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Mesenchymal Stem Cell Transfusion as a Novel Immunosuppressive Regimen with Possible Induction of Microchimerism

Gamal Saadi[1] • Mervat El-Ansary[2] • Samah Abd El-Hamid[2*] • Iman Abd El-Aziz[3]

[1] Department of Internal Medicine and Nephrology, Kasr EL-Aini, Cairo University, Manial EL-Rouda, Cairo, Egypt
[2] Department of Clinical Pathology, Kasr EL-Aini, Cairo University, Manial EL-Rouda, Cairo, Egypt
[3] Department of Clinical Pathology, Theodor Bilharz Research Institute, Kornish Elnile Street, Warrak Elhadar, Giza, Egypt

Corresponding author: * **samah_cpath@yahoo.com**

ABSTRACT

Human mesenchymal stem cells (MSCs) have immunosuppressive capacities. Although their efficacy is currently studied in graft versus host disease (GVHD), their effect on alloreactivity in solid organ transplant (SOT) patients is unknown. Our work aimed to use allogeneic donor-specific MSCs (DS-MSCs) transfusion prior to renal transplantation as an immunosuppressive induction regimen. Our study included 4 groups of patients, all of which were diagnosed with chronic renal failure and had undergone renal transplantation. The first group included 7 patients that were induced by DS-MSCs. The second included 6 patients induced by antithymocyte globulin (ATG). The third included 6 patients induced by anti-CD25 while the 4th group included 7 patients who received no induction. The immunosuppressive regimen was cyclosporine (CsA), Mycophenolate mofetil (MMF) and prednisolone (PRD) for all patients. Bone marrow (BM) (90 ml) were aspirated from the iliac bone of related donors, to separate MSCs, then about 10 million MSCs placed in 10 ml saline were infused intravenously in 2 divided doses 1 week apart. Our results showed that the lowest mean serum creatinine level measured after 1, 3, and 6 months were in those patients who received pre-transplantation DS-MSC infusion (group I). Also rejection was less frequent in patients of group I. Microchimerism was detected after MSCs transfusion in one case of group I. We conclude that MSCs can escape immune recognition, can inhibit immune responses and prevent the development of cytotoxic T-cells so their transfusion may be used to treat organ allograft rejection and reduce the need for an immunosuppressive regimen after renal transplantation.

Keywords: rejection, renal transplantation, stem cells
Abbreviations: **ATG**, antithymocyte globulin; **BM**, bone marrow; **CRF**, chronic renal failure; **CsA**, cyclosporine; **DC**, dendritic cell; **GVHD**, graft versus host disease; **HLA**, human leucocyte antigen; **HSC**, hematopoietic stem cell; **IDO**, indoleamine 2,3-dioxygenase; **IL-10**, interleukin 10; **MC**, microchimerism; **MMF**, mycophenolate mofetil; **MSC**, mesenchymal stem cell; **NK**, natural killer; **PRD**, prednisolone; **SOT**, solid organ transplant; **TH**, T-helper; **TNF-α**, tumor necrosis factor; **αMEM**, alpha-modified Eagle's medium

INTRODUCTION

Our work aimed to use allogeneic donor-specific (DS) transfusion prior to renal transplantation as an immunosuppressive induction regimen.

Mesenchymal stem cells (MSCs) were first described by Friedenstein *et al*. (1968). They are a rare subset of stem cells residing in the bone marrow representing 0.001-0.01% of total bone marrow (BM) cells where they interact closely with hematopoietic stem cells (HSCs) and support their growth and differentiation. MSCs can be obtained easily from a BM aspirate and can be isolated and expanded through passages in plastic plates where they grow as adherent cells in appropriately enriched media, reaching confluence at time intervals related to plating density. MSCs do not express the hematopoietic cluster of differentiation 34 (CD34), CD14 and CD45, while they are positive for CD44, CD71, CD73, CD90, CD271 and CD105 (Yokoo *et al.* 2005; Meirelles and Nardi 2009). MSCs can differentiate into multiple mesenchymal and non-mesenchymal lineages, which make them a promising tool for tissue repair. In addition, MSC suppress many T, B and natural killer (NK) cell functions and may also affect dendritic cell (DC) activities (Uccelli *et al*. 2007). Due to their limited immunogenicity, MSCs are poorly recognized by human leucocyte antigen (HLA)-incompatible hosts (Le Blanc *et al*. 2003). Based on these unique properties, MSCs are currently subject to many investigations for their possible use in the treatment of immuno-mediated diseases (van Laar and Tyndall 2006; Bell 2008; Kong *et al*. 2009).

MSCs derived from BM and other tissues modulate the immune system through interaction with a broad range of immune cells including T and B lymphocytes, NK cells and DCs (Noel *et al.* 2007; Tabera *et al.* 2008; Di Ianni *et al.* 2008). These immunomodulatory properties of MSCs have been the basis for their use in treating conditions characterized by immunologic dysregulation such as Crohn's disease and graft versus host disease (GVHD) after allogeneic HSC transplantation. By extrapolation, the same immunomodulatory properties might be potentially useful for prevention or treatment of solid organ transplantation (SOT) rejection (Nauta and Fibbe 2007; Crop *et al.* 2009).

As early as 2000, it was suggested that immunomodulatory properties of MSCs could be exploited in SOT for prevention and/or treatment of organ rejection (Devine and Hoffmann 2000; Popp *et al.* 2008). In contrast to most current pharmacologic agents that target only a single pathophysiological pathway, MSCs potentially work through multiple mechanisms and have the potential to affect immunologic, inflammatory, vascular and regenerative pathways (Brooke *et al.* 2007).

Thus, harnessing both their immunomodulatory capabilities in the potential treatment of acute rejection after SOT and their ability for tissue repair MSCs became an interes-

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ting domain for further research. Their ease of production combined with their apparent lack of need for HLA matching could also have significant implications for the therapeutic application of MSCs because previously expanded and cryopreserved MSCs derived from unrelated healthy donors can potentially be available for acutely ill patients in a timely manner. However, to date, results reported with preclinical animal models have been conflicting, and further research is needed to clarify the use of MSCs in SOT. Currently, prospective randomized phase III studies in Europe and United States are in progress to further define the therapeutic potential of MSCs for promotion of HSC engraftment and/or treatment/prevention of acute GVHD after allogeneic HSC transplantation (Giordano *et al*. 2007). Despite all the encouraging results so far, the clinical use of MSCs is still not a standardized and accepted form of cell therapy for treatment or prevention of GVHD. Finally, culture-expanded BM-derived MSCs have been used in several small phase I and II trials for a variety of nonhematological indications including treatment of patients with metachromatic leukodystrophy and Hurler disease, osteogenesis imperfecta, myocardial infarction, amyotrophic lateral sclerosis, and Crohn's disease (Mazzini *et al.* 2006; Duijvestein *et al.* 2008).

Microchimerism (MC) refers to the presence of a limited number of nonhost cells in the body of an individual. These cells can enter via blood transfusion and organ transplantation or naturally through pregnancy. Chimeric cells engraft in the host body, develop, proliferate, and are accepted by the immune system as self. These include stem cells that enter the maternal body during fetal stages. These stem cells are also postulated to be helpful reservoirs in protecting the host body (Artlett 2005).

MC has been considered a risk factor in autoimmune disease induction (Nelson 1998). MC has been investigated in different autoimmune disorders, such as systemic sclerosis, systemic lupus erythematosus, autoimmune thyroid diseases, primary biliary cirrhosis and juvenile inflammatory myopathies (Sarkar and Miller 2004). However, today it is a natural phenomenon, establishment and persistence of engrafted donor cell in the recipient body is a sign of transplantation success (Bettens *et al*. 2005), the earliest engrafting cells being fetal mesenchymal stem cells (MSCs). MSCs have two notable features. For the fetus, MC appears to be an effective factor in maternal tolerance induction toward the fetal graft and for the mother; these novel fetal cells might be useful in disease conditions occurring after pregnancy (Lapaire *et al*. 2007). Also monitoring of donor chimerism in sorted $CD34^+$ peripheral blood cells allows the sensitive detection of imminent relapse after allogeneic stem cell transplantation (Bornhäuser *et al.* 2009).

Our study hypothesized down-regulation of the immune response and preservation of graft function after human renal transplantation by using expanded MSCs *in vitro* and compare these effects with different immunosuppressive drugs.

MATERIALS AND METHODS

Subjects

The present study included 26 patients, were divided into 4 groups. All groups were diagnosed as chronic renal failure (CRF) and had undergone renal transplantation. These patients were selected among cases of a private clinic; a written informed consent was taken from all.

Group I (induction by DS-MSCs) included 7 patients: 5 males and 2 females whose ages ranged from 15 to 50 years;

Group II (induction by ATG) included 6 patients: 4 males and 2 females whose ages ranged from 14 to 55 years;

Group III (induction by Anti CD25) included 6 patients: 4 males and 2 females whose ages ranged from 23 to 59 years;

Group IV (no induction; control group) included 7 patients: 5 males and 2 females whose ages ranged from 21 to 48 years.

The immunosuppressive regimen was CsA, MMF and PRD for all patients.

Methods

Sampling

90 ml BM were aspirated from the iliac bone of related donors under local anesthesia and placed in sterile tubes containing preservative-free heparin (Sigma-Aldrich, St. Louis, USA).

Separation of mononuclear cells

The bone marrow aspirate was diluted at a ratio of 6: 1 with phosphate buffer saline (PBS) with 2 mM EDTA (30 ml BM aspirate + 5 ml PBS/EDTA buffer). The MNCs were separated under aseptic conditions using a Ficoll Hypaque density gradient (density 1.077, GibcoBRL, Grand Island, NY, USA) by centrifugation at 1800 rpm for 20 min then the MNCs were plated in 40 ml alpha-modified Eagle's medium (αMEM), 10 ml fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (10 mg/ml), 0.5 ml amphotericin B (all from GibcoBRL) and 10 ng/ml basic fibroblast growth factor (b-FGF) (R&D System, Minneapolis, MN) and were incubated at 37°C in a humidified atmosphere containing 5% CO_2 (DiGirolamo *et al.* 1999). After one day, non adherent cells were removed and adherent cells were cultured in the presence of mesenchymal media for 3 weeks changed every 1 week (Cambrex BioScience, Nottingham, UK). After reaching 80% confluence the MSCs were harvested by incubation with trypsin/EDTA (Gibco BRL) and counted on a hemocytometer (Newbauer, Germany). Then 10 million MSCs were placed in 10 ml saline and were infused intravenously in 2 divided doses 1 week apart (**Fig. 1**).

Flow cytometry

Surface expression of MSCs using anti-CD271 and anti-CD34 monoclonal antibodies (mAbs) were analyzed using flow cytometry. MSCs (2×10^5 cells) were suspended in PBS containing 1% BSA and were stained with fluorochrome-conjugated mAbs for 20 min on ice (anti-mouse mAanti-CD271 and mAanti-CD34; BD Biosciences, MN, USA). Flow cytometric analysis was performed using a FACSCaliber (BD Biosciences) equipped with CellQuest Software. 10000 cells were passed in front of the laser for each sample. Each sample was analyzed in duplicate. A cut off value at 20% was set to categorize samples as positive. Negative CD34 and positive CD271 expression occurred (**Figs. 2, 3**).

Follow up

There was a follow up of patients after 1, 3 and 6 months by laboratory assessment of kidney function tests including serum creatinine level (enzymatic creatinine assay) (Junge *et al*. 2004) and creatinine clearance level (according to Cockcroft and Gault's equation) utilizing the adjusted body weight to calculate an estimated creatinine clearance (Cockcroft and Gault 1976). Results are presented in **Table 1**.

Statistical analysis of data

Quantitative values were expressed as mean ± S.D, and were compared using the *t*-test for 2 groups and one multifactorial analysis of variance (ANOVA) test for > 2 groups. Qualitative data were compared using the χ^2 test. $P < 0.05$ was considered to be significant and $P < 0.01$ was considered highly significant. SPSS 12 statistical package was used for analyses.

Declaration of ethics

This study was approved by the review board of our hospital (FWA 00010609), and written informed consent was obtained from all patients according to Helsinki guidelines of research ethics.

RESULTS AND DISCUSSION

Transplantation of organs, such as kidney, heart, liver, and lung, has now become a standard therapy for diseases that result in organ failure. While current transplant programs in all fields are enormously successful, the need for ongoing immunosuppression results in significant morbidity due to infectious complications and potential oncogenicity and continues to drive research into novel immunosuppressants and protocols with the goal of achieving transplant tolerance. Clinical interest has arisen in using the immunosuppressive capacities of MSCs to prevent/control GVHD after HSC transplantation (Le Blanc *et al*. 2008). Details of an understanding of the immunological processes underlying SOT rejection have been reviewed (LaRosa *et al.* 2007; Merad *et al*. 2007).

Immunosuppression post-SOT differs from that required in HSCT in that the key goal is to prevent the alloimmune response against the graft with no need to balance this against a graft-vs-tumor effect. Although current immunosuppressive drugs are very effective at attenuating alloimmune responses, MSCs do offer some potential advantage in that they may allow more specific targeting of the immunoinhibitory effect. MSCs migrate to sites of inflammation and in an animal study were shown to migrate to cardiac allografts undergoing chronic rejection (Wu *et al*. 2003). Thus, MSCs may be able to deliver localized immunosuppression thereby minimizing the systemic complications of nonspecific immunosuppressants and offering a novel cellular immunosuppressant therapy.

MSCs mediate their immuno-modulatory effects by interacting with cells from both the innate (DCs and NK cells) and adaptive immunity systems (T-cell) (Aggarwal and Pittenger 2005; Tabera *et al.* 2008). MSC inhibition of tumor necrosis factor (TNF)-α secretion and promotion of interleukin (IL)-10 secretion may affect DC maturation state and functional properties, resulting in skewing the immune response toward an antiinflammatory/tolerant phenotype. Alternatively, when MSCs are present in an inflammatory microenvironment, they inhibit interferon (IFN)-γ secretion from T-helper (TH)1 and NK cells and increase IL-4 secretion from TH2 cells, thereby promoting a TH2 →TH1 shift. It is likely that MSCs also mediate their immuno-modulatory actions by direct cell-cell contact by secreted factors (Krampera *et al*. 2006).

As with GVHD, DC have a crucial role in the pathogenesis of solid organ rejection, with studies identifying key roles for various DC subsets and the potential for DC depletion as a therapeutic immunosuppressive therapy (Athanassopoulos *et al*. 2005). MSCs have significant effects on DC function by altering DC maturation and skewing their function toward a regulatory phenotype. However, all the current data has been gained from *in vitro* studies and further studies in humans are required to determine if MSCs are able to modulate DC maturation *in vivo* and function in a transplant setting. Soluble mediators produced by MSC may also have an important immunosuppressive role in SOT (Tabera *et al.* 2008). MSCs produce indoleamine 2,3-dioxygenase (IDO) which, in animal studies, has been identified as a potential immunomodifier in SOT (Brandacher *et*

Table 1 Clinical and laboratory data of all groups.

	Patients (n= 26)			
	Group 1 (No. 7)	**Group II (No. 6)**	**Group III (No. 6)**	**Group IV (No. 7)**
Clinical data				
Age (years)				
Range	15-50	14-55	23-59	21-48
Mean ± SD	30.29 ± 13.90	30.29 ± 13.90	30.29 ± 13.90	54.33 ± 12.99
Gender				
Males (No.; %)	5; 71%	4; 67%	4; 67%	5; 71%
Females (No.; %)	2; 29%	2; 33%	2; 33%	2; 29%
Laboratory data				
Creatinine (mg/dl) after 1 month				
Range	0.6-1.3	0.7-1.4	0.9-1.97	0.6-4.2
Mean ± SD	0.98 ± 0.23	1.03 ± 0.27	1.25 ± 0.40	1.7 ± 1.27
Creatinine (mg/dl) after 3 months				
Range	0.61-1.24	0.6-1.2	0.9-1.75	0.74-3.12
Mean ± SD	0.93 ± 0.22	0.95 ± 0.23	1.35 ± 0.33	1.6 ± 0.91
Creatinine (mg/dl) after 6 months				
Range	0.8-1.1	0.68-1.6	0.9-1.8	0.9-2.25
Mean ± SD	0.94 ± 0.15	1.09 ± 0.30	1.34 ± 0.38	1.5 ± 0.53
Rejection (No.; %)	1; 14%	2; 33%	2; 33%	4; 57%
+ve cross match (No.; %)	2; 28%	2; 33%	2; 33%	0; 0%

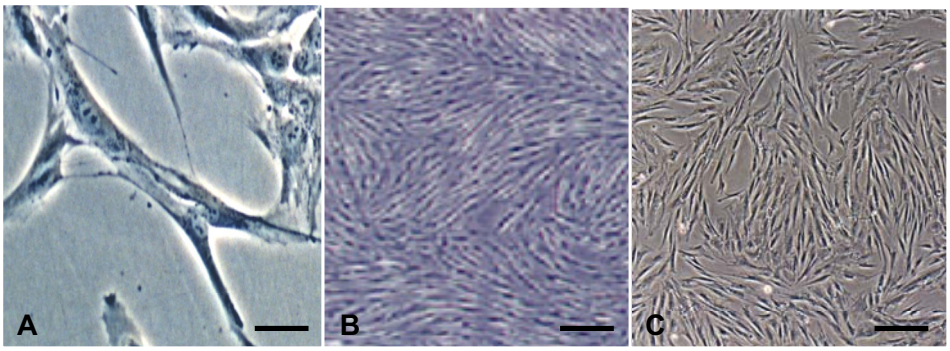

Fig. 1 MSCs in culture. (**A**) Adherent MSCs on plastic wall after separation of MNCs and addition of αMEM, fetal bovine serum (FBS) and basic fibroblast growth factor (b-FGF) then incubation at 37°C in a humidified atmosphere containing 5% CO_2. After one day, non adherent cells were removed leaving adherent cells; (**B**) Expanded MSCs with 90% confluence after culturing in the presence of mesenchymal media for 3 weeks changed every 1 week; (**C**) Passaged MSCs were harvested by incubation with trypsin/EDTA and counted on a hemocytometer then transfused intravenously to the patients.

Table 2 Statistical comparison of CD271 percentage in all patients before and after MSCs culture using a *t*-test.

	Before culture	After culture	*P* value
CD271 (%)			
Range	5.00- 9.00	75.00- 95.00	0.001
Mean ± SD	6.62 ± 1.55	83.31 ± 6.09	HS

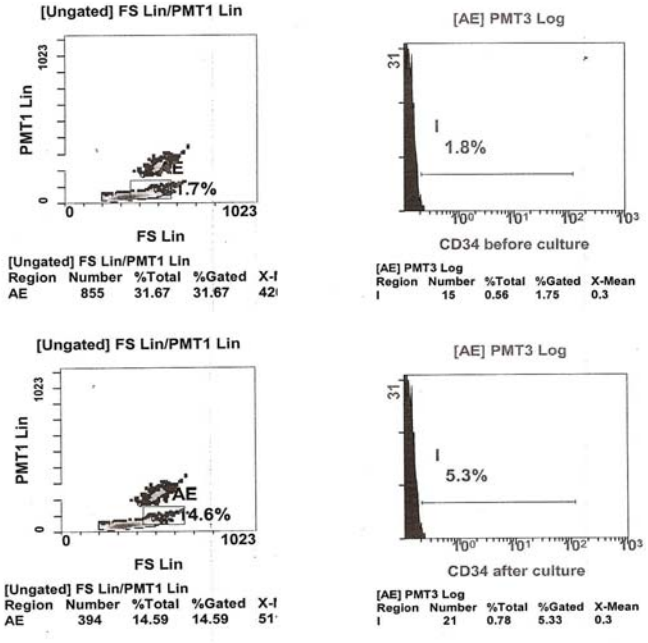

Fig. 2 CD34 expression before and after culture by flow cytometry, revealing negative expression after culture.

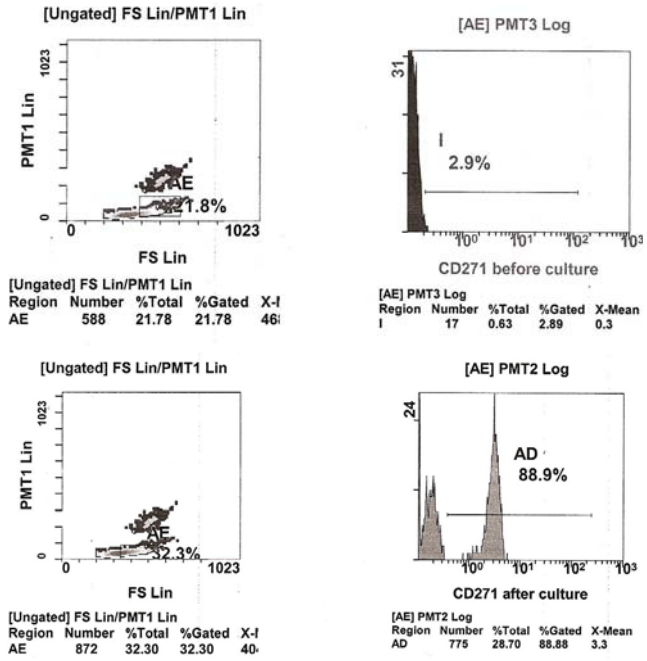

Fig. 3 CD271 expression before and after culture by flow cytometry, revealing positive expression after culture.

al. 2007). Alexander *et al*. (2002) showed that overexpression of IDO in murine pancreatic islets was associated with significant prolongation of islet graft survival that could be attributed to the depletion of tryptophan, resulting in decreased T-cell proliferation. Interestingly, a study by Feunou *et al*. (2007) demonstrated that IDO could be activated by Treg cells resulting in production of DC with immune regulatory activity. This study suggests that IDO may have additional alloinhibitory effects independent of its inhibition of T-cell proliferation. It has not yet been determined if any of the effects of MSC on DC are mediated by MSC production of IDO (Feunou *et al*. 2007).

Our work aimed to use transfusion of allogeneic DS-MSCs prior to renal transplantation as an immunosuppressive induction regimen.

Our study included 26 patients that were divided into 4 groups of patients; all groups were diagnosed as CRF and had undergone renal transplantation. MSCs were harvested for IV infusion into patients after separation of MNCs from BM samples and its cultivation in the presence of αMEM, FBS, antibiotics, antifungal and basic fibroblast growth factor (**Fig. 1**). Our results revealed positive CD271 and negative CD34 expression after MSCs culture analyzed by flow cytometry (**Figs. 2, 3**). CD271 expression showed a highly statistically significant difference before and after MSC culture in all patients with an increase in CD271 levels at the end of culture ($P < 0.01$) (**Table 2**). There was also a statistically significant difference in mean serum creatinine levels between different groups after 6 months ($P < 0.05$) (**Table 3**). However, there was no statistically significant difference in mean serum creatinine levels between different groups after 1 and 3 months ($P > 0.05$) (**Table 3**).

Our study also showed less frequent rejection in patients of group I who received allogeneic transfusion of DS-MSCs prior to renal transplantation as an immunosuppressive induction regimen (**Table 4**). These results are consistent with those of Bartholomew *et al*. (2002), who found that MSCs have immunosuppressive properties and delay skin graft rejection. In a further study in a rat cardiac allograft model, systemic administration of MSC also significantly improved the survival of treated rats (Zhou *et al*. 2006). GVDH is a form of rejection, where transplanted

Table 3 Statistical comparison between different groups as regards serum creatinine levels after 1, 3 and 6 months following ANOVA.

	Patients (n= 26)				
	Group 1 (No. 7)	Group II (No. 6)	Group III (No. 6)	Group IV (No. 7)	*P* value
Creatinine (mg/dl) after 1 month					
Range	0.6-1.3	0.7-1.4	0.9-1.97	0.6-4.2	0.27
Mean ± SD	0.98 ± 0.23	1.03 ± 0.27	1.25 ± 0.40	1.7 ± 1.27	NS
Creatinine (mg/dl) after 3 months					
Range	0.61-1.24	0.6-1.2	0.9-1.75	0.74-3.12	0.08
Mean ± SD	0.93 ± 0.22	0.95 ± 0.23	1.35 ± 0.33	1.6 ± 0.91	NS
Creatinine (mg/dl) after 6 months					
Range	0.8-1.1	0.68-1.6	0.9-1.8	0.9-2.25	0.04
Mean ± SD	0.94 ± 0.15	1.09 ± 0.30	1.34 ± 0.38	1.5 ± 0.53	S

Table 4 Statistical comparison between different groups as regards frequency of rejection after 1, 3 and 6 months using a χ^2 test.

	Patients (n= 26)				
	Group 1 (No. 7)	Group II (No. 6)	Group III (No. 6)	Group IV (No. 7)	*P* value
Rejection (No; %)	1; 14%	2; 33%	2; 33%	4; 57%	0.69
No rejection (No; %)	6; 86%	4; 67%	4; 67%	3; 43%	NS

cells begin to attack host tissues and organs, such as the digestive tract, skin, and liver. It is important to find effective ways to eliminate or at least minimize such serious transplant side effects (Cohen and Sudres 2009). Le Blanc *et al*. (2004) reported that coinfusion of *ex vivo* expanded MSC and HSC in transplant patients leads to a lower incidence of severe GVHD. Also, Vanikar *et al*. (2010) stated that co-transplantation of MSC and HSC in renal transplantation lead to donor hypo-responsiveness. There have been case reports of MSCs applied as a third party haploidentical treatment strategy to reduce severe acute GVHD and graft failure in recipients of HSCT (von Bonin *et al*. 2009). In contrast to our result, Inoue *et al*. (2006), in a major mismatch cardiac model, found that MSC had no effect on allograft outcomes despite *in vitro* inhibition of alloimmune responses. There is no clear reason for the observed differences in outcomes, but notably, improved survival in the initial study was not associated with tolerance, suggesting that MSCs alone are unlikely to provide sufficient immunosuppression for vascularized transplants. Thus, while there have been significant advances in our understanding of the immunomodulatory properties and function of MSCs, these have mainly been demonstrated *in vitro* and further clinical studies are required to evaluate their potential use for clinical SOT (Zhang *et al*. 2009).

MC is defined by the presence of circulating cells, bidirectionally transferred from one genetically distinct individual to another. It occurs either physiologically during pregnancy, or iatrogenically after blood transfusion and organ transplants (Adams and Nelson 2004). The migrated cells may persist for decades. MC was documented in one patient of group I after DS-MSCs transfusion by examination of HLA class II antigens (HLA-DR) by a molecular biology technique. Before transfusion of MSCs HLA typing of the patient was DR7, DR13 (6), DR53, DR52. HLA typing of related donor was DR4, DR13 (6), or DR14 (6), DR52, DR53. After transfusion of MSCs HLA typing of the patient became DR4, DR7, DR13 (6), DR52, DR53. This haemopoietic MC confirms the persistence of donor cells in recipients of kidney transplant and is important for the development and maintenance of immunological tolerance.

CONCLUDING REMARKS

MSCs, by virtue of escaping immune recognition, can inhibit immune responses and prevent the development of cytotoxic T-cells. Thus transfusion of MSCs may overcome rejection of organ allograft and minimize the need for an immunosuppressive regimen after renal transplantation.

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