Allogeneic mesenchymal stem cell and mesenchymal stem cell-differentiated chondrocyte suppress the responses of type II collagen-reactive T cells in rheumatoid arthritis

Z. H. Zheng, X. Y. Li, J. Ding, J. F. Jia and P. Zhu

Objective. Rheumatoid arthritis (RA) is a T-cell-mediated systematic disease and is usually accompanied by articular cartilage damage. In the present study, we explored the effects of bone marrow-derived mesenchymal stem cells (MSCs) and MSC-differentiated chondrocytes (MSC-chondrocytes) on the responses of antigen-specific T cells in RA to type II collagen (CII) to evaluate the potential therapeutic value of MSCs in RA treatment.

Methods. The effects of both MSCs and MSC-chondrocytes on the proliferation, activation-antigen expression (CD69 and CD25) and cytokine production [interferon-γ (IFN-γ), interleukin (IL)-10 and IL-4] of CII-reactive T cells in RA patients were investigated with the stimulation of CII or otherwise. CD3/annexin V staining was used to evaluate T-cell apoptosis in the inhibition. The role of transforming growth factor-β1 (TGF-β1) underlying the inhibition was also investigated.

Results. MSCs failed to elicit positive responses of CII-reactive T cells, whereas they significantly suppressed CII-stimulated T-cell proliferation and activation-antigen expression in a dose-dependent fashion without inducing T-cell apoptosis. The inhibition was observed even after MSCs were added as late as 3 days after the initiation of stimulation. Moreover, MSCs inhibited both CD4+ and CD8+ T cells from producing IFN-γ and TNF-α, while they up-regulated the levels of IL-10 and restored the secretion of IL-4. TGF-β1 was confirmed to play a critical role in the inhibition. Throughout our study, MSC-chondrocytes shared similar properties with MSCs.

Conclusion. Both MSCs and MSC-chondrocytes suppressed CII-reactive T-cell responses to CII in RA, which suggested that MSCs could be a potential candidate for RA treatment in future if further confirmed in vivo.

Key WORDS: Mesenchymal stem cells, Type II collagen, T lymphocyte, Rheumatoid arthritis, Immunosuppression.

Introduction

Bone marrow (BM)-derived mesenchymal stem cells (MSCs) are multipotential cells present in the BM at a low frequency (1/10^5) and are capable of differentiating into chondrocytes, osteocytes, myocytes and adipocytes under appropriate culture conditions, both in vitro and in vivo [1–6]. The facility for isolation and expansion of MSCs in vitro without losing their phenotype or multi-lineage potential has made the use of MSCs for tissue engineering and repair medicine burgeon in the last 10 yrs.

Further interest in the clinical application of MSCs has been generated by the observation that MSCs can exert profound immunosuppression by inhibiting T-cell activities in vitro [7–10]. This property has been exploited in vivo by i.v. injecting of MSCs to major histocompatibility complex-mismatched baboons, which resulted in the prolonged survival of an allogeneic skin implant in recipient baboons [11]. A recent case even reported that systemic infusion of MSCs suppressed a grade IV graft-versus-host disease (GVHD) in a 9-yr-old child who had received a BM transplant [12]. On the basis of these findings, some researchers employed MSCs to treat experimental autoimmune encephalomyelitis (EAE) and alleviated this T-cell-mediated auto-immune disease in an animal model [13].

Rheumatoid arthritis (RA) is another T-cell-mediated auto-immune disease. Both antigen-activated CD4+ T helper 1 (Th1) and CD8+ T cells were reported to be involved in RA pathogenesis [14–18]. Type II collagen (CII), a major component of hyaline cartilage acting as an auto-antigen in RA, has been well established [19, 20]. When triggered by antigenic peptides, T cells stimulate monocytes, macrophages and synovial fibroblasts to produce interleukin (IL)-1, IL-6 and tumour necrosis factor-α (TNF-α) and to secrete matrix metalloproteinases by secreting pro-inflammatory cytokines such as interferon-γ (IFN-γ) and TNF-α [15, 21] as well as cell-surface signalling CD69 and CD11 [22]. Activated T cells also expressed osteoclastogenesis-stimulating osteoprotegerin ligands [23] and stimulated B cells to release immunoglobulins [24]. All of these contribute to the propagation of the systemic immunological disorder, eventually resulting in the destruction of joints [25].

Many therapeutic strategies for RA treatment have been developed that aim at inhibiting antigen-reactive T cell activities and antagonizing pro-inflammatory cytokines [26]. Three anti-TNF-α drugs (etanercept, infliximab and adalimumab) have been proven to be effective and safe in RA treatment [27]. Administration of anti-inflammatory cytokines IL-4 and IL-10 has also been reported as an effective approach to protect against arthritis [28, 29]. But so far, there is no one approach that can both alleviate the arthritis through inhibiting T-cell activation and have reparative capacity in repairing the destructed cartilage.

In this regard, we explored the therapeutic potentials of MSCs in RA in this study. CII was used as a stimulus and the effects of MSCs on the responses of CII-reactive T cells from both peripheral blood (PB) and synovial fluid (SF) of RA patients were evaluated. We also investigated whether MSC-differentiated chondrocytes (MSC-chondrocytes) possess the same immunological properties as MSCs to explore their cartilage-repairing potentiality in the inflammation-infiltrated RA joints for preventing cartilage re-destruction, which has not been well-studied.

Materials and methods

Culture of MSCs

For MSC isolation, 10–20 ml heparinized BM aspirates were collected from the iliac crests of five healthy donors (three men...
and two women, aged 20–35 yrs) following informed consent, which had also been approved by the Ethics Committee at Xijing Hospital. MSCs were cultured as previously described [1]. Briefly, mononuclear cells were isolated from Percoll-separated BM and re-suspended in medium consisting of Dulbecco’s modified Eagle’s medium-low glucose (DMEM-LG; Gibco-BRL, Life Technologies, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin. Mononuclear cells were plated at 10^5 cells/75 cm^2 in flasks (Corning, Acton, MA, USA), and the cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. When cultures reached 90% confluence, cells were trypsinized (0.05% trypsin without EDTA) and replated at 5 x 10^5 cells/75 cm^2 in flasks. After two passages, the cells were harvested and stained with fluorescein-conjugated monoclonal antibody against CD14, CD29, CD34, CD44, CD45, CD80, CD86, CD106, HLA-ABC and HLA-DR (BD Pharmingen, San Diego, CA, USA), CD90 and CD105 (Serotec, Oxford, UK) followed by analysing with flow cytometry (FACSort; BD, San Jose, CA, USA). The capacity of MSCs that differentiate along adipogenic and osteogenic lineages was also evaluated as previously described [1].

**Preparation of MSC-differentiated chondrocytes**

To promote chondrogenic differentiation, 10^5 mesenchymal cells were centrifuged in a 15 ml polypropylene tube, and the pellets were cultured in chondrogenic media as previously described [1]. Three weeks later, pellets were harvested and sections of paraffin-embedded pellets were stained with toluidine blue for identification. Then, pellets were minced and washed twice in Hanks’ balanced salt solution (HBSS). MSC-chondrocytes were released by overnight digestion at 37°C in FBS-free DMEM-LG medium consisting of 0.2% crude type IV collagenase (Gibco-BRL) and collected by density gradient centrifugation on Ficoll-Hypaque (1.077 g/ml; Sigma, St Louis, MO, USA). Cell viability was 95% by trypan blue exclusion.

Properties of chondrocytes have been reported to be invariably lost during monolayer culture, which is referred to as de-differentiation [30]. To determine the influence of 5 days of monolayer culture in our study on MSC-chondrocytes, immunostaining for CII were performed in monolayer-cultured MSC-chondrocytes using mouse anti-human CII (NeoMarkers, Fremont, CA, USA) followed by fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Sigma) as previously described [31].

**Patients**

Informed consents were obtained from 37 RA patients (5 men and 32 women) who met the 1987 revised criteria of the American College of Rheumatology. Ethics approval was granted for this study by the Ethics Committee at Xijing Hospital. The mean age of the RA patients was 51.7 yrs and the mean disease duration was 36.1 months (range 5.0–50.3 months). All the patients involved had received no treatment or were treated only with non-steroidal anti-inflammatory drugs and had clinically active synovitis at the time of arthrocentesis. Paired samples of PB and SF were obtained after a 48 h medication washout period. According to the previous methods [22], we tested the T-cell proliferation response to CII in these RA patients and found that 18 of the 37 RA patients (48.6%) had positive T-cell responses to CII in both PB and SF, and only these T cells that responded positively to CII were involved in the study. T-cell proliferative responses were considered positive if the stimulation indices (SIs) were >2. SI was calculated as the ratio of counts per minute (c.p.m.) in the presence of antigens to c.p.m. without antigens.

**Cell isolation**

Heparinized PB was collected under sterile conditions and diluted 1:1 with DMEM-LG. SF from RA patients with knee joint effusions was collected by arthrocentesis into sterile tubes and diluted to 1:5 with HBSS. Mononuclear cells in PB (PBMCs) and SF (SFMCs) were isolated by density gradient centrifugation on Ficoll-Hypaque (1.077 g/ml). Cell viability was 95% by trypan blue exclusion. Mononuclear cells were then separated immunomagnetically into T cells and non-T cells using anti-CD3 high-gradient microbeads (Miltenyi Biotec, Auburn, CA, USA). Non-T cells were used as antigen presenting cells (APCs).

**Preparation of CII**

Lyophilized native chicken CII (Chondrex, Inc., Redmond, WA, USA) was dissolved in 0.1N acetic acid at 1 mg/ml, dialysed against 50 mM Tris, 0.2M NaCl and then sterilized by filtering through a 0.2μm micropore filter. According to our previous study, the optimal final concentration of CII that we used was 400 μg/ml [32].

**Proliferation assay**

Non-T cells, MSCs and MSC-chondrocytes were all irradiated (30 Gy) before being cultured with T lymphocytes as previously described [8]. Each culture was performed in triplicate at 2 x 10^5 cells/well for T cells in 96-well round-bottom microtitre plates (Corning) in a total volume of 0.2 ml DMEM-LG supplemented with 10% FBS. Non-T cells, acted as APCs, were mixed with T-cell at the ratio of 1:1. Then, MSCs or MSC-chondrocytes were added to the plates at different ratios to T cells (0.1:1, 0.2:1 and 1:1, respectively) with the stimulation of CII or otherwise. The group in which T cells were cultured only with non-T cells served as negative controls. The plates were incubated in a humidified atmosphere of 5% CO2 at 37°C for 5 days. MSCs or MSC-chondrocytes were added on day 3 (1:1 to T cells) to the 5-day-old culture to explore the effects of MSCs and MSC-chondrocytes on T cells when they were added late.

Twelve hours before the end of culture, 1 μCi of 3H-thymidine (NEN Life Science Products, Boston, MA, USA) was added to each well. Cells were harvested onto nitrocellulose, and the radioactivity incorporated was counted in a scintillation counter. The T-cell proliferation was represented as the incorporated radioactivity in c.p.m. and the results were expressed as c.p.m. ± s.d. of the mean. The inhibition capacity was calculated by the following formula: 1 – (proliferation of CII-stimulated T cells in the presence of MSCs or MSC-chondrocytes) (c.p.m.)/(proliferation of T cells cultured with CII alone) (c.p.m.) x 100%. All experiments in our study including the following study were performed independently at least three times for each point described.

**Activation assay**

T-cell activation assays were performed in 24-well round-bottom plates (Corning) in a total volume of 1 ml DMEM-LG. T cells and non-T cells were added, respectively, at 1 x 10^5 cells/well. MSCs or MSC-chondrocytes were mixed at different ratios (0.1:1, 0.2:1 and 1:1, respectively) with the stimulation of CII or otherwise. The concentrations of CII used were 25 μg/ml (C11) for 5 days. MSCs or MSC-chondrocytes were mixed with 10% FBS. Non-T cells, acted as APCs, were mixed with T-cell at the ratio of 1:1. Then, MSCs or MSC-chondrocytes were added to the plates at different ratios to T cells (0.1:1, 0.2:1 and 1:1, respectively) with the stimulation of CII or otherwise. The group in which T cells were cultured only with non-T cells served as negative controls. The plates were incubated in a humidified atmosphere of 5% CO2 at 37°C for 5 days. MSCs or MSC-chondrocytes were added on day 3 (1:1 to T cells) to the 5-day-old culture to explore the effects of MSCs and MSC-chondrocytes on T cells when they were added late.

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**Cytokine quantification**

After 5 days of co-culture (ratios of MSCs and MSC-chondrocytes to T cells, 1:1) with or without CII-stimulation, fresh supernatants were collected. Quantitative analyses of IL-4, IL-10, IFN-γ and TNF-α production were performed by enzyme-linked immunoassorbent assay (ELISA) using commercially available kits (R&D Systems, Minneapolis, MN, USA). Supernatants of MSCs,
MSC-chondrocytes and T cells that were cultured alone served as controls. The detection limits were 3 pg/ml for IL-4, 15 pg/ml for IL-10, 4 pg/ml for IFN-γ and 7 pg/ml for TNF-α.

Intracellular cytokine assay
To further identify whether MSCs and MSC-chondrocytes selectively exert their inhibitory effects on T-cell subsets, intracellular cytokines secreted by CD4+ and CD8+ T cells were detected. MSCs or MSC-chondrocytes were added at 1:1 ratio to T cells in 24-well round-bottom plates. We activated T cells with CII for 12 h before analysis. During the last 4 h of the 12 h stimulation, Brefeldin A (Sigma) was added to the culture to block cytokine secretion. After being harvested, the cells were stained with peridinin chlorophyll protein (Percp)-labelled CD4 or CD8 monoclonal antibody and FITC or phycoerythrin (PE)-conjugated monoclonal antibodies against IL-4/IFN-γ, IL-10 and TNF-α (BD Pharmingen). Then, the cells were analysed by flow cytometry within CD4+ and CD8+ populations.

Apoptosis evaluation
The proportions of apoptotic T cells were evaluated after 5 days of co-culture (1:1 to MSCs and MSC-chondrocytes, respectively) to investigate the effects of MSCs and MSC-chondrocytes on T-cell apoptosis. According to the previously described method [33], T cells were double-stained with PerCP-labelled CD3 monoclonal antibody and FITC-labelled annexin V. After being counterstained with propidium iodide (PI), cells were analysed by flow cytometry and the total annexin V-positive cells were analysed within CD3+ populations.

Determination of the influences of transforming growth factor-β1 (TGF-β1) on the inhibition
TGF-β1 is well known to be the most potent inhibitor of lymphocyte function and can be secreted by MSCs. To explore whether MSCs still secreted TGF-β1 after differentiation into chondrocytes, both fresh supernatants of irradiated MSCs and MSC-chondrocytes (1 × 10⁶ cells/well in 24-well plates in 1 ml FBS-free DMEM-LG with the stimulation of CII or otherwise) were collected after 3 days of culture followed by assaying with an ELISA kit (Quantikine TGF-β1, R&D Systems). The detection limit of the kit was 7 pg/ml.

Then, we further identified TGF-β1 responsible for the inhibitory effects of MSCs and MSC-chondrocytes. In brief, rhTGF-β1 (R&D System; 500 ng/ml) was added to the culture containing only T cells and non-T cells (1:1) with the stimulation of CII to observe its effects on the T-cell proliferation, activation and cytokine secretion, as described earlier in this article. As controls, monoclonal antibody anti-rhTGF-β1 (R&D System; 1 µg/ml) as added to the CII-stimulated culture in the presence of MSCs and MSC-chondrocytes (the ratios of MSCs and MSC-chondrocytes to T cells were 1:1). Furthermore, the time course of suppression exerted by TGF-β1, MSCs and MSC-chondrocytes was also evaluated, respectively, by observing their effects on CII-stimulated T-cell proliferation during 5 days of culture.

Statistics
Results were expressed as mean ± s.d. of the mean. Differences between experimental conditions were analysed by t-test (paired when possible). P-value <0.05 was considered statistically significant.

Results
Culture and differentiation of MSCs
MSCs were successfully isolated and expanded from all the five donors. These populations were uniformly negative for the expression of CD14, CD34, CD45, CD80, CD86 and HLA-DR but expressed as CD29, CD44, CD90, CD105, CD106 and HLA-ABC (data not shown). MSCs also retained the capacity to differentiate into osteocytes and adipocytes (Fig. 1A and B). After 3 weeks of induction, the pellets of MSCs showed a chondrogenic morphology when stained with toluidine blue (Fig. 1C). MSC-chondrocytes still expressed CII during 5 days of monolayer culture (Fig. 1D), suggesting that MSC-chondrocytes still retained some properties of chondrocytes and would not dedifferentiate into MSCs in short-term culture in our study.

MSCs and MSC-chondrocytes failed to elicit T-cell proliferation
T cells isolated from RA patients responded positively to CII in both PB (mean c.p.m. 5240 vs 2491, P = 0.003) and SF (mean c.p.m. 8560 vs 2935, P = 0.001). Compared with the control in which T cells were cultured only with non-T cells, no significant proliferation of T cells was observed against allogeneic MSCs and MSC-chondrocytes, even when MSCs and MSC-chondrocytes were added to T cells at the ratio of 1:1, demonstrating that allogeneic MSCs and MSC-chondrocytes were not recognized by antigen-specific T cells from RA patients (Fig. 2A).

MSCs and MSC-chondrocytes inhibited CII-reactive T-cell proliferation
At the ratio of 1:1, CII-stimulated T-cell proliferation was significantly inhibited by allogeneic MSCs [85.9±4.2% for T cells from PB (P<0.001), 84.4±3.8% for T cells from SF (P<0.001)] and MSC-chondrocytes [80.5±4.7% for T cells from PB (P<0.001), 81.1±4.6% for T cells from SF (P<0.001)]. The inhibition was dose dependent when the ratios of MSCs and MSC-chondrocytes to T cells decreased from 0.2:1 to 0.1:1 (Fig. 2B). In addition, T-cell proliferation was also significantly inhibited by the delayed addition of MSCs [64.4±5.3% for T cells from PB (P=0.001), 69.6±7.9% for T cells from SF (P=0.001)] and MSC-chondrocytes [55.2±6.4% for T cells from PB (P=0.002), 56.9±8.6% for T cells from SF (P=0.001)] (Fig. 2C). MSCs derived from the five different donors all possessed inhibitory effects on CII-reactive T cells, though the inhibitory capacity was different (ranging from 73.6±6.2% to 95±7.9% for T cells from PB, 75.1±7.4% to 94±8.1% for T cells from SF, at 1:1 ratio to T cells, data not shown). However, MSCs and MSC-chondrocytes derived from the same donor exhibited similar inhibitory capacity at the same ratio to T cells (Fig. 2B and C).

MSCs and MSC-chondrocytes suppressed CII-reactive T-cell activation
Allogeneic MSCs and MSC-chondrocytes did not stimulate CD3+ T cells to increasingly express activation antigens CD69 and CD25 (IL-2 receptor), further suggesting that they could escape the recognition by CII-reactive T cells. When added to the cultures in the absence of MSCs or MSC-chondrocytes, CII significantly up-regulated both the percentages and mean fluorescent intensity (MFI) of activation-antigens CD69 and CD25 on CII-reactive T-cell surface, which is consistent with our previous study [32] (data not shown). The addition of MSCs and MSC-chondrocytes resulted in dose-dependent inhibitory effects on the MFI of CD69 and CD25, with a peak at 1:1 ratio (to T cells) and with a peak at 1:1 ratio (to T cells from both PB and SF: P<0.01 in the presence of both MSCs and MSC-chondrocytes, Fig. 3). However, any doses of MSCs and MSC-chondrocytes in our study (0.1:1, 0.2:1, 1:1 to T cells) could not down-regulate the percentages of CD69 and CD25 on T cells until the ratios of MSCs and MSC-chondrocytes to T cells increased higher than 10:1 (data not shown). All these data suggested that the reduction of T-cell activation specifically
required the abundant presence of MSCs and MSC-chondrocytes (Fig. 3).

**MSCs and MSC-chondrocytes regulated cytokine production by CII-reactive T-cell**

IL-4, IL-10, IFN-γ and TNF-α were not detected in the supernatants of MSCs and MSC-chondrocytes whether with the stimulation of CII or not. Allogeneic MSCs and MSC-chondrocytes also did not stimulate T cells to produce pro-inflammatory cytokines IFN-γ and TNF-α compared with the control in which T cells were cultured only with non-T cells, whereas MSCs and MSC-chondrocytes significantly suppressed CII-stimulated T cells from producing pro-inflammatory cytokines IFN-γ (for T cells from both PB and SF: \(P < 0.01\), Fig. 4A) and TNF-α (for T cells from both PB and SF: \(P < 0.01\) Fig. 4B). With the stimulation of CII, the levels of IL-4 significantly decreased as previously reported (data not shown) \([15]\), but the decreasing IL-4 was significantly restored by MSCs and MSC-chondrocytes (for T cells from both PB and SF: \(P < 0.01\), Fig. 4C). As for IL-10, whether simulated by CII or not, it was significantly elevated by MSCs and MSC-chondrocytes (for T cells from both PB and SF: \(P < 0.01\), Fig. 4D).

**MSCs and MSC-chondrocytes exerted suppressive effects through TGF-β1**

For all the five donors, MSC-chondrocytes secreted equivalent quantities of TGF-β1 to that of MSCs (mean 2152.94 pg/ml vs 2215.43 pg/ml, \(P = 0.495\); Fig. 7A) even with the stimulation of CII (data not shown). When added to the co-culture, commercially available rhTGF-β1 alone still suppressed the proliferation, activation and production of IFN-γ and TNF-α by CII-stimulated T cells (from both PB and SF) while promoting these T cells to secrete IL-10 and restoring their abilities to produce IL-4 to the same extent as MSCs and MSC-chondrocytes did. In addition, neutralizing the monoclonal antibody anti-rhTGF-β1 partly prevented MSCs and MSC-chondrocytes from exerting suppressive effects (Table 1, a representative result, in which T cells were...
derived from SFMCs). These data suggested that TGF-β1 may play an important role in the inhibition, which was further confirmed by the time course of suppression as shown in Fig. 7B (a representative result in which T cells were derived from SFMCs). Compared with the control that T cells stimulated only with CII, rhTGF-β1 significantly suppressed T-cell proliferation from the beginning of the culture while MSCs exerted significantly inhibitory effects until 3 days later after the initiation of stimulation. However, at the end of the culture, MSCs showed higher inhibitory ability than that of rhTGF-β1 (mean c.p.m. 3703 vs 4932, \( P = 0.012 \)), which might be associated with the accumulation of TGF-β1 secreted persistently by MSCs in culture. Though not shown in Fig. 7B, MSC-chondrocytes exhibited similar properties as MSCs.

Discussion

In this report, we demonstrated that MSCs could exert profound suppressive effects on CII-reactive T cells from RA patients without inducing T-cell apoptosis. We were also glad to observe that MSCs could, exert inhibitory effects even after differentiating into chondrocytes. As T cells are believed to play a critical role in orchestrating the inflammatory response in RA patients and suppression of T-cell activation is of major importance in RA treatment, MSCs could be used as a more intensive immunosuppression approach in the treatment of RA based on our results. In our study, MSCs were found to exert inhibitory effects on T cells through various ways: they directly inhibited the T-cell proliferation and the expression of CD69, suppressed Th1/Tc1 cells in secreting IFN-γ and TNF-α, thus inhibiting the activities of other inflammatory cells and the
production of other pro-inflammatory cytokines, in sequence, arresting the process of inflammation and alleviating arthritis. On the other hand, MSCs had also been found to elevate the secretion of IL-10 and reverse the low-level IL-4 secreted by CII-reactive T cells, which enhanced the anti-inflammation effect of MSCs. It is well known that both IL-4 and IL-10, two anti-inflammatory cytokines, are found to lower production in RA patients [34, 35], and the elevation of these two cytokines could be helpful for suppressing T-cell activation and inhibiting those pro-inflammatory cytokines production, thus alleviating arthritis and preventing cartilage damage in RA [36–38]. IL-10 has even been reported to have reversed the cartilage degradation [37, 39]. Delayed addition of MSCs still maintained their inhibition capacity, suggesting that the transplantation of these cells during the development of RA may be practicable and effective.

Besides systematical therapy, articular cartilage repair is another important aspect of RA treatment. Although MSC-based tissue engineering has obtained satisfactory results in repairing the articular cartilage defects in osteoarthritis (OA) models [6], there have been few studies on the potentials of MSC-chondrocytes in articular cartilage repair of RA. It is well known that OA has a different pathogenesis from that of RA and that cartilage defects in OA result from degeneration but not from inflammation. One difficulty in cartilage repair in RA joints is that regenerated cartilage would be re-destructed in inflammatory context of RA joints. Our findings that MSC-chondrocytes possessed the same immunological properties as those of MSCs suggest that when implanted into RA joints, MSC-chondrocytes might suppress inflammatory factors and prevent re-destruction while exerting their repairing effects.

Autologous stem cell transplantation has been used to treat some auto-immune diseases [40]. However, RA patients derived stem cells are recently considered to have some functional defects [41, 42]. Then, allogeneic healthy MSCs naturally become an optimal substitute in therapy for they could be ‘ignored’ by recipients even after differentiation, as observed in our study, which might be due to the negative expression of MHC class II molecules as well as co-stimulatory molecules such as CD80 and CD86 [43, 44]. The safety of transplantation is further enhanced by our observation that allogeneic MSCs will not elicit T-cell apoptosis while exerting inhibitory effects.

As far as the mechanism underlying the immunosuppression is concerned, it is conflicting and remains to be clarified. However, we still identified that TGF-β1 played an important role in the inhibition by observing that rhTGF-β1 exerted similar inhibitory effects as MSCs and MSC-chondrocytes did, while MSCs-
MSC-chondrocytes-induced inhibition was partly antagonized by anti-TGF-β1 monoclonal antibody. In addition to further confirming the role that TGF-β1 played in the inhibition, our data about the time cause of the suppression also suggested that MSCs may be used as a more potent therapeutic approach than the single application of rhTGF-β1 in RA treatment, for they could persistently secrete TGF-β1. After all, previous study has been reported that systemic administration of TGF-β1 to mice inhibits collagen-induced arthritis (CIA) [45]. Throughout our study, MSC-chondrocytes shared the same inhibitory capacity with MSCs including TGF-β1 production, indicating that the conditional differentiation might not impair the immunological properties of MSCs.

However, efficient engraftments are prerequisites for stromal cell-based therapy. We found that MSCs and MSC-chondrocytes exerted inhibitory effects in a dose-dependent manner, demonstrating that sufficient doses of MSCs are essential for the treatment. The i.v. administration might disperse cells and prevent them from exerting immunosuppressive effects, which might lead to the failure of therapy. On the other hand, TGF-β1 was shown to be site- and context-dependent in the regulation of RA [45, 46], suggesting that therapeutic effects of using MSCs may also be influenced by the status of patients with RA.

In conclusion, our findings support the use of MSCs in intensive treatment of RA for their immune tolerance and profound suppression on CII-reactive T cells even after being induced to differentiate into chondrocytes. In spite of the hard work to be done before clinical practice, the study presented here opens a new perspective for the treatment of RA.
Fig. 7. Comparison of TGF-β1 secretion by MSCs and MSC-chondrocytes and time course of the suppression after the single application of rhTGF-β1 and MSCs. (A) After 3 days of culture in FBS-free medium, MSC-chondrocytes secreted similar quantities of rhTGF-β1 to that of MSCs (n = 5). Data were presented as mean pg/ml ± s.d. of triplicates of three separate experiments for MSCs/MSC-chondrocytes from all five donors. (B) Compared with the no-MSC controls that T cells cultured with APCs (1:1) with the stimulation of CII, rhTGF-β1 alone significantly suppressed CII-stimulated T-cell proliferation throughout 5 days of culture while MSCs began to exert significantly inhibitory effects on day 3. Data were presented as mean c.p.m. ± s.d. of triplicates of three separate experiments. *P < 0.01 and **P < 0.01 vs no-MSC controls for MSCs groups and rhTGF-β1 groups, respectively, in the presence of CII.

Table 1. The influences of TGF-β1 and anti-rhTGF-β1 antibody on the inhibition

<table>
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<th>Groups</th>
<th>Proliferation Percentage of maximal response</th>
<th>CD69 (MFI)</th>
<th>CD25 (MFI)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
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<tr>
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<td>100 ± 9</td>
<td>351 ± 69</td>
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<td>12 ± 1</td>
<td>540 ± 12</td>
<td>144 ± 19</td>
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<tr>
<td>SFMC + CII + TGF-β1</td>
<td>34 ± 9</td>
<td>233 ± 36</td>
<td>693 ± 39</td>
<td>16 ± 2</td>
<td>97 ± 14</td>
<td>224 ± 8</td>
<td>47 ± 5</td>
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<tr>
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<td>19 ± 9</td>
<td>103 ± 29</td>
<td>427 ± 37</td>
<td>17 ± 3</td>
<td>26 ± 4</td>
<td>180 ± 9</td>
<td>37 ± 3</td>
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<tr>
<td>SFMC + CII + MSCs + anti-TGF-β1</td>
<td>85 ± 5</td>
<td>291 ± 23</td>
<td>1107 ± 60</td>
<td>13 ± 3</td>
<td>109 ± 16</td>
<td>411 ± 13</td>
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<td>16 ± 2</td>
<td>28 ± 4</td>
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</tbody>
</table>

After 5 days of culture, rhTGF-β1 exerted similar inhibitory effects on the T-cell proliferation, activation, and cytokine production as MSCs and MSC-chondrocytes did, while monoclonal antibody anti-rhTGF-β1 neutralized the suppressive effects of MSCs and MSC-chondrocytes. Data were expressed as mean ± s.d. of triplicates of three separate experiments.

1Statistically different as compared with the group that was only stimulated with CII (P < 0.01).

Rheumatology key messages

- MSCs inhibited the responses of CII-reactive T cells from RA even after differentiation.
- MSCs could be used as a potential therapeutic approach for RA treatment in future.

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