Human Umbilical Cord Mesenchymal Stem Cell Therapy for Patients with Active Rheumatoid Arthritis: Safety and Efficacy

Liming Wang[1*], Lihua Wang[2*], Xiuli Cong[3], Guangyang Liu[3], Jianjun Zhou[1], Bin Bai[1], Yang Li[1], Wen Bai[1], Ming Li[1], Haijie Ji[3], Delin Zhu[3], Mingyuan Wu[4,5,#] and Yongjun Liu[3,5,#]

[*]Co-first authors, [#]Co-corresponding authors

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Authors: [1]Liming Wang, M.D., Ph.D., Email: wanglm@fmmu.edu.cn; Jianjun Zhou, M.D., Ph.D., Email: zhoujianjun196512@163.com; Bin Bai, M.D., Email: baibin513127@163.com; Yang Li, M.D., Email: doctlyd@sina.com; Wen Bai, M.D., Email: bai_wen_323@126.com; Ming Li, M.D., Email: Wendyyao8105@hotmail.com: Cell Therapy Center, 323 Hospital of Chinese People's Liberation Army, 6 West Jianshe Road, Xi'an city, China, Telephone: +86-29-84756502, Fax number: +86-29-85513262; [2]Lihua Wang, M.D., Email: wanglihua1168@163.com: The State Key Laboratory of Experimental Hematology, Institute of Hematology and Hospital of Blood Diseases, Graduate School of Chinese Academy of Medical Sciences and Peking Union of Medical College, 288 Nanjing Road, Tianjin, China, Telephone: +86-22-58775766-6108, Fax number: +86-22-58775779; [3]Xiuli Cong, M.D., Ph.D., Email: cxl.sherry@163.com; Guangyang Liu, Ph.D., Email: liugy04@163.com; Haijie Ji, Ph.D., Email: jihaijie82@hotmail.com; Delin Zhu, Ph.D., Email: delzhu@126.com; Yongjun Liu, M.D., Ph.D., Email: yongjunliu8959@163.com: Alliancells Institute of Stem Cells and Translational Regenerative Medicine of Zhongyuan Union Stem Cell

Bioengineering Co Ltd, No.45 East Nine Road, Special Economic Zone of KongGang, Tianjin, China; [4]Mingyuan Wu, M.D., Ph.D., Email: mingyuan-wu@ouhsc.edu: Harold Hamm Diabetes Center and Section of Endocrinology and Diabetes in the Department of Internal Medicine, and Department of Physiology, University of Oklahoma Health Sciences Center, 941 Stanton L. Young Blvd., Oklahoma City, OK, 73104, USA; Telephone: 405-271-3270; Fax: 405-271-3973. [5]Mingyuan Wu and Yongjun Liu, School of Life Sciences and Technology, Tongji University, 1239 Siping Road, Shanghai, 200092, China.

Address correspondence and reprint request to Yongjun Liu, Alliancells Institute of Stem Cells and Translational Regenerative Medicine of Zhongyuan Union Stem Cell Bioengineering Co Ltd, No.45 East Nine Road, Special Economic Zone of KongGang, Tianjin, 300381, China. Telephone: +86-2258775779, Fax number: +86-2258775779, Email: yongjunliu8959@163.com or Mingyuan Wu, University of Oklahoma Health Sciences Center, 941 Stanton L. Young Blvd., Oklahoma City, OK 73104, USA; Telephone: 405-271-3270; Fax: 405-271-3973, Email: mingyuan-wu@ouhsc.edu

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Abbreviations: MSCs: Mesenchymal Stem Cells; UC-MSCs: Umbilical Cord Mesenchymal Stem Cells; RA: rheumatoid arthritis; ACR response: the American College of Rheumatology response; DAS28: the 28-joint disease activity score; HAQ: the Health Assessment Questionnaire; TNF-α: Tumor Necrosis Factor-Alpha; IL-6:

Interleukin-6; Tregs: $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells; NSAIDs: Non-Steroidal Anti-Inflammatory Drugs; DMARDs: Disease Modifying Anti-Rheumatic Drugs;

Abstract

This study was designed to assess the safety and efficacy of human umbilical cord mesenchymal stem cells (UC-MSCs) in the treatment of rheumatoid arthritis (RA). In this ongoing cohort, 172 patients with active RA who had inadequate responses to traditional medication were enrolled. Patients were divided into two groups for different treatment: Disease modifying anti-rheumatic drugs (DMARDs) plus medium without UC-MSCs, or DMARDs plus UC-MSCs group (4×10^7 cells per time) via intravenous injection. Adverse events and the clinical information were recorded. Tests for serological markers to assess safety and disease activity were conducted. Serum levels of inflammatory chemokines/cytokines were measured and lymphocyte subsets in peripheral blood were analyzed. No serious adverse effects were observed during or after infusion. The serum levels of tumor necrosis factor-alpha and interleukin-6 decreased after the first UC-MSCs treatment ($p<0.05$). The percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells of peripheral blood was increased ($p<0.05$). The treatment induced a significant remission of disease according to the American College of Rheumatology improvement criteria, the 28-joint disease activity score, and the Health Assessment Questionnaire. The therapeutic effects maintained for 3-6 months without continuous administration, correlating with the increased percentage of regulatory T cells of peripheral blood. Repeated infusion after this period can enhance the therapeutic efficacy. In comparison, there were no such benefits observed in control group of DMARDS plus medium without UC-MSCs. Thus our data indicate that treatment with DMARDs plus UC-MSCs may provide safe, significant and persistent

clinical benefits for patients with active RA.

Introduction

Rheumatoid arthritis (RA) is characterized mainly by synovial inflammation and hyperplasia, cartilage/bone damage, and systemic comorbidities [1]. The potential pathogenic mechanisms that initiate and lead to the development of RA include genetic–environmental interactions, synovial immunologic processes and inflammation, and a loss of immunological self-tolerance. The induction and maintenance of immunological self-tolerance depends on the self-reactive clones during adult life [2], and regulatory T cells play an important role in suppression of autoimmune pathology. Therefore, to enhance the function of regulatory T cells component could be a valuable therapeutic strategy for treating RA [3].

It has been reported that pro-inflammatory cytokines and chemokines play an essential role in RA [4]. Biological agents aiming at those cytokines have been tested clinically, such as recombinant human tumor necrosis factorreceptor-Fc fusion protein [5,6] and recombinant human interleukin-1 receptor antagonist [7]. However, there were moderate-severe side effects observed including relapse and an increased susceptibility to infections [8]. Moreover, such symptomatic therapies were unable to promote the reconstruction of immune tolerance.

Mesenchymal stem cells (MSCs) are multipotent cells existing in many fetal [9] and adult tissues [10], which can replicate as undifferentiated cells and potentially differentiate to lineages of mesenchymal tissues [11]. MSCs can also modulate several immune functions through interplay with cells from both innate and adaptive immune systems. Furthermore, after administration *in vivo*, MSCs can migrate to injured tissues,

where they can restrain the release of pro-inflammatory cytokines and facilitate the survival of damaged cells, and also induce peripheral tolerance [12]. Clinical studies have confirmed that MSCs have clinical benefits in severe acute graft-versus-host disease [13] and in different autoimmune diseases, such as systemic lupus erythematosus [14]. Previous studies found that MSCs may treat RA and many mechanisms were explored [15-17]. However, there were no comprehensive reports regarding the effects of UC-MSCs on patients with RA, who were with recurrent symptoms after long-term treatment with regular strategies in clinics.

We are conducting this cohort to evaluate safety and efficacy of UC-MSCs in the treatment of RA, along with discovery of the possible mechanisms. Here we demonstrated that (1) UC-MSCs treatment was safe without major side effects during and after infusion. (2) Treatment with DMARDs plus UC-MSCs was more efficacious than DMARDs plus medium without UC-MSCs. (3) Continuous UC-MSCs treatment maintained clinical benefits. (4) Clinical benefits are likely resulting from anti-inflammation, immune-modulatory, and immune-tolerance induction.

Materials and methods

Patients

According to the American Rheumatism Association's diagnostic criteria [18], RA patients were enrolled and a written informed consent was provided in accordance with the Declaration of Helsinki. The study was registered in *ClinicalTrials.gov* (identifier: NCT01547091), approved by the ethic committee of 323 hospital of Chinese People's Liberation Army.

All patients' condition could not be well controlled by multiple traditional chemotherapies including disease modifying anti-rheumatic drugs, non-steroidal anti-inflammatory drugs, steroid and biological treatment. An inadequate response was defined as discontinuation of traditional medication therapy due to lack of effect and/or serious side effects. Patients should have active disease despite treatment with DMARDs at enrollment, defined as more than three painful joints, accompanied by joint swelling and tenderness and at least one of the following situations: erythrocyte sedimentation rate ≥45 mm/h, C-reactive protein ≥of at least 15 mg/dl or morning stiffness lasted for at least 1 h.

Treatment protocol

All patients continued to receive DMARDs: small doses of disease modifying anti-rheumatic drugs individually: methotrexate at 7.5-10 mg/week, and/or leflunomide at 10 mg/d, and/or hydroxychloroquine at 200 mg/d. Con-comitant therapy with stable and proper doses of NSAIDs were permitted.

UC-MSCs were obtained from Alliancells Institute of Stem Cells and Translational

Regenerative Medicine using the established protocol [19] and meet the eligible criteria for clinical use [20]. Patient either received 4.0×10^7 of UC-MSCs in 40 ml stem cell solvent [21] as treatment, or 40 ml stem cell solvent without UC-MSCs as control, via intravenous infusion.

In detail, 172 RA patients were enrolled and allocated into two groups (**Schema 1**). One group is DMARDs group (n=36, treated by DMARDs plus medium without UC-MSCs); another is DMARDs plus UC-MSCs group (n=136, treated by DMARDs plus UC-MSCs). 136 patients from treatment group were enrolled from 2010 and 36 patients from control group were enrolled since late 2012. Therefore, the control population was accessed at a time different from the treated population. However, all patients were enrolled and studied at the same institution, and were not part of any other formal randomized controlled trials. In addition, DMARDs plus UC-MSCs group was divided into 3 groups according to different intervals after the first treatment **(Table 1)**. Group 1 has 76 patients for 3 months' interval; group 2 has 45 patients for 6 month's interval; group 3 has 15 patients for over 8 months' interval. In addition, 24 among 76 patients in group 1 were treated by UC-MSCs twice with 3 months' interval.

Safety evaluation was performed before and after UC-MSCs or medium without UC-MSCs administration by monitoring physical examination, liver and kidney function, chest radiography and electrocardiograph. Standard hematological and biochemical tests and urine analysis were performed as well. Adverse events were recorded individually.

Assessment of disease status was composed of a complete count of tender and

swollen joints [4], the 28-joint disease activity score (DAS28) [22] and the Health Assessment Questionnaire (HAQ) [23]. The other indices of disease activity included C-reactive protein, erythrocyte sedimentation rate, duration of morning stiffness, patient's and physician's global assessments, rheumatoid factor titers [4] and anti-cyclic citrullinated peptide antibody.

Study End Points

The primary efficacy end points were the American College of Rheumatology (ACR) 20 and ACR50 responses [24] in disease activity at different time points. Other efficacy end points were ACR70 response, the 28-joint disease activity score (DAS28) and the percentage changes from baseline in the Health Assessment Questionnaire (HAQ).

T regulatory cells detection and Intracellular Cytokine Staining for Th1/Th2

Peripheral blood samples of RA patients were collected and analyzed with BD Multitest™ IMK kit. Regulatory T cells were stained with anti-CD4-fluorescein isothiocyanate, anti-CD25-Allophycocyanin and anti-Foxp3-PE (eBioscience). Th1/Th2 test was carried out using Fast Immune Intracellular Cytokine Staining Procedure. Data was acquired and analyzed by FACS Caliber (Becton Dickinson).

Multiplex Cytokine Assay

A bead-based multiplex cytokine assay was custom-designed for the quantification of the following cytokines: IL-1β, IL-4, IL-6, IL-10, IL-17A, TNF-α, IFN-γ, RANTES, and TGF-β1. Assays were performed according to instructions and read with a Luminex 200 system (Millipore Corporation).

Statistical analysis

All continuous variables were subjected to descriptive statistics. Changes between baseline and end point were compared by the Wilcoxon signed rank test. Rates of ACR response criteria were analyzed by logistic regression. Data for safety evaluation before and after the treatment were compared by paired *t-test*. All statistical tests were two sided, and the significance level was set as $p<0.05$. All analyses were conducted by SPSS 17.0 (SPSS Inc).

Results

Safety evaluation

No patients showed acute serious side-effects either during or after UC-MSCs infusion, and there were 6 cases of 136 patients (4%) showing mild adverse effects during the infusion, such as chill and/or fever (<38.5 ˚C), which disappeared within 2 hours without any treatments.

26 cases of 87 individuals (30%) presenting anemia with average hemoglobin level of 99 g/L returned to normal after UC-MSC treatment. Levels of serum total protein and globulin were decreased (from 71 to 69 g/L and from 32 to 29 g/L, on average, respectively, **Table 2**), which were in consistent with the lessened titers of rheumatoid factor and anti-cyclic citrullinated peptide antibody. No major abnormal findings in hematologic or serum chemical profiles were found in the study. In addition, clinical profiles between DMARDs plus medium without UC-MSCs group as controls (n=36) and DMARDs plus UC-MSCs group (n=58) were shown in **Table 3**, indicating a similar baseline between two groups before the treatment. DMARDs plus UC-MSCs showed decreased levels of total protein and globulin and increased levels of albumin and hemoglobin compared to control group. The increased levels of albumin and hemoglobin may be related to the improved liver function and the decreased incidence of gastro-intestinal tract bleeding.

Efficacy determination

Changes of symptoms:

All patients have shown improvements in the diet, sleep and physical strength as

early as 2 weeks after the cell therapy based on patients' reports. In comparison, there was no such improvement in control group. In addition, the clinical response to UC-MSCs treatment was rapid with the physical evidence after administration of UC-MSCs. The joint pain and swelling were alleviated within 12 h, and was maintained through the period of the study.

Clinical benefits before and after the treatments:

3 months after DMARDs plus UC-MSCs treatment, significant decreased levels of HAQ and DAS28 scores were observed, indicating an improvement of clinical behaviors. Further tests showed decreased level of CRP and RF and increased levels of percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells (**Fig 1 A-E)**. In comparison, there was no significant change of the scores of HAQ and DAS28 in control group with DMARDs plus medium without UC-MSCs. In parallel, DMARDS did not alter serum levels of CRP, RF, and percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells in peripheral blood (**Fig 1 A-E**).

Data from Fig 1 provided core message that DMARDs plus UC-MSCs showed encouraging clinical benefits in the treatment with refractory RA resulting from decreased level of systemic inflammation and increased level of immune tolerance.

The scores of HAQ and DAS28 showed steady reduction with continuous and repeated treatments (twice with 3 months' interval) (**Fig 2 A-B**). The number of joints with tenderness and swelling was significantly reduced along with the alleviated symptoms. After two cycles of treatment, 58% (14 in 24) patients achieved ACR20, 13% (3 in 24) achieved ACR50, and 13% (3 in 24) patients achieved ACR70,

respectively.

In addition, DMARDs plus UC-MSCs group was divided into 3 sub-groups according to different intervals after the first treatment (Group 1 has 76 patients for 3 months' interval; group 2 has 45 patients for 6 month's interval; group 3 has 15 patients for over 8 months' interval). HAQ Score, DAS28 Score and ACR were re-analyzed among three groups. We observed a reduced level of HAQ in three groups. However, there was no statistical difference in group 3 (**Fig 3A**). A significant decrease of DAS28 was observed in three groups (**Fig 3B**). In detail, 50% (38 in 76) of patients achieved remission and 25% (19 in 76) staying in a low-active period in group 1; 49% of patients (22 in 45) achieved remission and 38% (17 in 45) in a low-active period in group 2; 53% (8 in 15) of patients achieved remission with 27% (4 in 15) in group 3. To further assess the therapeutical consequences, ACR data were further analyzed as shown in **Fig 3C**. In detail, 36% (27 in 76) patients achieved ACR20, 28% (21 in 76) achieved ACR50, and 12% (9 in 76) patients achieved ACR70 in group 1. In comparison, there were only 14% patients (5 in 36) achieved ACR20 in DMARDs plus medium without UC-MSCs group (data not shown). In group 2, 47% (21 in 45) patients achieved ACR20, 20% (9 in 45) achieved ACR50, and 4% (2 in 45) patients achieved ACR70; In group 3, 33% (5 in 15) patients achieved ACR20, 7% (1 in 15) achieved ACR50, and 7% (1 in 15) patients achieved ACR70.

Potential mechanisms

It has been well documented that anti-inflammation is an important mechanism in treatment of autoimmune diseases. Level of CRP, a maker of inflammation, was

decreased after the treatment **in Fig 4A.** In addition, levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, were tested as shown in **Fig 5**. Compared with the level from normal donors, patients with RA showed increased levels of IL-6 and TNF-α. One time of UC-MSCs administration induced reduced levels of two pro-inflammatory factors (**Fig 5 A-B**). Another marker, RF, was also showed a decreased trend, particularly, at 3 months after the treatment (**Fig 4B**). Information from **Fig 4-5** indicated that UC-MSCs might clear up circulating inflammatory and other rheumatoid-related factors.

Although there were no significant changes in the percentages of $CD3^+$ cells, $CD4^+$ cells and $CD8^+$ cells before and after treatment (data not shown), the percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells in peripheral blood was significantly increased after DMARDs plus UC-MSCs treatment (by 25 % in 3 months, by 34% in 6 months, by 14% in 8 months) in **Fig 6A** and repeated infusion maintained this level (from 3.78% to 5.12%, then to 5.81% in **Fig 6B**). In addition, the change of regulatory T cells was found to be associated with the clinical benefits, indicating that the patients showing higher levels of regulatory T cells after UC-MSCs treatment may achieve better clinical benefits (**Fig 6C**). Furthermore, the level of IL-4 was increased, which was in consistent with the decreased ratio of Th1/Th2 cells (**Fig 6D**).

Discussion

Core message from current study is that DMARDs plus UC-MSCs administration was safe and effective in reducing disease activity for a long period in patients with refractory RA than controls receiving DMARDs plus medium without UC-MSCs. No major toxicities were observed during and after UC-MSCs administration. In UC-MSCs group, evidence of clinical benefits were obtained and the improvements of clinical manifestations were likely related to the decreased expression levels of various inflammatory cytokines and chemokines, the increased percentage of regulatory T cells in peripheral blood, and the upregulated IL-4-producing Th2 cells, suggesting anti-inflammation along with the improved immune-modulation and the induced immune-tolerance are likely to be major potential mechanisms.

RA, the pathogenesis of which is still unclear, can not only lead to joints deterioration, but also cause damages to multiple tissues and organs [1]. Joints damages cannot be repaired by the traditional medications, which have been used to treat RA patients for many years despite of their obvious and unavoidable side effects [5]. Most of those patients got relapse and progress after certain period of disease stability. Thus, it is critical and necessary to find a new method to improve the therapeutic outcomes.

A large number of active cytokines, such as TNF-α [25], IL-1 [26] and IL-6 [27], have been found in the joints of RA patients, which may influence the disease processes and result in articular damages and the co-morbidities of RA [28]. In recent decades, targeted therapy has been developed, for instance, the etanercept (TNF-α competitive inhibitor) [5] and infliximab (TNF-α monoclonal antibodies) [29] were used to treat RA

patients. However, Giles [30] found that TNF-α inhibitor could make patients more susceptible to surgical infections without exerting a positive effect on joint repair and cessation of therapy may increase the disease activity [6].

MSCs have been reported to have the capacity of modulating immune responses and healing damaged tissues and organs. Previous studies [15-17,31] have found that MSCs could be a new effective therapeutic approach for autoimmune arthritis [17]. However, MSCs from RA patients could not maintain high clonogenic potential and proliferative capacity as normal MSCs [32], making allogenic MSCs as a possible way to help defective self-MSCs to achieve clinical benefits. With distinct advantages of UC-MSCs including accessibility, higher proliferation capacity and lower immunogenicity [19] when compared to bone marrow derived-MSCs, UC-MSCs were chosen in this clinical trial.

UC-MSCs were well tolerated as described in previous reports [33] by showing no anaphylaxis and no severe gastrointestinal side effects. In our study, no major abnormalities were observed in serum chemical profiles, including liver and kidney functions during or after the UC-MSCs treatment, revealing that UC–MSCs infusion was safe and feasible to treat active RA.

Rapid clinical response to UC-MSCs treatment was also noticed in this trial. The joint pain, swelling and stiffness in patients were relieved within 12 h post-treatment. The possible mechanism may be that MSCs can chemotaxis to the damaged organ rapidly and accumulate there [34-37], subsequently secrete several soluble immunosuppressive factors constitutively and express a variety of receptors for

inflammatory factors under the state of inflammation [12]. In UC-MSCs group, the disease activity was reduced significantly assessing by DAS28 compared to the condition before treatment. Moreover, clinical utility was defined by ACR20 response and the higher magnitude responses (ACR50 and ACR70) appeared after the first treatment. In addition, the hematology profiles returned to normal level and autoantibodies titers also declined. We further observed that UC-MSCs treatment increased the patients' compliance to DMARDs by alleviating the side effects of these drugs. The second cycle of treatment resulted in better clinical benefits and improved the quality of life more obviously for RA. These data suggested that RA patients, showing refractory to traditional treatments, may achieve significant improvements after UC-MSCs treatment. It was inferred that the response rate of MSCs was not related to the donor HLA-match. As it has been proven that the response rate of MSCs for treatment of steroid-resistant, severe, acute graft-versus-host disease was not related to the donor HLA-match [33]. They have investigated immune responses to allogeneic MSC infused into HSCT recipients. The recipients given MSCs showed no response to infused MSCs before and up to 6 months after infusion, the infused MSCs are only weakly immunogenic in humans and validate the clinical use of MSCs from HLA-mismatched donors. It has been shown that patients infused with MSCs that are HLA haploidentical or completely HLA mismatched with the stem cell donor and recipient show no immunological memory to the infused MSCs [38]. Undifferentiated and differentiated MSCs do not elicit allo-reactive lymphocyte proliferative responses and modulate immune responses. So MSCs can be transplantable

between HLA-incompatible individuals [39].

It was known that the cytokines arising from numerous synovial cells were central to RA pathogenesis [1], and MSCs can express various receptors for inflammatory factors [40] which might combine with the corresponding inflammatory factors to reduce inflammation in RA patients. Immunoregulatory role of MSCs may associate with the occurrence of inflammatory mediators [41]. In this study, the serum levels of TNF-α and IL-6 significantly decreased in parallel with the deduction of serum C-reactive protein after treatment, indicating anti-inflammation was one of the major mechanisms of MSCs. Furthermore, MSCs were recently reported to suppress effector T cells and inflammatory responses and have emerged as attractive therapeutic candidates for immune disorders [16]. UC-MSCs administration in this study significantly increased IL-4 expression secreted by Th2 cells and the percentage of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells in peripheral blood. The increased level of regulatory T cells was positively correlated with the improvement of disease status, especially the ACR responses, which made the level of regulatory T cells to be one of the important clinical indices for the evaluation of the efficacy of UC-MSCs treatment [17]. In addition, MSCs might provide a multitude of trophic factors with various properties, thereby reducing tissue injury, protecting tissue from further degradation and thus enhancing tissue repair [42].

This is the first investigation of the safety and efficiency of UC-MSCs in treatment of RA patients. However, there is one limitation of current study: all patients were recruited and treated from single center. Therefore, a larger multiple center study will be

necessary to further confirm current finding. In addition, relevant joint imaging data before and after MSC infusion should be collected and analyzed. In spite of these, this study was also valuable, since all patients were from the failed traditional medication treatment and obtained significant improvements including symptom alleviation and cytokines decrease after UC-MSCs treatment.

Overall, our study confirmed the safety and efficacy of UC-MSCs infusion in active RA patients. The therapeutic effects can maintain for at least 3 months, and repetitive treatment would stabilize the clinical outcomes and improve the patients' quality of life, which was significantly correlated with the increased percentage of regulatory T cells in peripheral blood. Thus, UC-MSCs are suitable application in the clinic and provide an additional choice to many RA patients.

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Author Disclosure Statement

No competing financial interests exist.

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Figure 1. Scores of HAQ, DAS28, CRP, RF and regulatory T cells before and after 3-month treatment between DMARDs plus medium without UC-MSCs and DMARDs plus UC-MSCs groups. A. HAQ score was evaluated; **B.** DAS28 score was determined; **C.** Serum level of CRP was evaluated; **D.** Serum level of RF was determined; **E.** Percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells of $CD4^+$ T cells was tested. Open column represents the value before treatment and dark column means the data after treatment. *$p<0.05$, before *vs.* after treatment; $^{\#}p<0.01$, before *vs.* after treatment; $^{\Psi}p<0.05$, after the first treatment *vs.* after the second treatment; $^{\&}p<0.01$, after the first treatment *vs.* after the second treatment. n=36 in DMARDs plus medium without UC-MSCs group; n=58 in DMARDs plus UC-MSCs group.

Figure 2. Scores of HAQ and DAS28 were evaluated after twice of UC-MSCs treatment. A. HAQ score was evaluated; **B.** DAS28 score was evaluated. Open column represents the value before the treatment; gray column represents the data after the first treatment; dark column means the data after the second treatment. *$p<0.05$, $^{\#}p<0.01$ before treatment *vs.* after the first or second treatment; $^{\Psi}p<0.05$, after the first treatment *vs.* after the second treatment; $^{\&}p<0.01$, after the first treatment *vs.* after the second treatment (n=24).

Figure 3. Scores of HAQ, DAS28 and ACR20, 50, 70 response rates at three groups in DMARDs plus UC-MSCs group respectively. A. HAQ score was evaluated before and after first UC-MSCs treatment in three groups; **B.** DAS28 score was evaluated;

open column represents the value before the first UC-MSCs treatment; dark column means the data after the first UC-MSCs treatment. $^{\#}p<0.01$, before *vs*. after treatment; **C.** Proportion of patients with American College of Rheumatology 20%, 50%, 70% response rates in three groups after the first UC-MSCs treatment. Group 1: 3 months after the first UC-MSCs treatment (n=76); group 2: 6 months after the first UC-MSCs treatment (n=45); group 3: over 8 months after the first UC-MSCs treatment (n=15).

Figure 4. CRP and RF levels were measured at three groups in DMARDs plus UC-MSCs group respectively. A. Serum level of CRP; **B.** Serum level of RF. $^{\#}p<0.01$ before *vs.* after treatment. Open column represents the value before the first UC-MSCs treatment; dark column means the data after the first UC-MSCs treatment. Group 1: 3 months after the first UC-MSCs treatment (n=76); group 2: 6 months after the first UC-MSCs treatment (n=45); group 3: over 8 months after the first UC-MSCs treatment (n=15).

Figure 5. Cytokine milieu of the serum from patients with RA was measured at 1 week, 3 months, and 6 months in DMARDs plus UC-MSCs group respectively. A. Serum level of IL-6; **B.** Serum level of TNF-α. Open column represents the value before the first UC-MSCs treatment; dark column means the data after the first UC-MSCs treatment. 1 W means 1 week after the first UC-MSCs treatment (n=41); 3 M means 3 months after the first UC-MSCs treatment (n=30); 6 M means 6 months after the first UC-MSCs treatment (n=34). $^{*}p<0.05$, $^{\#}p<0.01$, before *vs.* after treatment.

Figure 6. Percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells to total $CD4^+$ T cells in peripheral blood, the relationship between changes of ACR response and regulatory T cells percentage, and Th1 and Th2-type responses measuring by IFN-γ and IL-4. A. The percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells of $CD4^+$ T cells**;** gray column represents normal value; open column means the data before the treatment; dark column means the data after the first treatment. *p<0.05, before *vs.* after the first treatment. Group 1: 3 months after the first treatment (n=34); group 2: 6 months after the first treatment (n=19); group 3: over 8 months after the first treatment (n=6). **B.** Percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells to total $CD4^+$ T cells before and after first and second treatments; n=12, open column represents the value before the treatment; gray column represents the data after the first treatment; dark column means the data after the second treatment. **C.** Correlation between changes of regulatory T cells and ACR response rates; *p<0.05, the ACR rates of increased regulatory T cells patients (n=26) *vs.* the ACR rates of decreased regulatory T cells patients (n=21). **D.** Effects of UC-MSCs on Th1 and Th2-type responses measured by IFN-γ and IL-4. 3 M represents group 1 after the first UC-MSCs treatment (n=21), over 6 M represents group 2 and group 3 after the first UC-MSCs treatment (n=20). *p<0.05, before *vs.* after treatment. Open column represents the value before the treatment; dark column means the value after the treatment in group 2 and group 3 in DMARDs plus MSCs group.

Schema 1

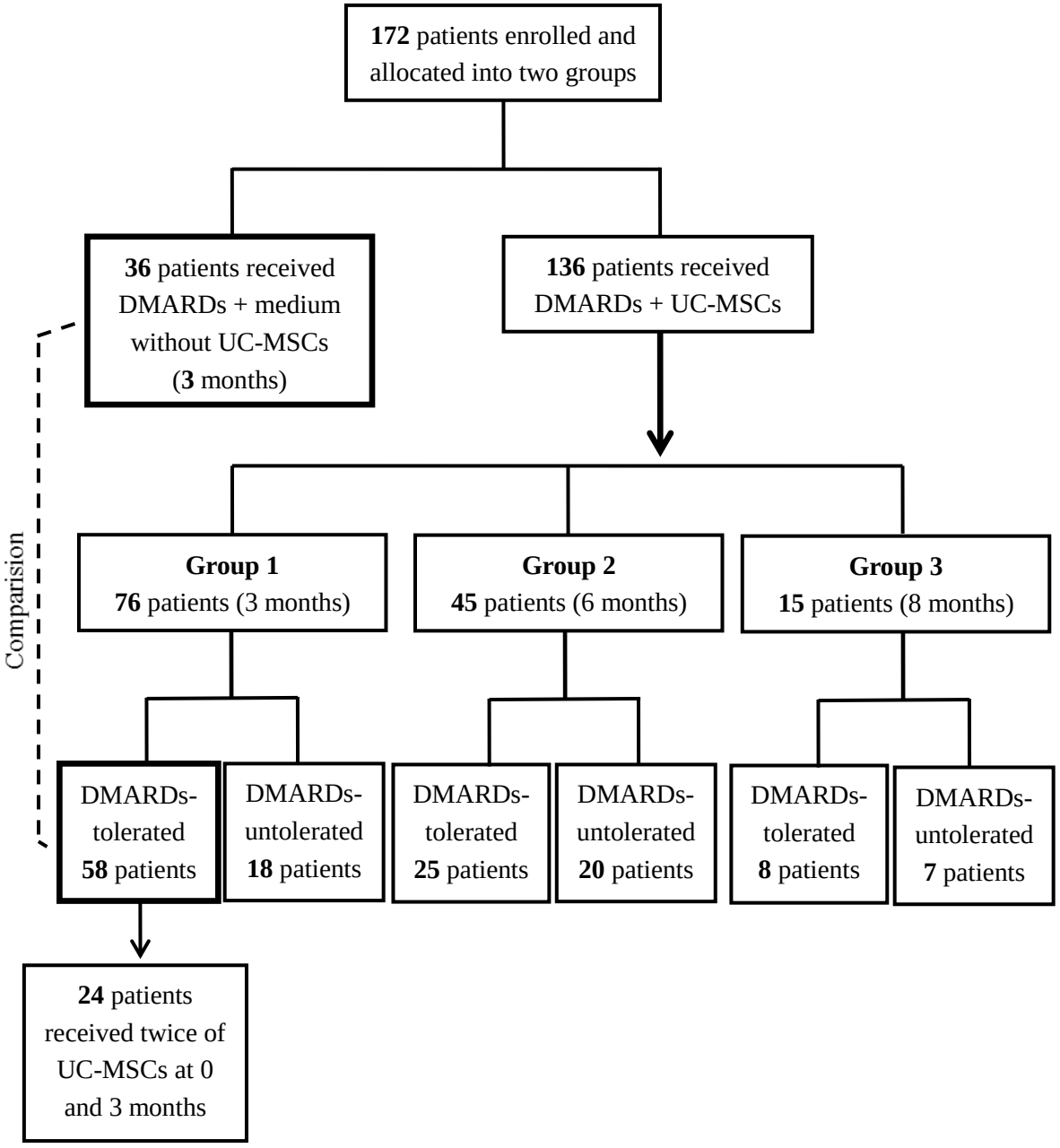


Table 1. Demographic and clinical characteristics of Patients in DMARDs plus UC-MSCs Group before the Treatment.

	Group 1	Group 2	Group 3
Patients No.	76	45	15
Female No. (%)	68 (89)	44 (98)	15 (100)
Mean age (yr)	47.0	44.6	46.2
Duration of disease No. (%)			
≤2 yr	15 (20)	4 (9)	1 (7)
2–5 yr	17 (22)	11 (24)	3 (20)
>5 yr	44 (58)	30 (67)	11 (73)
Disease status			
HAQ§	0.71	0.64	0.58
DAS 28#	5.75	5.55	5.31
Previous Medication No. (%)			
DMARDs*	59 (78)	25 (56)	8 (53)
Biologics	9 (12)	1 (2)	1 (7)
NSAIDs†	76 (100)	45 (100)	15 (100)
Steroids	25 (33)	9 (20)	4 (27)

HAQ§: the Health Assessment Questionnaire; DAS 28#: the 28-joint disease activity score; DMARDs*: disease-modifying antirheumatic drugs; NSAIDs†: nonsteroidal anti-inflammatory drugs.

Table 2. Safety Evaluation on Patients in DMARDs plus UC-MSCs Group.

Measures (normal value range)	Before Treatment	After Treatment
Total protein (60-80g/L)	71.07 ± 8.01	68.87 ± 8.71$^{\#}$
Albumin (40-55g/L)	38.79 ± 4.69	40.15 ± 6.16*
Globulin (20-40g/L)	32.23 ± 7.87	28.79 ± 7.29*
Cholesterol (2.86-5.98mmol/L)	4.17 ± 0.87	4.29 ± 0.95
Triglyceride (<1.7mol/L)	1.34 ± 0.69	1.37 ± 0.69
Creatinine（45-104 μmol/L）	45.21 ± 12.26	48.30 ± 15.17$^{\#}$
Blood urea nitrogen (1.43-7.14 mmol/L)	5.15 ± 1.70	5.19 ± 2.66
Fasting blood glucose (3.15-6.19mmol/L)	4.76 ± 1.20	4.73 ± 0.89
White blood cell (4-10)×10^{9}	6.79 ± 4.56	5.99 ± 2.03
Hemoglobin (110-150g/L)	104.24 ± 19.11	109.01 ± 14.50$^{\#}$
Platelet (100-300)×10^{9}	261.38 ± 93.43	232.61 ± 86.87$^{\#}$

Value: Mean ± SD, t-test, $^{*}p<0.05$, $^{\#}p<0.01$, n=136

Table 3. Safety Evaluation on Patients between DMARDs plus medium without UC-MSCs and DMARDs plus UC-MSCs

Measures (normal value range)	DMARDs+MEDIUM		DMARDs+UC-MSCs	
	Before Treatment	After Treatment	Before Treatment	After Treatment
Total protein (60-80g/L)	70.15±3.36	70.30±3.55	70.55±8.25	68.55±10.46
Albumin (40-55g/L)	38.18±4.52	37.61±4.22	38.21 ±4.64	39.69±4.95
Globulin (20-40g/L)	31.97±4.23	32.69±3.99	32.35 ±8.52	29.42±8.14$^{\#}$
Cholesterol (2.86-5.98mM)	4.17 ±1.13	4.19±1.21	4.27 ±0.85	4.37±0.97
Triglyceride (<1.7mol/L)	1.51±0.74	1.49±0.76	1.51±0.71	1.51±0.77
Creatinine(45-104 μM)	45.44±12.20	45.97±8.38	46.32±11.70	50.72±16.11*
Blood urea nitrogen (1.43-7.14 mM)	5.11±1.42	5.15±1.51	5.21±1.73	5.39±1.73
Fasting blood glucose (3.15-6.19mM)	4.80±0.98	4.81±0.80	4.71±0.98	4.66±0.90
White blood cell (4-10)×10^{9}	6.19±1.47	5.96±1.04	6.32±1.89	6.00±2.18
Hemoglobin (110-150g/L)	103.81±19.09	103.56±16.39	105.09±18.71	112.09±14.50$^{\#}$
Platelet (100-300)×10^{9}	252.33±76.83	241.25±65.75	265.88±90.96	222.88±97.23$^{\#}$

Value: Mean ± SD, t-test, $^{*}p<0.05$, $^{\#}p<0.01$.

DMARDs plus medium without UC-MSCs: n=36; DMARDs plus UC-MSCs: n=58.

Figure 1

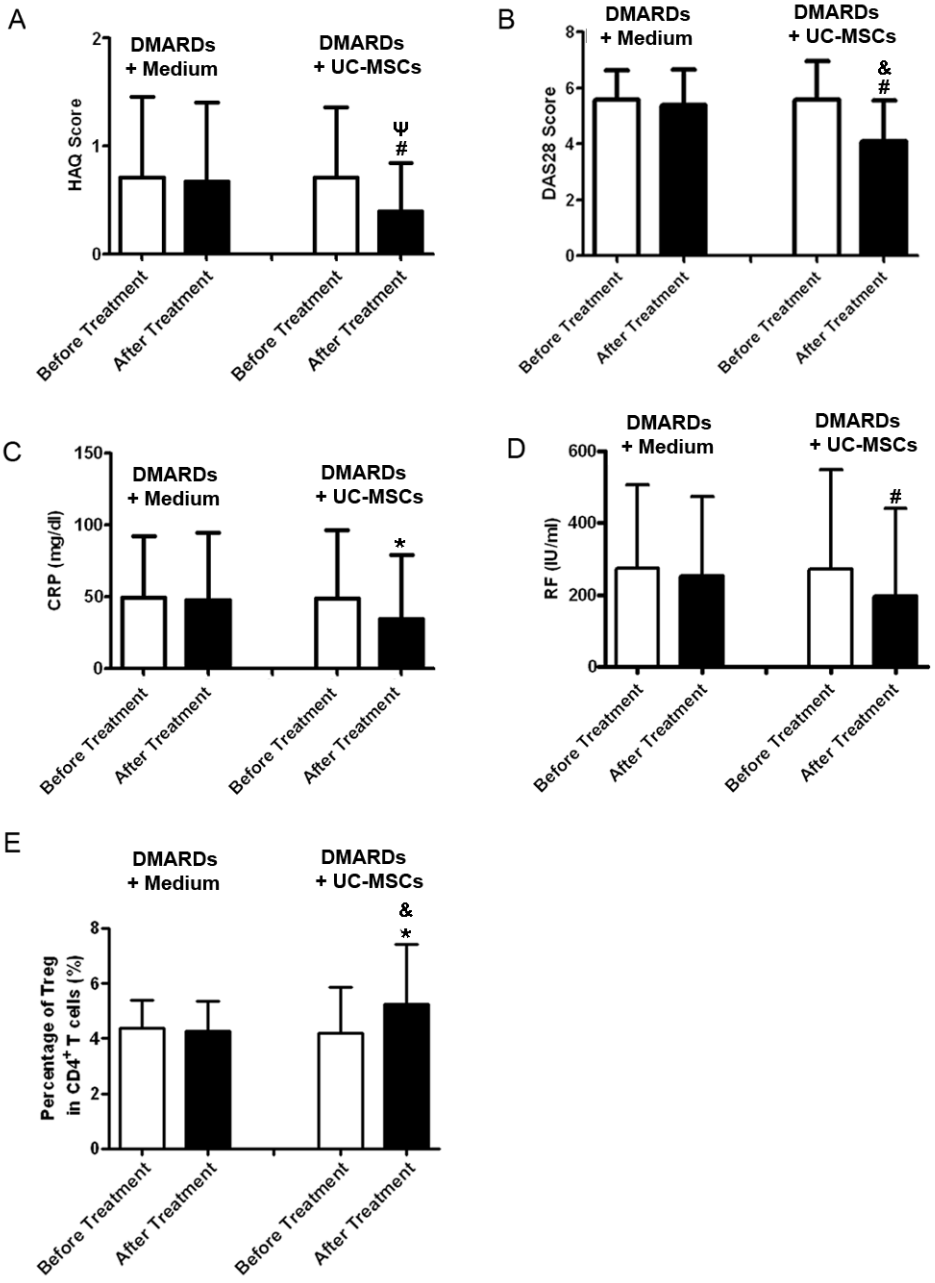


Figure 2

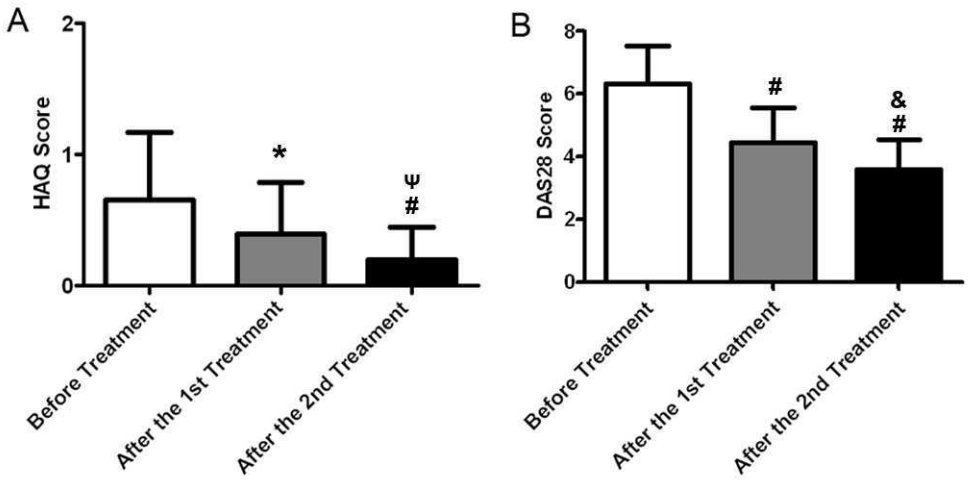


Figure 3

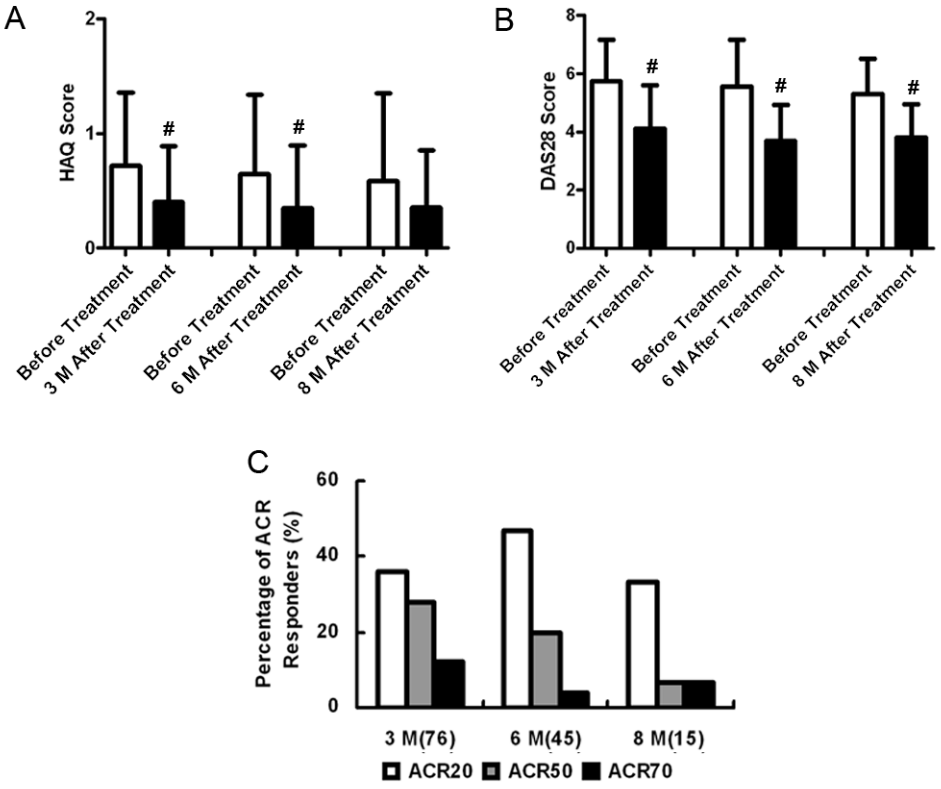


Figure 4

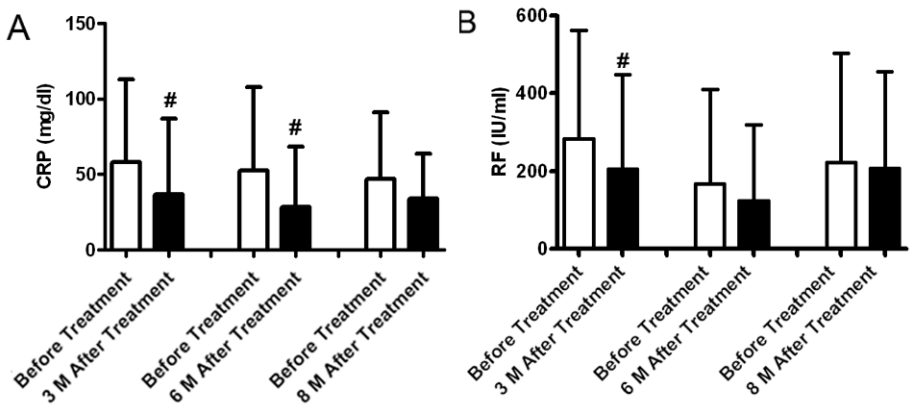


Figure 5

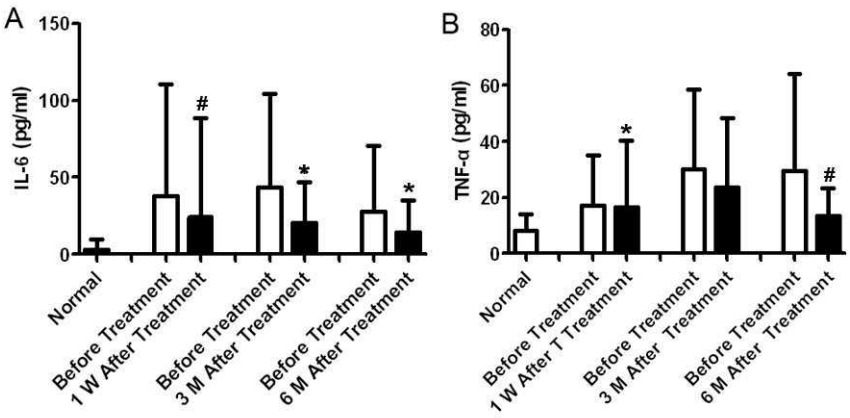
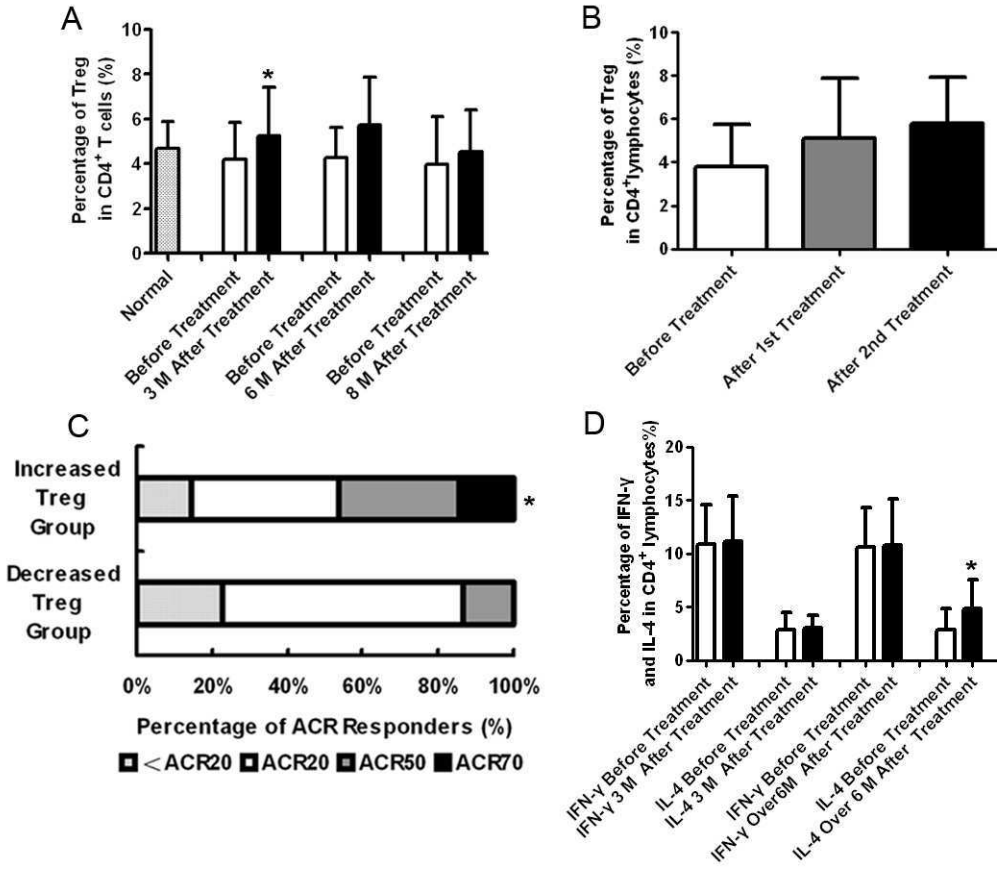


Figure 6



Methods for Supplementary Experiments:

1. **The percentage of B cells:** Peripheral blood samples from responders and non-responders of RA patients were collected before and after UC-MSCs treatment. The percentage of B cells were conducted based on the instruction provided by BD Multitest™ IMK kit. Data was acquired and analyzed by FACS Caliber (Becton Dickinson).

2. **Cytokine levels in peripheral blood mononuclear cell:** Cytokine levels (TNF-α and IL-6) of peripheral blood mononuclear cell samples were tested from patients with DMARDs plus medium without UC-MSC or plus UC-MSCs as described in previous report [1,2]. In detail, EDTA-blood was collected from patients and healthy controls before treatment and, 1 week, and 3 months after the treatment. PBMCs were isolated by Ficoll gradient centrifugation. The cells were then washed for three times with pyrogen free phosphate buffered saline (PBS) and suspended in RPMI 1640 medium containing 5% fetal calf serum, 50 μM β-mercaptoethanol, 25 mM Hepes, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. PBMCs were stimulated in a concentration of 2×10^5 cells/200 μl/well in 96-well plate with 10 μg/ml phytohaemagglutinin (PHA) for 24 hours in U bottomed plates at 37˚C. Supernatants and cells were collected 24 hours after the stimulation and the samples were analyzed TNF-α and IL-6 by Enzyme linked immunosorbent assay (ELISA).

Results for Supplementary Experiments:

Peripheral blood samples from responders and non-responders of RA patients were collected before and after UC-MSCs treatment. There were no significant different of percentage of B cells among groups (**S-Fig1**).

Cytokine levels of peripheral blood mononuclear cell samples from patients with DMARDs plus medium without UC-MSC or plus UC-MSCs were detected. The data showed that DMARDs plus UC-MSCs significantly reduced levels of TNF-α and IL-6, 1 week and 3 months after the treatment. In comparison, DMARDs plus medium without UC-MSC did not receive such achievement (**S-Fig2**).

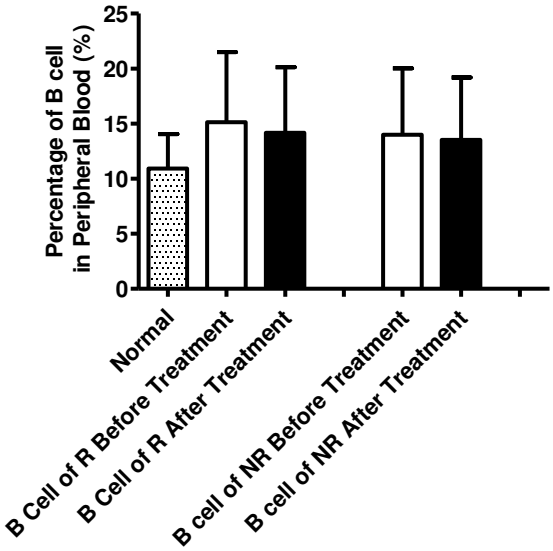
Supplementary Figure Legends

S-Figure 1. The percentage of B cells before and after UC-MSCs treatment. There was no significant difference between before and after UC-MSCs treatment from responders and non-responders on the percentages of B cells in peripheral blood by flow cytometry. Open column means the data before the treatment and dark column means the data after the treatment [R (responders, n=57); NR (non-responders, n=19)].

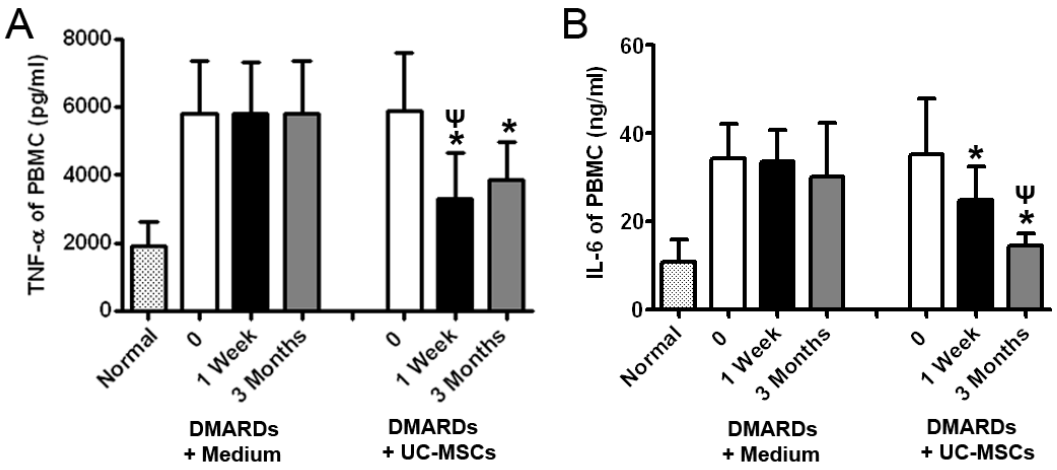
S-Figure 2. Cytokine secretion by peripheral blood mononuclear cell from patients with DMARDs plus medium without UC-MSC or plus UC-MSCs before and after treatment. A. TNF-α secretion after 24 h of incubation with PHA; **B.** IL-6 secretion after 24 h of incubation with PHA. Open column represents the value before the treatment; dark column means 1 week after treatment; gray column means 3 months after treatment. (DMARDs + medium without UC-MSCs: n=6; DMARDs + UC-MSCs: n=6, $^{*}p<0.05$ before treatment *vs.* after 1 week or 3 months treatment analyzed by the Wilcoxon signed rank test; $^{\Psi}p<0.05$ after treatment between two groups analyzed by Mann-Whitney test).

Supplementary Figures

S-Figure 1



S-Figure 2



Supplementary references:

1. Nissinen, R., et al., *Cytokine and chemokine receptor profile of peripheral blood mononuclear cells during treatment with infliximab in patients with active rheumatoid arthritis.* Ann Rheum Dis, 2004. 63(6): p. 681-7.
2. Ruschen, S., W. Stellberg, and H. Warnatz, *Kinetics of cytokine secretion by mononuclear cells of the blood from rheumatoid arthritis patients are different from those of healthy controls.* Clin Exp Immunol, 1992. 89(1): p. 32-7.