Safety and Efficacy of the Intravenous Infusion of Umbilical Cord Mesenchymal Stem Cells in Patients With Heart Failure: A Phase 1/2 Randomized Controlled Trial (RIMECARD Trial)

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ABSTRACT

Rationale: Umbilical cord-derived mesenchymal stem cells (UC-MSC) are easily accessible and expanded in vitro, possess distinct properties, and improve myocardial remodeling and function in experimental models of cardiovascular disease. While bone marrow-derived mesenchymal stem cells (BM-MSCs) have been previously assessed for their therapeutic potential in individuals with heart failure and reduced ejection fraction (HFrEF), no clinical trial has evaluated UC-MSCs in these patients.

Objective: Evaluate the safety and efficacy of the infusion of UC-MSC in patients with chronic stable HFrEF.

Methods and Results: HFrEF patients under optimal medical treatment were randomized to intravenous infusion of allogenic UC-MSCs (Cellistem, Cells for Cells S.A., Santiago, Chile) ($1x10^6$ cells/Kg) or placebo (n=15 per group). UC-MSCs *in vitro*, compared to BM-MSCs, displayed a 55-fold increase in the expression of Hepatocyte Growth Factor (HGF), known to be involved in myogenesis, cell migration and immunoregulation. UC-MSC treated patients presented no adverse events related to the cell infusion and none of the patients tested at 0, 15 and 90 days presented alloantibodies to the UC-MSCs (n=7). Only the UC-MSC treated group exhibited significant improvements in left ventricular ejection fraction at 3, 6 and 12 months of follow-up assessed both through transthoracic echocardiography (p=0.0167 versus baseline) and cardiac magnetic resonance imaging (p=0.025 versus baseline). Echocardiographic LVEF change from baseline to month 12 differed significantly between groups (+7.07±6.22% vs +1.85±5.60, p=0.028). In addition, at all follow-up time points, UC-MSCs treated patients displayed improvements of NYHA functional class (p=0.0167 versus baseline) and MLHFQ (p<0.05 versus baseline). At study completion, groups did not differ in mortality, heart failure admissions, arrhythmias or incident malignancy.

Conclusions: Intravenous infusion of UC-MSC was safe in this group of patients with stable HFrEF under optimal medical treatment. Improvements in left ventricular function, functional status and quality of life were observed in patients treated with UC-MSCs.

Trial registration number: NCT01739777.
Registry URL: https://clinicaltrials.gov/ct2/show/NCT01739777

Keywords:
Multipotent stem cells/transplantation; stem cell transplantation/methods; heart failure; cardiomyopathy; clinical trial; mesenchymal stem cells.

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Nonstandard Abbreviations and Acronyms:

HF	Heart Failure
HFrEF	Heart Failure with reduced ejection fraction.
MSC	Mesenchymal stem cells
BM-MSC	Bone marrow-derived mesenchymal stem cells
UC-MSC	Umbilical cord-derived mesenchymal stem cells
LVEF	Left ventricular ejection fraction
LVEDV	Left ventricular end-diastolic volume
LVESV	Left ventricular end-systolic volume
NYHA	New York Heart Association
TNM	Staging of Malignant Tumors (Tumor, Node, Metastasis)
MLHFQ	Minnesota Living with Heart Failure Questionnaire
KCCQ	Kansas City Cardiomyopathy Questionnaire
BNP	Brain natriuretic peptide
HSCRP	High sensitivity C-reactive protein
VEGF	Vascular endothelial growth factor
HGF	Hepatocyte growth factor

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INTRODUCTION

Stem cell therapy has been under evaluation as a treatment for heart failure (HF) with reduced ejection fraction (HFrEF) for more than a decade. Experimental studies report improvements in cardiac function and regeneration of damaged heart tissue through mechanisms including transdifferentiation, cell fusion and paracrine modulation[1,2]. In human disease, recent reviews suggest that stem cell therapy is safe and associated with moderate clinical benefits in survival, left ventricular function and quality of life of HFrEF patients[3–6]. Clinical trials in patients with chronic ischemic or non-ischemic disease have assessed a range of cellular products and delivery routes. These include autologous or allogenic bone-marrow mononuclear cells and mesenchymal stem cells (MSC), administered by intramyocardial injections, percutaneous intracoronary infusion and exceptionally peripheral intravenous infusion[3,4,6]. However, after decades of basic and clinical research, overall benefit and the best cell source and route of administration remain unsettled.

MSCs are multipotent cells with low immunogenic potential that can be isolated from adult tissues including bone marrow, adipose tissue, and umbilical cord among other sources. The niche of origin represents an essential factor when evaluating biological differences between cell types, since MSC properties can be highly influenced by microenvironmental changes[1,7]. Most experimental and clinical studies have utilized bone-marrow derived MSC (BM-MSC), nonetheless these cells present disadvantages for clinical application, including an invasive harvesting procedure and a decreased proliferation and differentiation potential related to donor age and comorbidity[8]. In contrast, umbilical cord MSCs (UC-MSC) are easily attainable and expanded in vitro, have less cellular aging, and are devoid of ethical concerns. Preclinical studies have demonstrated that UC-MSC can express cardiac-specific molecules (troponin-I, connexin-43), differentiate into cardiomyocyte-like and endothelial cells in vitro, and also exert paracrine effects that enhance vascular regeneration and cardiomyocyte protection. Such actions might underly the improvement in cardiac function observed in animal models of chronic ischemic cardiomyopathy and dilated cardiomyopathy in response to UC-MSCs[9–14]. The aim of this prospective, randomized, double blinded placebo–controlled trial was to evaluate the safety and efficacy of a well characterized source of UC-MSCs administered intravenously in patients with chronic HFrEF.

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METHODS

Study design and patient population.

The RIMECARD trial was a phase 1/2, randomized, double-blind, placebo-controlled clinical trial. The study was conducted at Clínica Santa Maria and Clínica Dávila, Chile. Participants were referred from these private healthcare centers or public hospitals, and randomized between December 2012 and June 2014. The experimental design was approved by the ethics committee at both participant health centers and the Chilean Metropolitan Health Service. Before enrolment, all patients agreed to participate and signed an informed consent approved by the institutional review board. This study was registered in Clinicaltrials.gov (NCT01739777).

Inclusion criteria were: 1) 18–75 years of age. 2) Chronic HFrEF with New York Heart Association (NYHA) classification I-III and left ventricular ejection fraction (LVEF) ≤40% at echocardiographic assessment. 3) All patients had to be under optimal medical management for at least 3 months prior to randomization, which encompassed class I guideline-recommended therapies (angiotensin converting enzyme inhibitor or angiotensin receptor blocker, beta blocker and mineralocorticoid receptor blocker) at maximal tolerable dosages. Ivabradine and sacubtril/valsartan were not included given their recent introduction in our country. Exclusion criteria were: 1) End-stage HFrEF defined as patients with ACCF/AHA stage D (candidates for specialized interventions including heart transplantation and mechanical assistance) or terminal heart failure (advanced heart failure with poor response to all forms of treatment, frequent hospitalizations and life expectancy <6 months). 2) Recurrent myocardial ischemia defined as any type of acute coronary syndrome 3 months prior to enrollment. 3) Uncontrolled ventricular tachycardia defined by sustained ventricular tachycardia, including electrical storm and/or incessant ventricular tachycardia with no response to antiarrhythmic medication. 4) Malignant disease with life expectancy <1 year according to TNM classification. 5) Manifest ventricular asynchrony defined by intraventricular asynchrony at qualitative echocardiographic assessment (ondulating systolic movement beginning at the interventricular septum and extending to other left ventricular segments, with late activation of LV lateral wall). Patients with LBBB without manifest ventricular asynchrony were allowed to enroll. 6) Hematologic disease: anemia (hemoglobin ≤9.5g/dl); leukopenia (<4000/µL); thrombocytopenia (<75000/uL); myeloproliferative disorders, myelodysplastic syndrome, acute or chronic leukemia and plasma cell dyscrasias (multiple myeloma, amyloidosis). 7) Recent cerebrovascular disease (<3 months). 8) Serum creatinine >2.26mg/dL (>200µmol/L). 9) Atrial fibrillation without optimal heart rate control in the last 3 months. Every patient assessed for eligibility was subject to coronary angiography and exercise stress test to guarantee the stability of their coronary disease, and rule out signs of ischemia prior to inclusion into the protocol. Hence the patients with ischemic cardiomyopathy had predominantly scar.

Eligible patients were enrolled in a 1:1 randomization to intravenous infusion of UC-MSCs or placebo. The randomization list was computer generated by a person unrelated to the study. All patients were assessed at baseline and at the pre-established follow-up points of 3, 6 and 12 months. These evaluations consisted of a clinical assessment for adverse events and NYHA functional classification; Minnesota Living with Heart Failure Questionnaire (MLHFQ) and Kansas City Cardiomyopathy Questionnaire (KCCQ); laboratory testing including complete blood count, liver and renal function tests, brain natriuretic peptide (BNP) and high sensitivity C-reactive protein (HSCRP); resting ECG, signal averaged ECG, 24-hour ECG Holter monitoring; transthoracic echocardiography, cardiac magnetic resonance (CMR) and cardiopulmonary exercise test. Technical specifications regarding quality of life questionnaires, echocardiography, CMR and cardiopulmonary exercise tests are provided below. Clinical researchers, study nurses and patients were blinded to treatment allocation.

Preparation, characterization and infusion of UC-MSC.

UC-MSC treatments were processed in an ISO 9001:2015 certified GMP type Laboratory (*Cells for Cells*, Santiago, Chile), under good manufacturing practice (GMP) conditions according to the Food and Drug Administration (FDA) Guidance for industry (Current good tissue practice (CGTP) and additional requirements for manufacturers of human cells, tissues, and cellular and tissue-based products (HCT/Ps). Umbilical cords were obtained from full-term human placentas by caesarean section after informed consent, from healthy donors, and were aseptically stored in sterile phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin and 100 µg/mL streptomycin (Gibco, Gran Island, USA). Within 3 hours of birth, the umbilical cord was sectioned and washed with PBS and antibiotics. Wharton's jelly was dissected into small fragments (1–2 mm^2 pieces), seeded in 100 mm culture plates and maintained in Minimum Essential Medium Eagle (MEM) Alpha Modifications (Alfa-MEM) high glucose (Gibco, Gran Island, USA) - supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA), 1% penicillin/streptomycin and 2 mM L-glutamine (L-G) (Gibco, Gran Island, USA). At 48 hours, non-adherent cells were removed, washed with PBS and culture medium was replaced with fresh medium every 3 days. When the cell culture reached 70-80% confluence, cells were detached by treatment with TrypLE TM Express (Gibco, Gran Island, USA) and reseeded at a density of 2,500 cells per cm^2 into 500 cm^2 flasks (Nunc, Denmark). At passage 3, UC-MSC were characterized according to the International Society for Cellular Therapy Guidelines[15], harvested and cryopreserved in Profreeze (Lonza, Walkersville, USA) following the manufacturer's instruction. In vitro tests (described in the online supplement) were performed to further characterize the UC-MSC used in the trial, including cell size and doubling time, senescence markers, cardiomyogenic differentiation potential, paracrine and immunomodulatory activity, and migration capacity of UC-MSCs as compared with BM-MSCs. BM-MSCs were obtained from a 18 year old healthy male undergoing surgery due to hip trauma, and 2 iliac crest samples that were from a female and a male healthy donor, aged respectively 23 and 30 yrs, purchased from Lonza. None had cardiovascular diseases.

According to the amount of cells required in each case, cryopreserved vials were thawed and expanded until passage 5-6 using Alfa-MEM supplemented with 10% AB plasma. HLA typing for these cells was assessed by PCR for HLA Class I (A, B, C) and Class II (DP, DQ, DR). The release criteria for clinical use of UC-MSCs included the absence of: macroscopic clumps, contamination by pathogenic microorganisms (bacteria, mycoplasma, syphilis, HBV, HCV, HIV, CMV and fungi) or endotoxin (≤0.5 EU/mL) and a viability >80%, with an identity and purity pattern characterized by positivity (≥95%) of CD73, CD90 and CD105, and negative expression (≤2%) of CD45, CD34, CD14 and HLA-DR. A total of $1x10^6$ UC-MSC/kilogram of body weight were re-suspended in a final volume of 100 ml of AB plasma. The placebo group received 100 ml of autologous plasma. Patients received premedication with intravenous hydrocortisone 100 mg and chlorphenamine 10 mg, complying the local protocol for prevention of allergic and non-hemolytic transfusion reactions. After 30 minutes they were infused with UC-MSCs or placebo at 2 ml/min via peripheral vein, under noninvasive monitoring of vital signs.

Study endpoints.

The primary safety endpoints encompassed immediate adverse events after intravenous infusion of UC-MSC or placebo; incidence of overall death, major cardiovascular events (defined by the combined outcome of cardiovascular deaths, hospital admission due to decompensated HF, non-fatal myocardial infarction), and other adverse events including stroke, sustained ventricular arrhythmias and incident malignancy. The humoral immune response to infused allogeneic UC-MSCs was tested in a group of 12 patients (7 treated with UC-MSC, 5 receiving placebo) at days 0, 15 and 90 of infusion using Luminex 200 (Kashi Clinical Laboratories Inc. Portland, OR).

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The primary efficacy endpoint was change in LVEF in echocardiography[16]. Secondary efficacy endpoints included changes in left ventricular end-systolic volume (LVESV) and end-diastolic volume (LVEDV) at echocardiography; LVEF, LVESV and LVEDV in CMR; NYHA functional classification; quality of life questionnaires overall scores; maximum peak oxygen consumption (peak VO2) and ventilatory efficiency (VE/VCO2 slope) assessed through cardiopulmonary exercise test; BNP and HSCRP.

Transthoracic echocardiography.
Transthoracic echocardiography was performed by 2 experienced cardiologists, blind from treatment allocation, from both participating centers. Studies were performed in Vivid 7 Dimension Cardiovascular Ultrasound System (General Electric Healthcare, USA). LVEF was measured through modified Simpson biplane method, LVESV and LVEDV were measured at parasternal long axis in four and two chamber. Chamber quantifications, diastolic dysfunction and global longitudinal strain were measured according to recommendations of the American Society of Echocardiography[17,18].

Cardiac Magnetic Resonance.
Cardiac magnetic resonance (CMR) studies were performed on a 1.5 Tesla magnetic resonance system using cardiac phased-array SENSE coil with 5 channels (Philips Achieva, Netherlands). All scans were obtained by a single operator and at a single institution (Clinica Davila, Chile). The imaging protocol included axial, coronal and sagittal scout images to localize the heart; afterwards balanced SSFP cine ECG-gated sequence in four-chamber, three-chamber, long axis and short axis planes were performed for LV functional assessment. Images were transferred to the workstation (Philips Extended MR Workspace, 2.6.3.5, Netherlands) for post-processing. Global LV function was quantified by radiologists blinded to treatment allocation, using Segment v1.9 software (Medviso AB, Sweden)[19]. Endocardial and epicardial contours were drawn on short-axis end-diastolic and end-systolic images by radiologists, blinded to treatment allocation and affiliated to an independent institution (Hospital Clinico Universidad de Chile, Chile). Papillary muscles and endocardial trabeculations were included into LV volume. A total of 8 to 12 short-axis segments were needed to encompass the entire left ventricle.

Cardiopulmonary exercise test.
Standardized symptom-limited cardiopulmonary test exercise protocols with treadmill or cycle ergometry were performed, based on availability of the technique al each healthcare center of recruitment. Gas exchange measurements analyzed for each breathing cycle were performed using metabolic charts. Exercise capacity variables including Peak VO2, VE/VCO2, METS, oxygen consumption at anaerobic threshold, peak respiratory exchange ratio and exercise time were recorded.

Quality of life questionnaires.
Patients answered validated translations of MLHFQ and KCCQ. MLHFQ is a 21-item self-administered questionnaire assessing the patients' perception of the effects of CHF on physical, socioeconomic and psychological aspects of their life[20]. Scores range between 0 and 105, higher scores indicate worst quality of life[20]. KCCQ is a 23-item self-administered questionnaire addressing specific health domains pertaining to heart failure: physical limitation, symptoms, quality of life, social limitation, symptom stability, and self-efficacy[21]. The first 4 domains combine into a clinical summary score. Scores range from 0 to 100, higher scores point to lower symptom burden and better quality of life[21].

Statistical analysis.
Continuous data are expressed as mean ± SD, and categorical data as absolute number. Categorical data was compared using Pearson's chi-squared test. Continuous data was assessed by Shapiro–Wilk test for normality. Comparison between groups at baseline was assessed through unpaired t-test or Mann-Whitney U-test according to normality. Intra-individual comparison of continuous variables at baseline with those at follow-up was performed with paired t-test or Wilcoxon rank sum test according to normality. Statistical significance was assumed at a value of $p<0.05$. For comparisons of various post-treatment evaluations

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versus baseline, Bonferroni alpha correction was performed and statistical significance was assumed at a value of $p<0.0167$. CMR studies were additionally analyzed through a mixed effect maximum likelihood regression. In vitro data are expressed as mean ± SEM, and were compared using one-way analysis of variance followed by Bonferroni correction. A value of $p<0.05$ was considered statistically significant. Analyses were performed with IBM SPSS Statistics 20.0 (IBM Corp, USA) and STATA 12.0 (StataCorp, USA).

RESULTS

Characterization of the UC-MSCs.

UC-MSC and BM-MSC were grown and characterized for surface markers as described above. Their capacity to differentiate to mesodermal lineages was confirmed under specific osteogenic, chondrogenic and adipogenic differentiation conditions (Online Figure I). Cell size, doubling time and senescence markers can be seen in the online Figure II.

Cardiac differentiation potential.

Treatment with 5-AZA for 25 days induced cardiomyogenic differentiation of UC-MSCs, revealed by the expression of specific markers including transcription factors involved in myogenesis (NKx2.5, GATA-4, MEF2C) and other genes (MYH7B, GJA1, TNNT2). The expression of all 6 genes was induced in both cell sources, though BM-MSCs exhibited higher mRNA levels as shown by RT-PCR ($p<0.001$) (Figure 1A). Conexin-43 staining also had greater expression in BM-MSCs than in UC-MSCs (24.33±1.84% versus 17.42±1.43%), ($p=0.018$). In contrast, Troponin expression seemed increased in UC-MSCs (23.47±7.94% versus 9.06±2.61% for BM-MSCs), but did not reach significance ($p=0.166$). (Figure 1B). Beating was not observed in MSCs after induction with 5-AZA.

Paracrine profile.

UC-MSCs showed a higher TGF-β3 gene expression in comparison with BM-MSCs ($p<0.001$) but VEGF expression levels in comparison with BM-MSCs was not significantly different (Figure 1C). Of note, UC-MSCs showed a 55 fold higher expression of HGF in comparison with BM-MSCs ($p>0.0001$) (Figure 1C), that in some cases showed undetected levels of HGF. Comparative quantification of IDO activity, IL6, TGF-β1, PGE2, HLA-G and PDL-1 at basal and stimulated condition of UC-MSCs and BM-MSCs can be seen at online Figure III.

Immunomodulatory effects.

The immunosuppressive properties of UC-MSCs were assessed by evaluating their effect on the proliferative response of peripheral blood mononuclear cells (PBMCs) following PHA stimulation *in vitro*. UC-MSCs exhibited a similar inhibitory effect on T cell proliferation compared to BM-MSCs at the 1:10 ratio, inhibition percentages were 21.53%±3.85% and 23.96±4.50 for UC-MSCs and BM-MSCs, with respect to PHA-induced proliferation in the absence of MSCs ($p<0.005$ versus control) (Figure 2A). T helper 1, T helper 2 and cytotoxic T cells exhibited a tendency to decrease their proliferation after co-cultured with UC-MSCs or BM-MSCs ($p>0.05$). No effect of the MSC co-cultures was observed on regulatory T cell proliferation (Figure. 2B).

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Migration profile in response to HFrEF patient's serum.

The percentage of migrating cells was significantly higher in UC-MSCs compared with BM-MSCs in response to HFrEF patient's serum (41.18±6.53 vs 29.67±8.35; p<0.01) (Figure 3).

Patient population.

From December 2012 to June 2014, 65 patients were assessed for eligibility, 30 patients underwent randomization (n=15 per group) (Figure 4). Baseline characteristics of the UC-MSC and placebo patients did not differ in terms of demographic variables, cardiovascular risk factors, NYHA class and electrocardiography (Table 1). Ischemic cardiomyopathy was the predominant etiology of HFrEF (21 patients, 70%). There were no differences between groups concerning therapeutic agents that modify cardiac remodeling. No patient had cardiac implantable electronic devices. One patient from each group had left bundle branch block, although none presented manifest ventricular asynchrony at baseline. Patients treated with placebo presented higher BNP levels and 25% greater LVEDV at baseline (p<0.05).

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Safety.

There were no acute adverse events associated to the infusion of allogenic UC-MSC or placebo. None of the tested individuals (7 treated with UC-MSC and 5 receiving placebo) developed alloantigen directed antibodies post infusion. Of note, one female patient with baseline reactivity to 52 different HLA specificities prior to UC-MSC, lost reactivity to 16 of these specificities at day 90. Furthermore, since we typed the infused cells, we could detect that only 21% of specificities not expressed on the infused MSCs disappeared, as opposed to 100% of those present on the infused MSCs (p=0.004). Our data not only confirm the absence of humoral immune reaction to UC-MSCs, but also suggest that MSCs preferentially suppress reactivity to their own HLA molecules.

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Clinically relevant events throughout the 12 months of follow-up are shown in Table 2. The deceased patient from the placebo group had an acute myocardial infarction at 5 months of follow-up. The patient from the UC-MSC presented an acute lymphocytic leukemia at 5 months from intravenous infusion of UC-MSC, lacking clinical and laboratory elements suggestive of leukemia at baseline and at 3 months of follow-up. One patient from the placebo group developed a malignant melanoma. Concerning major cardiovascular events, three patients from the placebo group and one from the UC-MSC had hospitalizations due to decompensated heart failure, only one patient experienced an acute coronary syndrome in the placebo group. None of the patients had an acute ischemic stroke. No new-onset supraventricular arrhythmias, sustained ventricular arrhythmias, atrioventricular blocks or bundle branch blocks were diagnosed during follow-up, and none were observed at ECG Holter monitoring. There was an increase in the amount of premature ventricular complexes at 24-hour ECG monitoring in the placebo group at follow-up, albeit without changes in mean Lown classification (Online Table I). No noteworthy variations were observed in time or frequency domains at follow-up. No thoracic ectopic tissue formation was observed in CMR at completion of this study. No significant abnormalities were seen in complete blood counts, renal and liver function during monitoring points.

Cardiac imaging.

Echocardiographic parameters evaluated at baseline and follow-up are depicted in Table 3. Compared to baseline, there were improvements in LVEF in the UC-MSC treated group that began at 3 months of follow-up (+3.71±5.01%, p=0.010), and continued at 6 months (+5.43±4.99%, p=0.001) and 12 months (+7.07±6.22%, p=0.001). There were no changes in left ventricular volumes. The placebo group

showed no major differences in these variables. The change of LVEF from baseline to month 12 differed significantly for both groups (+7.07±6.22% vs +1.85±5.60%, p=0.028).

CMR measurements are shown in Table 3. Patients treated with intravenous infusion of UC-MSCs presented an increase of LVEF (p=0.0003) and LVEDV (p=0.012) (Figure 5). The most significant improvements of LVEF was at 6 months of follow-up (+4.67±4.51; p=0.005). There was an increase in LVEDV in the UC-MSC group at 12 months (p=0.033). We observed no changes in LVEF or left ventricular volumes in the placebo group (n=13). One patient from the placebo group withdrew consent for CMR.

Functional status, quality of life and clinical biomarkers.

Results are summarized in Table 3. There were substantial improvements in NYHA class in patients treated with UC-MSC, starting at 3 months (-0.54±0.56; p=0.011), which remained at 12 months follow-up (-0.62±0.46; p=0.003). Only the UC-MSC group experienced improvements in MLHFQ from baseline to all follow-up points ($p<0.05$). Both groups experienced an initial improvement of KCCQ clinical summary at 3 and 6 months of follow-up, with persistence of improvement at trial completion only in the UC-MSC treated group (p=0.014). Patients treated with UC-MSC exhibited an improvement in VE/VCO2 at 12 months (-1.89±3.19; p=0.023 versus baseline), while no differences were observed in peak VO2. We found no differences in other exercise capacity variables including METS, oxygen consumption at anaerobic threshold, peak respiratory exchange ratio and exercise time after cell therapy (Online Table II). We observed a slight decrease in BNP levels in the group treated with UC-MSC at 3 and 12 months of follow-up.

DISCUSSION

RIMECARD is the first randomized, double-blind, placebo controlled clinical trial with intravenous infusion of allogenic UC-MSC in patients with chronic HFrEF. Intravenous infusions of UC-MSC are safe in this population and suggests benefits in surrogate clinical endpoints including LVEF, functional status and quality of life in HFrEF patients receiving this form of systemic stem cell therapy.

MSC-based therapies have been considered overall safe procedures. A recent systematic review of 36 prospective clinical trials for several clinical conditions, including myocardial infarction and chronic cardiomyopathy, did not detect an association between intravascular infusions of MSCs and the risk of acute infusion toxicity, organ system complications, infection, death or malignancy in treated patients[22]. Systematic reviews in HF population actually describe an association between stem cell therapy and a reduction of mortality and major cardiovascular events, albeit most of the analyzed studies used intramyocardial injection or percutaneous intracoronary infusion of bone marrow mononuclear cells[4,5]. There is limited experience regarding intravenous administration of MSCs in patients with cardiovascular diseases, mainly due to safety concerns regarding the entrapment of donor cells in pulmonary circulation and apprehensions on their therapeutic efficacy in a context of low cardiac engraftment. A phase 2 study by Hare et al, supports the safety of intravenous administration of allogenic BM-MSC (up to $5x10^6$ cells/kg) in acute myocardial infarction[23]. At 6 months of follow-up, MSC-treated patients had similar adverse event rates, a trend towards decreased in hospitalization rate and a decrease in arrhythmic events versus placebo[23]. Additionally, there were benefits in pulmonary function at 6 months and lack of evidence of pulmonary ectopic formations in CMR studies performed at 12 months[23]. A recent cross-over phase 2 clinical trial by Butler et al, assessed the safety of the intravenous administration of ischemia tolerant allogenic BM-MSC versus placebo in patients with non-ischemic cardiomyopathy[24]. At 90 days of follow-up, this trial reported no differences in death, hospitalizations and serious adverse events between groups[24]. Considering both studies and our results, the intravenous delivery of UC-MSCs appears safe in HFrEF population.

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Intravenous UC-MSC was not associated with a decrease in the incidence of ventricular arrhythmias, unlike the study by Hare et al; a difference that could be due to several reasons including different patient populations, MSC dosages and monitoring time points.

Our trial displayed improvements in LVEF in patients receiving intravenous UC-MSC treatment, albeit no noteworthy reductions in LVESV or LVEDV were observed. Randomized clinical trials with autologous and allogenic MSC have reported differing results regarding evolution of left ventricular systolic function and volumes[24–32]. In the dose-escalation POSEIDON trial, patients with ischemic HF that received transendocardial injections of autologous and allogenic BM-MSC showed non-clinically relevant improvement on LVEF within 13 months (mean increase +1.96%, p=0.11, n=27)[25]. In the later POSEIDON-DCM trial, a phase I/II randomized clinical trial in patients with non-ischemic dilated cardiomyopathy comparing transendocardial injections of allogenic versus autologous BM-MSC ($100x10^6$ cells), an increase in LVEF was described for patients receiving allogenic BM-MSC at 12 months (+8.0%; p=0.004; n=18), while patients with autologous BM-MSC exhibited non-significant changes (+5.4%; p=0.116; n=16); there were no changes in ventricular volumes for both groups[30]. In the C-CURE trial, the group of ischemic HFrEF patients treated with a combination of autologous BM-MSC exposed to a cytokine cocktail for cardiogenic differentiation (mean dose 733 $x10^6$ cells, n=32), presented noteworthy improvements in LVEF (+6.8%; p<0.0001) and LVESV (-16ml; p<0.0001) at 6 months follow-up[26]. In the TAC-HFT, ischemic HF patients receiving intra-myocardial injections of autologous BM-MSC (100-$200x10^6$ cells, n=19) showed non-significant trends towards improvement in LVEF, LVESV and LVEDV at 12 months[27]. In the MSC-HF trial, ischemic HF patients receiving intra-myocardial injections of autologous BM-MSC (mean dose $77.5x10^6$ cells, n=40) exhibited an increase in LVEF (+5.0%; p<0.0001), and a decrease in LVESV (-7.6ml; p=0.001), while no changes in LVEDV were observed at 6 months follow-up[28]. A phase 2 dose-escalation study in patients with HFrEF performed by Perin et al, assessing transendocardial injections of immunoselected allogenic BM-MSC (25, 75 and 150 $x10^6$ cells, n=15 per group), revealed no differences in LVEF at 12 months of follow-up, although the 150 $x10^6$ MSC group had a significant reduction in LVESV and LVEDV at 6 months and a non-significant decrease of both ventricular volumes at 12 months[32]. In a randomized trial by Zhao et al in patients with decompensated HFrEF, individuals receiving intramyocardial injections of allogenic UC-MSC (n=30) presented improvements in LVEF (+19.0±6.8%; p<0.01) and LVESV (-13.14±10.62ml; p<0.05) at 6 months[29]. In the recent trial by Butler et al, HFrEF patients receiving ischemia-tolerant allogenic BM-MSC ($1.5x10^6$ cells/kg, n=10) experienced a significant increase in LVEF (+2.31%; p=0.02) and reductions in LVEDV (-17.86ml; p=0.04) and LVESV (-16.60ml; p=0.02) at 3 months[24]. Remarkably, the ixCELL-DCM trial reported a reduction in the combined outcome of all-cause mortality and cardiovascular admissions (RR 0.63, 95%CI 0.42–0.97; p=0.0344), in patients with symptomatic HFrEF receiving transendocardial injections of ixmyelocel-T (n=58), a multicellular therapy produced from autologous bone marrow mononuclear cells -with selective expansion of MSC and macrophages,- versus placebo (n=51)[31]. These patients receiving ixmyelocel-T experienced no change in LVEF or ventricular volumes[31]. The CHART-1 trial, showed neutral results regarding composite and individual outcomes, including all-cause mortality, worsening heart failure events, and surrogate endpoints (LVEF, LVESV, LVEDV, MLHFQ), in HFrEF patients with ischemic cardiomyopathy receiving intramyocardial injections of cardiopoietic cells (MSC; n=120) versus sham procedures (n=151)[33]. Exploratory analysis from CHART-1 suggest a benefit in treated individuals with baseline LVEDV >200 ml; unlike our trial, in which most treated patients had lower baseline LVEDV. A recent retrospective cohort of 2166 outpatients with HF by Kalogeropoulos et al, concluded that patients who experienced recovery of LVEF (defined as current LVEF>40% but any previously documented LVEF≤40% by transthoracic echocardiography), had fewer all-cause mortality (RR 0.71; 95% CI, 0.55-0.91), cardiovascular hospitalizations (RR 0.50; 95% CI, 0.35-0.71) and HF-related hospitalization (RR 0.48; 95% CI, 0.30-0.76) compared to patients with HFrEF or HF with preserved LVEF. In the POSEIDON-DCM trial, such recovery of LVEF was achieved by 46.7% of patients receiving allogenic BM-MSC and 22.2% of patients treated with autologous BM-MSC[30], whereas in our study this occurred in 50% (7/14) of UC-MSC treated individuals versus 7.1% (1/14) of the placebo group at month

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12 (p=0.0365). Albeit ours is a small series, only the UC-MSC treated group exhibited significant improvements in left ventricular ejection fraction at 3, 6 and 12 months of follow-up, both by transthoracic echocardiography (p=0.0167 versus baseline) and cardiac magnetic resonance imaging (p=0.025 versus baseline). This suggests our patients might experience benefits regarding major clinical outcomes, although this observation requires verification in a larger phase 3 clinical trial.

Improvements in NYHA and quality of life questionnaires were observed in the UC-MSC group also, in agreement with results from other MSC-based therapy clinical trials in HF[24–28,30]. Interestingly at 12 months of the POSEIDON-DCM trial, the groups receiving allogenic BM-MSC had 66.7% of patients with improved NYHA functional class and a substantial decrease in mean MLHFQ scores, while patients receiving autologous BM-MSC exposed only a trend towards improvement[30]. We appreciated a low concordance between improvement on NYHA classification and performance at cardiopulmonary exercise test, a phenomena previously described[34]. Cardiopulmonary exercise tests have been seldom performed in cell therapy trials, and with wide-ranging results. Regarding MSC therapies, to our knowledge only the POSEIDON and TAC-HFT assessed peak VO2, describing no changes for this outcome in patients treated with autologous BM-MSC[25,27]. We did not observe changes in this variable, although we identified a modest improvement in ventilatory efficiency in patients treated with UC-MSC at 12 months. Recent evidence suggests VE/VCO2 is an excellent marker of severity and prognosis of HF, better than peak VO2 at reflecting the complex interplay of pulmonary, cardiac, and peripheral manifestations in HF population[35,36]. The lack of major benefits in cardiopulmonary performance can be attributed to several factors. Honold et al, in a sub-analysis of patients with poor, moderate and conserved cardiopulmonary test results prior to cell therapy documented that patients with lowest initial exercise capacity showed largest improvements in peak VO2 and VE/VCO2 after intracoronary stem cell infusion[37]. Our patients had slight alterations at baseline, therefore limited benefits could be anticipated.

A range of mechanisms have been proposed to explain the clinical benefit observed in HF patients treated with MSC, including reductions in myocardial cell apoptosis, modulation of inflammation, myocardial fibrosis, neovascularization and increased cell differentiation[13]. Incorporation of MSCs into tissues is regulated by multiple processes including cell recruitment, migration and adhesion[38]. The higher migration of UC-MSCs in response to HFrEF patient serum, herein described, is compatible with the notion that this cell type might sense biological cues that are contributory to their therapeutic effect by systemic delivery.

In our study, UC-MSCs and BM-MSCs expressed cardiomyogenic differentiation potential, although BM-MSCs presented a more favorable profile of transcription factors related to cardiac differentiation. Despite early reports describing cell engraftment and differentiation in animal models of HF, later studies evidence retention rates below 0.5% after 4 days of intramyocardial injections of BM-MSC[39], which seem insufficient to account for the magnitude of clinical benefit. Mounting evidence rather suggests the reparative actions of MSCs rely on paracrine modulation[1,2]. The comparative results of the paracrine factors assessed in this work, point to a significant advantage of UC-MSCs over BM-MSCs. The most striking difference was the prominent expression of HGF in UC-MSC from all tested donors, while BM-MSCs showed low to undetectable levels. Remarkably, several studies in chronic ischemic or non-ischemic HF animal models, have reported that gene transfection of HGF promotes angiogenesis and decreases fibrosis and apoptosis, attenuating cardiac remodeling and improving myocardial remodeling, perfusion and contractile function[40–44]. Furthermore, MSCs share several biological properties with endothelial cells, enabling them to contribute to angiogenesis. Preclinical data from several groups including ours, suggest UC-MSC can enhance angiogenesis by promoting the formation of capillary-like structures in vitro or increasing capillary density in vivo, through upregulation of various proangiogenic factors and chemokines including VEGF, angiopoietin and MCP-1 among others[12,13,45,46]. Liu et al have described that UC-MSC intracoronary and intravenous infusion was associated with a promotion of angiogenesis through paracrine modulation and perhaps endothelial cell differentiation, an augmented myocardial perfusion and enhancement of collateral vessel development in a porcine model of a chronic

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myocardial ischemia[14]. In the same study, animals treated with UC-MSC had improved LVEF and a reduction of myocardial fibrosis and apoptosis[14]. Moreover, allogenic MSCs can improve endothelial function and vascular reactivity through stimulation of endothelial progenitor cell mobilization in HF patients[30,47]. Interestingly, the PROMETHEUS trial assessed the impact of intramyocardial injections of autologous BM-MSC into the akinetic non-revascularized myocardial segments of patients with chronic ischemic cardiomyopathy, reporting an improvement in myocardial perfusion and functional recovery, and subsequently an improvement in global left ventricular function[48].

Limitations.

The assessment of differences in major cardiovascular outcomes and surrogate efficacy outcomes was underpowered due to the small number of participants from each patient group. Post hoc analysis considering echocardiographic assessment of LVEF at 12 months revealed an estimated power of 71%. This discouraged further analysis to discriminate responders from non-responders to therapy or differences regarding cardiomyopathy substrate. Differences in left ventricular volumes at baseline, in spite of randomization, could bias efficacy results in favor of UC-MSC. However the sub-analysis of CHART-1 is reassuring in that most benefit in response to treatment occurred precisely in patients with higher baseline LVEDV, suggesting such bias might not be in favor of our UC-MSC group. We could not perform myocardial perfusion and fibrosis measurements due to non-contrast CMR imaging and software restraints, nonetheless these had not been considered as secondary endpoints of the study.

Conclusions.

Intravenous infusion of UC-MSC was feasible and safe in this group of patients with HFrEF under otherwise optimal medical therapy. Allogenic UC-MSC treatment induced no humoral immune response in tested individuals. The intervention resulted in a significant improvement in left ventricular function, functional status and quality of life. These findings suggest UC-MSC could have an impact on clinical outcomes, supporting further testing through large clinical trials.

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PLG and FA received stipends from Cells for Cells. MK is CSO of Cells for Cells and Consorcio Regenero. The other authors indicated no potential conflicts of interest.

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FIGURE LEGENDS

Figure 1. UC-MSCs and BM-MSCs displayed different cardiac differentiation potential and paracrine factors profile. Cardiac differentiation was induced in UC-MSCs and BM-MSCs by cultured with 5-aza 10uM during 25 days. Cardiac differentiation potential was evaluated through mRNA relative expression of cardiac gene (NKX2-5, GATA-4, MEF2C, MYH7b, GJA1 and TNNT2) by real time PCR with B2M as a housekeeping gene (A) and by detection of cardiac proteins using indirect immunofluorescence staining troponin and connexin-43 (B), the respective graphs show the quantification of positive cells in the each staining. TGFβ3 expression was quantitated by RT-qPCR (C). VEGF and HGF levels were evaluated by ELISA assay (C). Data shown in the graphs are the mean±SEM of at least three individual experiments. *$p<0.05$, *** $p< 0.001$, UC-MSCs compared to BM-MSCs. + $p<0.05$, ++ $p<0.001$ UC-MSC-4 compared to UC-MSCs-1, 2 and 3.

Figure 2. UC-MSCs and BM-MSCs display the same suppressive capacities to inhibit proinflammatory T-cells. PHA-activated hPBMC obtained from dilated cardiomyopathy patients with heart failure and reduced ejection fraction (HFrEF), labeled with CFSE were co-culture with or without MSCs at a 1:10 ratio (MSCs:hPBMC). A) T-cell proliferation was evaluated by the reduction in CFSE intensity at 72 hours after culture, the graphs in the left is a representative CFSE proliferation panel (light color histogram represent activated PBMCs and dark color histogram to activated PBMC co-cultured with MSCs). B) Th1, Th2, CD8 and regulatory T cells subsets analysis from co-culture of PBMC and MSCs. Results are represented as mean±SEM of at least 3 independent experiments using at least 3 different donors for hPBMC (healthy donor and DCM patient), UC-MSCs and BM-MSCs. *** $p<0.001$ UC-MSCs or BM-MSCs with respect to PHA.

Figure 3. UC-MSCs possess a superior migration capacity compared with BM-MSCs. Migration capacity of MSCs was evaluated by transwell assay in response to serum from HfrEF patients after 16h. The pictures show the representative staining with violet crystal and the left graph the quantification of % of migrated cells under the different conditions. Data shown in the graphs are the mean±SEM of at least three serum donor, UC-MSCs and BM-MSCs. *$p<0.05$ UC-MSCs vs BM-MSCs.

Figure 4. Study Flow Chart.

Figure 5. Changes in cardiac magnetic resonance imaging measurements from baseline to 12 months post treatment in studied groups. A. Left ventricular ejection fraction (LVEF). B. Left ventricular end-diastolic volume (LVEDV). C. Left ventricular end-systolic volume (LVESV). Continuous line represents UC-MSC group (n=14 per protocol). Dashed line represents placebo group (n=13 per protocol; withdrawal of consent from 1 patient). Statistical analysis are based on mixed effect maximum likelihood regression between baseline and follow-up measures for each group and variability between groups.

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NOVELTY AND SIGNIFICANCE

What Is Known?

- Intracoronary and intramyocardial cell therapy, mainly with allogenic bone marrow mesenchymal stromal cells (BM-MSC), has shown to be safe and potentially effective in heart failure patients, even if low levels of cell engraftment are expected, suggesting a paracrine mechanism of action.

- Umbilical cord mesenchymal stromal cells (UC-MSC) are of easier access and *in vitro* expansion, and exhibit superior angiogenic and paracrine effects compared with BM-MSC, but their systemic administration in human heart failure patients has not been tested.

What Information Does This Article Contribute?

- This is the first double blind randomized placebo controlled trial of the intravenous administration of umbilical cord derived mesenchymal stromal cells (UC-MSC´s), confirming this a feasible and safe treatment in patients with ischemic and non-ischemic heart failure.

- The UC-MSCs employed in this trial exhibited superior clonogenicity, migration and paracrine capacities *in-vitro* and less senescence when compared with bone marrow derived MSCs (BM-MSCs).

- UC-MSC treatment was associated with significant improvements in ventricular systolic function, NYHA functional classification and quality of life indexes.

Cell therapy has been evaluated in cardiovascular diseases for more than a decade without reaching consensus regarding optimal cell source or method of application. Trials using BM-MSCs administered through invasive local implantation have suggested positive results, and have indicated that allogenic cell sources may be superior to autologous MSCs in aged patient population, usually with comorbid disease. Herein, we report the first randomized placebo controlled clinical trial using UC-MSCs intravenously in patients with heart failure and reduced ejection fraction of both ischemic and non-ischemic etiology. The results show that systemic administration of UC-MSCs is safe in these patients and point to significant improvements in functional capacity, quality of life and left ventricular ejection fraction. Moreover, we show this highly accessible and allogenic cell source of younger origin than BM-MCSs, displayed biologic and paracrine advantages, and exerted long-term (12 months) clinical effects via intravenous administration. This route of administration simplifies therapy, decreases costs of the procedure, allows exploration of repeated dosages, and should be tested further with UC-MSCs in larger trials assessing long-term clinical endpoints.

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Table 1. Baseline Characteristics

Characteristics	Placebo (n = 15)	UC-MSC (n = 15)	p
Age	57.20±11.64	57.33±10.05	NS
Gender (male %)	14 (93.3)	12 (80.0)	NS
Ischemic cardiomyopathy (%)	11 (73.3)	10 (66.7)	NS
Arterial hypertension (%)	8 (53.3)	7 (46.7)	NS
Diabetes (%)	7 (46.7)	5 (33.3)	NS
Dyslipidaemia (%)	6 (40.0)	7 (46.7)	NS
Smoking (%)	4 (26.7)	7 (46.7)	NS
Obesity (%)	8 (53.3)	6 (40.0)	NS
BMI	29.52±4.00	29.12±2.88	NS
Medication			
Aspirin (%)	9 (60.0)	14 (93.3)	0.031
Clopidogrel (%)	1 (6.7)	3 (20.0)	NS
Acenocumarol (%)	9 (60.0)	2 (13.3)	0.008
ACEI or ARB (%)	15 (100)	15 (100)	NS
Beta blockers (%)	15 (100)	15 (100)	NS
Spironolactone (%)	13 (86.7)	13 (86.7)	NS
Other vasodilators	2 (13.3)	1 (6.7)	NS
Digitalis (%)	1 (6.7)	4 (26.7)	NS
Other antiarrhythmic (%)	2 (13.3)	1 (6.7)	
Diuretics (%)	10 (66.7)	9 (60.0)	NS
Metformin (%)	7 (46.7)	4 (26.7)	NS
Other oral antidiabetics (%)	2 (13.3)	0 (0.0)	NS
Insulin (%)	1 (6.7)	2 (13.3)	NS
Statins (%)	12 (80.0)	11 (73.3)	NS
NYHA class	1.67±0.49	2.03±0.61	NS
Laboratory			
GFR (mL/min/1.73 m2)	76.18 ±24.36	81.91±15.69	NS
Haemoglobin (mg/dl)	14.33±1.13	14.29±1.35	NS
C-reactive protein (mg/L)	1.65±1.41	1.84±1.42	NS
Brain natriuretic peptide (pg/mL)	767.45±481.02	451.61±495.14	0.015
Electrocardiogram			
Sinus rhythm (%)	14 (93.3)	14 (93.3)	NS
LBBB (%)	1 (6.7)	1 (6.7)	NS
LAFB (%)	3 (20.0)	5 (33.3)	NS
RBBB (%)	4 (26.7)	2 (13.3)	NS
Echocardiography			
LVEF (%)	31.49±4.71	33.00±6.18	NS
LVESV (mL)	136.53±35.32	108.93±38.65	NS
LVEDV (mL)	202.07±45.79	161.80±53.13	0.034
Restrictive diastolic dysfunction (%)	3 (20.0)	1 (6.7)	NS
Moderate mitral regurgitation (%)	2 (13.3)	1 (6.7)	NS

BMI: Body mass index. ACEI: Angiotensin converting enzyme inhibitors. ARB: Angiotensin II Receptor Blockers. NYHA: New York Heart Association. GFR: Glomerular filtration rate according to CKD-EPI formula. LBBB: Left bundle branch block. LAFB: Left anterior fascicular block. RBBB: Right bundle branch block. LVEF: Left ventricular ejection fraction. LVESV: Left ventricular end-systolic volume. LVEDV: Left ventricular end-diastolic volume. NS: $p>0.05$ between groups.

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Table 2. Incidence of clinically relevant events at 12 months of follow-up

	Placebo (n = 15)	UC-MSC (n = 15)	p
Overall Deaths	1	1	NS
Cardiovascular deaths	1	0	NS
Hospitalizations	4	1	NS
Heart Failure	3	1	NS
Myocardial infarction	1	0	NS
Incident malignancy	1	1	NS
Nonsustained ventricular tachycardia	7	7	NS

NS: $p>0.05$ between groups.

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Table 3. Primary and secondary efficacy outcomes at baseline and follow-up points

Variable	Group	n	Baseline	3 months	6 months	12 months
TTE LVEF	**Placebo**	14	31.53±4.89	33.00±7.24	32.79±7.76	33.39±7.38
	UC-MSC	14	33.50±6.09	37.21±6.80‡	38.93±5.74‡	40.57±8.19‡
TTE LVESV	**Placebo**	14	134.3±35.5	126.4±39.8	128.4±43.4	131.1±42.0
	UC-MSC	14	110.1±37.6	99.1±39.0	104.4±42.8	100.5±36.8
TTE LVEDV	**Placebo**	14	199.2±46.1	188.8±42.1†	189.1±45.4	191.7±43.5
	UC-MSC	14	168.1±48.5	161.6±43.9	167.0±56.6	161.4±48.6
CMR LVEF	**Placebo**	13	29.62±6.53	28.80±6.55	30.66±7.65	31.31±7.10
	UC-MSC	14	32.64±8.42	35.93±9.83†	38.41±12.00‡	37.43±10.44†
CMR LVESV	**Placebo**	13	175.2±56.8	170.9±39.8	167.8±50.5	179.0±52.6
	UC-MSC	14	130.2±42.8*	130.8±62.1	121.3±46.2	133.9±62.1
CMR LVEDV	**Placebo**	13	245.9±60.1	207.3±75.4	241.0±56.0	257.8±54.1
	UC-MSC	14	185.5±50.0*	197.7±67.2	190.8±48.2	210.0±67.2†
NYHA	**Placebo**	14	1.71±0.48	1.50±0.62	1.43±0.55†	1.46±0.63
	UC-MSC	14	2.07±0.62	1.57±0.61‡	1.50±0.59‡	1.43±0.63‡
MLHFQ	**Placebo**	14	37.42±22.22	29.04±18.39	26.86±22.93	27.07±20.36
	UC-MSC	14	53.21±30.25	30.50±23.76†	27.07±21.54‡	31.21±26.66†
KCCQ–CS	**Placebo**	14	69.92±21.24	78.08±15.94†	78.64±18.46†	75.46±22.43
	UC-MSC	14	57.48±25.33	73.22±22.89†	74.99±20.70†	72.82±24.10‡
VO2 peak	**Placebo**	14	17.56±5.04	18.14±5.32	17.85±4.92	18.16±4.70
	UC-MSC	14	18.11±4.67	18.52±4.28	18.59±4.84	17.88±4.11
VE/VCO2	**Placebo**	14	34.42±5.12	34.19±6.01	33.61±6.28	33.42±6.74
	UC-MSC	14	34.06±8.53	32.11±5.99	32.41±5.18	32.17±7.41†
BNP	**Placebo**	14	731±477	654±468	681±499	892±801
	UC-MSC	14	474±507*	355±443‡	452±586	394±535†
HSCRP	**Placebo**	14	1.63±1.46	1.78±1.86	1.92±1.85	1.67±1.09
	UC-MSC	14	1.68±1.33	3.15±3.60	5.15±15.94	2.15±2.58

TTE: Transthoracic Echocardiogram. CMR: Cardiac Magnetic Resonance. LVEF: Left ventricular ejection fraction (%). LVESV: Left ventricular end-systolic volume (ml). LVEDV: Left ventricular end-diastolic volume (ml). NYHA: New York Heart Association. MLHFQ: Minnesota Living with Heart Failure Questionnaire. KCCQ-CS: Kansas City Cardiomyopathy Questionnaire Clinical Summary. VO2 peak: Maximal oxygen consumption (ml/Kg/min). VE/VCO2: Minute ventilation to carbon dioxide production ratio. BNP: Brain natriuretic peptide (pg/ml). HSCRP: High sensitivity C-reactive protein (mg/L). *p<0.05 versus placebo. †p<0.05 versus baseline. ‡p<0.0167 versus baseline.

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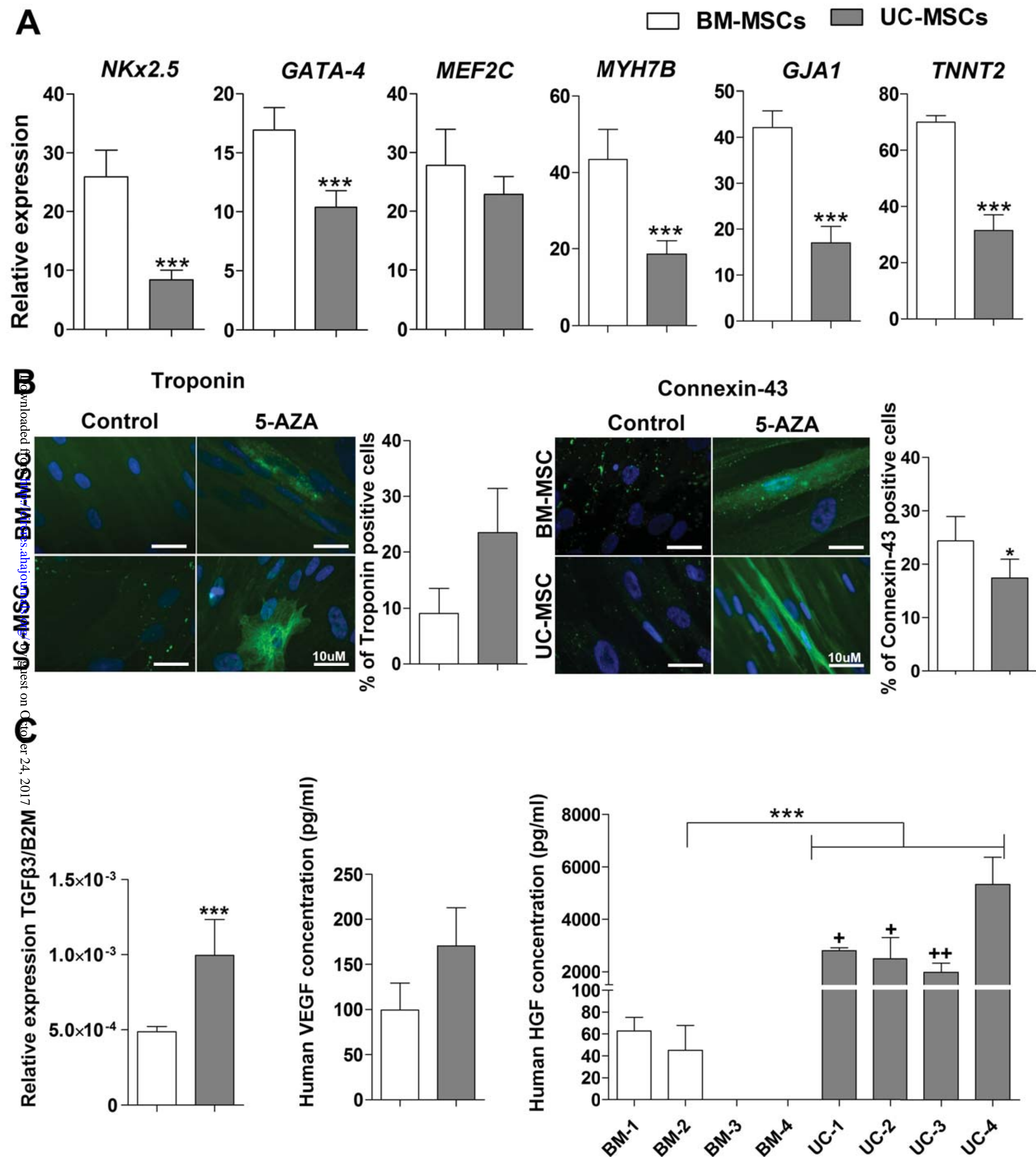

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FIGURE 2

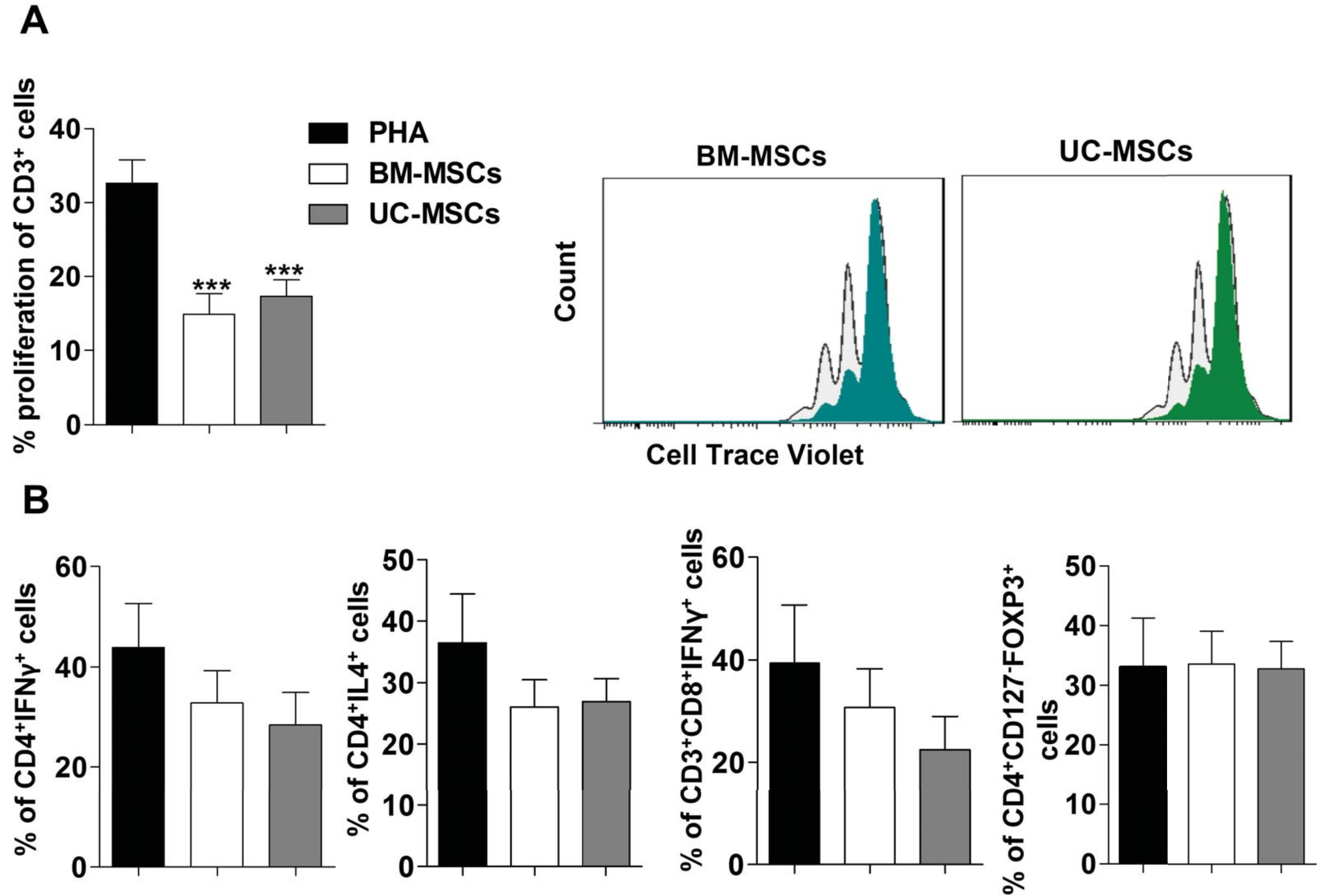

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FIGURE 3

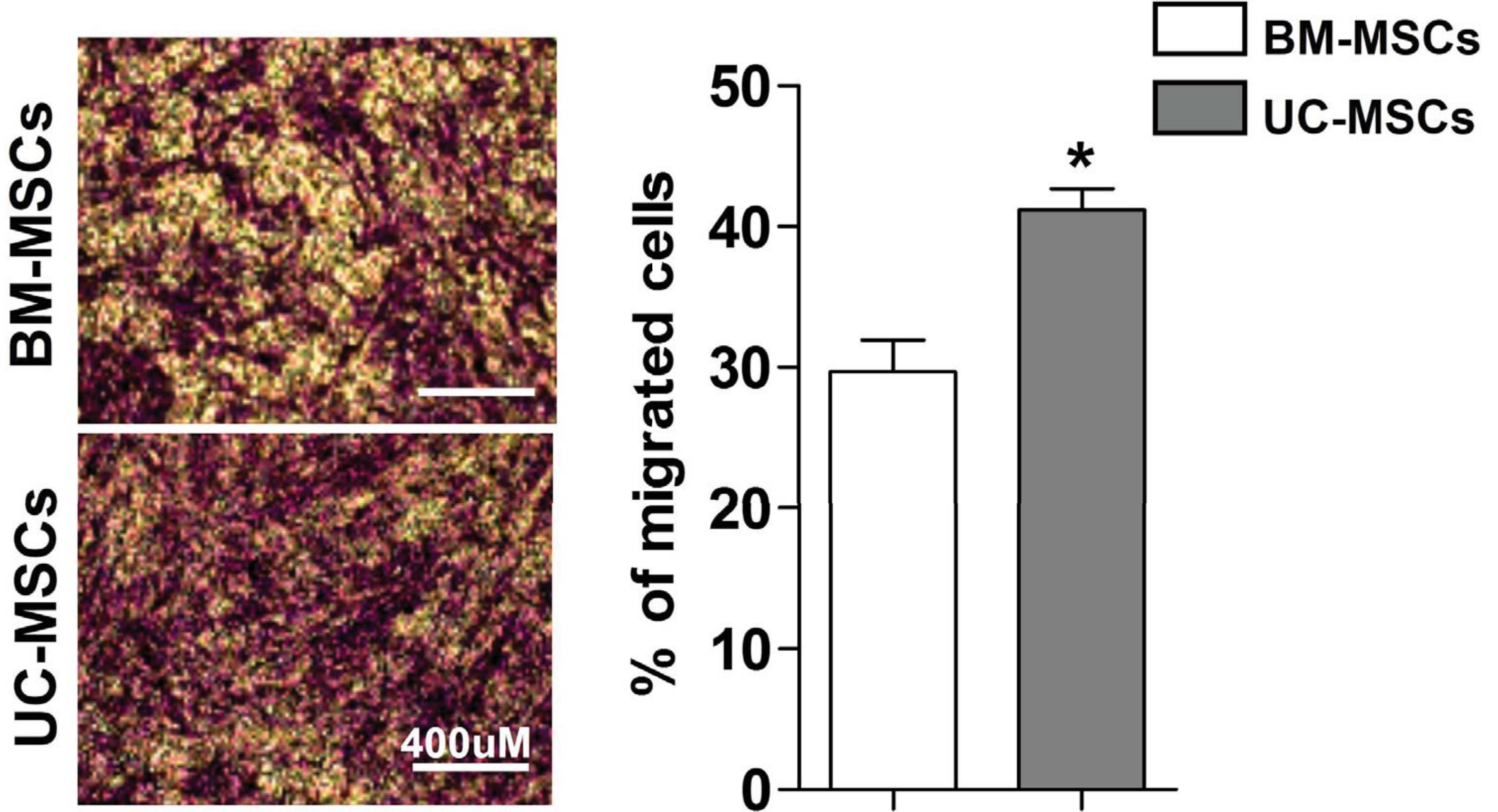

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FIGURE 4

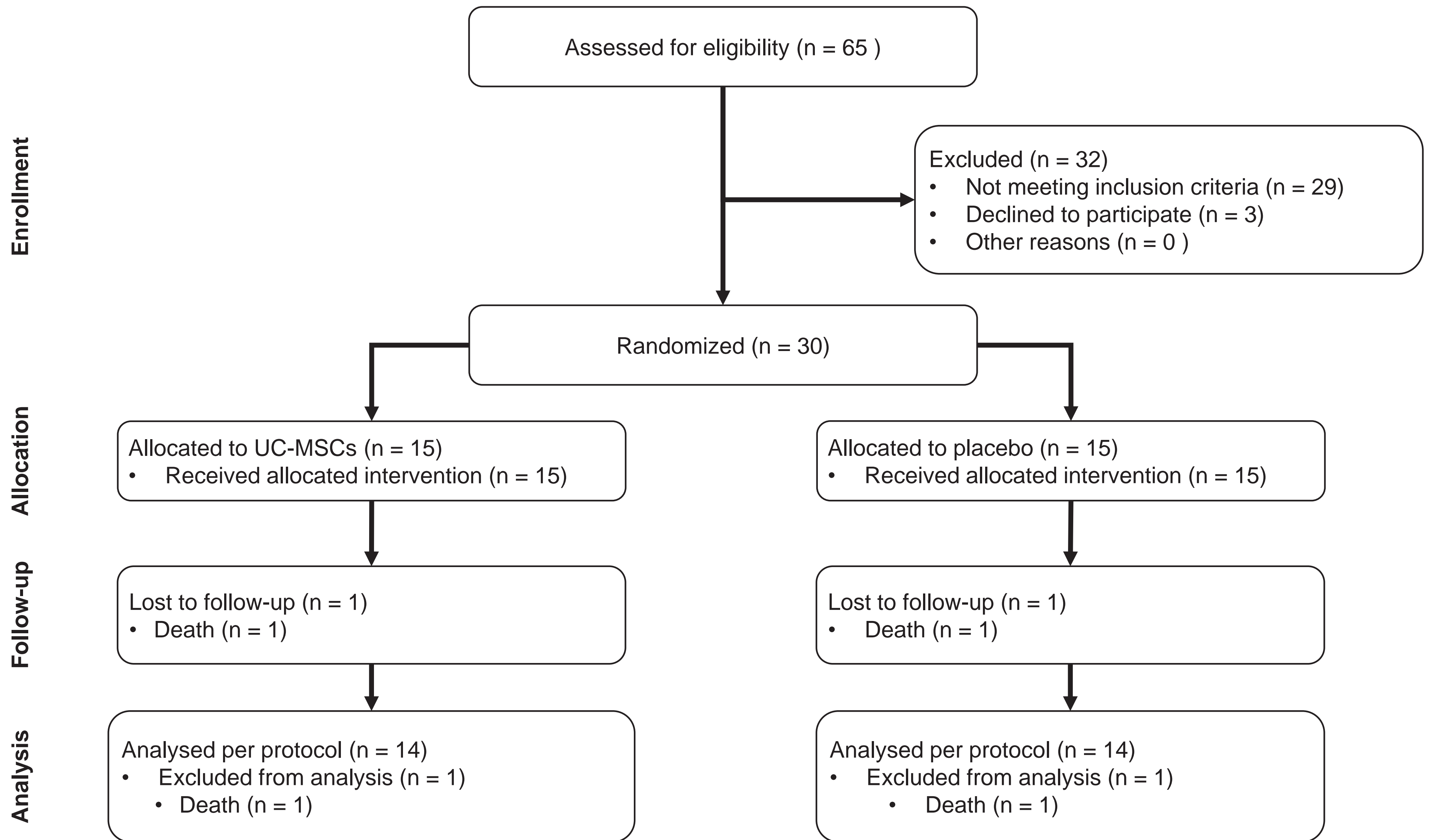
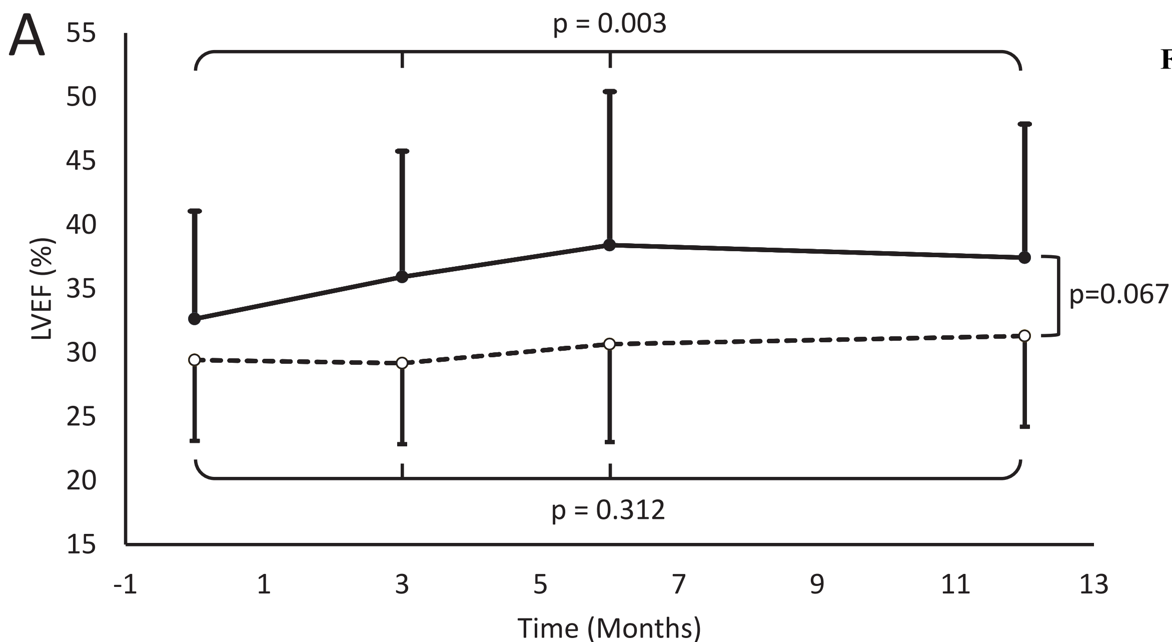
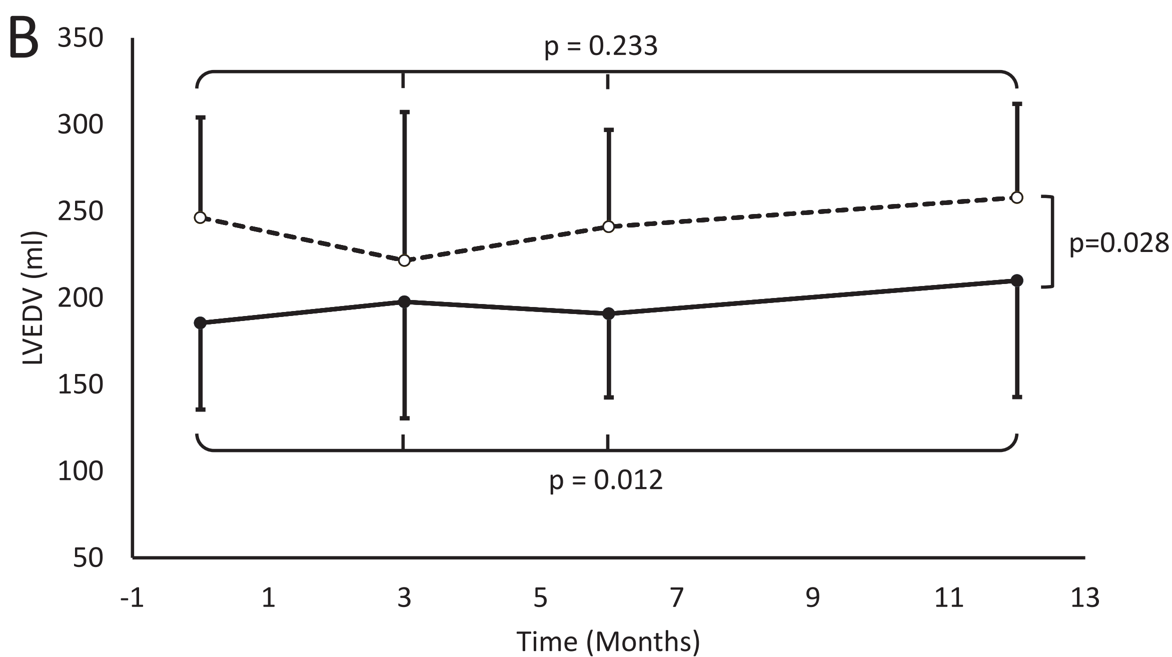
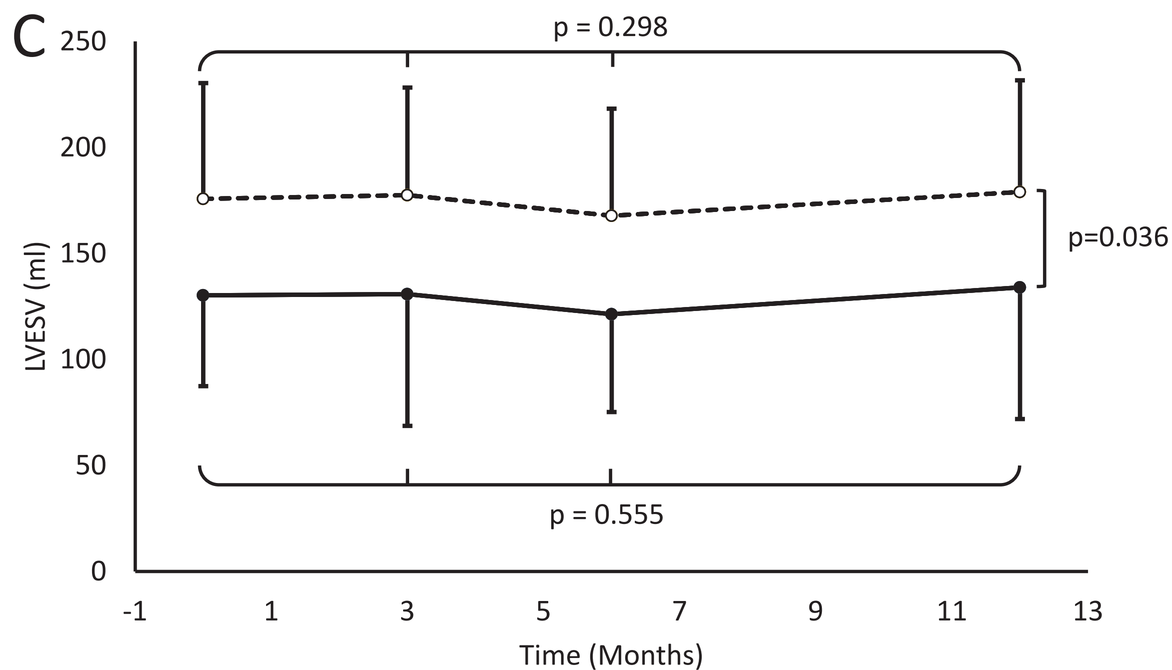


FIGURE 5







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Safety and Efficacy of the Intravenous Infusion of Umbilical Cord Mesenchymal Stem Cells in Patients With Heart Failure: A Phase 1/2 Randomized Controlled Trial (RIMECARD Trial)

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ONLINE SUPPLEMENT

EXPANDED METHODS

Automated cell counting, size and characterization:

Prior to the release of each batch of cell-therapy, preparations were assessed using a Countess™ (Invitrogen, USA) automated cell counter for viability, cell count and size measurements using trypan blue staining for viability combined with advanced image analysis. After setting the cell mode parameters for MSC cell type (circularity, maximum and minimum cell size), UC-MSC cell samples were mixed with trypan blue (1:1) and loaded onto the cell counting chamber slide. Each sample data was stored and then analyzed.

Doubling time

Data were collected from each cell culture process performed under GMP conditions. Doubling time was calculated based on initial cell number, culture time (hours) and final number of cells harvested according to the described formula (Roth V. 2006 Doubling Time Computing, available from: http://www.doubling-time.com/compute.php): Doubling Time = duration x log (2) / [log (final concentration) −log (initial concentration)]. The range of analyzed data was the following:

Cell seeding density: 1400 – 4900 cells/cm2
Initial cell number seeded: 1.4 – 62 million
Culture days: 3-8 days
Harvested cell number: 8.5 – 336 millions

Senescence-associated beta-galactosidase assay

UC-MSC and BM-MSC were cultured and harvested under standard conditions. To perform the assay using Senescent cells histochemical staining kit (sigma #CS0030), 10.000 cells per well were seeded in 24-well plates. After 5hrs, the staining mixture was added for detection of SA-β-galactosidase and incubated overnight at 37°C following the manufacturer's instructions. Positive stained cells were counted and the percentage of cells expressing SA-β-galactosidase (senescent cells) calculated over the total cell number of each sample.

Cardiomyogenic differentiation

For cardiomyogenic differentiation, cells in passage 3 were seeded at 60% of confluence and cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose (Gibco, USA) supplemented with 5% heat-inactivated FBS (Gibco, USA) with 1% penicillin/streptomycin and 2 mM L-G and 10 uM 5-azacytidine (Sigma-Aldrich, USA). The media was removed and replaced for fresh media every 3 days. At day 20 relative expression of NKX2-5, GATA-4, MEF2C, MYH7b, GJA1 and TNNT2 were measured. RNA extraction was performed using the RNeasy mini kit (Qiagen, USA) and complementary DNA was synthesized in a 20 µl reaction mixture using SuperScript III First-Strand Synthesis for RT-PCR (Invitrogen, USA). RT-qPCR was performed using SYBR Green Reagents (QPCR Master Mix, Agilent Technologies). All primer sets were previously screened for efficiency and their sequences were: B2M (F:5´ TCAGGTTTACTCACGTCATCC 3´, R:5' ACACGGCAGGCATACTCATC 3´), GATA-4 (F: 5´AAACGGAAGCCCAAGAACCT 3´, R: 5´ ACTGAGAACGTCTGGGACAC 3´), NKX2-5 (F: 5´ TGTCCACGCTGCATGGTATC 3´, R:5´GATCACTCATTGCACGCTGC3´), MEF2C (F: 5´ CCAACTTCGAGATGCCAGTCT 3´, R:5´ GTCGATGTGTTACACCAGGAG 3´), MYH7B (F:5'

GCAATAAAAGGGGTAGCAGAGC 3', R:5'GACTCCCCAAGTTCACTCACAT3'), TNNT2 (F:5' CTGGCCATTGACCACCTGAA 3', R:5'GCTGCTTGAACTTCTCCTGC3'), GJA1 (F:5' TCTCTCATGTGCGCTTCTGG 3', R: 5' TGACACCATCAGTTTGGGCA 3'). Data were expressed as relative mRNA level of specific gene using the 2-ΔΔCT method and normalized with the Beta-2 microglobulin (B2M) housekeeping gene. Additionally, differentiation was confirmed by indirect immunofluorescence using Anti-Cardiac Troponin I antibody (ab47003, Abcam), Anti-Connexin 43/GJA1 (ab47368, Abcam) and Goat Anti-Rabbit IgG FITC (ab6717, Abcam). Cells were both fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature and permeabilized with 0.3% Triton X-100 for 10 minutes. Nonspecific binding was blocked by PBS plus 5% serum albumin bovine serum (BSA) (Sigma) for 60 minutes. Primary antibodies were incubated overnight at 4°C and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 2 hours at room temperature. Nuclei were counterstained with 4´, 6´diamino-2-phenylindole (Sigma) for 1 minute. Images were obtained with a NIKON ECLIPSE TE2000-fluorescence microscopy and Nikon Sight DSU2 camera.

Measurements of Paracrine Factors

To compare the secretion levels of growth factors between BM–MSCs and UC–MSCs, $3X10^4$ cells were plated in serum-free medium in 6-well plates. After 24 hours of incubation, the conditioned medium was collected, and the secreted levels of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) were measured using the DuoSet ELISA Development System (R&D Systems, Minneapolis,MN). In addition, relative expression of *TGFβ1* was measured using the RNeasy mini kit (Qiagen, USA) to RNA extraction and complementary DNA was synthesized in a 20 µl reaction mixture using SuperScript III First-Strand Synthesis for RT-PCR (Invitrogen, USA). RT-qPCR was performed using SYBR Green Reagents (QPCR Master Mix, Agilent Technologies). All primer sets were previously screened for efficiency and their sequences were: *B2M* (F:5´ TCAGGTTTACTCACGTCATCC 3´, R:5' ACACGGCAGGCATACTCATC 3´), *TGFβ1* (F:5′ACAATTCCTGGCGATACCTCAGCA3′, F:5′TGCAGTGTGTTATCCCTGCTGTCA3′).

IDO activity

UC-MSCs and BM-MSCs cells were stimulated with 20 ng/mL of IFNγ and 10 ng/mL of IL-1β or with 100 ng/mL of IFNγ during 48 hours in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin (all reagents from Gibco, Gran Island, USA) and 100 ug/mL of L-tryptophan (Sigma-Aldrich, St Louis, MO,USA). IDO enzyme activity was measured determining the kynurenine content in the cell supernatant as previously reported[1,2].

IL6, TGF-β, PGE2, PDL-1 and HLA-G quantification

IL6, TGF-β and PGE2 were quantified in the supernatants of BM-MSCs and UC-MSCs stimulated for 48 hours in the absence and presence of 20 ng/mL IFN-γ and 10 ng/ml IL-1β in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin (all reagents from Gibco, Gran Island, USA), using an enzyme- linked immunosorbent assay (ELISA) from R&D Systems (R&D Systems, Minneapolis, MN,USA) following the manufacturer instructions. PDL-1 and HLA-G detection was performed using flow cytometry (FACSCanto™, BD Bioscience). BM-MSCs and UC-MSCs were collected and stained with specific antibodies (BD Pharmingen, San Jose, CA, USA) according to previously published staining procedures[3]. Briefly, The collected cells were resuspended in 100 μl of FACS buffer (PBS 1X, 0.2% BSA, 0.01% sodium azide) and incubated for 20 minutes at 4°C with the

appropriate fluorescently labeled monoclonal antibody directed against lymphocyte surface markers (BD Biosciences, CA, USA), washed and resuspended in FACS buffer, and analyzed by the FACS Canto II cytometer using the FACS Diva software (BD Biosciences, CA, USA). The viability was determined using LIVE/DEAD®Fixable dead cell stain kit (Invitrogen, CA, USA) according to the manufacturer's protocol. The data acquired was analyzed using the FlowJo software (Tree Star, Ashland, OR, USA).

T-Cell Subset Proliferation Assays

The proliferation of different T-cell subsets from 4 HFrEF patients included in this trial was performed in vitro to evaluate the immunomodulatory effect of UC-MSCs and BM-MSCs. Human peripheral blood mononuclear cells PBMCs were isolated by Ficoll-Paque Plus (GE Healthcare, Amersham, UK) (1.077 g/ml) density-gradient according to manufacturer's instruction. PBMCs were stained with carboxyfluorescein succinimidyl ester (CFSE; Life Technologies, Carlsbad, CA) following the manufacturer's protocol, and co-cultured with MSCs in 96-well plates at a 1:10 ratio (MSCs:hPBMC) in Roswell Park Memorial Institute (RPMI) medium (ThermoFisher, USA) supplemented with 10% FBS, 1% L-G, 1% nonessential amino acids (Sigma-Aldrich, USA), 100mM sodium pyruvate (Sigma, USA), 25 mMb-mercaptoethanol (Gibco, NY), and 15mg/ml phytohemagglutinin (PHA) (Sigma, USA). After 72 hours, PBMC were stimulated for 4 hours with 50 ng/ml phorbolmyristate acetate (PMA) (Sigma Aldrich) and 1μg/ml ionomycin (Sigma-Aldrich) in the presence of Brefeldin A (Biolegend, San Diego, Ca, USA). For surface staining, cells were incubated with antibodies against human CD4, CD8, CD3 and CD25 (BD Biosciences, USA) in the dark at 4°C for 30 min. Intracellular staining was performed using the BD Cytofix/Cytoperm solution, according to the manufacturer's protocol with antibodies against human IL-17, IL4 and IFNγ (eBioscience, USA). For transcriptional factor evaluation, we assessed FoxP3 expression with staining buffer and specific antibodies (eBioscience, USA) according to the manufacturer's protocol. Cells were acquired using a FACS Canto II Flow cytometer (BD Biosciences) and analyzed with the FlowJo software (Tristar, Stanford) for phenotype and proliferation, calculated by the decrease in CFSE fluorescence.

Migration capacity

Cell migration assays were performed with the Transwell two-chamber cell culture method (Corning, Cambridge, MA) with an 8 μm pore polycarbonate membrane. The uppermost side of the Transwell membrane was coated with 0.1% gelatin in PBS (Sigma-Aldrich, St. Louis, MO) for 2 h at 37℃. UC-MSCs or BM-MSCs were seeded at a density of 15.000 cells per 100 μl of DMEM 1% P/S, 0.1%FBS in the upper chamber of the Transwell apparatus. Cells were allowed to migrate toward medium (500 μl) in the lower chamber containing DMEM alone or supplemented with 5% of serum isolated from the HFrEF patients. The Transwell system was incubated for 16h at 37℃ in a humidified atmosphere containing 5% CO2. After incubation, non-migratory cells were carefully removed from upper face of the Transwell insert with a cotton swab. The attached cells remaining on the Transwell insert were fixed with 70% methanol and stained with 1% crystal violet in 20% methanol for 1 h. After washing, the stained cells that migrated from the upper to the lower side of the membrane were counted under an inverted bright-field microscope at 20X magnification. The number of migrated cells was expressed as the percent change from the control value (DMEM alone). Each experiment was performed in biological and experimental triplicate.

SUPPLEMENTAL RESULTS

Cellular size

Parameters defined for the MSC setting of the Countess™ (Invitrogen) automated cell counter included control of circularity and maximum-minimum cell size (applied gate 10um - 28um). The analysis of 26 therapy releases resulted in an average size measurement of UC-MSC´s of 17.1 ± 3.5 μm.

Doubling time

The doubling time (DT) data were collected from each of the cell culture passage performed under GMP conditions. The assessment of 50 samples resulted in an average DT of 32.6 ± 8.8 hours.

Senescence-associated beta-galactosidase assay

UC-MSC and BM-MSC senescence according to the expression of SA-β-galactosidase was evaluated under standard culture conditions normalized for passage and culture duration. UC-MSC showed around 2 fold less senescent cells than BM-MSC´s (Online Figure II).

Quantification of IDO activity, IL6, TGF-β1, PGE2 and PDL-1 at basal and stimulated condition

The expression of these mediators involved in the regenerative and suppressive effects of MSCs was evaluated for both cell sources (BM or UC-MSCs) in baseline conditions and after proinflammatory stimulus with IFNγ and IL-1β at optimal conditions (20 and 10 ng/ml respectively). UC-MSCs expressed similar levels of PDL-1, HLA-G as well as IDO activity compared to BM-MSCs under the different culture conditions. Furthermore, both MSC sources responded to the IFNγ + IL-1β stimulated conditions by an increased protein expression levels of IL-6, TGF-β1 and PGE2 (Online Figure III). Of interest, UC-MSCs expressed higher constitutive levels of PGE2 and TGF-β1, a molecule known for the induction of T regulatory (Treg) CD4+ cells and inhibition of NK function.

ONLINE FIGURE LEGENDS

Online Figure I. UC- MSCs expressed all the common MSC markers and demonstrate capacity to differentiate into chondrogenic, osteogenic and adipogenic lineage. (A): UC-MSCs were stained with labeled monoclonal antibodies against known MSC surface markers (blue) and their respective isotypes (red); the cells were analyzed by flow cytometry. All UC-MSCs were positive for CD105, CD73, CD90, CD44, CD146, CD49a and HLA-ABC but negative for CD14, CD34, CD31, CD45, and HLADR. (B) Representative images of UC-MSC differentiation after specific inductions and staining: adipocytes (Oil Red O), osteocytes (alizarin red), and chondrocytes (safranin O). Scale bars = 200 mm. All data are presented as mean ± SEM (n=3) of a minimum of 4 donors. Abbreviations: MSCs, mesenchymal stem cells; UC, umbilical cord.

Online Figure II. Size, doubling time and senescence of UC-MSCs. A. Cell preparations were counted using an automated cell counter assessing viability, cell counting and size measurements using the trypan blue method of dead-cell staining combined with advanced image analysis. The parameters for MSC setting included setting of the circularity and maximum and minimum cell size (applied gate 10um - 28um). The analysis of 26 therapy releases resulted in the average size measurement of 17.1±3.5 μM. B. The doubling time (DT) of UC-MSC. The data were collected from each cell culture process performed under GMP conditions. Doubling time was calculated based on initial cell number, culture time (hours) and final number of cells harvested. The assessment of 50 samples resulted in the doubling time of 32.6± 8.8 hours. C. Senescence-associated beta-galactosidase. UC-MSC and BM-MSC (n=3) were cultured and harvested under standard conditions. Positive stained cells for SA-β-galactosidase were counted and the percentage of cells expressing SA-β-galactosidase calculated over the total cell number of each sample analyzed. Under normalized culture condition (number of passages, and duration of culture), UC-MSC showed around 2 folds less senescent cells.

Online Figure III. BM and UC-MSCs immunoregulatory response to cytokine activation and expression of immunosuppressive molecules. A) IDO activity measured by kynurenine production. B) PDL-1 and HLA-G expression levels determined by flow cytometry (left), representative cytometry plots (right). C) PGE2, TGFβ1 and IL6 expression levels measured by ELISA. IDO activity were quantified in the supernatants of MSCs cultured in the absence (control group) or presence of 20 ng/ml IFNγ and 10ng/ml IL1β or 100 ng/ml of IFNγ. PDL-1, PGE2, TGF-β1 and IL6 expression levels were quantified on MSCs cultured in the absence (control group) or presence of 20 ng/ml IFNγ and 10ng/ml IL1β. Results are represented as mean±SEM of three independent experiments using each time three different UC-MSCs and BM-MSC donors. *, $p<.05$; **, $p<.01$; ***, $p<.005$ compared to MSCs control group without proinflammatory cytokines treatment. #, $p<.05$; ##, $p<.01$, UC-MSCs compared to BM-MSCs. Abbreviations: UC-MSCs, umbilical cord-derived mesenchymal stem cells; BM-MSCs, bone marrow-derived mesenchymal stem cells; IL, interleukin; IFN, interferon; IDO, indoleamine 2,3 dioxygenase; PDL-1, programmed cell death-ligand; HLA-G, histocompatibility antigen, class I, G**;** MFI, mean fluorescence intensity.

Online TABLES

Online Table I. Electrophysiological assessment of arrhythmogenic potential.

Variable	Group	N	Baseline	3 months	6 months	12 months
QTC	**Placebo**	14	453.9±32.1	442.1±30.6	446.6±36.4	449.4±29.7
	UC-MSC	14	437.5±43.5	449.8±27.3	449.5±36.3	440.9±30.0
Lown class	**Placebo**	14	2.57±1.50	2.36±1.50	1.85±1.46	2.14±1.46
	UC-MSC	14	1.43±1.16*	1.71±1.27	1.57±1.22	1.50±1.09
PVC	**Placebo**	14	550±1090	904±1535‡	1526±3657	1875±3052†
	UC-MSC	14	385±970	394±669	1294±1955	967±2100
LP §	**Placebo**	9	0.67±0.86	0.78±0.83	0.22±0.67	0.44±0.88
	UC-MSC	10	1.20±1.13	0.70±0.95	0.80±0.92	0.30±0.67†
SDNN	**Placebo**	14	35.35±17.53	36.96±20.21	39.43±31.19	43.94±27.89
	UC-MSC	14	52.89±52.06	42.29±47.91‡	49.43±60.84	43.25±49.52
RMSSD	**Placebo**	14	28.10±21.50	34.06±23.48	30.21±20.13	34.06±24.03
	UC-MSC	14	62.85±99.90	43.89±71.04	58.58±88.72	65.70±97.69
HRVTI	**Placebo**	14	11.76±4.86	9.54±3.82†	12.12±6.78	11.34±5.18
	UC-MSC	14	11.97±6.53	11.04±7.08	10.77±8.92	20.45±36.22
LF	**Placebo**	14	103.3±47.6	89.51±41.17	97.26±78.28	95.40±54.58
	UC-MSC	14	111.2±34.3	111.0±46.6	108.65±64.08	99.12±36.51
HF	**Placebo**	14	104.7±52.5	126.0±80.6	95.6±60.3	109.2±78.7
	UC-MSC	14	160.9±88.4	138.7±87.1	167.9±103.2	149.1±99.6
LF/HF ratio	**Placebo**	14	1.32±1.12	2.04±3.75	2.09±3.77	1.32±1.16
	UC-MSC	14	1.24±1.52	1.51±1.86	1.03±0.82	1.04±0.77

QTC: Corrected QT segment according to Bazett's formula. PVC: Total number of premature ventricular contractions in 24-hour ECG Holter test. LP: Number of late potentials criteria according to signal averaged ECG. SDNN: Standard deviation of NN intervals. RMSSD: Root mean square of successive differences. HRVTI: Heart Rate Variability Triangular Index. LF: Low Frequency ranges. HF: High Frequency ranges. *$p<0.05$ versus placebo. †$p<0.05$ versus baseline. ‡$p<0.0167$ versus baseline. § Patients with bundle branch block or atrial fibrillation were excluded from this analysis.

Online Table II. Additional parameters of exercise capacity assessed at cardiopulmonary test.

Variable	Group	N	Baseline	3 months	6 months	12 months
METS	**Placebo**	14	5.01±1.43	4.76±1.47	5.10±1.40	5.35±1.46
	UC-MSC	14	5.17±1.34	5.40±1.13	5.31±1.38	5.11±1.18
VT	**Placebo**	14	12.42±3.29	12.44±4.00	12.45±2.88	13.52±3.02
	UC-MSC	14	12.32±3.43	13.60±5.36	13.30±5.99	13.52±5.49
RER	**Placebo**	14	1.22±0.20	1.13±0.09†	1.16±0.09	1.13±0.10
	UC-MSC	14	1.13±0.10	1,13±0.09	1.09±0.08	1.09±0.09
Exercise time (min)	**Placebo**	14	6:41±1:24	6:17±1:52	6:18±1:35	7:03±2:11
	UC-MSC	14	6:51±2:40	6:35±2:05	6:48±2:23	6:46±2:15

VT: VO2 at anaerobic threshold (ml/Kg/min). RER; peak respiratory exchange ratio. *p<0.05 versus placebo. †p<0.05 versus baseline.

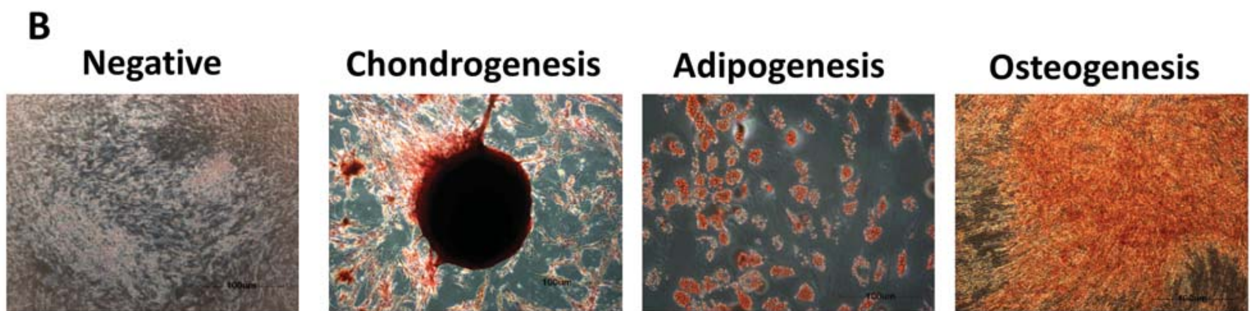
ONLINE SUPPLEMENT REFERENCES

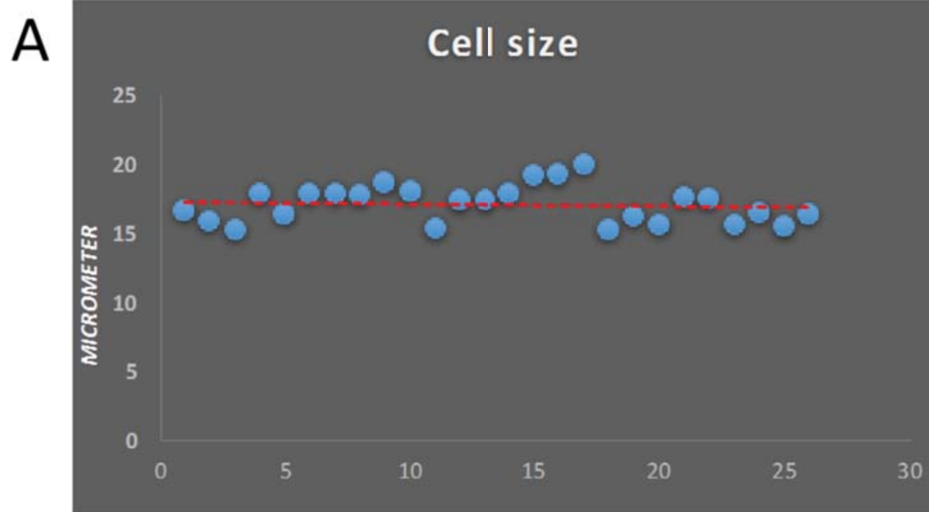
1. Däubener W, Hucke C, Seidel K, Hadding U, MacKenzie CR. Interleukin-1 inhibits gamma interferon-induced bacteriostasis in human uroepithelial cells. *Infect Immun*. 1999;67:5615–20.
2. Luz-Crawford P, Torres MJ, Noël D, Fernandez A, Toupet K, Alcayaga-Miranda F, Tejedor G, Jorgensen C, Illanes SE, Figueroa FE, Djouad F, Khoury M. The immunosuppressive signature of menstrual blood mesenchymal stem cells entails opposite effects on experimental arthritis and graft versus host diseases. *Stem Cells*. 2016;34:456–69.
3. Alcayaga-Miranda F, Cuenca J, Martin A, Contreras L, Figueroa FE, Khoury M. Combination therapy of menstrual derived mesenchymal stem cells and antibiotics ameliorates survival in sepsis. *Stem Cell Res Ther*. 2015;6:199.

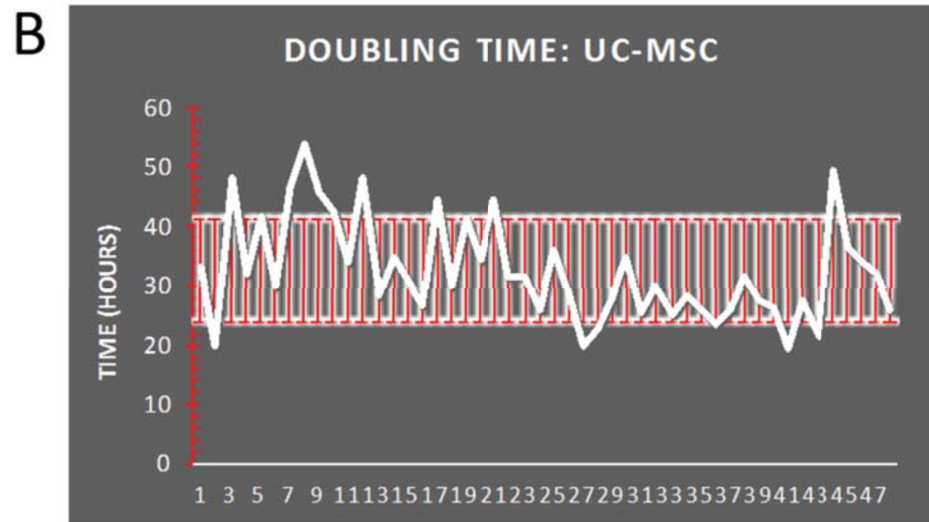
A

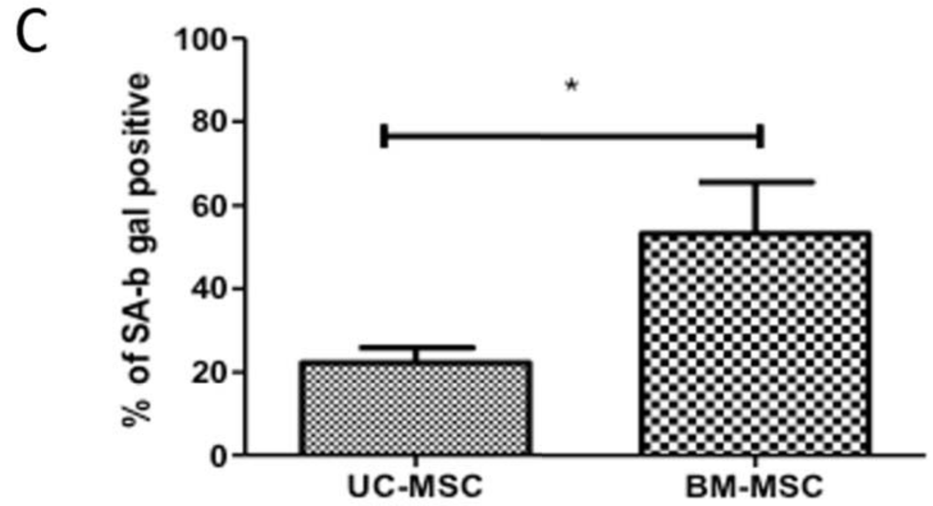
Marker	Fluorochrome	% positive
CD105	PE	99,8%
CD73	PECy7	99,8%
CD90	PECy7	99,9%
CD44	FITC	98%
CD146	PECy7	99,8%
HLA-ABC	APC	99,8%
CD49a	PE	98%
CD45	PE-Cy7	0.023 %
CD34	APC	0.169%
CD14	PE	0.431%
HLA-DR	FITC	0.072%
CD31	FITC	0.0%

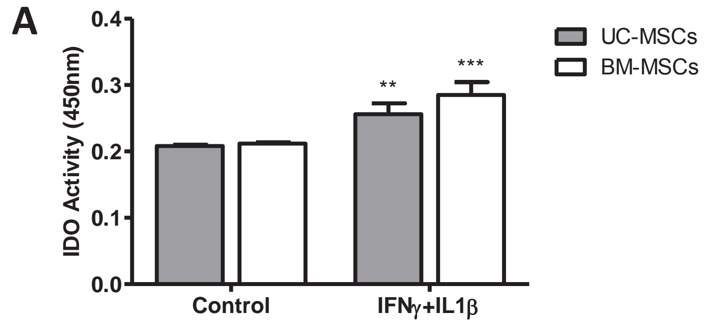
0 10^2 10^3 10^4 10^5

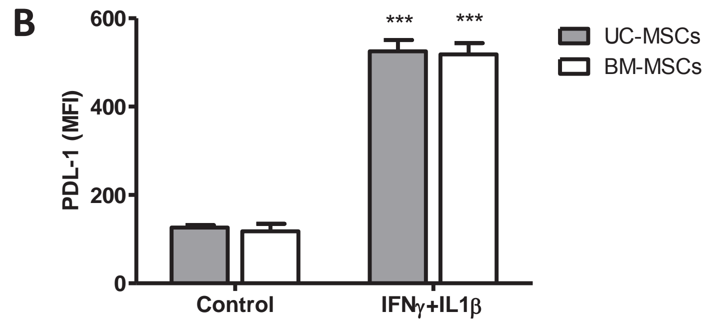


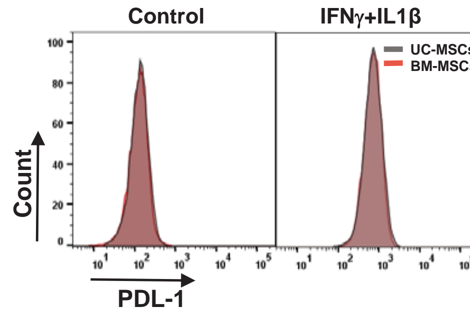

A
Cell size
MICROMETER
25
20
15
10
5
0
0
5
10
15
20
25
30

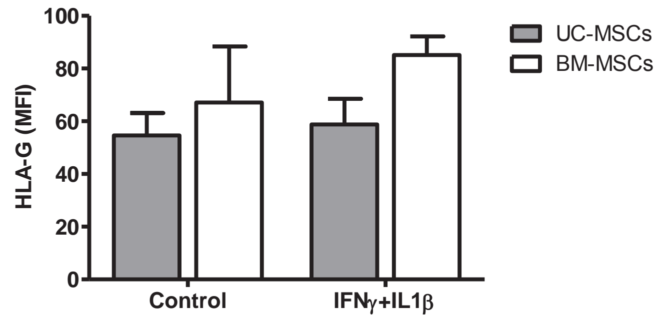

B
DOUBLING TIME: UC-MSC
TIME (HOURS)
60
50
40
30
20
10
0
1 3 5 7 9 11131517192123252729313335373941434547

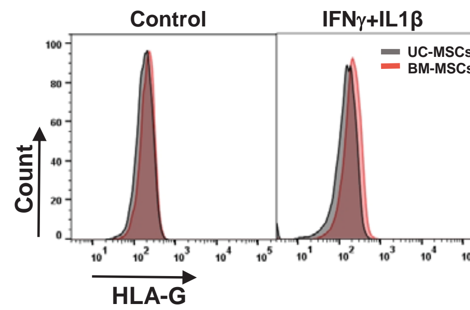

C
% of SA-b gal positive
100
80
60
40
20
0
*
UC-MSC
BM-MSC

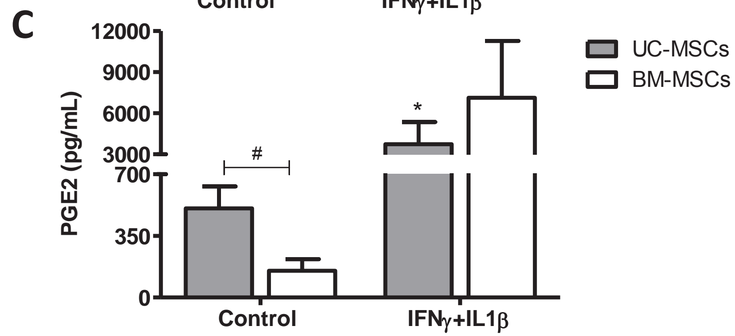

A
UC-MSCs
BM-MSCs
IDO Activity (450nm)
0.4
0.3
0.2
0.1
0.0
**
***
Control
IFNγ+IL1β

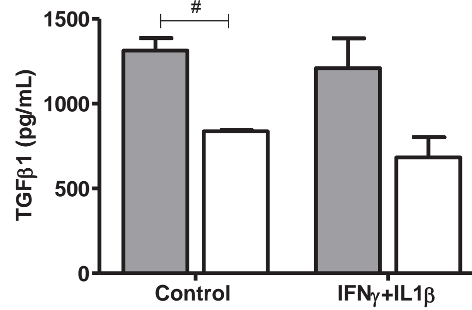

B
UC-MSCs
BM-MSCs
PDL-1 (MFI)
600
400
200
0
***
***
Control
IFNγ+IL1β

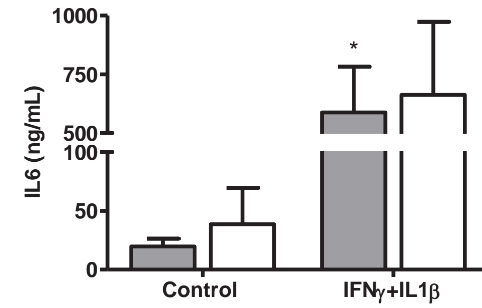

Control
IFNγ+IL1β
UC-MSCs
BM-MSCs
Count
PDL-1


UC-MSCs
BM-MSCs
HLA-G (MFI)
100
80
60
40
20
0
Control
IFNγ+IL1β


Control
IFNγ+IL1β
UC-MSCs
BM-MSCs
Count
HLA-G


C
UC-MSCs
BM-MSCs
PGE2 (pg/mL)
12000
9000
6000
3000
700
350
0
#
*
Control
IFNγ+IL1β


TGFβ1 (pg/mL)
1500
1000
500
0
#
Control
IFNγ+IL1β


IL6 (ng/mL)
1000
750
500
100
50
0
*
Control
IFNγ+IL1β