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ORIGINAL ARTICLE

In vivo effect of bone marrow-derived mesenchymal stem cells in a rat kidney transplantation model with prolonged cold ischemia

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Summary

Brain death and prolonged cold ischemia are major contributors to the poorer long-term outcome of transplants from deceased donor kidney transplants, with an even higher impact if expanded criteria donors ('marginal organs') are used. Targeting ischemia-reperfusion injury-related intragraft inflammation is an attractive concept to improve the outcome of those grafts. As mesenchymal stem cells (MSCs) express both immunomodulatory and tissue repair properties, we evaluated their therapeutic efficacy in a rat kidney transplant model of prolonged cold ischemia. The *in vitro* immunomodulatory capacity of bone marrow-derived rat MSCs was tested in co-cultures with rat lymph node cells. For *in vivo* studies, Dark Agouti rat kidneys were cold preserved and transplanted into Lewis rats. Syngeneic Lewis MSCs were administered intravenously. Transplants were harvested on day 3, and inflammation was examined by quantitative RT-PCR and histology. Similarly to MSCs from other species, rat MSCs *in vitro* also showed a dose-dependent immunomodulatory capacity. Most importantly, *in vivo* administration of MSCs reduced the intragraft gene expression of different pro-inflammatory cytokines, chemokines, and intercellular adhesion molecule-1. In addition, fewer antigen-presenting cells were recruited into the renal allograft. In conclusion, rat MSCs ameliorate inflammation induced by prolonged cold ischemia in kidney transplantation.

Introduction

As a result of organ shortages, transplants using so-called 'marginal' organs have increased during the last two decades despite having reduced viability and increased immunogenicity compared with living donor renal allografts [1,2]. Prolonged ischemia and preinjury of donor organs are major contributors to the development of primary failure, delayed graft function and enhanced immunogenicity in kidney transplantation, having a major impact on long-term outcome [3,4]. Many studies have shown that ischemia/reperfusion injury (IRI) induces an acute inflammatory process resulting from multiple interactions between danger-associated molecular pattern (DAMPs) and their respective ligands on immune cells [5,6]. The inflammation process leads to activation of the endothelium with increased impermeability and expression of various adhesion molecules [7]. IRI-related acute

inflammation causes acute organ damage and more importantly strengthens the host immune response by enhancing graft immunogenicity through activation of intragraft antigen-presenting cells and supporting infiltration by immune cells by up-regulation of major histocompatibility complex (MHC) class II (MHC II) antigens, intracellular adhesion molecule-1 (ICAM-1), P- and E-selectin [8–10].

Using 'marginal organs' from deceased donors further amplifies this problem, as they are more susceptible to the consequences of IRI-related inflammation. Enhanced immunogenicity may also explain their higher incidence of acute rejection and poorer long-term function. Targeting inflammation by donor-pretreatment with heme oxygenase-1-inducing compounds or steroids improves outcomes of marginal allografts [11,12], but has several logistic and ethical problems, indicating the need for novel solutions.

Mesenchymal stem/stromal cells (MSCs) are multipotent stem cells located within the stroma of the bone marrow and other organs [13]. Recently, numerous *in vitro* studies showed that MSCs have low immunogenicity [14] and have immunomodulatory potential caused by suppressing immune cell functions and affecting dendritic cell (DC) activity [15–17]. Immunomodulation efficacy has also been shown *in vivo* during graft versus host disease [18], autoimmune disease [19] and solid organ transplantation [20]. Another unique property of MSCs is their tissue repair potential, which is attributed to their migration and differentiation capacity and ability to secrete various growth factors [21–23]. Recently, therapeutic applications of MSCs have been reported for cardiovascular diseases [21,24] and treatments following kidney injury [25–27].

In this study, we focused on the immunomodulatory potential of MSCs and hypothesized that they have an anti-inflammatory therapeutic effect in transplanted kidney allografts exposed to prolonged cold ischemia. After verifying the immunomodulatory features of rat MSCs *in vitro,* we attempted to evaluate the influence of MSC administration in a rat model of kidney transplantation after prolonged cold ischemia. In order to better mimic the clinical situation, a transplant model with highly dissimilar strains [Dark agouti (DA) to Lewis (LEW)] was used.

Materials and methods

Animals

Adult male Lewis (LEW; $RT1^{l}$) rats weighing 200–250 g were used as recipients of kidney grafts and Dark Agouti (DA; $RT1^{av1}$) rats (Harlan-Winkelmann, Borchen, Germany) weighing 200–250 g served as kidney donors. All animal experiments were performed with local authority approval (Landesamt für Gesundheit und Soziales, Berlin, Germany).

Isolation and characterization of MSC

MSCs were harvested from bone marrow of femurs and tibias from adult male LEW or DA rats by centrifugation of the bone shaft to collect the cells into α-modified Eagle's medium (α-MEM; PAA, Pasching, Austria) containing 10% fetal calf serum (FCS; Biochrom AG, Berlin, Germany), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (Life Technologies, Karlsruhe, Germany). After centrifugation and resuspension in complete α-MEM, cells were filtered through a 70 μm sieve (Falcon, Oxnard, USA) and placed in 75 cm^2 culture flasks at a density of 7.5×10^6 MSCs/ml. Nonadherent cells were removed after 72 h by media replacement. Upon reaching 80–90% confluence, adherent cells were trypsinized (1% trypsin–EDTA, Life Technologies), washed and placed into new flasks. The absence of contaminating $CD45^+$ cells was confirmed by flow cytometry. MSCs from passage 4–8 were used for all experiments. Expression of surface markers was tested by FACS analysis using specific mouse monoclonal antibodies for rat surface markers CD90 (eBiosciences, San Diego, USA), CD45 (AbDSerotec, Düsseldorf, Germany), MHC I, MHC II, CD80, CD86, ICAM-1 and vascular adhesion molecule (VCAM) (all BD Biosciences Pharmingen, San Diego, USA) followed by staining with a PE-labeled donkey-anti-mouse-IgG specific antibody (Dianova, Hamburg, Germany). FACS measurement was performed on FACSCanto™II (Becton Dickinson, San Jose, USA). FlowJo software (TreeStar Inc., Ashland, USA) was used to analyze all flow cytometry data.

Differentiation of rat MSCs

The differentiation of rat MSCs (passage 3) into osteocytes, adipocytes and chondrocytes was performed as described previously [13]. Briefly, for osteogenesis 6×10^3 MSCs/cm^2 were seeded and cultured for 28 days in Dulbecco's Modified Eagle Medium (DMEM)/10% FCS/100 μg/ml streptomycin/100 U/ml penicillin (all Biochrom AG) with osteogenic supplements 50 μM L-ascorbic acid-2-phosphate, 100 nM dexamethasone and 10 mM β-glycerophosphate (all Sigma-Aldrich, Steinheim, Germany). Alkaline phosphatase expression and bone mineralization were confirmed by Sigma fast BCIP/NBT (Sigma-Aldrich) and Von Kossa staining. Adipocytes were identified by Oil red O (Sigma-Aldrich) after treatment of postconfluent MSCs with induction medium consisting of high-glucose DMEM/10% FCS/1 μM dexamethasone/0.2 mM indomethacin/0.5 mM 3-isobutyl-1-methylxanthine (all from Sigma-Aldrich)/10 μg/ml insulin (Novo Nordisk, Mainz, Germany), and maintenance medium containing DMEM, FCS, antibiotics and 10 μg/ml insulin.

To induce chondrogenic differentiation, 2.5×10^5 rat MSCs were centrifuged into a dense pellet and cultured in medium consisting of DMEM, ITS + 1, 100 nM dexamethasone, 0.17 mM L-ascorbic acid-2-phosphate (all Sigma-Aldrich) and 10 ng/ml transforming growth factor-β3 (TGFβ3; R&D Systems, Wiesbaden, Germany). To assess chondrogenesis, pellets were embedded in carbowax (Sakura Finetek, Torrance, USA) and cryosections (8 μm) were stained with Alcian Blue (Sigma-Aldrich) and counterstained with nuclear fast red.

T-cell proliferation assay with MSCs

LEW MSCs or LEW primary dermal fibroblasts as a control cell type were seeded in 96-well flat-bottom plates (Sarstedt, Nümbrecht, Germany) in 200 μl α-MEM/10% FCS at various cell densities, of 1.5×10^5, 3×10^4, 6×10^3 or 1.2×10^3 cells per well and cultured for 24 h at 37 °C, 5% CO_2. After 24 h, lymph nodes were isolated from LEW and DA rats and were homogenized using a 100 μm cell strainer into a single cell suspension in phosphate buffered saline (PBS). LEW lymph node cells were washed twice and resuspended at a density of 1×10^7 cells/ml in PBS and labeled with a final concentration of 2 μM carboxy-fluoresceindiacetatesuccinimidyl ester (CFDA-SE; Invitrogen, Karlsruhe, Germany) with gentle mixing for 4 min at room temperature. Unbound CFDA-SE was quenched by adding an equal volume of FCS followed by three washes with Roswell Park Memorial Institute (RPMI) medium/10% FCS.

For the mixed lymphocyte reaction (MLR), CFDA-SE-labeled LEW lymph node cells (3×10^5/well) as the responder and 30 Gy γ-irradiated DA lymph node cells (3×10^5/well) as the stimulator were seeded in 96-well plates alone or in wells preseeded with MSCs or fibroblasts, with cell ratios of 1:2, 1:10, 1:50 and 1:250 at a final volume of 200 μl/well RPMI medium/2% autologous rat serum/10% FCS in humidified atmosphere at 37 °C and cultured for 4 days. All lymphocytes were harvested, washed in cold PBS/2% FCS/0.1% sodium azide and thereafter co-stained with mouse antibodies specific for rat CD4-APC, CD8a-PerCP and T-cell receptor (TCR)-PE (all BD Biosciences Pharmingen). FACS measurement and analysis was performed with FACS-Calibur™ and CellQuest software (both Becton Dickinson) was used to identify proliferation of lymphocyte subpopulations.

Transwell cultures

Syngeneic (LEW) or allogeneic (DA) MSCs (each 2×10^5/well) were seeded into the lower chambers of 24-well plates with a 3 μm pore membrane (Corning Life Sciences, Lowell, USA) 24 h before beginning the MLR assay. CFDA-SE-labeled LEW lymph node cells and irradiated DA cells (each 2×10^6/well) were co-cultured in the upper chamber in a total volume of 1.5 ml RPMI medium/10% FCS/2% autologous serum. After 4 days, cells from the upper chamber were harvested and analyzed by FACS as described above. Supernatants were collected and TNF-α and IFN-γ levels were measured using ELISA kits (eBioscience).

Sample preparation and total RNA isolation

After kidney grafts were harvested, the upper half of the graft was immediately frozen in liquid nitrogen and stored at −80 °C. Thawed tissues were placed in 3 ml lysis buffer (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and then homogenized with a tissue homogenizer (Janke & Kunkel, Staufen, Germany). RNA for real-time RT-PCR was extracted with the NucleoSpin RNA II Kit (Macherey-Nagel GmbH & Co. KG) according to the manufacturer's instructions.

Real-time RT-PCR

Total RNA was reverse transcribed into cDNA as described elsewhere [28] and subjected to quantitative real-time RT-PCR utilizing the GeneAmp®5700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). The Taqman-PCR reactions for CD3, CD25, ICAM-1, MHC II, IFN-γ, TNF-α and interleukin (IL)-1β (synthesized by Metabion, Martinsried, Germany) were performed in a final volume of 25 μl containing 1 μl cDNA, 12.5 μl Mastermix (TaqMan Universal PCR Mastermix; Applied Biosystems), 1 μl fluorogeneic hybridization probe, 6 μl primer mix and 5.5 μl PCR-grade water. For CCL19 and CCL21, the analysis was performed using the SYBR®Green PCR Kit (Applied Biosystems). The sequences for all forward and reverse primers as well as noncommercial probes are shown in Table 1. For ICAM-1, an assay-on-demand system was used (Applied Biosystems). Expression of the housekeeping gene β-actin was used for normalization before relative expression levels of the target gene mRNA were calculated by the formula ($2^{-\triangle Ct}$).

Kidney transplantation

Male DA kidneys were harvested and perfused with University of Wisconsin solution (Charité, Berlin, Germany) and kept at 4 °C for 30 min or 24 h. Then kidney grafts were transplanted orthotopically into male LEW recipients using standard microsurgical techniques. Contralateral kidneys were removed immediately after implantation of the left kidney graft. Cyclosporine A (CsA, Sandimmun®; Novartis, East Hannover, USA) was

Table 1. Applied primers and probes used for real-time RT-PCR analysis.

Gene	Forward primer	Reverse primer	Probe
CD3	5′-caa aga aac taa cat gga gca ggg-3′	5′-ctt ttt gct ggg cca tgg t-3′	5′-agg ttt ggc tgg cct ctt cct ggt g-3′
CD25	5′-cac agt ctg tgt acc agg aga acc t-3′	5′-cca cga agt ggt aga ttc tct tgg-3′	5′-cag gtc act gca ggg agc ccc c-3′
MHC class II	5′-ggt tga gaa cag caa gcc agt c-3′	5′-ggt gag gta agc cat ctt gtg g-3′	5′-tga gac cag ctt cct ttc caa ccc tga-3′
TNF-α	5′-tcg agt gac aag ccc gta gc-3′	5′-ctc agc cac tcc agc tgc tc-3′	5′-cgt cgt agc aaa cca cca agc aga-3′
IFN-γ	5′-cag gtc act gca ggg agc ccc c-3′	5′-ttc att gac agc ttt gtg ctg g-3′	5′-cgc caa gtt cga ggt gaa caa ccc-3′
IL-1β	5′-acc aaa aat gcc tcg tgc tgt ct-3′	5′-tgt tgg ctt atg ttc tgt cca ttg-3′	5′-acc cat gtg agc tga aag ctc tcc acc-3′
CCL19	5′-gcc ttc cgc tac ctt ctt atc ca-3′	5′-agc ccc tta gtg tgg tga aca c-3′	–
CCL21	5′-cca tcc cag caa tcc tgt tc-3′	5′-cct cag ggt ttg cgc ata-3′	–
β-Actin	5′-gta caa cct cct tgc agc tcc t-3′	5′-ttg tcg acg acg agc gc-3′	5′-cgc cac cag ttc gcc atg gat-3′

administered for 2 days at a dosage of 1.5 mg/kg/day. Either 2.5×10^6 or 5.0×10^6 LEW MSCs were injected intravenously at three time points: 7 days before, immediately after, and 1 day after transplantation. Three days after transplantation, grafts were harvested for RT-PCR and histological analysis ($n = 6$ per group per ischemic interval and dosage of injected MSCs; Fig. 1). Operated animals appeared healthy until just before graft harvest without surgical complications during the observation period.

Immunohistology

Immunohistochemistry was performed as described elsewhere [29] using primary mouse anti-rat specific antibodies against CD4, CD8, CD68, OX62 (all AbDSerotec), MHC II or an IgG isotype-identical control antibody (both BD Biosciences Pharmingen).

Statistical analysis

All values are reported as means ± SEM. The data within groups were compared using Student's *t*-test and Mann–Whitney *U*-test. The difference was considered to be significant when $P < 0.05$.

Results

Rat MSCs express typical marker and differentiation profiles

LEW bone marrow-derived MSCs strongly expressed CD90, MHC I, and ICAM-1, and moderately expressed CD80, whereas MHC II, TCR, CD45, CD86 and VCAM were absent from the cell surface as shown by flow cytometric analysis (Fig. 2a). Culture expanded MSCs were also tested for their multilineage differentiation potential. *In vitro* tests using appropriate inductive culture conditions promoted differentiation of MSC toward osteogenic, adipogenic and chondrogenic lineages (Fig. 2b), confirming they possess typical MSC features.

Rat MSCs dose-dependently inhibit immune cell proliferation

Proliferation assays based on CFDA-SE labeling demonstrated low immunogenicity of allogeneic DA MSCs with

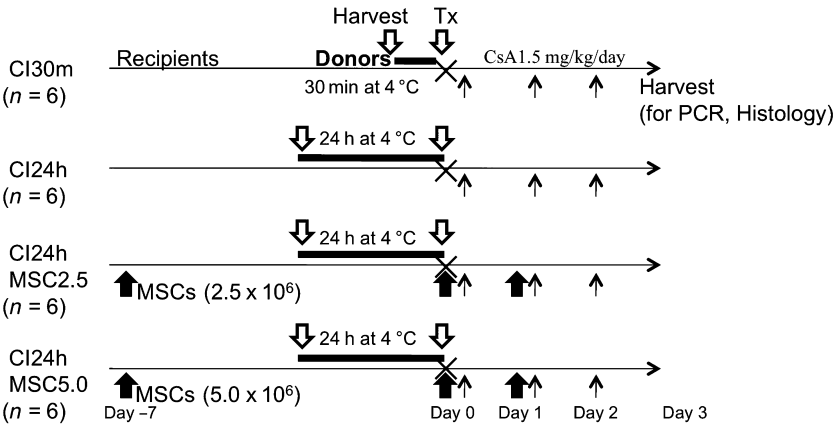

Figure 1 Experimental flow chart for *in vivo* study design. Each group consisted of six animals. Male Dark Agouti (DA, $RT1^{av1}$) kidneys were removed, perfused with University of Wisconsin solution and kept at 4 °C for 30 min or 24 h. Thereafter, kidney grafts were transplanted orthotopically into male Lewis (LEW, RT1) recipients. Cyclosporine A (CsA) treatment was given daily at a dosage of 1.5 mg/kg/day. 2.5×10^6 or 5.0×10^6 of LEW mesenchymal stem cells (MSCs) were intravenously injected three times: 7 days before, immediately after and 1 day after transplantation. Three days after transplantation, grafts were harvested for qRT-PCR and histological analysis.

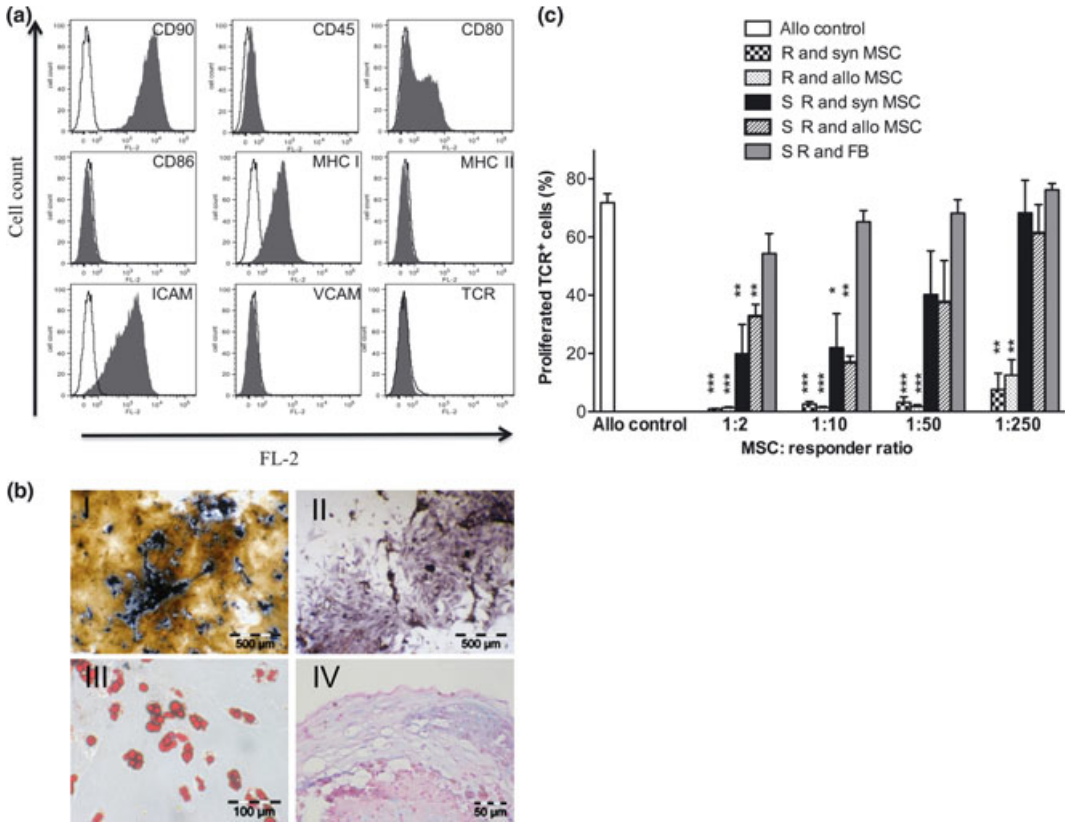

Figure 2 Characteristics of rat bone marrow-derived mesenchymal stem cells (MSCs). (a) Flow cytometric analysis of LEW bone marrow-derived mesenchymal stem cell population after passage five. MSCs are positive for CD90, MHC class I, CD80, and ICAM-1, but negative for MHC class II, CD86, CD45, VCAM and T-cell receptor. Representative FACS histograms of $n = 6$ are shown. (b) Representative microphotographs demonstrate the osteogenic (I: von Kossa staining and II: Alkaline Phosphatase staining), adipogenic (III: Oil Red O staining) and chondrogenic (IV: Alcian blue staining) differentiation of LEW MSCs. (c) T-cell proliferation assay was performed in the presence of various concentrations of syngeneic (syn) or allogeneic (allo) rat bone marrow-derived MSCs using CFDE-SE-labeled lymph node cells (3×10^5) from the LEW rat as the responder (R) and 30 Gy γ-irradiated lymph node cells (3×10^5) from the DA rat as the stimulator (S) for the mixed lymphocyte reaction (MLR) setting. LEW fibroblasts (FB) were seeded as a control. The percentage of proliferated TCR^+ cells was determined by FACS analysis after 4 days co-culture. Allogeneic MSCs exhibited low immunogenicity, similar to syngeneic MSCs. Both MSC types reduced proliferation responses compared with the normal MLR setting without MSCs (white bar) as well as fibroblasts control (gray bar). Results are presented as mean ± SEM of three independent experiments. *, ** and *** indicates statistical significance (at least $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively) versus control cultures (allo control without MSCs).

proliferation levels comparable to that of LEW responder lymph node cells alone. MLR co-cultures with both syngeneic (LEW) and allogeneic (DA) MSC types showed significantly reduced proliferation responses at MSC:responder ratios of 1:2 and 1:10 for all TCR^+ cells in contrast to the MLRs with fibroblasts (Fig. 2c). In addition, the allo-specific proliferation response of both $CD4^+$ and $CD8^+$ T cell subpopulations was reduced with MSCs present. However, in the transwell setting, the inhibitory effect of MSCs on T cell proliferation was absent (Fig. 3a).

MSCs express anti-inflammatory properties

To evaluate the potential anti-inflammatory properties of rat MSCs, supernatants from MLR/MSC co-cultures were analyzed by ELISA. In the presence of MSCs we detected significantly decreased levels of the key inflammatory cytokines, TNF-α and IFN-γ, compared with those without MSCs. In the transwell setting, this cytokine release inhibition was abolished for TNF-α, but still partially observed for IFN-γ (Fig. 3b). In addition, we ascertained that MSCs are able to diminish TLR-triggered TNF-α release in LPS stimulated co-cultures with rat macrophages (data not shown).

MSCs ameliorate IRI-induced early inflammation process in allografted kidneys *in vivo*

To investigate the immunomodulatory potential of MSCs *in vivo*, isolated MSCs were applied in a 'marginal' kidney

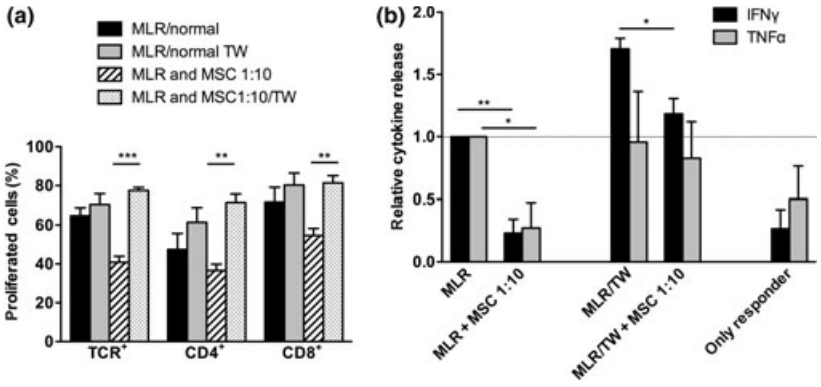

Figure 3 Abolished inhibitory effects of mesenchymal stem cells (MSCs) in transwell cultures. LEW MSCs were seeded into the lower chamber of 24-well plate 24 h before the beginning of the culture. CFDE-SE-labeled LEW lymph node cells (2×10^6) and irradiated DA lymph node cells (2×10^6) were co-cultured in the upper chamber of a transwell plate for 4 days. (a) Normal mixed lymphocyte reaction (MLR normal) assays with MSCs at a 1:10 ratio exhibited inhibition of T-cell proliferation (shaded bar), whereas in transwell assays the inhibitory effects of MSCs were abolished (dotted bar). (b) ELISA tests showed decreased levels of TNF-α and IFN-γ in the supernatants from normal MLR with MSCs compared with those without MSCs. However, in the transwell setting MSC induced inhibition was abrogated completely in TNF-α and partially in IFN-γ release. Data are recalculated as a relative value to normal MLR (set = 1, dashed line) and presented as mean ± SEM of three independent experiments; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

transplantation model: prolonged cold ischemia of the DA donor kidney grafted into a high-responder fully MHC-mismatched LEW recipient. First we compared kidney grafts undergoing short and long CI without MSC administration at day 3 after transplantation to evaluate the effect of CI on intragraft inflammation (Fig. 4). Confirming recent data by our group that long CI induces up-regulation of several candidate markers, like pro-inflammatory cytokines and cell adhesion molecules in a weaker strain combination [11], here we measured in our strong model following long CI a significant increase of intragraft TNF-α, IFN-γ, CD25 ($P < 0.05$) and ICAM-1 ($P < 0.01$) mRNA expression compared with short CI (Fig. 4a–c; $n = 6$ per group). A similar trend, but not statistically significant, was observed regarding the transcription of IL-1β, chemokine ligand (CCL)19 and CCL21, and early T cell infiltration (CD3), whereas MHC class II expression was similar between groups (Fig. 4a–c).

To evaluate potential beneficial effects on candidate marker gene expression, we applied either 2.5 or 5.0×10^6 syngeneic LEW MSCs intravenously at three time points (Fig. 1). Low-dose (2.5×10^6 per application time point) MSC administration had only a marginal impact on intragraft inflammation; only CCL21 ($P < 0.01$) was significantly decreased compared with untreated controls within the long CI group. Notably, the high-dose MSC administration group displayed a distinctively decreased mRNA expression level for TNF-α, IFN-γ, IL-1β, MHC II, CD3, CD25, ICAM-1, CCL19 and CCL21 ($P < 0.05$, Fig. 4a–c). For other markers (e.g. IL-6 and interferon gamma induced protein-10) we could not detect any significant changes in intragraft expression levels induced by high-dose MSC administration (data not shown).

In addition, we also performed immunohistological analysis of the harvested kidney grafts ($n = 6$ per group) and evaluated the effect of MSC administration on cellular infiltration levels, especially in the kidney cortex. A significant inhibition of macrophage ($CD68^+$) and DC ($Ox62^+$) infiltration was observed in the high-dose MSC group (Fig. 5). In contrast, the early graft infiltration of $CD4^+$ and $CD8^+$ T cells as well as the intragraft MHC II expression was unchanged by MSC application (Fig. 5).

Despite prolonged cold ischemia, serum creatinine levels were only moderately increased (ca 1.7–2.2 mg/dl) at day 1 post-transplantation in all animals but dropped to normal levels (<0.8 mg/dl) within 3 days. MSC therapy did not change this result (data not shown). Without immunosuppression, graft survival in the very strong DA to LEW model is only 7.2 ± 0.37 days (mean ± SEM) days. Short-term treatment with low-dose CsA (1.5 mg/kg for 10 days after transplantation) prolonged mean graft survival to 117.57 ± 32.00 days, but peri-transplant MSC treatment did not further improve the survival time.

Discussion

Ischemia/reperfusion injury-induced intragraft inflammation is a key factor of renal allograft immunogenicity, explaining the poorer outcome of 'marginal organs' from deceased donors [30]. Targeting inflammation by donor-pretreatment with heme oxygenase-1 inducing drugs or carbon monoxide improved the short-term and long-term outcome of renal 'marginal' allografts [31–34]. MSCs with

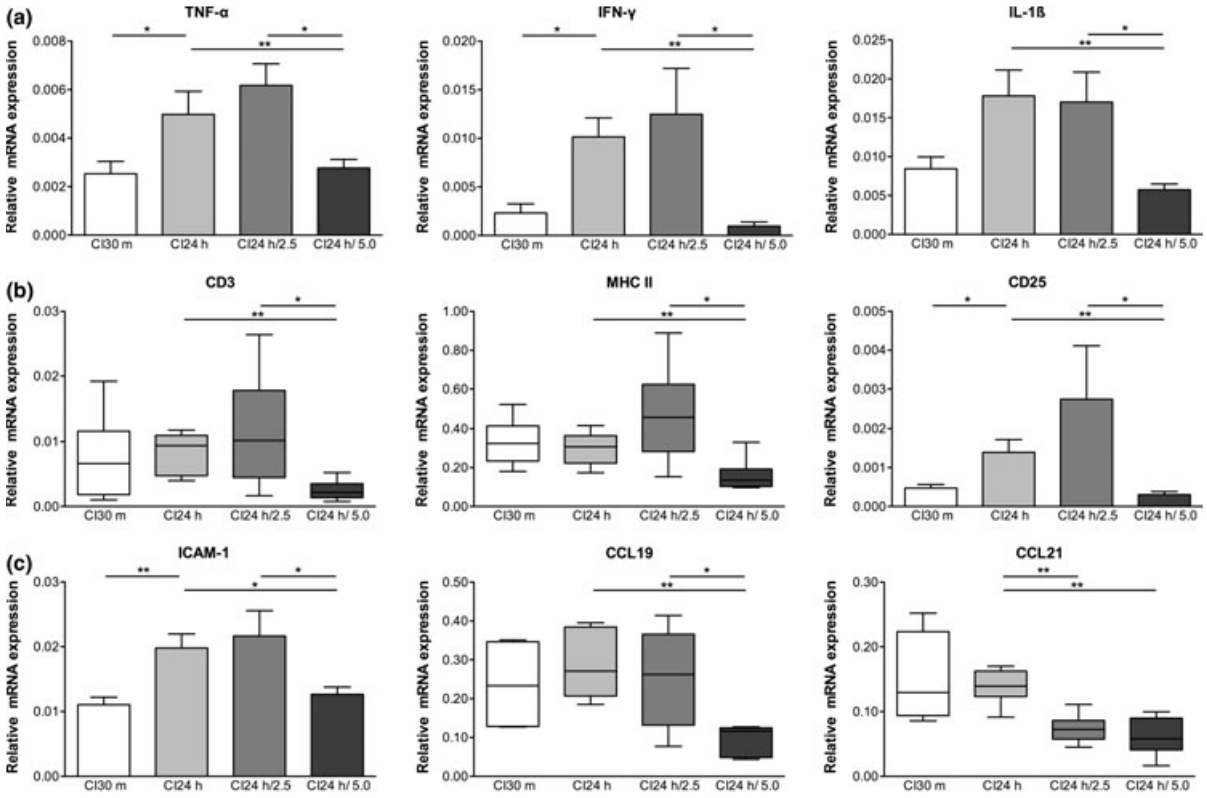

Figure 4 mRNA expression in kidney graft 3 days after transplantation. (a) Grafts transplanted after prolonged cold ischemic time of 24 h (CI24h) exhibited increased levels of TNF-α and IFN-γ compared with levels measured after short cold ischemia of 30 min (CI30m) (*$P < 0.05$). Three times administration of high-dose (5×10^6) mesenchymal stem cells (MSCs) led to decreased levels of TNF-α, IFN-γ and IL1-β expression in the grafts subjected to long cold ischemia compared with those without MSC administration (**$P < 0.01$). (b) Long cold ischemia led to enhanced level of CD25 expression (*$P < 0.05$), and the grafts from the rats treated with high-dose MSCs led to significant decreases in MHC II, CD3 and CD25 expression in the grafts (**$P < 0.01$). (c) Intracellular adhesion molecule-1 (ICAM-1) expression was enhanced in the grafts subjected to long cold ischemia (**$P < 0.01$). Kidney grafts of high-dose MSC group showed decreased levels of ICAM-1 (*$P < 0.05$), CCL19 and CCL21 expression (**$P < 0.01$). The box-and-whisker plot demonstrates the minimum, and 5th to 95th percentile of each marker; ($n = 6$).

their broad immunomodulatory and tissue protective properties might be an ideal candidate for conditioning the recipient environment to combat IRI in transplanted organs. Therefore, we addressed in this study whether the administration of MSCs might protect kidneys from IRI following long-term CI.

Although numerous IRI-related studies describe protective effects of MSCs upon warm ischemic damage [25–27,35] or cold ischemia in chronic kidney transplantation models [36,37], few groups focused on the relationship between IRI and allo-reactive responses [38,39]. With the objective to investigate MSC efficacy in a more clinically relevant model, we chose the strongest and best-established acute rat kidney rejection model (DA–LEW) with prolonged cold IRI.

The key steps of IRI in the early phase following the exposure of pattern-recognition receptors on intragraft macrophages, DCs and some tissue cells to DAMP-ligands are: (i) the induction of pro-inflammatory cytokines, that further amplify the innate responses of macrophages and DCs, (ii) attracting additional immune cells (monocytes/macrophages, granulocytes, NK cells, preformed memory T/B cells) from the circulation to the inflamed graft that boost inflammation by further cytokine release, e.g. TNF-α and IFN-γ and (iii) the enhanced recruitment and activation of DCs leading to their migration into regional lymph nodes and spleen, inducing allo-reactivity.

Analyzing the effects of MSC application within this corresponding early time span we demonstrated an impact on IRI-related inflammation on all three processes described above: (i) downregulation of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β), (ii) inhibition of adhesion molecules (ICAM-1) resulting in diminished infiltration by macrophages ($CD68^+$) and $CD3^+$ T cells, particularly of activated $CD25^+$ cells and (iii) prevention of IRI-induced release of DC-attracting chemokines (CCL19 and 21) resulting in diminished infiltration by DCs ($Ox62^+$).

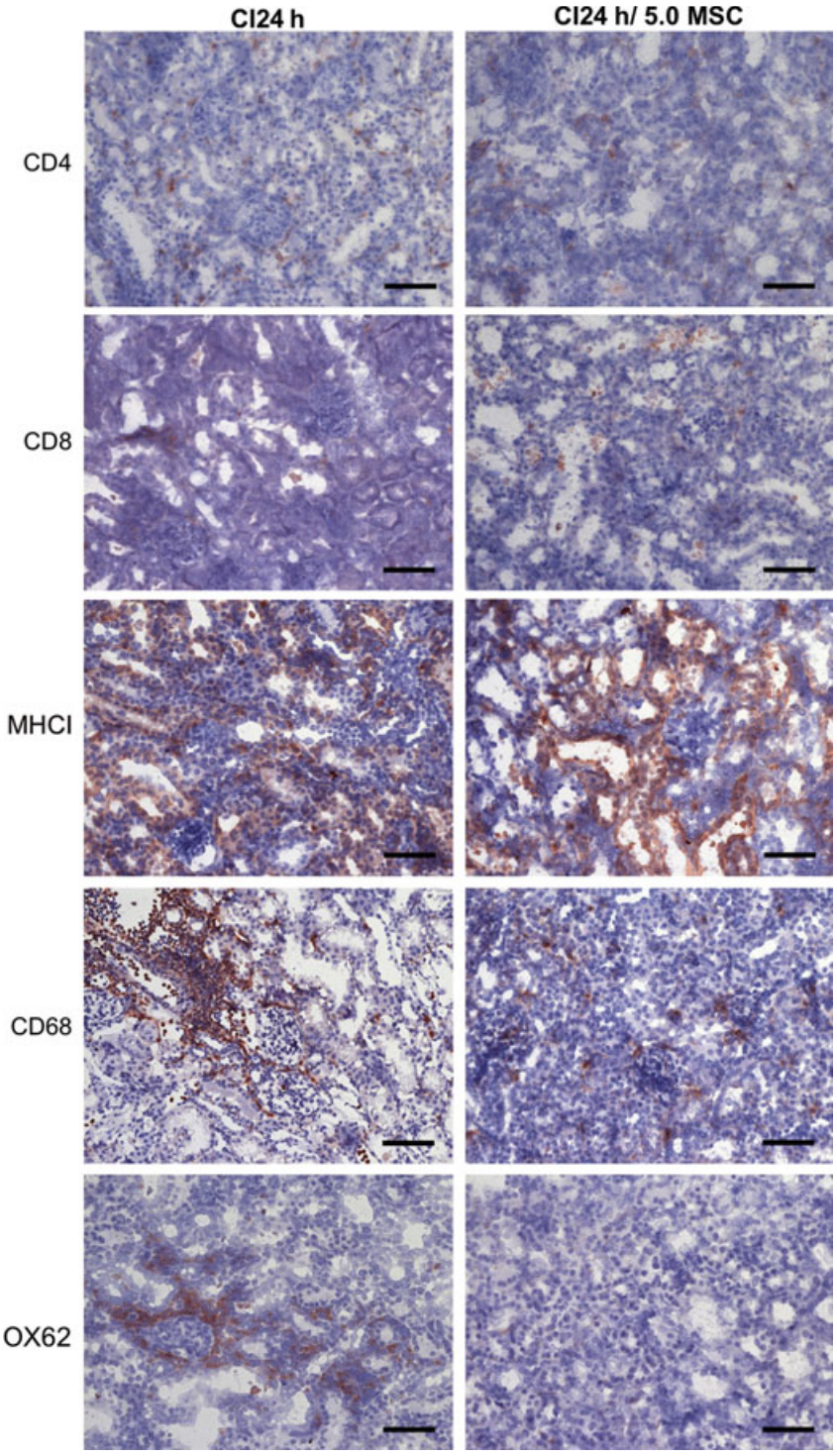

Figure 5 Immunohistological analysis of the kidney grafts 3 days post-transplantation. Cryosections (5 μm) of kidney grafts subjected to 24 h cold ischemia (CI24h) in the absence of mesenchymal stem cells (MSCs) administration (left panel), or with MSCs administration at three time points (right panel) were stained with specific antibodies against CD4, CD8, major histocompatibility complex (MHC) class II, CD68, OX62 (brown staining). Counterstaining was performed with hematoxylin (blue staining). No difference was observed in graft infiltration of CD4, CD8 and MHC II expressing cells between both treatment groups. However, grafts of MSC-treated rats exhibited decreased infiltration of CD68 and OX62 positive cells compared with those of untreated rats; (n = 6). Scale bars represent 100 μm.

Several studies have demonstrated that prolonged CI of allografts results in impaired organ function immediately after transplantation and that an initial damage strongly influences both early and late functional survival [40,41]. IRI may enhance the expression of MHC molecules in renal epithelium and could thereby trigger allogeneic acute/chronic rejection [34].

Consistent with previous reports in weaker models [11,42] we found a negative impact of prolonged CI on intragraft inflammation even in our high-responder/mismatch combination. ICAM-1 is known to be expressed in the graft epithelium following IRI through up-regulation of IL-1β and TNF-α [43], and increases during allograft rejection [44]. Therefore, in our IRI model of allogeneic kidney transplantation, ICAM-1 up-regulation might be associated with both allo-antigen induced immune response and antigen-independent factors. Inconsistent with previous reports [11], we observed increased CD25 but not MHC II mRNA expression after a prolonged cold ischemic time in damaged grafts, suggesting IRI might boost activated allo-reactive T-cell migration to the graft.

As shown in reports using syngeneic settings, in our allogeneic model we showed that the administration of high-dose MSCs results in down-regulation of pro-inflammatory cytokines IL1-β, TNF-α and IFN-γ in kidney grafts (Fig. 4a). Moreover, MHC II, CD3 and CD25 (Fig. 4b) expression was significantly diminished indicating a decreased immunogenicity of transplanted kidney and inhibited migration of activated T cells or possibly regulatory T cells necessary to mediate the immunomodulatory function [45]. Interestingly, MSC administration also inhibits the expression of chemokine receptor (CCR)7-ligands CCL19 and CCL21 (Fig. 4c), both involved in migration of lymphoid cells such as leukocytes and DCs. CCL19 and CCL21 are able to attract CCR7-positive naive and memory T cells that support their co-localization with DCs. Therefore, down-regulation of these chemokines might contribute to diminished graft immunogenicity.

As a possible mechanism, we speculate that MSC administration suppresses the production of pro-inflammatory cytokines, induces the down-regulation of adhesion molecules and chemokines, and thereby inhibits the migration of immune cells. IFN-γ is well known to induce MHC II expression and can also be amplified by TNF-α produced by other cells [46]. ICAM-1 expression could be affected by TNF-α and IFN-γ production and it has also been reported that MSCs induce down-regulation of ICAM-1 on co-cultured fibroblasts [47].

Following down-regulation of pro-inflammatory cytokines, both MHC II expression and subsequent cell migration into the injured kidney might be suppressed by MSC administration. Our immunohistological data showing reduced infiltration of antigen-presenting cells (Fig. 5) further support this presumed mechanism. Corresponding effects of MSC administration including reduced infiltration of antigen-presenting cells and diminished intragraft ICAM-1 expression were recently described in an allogeneic rat heart transplantation model [48].

However, the exact immunomodulatory mode of MSC action still remains unclear. Some reports have demonstrated that direct cell-cell contact is required for suppression [49], whereas others have shown that the suppressive activity rather depends on soluble factors [14,50,51]. Recent reports propose a potential role of IL-1 receptor antagonist expressed by MSCs subpopulations [52,53]. Data by Ortiz *et al.* showing the inhibition of TNF-α and IL-1 following MSC administration in a model of lung injury preventing lymphocytes and neutrophils from migrating into the injured lung are consistent with our results [52]. Other paracrine effects proposed to explain MSC mechanisms are the release of microvesicles and transfer of mRNA or microRNA [54]. In our model, MSCs did not inhibit T cell proliferation in the *in vitro* transwell setting, but their suppressive effect on IFN-γ release was still partially observed. Therefore, the combined effects of cell-cell contact along with soluble factors [55], might be responsible for MSC-related immunomodulation and would also explain the reduced TNF-α release in LPS stimulated rat macrophage cultures (data not shown).

To examine the mechanism of MSC action *in vivo* we analyzed the migration of intravenously injected MSCs labeled with Dil. Most of the cells were trapped in the lungs, whereas few cells were observed in the damaged kidney (data not shown), confirming data by others [56,57]. In contrast to the clear contact dependency measured *in vitro*, the *in vivo* condition appears to be different. Other groups have suggested that either paracrine actions or differentiation into functional renal cells might be responsible [52,58–60] for MSC effects in models of acute and chronic diseases. Recently, kidney transplantation studies in a mouse model support the role of indoleamine 2,3-dioxygenase expression accompanied with an induction of regulatory T cells in mediating the immunomodulatory MSC function *in vivo* [45]. Our *in vitro* results comparing the pro-inflammatory cytokine release in MLR/MSC co-cultures with direct cell contact or in separate compartments support the involvement of paracrine factors as well as direct interaction (Fig. 3). Moreover, the preferential effects observed using higher dose MSC administration *in vivo* might be because of higher cell counts reaching the grafted kidney after being initially trapped within lung tissue and/or releasing higher levels of paracrine acting mediators.

The failure to induce tolerance and long-term graft survival by MSC application in our model might be caused

either by the strong MHC mismatch combined with the long-term ischemic damage and/or injecting syngeneic MSC in contrast to other studies using allogeneic cells and weaker models of MHC disparity [61,62].

In conclusion, we have demonstrated that MSC therapy ameliorates the negative impact of prolonged ischemia on intragraft inflammation and immunogenicity in a very strong, clinically relevant model of rat kidney transplantation at early time points. It suggests the potential power of MSC therapy for improving outcomes of future marginal organ transplants. However, we see the greater potency of MSCs in combating undesired inflammation related to IRI than in tolerance induction.

Authorship

YH: acquired and analyzed the data, participated in research design and drafted the manuscript. MS: acquired and analyzed the data and made critical revision of the manuscript. JR, TD: acquired and analyzed data and drafted part of the manuscript. JL: participated in data analysis and made critical revision of the manuscript. KK: participated in research design and made critical revision of the manuscript. AR-S: made critical revision of the manuscript. PR, H-DV: participated in research design and made critical revision of the manuscript. MS: conceived and participated in research design, interpreted the data, and drafted the manuscript.

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