Validation of Contrast-Enhanced Magnetic Resonance Imaging to Monitor Regenerative Efficacy After Cell Therapy in a Porcine Model of Convalescent Myocardial Infarction

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Background—Magnetic resonance imaging (MRI) in the Cardiosphere-Derived autologous stem cells to reverse ventricular dysfunction (CADUCEUS) trial revealed that cardiosphere-derived cells (CDCs) decrease scar size and increase viable myocardium after myocardial infarction (MI), but MRI has not been validated as an index of regeneration after cell therapy. We tested the validity of contrast-enhanced MRI in quantifying scarred and viable myocardium after cell therapy in a porcine model of convalescent MI.

Methods and Results—Yucatan minipigs underwent induction of MI and 2–3 weeks later were randomized to receive intracoronary infusion of 12.5 × 10⁶ mismatched allogeneic CDCs or vehicle. Allogeneic CDCs induced mild local mononuclear infiltration but no systemic immunogenicity. MRI revealed that allogeneic CDCs attenuated remodeling, improved global and regional function, decreased scar size, and increased viable myocardium compared with placebo 2 months post-treatment. Extensive histological analysis validated quantitatively the MRI measurements of scar size, scar mass, and viable mass. CDCs neither altered gadolinium contrast myocardial kinetics nor induced changes in vascular density or architecture in viable and scarred myocardium. Histology demonstrated that CDCs lead to cardiomyocyte hyperplasia in the border zone, consistent with the observed stimulation of endogenous regenerative mechanisms (cardiomyocyte cycling, upregulation of endogenous progenitors, angiogenesis).

Conclusions—Contrast-enhanced MRI accurately measures scarred and viable myocardium after cell therapy in a porcine model of convalescent MI. MRI represents a useful tool for assessing dynamic changes in the infarct and monitoring regenerative efficacy. (Circulation. 2013;128:2764-2775.)

Key Words: adult stem cells ◼ allogeneic transplantation ◼ cell transplantation ◼ magnetic resonance imaging ◼ myocardial infarction ◼ regeneration

Clinical Perspective on p 2775

Cell therapy has emerged as a potential therapeutic strategy for ischemic cardiomyopathy. Although the paradigm of administering bone marrow-derived cells in the setting of acute or recent myocardial infarctions (MIs) has proven to be safe, efficacy has been inconsistent.¹² Early clinical experience with autologous heart-derived progenitor cells has been more encouraging.¹³ In the Cardiosphere-Derived autologous stem cells to reverse ventricular dysfunction (CADUCEUS) trial, intracoronary infusion of autologous cardiosphere-derived cells (CDCs)⁵ in post-MI patients with left ventricular (LV) dysfunction decreased scar size, increased viable myocardium, and improved regional function, as measured by contrast-enhanced magnetic resonance imaging (MRI).³ Contrast-enhanced MRI has been extensively validated (and is considered the gold standard imaging modality) for the quantification of necrotic/scared and viable myocardium in the setting of acute or chronic MI.²⁻⁶⁻⁸ Thus, MRI represents a potentially useful tool for monitoring regenerative efficacy, as it affords the unique

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ability to quantify, rigorously and independently, scar mass and viable myocardial mass in human subjects before and after cell therapy interventions. However, its validity in characterizing tissue viability after cell administration has been called into question. Without evidence, concerns have been raised that cell therapy may promote changes in vessel density or architecture (e.g., increase in wall thickness or decrease in vascular permeability) that could affect gadolinium (Gd)-contrast myocardial kinetics (in the form of accelerated contrast wash-out [a phenomenon that has been described in non-cardiac tissues] or decreased contrast extravasation, respectively), therefore compromising the ability of contrast-enhanced MRI to distinguish scar from viable myocardium.

Here, we sought to test the validity of contrast-enhanced MRI to distinguish, and accurately measure, scarred and viable myocardium after cell therapy. We used a porcine model of intracoronary infusion of allogeneic CDCs, which enabled us to concurrently investigate the safety and efficacy of allogeneic heart-derived cells without immunosuppression in a clinically-relevant model of convalescent MI.

Methods

All animal studies were performed in an American Association for Accreditation of Laboratory Animal Care-accredited facility with approval from the Institutional Animal Care and Use Committee of the Cedars-Sinai Medical Center (IACUC 3661). The experimental protocol is depicted schematically in Figure 1. A total of 26 minipigs were studied: 3 completed the 24-hour retention study (Figure 1A), 10 completed the validation study (Figure 1B), 5 completed the 2-month engraftment study, 4 were excluded per protocol for procedural mortality, 3 were used for allosensitization protocols, and 1 served as CDC donor.

Cell Culture

Allogeneic CDCs were grown from a freshly-explanted heart obtained from 1 male Sinclair minipig (Sinclair Bioreources) (for detailed Methods see the online-only Data Supplement). For 24-hour retention analysis, CDCs were transduced with an adenoviral vector carrying the firefly luciferase gene 3 days before infusion. To the 1st diagonal branch) for 2.5 h (for detailed Methods see the online-only Data Supplement). Two to 3 weeks later, pigs were randomized to receive 12.5 million CDCs (in 10 ml of Cryostor CS10 containing 45 μg/ml nitroglycerin and 180 U/ml heparin) or vehicle. Intracoronary infusion was performed via an over-the-wire balloon catheter, placed in the mid-left anterior descending artery. CDCs or vehicle solution were infused in 3 cycles of intermittent balloon inflation. Minipigs were euthanized either 24 hours post-infusion, to measure short-term cardiac retention of administered cells (n=3), or 2 months later (n=15; 10 completed the validation study, 5 completed the 2-month engraftment study).

In Vivo Cardiac MRI

Baseline (2–3 weeks post-MI, before intracoronary infusion) and end point (2 months post-infusion) contrast-enhanced cardiac MRI was performed to measure scar mass, viable myocardial mass (i.e., total mass minus scar mass), scar size (scar mass divided by total mass), volumes, global function, and regional function of the LV. All MRI studies were performed on a 3.0 T clinical MRI scanner (Siemens MAGNETOM Verio, Erlangen, Germany). Typical in-plane resolution was 1.3×1.3 mm, and slice thickness was 6 mm, with no gaps. Global LV function, regional systolic thickening, and regional end-systolic thickness were assessed using ECG-gated, breath-hold, cine steady-state free precession acquisitions. Minipigs undergoing baseline MRI subsequently received an IV injection of Gd-based contrast agent gadoversetamide (OptiMARK, Coviden Imaging Solutions, Hazelwood, MO; 0.2 mmol/kg body weight), and, 8 minutes later, delayed contrast-enhanced images were acquired with an ECG-gated, breath-hold, interleaved, 2D-Turbo FLASH sequence. The inversion time was adjusted by the scanner operator to null signal from non-infarcted remote myocardium. Subsequently, minipigs received a second IV injection of Gd-contrast (0.2 mmol/kg body weight) and 8 minutes later delayed contrast-enhanced images were acquired to assess scar size as described above for baseline MRI. Thirty minutes after the second contrast injection, a TI scout was performed to ensure sufficient contrast clearance. Subsequently, minipigs received a third IV injection of Gd-contrast (0.2 mmol/kg body weight) and were euthanized 15 minutes later to perform ex vivo MRI of the heart.

Ex Vivo Cardiac MRI and Histology

Hearts from minipigs sacrificed 2 months post-infusion underwent ex vivo MRI. The heart was removed 30 minutes post Gd-DTPA administration, thoroughly rinsed in ice-cold saline to remove any residual blood, and suspended by sutures in a plastic container filled with saline. 3D Turbo FLASH images were acquired with a resolution of 1×1×1 mm. After image acquisition, the heart was sectioned into 1-cm-thick short-axis slices, which were incubated with 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 20 min at 37°C to stain viable myocardium. Each slice was photographed with a digital camera, and infarct size was determined as the percentage of LV volume by manual tracing by a researcher blinded to treatment allocation. To evaluate regional function, each short-axis slice was divided into 6 segments, using the right ventricular insertion as a reference point. Scar size from late Gd-enhanced cardiac MR images was defined based on the full-width half-max criterion to delineate scarred myocardium.

Figure 1. Study protocol. Schematic depiction of the 24-hour retention study (A) and the validation study (B).

Ex Vivo Cardiac MRI and Histology

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Immune rejection in the heart was investigated by hematoxylin and eosin staining; analysis was performed by an experienced cardiac pathologist blinded to treatment allocation (D.L.), and rejection was graded according to the International Society for Heart & Lung Transplantation (ISHLT) grading system, used in clinical practice to diagnose solid organ transplant rejection. Morphometric analysis was performed with Masson’s trichrome staining. Vascular density and architecture were investigated by fluorescent immuno- histochemistry; arterioles and arteries were defined as smooth muscle–coated vessels with external vascular diameter (lumen + vascular walls) <75 μm and >75 μm, respectively. Myocyte cross-sectional area was investigated by fluorescent immunohistochemistry; cardiomyocytes were accepted for size measurement if they met the following criteria: (1) cellular cross-sections present, (2) visible nuclei located close to the cell center, and (3) intact cell borders. Cardiomyocyte cell-cycling and activation of cardiac progenitors were investigated by fluorescent immunohistochemistry. In all histological analyses, peri-infarct (border zone) area was defined as the region at the edges of the scar (comprising areas of both viable and scarred myocardium). For detailed histological methods see the online-only Data Supplement.

Evaluation of 24-Hour Cardiac Retention and 2-Month Cardiogenic Engraftment

Evaluation of 24-hour cardiac retention was performed with the ex vivo luciferase assay17 (Figure IA in the online-only Data Supplement). Two-month cardiogenic engraftment was investigated by fluorescence in situ hybridization for the male Y chromosome (Figure II in the online-only Data Supplement) 2 months after sex-mismatch cell transplantation (male cells infused into infarcted female recipients; for details see the online-only Data Supplement).

Histocompatibility

To assess histocompatibility, low-resolution swine leukocyte antigen (SLA) typing was performed on the donor and recipient minipigs as described in the online-only Data Supplement.

Circulating Donor-Specific Antibodies

To evaluate humoral immune response, recipient minipig serum samples (obtained at baseline, 1 day, 1 week, 2 weeks, and 2 months postinfusion) were screened for circulating anti-donor IgG antibodies by flow cytometry.18 An allosensitization protocol was performed to provide positive controls for the flow cytometry assay. Peripheral bone marrow mononuclear cells (PBMCs) were harvested from a donor farm pig and injected intradermally and subcutaneously into the pinnae of recipient Yucatan minipigs (n=2, 190 million PBMCs injected per pig). Serum samples collected 2 weeks post PBMCN injection served as positive controls for allosensitization.

Statistical Analysis

Results are presented as mean±SD in the text and as mean±SEM in the figures. For continuous measures, differences between 2 groups (controls, CDC-treated) were tested using independent samples t test. Comparisons of changes from baseline within groups were tested using paired samples t test. Comparisons of changes from baseline within groups were tested using independent samples t test. Comparisons of changes from baseline within groups were tested using independent samples t test. Comparison of rejection were analyzed using a generalized estimating equations model with an autoregressive correlation structure. Pearson correlations and the Bland-Altman analysis method were used to compare MRI measurements against postmortem histological measurements. All tests were 2-sided. No multiplicity adjustment for multiple comparisons was performed. A P value of <0.05 was considered statistically significant.
Functional and Structural Benefits After Infusion of Allogeneic CDCs

To assess efficacy of allogeneic CDCs, minipigs underwent cardiac MRI before infusion (2–3 weeks post MI) and 2 months later. Allogeneic CDCs resulted in preservation of LV ejection fraction ($\Delta: -0.5\pm3.2$, $P=0.73$ within group), whereas LV ejection fraction decreased in control animals ($\Delta: -9.9\pm1.3\%$, $P<0.001$ within group, $P=0.001$ between groups) (Figure 2A–2C). Movies I–IV in the online-only Data Supplement). In addition, allogeneic CDCs attenuated LV remodeling: CDC-treated animals exhibited a smaller increase in end-systolic volume (ESV) ($\Delta: 10.9\pm7.4$ ml) compared with controls ($\Delta: 26.6\pm13.2$ ml, $P=0.048$ between groups; Figure 2E). End-diastolic volume (EDV) increased in both CDC-treated animals ($\Delta: 17.7\pm10.5$ ml, $P=0.020$ within group) and controls ($\Delta: 30.5\pm19.8$ml, $P=0.026$ within group, $P=0.24$ between groups; Figure 2D).

Regional function was assessed in infarcted and noninfarcted myocardial segments, after visual inspection of corresponding late Gd-enhanced images. Two months post-infusion, CDC-treated infarcted myocardial segments displayed increased systolic thickening ($P<0.001$ between groups), and increased end-systolic thickness ($P=0.025$ between groups), compared with infarcted segments from placebo-treated animals (Figure 2F and 2G). In addition, regional function in the noninfarcted myocardial segments was improved in CDC-treated animals compared with controls, consistent with attenuation of LV remodeling (Figure 2F and 2G).

Figure 3A shows representative late Gd-enhanced MRI acquisitions of hearts in short-axis section at end-diastole. Gd-contrast agent accumulates in the infarct scar (as a result of a larger distribution volume due to increased extracellular space in the scar compared with normal myocardium$^{19,20}$). In the CDC-treated minipig, the infarcted wall thickness was preserved 2 months post-infusion; importantly, the scar decreased in transmurality, whereas viable myocardial mass increased. In contrast, the placebo-treated minipig was characterized by infarct thinning and expansion, with no evidence of an increase in viable myocardium over the same time period (Figure 3A).

Two months post-infusion, MRI analysis revealed that scar size remained unchanged in controls ($\Delta: 0.4\pm0.9\%$, $P=0.33$ within group) but decreased in CDC-treated animals ($\Delta: -3.6\pm2.4\%$, $P=0.026$ within group, $P=0.007$ between groups; Figure 3B), resulting in significantly smaller scar size in CDC-treated animals (9.2$\pm$0.8%) compared with controls (14.6$\pm$3.2% [controls], $P=0.007$ between groups; Figure 3E). Although scar size is a conventional measure of myocardial viability, cardiac MRI can quantify independently the individual components of scar mass and viable myocardial mass, enabling more
discerning insight into mechanism. Scar mass decreased in CDC-treated animals ($\Delta$: $-1.4 \pm 1.4$ g), but not in controls ($\Delta$: $0.8 \pm 0.6$ g, $P=0.012$ between groups; Figure 3C), resulting in a trend towards smaller end point scar mass in CDC-treated animals ($6.6 \pm 0.7$ g) compared with controls ($9.7 \pm 3.5$ g, $P=0.093$ between groups; Figure 3F). In addition, CDC-treated animals exhibited significant increases in viable myocardial mass ($\Delta$: $10.7 \pm 3.6$ g) compared with controls ($2.7 \pm 2.1$ g, $P=0.003$ between groups) over 2 months (Figure 3D), resulting in greater end point viable mass ($65.8 \pm 4.5$ g) compared with controls ($55.5 \pm 5.0$ g, $P=0.010$ between groups; Figure 3G).

A complete list of MRI-measured parameters for each experimental animal is provided in Table I in the online-only Data Supplement.

Comparison of Cardiac MRI With Histology for Assessment of Scarred and Viable Myocardium After Cell Therapy

Postmortem histological analysis confirmed the MRI results, not just qualitatively but also with quantitative accuracy. Figure 4A shows representative short-axis cardiac slices after incubation with TTC, and Figure 4B shows representative sections from the infarcted wall stained with Masson’s trichrome. CDC-treated hearts consistently exhibited significant amounts of viable myocardium in the infarcted wall, most often in the form of endocardial and epicardial muscular layers surrounding the scar but also in the form of islets of viable myocardial tissue interspersed between the collagen fibers. In contrast, in control minipigs the scar was homogeneous and largely transmural (Figure 4A, 4B, and 4F). The increased amount of viable myocardium in the infarct region after cell therapy is consistent with the improved regional contractility of infarcted segments in CDC-treated animals compared with controls (Figure 2F and 2G). Histological measurement of scar and viable myocardium demonstrated decreased scar transmurality in CDC-treated animals ($44.1 \pm 21.3\%$ vs $79.3 \pm 25.2\%$, $P=0.044$), smaller scar size ($9.1 \pm 1.2\%$ vs $14.0 \pm 2.9\%$, $P=0.009$), and increased viable myocardial mass ($68.7 \pm 4.7$ g vs $55.2 \pm 4.9$ g, $P=0.002$) in CDC-treated animals compared with infarcted controls (Figure 4C–4F), yielding virtually identical results to those obtained from contrast-enhanced MRI (Figure 3E–3G). A complete list of histological measurements of scar size, scar...
mass and viable mass for each experimental animal is provided in Table II in the online-only Data Supplement.

To further evaluate the ability of contrast-enhanced MRI to accurately measure scarred and viable myocardium after cell therapy, all TTC-stained cardiac slices were matched with their corresponding late Gd-enhanced images (from both the in vivo and ex vivo MRI datasets). Figure 5A provides representative examples of this analysis for a CDC-treated and a control minipig. In both cases, areas of hyperenhancement in cardiac MRIs correspond faithfully to regions of scarred myocardium in histological slices. Importantly, MRI reveals significant amounts of viable myocardium within the infarct region of the CDC-treated heart, a finding that is confirmed by cardiac histology: the endocardial and epicardial rims of non-hyperenhanced tissue in the infarcted wall in contrast-enhanced MRIs are virtually identical to the endocardial and epicardial TTC-positive muscular layers surrounding the scar in histological slices. In contrast, the control heart is characterized by a dense transmural scar, and no viable myocardium can be detected in the infarct area by either MRI or histology. Figure 5B–5D shows quantitative correlation analysis between the various MRI parameters and the corresponding histological values; the measures of scar size (as % of isolated cardiac slices and as % of the LV), LV scar mass, and LV viable mass all correlate strongly, with a slope close to the line of identity. Bland-Altman analysis demonstrated excellent agreement between MRI and post-mortem histology (Figure VI in the online-only Data Supplement).

**MR Imaging of Gd-Contrast Kinetics in Cell-Treated Myocardium**

To assess whether cell therapy fundamentally alters myocardial contrast uptake or wash-out, we compared Gd-contrast kinetics in various regions infused with cells to the kinetics in the remote (noninfarcted, non–cell treated) myocardium. A series of dynamic late Gd-enhanced images were acquired with a fixed TI (Figure 6A), and signal intensity of specific areas (collagenous scar, viable myocardium in the infarcted...
Figure 5. Comparison of cardiac MRI with histology for assessment of scarred and viable myocardium after cell therapy. A, 2% 2,3,5-triphenyltetrazolium chloride (TTC)-stained cardiac slices matched with their corresponding delayed contrast-enhanced MRI images (from both the in vivo and ex vivo MRI datasets) for a cardiosphere-derived cell (CDC)-treated and a control minipig. Areas of hyperenhancement in cardiac MRIs correspond excellently to regions of scarred myocardium in histological slices. The treated minipig MRI demonstrates endocardial and epicardial rims of nonhyperenhanced tissue in the infarcted wall, which correspond excellently to endocardial and epicardial 2% TTC-positive muscular layers surrounding the scar in histological slices. The control heart is characterized by a transmural scar, and no viable myocardium can be detected in the infarct area by either MRI or histology. B, Correlation of MRI measurements of scar size as % of isolated cardiac slices (for in vivo and ex vivo MRI), scar size as % of left ventricle (LV), LV scar mass, and LV viable mass with the corresponding histological measurements.
region, viable myocardium at the border zone, remote myocardium) was measured at various time points after contrast administration, resulting in the generation of site-specific Gd-contrast curves (Figure 6B; pooled data from all CDC-treated minipigs). Analysis using a linear mixed effects model demonstrated that the only site in which Gd kinetics were (predictably) different compared with remote healthy myocardium was the infarct scar. Importantly, Gd-contrast kinetics were virtually identical in cell-treated viable (nonhyperenhanced) areas and in the remote non–cell treated healthy myocardium.

Vascular Density and Architecture
To investigate whether cell therapy with CDCs can induce vascular changes in the treated (scared or viable) myocardium, we studied vessel density and architecture in the collagenous scar, in viable myocardium in the infarcted wall, and in the remote (noninfarcted, non–cell treated) myocardium. As expected,21,22 capillary density was low in the collagenous scar (117.7±32.9 vessels/mm²) and in the border zone (525.3±274.3 vessels/mm²) as compared with remote myocardium (1344.4±356.5 vessels/mm²). Whereas total capillary density was increased in the border zone (defined as the region at the edges of the scar comprising areas of both scarred and viable myocardium) of CDC-treated hearts (721.9±192.5 vessels/mm² vs 328.8±188.8 vessels/mm², P=0.012; Figure 7B), no differences in capillary, arteriolar or arterial density in scarred or viable myocardium could be detected between CDC-treated animals and controls (Figure 7C). Thus, the increased total capillary density in the border zone of CDC-treated hearts is a result of the increased viable mass and decreased scar mass observed in the infarct border zone after CDC therapy, and cannot be attributed to differences in vascular density of scarred or viable myocardium between CDC-treated hearts and controls. Vessel architecture (as quantified by lumen diameter [Figure 7D], wall thickness, endothelial layer thickness, smooth muscle layer thickness [Figure 7E], and lumen/wall ratio [Figure 7F]) was similar in CDC-treated and control hearts.

Stimulation of Endogenous Cardiac Regeneration by Allogeneic CDCs
To investigate whether the increased viable myocardium observed after CDC-therapy was a result of myocyte hypertrophy, we measured cardiomocyte cross-sectional area in the infarct and peri-infarct area. Myocyte size was significantly smaller in CDC-treated animals compared with controls (Figure 8A), thus excluding myocyte hypertrophy as a contributor to the increase in viable myocardium. The conjunction of increased viable mass and smaller myocyte size are indicative of cardiomycyte hyperplasia after cell therapy. With regard to the latter, we found that transplantation of CDCs upregulated cardiomycyte cycling (Figure 8B) and increased the number of small round TnI+ cells (previously defined as putative myocyte progenitors)22,23 in the infarct and peri-infarct area (Figure 8C), confirming previous reports.18,24 Although cardiomyogenesis is likely to play a role in the reduction of cardiomycyte area, attenuation of adverse remodeling (which involves cardiomycyte hypertrophy) may potentiate the reduction in myocyte size in the infarct and peri-infarct area of CDC-treated animals. Supporting this conjecture, myocyte cross-sectional area in the remote myocardium tended to be lower as well (consistent with relief of wall stress), although the differences did not reach statistical significance (Figure VII in the online-only Data Supplement).

Discussion
Heart-derived cells are particularly promising for cardiac repair and regeneration. In the CADUCEUS trial, intra-coronary infusion of autologous CDCs decreased scar size, increased viable myocardium, and improved regional myocardial function, as measured by MRI.1 An interim MRI analysis of the still-ongoing cardiac Stem Cell Infusion in Patients with...
Ischemic cardiomyopathy (SCIPIO) trial (in which MRI was performed only in treated, primarily nonrandomized patients) showed similar results. Although late Gd-enhanced cardiac MRI has been extensively validated for the quantification of necrotic/fibrotic and viable myocardium in acute/chronic MI, its validity to accurately characterize tissue viability...
after cell therapy has been questioned\(^\text{10}\): cell administration may increase vascular wall thickness, resulting in decreased vessel permeability and attenuation of Gd-contrast extravasation in cell-treated myocardial regions, or stimulate angiogenesis, leading to enhanced drainage of Gd-contrast from the cell-treated myocardium (a phenomenon that has been described in hypervascular hepatocellular carcinomas).\(^\text{11}\)

Any of these confounding factors, if operative, would result in altered Gd-contrast myocardial kinetics (in the form of decreased contrast extravasation or accelerated wash-out) that could compromise the fidelity of contrast-enhanced MRI.\(^\text{10}\)

We sought to validate experimentally the ability of contrast-enhanced MRI to distinguish and accurately measure scarred from viable myocardium after cell therapy. We find that CDC infusion does not change vascular density or architecture in scarred and viable myocardium, neither does it result in altered Gd-contrast myocardial tissue kinetics. Importantly, using postmortem histology as the gold standard, we demonstrate that contrast-enhanced MRI readily distinguishes viable and scarred myocardium and provides accurate measurements of scar size, scar mass, and viable myocardial mass in cell-treated hearts.

The present work differs from previous human\(^\text{3}\) and porcine\(^\text{12}\) studies of CDCs in that the previous studies used autologous cells. The effects of allogeneic CDC therapy reported here are qualitatively similar, and at least as impressive quantitatively in terms of regenerative and functional efficacy; however, a head-to-head comparison of allogeneic versus autologous cells was beyond the scope of this study. The greater increase in EF observed in this study may be attributable to one of several factors: (1) increased efficacy of allogeneic cells (not evident in previous rat studies, which showed equivalence of intramyocardially-injected allogeneic and syngeneic CDCs\(^\text{18}\) or cardiospheres)\(^\text{35}\); (2) the earlier administration of CDCs here (2–3 weeks post-MI, versus 4–5 weeks in our autologous porcine study\(^\text{12}\) or 1.5–3 months post-MI in humans).\(^\text{3}\)

A comparison of EF values in the present study versus our previous porcine study\(^\text{12}\) reveals higher baseline EF values in both groups (a finding consistent with the earlier time point of baseline imaging) and a larger decrease in EF in the control...
group over the period of 2 months in the present study; EF of the treated group is preserved over time in both studies. These data may hint that administration of CDCs earlier in the remodeling process may offer increased functional benefit; and (3) the absence of concomitant anti-remodeling therapies (which in the human setting leave little room for EF improvement). Allogeneic CDCs induced a mild local immune reaction in the heart with no signs of immune-related myocardial damage. Importantly, no circulating anti-donor antibodies could be detected, predicting that no development of panel reactive antibodies would occur in the human setting (at least after a single administration of allogeneic cells). The efficacy of allogeneic cells is rationalized by their indirect mechanism of benefit, which relies on activation of endogenous reparative and regenerative pathways (increased cardiomyocyte cycling, upregulation of endogenous progenitors, angiogenesis), rather than long-term engraftment and differentiation of transplanted cells (no donor cells could be detected in the recipient myocardium 2 months postadministration).

Although cardiac MRI cannot distinguish cardiac hypertrophy from hyperplasia, postmortem histological analysis ruled out myocyte hypertrophy as a contributor to the increase in viable myocardium observed after CDC therapy; myocyte size was actually smaller in the infarct and peri-infarct area of CDC-treated animals compared with controls, a finding consistent with attenuation of remodeling-associated cardiomyocyte hypertrophy and birth of new (smaller) myocytes after cell therapy. The latter likely occurs through differentiation of endogenous progenitors (visualized here as small round Tn+ cells) and induction of resident cardiomyocyte proliferation in the border zone, in agreement with results from fate-mapping studies.

Limitations
Our study has several limitations. First, delayed contrast enhancement images were acquired 8 minutes post-Gd administration. Even though the full-width half-max technique (used here) has been shown to provide accurate measurement of infarct size as early as 6 minutes post contrast administration, standardized protocols issued by the Society for Cardiovascular Magnetic Resonance advocate waiting at least 10 minutes before acquisition of delayed contrast enhancement images. Second, allogeneic CDCs were derived from a single healthy donor minipig; we did not investigate interdonor variability in cell immunogenicity or potency. Finally, we did not investigate the safety and efficacy of repeat administrations of allogeneic CDCs. Careful preclinical studies of safety and efficacy will be required before repeat dosing with allogeneic cells can be contemplated.

Conclusions
In conclusion, we validate the ability of late Gd enhancement MRI to accurately measure scarred and viable myocardium after cell therapy, supporting the utility of contrast-enhanced MRI for assessing dynamic changes in the infarct and monitoring therapeutic regenerative efficacy. We also demonstrate that intracoronary infusion of allogeneic CDCs without immunosuppression is safe, improves heart function, and indirectly promotes cardiac regeneration in a clinically-relevant porcine model of convalescent MI. The safety and efficacy of allogeneic CDCs in human subjects with LV dysfunction post-MI is currently being tested in the phase 1/2 randomized double-blind, placebo-controlled ALLSTAR trial (ALLogeneic heart STem cells to achieve myocardial Regeneration; NCT01458405), which uses infarct size assessed by MRI as its primary efficacy end point.

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Disclosures
Drs E. Marbán and L. Marbán own equity in Capricor Inc. Dr Malliáras is a consultant for Capricor Inc. The authors report no conflicts.

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**CLINICAL PERSPECTIVE**

Magnetic resonance imaging (MRI) in the CArdiosphere-Derived aUtologous stem Cells to reverse ventricuLar dySyFunction (CADUCEUS) trial revealed that autologous cardiosphere-derived cells (CDCs) decrease scar size and increase viable myocardium post-myocardial infarction (MI). However, the validity of contrast-enhanced MRI in characterizing tissue viability after cell therapy has been called into question. Administered cells may promote changes in vessel density or architecture that could affect gadolinium-contrast myocardial kinetics, therefore compromising the ability of MRI to distinguish scar from viable myocardium in cell-treated hearts. We tested the validity of contrast-enhanced MRI to characterize myocardial tissue viability after intracoronary infusion of allogeneic CDCs in a porcine model of convalescent MI. We find that cell therapy with CDCs neither alters gadolinium contrast myocardial kinetics, nor induces changes in vascular density or architecture in viable and scarred myocardium. Using postmortem histology as the gold standard, we demonstrate that contrast-enhanced MRI readily distinguishes viable myocardium and scar and provides accurate measurements of scar size, scar mass, and viable myocardial mass in cell-treated hearts. Microscopic analysis ruled out myocyte hypertrophy as a contributor to the increase in viable myocardium observed after CDC therapy, and revealed that CDCs lead to cardiomyocyte hyperplasia in the border zone, consistent with the observed stimulation of endogenous regenerative mechanisms (cardiomyocyte cycling, upregulation of endogenous progenitors, angiogenesis). The present works validates MRI as a useful tool for assessing dynamic changes in the infarct and monitoring regenerative efficacy of cell therapy.
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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Culture of porcine CDCs

A Sinclair mini-pig heart from a ~4 months old male was obtained from Sinclair BioResources to create a master cell bank (MCB). The heart arrived on ice submerged in cardioplegic solution. Hearts were grossly dissected and biopsy-sized pieces (~25mg) were seeded to create explant derived cells (EDCs). After ~14 days, the EDCs were harvested to create a MCB. MCB vials were thawed and cultured as cardiospheres (CSps) in suspension culture. CDCs were grown by seeding CSps on tissue culture treated flasks, and passaged when confluent. Passage 5 CDCs were resuspended in CryoStor™CS10 (BioLife Solutions) with 100 U/mL heparin in cryobags (for a total dose of 12.5 M cells/10 mL), placed directly in a CryoMed controlled-rate freezer, and then transferred to liquid nitrogen. CDCs were thawed at the day of the infusion. Upon thawing, 100 U/mL heparin and 50 µg/mL nitroglycerin were added as excipients to each 10 mL dose.

MI creation and CDC infusion

On day 0, animals were premedicated with ketamine 20mg/kg IM, atropine 0.05mg/kg IM, and acepromazine 0.25mg/kg IM. Animals were subsequently induced with propofol 2-4mg/kg IV to effect, intubated, and maintained on isoflurane 2-3%. Amiodarone 10mg/kg IV loading dose, then 0.5mg/kg IV as needed and lidocaine 0.03µg/kg/min were given for ventricular arrhythmias. Heparin 100IU/kg IV was given for anticoagulation. Minipigs were subjected to an anteroseptal MI by inflation of an angioplasty balloon in the mid-left anterior descending artery (LAD) (distal to the 1st diagonal brach) for 2.5 h., followed by coronary reperfusion. Two to 3 weeks after MI creation, infarcted minipigs were re-anesthetised using the same protocol and drug regimen. Pigs were randomized to receive 12.5 million CDCs (in 10 mls of Cryostor™CS10 containing 45 µg/ml nitroglycerin and 180 U/ml heparin) or vehicle. Intracoronary infusion was performed via an over-the-wire balloon catheter, placed in the mid LAD. CDCs or vehicle solution were infused in 3 cycles of intermittent balloon inflation. Minipigs were euthanized either 24 hours post-infusion, to measure short-term cardiac retention of administered cells, or 2 months later.
Histocompatibility

To assess histocompatibility, low-resolution swine leukocyte antigen (SLA) typing was performed on the donor Sinclair minipig and all recipient Yucatan minipigs as described elsewhere (1,2). Divergent pig strains were used to simulate the typical allogeneic conditions in human transplantation where donor and recipient are frequently mismatched with regard to human leukocyte antigen. Complete SLA mismatch was confirmed if there was at least one class I and one class II antigen mismatch at the allele group level between donor and recipient.

Ex vivo luciferase assay

The ex vivo luciferase assay, a method with high sensitivity and reproducibility (3) (depicted schematically in Supplemental Figure 1a) was performed as described (4). In brief, CDCs were transduced with an adenoviral vector carrying the firefly luciferase gene 3 days prior to infusion. For each animal a separate standard curve was constructed, as transduction efficiency and transgene copy number per cell vary by experiment. Specifically, once the cell dose was allocated for infusion, an aliquot of cells (from the same batch of adenovirally-transduced cells) was cultured for an additional 24 hours. Twenty-four hours after cell delivery, the explanted LV was cut into 1g samples that were subsequently homogenized and analyzed for luciferase signal. The animal-specific standard curve was prepared by measuring the luciferase signal from pig myocardial samples from the left atrium (not infused with cells) spiked with known numbers of transduced CDCs.

Histology

To evaluate immune rejection in the heart, hematoxylin and eosin-stained sections from myocardial samples (fixed in 10% formalin, paraffin-embedded [FFPE]) obtained from the infarct and peri-infarct area (n=4-6 slides/heart) and the remote myocardium (n=2 slides/heart) were analyzed by an experienced cardiac pathologist blinded to treatment allocation (DL). Analysis was graded according to the International Society for Heart & Lung Transplantation (ISHLT) grading system, used in clinical practice to diagnose solid organ
transplant rejection. Morphometric analysis with Masson’s trichrome staining was performed on 8 μm sections from myocardial samples (FFPE) extending from the viable myocardium at the edges of the scar to the core of the collagenous scar (n=4 slides/heart). To study vascular density and architecture, 8 μm sections from samples (FFPE) obtained from the infarct, peri-infarct (n=4-6 slides/heart) and remote (n=2 slides/heart) areas underwent immunostaining for α-sarcomeric actinin (Sigma), α-smooth muscle actin (Abcam) and isolectin (Molecular probes). Arterioles and arteries were defined as smooth-muscle-coated vessels with external vascular diameter (lumen + vascular walls) <75 μm and >75μm respectively. To measure myocyte cross-sectional area, 8 μm sections from myocardial samples (FFPE) obtained from the infarct, peri-infarct (n=4-6 slides/heart) and remote (n=2 slides/heart) areas underwent immunostaining for α-sarcomeric actinin and wheat-germ agglutinin (Molecular probes) (to visualize cell borders). Cardiomyocytes were accepted for size measurement if they met the following criteria: (a) cellular cross-sections present (b) visible nuclei located close to the cell center and (c) intact cell borders (17). To measure cardiomyocyte cell-cycling, 8 μm sections from myocardial samples (FFPE) obtained from the infarct and peri-infarct area (n=4-6 slides/heart) underwent immunostaining for α-sarcomeric actinin and Ki67 (Thermo). To visualize myocyte progenitors, 8 μm sections from myocardial samples (FFPE) obtained from the infarct & peri-infarct area (n=4-6 slides/heart) underwent immunostaining for Troponin I (Sigma). Alexa Fluor-conjugated secondary antibodies (Molecular probes) were used and slides were counterstained for DAPI (Molecular probes). Sections were imaged using a confocal laser scan microscope (Leica Microsystems) and images were processed by Leica Application Suite software. Peri-infarct (border zone) area was defined as the region at the edges of the scar (comprising areas of both viable and scarred myocardium).

**Fluorescence In Situ Hybridization**

Two to 3 weeks post-MI infarcted female mini-pigs were intracoronarily infused with allogeneic CDCs derived from a male mini-pig donor to enable cell detection Fluorescence In Situ Hydridization (FISH). ZytoLight porcine X/Y Dual Color Probe and ZytoLight FISH-Tissue Implementation Kit (ZytoVision GmbH) were used to prepare slides and test for the porcine Y and X chromosomes. Sections obtained from 3 sites were used for FISH analysis: central infarct region, infarct border zone and normal myocardium Three to 6
slides (1 or 2 from each of the 3 sites) were selected for each of the animals for analysis. A simple preparation of male CDCs served as a positive control for the method (Supp Fig 2A).

On day one, slides were incubated for 10 minutes on a hot plate (VWR, Digital Heatblock) at 70°C, followed by two 10 minute incubations in xylene (Sigma). Next, the slides were incubated for 5 minutes each in 100%, 100%, 90%, and 70% ethanol. In order to keep the Heat Pretreatment Solution Citric Solution at 98°C, a coplin jar was kept in a hot water bath at boiling temperature. Immediately before placing slides in the jar, the Heat Pretreatment Solution Citric Solution was brought up to boiling temperatures in a microwave. The solution was placed inside the warmed up coplin jar along with the slides, slides were incubated at 98°C for 15 minutes. Slides were then washed twice for 2 minutes in deionized water and then water was blotted off. Pepsin solution was added drop-wise to slides and incubated for 10 minutes at 37°C in a humidity chamber. Slides were washed for 5 minutes in Wash Buffer SSC and for 1 minute in deionized water. Slides were dehydrated by placing in a series of ethanol concentrations, 70%, 90%, and 100% for 1 minute each. Slides were allowed to air dry. The remaining steps were performed in the dark to protect the probe. ZytoLight FISH probe (10µL) was pipetted onto the slide, a coverslip was placed over the probe and sealed with rubber cement. The slide was placed on a heat block at 72°C for 10 minutes. Finally, slides were incubated overnight in a humidity chamber at 37°C. On day two, slides continued to be protected from light. Rubber cement was carefully removed and the slides were placed in 1 X Wash Buffer at 37°C for 3 minutes to ensure coverslip removal. Slides were then washed twice in 1 X Wash Buffer at 37°C for 5 minutes. Slides were incubated in a series of ethanol concentrations, 70%, 90%, and 100% for 1 minute each. After being allowed to air dry, 1 drop (~30µL) of VECTASHIELD Mounting Medium with DAPI (Vector Laboratories) was added to the slide. A coverslip was placed over the mounting medium and sealed with nail polish. Slides were stored in the dark at 4°C until ready to analyze. Slides were analyzed using a confocal microscope (Leica TCS SP-X, Leica Microsystems CMS GmbH) with three filters, red, blue, and green. Images were analyzed using LAS AF Lite (Leica Microsystems CMS GmbH) and Image J.
References


Supp Fig 1. Twenty-four-hour cardiac retention of IC infused CDCs. A: Schematic protocol for transduction of CDCs with adenoviral luciferase and ex vivo luciferase assay. B: Twenty-four-hour cardiac retention of IC infused CDCs.
Supp Fig 2. Long-term engraftment of IC infused CDCs. **A:** Control preparation of male donor CDCs show both red and green fluorescence signals in each nucleus, corresponding to one X and one Y chromosome, respectively, in each cell. **B:** A male CDC (box) in the recipient myocardium at 2 weeks post-infusion.
**Supp Fig 4. Low resolution SLA genotyping.** Low resolution SLA genotyping revealed complete Class I (SLA-1, SLA-2, SLA-3) and Class II (DRB1, DQB1, DQA) mismatches between the donor minipig and CDC recipients.

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Supp Fig 5. Immune reaction to allogeneic CDCs. **A:** Increased focal lymphoplasmacytic infiltration (Grade 1R), deemed to be unrelated to the natural inflammatory response to the ischemic insult, was detected in the peri-infarct area of CDC-treated minipigs (16/22 slides) compared to placebo. **B:** Representative image of H&E-stained heart section revealing infiltrating cells localized within the interstitial space. **C, D:** Recipient minipig serum samples were screened for circulating anti-donor IgG antibodies by flow cytometry. No alloreactive antibodies were detected in any recipients of allogeneic CDCs at any time point. In contrast, in minipigs that were allosensitized by intradermal and subcutaneous PBMNC injections, high titers of circulating alloreactive IgG antibodies were detected at two weeks post-injection.
**Supp Fig 6. Bland-Altman analysis.** Bland-Altman analyses showing the level of agreement between MRI and post-mortem histology for measurement of scar size as % of isolated cardiac slices (A [in vivo MRI], B [ex vivo MRI]), scar size as % of the LV (C), scar mass (D) and viable myocardial mass (E). Dotted lines denote 95% limits of agreement.
Supp Fig 7. Cardiomyocyte cross-sectional area in the remote myocardium.

Myocyte area

\[ p:0.093 \]

\[ \mu m^2 \]

controls  CDCs
### Supp Table 1. A complete list of MRI-measured parameters for each experimental animal (EF: ejection fraction; EDV: End-diastolic volume; ESV: End-systolic volume; LV: left ventricle; base: baseline; mos: months).

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### Supp Table 2. A complete list of histological measurements of scar size, scar mass and viable mass for each experimental animal (TTC: 2,3,5-triphenyltetrazolium chloride).

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Supplemental Video 1. Video of short-axis cine acquisition of a CDC-treated pig at baseline (before CDC infusion).

Supplemental Video 2. Video of short-axis cine acquisition of a CDC-treated pig at 2 months post-CDC infusion.

Supplemental Video 3. Video of short-axis cine acquisition of a control pig at baseline (before vehicle infusion).

Supplemental Video 4. Video of short-axis cine acquisition of a control pig at 2 months post-vehicle infusion.