: rheumatoid arthritis; CIA: collagen induced arthritis; RA-FLSs: fibroblast-like synoviocytes of RA; NSAIDs: non-steroidal anti-inflammatory drugs; DMARDs: disease modifying anti-rheumatic drugs; Treg: regulatory T cells; Ths: T helper cells; BMSCs: bone marrow derived MSCs; UC-MSCs: umbilical cord-derived MSCs; AD-MSCs: adipose tissue derived MSCs; AIDs: autoimmune diseases; Teff: Effector T cells; CFSE: carboxyfluorescein succinimidyl ester; APCs: antigen presenting cells; RANKL: receptor activator of NF-κB ligand; TRAP: tartrate-resistant acid phosphatase; CFA: complete Freund's adjuvant; PDF: Primary Dermal Fibroblast; BMP6: bone morphogenetic protein 6; OCPs: osteoclast precursors.

**Declarations**

**Acknowledgments**

This study was supported by grants from the National Key R&D Program of China (2017YFA0105800 to S.G.Z), National Science Funds of China (81871224 and 82371817 to S.G.Z, 81960293 to Y.L and 81870481 to H.X), Natural Science Foundation of Gansu Province (20JR5RA3 to Y.L), Guangdong Basic and Applied Basic Research Foundation (2023A1515010002 to W.W), and Guangzhou Basic and Applied Basic Research Foundation (202201011085 to W.W).

**Ethics approvals:**

The donors of Human gingiva tissue samples had agreed the content of informed consents form in this project. The protocols were approved by the Institution Review Boards of The third affiliated hospital of Sun Yat-sen University (IRB: 201809). The title of this project was “Clinical study of human gingival mesenchymal stem cells treating rheumatoid arthritis”. Date of this approval was March 30th 2018.

All mice used in this project originated from Jackson Laboratory and maintained in specific pathogen-free (SPF) animal facility of Guangdong laboratory animals monitoring institute. Animal works were complied by National Institutes of Health (NIH) guidelines for the use of experimental animals. All protocols were approved by the Institution Review Boards of Guangdong laboratory animals monitoring institute (IRB:IACUC2019022). The title of this project was “Clinical study of human gingival mesenchymal stem cells in treating autoimmune inflammatory diseases”. Date of this approval was April 19th 2019.
Contributions

SGZ initiated the study, designed the experiments and wrote the paper. YH, DZ, XZ, YL, JD, WW, YX, JZ, SZ, JH, JY, SW, JW and ZC performed most of the experiments and statistical analyses. HX and SGZ edited the Manuscript.

Data sharing statement

Data available upon request. Correspondence and requests for materials should be addressed to SGZ(Song.Zheng@shsmu.edu.cn).

Declaration of interests

The authors declare that they have no competing interests.

References


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**Figures**
Figure 1

**RA-GMSCs share similar immunophenotypes with H-MGSCs.** RA-GMSCs or H-GMSCs from P3-P6 were prepared for surface marker detection using flow cytometry. (A) The flow data showed the percentages of surface markers associated to MSCs identification. (B) IDO, COX-1, COX-2, ROR2, IL-10 and TGF-β levels in RA-GMSCs or H-GMSCs were compared using RNA-seq data.
RA-GMSCs are effective in regulating T cell inflammatory responses and osteoclastogenesis.

RA-GMSCs or H-GMSCs were co-cultured in the established T cell proliferation, TNF-α secretion and osteoclastogenesis induction systems respectively. (A) GMSCs suppressed T cell proliferation. Fresh T cells were labeled with CFSE (1 μM) and co-cultured with GMSCs in the presence (baseline) of anti-mouse CD3ε (0.05 μg/ml). T cell proliferation was determined by the CFSE dilution ratios after 72 hours of culture. T cell proliferation summary data from GMSCs co-culture systems were shown. (B) Splenic enriched T cells were co-cultured with mitomycin devitalized APCs in the presence of anti-mouse CD3ε (1 μg/ml). RA-GMSCs or H-GMSCs were individually co-cultured in the system for 3 days. Representative FACS plots (left) and summary data (right) were shown. (C) CD11b+ cells were sorted from mice bone marrow and stimulated with M-CSF (50ng/ml) for 3 days. Osteoclasts were induced in the presence of M-CSF (50ng/ml) and RANKL (50 ng/ml) for another 6 days. RA-GMSCs or H-GMSCs were co-cultured in the system for the last 6 days, osteoclasts were detected using TRAP kit. The representative images (left) and summary data (right) were shown. The data indicate the Mean ± S.E.M of three independent experiments (n=3, *P<0.05, **P<0.01. NS, not significant. RA-GMSCs versus H-GMSCs at the same ratio).
Figure 3

RA-GMSCs alleviate pathologic severity in CIA model.

Bovine type II collagen (CII, 2mg/mL) was emulsified with Complete Freund's adjuvant (CFA) at a ratio of 1:1. And then, the emulsified mixture was injected intradermally into the tail of DBA1/J mice. Mice were divided into different groups randomly, with or without a single intravenous injection of 2 × 10⁶ RA-GMSC or H-GMSCs at day 14 after immunization. Mice were sacrificed and data were collected at day 60. (A) The experimental schedule was shown. (B-C) The disease incidences and clinical scores were shown. (D) The H&E, Toluidine Blue, and TRAP staining showed joint pathologic lesions, cartilage destruction and osteoclastogenesis degree in the CIA mice that received RA-GMSCs or H-GMSCs. The representative images (left) and summary data (right) of two independent experiments were shown. n=5 in each experiment. The data indicate the Mean ± S.E.M (**P<0.01, ***P<0.001. NS, not significant).
Figure 4

RA-GMSCs are potent in suppressing T cell immune responses and bone erosion in CIA inflammatory milieu.

The Treg cells, TNF-α+ T cells, Th17 cells and Th1 cells populations were detected using flow cytometry at the endpoints of experiments. (A-B) Treg (FoxP3+) and TNF-α+ Treg cells were shown. Representative FACS plots (left) and summary data (right) were shown. (C-D) Th17 (IL-17a+)/Th1 (IFN-γ+) ratios and TNF-α+ T cells were shown. Representative FACS plots (left) and summary data (right) were shown. (E) Bone resorption in RA-GMSCs or H-GMSCs treated CIA mice using micro-CT imaging. The representative
images (left) and summary data (right) of two independent experiments were shown. n=5 in each experiment. The data indicate the Mean ± S.E.M (**P<0.01, ***P<0.001. NS, not significant).

Figure 5

RA-GMSCs suppress RA FLSs tumor-like behaviors in vitro.

RA FLSs were isolated from RA patients. GMSCs were co-cultured with RA FLSs for proliferation, migration and invasion detections using the established systems. (A) RA FLSs proliferation was detected using the cck8 kit. Summary data from GMSCs co-culture systems were shown. (B-C) The migration or invasion abilities of RA FLSs were detected using Trans-well chambers. For migration assay, RA FLSs (8000 cells/well) were seeded in the upper chambers with serum-free DMEM. DMEM containing 10% FBS was used as a chemoattractant added in the lower wells. For the invasion assay, matrigel basement membrane matrix was coated in the chambers according to the manufacturer’s instructions before RA FLSs planted. RA-GMSCs or H-GMSCs were planted in the lower wells at the ratios of 1:1-1:100 for 3 days. The representative images (left) and summary data (right) were shown. The data indicate the Mean
± S.E.M of three independent experiments (n=3, *P<0.05, **P<0.01. NS, not significant. RA GMSCs versus H GMSCs at the same ratio).

Figure 6

RA-GMSCs protect cartilage from RA FLSs invasion in humanized synovitis model.

Freshed isolated normal human cartilages were transplanted with absorbable gelatin sponge subcutaneously into the back of NOD-SCID mice. After the insertion, RA FLSs (5×10^5 cells) were injected into the sponge. RA-GMSCs or H-GMSCs (2×10^6 cells) were injected into the RA FLSs transferred sponges. Mice were euthanatized at day 60, the implants were removed and fixed in 4% buffered formalin for H&E staining. The representative images (A) and summary data (B) of two independent experiments were shown. n=5 in each experiment. The data indicate the Mean ± S.E.M (**P<0.01, ***P<0.001. NS, not significant).

Supplementary Files

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