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Abstract

Intramuscular administration of mesenchymal stromal cells (MSCs) represents a therapeutic option for diabetic critical limb ischemia. Autologous or allogeneic approaches may be used but disease-induced cell dysfunction may limit therapeutic efficacy in the former. Our aim was to compare the efficacy of allogeneic and autologous MSC transplantation in a model of hindlimb ischemia in diabetes mellitus and to determine whether allogeneic transplantation would result in the activation of an immune response. MSCs were isolated from C57BL/6 (B6) and diabetic obese C57BKS^{db/db} mice. Phosphate-buffered saline (control group), and MSCs (1×10^6) from B6 (allogeneic group) or C57BKS^{db/db} (syngeneic group) were administered intramuscularly into the ischemic thigh of C57BKS^{db/db} mice following the induction of hindlimb ischemia. MSCs derived from both mouse strains secrete several angiogenic factors, suggesting that the potential therapeutic effect is due to paracrine signaling. Administration of allogeneic MSCs significantly improved blood perfusion as compared with the control group on week 2 and 3, post-operatively. In comparison with the control group, syngeneic MSCs significantly improved blood perfusion at week 2 only. There was no statistical difference in blood perfusion between allogeneic and syngeneic MSC groups at any stages. There was no statistical difference in ambulatory and necrosis score among the three groups. Amputation of toes was only observed in the control group (one out of seven animals). Alloantibody was detected in three out of the eight mice that received allogeneic MSCs but was not observed in the other groups. In summary, we demonstrated comparable efficacy after transplantation of autologous and allogeneic MSCs in a diabetic animal model despite generation of an immune response.

Keywords

Mesenchymal stem cells, diabetes mellitus, allogeneic transplantation, alloantibody

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Introduction

We have previously shown that mesenchymal stromal cells (MSCs) promote angiogenesis in both *in vitro* and *in vivo* models^{1–3}. Early clinical trials showed that administration of either autologous or allogeneic MSCs in patients with diabetes and critical limb ischemia is both feasible and safe with some evidence of efficacy^{4–8}. A direct comparison with an exploration of whether an immune reaction has been induced has not been studied in this cohort. Type 2 diabetes mellitus (T2DM) is associated with a reduction in MSC number and impaired MSC survival, proliferation, multipotency, homing ability and *in vivo* revascularization^{9–11}. Furthermore, there is evidence of impaired neovascularization in animal models of diabetes with db/db mice exhibiting greater impairment in neovascularization than observed in type I diabetic mice¹². Yan et al. showed that the wild type recipient mice that received MSCs from db/db mice exhibited less blood flow in the ischemic leg than MSCs derived from the wild type donor¹³. While this study explored the effect of the origin of the cells from diabetic or non-diabetic animals, the effect of transplantation into diabetic animals has not been reported, in particular, in the context of comparing autologous and allogeneic cell sources. The rationale of allogeneic transplantation is anchored on mitigating the risk of cell dysfunction related to diabetes mellitus and practical difficulties of MSC isolation following patient presentation to the hospital, and thus may provide the advantage of an ‘off the shelf’ product which is ready for use on patient presentation.

MSCs possess immunosuppressive properties, albeit, they may not be immune-privileged. A humoral immune response has been demonstrated following MSC administration, with an increasing effect, observed from syngeneic, allogeneic to xenogeneic transplantation¹⁴. Furthermore, allogeneic MSC transplantation is associated with alloantibody formation^{15,16}. Huang et al. showed that allogeneic (but not syngeneic) cells were eliminated from the heart by 5 weeks after implantation, and their functional benefits were lost within 5 months¹⁷. This immune response can attenuate the survival of subsequent administration of allogeneic MSCs^{15,18}. However, the possibility of allograft tolerance induced by alloantibodies demonstrated in these preclinical studies, has not yet been shown in humans¹⁹.

In this study, we hypothesized that allogeneic MSCs derived from wild type B6 mice would be more efficacious than syngeneic MSCs derived from C57BKS^{db/db} mice for therapeutic revascularization in C57BKS^{db/db} mice following induction of hindlimb ischemia. We also sought to determine the incidence of alloantibody formation and whether this would have an effect on the efficacy of allogeneic MSC therapy in C57BKS^{db/db} mice following the induction of hindlimb ischemia.

Methods

Experimental Design

Schematic representation of the study design is shown in Fig. 1. The surface markers and tri-lineage differentiation ability of MSCs isolated from adult wild type B6 mice and C57BKS^{db/db} mice were compared using flow cytometric analysis and standard differentiation (osteogenesis, adipogenesis and chondrogenesis) assays. Angiogenesis array was performed using the conditioned media derived from these MSCs. MSCs from either adult wild type B6 mice or C57BKS^{db/db} mice, or phosphate-buffered saline (PBS) were delivered intramuscularly to three groups of C57BKS^{db/db} mice following induction of hindlimb ischemia. Laser Doppler flow assessment, as well as, ambulatory and necrosis scores were performed weekly for 3 weeks on these three groups of mice. Percentages of toe necrosis and toe amputation were assessed. Finally, anti-B6 immunoglobulin (Ig)G antibodies in serum samples from the three groups of C57BKS^{db/db} mice were quantified.

Animals

Adult wild type B6 mice and C57BKS^{db/db} mice (8–12 weeks of age) were purchased from Charles River Labs (Mayo, Ireland). The mice were housed and equilibrated to the preclinical facility at the Regenerative Medicine Institute (REMEDI), National University of Ireland Galway (NUIG). All animal experiments were carried out with ethical approval from the Animal Care Research Ethics Committee (ACREC) of the National University of Ireland, Galway, and under appropriate individual and project authorizations from Health Products Regulatory Authority (HPRA) of Ireland (License number B342) and were performed in accordance with the Principles of Laboratory Animal Care.

Isolation and Expansion of Mouse MSCs

Bone marrow cell suspension was prepared with modification, as previously described²⁰. Briefly, femurs and tibiae were dissected and crushed with a pestle and mortar. The crushed bones were washed several times in HBSS+ (Hanks-balanced salt solution) supplemented with 2% fetal bovine serum (FBS), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and 1% penicillin/streptomycin), and the cell suspension was filtered through a cell strainer (Fisher Scientific, Dublin, Ireland) several times. Cell suspensions were collected and bone marrow fragments were crushed again. The suspension was filtered with a cell strainer, and collected by centrifugation at 280 g for 7 minutes at 4°C. The bone marrow cells were collected in the pellet and soaked in 1 ml of water (Sigma-Aldrich, Wicklow, Ireland) for 5–10 seconds to burst red blood cells, after which 1 ml of 2x PBS containing 4% FBS was added. The cells were resuspended in HBSS+, and the suspension was filtered through a cell strainer.

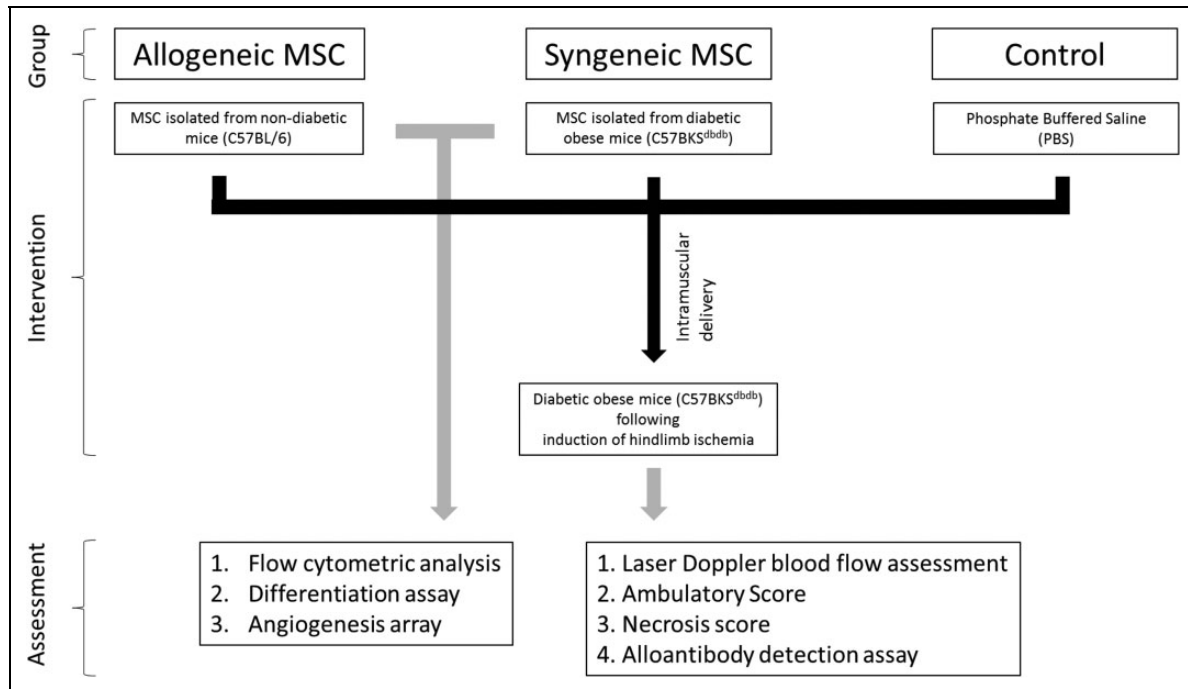


Fig. 1. Schematic representation of the study design. MSC: mesenchymal stromal cell.

MSC Culture Conditions

The cells were washed with PBS twice, digested with 0.25% trypsin for 2 minutes at 37°C to obtain a single cell suspension, then trypsin was neutralized with α -MEM medium. The cell suspension was then centrifuged at 800 g for 5 minutes and resuspended in a T75 flask (Corning Inc. Dublin, Ireland). The derived cell suspensions were plated on 10-mm plates in α -minimum essential medium (MEM) + GlutaMAX supplemented with 10% equine serum, 10% FBS and 100 units/ μ g penicillin/streptomycin (mMSC medium). Cells were incubated in a humidified incubator at 37°C supplemented with 21% O₂. At 3–4 day intervals, the medium was changed and non-adherent hematopoietic cells removed by gentle pipetting. At 10–14 days, when the primary culture was approximately 80% confluent, cells were detached with trypsin/ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, Wicklow, Ireland) for 5 min and seeded to new plates. Culture medium was changed every 3–4 days thereafter and subsequent passages were performed when cells reached 60 to 80% confluency.

Flow Cytometric Analysis

The cells were suspended in ice-cold PBS with 10% FBS at 10⁶ cells/ml, and then stained for 30 minutes on ice with the following monoclonal antibodies (mAbs): biotinylated F4/80; Allophycocyanin (APC)-conjugated CD29, platelet-derived growth factor receptor (PDGFR) α (APA5); fluorescein isothiocyanate (FITC)-conjugated Sca-1 (Ly6A/E),

CD45, CD49e; PE-conjugated CD31, CD44, CD73, CD90 and CD105. Biotinylated antibodies were visualized with FITC-conjugated streptavidin (BD Biosciences, BD Biosciences, Oxford, UK). All mAbs were purchased from eBioscience, San Diego, USA, except for CD90 (BD Biosciences). Flow cytometry analyses were performed on a dual-laser BD Accuri C6 flow cytometer. Propidium Iodide (PI) fluorescence was measured, and a live cell gate was defined that excluded the cells positive for PI. Additional gates were defined as positive or negative according to the isotype control fluorescence intensity.

Tri-Lineage Differentiation Assay

For chondrogenic differentiation, cultured cells were harvested by trypsinization at passage two to three. A total of 2.5 \times 10⁵ cells were transferred into a 15 ml conical tube and washed with MSC medium. The tube was centrifuged at 240 g for 5 minutes at room temperature, and the supernatant was aspirated. The cells were resuspended in 1 ml Incomplete Chondrogenic Medium (ICM) into 1.5 ml screwcap microtubes, centrifuged at 100 g for 5 minutes, and the medium was aspirated. The ICM consists of Dulbecco's modified Eagle's medium (DMEM) (high glucose) supplemented with 1 nM dexamethasone, 50 μ g/ml ascorbic acid 2-P, 40 μ g/ml L-proline, 1% Insulin Transferrin Selenium (ITS) plus supplement (BD Biosciences), 1 mM sodium pyruvate and 100 units/ μ g penicillin/streptomycin. The cells were resuspended in 500 μ l of Complete Chondrogenic Medium (CCM) and centrifuged

at 100 g for 5 minutes at room temperature. The CCM consists of ICM supplemented with transforming growth factor (TGF) β -3 (10 ng/ml; Peptidech, London, UK) and bone morphogenetic protein (BMP)-2 (100 ng/ml; Peptidech). The pellet was maintained with ICM or CCM changes every 3–4 days for 3 weeks. After 3 weeks, cell pellets were harvested, washed in Dulbecco's PBS (DPBS; Invitrogen, Dublin, Ireland) and stained with Safranin-O for sulfated proteoglycans.

For adipocyte differentiation, sub-confluent cells were cultured with three cycles of Adipogenic Induction Medium and Adipogenic Maintenance Medium. Adipogenic Induction Medium consists of DMEM (high glucose) supplemented with 1 μ M dexamethasone, 10 μ g/ml insulin, 200 μ M indomethacin, 500 μ M 3-isobutyl-1-methyl-xanthine (Sigma-Aldrich), 10% FBS and 100 units/ μ g penicillin/streptomycin. Adipogenic Maintenance Medium consists of DMEM (high glucose) supplemented with 10 μ g/ml insulin, 10% FBS and 100 units/ μ g penicillin/streptomycin. Each cycle consisted of feeding the sub-confluent cells with the induction medium for 3 days, followed by 3 days of culture in the maintenance medium. After 14 days of culture, the cells were fixed with 10% neutral buffered formalin for 30 minutes, and stained with Oil Red O (Sigma-Aldrich).

For osteocyte differentiation, sub-confluent cells were cultured with Osteogenic Differentiation Medium, which consists of α -MEM supplemented with 100 nM dexamethasone, 50 μ M ascorbic acid 2-P, 20 mM β -glycerophosphate, 50 ng/ml L-thyroxine, 9% FBS, 9% equine serum, 2 mM L-glutamine and 100 units/ μ g penicillin/streptomycin for 14 days. The cells were then fixed with 95% methanol for 10 min and stained with Alizarin Red (Sigma-Aldrich).

Angiogenesis Array

The MSC conditioned media derived from both wild type B6 mice and C57BKS^{db/db} mice were analyzed using the Proteome ProfilerTM Mouse Angiogenesis Array Kit (R&D Systems Inc, Abingdon, UK) according to the manufacturer's instructions. Briefly, each conditioned medium sample was mixed with a cocktail of biotinylated detection antibodies and incubated in nitrocellulose membranes which were spotted with 55 different angiogenesis-related antibodies in duplicate. Any protein/detection antibody complex present is bound by its cognate immobilized capture antibody on the membrane. Following a wash to remove unbound material, streptavidin-horseradish peroxidase and chemiluminescence detection reagents are added sequentially. The developed film was further scanned by Kodak Luminescent Image Analyzer LAS-4000 and densitometric analysis on the array image was performed using ImageJ software (NIH, Bethesda, MD, USA).

Hindlimb Ischemia and Cell Injection

Unilateral hindlimb ischemia was performed as previously described²¹. In brief, hindlimb ischemia was induced by ligation of the distal and proximal end of the left femoral artery.

The mice were anesthetized with ketamine and xylazine and maintained with isoflurane. Five intramuscular injections of 2×10^5 MSCs or PBS (40 μ l each) were administered into the left thigh (two medially and three posteriorly) following unilateral femoral artery ligation. The contralateral right hindlimb served as internal control within each mouse.

Laser Doppler Blood Flow Analysis

The ratio of the ischemic/normal hindlimb blood flow was measured using Laser Doppler Perfusion Imaging (LDPI; Moor Instrument Inc., Devon, UK) as previously described²¹. Briefly, a heating pad was used to maintain the body temperature of the animal during imaging. Blood perfusion changes were expressed as a ratio of ischemic left limb to non-ischemic right limb.

Preclinical Assessment of the Ischemic Limb

Preclinical assessment for ambulatory and necrosis scores were performed before and after induction of hindlimb ischemia and on a weekly basis for 3 weeks using a scoring system. Both the ambulatory score and necrosis score were used as a surrogate marker to mimic the clinical assessment of walking distance and ischemic damage as evidenced by the presence of ulcer, necrosis or gangrene in human in clinical practice, respectively. The ambulatory score consists of scores between zero to three depending on their mobility (0 = flexing the toes to resist traction on the tail similar to the non-operated foot; 1 = plantar flexion; 2 = no dragging but no plantar flexion; 3 = dragging of the foot). The necrosis score consists of scores between zero to four which takes into account the clinical appearance of the limbs which range from normal, mild to severe discoloration to limb necrosis (1 = plantar flexion, mild discoloration; 2 = no plantar flexion, mild discoloration; 3 = no plantar flexion, moderate-to-severe discoloration; 4 = any necrosis of the foot).

Alloantibody Detection Assay

Detection of anti-B6 IgG antibodies in serum samples from the three groups of mice was carried out using a flow cytometry-based assay. Blood samples were collected at the time of euthanasia, allowed to clot at room temperature for 4 hours then separated by centrifugation for 10 minutes at 500 g following which aliquots of serum were frozen at -80°C . For flow cytometric detection of anti-B6 antibody in serum, freshly prepared, erythrocyte-free B6 splenocytes were suspended at 8×10^6 cells/ml in fluorescent-activated cell sorting (FACS) buffer (1x PBS, 2% FBS, 0.05% NaN₃) and were incubated for 30 min at 4°C with either FACS buffer alone (negative control), purified anti-H-2K^b at various concentrations (positive control, clone AF6-88.5, BD Biosciences, Franklin Lakes, USA) or serum samples diluted 1:100 in FACS buffer. Next, splenocytes were washed twice in FACS buffer then incubated for 30 min at 4°C with goat anti-mouse

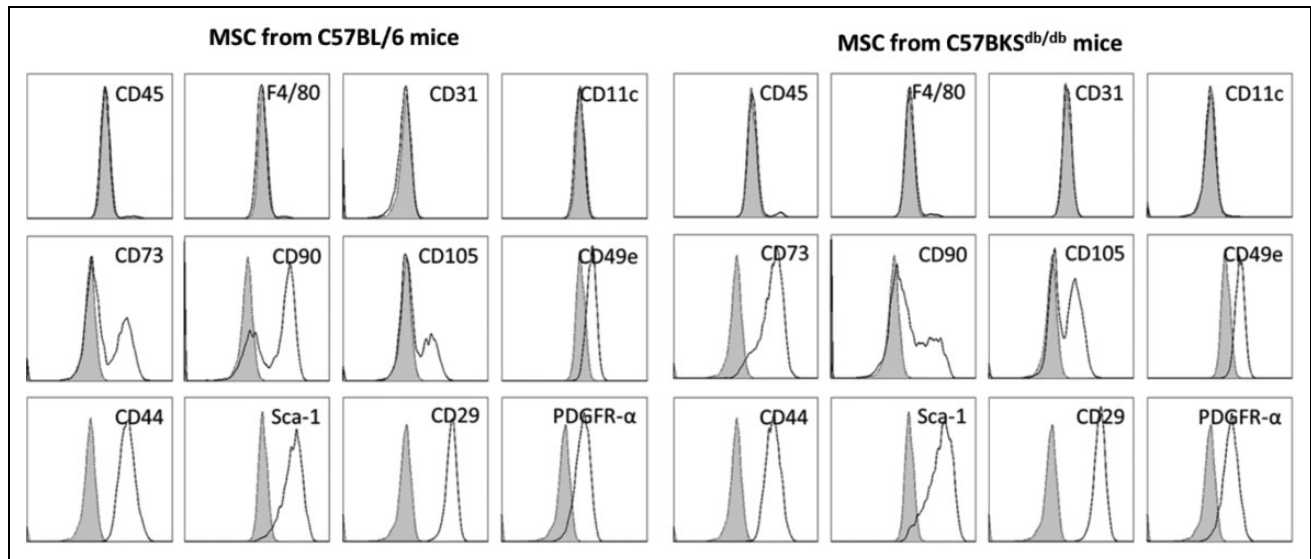


Fig. 2. Flow cytometric analysis revealed a uniform expression of a known MSC marker for mouse including CD44, Sca-1, CD29 and CD49e. Both cultures were also negative for macrophage (F4/80), endothelial (CD31) and hematopoietic (CD45) markers. A heterogeneous expression of known markers for cultured human MSCs (CD73, CD90, CD105) with a double peak distribution was observed in both cell cultures. The non-shaded and shaded peaks represent the positive and negative markers in each image. MSC: mesenchymal stromal cell.

IgG Fc F(ab)₂-FITC (Beckman Coulter, California, USA) at an optimized dilution of 1:400 in addition to anti-mouse T-cell receptor (TCR)-β-PE (BD Biosciences) to allow discrimination of T-cells and non-T-cells. Finally, stained splenocytes were again washed twice with FACS buffer, resuspended in 0.5 ml of FACS buffer and analyzed immediately on a Becton Dickinson FACSCanto™ flow cytometer. Analysis was carried out using FlowJo® software (TreeStar® Inc, Ashland, USA) with FITC fluorescence intensity of TCRβ-negative cells (B-cells) compared between negative control sample and individual serum-incubated samples.

Statistical Analysis

Data are expressed as mean ± standard deviation. Statistical analysis was performed by either a Student's *t* test for comparison between two groups or a one-way analysis of variance (ANOVA), followed by a Bonferroni test for comparison among the three groups using SPSS (version 24) (IBM, Dublin, Ireland). A value of *p* < 0.05 (two-tailed test) was considered statistically significant.

Results

MSC Culture and Characterization

MSCs were successfully isolated from bone marrow of both wild type B6 mice and C57BKS^{db/db} mice. These MSCs adhered to tissue culture plastic and demonstrated spindle shaped morphology. Flow cytometric analysis revealed a

uniform expression of known MSC markers for mouse including CD44, Sca-1, CD29 and CD49e²⁰. Cells from both wild type B6 mice and C57BKS^{db/db} mice were also negative for macrophage (F4/80), endothelial (CD31) and hematopoietic (CD45) markers. A heterogeneous expression of known markers for cultured human MSCs (CD73, CD90, CD105) with a double peak distribution was observed in both cell cultures (Fig. 2). Furthermore, they also demonstrated tri-lineage differentiation into chondrocytes, osteocytes and adipocytes when exposed to appropriate differentiation media (Fig. 3). There was no difference in the characteristics described above in MSCs derived from diabetic (C57BKS^{db/db}) and non-diabetic (B6) mice.

MSCs Derived from Both Mouse Strains Secrete Angiogenesis-Related Factors

The conditioned media from MSCs derived from both the B6 and C57BKS^{db/db} mice contained angiogenesis-related factors, supporting the notion that their potential therapeutic effect may be related to paracrine signaling. Differential expression of several angiogenesis-related factors including matrix metalloproteinase (MMP)-3, C-X-C motif chemokine ligand (CXCL)-16, CXCL-4, CINC-10, insulin-like growth factor-binding protein (IGFBP)-3, monocyte chemoattractant protein (MCP)-1, serpin e1, MMP-9, IGFBP-2, IGFBP-9, tissue inhibitor of metalloproteinases (TIMP)-1, pentraxin-3 and vascular endothelial growth factor (VEGF), was noted between MSCs derived from B6 and C57BKS^{db/db} mice (Fig. 4).

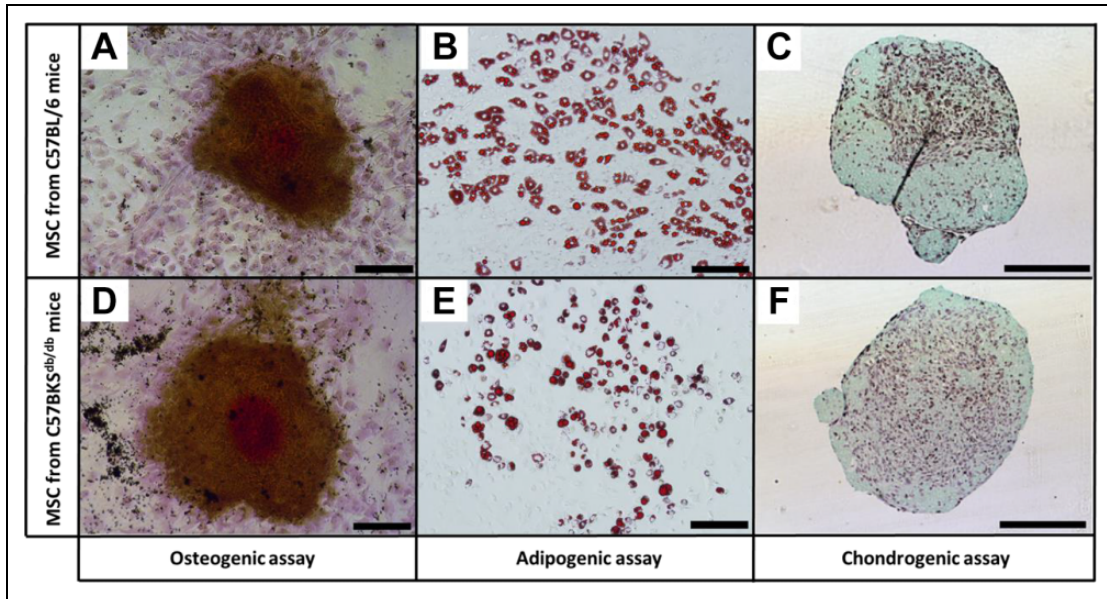


Fig. 3. MSCs isolated from C57BL/6 and C57BKS^{db/db} mice showed tri-lineage differentiation ability (differentiation assays: osteogenic assay: A, D; adipogenic assay: B, E; chondrogenic assay: C, F; MSCs isolated from C57BL/6 mice: A–C; and MSCs isolated from C57BKS^{db/db} mice: D–F). Scale bar: 10× magnification (bar = 100μm) for both the osteogenic and adipogenic assays and 4× magnification (bar = 200μm) for the chondrogenic assay.

MSC: mesenchymal stromal cell.

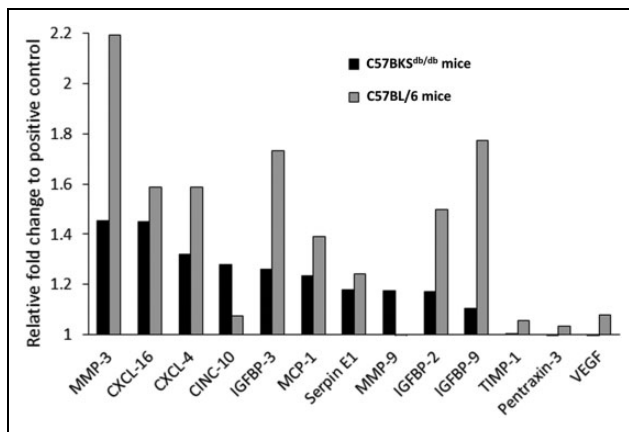


Fig. 4. Densitometry of the conditioned media from MSCs derived from both the C57BL/6 and C57BKS^{db/db} mice showed differential expression of angiogenesis-related factors (expressed as relative fold change to positive control).

CXCL: C-X-C motif chemokine ligand; IGFBP: insulin-like growth factor-binding protein; MCP: monocyte chemoattractant protein; MMP: matrix metalloproteinase; MSC: mesenchymal stromal cell; TIMP: tissue inhibitor of metalloproteinases; VEGF: vascular endothelial growth factor.

Laser Doppler Blood Flow Analysis

Representative LDPI showed dynamic changes in blood perfusion in the ischemic limb before, after and 1, 2 and 3 weeks following the induction of hindlimb ischemia (Fig. 5). In comparison with the control group, transplantation of MSCs

derived from the C57BKS^{db/db} mice significantly improved the blood flow in the C57BKS^{db/db} mice at weeks 2 and 3 post-operatively. The recipients of syngeneic MSCs demonstrated significant improvement in blood flow as compared with the control group at week 2 only. There was no significant difference in the hindlimb blood flow between the allogeneic and the syngeneic MSC-treated groups.

Preclinical Assessment of the Ischemic Limb

The ambulatory score was significantly improved in the groups which received both the allogeneic and syngeneic MSCs as compared with PBS at week 1 (Fig. 6). The necrosis score (which corresponds with the percentage of toe necrosis) was lower, albeit not statistically significant, for allogeneic MSCs as compared with syngeneic MSCs or PBS administration (Fig. 7). There was less toe necrosis, although not statistically significant, in the allogeneic MSC group as compared with other groups. The percentage of toe necrosis was 50% (4/8), 86% (6/7) and 86% (6/7) in the allogeneic, syngeneic and control groups respectively. Toe amputation occurred in one out of seven animals in the control group. None of the mice in the other two groups had toe amputation.

Alloantibody Detection

The flow cytometry-based assay used to detect anti-B6 IgG in mouse serum samples was shown to be capable of detecting concentrations of a purified H-2Kb monoclonal antibody

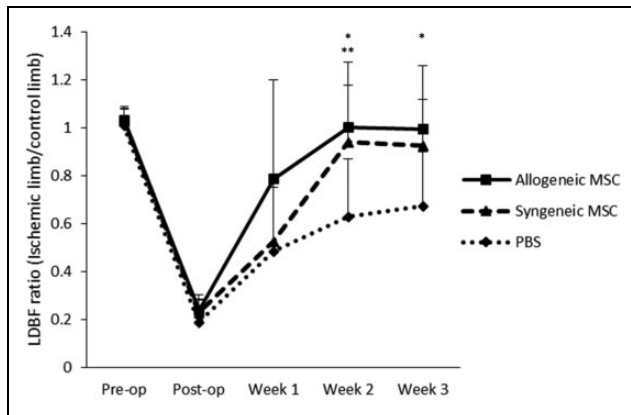


Fig. 5. Laser Doppler blood flow analysis showed that the recipient of allogeneic MSC group ($n = 8$) demonstrated significant improvement in blood flow as compared with the control group ($n = 7$) at 2 (1.00 ± 0.27 versus 0.63 ± 0.24 , $p < 0.05$) and 3 (0.99 ± 0.27 versus 0.67 ± 0.23 , $p < 0.05$) weeks post-operatively. However, the recipients of the syngeneic MSC group ($n = 7$) demonstrated a significant improvement in blood flow at week 2 only (0.94 ± 0.24 versus 0.63 ± 0.24 , $p < 0.05$), in comparison with the control group. There was no significant difference in the hindlimb blood flow between the allogeneic and the syngeneic MSC-treated groups. An asterisk (*) and double asterisks (***) denote significant statistical differences (Student's t test, $p < 0.05$) between the allogeneic versus PBS and syngeneic versus PBS groups, respectively. MSC: mesenchymal stromal cell; PBS: phosphate-buffered saline.

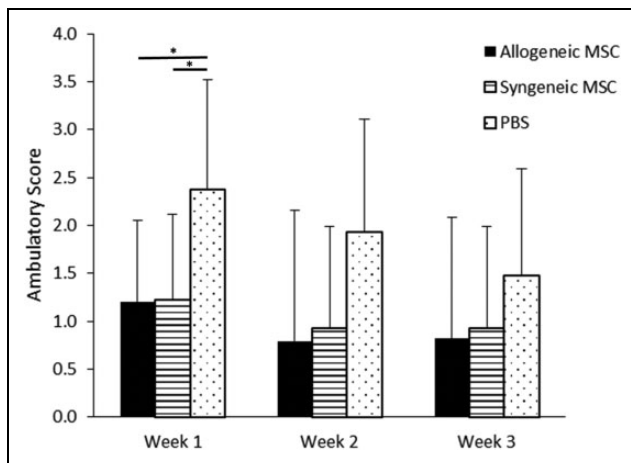


Fig. 6. The ambulatory score showed a significant improvement in the groups which received either the allogeneic ($n = 8$) and syngeneic MSCs ($n = 7$) as compared with PBS ($n = 7$) at week 1 (1.2 ± 0.9 versus 2.4 ± 1.2 , $p < 0.05$; and 1.2 ± 0.9 versus 2.4 ± 1.2 , $p < 0.05$, respectively). There was no statistical difference among the three groups at week 2 and 3. Error bars represent standard deviation. An asterisk (*) denotes significant statistical differences (Student's t test, $p < 0.05$) between any treatment groups. MSC: mesenchymal stromal cell; PBS: phosphate-buffered saline.

between 14 and 0.02 ng/ml. Using this assay, anti-B6 IgG antibodies were detected in sera from three out of the eight mice that received allogeneic MSCs and were undetectable

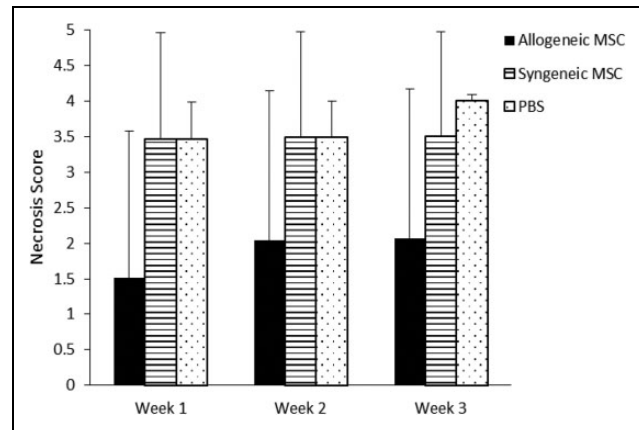


Fig 7. The necrosis score (which corresponds with the percentage of toe necrosis) showed no significant statistical difference (one-way analysis of variance, $p < 0.05$) between all three groups at all time points. Error bars represent standard deviation. MSC: mesenchymal stromal cell.

in sera from the groups that received saline and syngeneic MSCs (Fig. 8).

Discussion

Animal models of diabetes mellitus have been shown to have impaired revascularization with db/db mice exhibiting greater impairment in neovascularization than type I diabetic mice¹². MSC therapy has been shown to be safe with suggestion of efficacy in various preclinical (syngeneic, allogeneic and xenogeneic transplantation) and clinical (autologous and allogeneic transplantation) studies and has been previously summarized^{22,23}. Previously, Yan et al. have shown that transplantation of allogeneic MSCs derived from db/db mice or syngeneic MSCs derived from wild type mice into wild type recipients after induction of hindlimb ischemia was associated with a significantly impaired *in vivo* revascularization in the former group in comparison with the latter^{13,24}. However, the potential therapeutic effect of transplantation of syngeneic MSCs and allogeneic MSCs derived from diabetic and non-diabetic animals, respectively, into diabetic recipient animals is currently unknown. Therefore, the goal of this study was to compare in a head to head fashion in a diabetic animal model the effect of transplantation of MSCs isolated from syngeneic and thus diabetic animals with transplantation of MSCs isolated from non-diabetic allogeneic animals with hindlimb ischemia. Our study reflected the actual clinical setting whereby, both allogeneic and autologous MSCs can be obtained from healthy non-diabetic subjects and patients with T2DM, respectively, in order to be administered to patients with T2DM. In this study, we have demonstrated that intramuscular administration of allogeneic MSCs derived from wild type B6 mice is of comparable efficacy to that of syngeneic MSCs derived from C57BKS^{db/db} mice for therapeutic

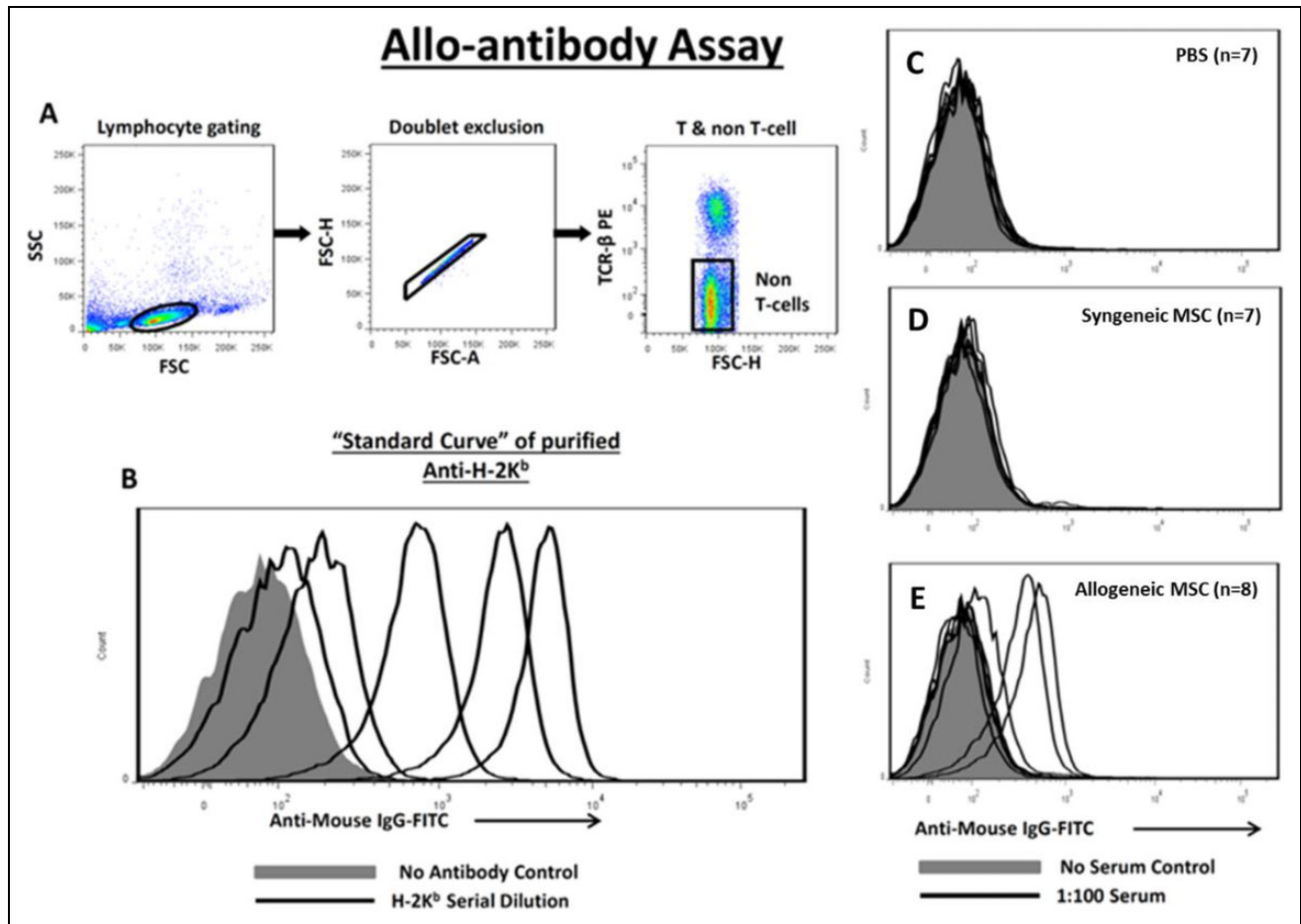


Fig 8. (A) Gating strategy for the flow cytometry-based assay using freshly-isolated C57BL/6 splenocytes: live cell gating, doublet discrimination and gating for non-T-cells. (B) Flow cytometry histograms showing relative staining of C57BL/6 splenocytes with anti-mouse IgG-FITC following incubation with five different concentrations (14, 2.8, 0.56, 0.11 and 0.02 ng/ml) of purified anti-H-2Kb (open histograms) or with no-antibody control (filled histogram). (C), (D) and (E): Flow cytometry histograms showing relative staining of C57BL/6 splenocytes with anti-mouse IgG-FITC following incubation with 1:100 dilutions of serum from groups of C57BKS^{db/db} mice with hindlimb ischemia that had received PBS (C), syngeneic MSCs (D) or allogeneic MSCs (E) or with no-antibody control (filled histograms). FITC: fluorescein isothiocyanate; Ig: immunoglobulin; MSC: mesenchymal stromal cell; PBS: phosphate-buffered saline.

revascularization following induction of hindlimb ischemia in C57BKS^{db/db} mice.

The other issue explored in this study was a comparison of MSCs isolated from diabetic and non-diabetic donors. As previously described by Morikawa et al.²⁰, we also showed that both the MSCs derived from diabetic (C57BKS^{db/db}) and non-diabetic (B6) mice exhibited comparable expression of known MSC markers for mouse including CD44, Sca-1, CD29 and CD49e, known markers for cultured human MSCs (CD73, CD90, CD105) with a double peak distribution, and a negative expression of macrophage (F4/80), endothelial (CD31) and hematopoietic (CD45) markers. They also demonstrated comparable tri-lineage differentiation into chondrocytes, osteocytes and adipocytes when exposed to appropriate differentiation media.

In this study, we showed that transplantation of MSCs derived from the C57BKS^{db/db} mice significantly improved

the blood flow in the C57BKS^{db/db} mice, at weeks 2 and 3, post-operatively, when compared with the control group. On the other hand, the recipients of syngeneic MSCs demonstrated significant improvement in blood flow as compared with the control group, at week 2 only. There was no significant difference in the hindlimb blood flow between the allogeneic and the syngeneic MSC-treated groups. Furthermore, this study also showed that the ambulatory score was significantly improved in the groups which received both the allogeneic and syngeneic MSCs as compared with PBS at week 1. In comparison with the syngeneic MSCs or PBS administration, the necrosis score (which corresponds with the percentage of toe necrosis) was lower, albeit not statistically significant, for allogeneic MSCs. In addition, there was less toe necrosis, although not statistically significant, in the allogeneic MSC group as compared with other groups. The percentage of toe necrosis was 50% (4/8), 86% (6/7) and

86% (6/7) in the allogeneic, syngeneic and control groups respectively. No toe amputation was observed in the two groups which received either allogeneic or syngeneic MSCs. Toe amputation occurred in one out of seven animals in the control group. Our flow cytometry-based method was able to detect anti-B6 IgG in mouse serum and samples were shown to be capable of detecting concentrations of a purified H-2Kb mAb between 14 and 0.02 ng/ml. Therefore, utilizing this assay, anti-B6 IgG antibodies were detected in sera from three out of the eight mice that received allogeneic MSCs and was undetectable in sera from the groups that received saline and syngeneic MSCs.

The conditioned media from MSCs derived from both the B6 and C57BKS^{db/db} mice contained angiogenesis-related factors including MMP-3, CXCL-16, CXCL-4, CINC-10, IGFBP-3, MCP-1, serpin E1, MMP-9, IGFBP-2, IGFBP-9, TIMP-1, pentraxin-3 and VEGF. IGFBP has been shown to modulate essential cellular processes including cell migration, senescence, autophagy, proliferation, survival, and angiogenesis²⁵. On the other hand, MMP involves in cell proliferation and apoptosis²⁶. Our findings are in keeping with other studies, which have assessed the paracrine effect of MSCs following specific stimulation. For instance, Ratushnyy et al., showed higher levels of serpin E1, IGFBP and VEGF following microgravity simulation²⁷. Park et al., showed that MSCs secrete higher levels of pentraxin-3 and VEGF in response to hypoxia²⁸. Maffioli et al., showed that both human and mouse MSCs secrete TIMP-1 in response to an inflammatory microenvironment²⁹. In contrast with our findings, Morris et al. showed that MSCs derived from patients with diabetes and peripheral arterial disease secrete higher levels of MCP-1 and CXCL-1³⁰. Dzhoyashvili et al. showed that MSCs derived from patients with coronary artery disease (CAD) secrete significantly higher levels of VEGF than those without CAD³¹. The VEGF level is also higher than those with CAD and T2DM, but not statistically significant, which is also in contrast with our preclinical finding³¹. Overall, the paracrine effect found in our study may explain the therapeutic effect of MSCs despite the presence of alloantibody.

We then explored whether allogeneic transplantation of MSCs would induce an immune response and whether this might influence therapeutic efficacy. Davies et al. and Yaochite et al. have shown that culture-expanded MSCs from donors with T1DM are phenotypically and functionally similar to healthy control MSCs with regard to their immunomodulatory response^{32,33}. In this study, we showed that allogeneic MSC transplantation did result in alloantibody production in 3 out of 8 animals, while, as expected, no anti-B6 antibody development was observed with syngeneic MSC transplantation. Furthermore, the therapeutic effect following allogeneic MSC transplantation was preserved despite the presence of alloantibody. These results raise a number of clinically relevant questions regarding the frequency and potential consequences of allogeneic MSC-induced anti-donor antibody and T-cell immune responses.

Firstly, although flow cytometry-based methods have been in use for several decades for the detection of donor-specific IgG antibodies in the field of human organ transplantation, they have been supplanted to a large extent by more sensitive assays based on detection of fluorescence on major histocompatibility complex (MHC) protein-coated beads³⁴. Thus, it is possible, in our experiments, that donor-specific IgG below the level of detection of the flow cytometry assay was present in a larger proportion of the allogeneic MSC-treated animals. Indeed, in a separate study involving single and repeated intramuscular injections of allogeneic (B6) MSCs into healthy BALB/C mice, we observed detectable anti-B6 IgG in sera from the majority recipient animals 3 weeks after the initial cell injections³⁵. In that study, the serum titers of anti-donor IgG induced by allogeneic MSCs were comparable with those induced by injection of highly immunogenic B6 splenocytes but were characterized by a predominance of IgG1 isotypes compared with an IgG2a/IgG3 predominance of the antibody response to splenocytes. In keeping with this IgG1-skewed phenotype, the anti-donor antibodies induced by intramuscular B6 MSCs showed more variable capacity for complement-mediated lysis of B6 target cells *in vitro* compared with B6 splenocyte-induced anti-donor antibodies³⁵.

Finally, we also observed that allogeneic MSC-induced anti-donor IgG was suppressed by co-administration of a short course of the T-cell-specific immunosuppressive drug tacrolimus³⁵, indicating that B-cell responses to MSC-delivered allo-antigen are T-cell-dependent and, thus, likely to occur in the context of indirect allo-antigen presentation by dendritic cells to donor-specific CD4⁺ T-cells³⁶.

The important question of whether the anti-donor antibody response to allogeneic MSCs has the potential to cause immunological 'rejection' of the implanted MSCs (or of subsequent doses of allogeneic MSCs) remains unclear. Theoretically, this could occur directly through activation of the classical complement activation pathway, through complement-mediated activation of innate immune cells such as neutrophils, macrophages and natural killer cells or through concomitant anti-donor CD4⁺ and CD8⁺ T-cell responses^{17,37-39}. Our observation of equivalent efficacy of autologous and allogeneic MSCs in the hindlimb ischemic model suggests that anti-donor immunogenicity did not compromise the beneficial effect of a single injection of intramuscular B6 MSC in db/db recipients. Furthermore, in separate experiments, we have observed that multiple intramuscular injections of allogeneic MSCs into healthy mouse recipients was associated with donor-specific CD4⁺ and CD8⁺ T-cell hypo-responsiveness (Alagesan and Griffin, 2014) consistent with a potential tolerizing effect on anti-donor cellular immune responses. In contrast, however, Seifert et al. showed that pretreatment with intravenous donor-specific allo-MSCs resulted in heightened anti-donor T-cell and B-cell immune responses and accelerated transplant rejection in a rat kidney allograft model²⁷. Similarly, Schu et al. showed that the sensitization induced by

intravenous injection of allogeneic MSC transplantation in rats is followed by an accelerated clearance of a repeated allogeneic MSC transplantation¹⁵. Huang et al. also showed the accelerated elimination of intra-myocardially-injected allogeneic MSCs from the heart with resultant loss of functional benefit following sensitization in a rat model of myocardial infarction¹⁷. Thus, while there is now a large body of animal model evidence to indicate that allogeneic MSCs induce donor-specific humoral and cellular immune responses, it is likely that the impact of these immune responses on the therapeutic efficacy of the administered cells is dependent on a range of factors including route of administration, cell dose and dose frequency, polarization of the adaptive immune response and the exact therapeutic mechanism of action¹⁹. Notably, for many of the clinical conditions that are being targeted by both autologous and allogeneic MSC therapy, the therapeutic benefits occur despite a lack of engraftment for longer than 24–48 hours^{40,41}.

The sensitization of recipients of allogeneic MSCs has also been recently demonstrated in a clinical trial, but not in the setting of patients with critical limb ischemia²⁸. Furthermore, repeated allogeneic MSC administration is feasible and with suggestion of efficacy in several early clinical trials^{29–31}. For example, Forbes et al. showed that repeated intravenous infusions of allogeneic MSCs (2×10^6 cells/kg body weight) weekly for 4 weeks improved clinical scores in patient with refractory luminal Crohn's disease²⁹. Kurtzberg et al. showed that biweekly intravenous infusions of allogeneic MSCs (2×10^6 cells/kg for 4 weeks, with an additional 4 weekly infusions after 28 days if needed) significantly improved survival at day 100 in patients with severe steroid-refractory acute graft-versus-host disease³⁰. Weiss et al. showed that 4-monthly infusions of allogeneic MSCs (100×10 cells/infusion) in patients with moderate-to-severe chronic obstructive pulmonary disease was safe and associated with a significant decrease in levels of circulating C-reactive protein (CRP) in patients who had elevated CRP levels at study entry³¹. Hare et al. showed sensitization of 2 out of 15 patients which was only apparent after 6 months²⁸. Although not yet examined in human clinical trials, Isakova et al. showed that the nature and magnitude of allo-immune response in rhesus macaques can be significantly influenced by the extent of the mismatch of MHC class I and II, between the MSC donor and recipient³². They also showed a significant inverse correlation between MSC dose and the degree of MSC engraftment³². Our results in mice, therefore, have relevance to a range of reported and ongoing clinical applications of allogeneic MSC therapy in critical limb ischemia as well as other acute and chronic diseases.

The group sizes were relatively small ($n = 7-8$), which represents a potential limitation of this study. The significant results reported are based on statistical testing that assumes normal distribution. Thus, while the analyses revealed clear differences between MSC-treated and control groups for

some key parameters, very accurate calculation of the true magnitude and variability of these differences would require substantially larger group sizes. Indeed, ongoing work in our laboratory in support of clinical translational goals for allogeneic stromal cell therapy in CLI is now using group sizes up to 18 animals.

Conclusions

This study highlighted the feasibility and efficacy of allogeneic and syngeneic MSC transplantation in C57BKS^{db/db} mice following induction of hindlimb ischemia, and suggests that either an autologous or allogeneic approach is reasonable. Furthermore, the therapeutic efficacy of allogeneic MSC transplantation was evident despite activation of an immune response.

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Ethical Approval

All animal experiments were carried out with ethical approval from the Animal Care Research Ethics Committee (ACREC) of the National University of Ireland, Galway, and under appropriate individual and project authorizations from Health Products Regulatory Authority (HPRA) of Ireland (License number B342) and were performed in accordance with the Principles of Laboratory Animal Care.

Statement of Human and Animal Rights

All experimental procedures and protocols were approved by the Institutional Ethical Committee/ACREC and the Department of Health and Children, Ireland (License number B342) and were performed in accordance with the Principles of Laboratory Animal Care.

Statement of Informed Consent

Statement of Informed Consent is not applicable for this article.

Declaration of Conflicting Interests

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this

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