The handle [http://hdl.handle.net/1887/38594](http://hdl.handle.net/1887/38594) holds various files of this Leiden University dissertation

**Author:** Haan, Melina C. den  
**Title:** Cell therapy in ischemic heart disease models: role of inflammation, paracrine factors and hypercholesterolemia  
**Issue Date:** 2016-03-23
Discrepant results of experimental human mesenchymal stromal cell therapy after myocardial infarction: are animal models robust enough?

Melina C. den Haan, MD;¹,*
Vanessa-Leigh van Zuylen, MSc, MD;¹,²,*
Niek J. Pluijmert, MD;¹, Cindy I. Schutte, BSc;¹,
Willem E. Fibbe, MD, PhD;², Martin J. Schalij, MD, PhD;¹,
Helene Roelofs, PhD; ², Douwe E. Atsma, MD, PhD;¹,*

¹Department of Cardiology, Leiden University Medical Center, Leiden, the Netherlands, ²Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands
# Both authors contributed equally to this study

Submitted for publication
Abstract

Background: Human mesenchymal stromal cells (MSCs) have been reported to preserve cardiac function in myocardial infarction (MI) models. Previously, we found a beneficial effect of intramyocardial injection of unstimulated human MSCs (uMSCs) on cardiac function after permanent coronary artery ligation. In the present study we aimed to extend this research by investigating the effect of intramyocardial injection of human MSCs pre-stimulated with the pro-inflammatory cytokine interferon-gamma (iMSCs), since pro-inflammatory priming has shown additional salutary effects in multiple experimental disease models.

Methods: MI was induced in NOD/Scid mice by permanent ligation of the left anterior descending coronary artery. Animals received intramyocardial injection of uMSCs, iMSCs or phosphate-buffered saline. Sham-operated animals were used to determine baseline characteristics. Cardiac performance was assessed after 2 and 14 days using 7-Tesla magnetic resonance imaging and pressure-volume loop measurements. Histology and q-PCR were used to confirm MSC engraftment in the heart.

Results: Both uMSC and iMSC therapy had no significant beneficial effect on cardiac function or remodeling in contrast to our previous studies.

Conclusions: Animal models for cardiac MSC therapy appear less robust than initially envisioned.
Introduction

Cardiovascular disease (CVD) is the main mortality cause in the western world (1). Over the past decade, studies have explored the potential of cell therapy as a novel treatment option for coronary artery disease. Various cell types including bone marrow-derived mononuclear cells (2), human mesenchymal stromal cells (MSCs) (3-5), embryonic stem cells (6), induced pluripotent stem cells (7), skeletal myoblasts (8) and cardiac progenitor cells (9) have been studied, showing varying results in both animal models and in patients (10-12). MSCs constitute an attractive therapeutic cell type in view of their immunomodulatory and anti-inflammatory properties, easy expandability and their ability to support tissue regeneration (13, 14). The reported improvement in heart function by MSC administration seems mostly attributed to paracrine mechanisms (15), leading to neoangiogenesis (16), anti-apoptotic effects (17) and attenuation of the ventricular remodelling process (18). Previously, we reported beneficial therapeutic effects of injection of unstimulated human MSCs (uMSCs) from CVD patients in a myocardial infarction (MI) model in NOD/Scid mice (4, 19). In the present study we aimed to extend this research by assessing the effect of pre-stimulating the human MSCs (iMSCs) with the pro-inflammatory cytokine interferon gamma (IFN).

Recent studies in animal models of graft–versus-host disease (GVHD) and colitis indicated that pre-stimulation of MSCs with IFN enhances their therapeutic effect (20, 21). The mechanism of this reported therapeutic benefit is not known but the role of immunomodulatory proteins that are upregulated by IFN including indoleamine 2,3 dioxygenase (IDO) and inducible nitrix oxide synthase, is currently being investigated (20-25). We hypothesized that IFN pre-stimulation of MSC also enhances their beneficial effect on cardiac function in a NOD/Scid model of MI. Also, as MI causes the influx of inflammatory cells into the injured myocardium (26), we investigated whether the inflammatory cell influx was altered by uMCs and iMSCs administration.

Materials and Methods

Details about the materials and methods are described in the Supplemental Materials and Methods at the end of this chapter.

Animals. All experiments were approved by the Committee on Animal Welfare of the Leiden University Medical Center (LUMC) and conformed to the Guide for the Care and Use of Laboratory Animals as stated by the U.S. National Institutes of Health. To avoid rejection of transplanted human cells, 8- to 10-weeks-old male NOD/Scid mice (Charles River Laboratories, Maastricht, the Netherlands) were used.

Primary cultured MSCs. Bone marrow aspiration procedures were performed in accordance with the Helsinki Declaration and were approved by the ethics committee
All MSC donors provided informed consent. Bone marrow-derived MSCs were obtained from three non-cardiac patients undergoing orthopedic surgery. IFN stimulation of MSCs was performed by adding 500U/ml IFN (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) to the culture medium for 7 days.

Immunophenotyping of cultured MSC was performed using the following primary antibodies: CD90, CD73, MHC-I, CD34, CD45, CD31, CD80, MHC-II (BD Biosciences, San Diego, USA), and CD105 (Ancell Corp., Bayport, MN, USA). MSCs from passages 4 to 5 were used for transplantation experiments after lentiviral transduction with a human vector expressing the enhanced green fluorescent protein (eGFP) gene. The cells transduced with lentivirus for eGFP, transmitted the eGFP signal in the FITC channel of the FACSCanto II (BD Biosciences, San Diego, CA, USA).

In vitro differentiation. To test the ability of uMSCs and iMSCs to differentiate into osteogenic and adipogenic lineages, cells were incubated in appropriate differentiation media. MSCs were stained for alkaline phosphatase activity with Fast Blue (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) and for calcium deposition with Alizarine Red (MP Biomedicals LLC, Illkirch Cedex, France). Formation of lipid droplets was visualized with Oil-red O staining (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands).

Suppression of PBMC proliferation by MSCs. Cultured MSCs were plated in graded doses in 96-well flat-bottom plates (Corning, Life Sciences). Human peripheral blood mononuclear cells (PBMC) isolated from buffy coats (1.0 x 10^5/well) were added to the MSCs and stimulated with human T-activator CD3/CD28 dynabeads (Invitrogen Corp., Paisley, UK) in a bead:cell ratio 1:5. The cultures were harvested on a glass fiber filter and thymidine incorporation was measured with a liquid scintillation counter (Wallac, Turku, Finland).

MI induction and Cell injection. Mice received buprenorphine subcutaneously before surgery and again 12 hours after surgery. MI was induced as described previously (9). Briefly, animals were anesthetized with 5% isoflurane for induction, subsequently intubated and kept anesthetized with 1.5-2% isoflurane in oxygen for the remainder of the surgical procedure. After a left thoracotomy, the left anterior descending (LAD) coronary artery was ligated 1 mm caudally from the tip of the left auricle using a 7-0-prolene suture (Johnson and Johnson, New Brunswick, NJ, USA).

Five minutes after LAD ligation the animals received either 2 x 10^5 uMSCs in 15 μL phosphate-buffered saline (PBS) (uMSC group), 2 x 10^5 iMSCs in 15 μL PBS (iMSC group) or 15 μL PBS containing no cells (PBS group). Intramyocardial injections were performed at 3 sites in the infarcted area (5 μL per site).

Sham-operated animals were operated in parallel to determine baseline characteristics (Sham group).

Cardiac Magnetic Resonance Imaging (MRI). Cardiac parameters were assessed 2 and 14 days post-MI using a 7-Tesla MRI (BrukerBiospin, Ettlingen, Germany). Mice were
pre-anesthetized as described above and kept anesthetized with 1.5-2% isoflurane in oxygen for the remainder of the procedure. All data were analysed with the MASS for Mice software package (Leiden, the Netherlands). The endocardial and epicardial borders were delineated manually (uMSC group n=12, iMSC group n=7, PBS group n=7, Sham group n=10), after which left ventricular (LV) end-diastolic volume (EDV), LV end-systolic volume (ESV) and ejection fraction (EF) were computed.

**Pressure-Volume (PV) measurements.** Fifteen days after MI, mice were anesthetized again as described above and kept anesthetized with 1-1.5% isoflurane in oxygen for the remainder of the procedure. A 1.2F pressure-conductance catheter (ScisenseInc, London, Canada) was introduced via the right carotid artery and positioned in the LV. The conductance catheter was connected to a PV control unit FV 896B (ScisenseInc, London, Canada). Parallel conductance and LV pressure-volume signals were measured as described previously (9). All data were acquired using Powerlab 8/30 Model ML870 (ADInstruments, Spechbach, Germany) and LabChart 7 software (ADInstruments, Spechbach, Germany). Data were analyzed off-line (uMSC group n=7, iMSC group n=7, PBS group n=5, Sham group n=7).

**Histology.** At day 15 post-MI, mice were weighed, sacrificed after PV loop measurements and hearts and lungs were removed. Lungs were weighed immediately after excision, freeze-dried for 24 hours and then weighed again. The wet weight/dry weight ratio was used as a measure of pulmonary congestion.

Per group 5 hearts were fixed by immersion in buffered 4% paraformaldehyde and embedded in paraffin. Serial transverse sections of 5 μm were cut along the entire long axis of the LV for (immuno)histological analyses. MSC engraftment was detected by immunostaining with a rabbit anti-GFP antibody (A11122, Invitrogen, Paisley, UK), followed by a biotinylated goat anti-rabbit IgG (E0432, Dako, Glostrup, Denmark) and a Qdot 655 streptavidin-conjugated (Q10121MP, Invitrogen, Paisley, UK) antibody.

**Real-time Polymerase Chain Reaction (PCR).** Per group, 5 hearts were used for DNA extraction to determine MSC engraftment rate by quantification of human genomic DNA in mouse hearts (27). DNA concentrations were measured using NanoDrop 1000 (NanoDrop products, Wilmington, DE, USA).

PCR reactions were performed in a volume of 10 μL, containing 5 μL Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 900 nM forward and reverse primers, 250 nM TaqManprobe and 50 ng of target template. Standard curves were generated by serially diluting human genomic DNA (Roche, Basel, Switzerland) in murine genomic DNA.

**Flow cytometry.** From each treatment group, mice not subjected to functional and histological analysis were used for flow cytometric analysis of cardiac inflammatory cell
invasion. Mice were sacrificed on days 1, 3, and 7 after MI. Each time point, 3 hearts were harvested per group. Sham animals were used as controls to determine base line characteristics. Total cardiac cell numbers were determined with a Sysmex cell counter (Sysmex America, Inc. Mundelein, Illinois, US). Single-cell suspensions were stained for flow cytometry with primary antibodies before analysis using a FACSCanto II (BD Biosciences, San Diego, CA, USA). The following antibodies were used: anti–CD90-APC, 53–2.1,–B220-APC, RA3-6B2, –CD49b-APC, DX5, –NK1.1-APC, PK136, –Ly-6G-APC, 1A8, CD11b-eFluor 450, M1/70,–CD11c-FITC, HL3, – I-A^b -FITC, AF6-120.1, –Ly-6C-PE, AL-21, –CD11c-PE, HL3 (all above antibodies are from BD Biosciences), –F4/80-FITC, CI:A3-1 (ABD Serotec, Kidlington, UK). Monocytes were identified as CD11b high (CD90/ B220/CD49b/NK1.1/Ly-6G) low (F4/80/I-A^b/CD11c) low Ly-6C high/low as previously described (26, 28). Macrophages were identified as CD11b high, F4/80 high. Dendritic cells were identified as CD11b, I-A^b and CD11c high. Neutrophils were identified as CD11b, Ly-6G high. The analysis of the acquired data was done with FlowJo software version 7.6.1 (Tree Star Inc. Ashland, OR, USA).

**Statistical Analysis.** Numerical values were expressed as means ± standard deviation (SD). Comparison of MRI parameters and inflammatory cells between the uMSC, iMSC, PBS and Sham groups was performed using two-way repeated-measures analysis of variance (ANOVA), with Bonferroni correction. Comparison of the remaining parameters was performed using one-way ANOVA with Bonferroni correction.

**Results**

**Characterization of MSCs.** The uMSCs in vitro expressed the established surface marker profile: CD90+, CD73+, MHC-I+, CD105+, and CD45-, CD34-, CD80- (Figure 1A). In addition, all (u/i)MSCs were able to differentiate into osteoblasts and adipocytes (Figure 1B) and demonstrated an inhibitory capacity on PBMC proliferation (Figure 1C), confirming the definition and behavior of true MSCs.

The transduction efficiency of the (u/i)MSCs used was 100% which was analyzed by bright light microscopy and flow cytometry (Figure 1D).

After IFN stimulation, iMSCs expressed MHC II (Figure 2A), while the other expressed surface markers were comparable to uMSC (data not shown). The in vitro differentiation capacity toward osteoblasts and adipocytes was similar between iMSCs and uMSCs for all MSC sources used (data not shown). The IFN stimulation upregulated the expression of the intracellular enzyme indoleamine 2,3dioxygenase (IDO) as shown by immunohistochemistry (Figure 2B).

**Effect of MSC therapy on cardiac function.** Two and 14 days after inducing MI, LVEF decreased in all MI groups when compared to Sham as assessed by MRI (Figure 3A). The EDV and ESV were significantly increased 14 days after MI in all MI animals.
Figure 1. Characterization of primary cultured MSCs. A: In vitro characterization of MSCs consisting of the specific surface marker antigen panel measured by flow cytometry. B: The differentiation capacity of MSCs towards osteoblasts shown by alkaline phosphatase activity and towards adipocytes shown by lipid droplets staining via Oil Red O. C: The inhibitory capacity of proliferation of activated peripheral blood mononuclear cells measured by 3H-thymidine uptake in counts per minute (CCPM). D: Bright light and fluorescence microscopy and flow cytometric analysis of eGFP labeling of lentivirally transduced MSCs.
Figure 2. Stimulation of MSCs with the pro-inflammatory cytokine interferon gamma (IFN). A: From left to right: Immunostaining of iMSCs for HLA-DR in red (PE) and nuclei in blue (DAPI) and expression of HLA-DR as measured by flow cytometry. B: Immunostaining of iMSCs for the immunomodulatory enzyme IDO in green (ALEXA 488 nm) and nuclei in blue (DAPI).

(Figure 3B and C). There were no differences between the uMSC group, the iMSC group or the PBS group for either EF, EDV or ESV at 2 or 14 days post MI.

These findings were confirmed by PV loop measurements 15 days after MI. LV EDV and ESV were significantly increased in all animals subjected to MI when compared to Sham. End-systolic pressure was significantly decreased in all MI groups, while the end-diastolic pressure was comparable for all four groups (Figure 3D). There were no differences between the uMSC, iMSC and the PBS group, indicating no beneficial effect of the uMSC or iMSC treatment on cardiac function.
Figure 3. Cardiac function as assessed by 7 T MRI. Cardiac function 2 and 14 days after myocardial infarction in uMSC and iMSC treated animals (uMSC and iMSC, resp.), PBS treated animals (PBS) and Sham-operated animals (Sham) (panel A-C). A: Left ventricular ejection fraction. B: End-diastolic volume. C: End-systolic volume. Data are expressed as mean ± SD. # = p<0.05 versus Sham. D: Pressure-Volume loops of all treatment groups at day 15 after MI. The oblique lines represent the end-systolic (Ees) and end-diastolic (Eed) pressure-volume relations.
Engraftment. Histological analysis showed that both uMSCs and iMSCs engrafted into the myocardium at 15 days after injection, predominantly in the infarcted anterolateral wall (Figure 4A). Quantitative assessment of histological sections revealed similar engraftment rates for the two cell groups (3.2% and 2.8% of injected cells for uMSC and iMSC, respectively, Figure 4B). In addition, we performed q-PCR for human ALU repeats to determine cell engraftment of the human MSCs in the mouse hearts. q-PCR showed a small, but not significantly different, percentage of human genomic DNA in both uMSC and iMSC treated animals (Figure 4C). Tissue from mice hearts injected with PBS and Sham-operated animals were used as control.

**Figure 4.** Assessment of engraftment of injected MSCs in mouse hearts 15 days after injection into the infarcted myocardium. A: Immunofluorescent staining of engrafted eGFP-labeled MSCs in red (Qdot 655) and nuclei in blue (hoechst 33342). B: Histological quantification of uMSC and iMSC engraftment (uMSC and iMSC resp.). PBS treated animals (PBS) and Sham-operated animals (Sham) were used as controls. C: Quantitative PCR for human genomic DNA in mouse hearts in animals treated with uMSC, iMSC, PBS and sham-operated animals. Data are expressed as mean ± SD. There were no significant differences between the uMSC and iMSC group.
**Pulmonary congestion and body weight.** Body weight was similar in all animals prior to surgery and did not significantly change between MI groups and the Sham-operated animals at day 15 post MI (Figure 5A). In addition, no significant differences were observed in the amount of lung fluid between all MI groups and the Sham group (Figure 5B).

![Figure 5.](image)

**Figure 5.** Physical parameters 15 days after myocardial infarction. Parameters in uMSC and iMSC treated animals (uMSC and iMSC, resp.), PBS treated animals (PBS) and Sham-operated animals (Sham). A: Weight loss. B: Amount of pulmonary fluid. Data are expressed as mean ± SD. There were no significant differences between the uMSC, iMSC, PBS and Sham group.

**Inflammatory cell influx in the infarcted heart.** In the MI groups the frequency of inflammatory cells in the heart did not significantly deviate from the Sham group. However, a trend of increased influx of neutrophils and non-inflammatory monocytes was found and a decreased presence of inflammatory monocytes, macrophages and dendritic cells on the first day after MI. This was not significantly different when comparing all MI groups to Sham animals. Most importantly, no difference between the PBS group and either of the 2 MSC treatment groups or between the groups themselves could be shown (Figure 6).

**Discussion**

The main findings of this study are: (1) stimulation of human bone marrow-derived MSCs with IFN does not alter their differentiation capacity; (2) uMSCs and iMSCs engraft in infarct myocardium and (3) neither treatment with uMSC nor iMSCs improves cardiac function after MI. Although we hereby confirmed the engraftment rate of our earlier findings, we now had discordant results with regard to cardiac function (4, 19).

In an attempt to explain why we could not reproduce our previous results, we compared the two study designs. For both studies, 8-10 week-old male NOD/Scid mice
Figure 6. Flow cytometric analysis of inflammatory cells in the heart 15 days after myocardial infarction. Analysis in uMSC and iMSC treated animals (uMSC and iMSC, resp.), PBS treated animals (PBS) and Sham-operated animals (Sham). Data are expressed as mean ± SD. There were no significant differences between the uMSC, iMSC, PBS and Sham group.

from Charles River Labs (Maastricht, NL) were used. Although physically at a different location, the mice were housed under identical conditions. The source of the MSCs used in the present study (orthopaedic surgery patients without known cardiac disease) differed from the MSCs we used previously (ischemic heart disease patients). However, the phenotypic (in vitro surface marker expression profile) and functional (osteogenic and adipogenic differentiation and immunomodulatory capacity) characteristics of the MSCs used in both studies were comparable. Non-cardiac patient derived-MSCs have also been tested in other experimental disease mouse models in which they showed therapeutic efficacy (20). The presence of injected MSC in the cardiac tissue was confirmed in both studies 2 weeks after MI by histology and human ALU repeats-PCR. Also, the levels of MSC engraftment were comparable (3.2% in the current study vs 4.1% in our previous study (4)).

The operating procedures were performed by different, although thoroughly trained and experienced operators. Baseline EF, EDV and ESV 2 days after MI were comparable in the two studies, indicating similar myocardial damage and loss of cardiac function. In the current study we used a 7-Tesla MRI, while in our previous study a 9.4-Tesla MRI was used and measurements were performed by a different, although experienced operator. However, studies about inter-operator differences report of excellent reproducibility of the cardiovascular MRI measurements of the LV volumes and mass (29, 30).
All together, we have encountered unexplained discrepant therapeutic efficacy of MSCs in the permanent ligation MI mouse model. The experimental conditions were overall the same, suggesting that this discrepancy is related to details that are not recognized as potential key parameters. Therefore, questions arise concerning the robustness of these intricate animal models to assess the efficacy of cell therapy using human cells.

Several other studies, both positive and negative, have been performed investigating the therapeutic potential of MSC infusion in cardiac disease models. These studies showed therapeutic benefit on cardiac function mostly via echocardiography or MRI and whilst the experimental protocols seem similar between studies, large variations exist between the reported parameters that were beneficially influenced by MSC infusion, which hampers a direct comparison between studies (5, 18, 31-36).

In contrast, debate exists about the therapeutic efficacy of MSC therapy in various experimental cardiac disease models, fuelled by a growing number of negative studies on MSC therapy (12, 17, 37-41). In a direct comparison between intramyocardial injection of various adult cell types in a mouse model of MI, using bone marrow-derived MSCs, bone marrow-derived mononuclear cells, skeletal myoblasts and fibroblasts, the MSCs did not show any beneficial effect on the preservation of cardiac function while the bone marrow-derived mononuclear cells did (12). Another study by van der Bogt et al confirmed this result in a comparable study of adipose tissue-derived MSC and bone marrow-derived MSCs where neither cell type was able to preserve cardiac function (37).

Our current results indicate that the reported therapeutic effects of MSCs may be easily obscured by unknown study parameters. This poses a major limitation on the use of animal models in further development of cell therapy for cardiac diseases.

**Conclusion**

Both uMSC and iMSC therapy have no significant beneficial effect on cardiac function or remodelling in a NOD/Scid mouse model of MI, in contrast to our previous studies. Animal models for cardiac MSC therapy appear less robust than initially envisioned.

**Funding.** This work was supported by the SmartCare project of the research program of the BioMedical Materials Program, co-funded by the Dutch Ministry of Economic Affairs.

The authors gratefully acknowledge the support of the TeRM SmartMix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science.

**Disclosure of conflicts of interest.** The authors confirm that there are no conflicts of interest.
Reference List

1. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, et al. Heart Disease and Stroke Statistics--2012 Update: A Report From the American Heart Association. Circulation 2012;125:e2-e220.

2. Nygren JM, Jovinge S, Breitbach M, Sawen P, Roll W, Hescheler J, et al. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med 2004;10:494-501.

3. Shake JG, Gruber PJ, Baumgartner WA, Senechal G, Meyers J, Redmond JM, et al. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. Ann Thorac Surg 2002;73:1919-25.

4. Grauss RW, Winter EM, van TJ, Pijnappels DA, Steijn RV, Rogers B, et al. Mesenchymal stem cells from ischemic heart disease patients improve left ventricular function after acute myocardial infarction. Am J Physiol Heart Circ Physiol 2007;293:H2438-H2447.

5. Dixon JA, Gorman RC, Stroud RE, Bouges S, Hirotsugu H, Gorman JH, III, et al. Mesenchymal cell transplantation and myocardial remodeling after myocardial infarction. Circulation 2009;120:S220-S229.

6. Leor J, Gerecht S, Cohen S, Miller L, Holbova R, Ziskind A, et al. Human embryonic stem cell transplantation to repair the infarcted myocardium. Heart 2007;93:1278-84.

7. Nelson TJ, Martinez-Fernandez A, Yamada S, Perez-Terzic C, Ikeda Y, Terzic A. Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. Circulation 2009;120:408-16.

8. Taylor DA, Atkins BZ, Hunspreugs P, Jones TR, Reedy MC, Hutcheson KA, et al. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. Nat Med 1998;4:929-33.

9. den Haan MC, Grauss RW, Smits AM, Winter EM, van TJ, Pijnappels DA, et al. Cardiomyogenic differentiation-independent improvement of cardiac function by human cardiomyocyte progenitor cell injection in ischemic mouse hearts. J Cell Mol Med 2012;16:1508-21.

10. van Ramshorst J, Bax JJ, Beeres SL, Dibbets-Schneider P, Roes SD, Stokkel MP, et al. Intramyocardial bone marrow cell injection for chronic myocardial ischemia: a randomized controlled trial. JAMA 2009;301:1997-2004.

11. Krause K, Jaquet K, Schneider C, Haupt S, Lioznov MV, Otte KM, et al. Percutaneous intramyocardial stem cell injection in patients with acute myocardial infarction: first-in-man study. Heart 2009;95:1145-52.

12. van der Bogt KE, Sheikh AY, Schrepfer S, Hoyt G, Cao F, Ransohoff KJ, et al. Comparison of different adult stem cell types for treatment of myocardial ischemia. Circulation 2008;118:S121-S129.

13. Williams AR, Hare JM. Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. Circ Res 2011;109:923-40.

14. van Zuylen VL, den Haan MC, Geutskens SB, Roelofs H, Fibbe WE, Schalij MJ, et al. Postmyocardial Infarct Inflammation and the Potential Role of Cell Therapy. Cardiovasc Drugs Ther 2015;29:59-73.

15. Gnecci M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 2008;103:1204-19.

16. Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 2004;94:678-85.

17. Uemura R, Xu M, Ahmad N, Ashraf M. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. Circ Res 2006;98:1414-21.
18. Arminan A, Gandia C, Garcia-Verdugo JM, Lledo E, Trigueros C, Ruiz-Sauri A, et al. Mesenchymal stem cells provide better results than hematopoietic precursors for the treatment of myocardial infarction. J Am Coll Cardiol 2010;55:2244-53.

19. Grauss RW, van TJ, Steendijk P, Winter EM, Pijnappels DA, Hogers B, et al. Forced myocardin expression enhances the therapeutic effect of human mesenchymal stem cells after transplantation in ischemic mouse hearts. Stem Cells 2008;26:1083-93.

20. Duijvestein M, Wildenberg ME, Welling MM, Hennink S, Molendijk I, van Zuylen VL, et al. Pretreatment with interferon-gamma enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis. Stem Cells 2011;29:1549-58.

21. Polchert D, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E, et al. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. Eur J Immunol 2008;38:1745-55.

22. Croitoru-Lamoury J, Lamoury FM, Caristo M, Suzuki K, Walker D, Takikawa O, et al. Interferon-gamma regulates the proliferation and differentiation of mesenchymal stem cells via activation of indoleamine 2,3-dioxygenase (IDO). PLoS One 2011;6:e14698.

23. Delarosa O, Lombardo E, Beraza A, Manchano-Corvo P, Ramirez C, Menta R, et al. Requirement of IFN-gamma-mediated indoleamine 2,3-dioxygenase expression in the modulation of lymphocyte proliferation by human adipose-derived stem cells. Tissue Eng Part A 2009;15:2795-806.

24. Francois M, Galipeau J. New insights on translational development of mesenchymal stromal cells for suppressor therapy. J Cell Physiol 2012;227:3535-8.

25. Hemeda H, Jakob M, Ludwig AK, Giebel B, Lang S, Brandau S. Interferon-gamma and tumor necrosis factor-alpha differentially affect cytokine expression and migration properties of mesenchymal stem cells. Stem Cells Dev 2010;19:693-706.

26. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med 2007;204:3037-47.

27. McBride C, Gaupp D, Phinney DG. Quantifying levels of transplanted murine and human mesenchymal stem cells in vivo by real-time PCR. Cytotherapy 2003;5:7-18.

28. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. Science 2009;325:612-6.

29. Bollache E, Redheuil A, Clement-Guinaudeau S, Defrance C, Perdrix L, Ladouceur M, et al. Automated left ventricular diastolic function evaluation from phase-contrast cardiovascular magnetic resonance and comparison with Doppler echocardiography. J Cardiovasc Magn Reson 2010;12:63.

30. Schuleri KH, Feigenbaum GS, Centola M, Weiss ES, Zimmet JM, Turney J, et al. Autologous mesenchymal stem cells produce reverse remodelling in chronic ischaemic cardiomyopathy. Eur Heart J 2009;30:2722-32.

31. Silva GV, Litovsky S, Assad JA, Sousa AL, Martin BJ, Vela D, et al. Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. Circulation 2005;111:150-6.

32. Huang XP, Sun Z, Miyagi Y, McDonald KH, Zhang L, Weisel RD, et al. Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. Circulation 2010;122:2419-29.

33. Behfar A, Yamada S, Crespo-Díaz R, Nesbitt JJ, Rowe LA, Perez-Terzic C, et al. Guided cardiopoiesis enhances therapeutic benefit of bone marrow human mesenchymal stem
35. Hassan F, Meduru S, Taguchi K, Kuppusamy ML, Mostafa M, Kuppusamy P, et al. Carvedilol Enhances Mesenchymal Stem Cell Therapy for Myocardial Infarction via Inhibition of Caspase-3 Expression. J Pharmacol Exp Ther 2012;343:62-71.

36. Zuo S, Jones WK, Li H, He Z, Pasha Z, Yang Y, et al. Paracrine effect of Wnt11-overexpressing mesenchymal stem cells on ischemic injury. Stem Cells Dev 2012;21:598-608.

37. van der Bogt KE, Schrepfer S, Yu J, Sheikh AY, Hoyt G, Govaert JA, et al. Comparison of transplantation of adipose tissue- and bone marrow-derived mesenchymal stem cells in the infarcted heart. Transplantation 2009;87:642-52.

38. Loffredo FS, Steinhauser ML, Gannon J, Lee RT. Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair. Cell Stem Cell 2011;8:389-98.

39. Deuse T, Peter C, Fedak PW, Doyle T, Reichenspurner H, Zimmermann WH, et al. Hepatocyte growth factor or vascular endothelial growth factor gene transfer maximizes mesenchymal stem cell-based myocardial salvage after acute myocardial infarction. Circulation 2009;120:S247-S254.

40. Carr CA, Stuckey DJ, Tatton L, Tyler DJ, Hale SJ, Sweeney D, et al. Bone marrow-derived stromal cells home to and remain in the infarcted rat heart but fail to improve function: an in vivo cine-MRI study. Am J Physiol Heart Circ Physiol 2008;295:H533-H542.

41. Flynn AW, Chen X, O’Connell E, O’Brien T. A comparison of the efficacy of transplantation of bone marrow derived mesenchymal stem cells and unrestricted somatic stem cells on outcome after acute myocardial infarction. Stem Cell Res Ther 2012;3:36.
Supplemental Materials and Methods

Animals. All experiments were approved by the Committee on Animal Welfare of the Leiden University Medical Center and conformed to the Guide for the Care and Use of Laboratory Animals as stated by the U.S. National Institutes of Health. To avoid rejection of transplanted human cells, 8- to 10-weeks-old male non-obese diabetic/severe combined immunodeficient (NOD/Scid) mice (Charles River Laboratories, Maastricht, the Netherlands) were used. All animals were housed in filtertop cages and were given standard diet and water with antibiotics and antymycotics ad libitum.

Primary cultured human Mesenchymal Stromal Cells (MSCs). Bone marrow-derived MSCs (unstimulated: uMSCs) were obtained from patients undergoing orthopedic surgery. The bone marrow mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (density: 1.077 g/cm3) and were plated at 1.3 x 10^5/cm^2 in DMEM-low glucose (DMEM-LG; Invitrogen Corp., Paisley, UK) supplemented with 10% fetal calf serum (FCS; Greiner Bio-one) and Penicillin/Streptomycin (P/S; Invitrogen Corp., Paisley, UK). Cultures were grown in 175 cm^2 flasks (Corning Life Sciences B.V., Schiphol-Rijk, The Netherlands) in a 37°C humidified incubator containing 5% CO_2 and the medium was refreshed every 3-4 days. When the spindle shaped MSC monolayer reached >80% confluence, cells were detached using trypsin/EDTA (Invitrogen Corp., Paisley, UK) and replated at a density of 4,000 cells per cm^2. Interferon-gamma (IFN) stimulation of MSCs (iMSCs) was performed by adding 500U/ml IFN (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) to the culture medium for 7 days, refreshing the medium within 3 days.

Immunophenotyping of cultured MSC (both uMSCs and iMSCs) was performed using the following primary antibodies: CD90, CD73, MHC-I, CD34, CD45, CD31, CD80, (BD Biosciences, San Diego, USA), and CD105 (Ancell Corp., Bayport, MN, USA). Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) and the data were analyzed with FlowJo software (version 7.6.3., Tree Star Inc. Ashland, OR, USA). MSCs from passages 4 to 5 were used for transplantation experiments after lentiviral transduction with a human vector expressing the enhanced green fluorescent protein (eGFP) gene which enabled ex vivo cell tracing via immunohistochemistry. The cells transduced with lentivirus for eGFP, transmitted the eGFP signal in the FITC channel of the FACSCanto II (BD Biosciences, San Diego, CA, USA). All sampling procedures were performed in accordance with the Helsinki Declaration and were approved by the ethics committee of Leiden University Medical Center (LUMC). All patients provided informed consent.

In Vitro Differentiation. For osteogenic differentiation uMSCs and iMSCs were grown to 80% confluency in 24-well culture plates and were stimulated for 21 days in osteogenic differentiation medium consisting of α-MEM (Invitrogen Corp., Paisley, UK) with
L-glutamin (200nM, Invitrogen Corp., Paisley, UK), P/S and 10% FCS supplemented with 10^{-7} M dexamethason, 50 μg/ml Vitamin C (both from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), and 5 mM β-glycerophosphate (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and stained for alkaline phosphatase activity with Fast Blue (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and for calcium deposition with Alizarine Red (MP Biomedicals LLC, Illkirch Cedex, France.) For adipogenic differentiation, MSCs were stimulated for 21 days in adipogenic differentiation medium consisting of α-MEM with L-glutamin (200nM, Invitrogen Corp., Paisley, UK), P/S and 10% FCS supplemented with 10^{-7} M dexamethason, insulin (10 μg/ml), indomethacin (5μM) and 3-isobutyl-1-methylxanthine (5μM) (all from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Formation of lipid droplets was visualized with Oil-red O staining (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands).

**Suppression of human peripheral blood mononuclear cell (PBMC) proliferation by MSCs.** cultured human MSCs were plated in graded doses in 96-well flat-bottom plates (Corning, Life Sciences) and allowed to adhere overnight. PBMC isolated from buffy coats (1.0 x 10^5/well) were added to the MSCs and stimulated with human T-activator CD3/CD28 dynabeads (Invitrogen Corp., Paisley, UK) in a bead:cell ratio 1:5. After 5 days of co-culture, cells were pulsed with [H]-thymidine (0.5 μCi/well) and incubated for 16 h at 37°C. The cultures were harvested on a glass fiber filter and thymidine incorporation was measured with a liquid scintillation counter (Wallac, Turku, Finland). Data were expressed as mean corrected counts per minute of triplicate co-cultures stimulated with anti-CD28/anti-CD3-coated Dynabeads (one bead/5 cells, Invitrogen) and were seeded in Iscove’s modified Dulbecco’s media (Invitrogen) supplemented with 5% human serum (Sanquin, Leiden, The Netherlands), and 5% FBS.

**Myocardial Infarction (MI) induction and Cell injection.** Mice received 100μL NaCl, containing 2 μg buprenorphine, subcutaneously before surgery and again 12 hours after surgery. Animals were anesthetized with 5% isoflurane for induction and kept anesthetized with 1.5-2% isoflurane in oxygen for the remainder of the surgical procedure. Mice were placed supine on a heating pad (34°C), intubated and ventilated using a rodent ventilator (model 845, Harvard Apparatus, Holliston, MA, USA) with 160 breaths per min and a stroke volume of 220 mL. A left thoracotomy was performed, followed by opening of the pericardial sac. The left anterior descending coronary artery (LAD) was visualized and ligated 1 mm caudally from the tip of the left auricle using a 7-0-prolene suture (Johnson and Johnson, New Brunswick, NJ, USA).

Ischemia was confirmed by myocardial blanching. One minute after LAD ligation animals received an intraperitoneal injection of lidocaine (6mg/kg) to prevent cardiac arrhythmias (1). Five minutes after LAD ligation animals received either 2×10^5 uMSCs in 15 μL phosphate-buffered saline (PBS) (uMSC group), 2×10^5 iMSCs stimulated
with IFN in 15 μL PBS (iMSC group), or 15 μL PBS containing no cells (PBS group).
Intramyocardial injections were performed at 3 sites in the infarcted area (5 μL per site). The chest was then closed in layers and animals were allowed to recover.

Sham-operated animals were operated in parallel, but without LAD ligation and intramyocardial injection, and were used to determine baseline characteristics (Sham group).

All surgical procedures and injections were performed by an investigator blinded to treatment.

**Cardiac Magnetic Resonance Imaging (MRI).** Cardiac parameters were assessed 2 and 14 days post-MI using a 7-Tesla MRI (Bruker Biospin, Ettlingen, Germany) equipped with a combined gradient and shim coil, which is inserted into the magnet bore. Mice were pre-anesthetized as described above and kept anesthetized with 1.5-2% isoflurane, and placed supine in an animal holder. A respiration detection cushion was placed underneath the thorax and connected to a gating module to monitor respiratory rate (SA Instruments, Inc., Stony Brook, NY, USA). Image reconstruction was performed using Bruker ParaVision 5.1 software.

*Left ventricular function.* Cardiac function was assessed at day 2 and 14. A high-resolution 2D cine sequence was used to acquire a set of 9 contiguous 1 mm slices in short-axis orientation covering the entire heart. Imaging parameters were: echo time of 1.49 ms, repetition time of 5.16 ms, field of view (26 mm)² and a matrix size of 144x192.

*Image analysis.* All MR image data were analysed with the MASS for Mice software package (Leiden, the Netherlands). The endocardial and epicardial borders were delineated manually by an investigator blinded to treatment (uMSC group n=12, iMSC group n=7, PBS group n=7, Sham group n=10). Subsequently, the end-diastolic volume, end-systolic volume and ejection fraction were computed.

**Pressure-Volume (PV) measurements.** Fifteen days after MI, mice were anesthetized again as described above and kept anesthetized with 1-1.5% isoflurane for the remainder of the surgical procedure. A 1.2F pressure-conductance catheter (standard; Scisense Inc, London, Canada) was introduced via the right carotid artery and positioned in the left ventricle (LV). The conductance catheter was connected to a PV control unit FV 896B (Scisense Inc, London, Canada) for online display and recording of LV pressure and volume signals. Parallel conductance was assessed by the hypertonic saline method using intravenous bolus injections of ~5 μL (2). The abdomen was opened to enable temporary preload reductions by directly compressing the inferior caval vein. All data were acquired using Powerlab 8/30 Model ML870 (ADInstruments, Spechbach, Germany) and LabChart 7 software (ADInstruments, Spechbach, Germany). Data were analyzed off-line by a blinded
investigator (uMSC group n=7, iMSC group n=7, PBS group n=5, Sham group n=7). LV PV signals were acquired in steady-state to quantify general hemodynamic conditions and generate PV loops.

**Histology.** At day 15 post-MI, mice were weighed, sacrificed after PV loop measurements under 5% isoflurane and their hearts and lungs were removed. Lungs were weighed immediately after excision, freeze-dried for 24 hours and then weighed again. The wet weight/dry weight ratio was used as a measure of pulmonary congestion.

Per group 5 hearts were fixed by immersion in buffered 4% paraformaldehyde and embedded in paraffin. Serial transverse sections of 5 μm were cut along the entire long axis of the LV for (immuno)histological analyses. Sections were deparaffinated and dehydrated in xylene and alcohol. Antigen retrieval was accomplished by heating in a microwave oven (98°C) in 0.01 M citric buffer of pH 6.0 for 12 minutes for all sections. Sections were incubated overnight at room temperature with primary antibodies and for 60 minutes with secondary antibodies.

**Engraftment rate.** MSC engraftment was detected by immunostaining with a rabbit anti-GFP antibody (A11122, Invitrogen, Paisley, UK), followed by a biotinylated goat anti-rabbit IgG (E0432, Dako, Glostrup, Denmark) and a Qdot 655 streptavidin-conjugated (Q10121MP, Invitrogen, Paisley, UK) antibody. Nuclei were visualized by Hoechst 33342 (Invitrogen, Paisley, UK).

The number of engrafted MSCs was assessed by counting the GFP-positive cells at a 20x magnification in every 10th serial section along the long axis of the heart. The number of counted cells was multiplied by 10 to obtain an estimate of the total number of engrafted cells in the heart. Subsequently this number was divided by the total number of transplanted MSCs (2×10⁵) and the result was multiplied by 100%.

**Real-time Polymerase Chain Reaction (PCR).** Mice were sacrificed 15 days post-MI to quantify the number of transplanted MSCs by real-time PCR (3). Per group 5 hearts were harvested, frozen in liquid nitrogen and stored at -80°C. Hearts were minced with fine scissors and suspended in lysis buffer (100 mM NaCl, 10 mM Tris-Cl pH 8, 25 mM EDTA pH 8, 0.5% SDS, 0.1 mg/ml proteinase K) and incubated at 55°C overnight, followed by incubation in 0.025 mg/ml ribonuclease at 37°C for 1 hour. DNA concentrations were measured using NanoDrop 1000 (NanoDrop products, Wilmington, DE, USA). PCR reactions were performed in a volume of 10 μL, containing 5 μL Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 900 nM forward and reverse primers, 250 nM TaqMan probe and 50 ng of target template. Reactions were incubated at 50°C for 2 min and 95°C for 10 min, and then amplified for 40 cycles. Each cycle comprised of an incubation step at 95°C for 15 s followed by 60°C for 1 min.

Standard curves were generated by serially diluting human genomic DNA (Roche, Basel, Switzerland) in murine genomic DNA. The sequence of the PCR primers and probe used for detection of human ALU repetitive sequences were
as follows: forward PCR primers, 5’-CATGGTGAAACCCCGTCTCTA-3’; reverse PCR primer, 5’-GCCTCAGCCTCCCGAGTAG-3’; TaqMan probe, 5’-FAM-ATTAGCCCGGCGTGTTGCG-TAMRA-3’.

**Flow cytometry.** From each treatment group, mice not subjected to functional and histological analysis were used for flow cytometric analysis of cardiac inflammatory cell invasion. Mice were sacrificed on days 1, 3, and 7 after MI (n = 3 mice per time point). Sham animals were used as controls to determine base line characteristics. Infarct tissue and healthy hearts were harvested, minced with fine scissors, and placed into a solution of 2% collagenase I\(^a\) (Sigma-Aldrich, Chemie BV, Zwijndrecht, The Netherlands) in PBS and shaken at 37 °C for 1 h. The cell suspension was then triturated through a nylon mesh and centrifuged in PBS at 300 g for 10 min at 4°C. Red blood cells in the cell pellet were lysed with lysis buffer (AZL, Leiden, The Netherlands), and the cells were washed in PBS and subsequently resuspended in medium containing IMDM (Lonza, Verviers, Belgium) supplemented with 2.5% fetal calf serum (FCS; Greiner Bio-one) and Penicillin/Streptomycin (P/S; Invitrogen Corp., Paisley, UK). Total cardiac cell numbers were determined with a Sysmex cell counter (Sysmex America, Inc. Mundelein, Illinois, USA). The resulting single-cell suspensions were stained for flow cytometry with primary antibodies for 30 minutes at 4°C in the dark and the cells were washed with PBS/1% human Albuman (Sanquin, Leiden, The Netherlands) before analysis using a FACSCanto II (BD Biosciences, San Diego, CA, US). The following antibodies were used: anti–CD90-APC, 53–2.1,–B220-APC, RA3–6B2, –CD49b-APC, DX5, –NK1.1-APC, PK136, –Ly-6G-APC, 1A8, CD11b-eFluor 450, M1/70,–CD11c-FITC, HL3, –I-A\(^b\)-FITC, AF6-120.1, –Ly-6C-PE, AL-21, –CD11c-PE, HL3 (all above antibodies are from BD Biosciences), –F4/80-FITC, C1:A3-1 (ABD Serotec, Kidlington, UK). Monocytes were identified as CD11b high (CD90/B220/CD49b/NK1.1/Ly-6G) low (F4/80/I-A\(^b\)/CD11c) low Ly-6C high/low as previously described (4, 5). Macrophages were identified as CD11b high, F4/80 high. Dendritic cells were identified as CD11b, I-A\(^b\) and CD11c high. Neutrophils were identified as CD11b, Ly-6G high. Monocyte and macrophage/dendritic cell numbers were calculated as the total cells multiplied by the percentage of cells within the monocyte/macrophage gate. The analysis of the acquired data was done with FlowJo software version 7.6.1 (Tree Star Inc. Ashland, OR, USA).

**Statistical Analysis.** Numerical values were expressed as means ± standard deviation. Comparison of MRI parameters and inflammatory cells between the uMSC, iMSC, PBS and Sham groups was performed using two-way repeated-measures analysis of variance (ANOVA), with Bonferroni correction. Comparison of the remaining parameters was performed using one-way ANOVA, with Bonferroni correction.
Reference List

1. Tarnavski O, McMullen JR, Schinke M, Nie Q, Kong S, Izumo S. Mouse cardiac surgery: comprehensive techniques for the generation of mouse models of human diseases and their application for genomic studies. Physiol Genomics 2004;16:349-60.

2. Steendijk P, Baan J. Comparison of intravenous and pulmonary artery injections of hypertonic saline for the assessment of conductance catheter parallel conductance. Cardiovasc Res 2000;46:82-9.

3. McBride C, Gaupp D, Phinney DG. Quantifying levels of transplanted murine and human mesenchymal stem cells in vivo by real-time PCR. Cytotherapy 2003;5:7-18.

4. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med 2007;204:3037-47.

5. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. Science 2009;325:612-6.
