

A Pilot Study of Mesenchymal Stem Cell Therapy for Acute Liver Allograft Rejection

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ABSTRACT

Acute allograft rejection remains common after liver transplantation despite modern immunosuppressive agents. In addition, the long-term side effects of these regimens, including opportunistic infections, are challenging. This study evaluated the safety and clinical feasibility of umbilical cord-derived mesenchymal stem cell (UC-MSC) therapy in liver transplant patients with acute graft rejection. Twenty-seven liver allograft recipients with acute rejection were randomly assigned to the UC-MSC infusion group or the control group. Thirteen patients received one infusion of UC-MSCs (1×10^6 /kg body weight); one patient received multiple UC-MSC infusions; 13 patients were used as controls. All enrolled patients received conventional immunosuppressive agents with follow-up for 12 weeks after UC-MSC infusions. No side effects occurred in treated patients. Four weeks after UC-MSC infusions, alanine aminotransferase levels had decreased markedly and remained lower throughout the 12-week follow-up period. Importantly, allograft histology was improved after administration of UC-MSCs. The percentage of regulatory T cells (Tregs) and the Treg/T helper 17 (Th17) cell ratio were significantly increased 4 weeks after infusions; in contrast, the percentage of Th17 cells showed a decreasing trend. In controls, the percentages of Tregs and Th17 cells and the Treg/Th17 ratio were statistically unchanged from the baseline measurements. Transforming growth factor beta 1 and prostaglandin E2 were increased significantly after UC-MSC infusions; by contrast, there were no significant changes in controls. Our data suggest that UC-MSC infusion for acute graft rejection following liver transplantation is feasible and may mediate a therapeutic immunosuppressive effect. *STEM CELLS TRANSLATIONAL MEDICINE* 2017;6:2053–2061

SIGNIFICANCE STATEMENT

This study aimed to evaluate the safety and clinical feasibility of umbilical cord-derived mesenchymal stem cell (UC-MSCS) therapy in liver transplant patients with acute graft rejection. No complications or side effects were observed. UC-MSC therapy can alleviate liver damage and improve allograft histology. Furthermore, the peripheral frequency of regulatory T cells (Tregs), the Treg/T helper 17 (Th17) cell ratio, and the levels of serum transforming growth factor beta 1 and prostaglandin E2 were significantly increased after UC-MSC transfusion.

INTRODUCTION

Currently available immunosuppressive agents have contributed to reducing acute rejection episodes and improving graft function following liver transplantation; however, recipients must usually adhere to a lifelong immunosuppressive regimen. Beside the toxicities of such regimens, such as the renal toxicity of cyclosporine and the neurotoxicity of tacrolimus, the risk for opportunistic infections and malignancies is markedly increased in organ transplant recipients receiving chronic immunosuppressive therapy [1]. Despite the use of potent agents, approximately 20%–40% of recipients suffer allograft rejection. Novel immunosuppression induction and maintenance protocols

with increased efficacy and minimal adverse effects are desirable.

Immunomodulatory cell-based therapies are emerging as innovative treatment options to promote the acceptance of solid organ allografts while potentially reducing the side effects associated with pharmacologic immunosuppression. Mesenchymal stem cells (MSCs) are multipotent progenitors present in bone marrow in both adult and fetal tissues [2, 3]. MSCs have regenerative, anti-inflammatory, and immunomodulatory properties achieved by regulating innate and adaptive immune responses, inhibiting the proliferation and function of T, B, and natural killer (NK) cells and the maturation of dendritic cells (DCs), and inducing the generation of regulatory T cells

Table 1. Characteristics of the enrolled patients

Parameters	UC-MSCs infusion (n = 14)	Control (n = 13)
Age at transplantation (years)	57 ± 12	55 ± 11
Gender (M/F), n (%)	13 (92.9)/1 (7.1)	12 (92.3)/1 (7.7)
ALT (U/L)	165 ± 69	158 ± 34
AST (U/L)	104 ± 70	94 ± 58
ALP (U/L)	280 ± 169	272 ± 149
GGT (U/L)	438 ± 307	583 ± 115
MELD (mean ± SD)	18 ± 8	18 ± 10
Blood transfusion volume (mean ± SD, mL)	1,483 ± 837	1,222 ± 908
RBC (mL)	917 ± 529	793 ± 624
FFP (mL)	417 ± 359	352 ± 285
PLT (mL)	150 ± 151	77 ± 101
Primary disease, n (%)		
CHB-related decompensated liver cirrhosis	8 (57.1)	6 (46)
CHB-related HCC	3 (21.4)	3 (23)
alcoholic liver cirrhosis	2 (14.3)	2 (15.4)
alcoholic liver cirrhosis + HCC	0	1 (7.7)
CHB+ alcoholic liver cirrhosis	1 (7.1)	0
PBC	0	1 (7.7)
Acute rejection (Banff), n(%)		
RAI: 3–4	6 (42.8)	6 (46)
RAI: 5–6	6 (42.8)	5 (38.4)
RAI: > 6	2 (14.3)	2 (15.4)
Opportunistic infection, n(%) (24-week follow-up)		
Cytomegalovirus	0	1 (7.7)
EB virus	0	1 (7.7)

The demographic parameters at baseline between the UC-MSCs infusion group and the control group are similar.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHB, chronic hepatitis B; EB, Epstein–Barr; F, female; FFP, freshly frozen plasma; GGT, gamma-glutamyl transpeptidase; HCC, hepatocellular carcinoma; M, male; MELD, model of end-stage liver disease; PBC, primary biliary cirrhosis; PLT, platelet; RAI, rejection activity index; RBC: red blood cells; UC-MSC, umbilical cord-derived mesenchymal stem cell.

(Tregs) [4, 5]. Some of these effects are mediated by soluble factors such as transforming growth factor beta (TGF- β) and prostaglandin E2 (PGE2) [4, 5]. Because of their immunosuppressive properties, MSCs are believed to play a role in the maintenance of peripheral tolerance and the induction of transplantation tolerance, and they are considered potential candidates for cellular therapy for graft versus host disease (GVHD) and autoimmune diseases, and for the prevention of transplant rejection [6]. Thus, MSCs offer new therapeutic opportunities to prevent and treat solid organ transplant rejection [7].

The *in vivo* immunomodulatory properties of MSCs were first described in a baboon model of skin transplantation [8]. In an allogeneic liver transplant rat model, it was demonstrated that MSCs inhibited acute rejection of allografts by expanding the circulating CD4⁺CD25⁺Foxp3⁺ Treg population in recipients [9]. In clinical trials, autologous MSCs have been used for induction therapy in

kidney transplant recipients [10, 11]. Recent reviews have investigated the rationale and potential use of MSC therapy in liver transplantation [12, 13]. A recent case study demonstrated that intraportal and intravenous infusion of multipotent adult progenitor cells after living-relative liver graft transplantation is clinically feasible [14]. However, clinical trials of umbilical cord derived-MSC (UC-MSC) therapy in liver transplantation have not been conducted. Our study aimed to examine the safety and clinical feasibility of UC-MSC infusion as a therapeutic option for liver allograft rejection.

MATERIALS AND METHODS

Patients

Twenty-seven patients undergoing their first cadaveric liver transplantation with an identical or compatible blood group graft were enrolled for this study. All patients received conventional immunosuppressive agents after liver transplantation, such as tacrolimus, corticosteroids, or mycophenolate mofetil (MMF), according to our center's practice guidelines and experience. Patients were considered to have acute rejection and were suitable for enrollment in the study if liver function tests did not respond to adjustment of immunosuppression, or there was liver damage showing recurrent rejection despite adjustments in the immunosuppression regimen. All recruited patients were randomly assigned into either the UC-MSC treatment group or the control group. Fourteen participants were treated with conventional immunosuppressive agents plus UC-MSCs, and 13 were treated with conventional immunosuppressive agents as controls. Patients in the following situations were excluded from the study: systemic infection; presence of severe renal, respiratory, or cardiac disease; lack of a supportive family; and unwillingness to sign informed consent. The characteristics of the enrolled patients are shown in Table 1. All the patients were followed up for 12 weeks. This clinical study was registered at the ClinicalTrials.gov site of the US National Institutes of Health (NCT01690247) and authorized by the General Logistic Ministry of Health, China. All participants provided written informed consent for participation in the study. No subjects refused to sign informed consent documents or dropped out during the clinical trial.

UC-MSC Preparation and Infusions

UC-MSCs were prepared in an approved good manufacturing practices (GMP)-compliant facility and identified as described previously [15]. In brief, with the written consent of maternity patients, fresh human umbilical cords were obtained after birth and collected in cold α -minimal essential medium (α -MEM; Gibco Invitrogen, Carlsbad, CA). The UC-MSCs were cultured and collected between the third and fourth passages for infusions and identified based on their capability for osteogenesis and adipogenesis and by flow cytometric analysis (these cells highly expressed CD44, CD90, CD73, and CD105 but did not express CD31, CD34, CD45, or HLA-DR; supplemental online Fig. 1). The UC-MSCs were negative for all tested contaminants before infusion, including *Mycoplasma*, Gram-positive and Gram-negative bacteria, and fungi. Endotoxin levels were below 5 EU/kg and viability was >80%. Freshly cultured UC-MSCs were infused in some patients; in others, frozen cells were thawed then cultured for 4–5 days to about 95% confluence, then prepared for infusion. Approximately 1.0 \times 10⁶/kg body weight UC-MSCs at the fourth

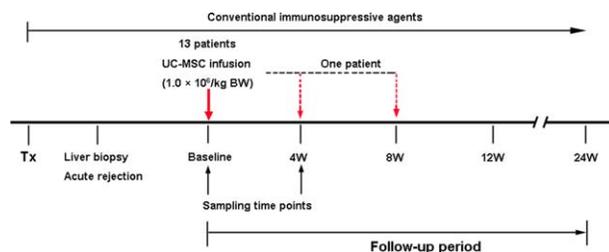


Figure 1. Protocol used for liver transplant recipients with acute allograft rejection in this study. All patients received conventional immunosuppressive agents. In addition, an UC-MSC infusion was given once to 13 patients. One patient who exhibited an abnormal alanine aminotransferase level and did not respond efficiently to immunosuppressive agents was given UC-MSCs three times (indicated with dotted lines) based on physician and patient preference. This regimen was allowed within the approved protocol. Clinical parameters were determined at baseline and at 4, 8, and 12 weeks during the follow-up period. Abbreviations: BW, body weight; Tx, transplantation; UC-MSC, umbilical cord-derived mesenchymal stem cell; W, weeks.

passage were suspended in saline and infused intravenously. One patient received three UC-MSC infusions at 4-week intervals.

Flow Cytometric Analysis

Peripheral blood samples were obtained from recipients 4 weeks after UC-MSC infusions. Flow cytometric analysis of Tregs and Th17 cells was performed as described previously [16]. Briefly, for immunostaining of intracellular interleukin (IL)-17A, two samples of freshly heparinized peripheral blood were incubated for 6 hours with phorbol-12-myristate-13-acetate and ionomycin (1 μ L/mL) in 800 μ L of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum. Monensin (0.4 μ M) was added during the first hour of incubation. Then, a Cytotfix/Cytoperm kit (BD Biosciences, San Jose, CA) and anti-CD3, anti-CD8, anti-IL-17, and anti-interferon gamma monoclonal antibodies (mAbs) were used with one sample and anti-CD4, anti-CD25, and anti-FoxP3 mAbs (clone PCH101, eBioscience, San Diego, CA) with the other sample, according to the manufacturers' protocols. For Treg analysis, anti-CD4, anti-CD25, and anti-histocompatibility leukocyte antigen (HLA)-DR mAbs were added to 200 μ L freshly heparinized blood samples and the samples were then permeabilized and fixed using a fixation/permeabilization kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. After permeabilization, cells were incubated with anti-FoxP3 mAb. The stained cells were examined on a FACSCalibur system (BD Biosciences) and the data analyzed using FlowJo software (FlowJo LLC, Ashland, OR).

Immunosuppression Regimen

All patients underwent an immunosuppressive regimen according to the center's practice, comprising basiliximab (20 mg/d i.v. during the procedure), corticosteroids (methylprednisolone 500–1,000 mg/d during the procedure then 200 mg/d on day 0 tapered by 40 mg/d to 40 mg/d, followed by prednisolone acetate p.o. 20 mg/d tapered by 5 mg/w to 5 mg/d and then to a maintenance dose of 5 mg/d for up to 3 months), MMF (0.75 g/d on day 1 post-transplantation), and tacrolimus (1 mg/d on day 2 post-transplantation with target trough blood levels of 8–12 ng/mL). Acute rejection episodes were treated by increasing the dose of tacrolimus or with methylprednisolone pulse therapy.

Enzyme-Linked Immunosorbent Assay

Blood plasma samples were collected before UC-MSC infusions and 4 weeks post-infusion. Levels of TGF- β 1 and PGE2 were examined using an enzyme-linked immunosorbent assay kit (BlueGene Biotechnology, Shanghai, China) following the manufacturer's instructions.

Histology and Immunohistochemistry

Paraffin-embedded sections of liver biopsy tissue were prepared and stained by hematoxylin and eosin (H&E), Van Gieson, Masson's trichrome (MTC), periodic acid–Schiff, reticulin, and immunohistochemical methods. Pathologists reviewing the biopsies were blinded to the study treatment groups.

Statistical Analysis

All data were analyzed using SPSS 13.0 for Windows software (IBM, Armonk, NY). Comparisons between individuals were made using the Mann-Whitney *U* test; comparisons within the same individual were made using the Wilcoxon matched pairs *t* test. Comparison of rates of histological improvement between two groups was analyzed using Fisher's exact test. For all tests, two-sided $p < .05$ was considered significant.

RESULTS

Safety of UC-MSC Infusions in Liver Transplant Recipients with Acute Rejection

The baseline characteristics of the patients are shown in Table 1. The most common primary disease in these recipients (14/27) was hepatitis B virus (HBV) infection-related decompensated liver cirrhosis. In clinical studies of UC-MSCs in liver transplantation, unwanted side effects of cell infusion must be assessed with the greatest care before planning large efficacy trials for acute rejection. In this study, we observed the patients for adverse events during 24 weeks of follow-up (Fig. 1), but blood samples were analyzed for 12 weeks. We monitored uric acid, creatinine, lactate dehydrogenase, and alkaline phosphatase levels before and after UC-MSC infusions and found that all parameters were within their respective normal ranges. No complications or side effects were observed in the UC-MSC treated patients during the 24-week follow-up period.

UC-MSCs Alleviate Liver Damage

To investigate the impact of UC-MSCs on liver damage with acute rejection, the liver damage parameters, namely the alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (GGT) levels, were monitored in all 27 patients throughout the 12-week follow-up period. ALT, AST, and TBIL were decreased significantly after UC-MSC infusions compared with the control over this time (Fig. 2). ALP and GGT showed downward trends after UC-MSC infusions; however, there was no statistical difference between the treatment and control groups. Furthermore, we examined histologic changes in liver allografts after UC-MSC infusions by H&E and MTC staining. Histologic improvements were observed in six patients (42.8%) 4 weeks after administration of UC-MSCs. No control patient was found with histologic improvement (Fig. 3A–3D, 3I, 3J). The rate of histologic improvement in the UC-MSC infusion group was significantly higher than that in the control group ($p = .016$). One patient's typical liver

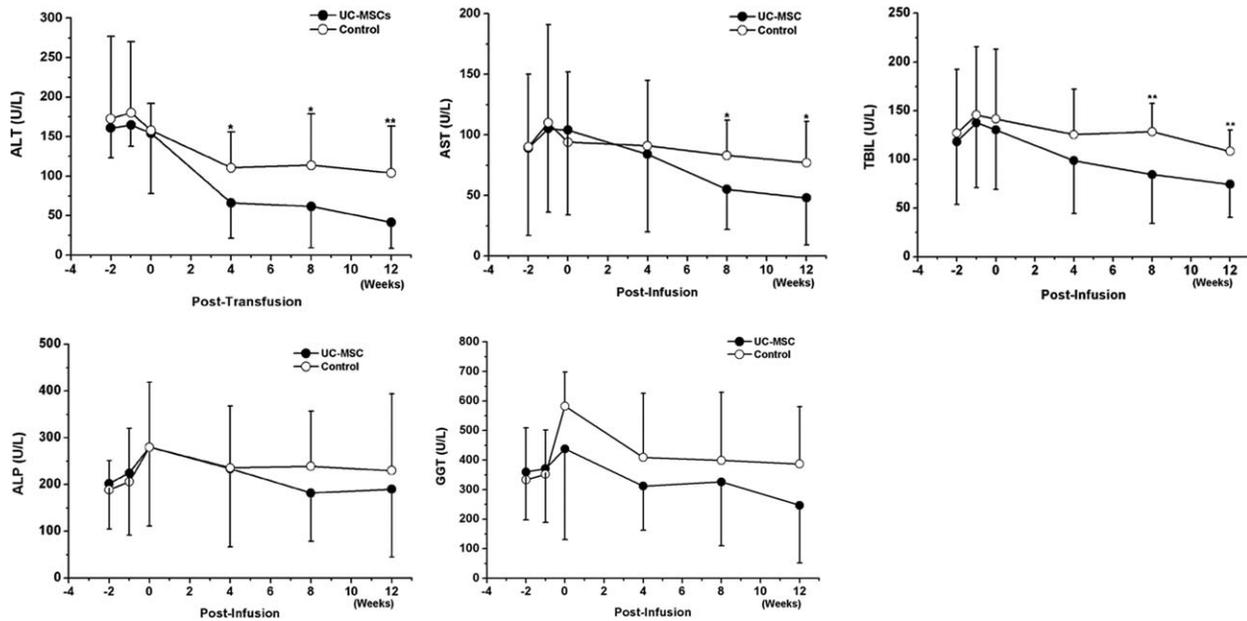


Figure 2. UC-MSCs alleviate liver damage in liver allograft recipients with acute rejection. ALT, AST, and TBIL levels decreased significantly after UC-MSC infusions ($n = 14$) compared with the control group ($n = 13$) during the 12-week follow-up period. ALP and GGT also showed downward trends after UC-MSC infusions; however, the treatment and control groups were not statistically different throughout the 12-week follow-up period. The error bars represent standard deviations. $*p < .05$, $**p < .01$ compared with UC-MSC therapy. Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; TBIL, total bilirubin; UC-MSC, umbilical cord-derived mesenchymal stem cell.

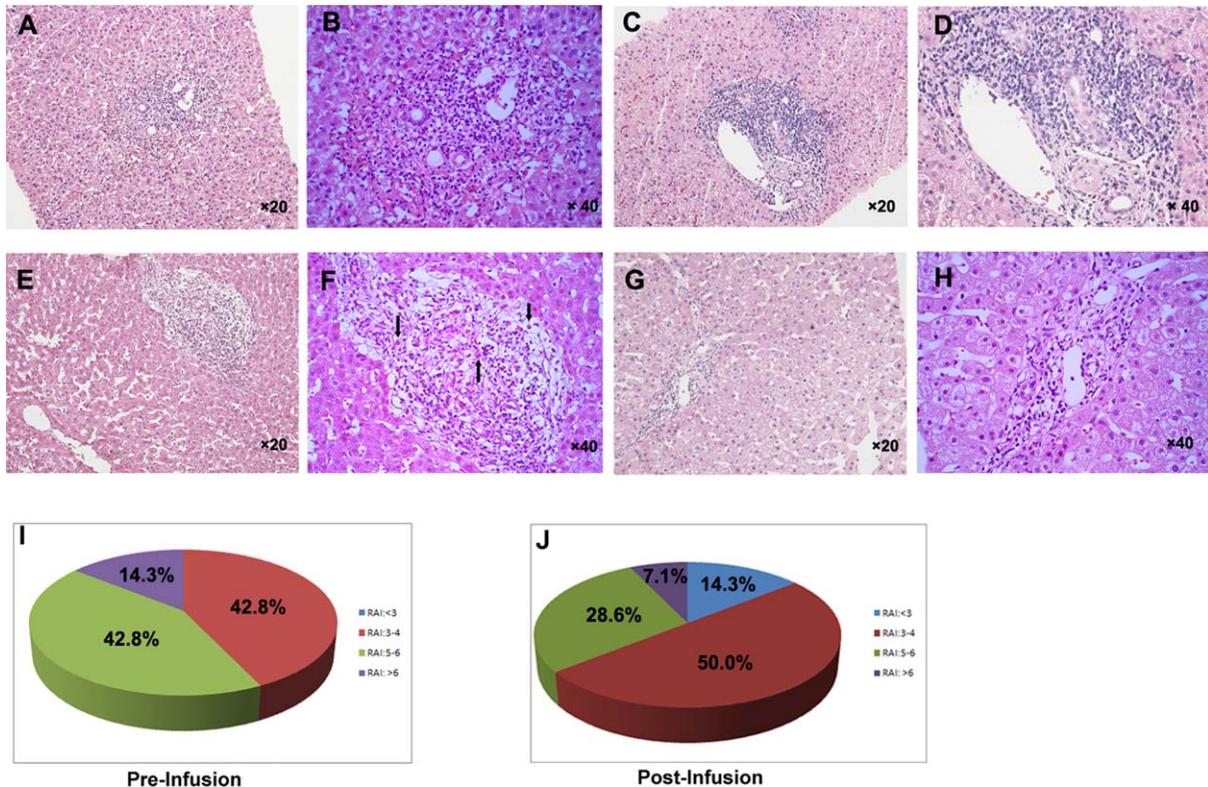


Figure 3. There were histologic improvements in liver allograft with acute rejection after umbilical cord-derived mesenchymal stem cell (UC-MSCs) infusions. Severe portal vein endotheliitis and bile duct damage in liver biopsy specimens were observed at baseline (RAIS:P1V2B2 = 5) (A, B) and at 4-week follow-up (RAIS:P2V2B2 = 6) (A, C): ×20 magnification; (B, D): ×40 magnification (C, D). (E, F): Severe portal vein endotheliitis and bile duct damage in liver biopsy specimen before UC-MSC therapy ([F]: arrow indicates eosinophils; RAIS:P2V2B2 = 6; [E]: ×20 magnification, [F]: ×40 magnification). (G, H): Mild portal inflammation and bile duct inflammation and damage were observed after UC-MSC therapy (RAIS:P1V1B1 = 3; [G]: ×20 magnification, [H]: ×40 magnification). (I, J): RAI level ratios in UC-MSC treatment group before (Pre-Infusion, [I]) and after (Post-Infusion, [J]) UC-MSC infusion. Abbreviations: P(n1)V(n2)B(n3), the scores for portal inflammation (n1), venous endothelial inflammation (n2), and bile duct inflammation damage (n3); RAI: rejection activity index; RAIS, rejection activity indexes.

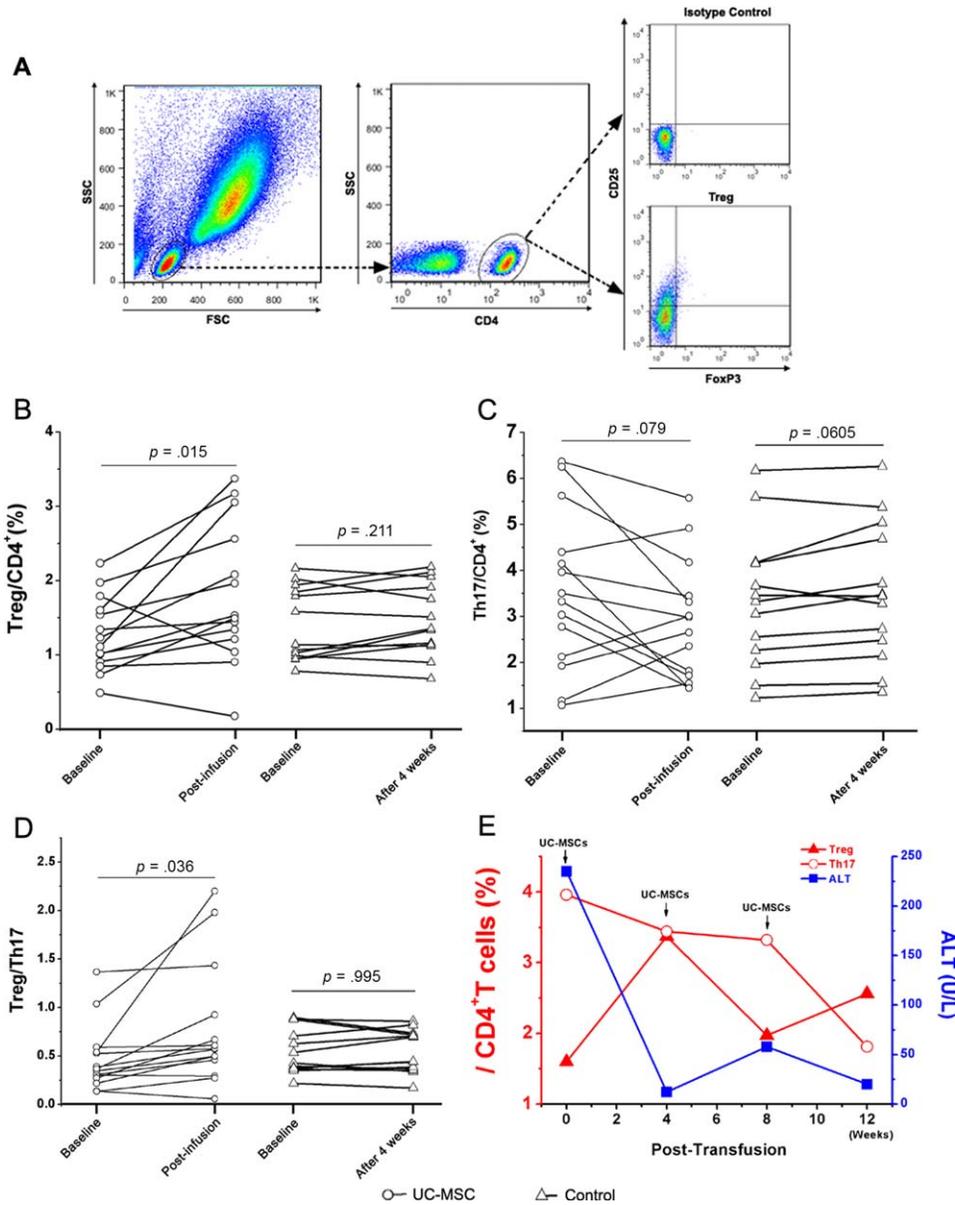


Figure 4. UC-MSCs upregulate Tregs and downregulate Th17 cells in liver allograft recipients with acute rejection. **(A):** A typical example of the fluorescence activated cell sorting strategy to obtain CD4⁺CD25⁺Foxp3⁺Treg cells is depicted. **(B):** The percentage of Tregs increased significantly 4 weeks after UC-MSC infusions (n = 14). In controls, the percentage of Tregs showed no significant change during the 4-week follow-up (n = 13). **(C):** The percentage of Th17 cells showed a decreasing trend 4 weeks after UC-MSC infusions, but the difference was not significant compared with preinfusion (n = 14). In controls, the percentage of Th17 cells showed no significant change during the 4-week follow-up period (n = 13). **(D):** A decrease in ALT levels accompanied the increase of Treg/Th17 ratio 4 weeks after UC-MSC infusions (n = 14). In controls, the Treg/Th17 ratio remained stable during the 4-week follow-up period (n = 13). **(E):** One patient received three UC-MSC infusions at 4-week intervals. ALT decreased to normal and the Treg level showed the same trend as ALT; by contrast, Th17 cells showed the opposite trend to ALT and Tregs. Abbreviations: ALT, alanine aminotransferase; FSC, forward scatter; SSC, side-scatter; Th17, T helper 17; Treg, regulatory T cell; UC-MSC, umbilical cord-derived mesenchymal stem cell.

histology before and after UC-MSC therapy is shown in Figure 3. The portal triads were obviously expanded by an inflammatory infiltrate that extended underneath the endothelium of the portal veins. The infiltrate in this case contained numerous eosinophils, which indicates severe bile duct damage (Fig. 3E, 3F). After UC-MSC infusions, improvement of liver allograft histology was observed. A minority of the portal spaces was involved and the inflammation was mild overall. Mild portal inflammation and bile duct inflammation and damage were evident (Fig. 3G, 3H).

UC-MSCs Upregulate Tregs and Downregulate Th17 Cells

A previous study indicated that MSCs may modulate immune responses via the induction of Tregs and modulation of the balance of Tregs and Th17 cells [17]. To investigate whether UC-MSC infusions impacted Tregs and Th17 cells in liver transplant recipients with acute rejection, the percentages of Tregs and Th17 cells in peripheral blood were analyzed 4 weeks after infusions. In treated subjects, the percentage of Tregs increased significantly at

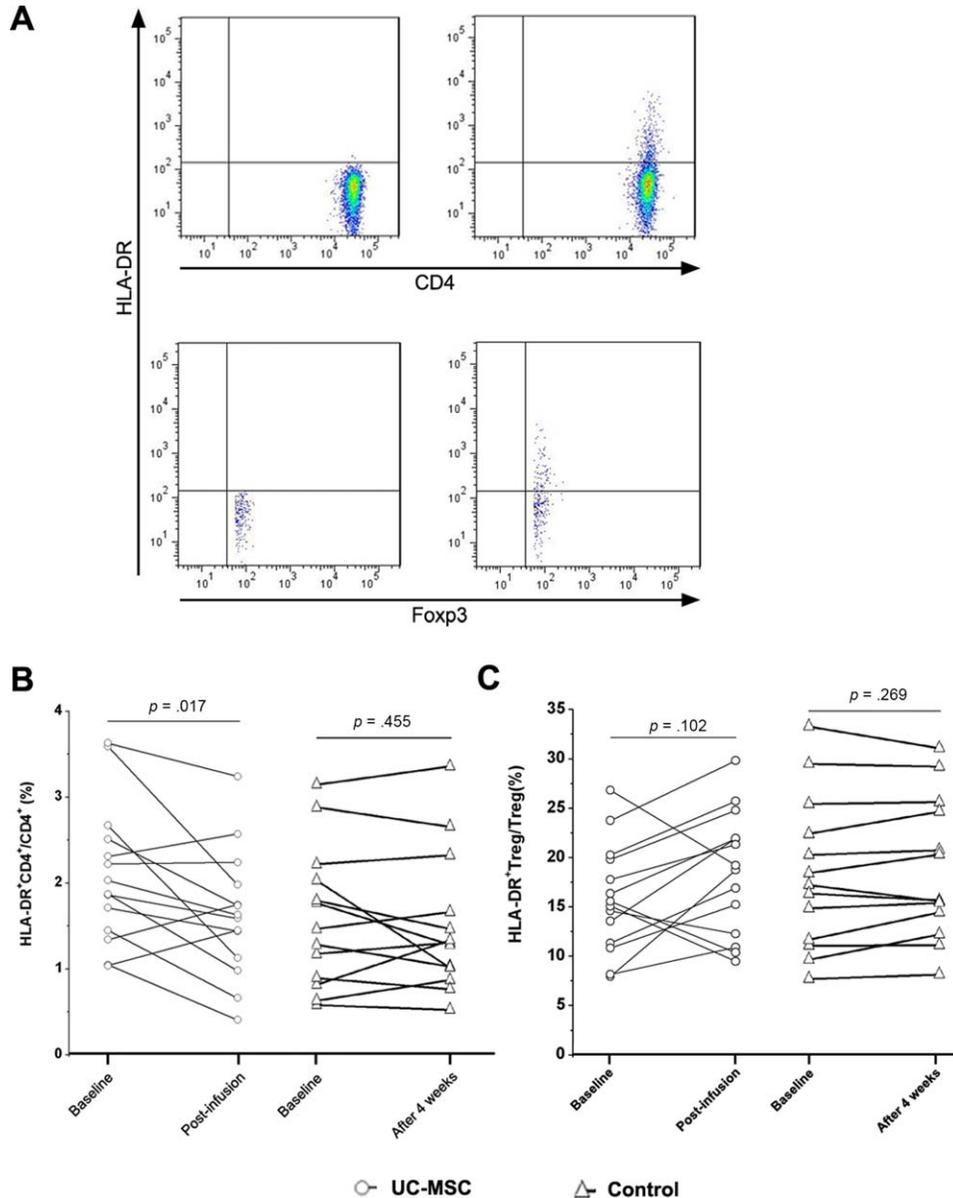


Figure 5. UC-MSCs downregulate CD4⁺ T-cell activation. **(A):** Representative fluorescence activated cell sorting profiles of HLA-DR expression on CD4⁺ T cells and Tregs (upper left: isotype controls; upper right: HLA-DR⁺CD4⁺T cells; lower left: isotype controls; lower right: HLA-DR⁺CD4⁺Treg cells). **(B):** HLA-DR expression in peripheral CD4⁺ T cells decreased significantly 4 weeks after UC-MSC infusions (n = 14). In controls, the HLA-DR expression in peripheral CD4⁺ T cells showed no significant change during the 4-week follow-up period (n = 13). **(C):** HLA-DR⁺ Treg levels showed an increasing trend after UC-MSC infusions, but the difference was not statistically significant (n = 14). In controls, HLA-DR⁺ Treg levels showed no significant change during the 4-week follow-up period (n = 13). Abbreviations, HLA, human leukocyte antigen; Treg, regulatory T cell; UC-MSC, umbilical cord-derived mesenchymal stem cell.

this time point ($p < .05$; Fig. 4A, 4B); by contrast, the percentage of Th17 cells showed a decreasing trend, but the difference was not significant compared with preinfusion ($p = .079$; Fig. 4C). Furthermore, an ALT level decrease accompanied the increase of the Treg/Th17 ratio 4 weeks after UC-MSC infusions (Fig. 4D). The percentage of Tregs and the Treg/Th17 ratio did show a statistical change at 4 weeks in the control group (Fig. 4B–4D). One patient received three UC-MSC infusions at 4-week intervals. Four weeks after the first infusion, ALT had decreased to normal; fluctuations of small amplitude occurred during the following 8 weeks. Interestingly, Treg levels showed the same trend as ALT; by contrast, Th17 levels showed the opposite trend to both ALT and Tregs

(Fig. 4E). In this patient, Treg levels were increased at week 4 after the first UC-MSC infusion and then were decreased at week 8. This may have been because the UC-MSC-induced circulating Treg had been redistributed, where the Tregs were inflating into the liver allograft. However, the exact reason warrants further exploration.

UC-MSCs Downregulate CD4⁺ T-Cell Activation

To better understand the mechanism by which CD4⁺ T cells and Tregs function in liver transplant recipients after UC-MSC treatment, activated markers of HLA-DR expression on CD4⁺ T cells and Tregs were analyzed. HLA-DR expression in peripheral CD4⁺ T

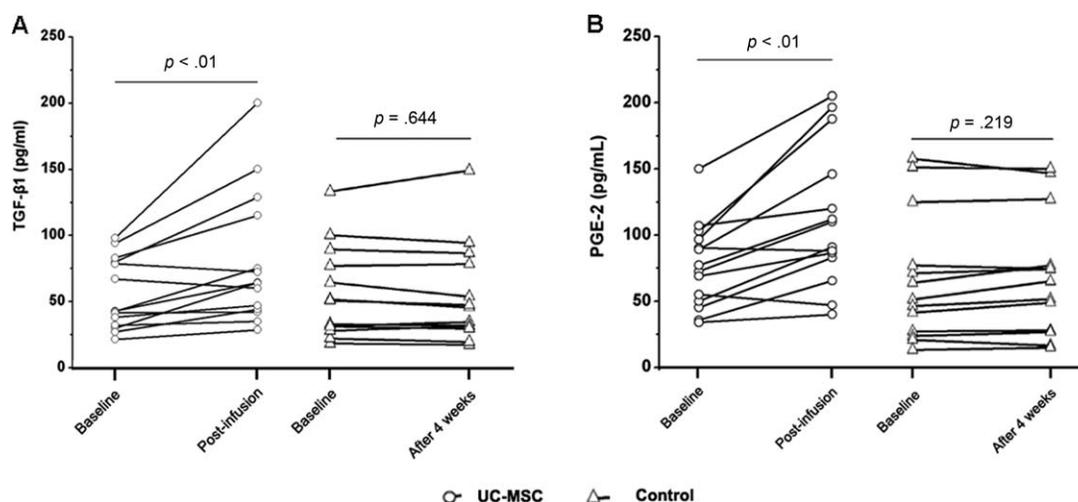


Figure 6. UC-MSCs elevate levels of TGF- β 1 and PGE2. Serum levels of TGF- β 1 (**A**) and PGE2 (**B**) were increased significantly 4 weeks after UC-MSC infusions ($n = 14$). There were no significant changes in serum TGF- β 1 or PGE2 levels during 4-week follow-up in controls ($n = 13$). Abbreviations: PGE2, prostaglandin E2; TGF- β 1, transforming growth factor beta 1; UC-MSC, umbilical cord-derived mesenchymal stem cell.

cells decreased significantly 4 weeks after UC-MSC infusions ($p = .017$; Fig. 5A, 5B), suggesting that UC-MSC treatment can suppress CD4⁺ T-cell activation. Levels of HLA-DR⁺ Tregs showed an increasing trend 4 weeks after infusions, but the difference was not statistically significant (Fig. 5A, 5C). In controls, no statistical difference was found for activated markers of HLA-DR expression on CD4⁺ T cells and Tregs during 4 weeks of follow-up (Fig. 5B, 5C).

UC-MSCs Result in Elevated Levels of TGF- β 1 and PGE2

MSCs can induce Tregs and modulate the proliferation of T, B, NK, NK T, and $\gamma\delta$ T cells and the maturation and function of monocyte-derived DCs via soluble factors including TGF- β 1 and PGE2. Our results show that the plasma levels of both TGF- β 1 and PGE2 in 12 of 14 infused patients were increased significantly 4 weeks after UC-MSC infusions (both $p < .01$; Fig. 6); these patients also showed elevation of Tregs. The serum levels of TGF- β 1 and PGE2 in the control group did not change significantly during 4 weeks of follow-up (Fig. 6).

DISCUSSION

MSCs have been shown to be effective in regulating the invoked immune response in settings such as tissue injury, GVHD, transplantation, and autoimmunity in several phase I, II, and III clinical trials [6], with no reports of significant adverse events associated with MSC transplantation. Until now, however, there has been no report on the safety or feasibility of MSC infusion in patients undergoing liver allograft transplantation. The main purpose of our study was to establish the safety and clinical feasibility of cell-based therapy with UC-MSCs for liver transplantation at the first acute rejection episode. Our data suggest that UC-MSC infusions improve liver allograft histology and suppress acute rejection in liver transplant recipients by upregulation of peripheral Tregs and the Treg/Th17 cell ratio. No side effects were observed.

MSCs have low immunogenicity and exert immunosuppressive effects by secreting soluble factors such as indoleamine 2,3-dioxygenase, TGF- β , PGE2, HLA-G, and inducible nitric oxide synthase [4]. These properties suggest great therapeutic potential for

MSCs in the context of cell-based therapy for conditions such as experimental autoimmune encephalomyelitis, rheumatoid arthritis, and diabetes, as demonstrated in several preclinical and clinical studies [18–20]. Because of their immunosuppressive properties, MSCs are believed to play a role in the maintenance of peripheral tolerance and the induction of transplantation tolerance, and they are considered potential candidates for cellular therapy for GVHD and autoimmune diseases and for the protection of solid organ grafts against rejection [21].

Although currently available immunosuppressive agents reduce the incidence of acute rejection, this remains as high as 20%–50% after liver transplantation. Also, the toxicity associated with these regimens is a long-standing clinical problem. The safety and clinical feasibility of bone marrow-derived MSC (BM-MSC) infusion have been demonstrated in several clinical trials in renal transplantation [10, 11, 22–25]. Some evidence has confirmed the efficacy of MSCs in reducing the incidence of acute rejection, decreasing the risk of opportunistic infection, and improving renal function, as well as enabling the safe use of a lower dose immunosuppression maintenance regimen [10, 25]. In the present study, we used UC-MSCs as an immunosuppressive agent to treat patients with acute rejection who did not respond to immunosuppression dose adjustments. No adverse effects were observed. This small study suggests that UC-MSC infusions may be considered safe for liver transplant recipients with acute rejection. Suppression of acute rejection by the alleviation of liver damage was determined via decreased ALT levels and histologic improvement after UC-MSC infusions. The study was not carried out for long enough to determine whether less infection resulted from MSC therapy.

It has been reported that MSCs induce kidney allograft tolerance by promoting the generation of CD4⁺CD25⁺FoxP3⁺ Tregs in vivo [26]. In animal kidney transplant models, MSC infusion leads to skewing of the balance between Tregs and effector/memory T cells toward a pro-tolerogenic profile, controlling effector/memory CD8⁺ T-cell proliferation and donor-specific CD8⁺ T-cell function [7]. The mechanism by which MSCs mediate their effects clinically remains, for the most part, unknown. Several researchers have demonstrated the mechanism by which MSCs regulate

alloreaction in organ transplantation. UC-MSCs constitutively express B7-H1, which is a negative regulator of T-cell activation, inhibiting the differentiation and maturation of monocyte-derived DCs and augmenting the generation of Tregs [27]. The induction of Tregs by MSCs involves not only direct contact between MSCs and CD4⁺ cells but also the secretion of soluble factors such as PGE2 and TGF- β 1 [28]. Autologous BM-MSC therapy for patients with HBV-related liver cirrhosis enhanced Tregs and decreased Th17 cells significantly, leading to an increased Treg/Th17 ratio and serum TGF- β levels and decreased IL-17, tumor necrosis factor alpha, and IL-6 [29]. In a heterotopic small bowel transplant rat model, BM-MSC infusions significantly attenuated acute cellular rejection while upregulating IL-10 and TGF- β expression and increasing Treg levels [30].

To date, only a few clinical protocols have included ex vivo immunologic studies to gain insight into the mechanistic effects of MSC-based therapy in liver transplant recipients. Our data show that Tregs were upregulated after infusions, whereas Th17 cells were downregulated. In addition, we evaluated the expression of the activated marker of HLA-DR on CD4⁺ T cells and Tregs in peripheral blood in all patients and found that the percentage of HLA-DR⁺ CD4⁺ T cells was significantly decreased after UC-MSC infusions. This finding suggests that downregulation of CD4⁺ T-cell activation may facilitate the suppression of alloreactive responses, which would be one of the mechanisms by which UC-MSCs regulate acute rejection. Moreover, levels of TGF- β 1 and PEG2 increased significantly after UC-MSC infusions, which may contribute to the induction of Tregs in peripheral blood.

In our clinical trial, we used UC-MSCs rather than autologous BM-MSCs. Compared with autologous BM-MSCs, UC-MSCs may be a better choice for clinical application. The collection of autologous BM-MSCs from liver transplant recipients would be harmful for the patients. In addition, the proliferative ability of BM-MSCs from patients with liver disease is deficient [31], whereas UC-MSCs can be obtained from discarded umbilical cords and can be produced on a larger scale [32]. Our studies have shown that infusion of human UC-MSCs is feasible and can improve liver function in liver failure, liver fibrosis, and primary biliary cirrhosis [16, 33,

34]. Importantly, UC-MSC treatment was safe and feasible for liver allograft recipients with acute rejection.

CONCLUSION

Our pilot study found that UC-MSC treatment upregulates the Treg/Th17 cell ratio, which may help to suppress acute rejection in liver transplant patients. Future large scale and randomized double-blinded control studies should be performed over longer periods of time to further ascertain the efficacy of UC-MSC treatment for liver allograft recipients with acute rejection.

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AUTHOR CONTRIBUTIONS

M.S.: conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; Z.L.: conception and design, provision of study material or patients, data analysis and interpretation; Y.W. and R.X.: provision of study material or patients, collection and/or assembly of data, data analysis and interpretation; Y.S., M.Z., X.Y., H.W., L.M., H.S., and L.J.: provision of study material or patients, collection and/or assembly of data; F.S.W.: conception and design, financial support, administrative support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

REFERENCES

- Vajdic CM, van Leeuwen MT. Cancer incidence and risk factors after solid organ transplantation. *Int J Cancer* 2009;125:1747–1754.
- Herzog EL, Chai L, Krause DS. Plasticity of marrow-derived stem cells. *Blood* 2003;102:3483–3493.
- Prockop DJ, Gregory CA, Spees JL. One strategy for cell and gene therapy: Harnessing the power of adult stem cells to repair tissues. *Proc Natl Acad Sci USA* 2003;100(Suppl 1):11917–11923.
- Shi M, Liu ZW, Wang FS. Immunomodulatory properties and therapeutic application of mesenchymal stem cells. *Clin Exp Immunol* 2011;164:1–8.
- Wang Y, Chen X, Cao W et al. Plasticity of mesenchymal stem cells in immunomodulation: Pathological and therapeutic implications. *Nat Immunol* 2014;15:1009–1016.
- Salem HK, Thiernemann C. Mesenchymal stromal cells: Current understanding and clinical status. *STEM CELLS* 2010;28:585–596.
- Cortinovich M, Casiraghi F, Remuzzi G et al. Mesenchymal stromal cells to control donor-specific memory T cells in solid organ transplantation. *Curr Opin Organ Transplant* 2015;20:79–85.
- Bartholomew A, Sturgeon C, Siatskas M et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 2002;30:42–48.
- Wang Y, Zhang A, Ye Z et al. Bone marrow-derived mesenchymal stem cells inhibit acute rejection of rat liver allografts in association with regulatory T-Cell expansion. *Transplant Proc* 2009;41:4352–4356.
- Tan J, Wu W, Xu X et al. Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: A randomized controlled trial. *JAMA* 2012;307:1169–1177.
- Perico N, Casiraghi F, Inrona M et al. Autologous mesenchymal stromal cells and kidney transplantation: A pilot study of safety and clinical feasibility. *Clin J Am Soc Nephrol* 2011;6:412–422.
- Obermajer N, Popp FC, Johnson CL et al. Rationale and prospects of mesenchymal stem cell therapy for liver transplantation. *Curr Opin Organ Transplant* 2014;19:60–64.
- Vandermeulen M, Grégoire C, Briquet A et al. Rationale for the potential use of mesenchymal stromal cells in liver transplantation. *World J Gastroenterol* 2014;20:16418–16432.
- Soeder Y, Loss M, Johnson CL et al. First-in-human case study: Multipotent adult progenitor cells for immunomodulation after liver transplantation. *STEM CELLS TRANSLATIONAL MEDICINE* 2015;4:899–904.
- Shi M, Zhang Z, Xu RN et al. Human mesenchymal stem cell transfusion is safe and improves liver function in acute-on-chronic liver failure patients. *STEM CELLS TRANSLATIONAL MEDICINE* 2012;1:725–731.
- Wang Y, Zhang M, Liu ZW et al. The ratio of circulating regulatory T cells (Tregs)/Th17 cells is associated with acute allograft rejection in liver transplantation. *PLoS One* 2014;9:e112135.
- Im KI, Park MJ, Kim N et al. Induction of mixed chimerism using combinatory cell-

based immune modulation with mesenchymal stem cells and regulatory T cells for solid-organ transplant tolerance. *Stem Cells Dev* 2014;23:2364–2376.

18 Papadopoulou A, Yiangou M, Athanasiou E et al. Mesenchymal stem cells are conditionally therapeutic in preclinical models of rheumatoid arthritis. *Ann Rheum Dis* 2012;71:1733–1740.

19 Shi Y, Hu G, Su J et al. Mesenchymal stem cells: A new strategy for immunosuppression and tissue repair. *Cell Res* 2010;20:510–518.

20 Pileggi A. Mesenchymal stem cells for the treatment of diabetes. *Diabetes* 2012;61:1355–1356.

21 Sensebé L, Krampera M, Schrezenmeier H et al. Mesenchymal stem cells for clinical application. *Vox Sang* 2010;98:93–107.

22 Reinders ME, de Fijter JW, Roelofs H et al. Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: Results of a phase I study. *STEM CELLS TRANSLATIONAL MEDICINE* 2013;2:107–111.

23 Perico N, Casiraghi F, Gotti E et al. Mesenchymal stromal cells and kidney transplantation: Pretransplant infusion protects from graft dysfunction while fostering immunoregulation. *Transpl Int* 2013;26:867–878.

24 Mudrabetu C, Kumar V, Rakha A et al. Safety and efficacy of autologous mesenchymal stromal cells transplantation in patients undergoing living donor kidney transplantation: A pilot study. *Nephrology (Carlton)* 2015;20:25–33.

25 Peng Y, Ke M, Xu L et al. Donor-derived mesenchymal stem cells combined with low-dose tacrolimus prevent acute rejection after renal transplantation: A clinical pilot study. *Transplantation* 2013;95:161–168.

26 Ge W, Jiang J, Arp J et al. Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stem cells associated with indoleamine 2,3-dioxygenase expression. *Transplantation* 2010;90:1312–1320.

27 Tipnis S, Viswanathan C, Majumdar AS. Immunosuppressive properties of human umbilical cord-derived mesenchymal stem cells: Role of B7-H1 and IDO. *Immunol Cell Biol* 2010;88:795–806.

28 English K, Ryan JM, Tobin L et al. Cell contact, prostaglandin E(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4+CD25(High) forkhead box P3+ regulatory T cells. *Clin Exp Immunol* 2009;156:149–160.

29 Xu L, Gong Y, Wang B et al. Randomized trial of autologous bone marrow mesenchymal stem cells transplantation for hepatitis B virus cirrhosis: Regulation of Treg/Th17 cells. *J Gastroenterol Hepatol* 2014;29:1620–1628.

30 Yang Y, Song HL, Zhang W et al. Reduction of acute rejection by bone marrow mesenchymal stem cells during rat small bowel transplantation. *PLoS One* 2014;9:e114528.

31 Zhong YS, Lin N, Deng MH et al. Deficient proliferation of bone marrow-derived mesenchymal stem cells in patients with chronic hepatitis B viral infections and cirrhosis of the liver. *Dig Dis Sci* 2010;55:438–445.

32 Lu LL, Liu YJ, Yang SG et al. Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. *Haematologica* 2006;91:1017–1026.

33 Zhang Z, Lin H, Shi M et al. Human umbilical cord mesenchymal stem cells improve liver function and ascites in decompensated liver cirrhosis patients. *J Gastroenterol Hepatol* 2012;27(Suppl 2):112–120.

34 Wang L, Li J, Liu H et al. A pilot study of umbilical cord-derived mesenchymal stem cell transfusion in patients with primary biliary cirrhosis. *J Gastroenterol Hepatol* 2013;28 (Suppl 1):85–92.



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