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Comparable therapeutic potential of umbilical cord mesenchymal stem cells in collagen-induced arthritis to TNF inhibitor or anti-CD20 treatment

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Abstract

Objective

The effects of mesenchymal stem cell (MSC) transplantation on established collagen-induced arthritis (CIA) were evaluated and compared to biologic therapies.

Methods

CIA was induced with the immunisation of type II collagen (CII) in DBA/1 mice. Human umbilical cord MSC, anti-TNF antibody, rhTNFR:Fc fusion protein and anti-CD20 antibody were respectively injected intraperitoneally into CIA mice.

Arthritis severity was assessed by clinical and histological scoring. The frequencies of lymphocytes in spleen were analysed, and serum concentrations of cytokines and autoantibody to CII were also measured. The ability of MSC to regulate the balance of T helper cell subsets in CII stimulated CIA CD4⁺ T cells was assessed in vitro.

Results

MSC treatment significantly decreased the severity of arthritis, which was comparable to biologic treatments. All the treatments down-regulated Th1 subset. Except anti-CD20 all the treatments decreased Th17 subset. MSC treatment enhanced the proportion of regulatory T (Treg) cells and inhibited the generation of T follicular helper (Tfh) cells.

The decrease in autoantibody level was detectable in all the treated groups. In vitro MSC induced Foxp3⁺ T cells, and down-regulated IL-17⁺, IFN γ ⁺ T cells and pathogenic IL-17⁺IFN γ ⁺ or IL-17⁺Foxp3⁺ T cells. MSC also reduced the secretion of IL-1 β , IL-6, IL-17 and TNF- α among collagen-specific T cells.

Conclusion

MSC show comparable effects to the known biologic treatments and correct immune imbalance in CIA.

MSC might provide a promising approach for the treatment of rheumatoid arthritis.

Key words

mesenchymal stem cells, immunomodulation, biologic therapies, collagen-induced arthritis

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by synovial inflammation and joint destruction. Inflammation in RA is caused by activation of T cells, B cells and macrophages, which release cytokines such as interleukin (IL)-1 β , IL-6, IL-17 and tumour necrosis factor (TNF)- α (1). Although numerous cells are involved in RA, the ongoing activation of T cells is central in perpetuating the process of immune responses, inflammation and tissue damage. It is also well established that CD4⁺ T cell subsets play an important role to maintain this process, in which Th1, Th17 and T follicular helper (Tfh) cells are pathogenic while regulatory T (Treg) cells control autoimmunity in RA (2-4).

For the treatment of RA, clinical drug development has progressed slowly, and none of them are curative. In recent years biologic agents have brought major therapeutic advances to the treatment of RA (5). Despite the clinical benefits of biologic therapies, there are still a considerable number of patients refractory to these treatments (6). Therefore the pursuits of novel therapies are badly needed. Mesenchymal stem cells (MSC) are the original cells of most connective tissues that have the potential to self-renew and differentiate. Besides their regenerative potential, MSC have potent immunomodulatory properties and therefore these cells have been suggested as an innovative therapeutic tool for rheumatic diseases including RA (7-9). MSC as a candidate drug for RA entered a phase II clinical trial (identifier: NCT01547091), and evidence of clinical benefits was obtained after MSC administration (10). Collagen-induced arthritis (CIA) has been widely accepted as a model for RA, because it shares several clinical and histologic features with RA. CIA development depends on both T and B cell immune responses to type II collagen (CII)-the major constituent of joint cartilage, which is the main site of inflammation in CIA and also in RA. The immune response to CII is characterised by both the stimulation of collagen-specific T cells and the production of high levels of specific antibodies (11).

In this study, the effect of MSC transplantation on established CIA was evaluated and compared with biologic therapies, including TNF inhibitors and B cell depletion. The feasibility of using MSC to correct immune imbalance and modulate cytokine levels were also explored in this *in vivo* setting.

Materials and methods

Animals

Male DBA/1J mice aged 6 to 8 weeks were obtained from Shanghai SLAC laboratory Animal Co., Ltd. All mice were maintained in specific pathogen free animal laboratory and were housed in a temperature controlled room (21 to 26°C) with a 12-hour alternating light/dark cycle. Mice were fed with standard mouse chow and water and were acclimated to their surroundings over one week to eliminate the effect of stress prior to the initiation of the experiments. All animal care and experimental procedures were approved by the Committee of Experimental Animal Administration of Nanjing University.

Materials and reagents

Bovine type II collagen (CII), complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were purchased from Sigma. Anti-mouse TNF monoclonal antibody (mAb) and anti-mouse CD20 mAb were purchased from eBioscience, and rhTNFR:Fc fusion protein was from Shanghai CP Guojian Pharmaceutical Co., Ltd. Antibodies (anti-CD4, anti-CD25, anti-CXCR5, anti-PD-1, anti-IL-4, anti-IL-17, anti-IFN γ , anti-FoxP3, anti-B220 and anti-CD138) used for flow cytometry were purchased from eBioscience. Mouse cytokine (IL-1 β , IL-6, IL-17, TNF α and IL-10) magnetic bead panel kit was obtained from Millipore. Mouse anti-CII antibody (IgG) ELISA kit was from Cayman. CII solution used for *in vitro* study was obtained from Chondrex. Cell isolation kit was from Miltenyi Biotec.

CIA induction and treatments

Arthritis was induced by immunising with CII as described previously (12). CII was dissolved in 50 mM acetic acid at 4 mg/ml overnight at 4°C and was emulsified with an equal volume

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of CFA or IFA in ice bath. The final concentration of CII was 2 mg/ml. CII emulsified in CFA was used for the first immunisation on day 0, and CII emulsified in IFA was used for the booster immunisation on day 21. Mice were immunised intradermally into the base of the tail with 50 µl of emulsion.

Treatment was initiated after the onset of disease (on day 28 after the first immunisation), when arthritis had become established (arthritis score ≥ 2). All the treatments were administered intraperitoneally. After obtaining informed consent, synovial tissues were obtained from traumatic patients without arthritis and umbilical cord samples were obtained from healthy pregnant donors. Umbilical cord mesenchymal stem cells (UC-MSC) and synovial fibroblast (FLS) were isolated and cultured as described previously (13, 14). On the basis of other studies (12, 15, 16), UC-MSC (5×10^6 /mouse), anti-TNF antibody (100 µg/mouse), anti-CD20 antibody (200 µg/mouse) were respectively injected into mice with a single injection on day 28. The control group was treated with PBS or FLS (5×10^6 /mouse). Alternative dose of anti-CD20 antibody (100 µg/mouse, once a week) and the dose of rhTNFR:Fc (4 mg/kg, three times a week) were also chosen according to the previous studies (17–19). 35 mice were assigned to seven groups of 5 mice each. The arthritis score in these mice was evaluated every three or four days. After treated for 3 weeks, all mice were sacrificed, and the hind limbs, spleen and blood samples were collected.

Clinical and histological evaluation of arthritis

Arthritis activity was evaluated by arthritis scores. Each limb was evaluated and scored (0 to 4 for each limb) and the scores were summed to yield an individual mouse score, with a maximum score of 16 (20).

4% paraformaldehyde fixed limbs were decalcified and paraffin embedded using standard histologic techniques. Tissue sections were prepared and stained with haematoxylin and eosin (H&E) to assess the histological scores. The extent of synovial inflammation and

bone/cartilage destruction was determined using a 0–4 scale according to the previous study (8).

Flow cytometric analyses

Spleens were dissected from mice and mononuclear cell suspensions were prepared after red blood cells were lysed. The following antibodies were used for surface staining: anti-mouse CD4-FITC, CD25-APC, PD-1-PerCP, CXCR5-APC, B220-PE and CD138-APC. Cytoplasmic staining (IL-17-APC, IL-4-PE or IFN γ -PE) and intracellular staining (FoxP3-PE) were performed as described previously (21). Data were acquired using a FACS calibur system (BD Biosciences) and analysed by Flowjo software.

Measurement of anti-CII antibodies and cytokine profile

Antibodies to CII antibodies in mouse serum were measured by ELISA according to the manufacturers' instructions. Serum levels of cytokines including IL-1 β , IL-6, IL-17, TNF- α and IL-10 were determined by Luminex 200™ system.

In vitro MSC suppression assay

To identify whether UC-MSC affected the balance of Th1/Th17/Treg cells and the production of cytokines in CD4 $^+$ T cells, CD4 $^+$ T cells were isolated from splenocytes at day 40 after CII immunisation. 5×10^5 CD4 $^+$ T cells were stimulated by CII (10 µg/ml) *in vitro* in the presence or absence of 5×10^4 UC-MSC (at a MSC: CD4 $^+$ T cell ratio of 1:10). After 72 hours, T cells were harvested and culture supernatants were also collected. Flow cytometric analyses were further performed as described before (22). Concentrations of IL-1 β , IL-6, IL-10, IL-17 and TNF α in supernatants of CII stimulated CD4 $^+$ T lymphocytes *in vitro* were also assessed by Luminex 200™ system.

Statistical analysis

All data were expressed as mean \pm SEM. Differences between groups were evaluated by one-way analysis of variance followed by post hoc Tukey's multiple comparison tests. *p*-values < 0.05 were considered to be significant. Anal-

yses and graphical representation were performed using GraphPad Prism™ software (Graphpad).

Results

MSC decreased the severity of arthritis and histological scores, comparable to TNF inhibitor or anti-CD20 treatment

MSC and biologic treatments such as anti-TNF antibody, rhTNFR:Fc and anti-CD20 antibody were designed and utilised in this study, and arthritis scores of CIA mice in every group were evaluated. The results showed that MSC, anti-TNF antibody, rhTNFR:Fc or anti-CD20 antibody (anti-CD20 * , 100 µg/week) treatment all significantly decreased arthritis scores compared to the control (PBS or FLS) group (Fig. 1A). Arthritis scores in different treated groups appeared to be comparable. At the endpoint of *in vivo* study, the control (PBS or FLS) group exhibited severe synovitis, synovial hyperplasia and joint erosion. The joints from anti-CD20 (200 µg), anti-CD20 * (100 µg/week) or MSC group had smooth articulation surfaces and mild synovitis (Fig. 1B). The joints from anti-TNF or rhTNFR:Fc group showed nearly normal morphology with the absence of synovitis. Histological scores were significantly decreased in all of the five treatment groups (Fig. 1C). The histological score in MSC group was parallel to that of anti-TNF, rhTNFR:Fc, anti-CD20 or anti-CD20 * group. These results suggested that MSC could engender comparable effects to ameliorate synovial inflammation and joint damage as those biologic treatments.

MSC down-regulated Th1, Th17 cells, and up-regulated Treg cells

We next examined the percentages of CD4 $^+$ T cell subsets in spleen of different treatment groups. We first confirmed that the percentage of Th1 (CD4 $^+$ IFN γ^+) and Th17 (CD4 $^+$ IL-17 $^+$) cells were significantly higher in CIA mice than normal mice, and Treg (CD4 $^+$ CD25 $^+$ FoxP3 $^+$) cell subset was reduced in CIA mice (data not shown), which were consistent to the previous study (23). In our study, MSC, anti-TNF, rhTNFR:Fc, anti-CD20 or anti-CD20 * treatment group could all

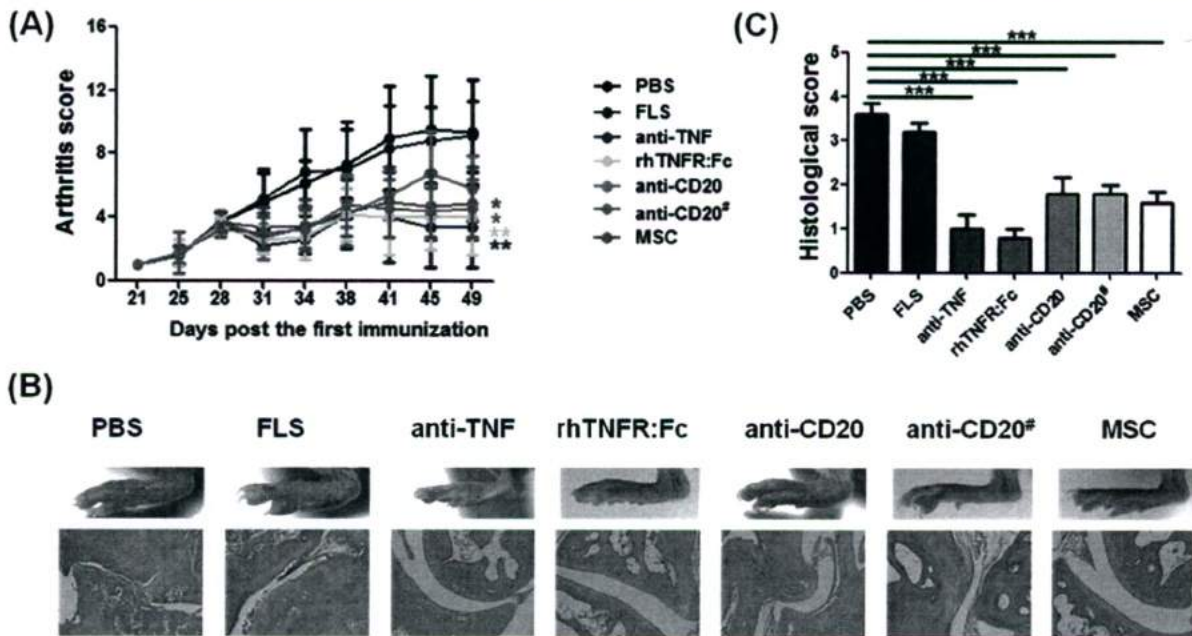


Fig. 1. MSC decreased the severity of arthritis and histological scores, comparable to TNF inhibitor or anti-CD20 treatment. (A) Arthritis scores in different collagen-induced arthritis (CIA) groups ($n=5$ in each group). CIA mice were treated by anti-tumour necrosis factor (TNF) (100 μ g), rhTNFR:Fc fusion protein (4 mg/kg, 3 times/week), anti-CD20 (200 μ g), anti-CD20^g (100 μ g/week) or umbilical cord mesenchymal stem cells (MSC) (5×10^6). The control groups were treated with PBS or human fibroblasts (FLS) (5×10^6). (B) H&E stained sagittal sections of ankle joints from CIA mice (photographed at $\times 200$). (C) The evaluation of histological scores of H&E stained sections in different CIA groups ($n=5$ in each group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

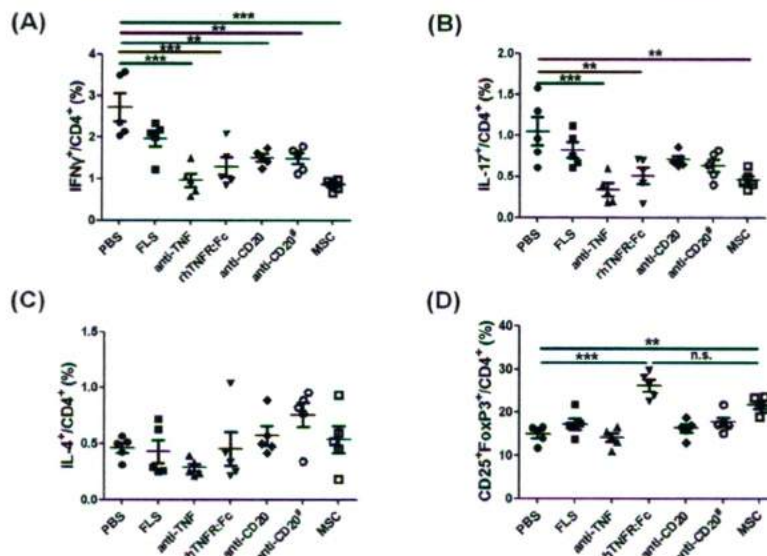


Fig. 2. The percentages of CD4⁺ T cell subsets in spleen of different CIA groups. The percentages of IFN- γ expression in CD4⁺ T (Th1) cells (A), IL-17 expression in CD4⁺ T (Th17) cells (B), IL-4 expression in CD4⁺ T (Th2) cells (C) or CD25⁺FoxP3⁺ in CD4⁺ T (Treg) cells (D) were determined via flow cytometric analysis ($n=5$ in each group). The graphs of flow cytometry were shown in supplementary Fig. S1. ** $p < 0.01$; *** $p < 0.001$; n.s.: no significant difference.

decreased the proportion of Th1 cells compared to the PBS group, however, there were no significant differences among these five treatment groups to

reduce Th1 cell subset (Fig. 2A). MSC, anti-TNF or rhTNFR:Fc treatment resulted in a significant reduction in the percentage of Th17 cells, but anti-CD20

treatments had no effect on Th17 cell subset (Fig. 2B). MSC and rhTNFR:Fc treatments showed comparable ability to up-regulate Treg cells (Fig. 2D). In addition, none of the treatments affected the proportion of Th2 (CD4⁺IL-4⁺) cells (Fig. 2C). Collectively, these results suggested that MSC showed comprehensive regulatory control of Th1, Th17 and Treg cells.

MSC reduced the proportion of Tfh cells and the levels of anti-CII antibodies

We investigated the effect of these treatments on Tfh and B cell populations. The results showed significantly more Tfh (CD4⁺CXCR5⁺PD-1⁺) cells in the spleens of control (PBS or FLS) mice than those of MSC treated mice. However, TNF inhibitors (both anti-TNF and rhTNFR:Fc) or anti-CD20 treatments showed no effect on Tfh cell subset (Fig. 3A). Anti-CD20 (200 μ g) or anti-CD20^g (100 μ g/week) treatment group depleted nearly half of the B (B220⁺) cells, and significantly decreased the percentage of plasma (B220⁺CD138⁺) cells and the serum levels of anti-CII

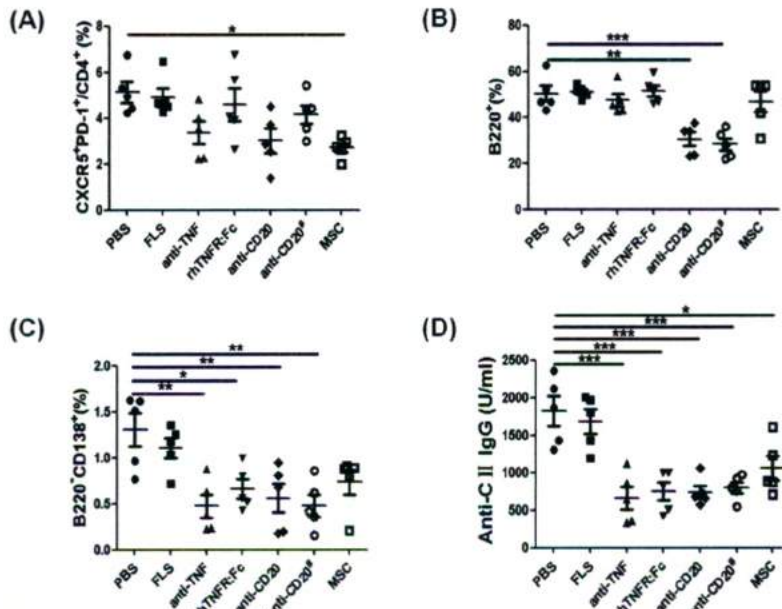


Fig. 3. The percentages of T follicular (T_{fh}) cells and B cell subsets and serum levels of anti-collagen II (CII) antibodies in different CIA groups. The percentages of CXCR5⁺PD-1⁺ in CD4⁺ T (T_{fh}) cells (A), B220 expression in splenocytes (B) or B220⁺CD138⁺ in splenocytes (C) were determined by flow cytometric analysis (n=5 in each group). (D) Serum levels of anti-CII antibodies were measured by ELISA (n=5 in each group). The graphs of flow cytometry were shown in supplementary Fig. S2. **p*<0.05; ***p*<0.01; ****p*<0.001.

antibodies (Fig. 3B-3D). Anti-TNF or rhTNFR:Fc treatment could lead to the reduction of plasma cells without affecting the percentage of B cells (Fig. 3B-3C). All of the five treatment groups significantly down-regulated the levels of anti-CII antibodies, but MSC treatment had no significant effect on the percentage of B cells or plasma cells (Fig. 3B-3D). These results suggested that MSC could reduce the production of autoantibodies *in vivo* comparable to the biologic treatments.

MSC decreased serum levels of IL-1 β and IL-6

We next examined the serum levels of pro-inflammatory cytokine IL-1 β , IL-6, IL-17, TNF α and protective cytokine IL-10 (24) in different treatment groups. The levels of IL-1 β were significantly decreased after anti-CD20 or MSC treatment (Fig. 4A). Among the treatment groups only MSC group reduced the serum levels of IL-6 (Fig. 4B). None of the five treatment groups had significant effect on IL-17 level,

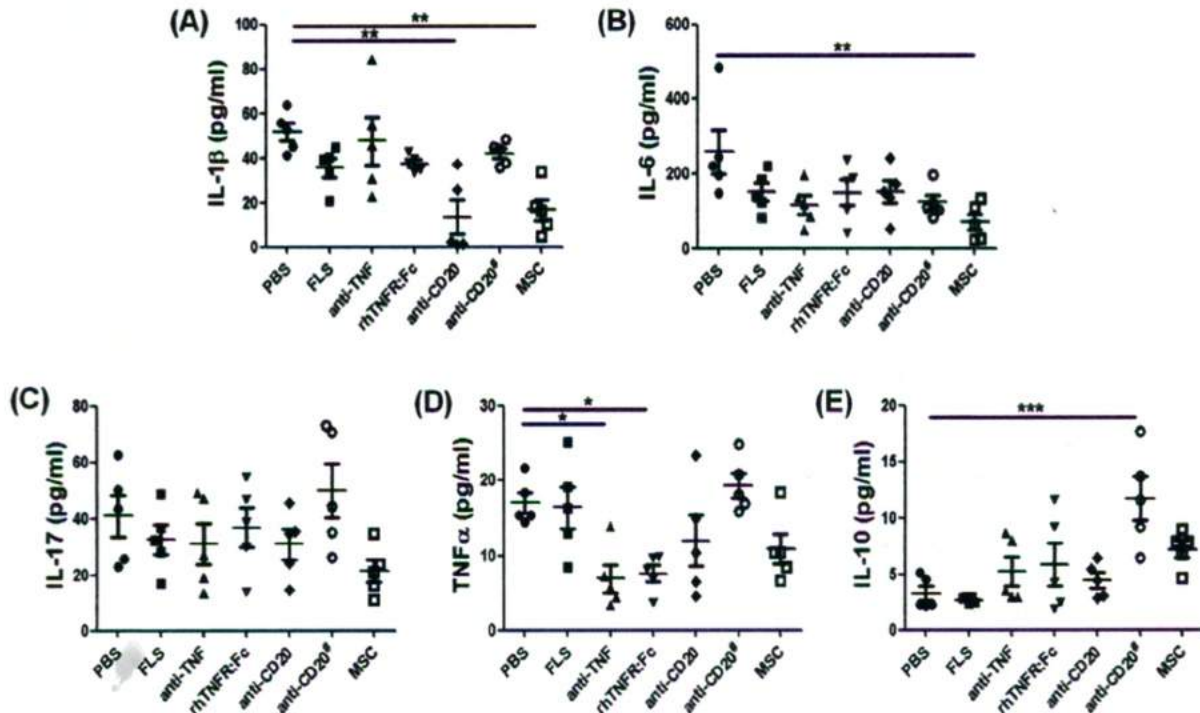


Fig. 4. Effects of different treatments on serum levels of cytokines. Luminex analysis shows the serum levels of pro-inflammatory cytokine IL-1 β (A), IL-6 (B), IL-17 (C), TNF α (D) and protective cytokine IL-10 (E) (n=5 in each group). **p*<0.05; ***p*<0.01; ****p*<0.001.

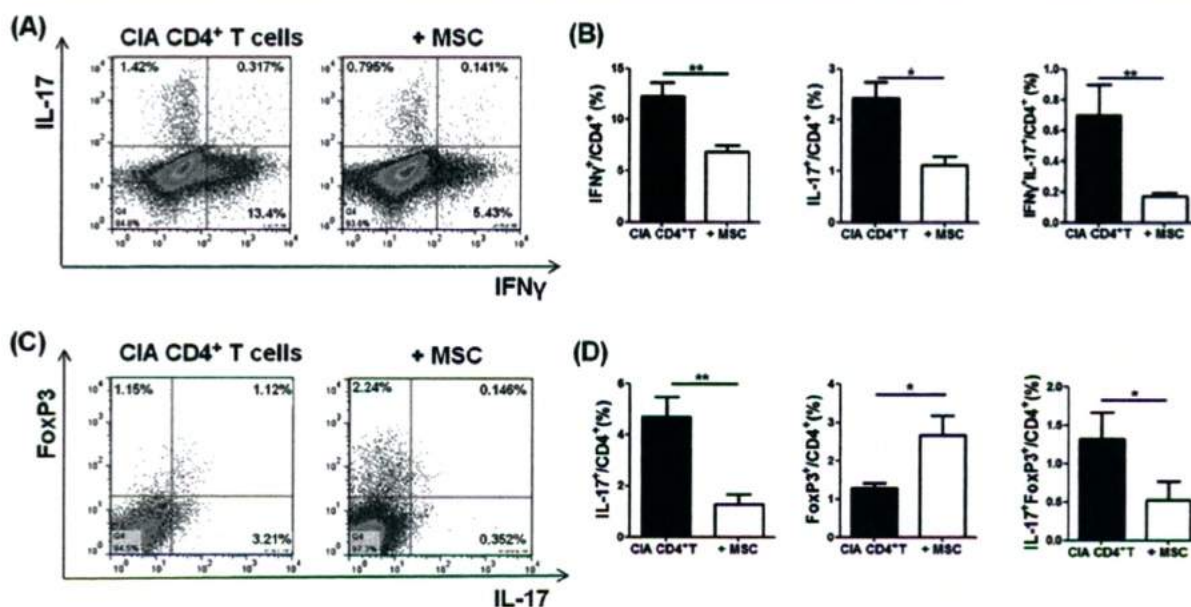


Fig. 5. MSC reduced IFN γ and IL-17 production and induced FoxP3⁺ Treg cells *in vitro* among collagen-specific CD4⁺ T cells. Isolated CD4⁺ T cells from CIA mice were stimulated by CII in the presence or absence of MSC (MSC: CD4⁺ T cells=1:10). After 72 hours, the percentages of IFN γ ⁺, IL-17⁺ and IFN γ ⁺IL-17⁺ in CD4⁺ T cells were determined via cytoplasmic staining plus flow cytometric analysis (A, B) (n=5); the percentages of IL-17⁺, FoxP3⁺ and IL-17⁺FoxP3⁺ in CD4⁺ T cells were determined via intracellular staining plus flow cytometric analysis (C, D) (n=4). * p <0.05; ** p <0.01.

and MSC group showed a decreased tendency in IL-17 level compared to control group (Fig. 4C). Anti-TNF and rhTNFR:Fc appeared to have the similar ability to reduce the serum levels of TNF- α (Fig. 4D). Despite that all of the five treatment groups showed an increased tendency in IL-10 level compared to control group, only the anti-CD20⁺ treatment significantly up-regulated IL-10 level (Fig. 4E).

MSC reduced IFN γ and IL-17 production and induced FoxP3⁺ Treg cells *in vitro* among collagen-specific CD4⁺ T cells

The *in vivo* results indicated that MSC treatment could effectively modulate the balance of CD4⁺ T cell subsets including Th1, Th17 and Treg cells. Previous studies showed that CD4⁺ T cell plasticity (Th17 cells convert to Th1 cells; Treg cells convert to Th17 cells) could be driven under the arthritic milieu, thus contributing to the pathogenesis of RA (21, 22). We next investigated whether MSC also had a role in CD4⁺ T cell plasticity. We re-stimulated CIA CD4⁺ T cells with CII to generate collagen-specific CD4⁺ T cells *in vitro* according to the previous study (25). In

the presence of MSC, IL-17⁺ or IFN γ ⁺ T cells were significantly reduced, and FoxP3⁺ T cells were up-regulated among collagen-specific CD4⁺ T cells. In addition, MSC also inhibited the generation of the intermediate phenotype IL-17⁺IFN γ ⁺ or IL-17⁺FoxP3⁺ T cells (Fig. 5). These results suggested that MSC might inhibit CD4⁺ T cell plasticity toward a proinflammatory phenotype, and this inhibition could be one of the possible ways for MSC to correct the immune imbalance (including Th1, Th17 and Treg cells) in CIA. We also investigated whether MSC could affect the cytokine secretion of CIA CD4⁺ T cells. In the co-culture system of MSC and collagen-specific CD4⁺ T cells, MSC significantly reduced the secretion of IL-1 β , IL-6, IL-17 and TNF- α *in vitro* (Fig. S3). The decreased level of IL-17 was consistent to the reduction of Th17 cells in this co-culture system, however, IL-10 level was not significantly affected or even in a decreased tendency in the presence of MSC.

Discussion

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease for which the non-conventional therapies

such as biologic agents have been dramatically developed. Previous studies demonstrating the central role of TNF- α have been translated into the development of blocking drugs. rhTNFR:Fc is a recombinant fusion protein, which links the soluble TNF receptor to the Fc portion of immunoglobulin G (IgG), and anti-TNF antibody is the fully monoclonal antibody directly binding to TNF (26). For early RA patients displaying high disease activity and poor prognosis, TNF inhibitors could be used as an immediate, first-line therapy (27). However, 30~50% of patients do not respond to TNF inhibitors, and TNF inhibitors do not provide long-term effectiveness in most patients. B cells are involved in the production of autoantibodies as well as in the induction of T cell activation and production of pro-inflammatory cytokines in RA (28). CD20 is expressed by B cells at different stages (from pre-B cell to mature stages) but not by haematopoietic stem cells or plasma cells. Anti-CD20 treatment was approved originally for the treatment of non-Hodgkin lymphoma and then approved as a second-line therapy (after failure of anti-TNF therapy) in patients with moderate to severe

RA (29). The degree of B-cell depletion correlates with response to the drug, and only 60% of RA patients respond to anti-CD20 treatment clinically (6).

New treatments for RA are still needed because a considerable portion of patients fail to respond to these biologic therapies or develop drug resistance or toxicity (6, 30). Mesenchymal stem cells (MSC) exhibit multiple immunomodulatory effects. These properties make them well-suited to serve as a candidate for a new approach in the prevention and treatment of RA. Umbilical cord MSC (UC-MSC) may provide safe and persistent clinical benefits for patients with active RA (10). In the present study, we compared the effect of UC-MSC transplantation with biologic therapies in RA animal models. We demonstrate that UC-MSC significantly attenuated inflammatory arthritis in collagen-induced arthritis (CIA) and showed comparable effect with TNF inhibitors or anti-CD20 treatment.

In RA, the breach of immune tolerance culminates in severe joint damage including the destruction of the synovial membrane, cartilage and bone. CD4⁺ T cells play an important role in orchestrating and maintaining this immune imbalance and inflammatory responses in RA. Therefore, inhibiting T cell response is very important in the treatment of RA. It was reported that the prevalence of Th1 cell response is associated with arthritis induction and inflammation, while Th2 cell response are found to be dominant during the remission stage of RA (31). Previous studies suggest that MSC might restore the balance between Th1 and Th2 cells in CIA (32, 33). Our data showed all of the treatments including biologic therapies and MSC inhibited the production of Th1 cells, but these treatments did not affect the Th2 subset. TNF inhibitors including rhTNFR:Fc and anti-TNF antibody reduced the proportion of Th1 and Th17 cells *in vivo*, which is consistent to the previous study (34). Compared to TNF inhibitors, MSC showed equivalent ability to decrease Th1 and Th17 cells. Furthermore, in MSC treated mice, the proportion of Treg cells was elevated. Treg cell subset could specifically suppress pro-inflammatory Th1 and Th17

cells (35). It has also been established that the imbalance of Th17/Treg cells is more important than the Th1/Th2 cell imbalance in the pathogenesis of RA (36, 37). So identifying the change of Th17 and Treg cells could be more meaningful for the evaluation of different treatments. In our results, rhTNFR:Fc and MSC groups appeared to have the similar ability to correct the imbalance of Th17 and Treg cells, thus exerting the comparable inhibition of arthritis progression with arrest of joint damage. Besides that, recent studies demonstrated that the plasticity of CD4⁺ T cell subsets is of pathological importance in the development of autoimmune diseases including RA (38). Th17 phenotype may be unstable and Th17 cells may convert to Th1 cells in human arthritis (21); Treg cells also showed instability in the generation of pathogenic Th17 cells in RA (22). Our *in vitro* experiments showed MSC inhibited the generation of IL-17⁺ or IFN γ ⁺ T cells, induced Foxp3⁺ T cells, and also reduced intermediate phenotype IL-17⁺IFN γ ⁺ or IL-17⁺Foxp3⁺ T cells. These results indicated that MSC might have a role in preventing the pathogenic plasticity of CD4⁺ T cell subsets in arthritic milieu, which further contributes to the immune correction in CIA.

Autoimmune diseases are characterised by the loss of immune tolerance and generation of autoreactive B cells that produce autoantibodies. T follicular helper (Tfh) cells play an important role in the selection of B cell clones in germinal centers (GCs). Increased frequency of circulating Tfh cells in RA patients have been reported (39). Among the treatments we examined in this study only MSC significantly inhibited the generation of Tfh cells in CIA. Anti-CD20 treatment inhibited the generation of B cells, plasma cells and autoantibodies. All of the treatments resulted in the comparable reduction of autoantibodies. Since MSC did not alter the percentage of B or plasma cells, MSC might act through the inhibition on Tfh cells to down-regulate autoantibodies. Previous studies showed TNF inhibitors could modulate the humoral response by affecting B cell subsets in CIA (18). In our study the two TNF in-

hibitors rhTNFR:Fc and anti-TNF decreased the production of plasma cells but had little effect on total B cells.

In addition to the systemic modulation on T and B cell response, MSC also modulated the production of cytokines in CIA. IL-1 β has been implicated as a pro-inflammatory role in both RA and CIA, and inhibition with an IL-1 receptor antagonist results in amelioration of arthritis (40). IL-17 is mainly produced by Th17 cells and has a synergistic effect with TNF- α and IL-6 in promoting disease progression (24). These cytokines cause local joint damage through increased production of metalloproteinases. IL-1 β , IL-6 and TNF- α also leak out to the blood stream resulting in systemic inflammation. In our *in vitro* experiments, MSC inhibit CII stimulated T cells to secrete pro-inflammatory cytokine including IL-1 β , IL-6, IL-17 and TNF- α . *In vivo* MSC significantly decreased the serum levels of IL- β and IL-6, but did not show significant effect on levels of IL-17, IL-10 and TNF- α . TNF inhibitors suppressed the generation of TNF- α , while anti-CD20 treatments down-regulated IL-1 β and up-regulated anti-inflammatory cytokine IL-10 in our *in vivo* study. These results indicating that MSC and these biologic therapies have distinct modulation on cytokine levels in CIA.

In this study we used human umbilical cord derived MSC. UC-MSC are now considered an alternative source of mesenchymal stem cells and could be used in long-term clinical trials. The positive MSC effect was shown to be MHC-independent and xenogenic or allogeneic MSC provided similar benefits in experimental arthritis (20, 32, 41, 42). These benefits are likely resulting from anti-inflammation, immune-modulatory, and immune-tolerance induction. Compared to the biologic agents targeting cytokines or lymphocytes, MSC also educated other cells such as Treg cells to inhibit the pathogenic immune reaction. Therefore, cell therapy using MSC might possess some superiority in systemic immunomodulation and induce longer-lasting remission in RA. Long-term clinical trials are further needed for comparative studies of different treatments in RA.

Collectively, this study shows that MSC could exert comparable therapeutic effect to TNF inhibitors and anti-CD20 treatments in established arthritis. MSC administration in CIA was able to decrease the arthritis score and the histological score accompanied by effectively correcting immune imbalance and modulating cytokine levels. These results suggest that manipulation of MSC may provide an alternative therapeutic approach for the treatment of RA patients, and the clinical applications of MSC in RA should be evaluated further.

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