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PRE-CLINICAL RESEARCH

# Allogeneic Cardiospheres Safely Boost Cardiac Function and Attenuate Adverse Remodeling After Myocardial Infarction in Immunologically Mismatched Rat Strains

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**Objectives** We sought to characterize the immunologic profile of allogeneic cardiospheres, which are 3-dimensional, self-assembling, cardiac-derived microtissues, and to evaluate their safety and efficacy in repairing ischemic heart tissue.

**Background** Intramyocardial injection of autologous cardiospheres ameliorates remodeling and improves global function in infarcted myocardium. It is as yet unknown whether allogeneic cardiospheres are similarly effective without eliciting deleterious immune reactions.

**Methods** We expanded cardiospheres from male Wistar Kyoto rat hearts and injected them surgically in the peri-infarct zone of Wistar Kyoto (syngeneic group, n = 28) and Brown Norway female rats (allogeneic group, n = 29). Female rats from both strains (n = 37) injected with normal saline served as controls.

**Results** In vitro, cardiospheres expressed a low immunogenic profile and inhibited proliferation of alloreactive T cells. In vivo, cell engraftment was similar in the syngeneic and allogeneic groups 1 week and 3 weeks after transplantation. Reductions in scar size and scar collagen content and increases in viable mass in the risk region were accompanied by improvements in left ventricular function and attenuation of left ventricle remodeling that were sustained during 6 months of follow up. Transplantation of allogeneic cardiospheres increased tissue expression of the regenerative growth factors vascular endothelial growth factor, hepatocyte growth factor, and insulin-like growth factor-1, stimulating angiogenesis. Syngeneic and allogeneic cardiospheres attenuated the inflammatory response observed histologically in the peri-infarct region.

**Conclusions** Allogeneic cardiospheres increase viable myocardium, decrease scar, improve function, and attenuate adverse remodeling in the infarcted rat heart, without deleterious immunological sequelae. These observations lay the groundwork for developing cardiospheres as a novel off-the-shelf microtissue product for myocardial regeneration. (J Am Coll Cardiol 2013;61:1108–19) © 2013 by the American College of Cardiology Foundation

Heart-derived cell products appear to be particularly promising from a therapeutic perspective (1–5). Among these, cardiosphere-derived cells (CDCs) have been found to reduce scar while producing new functional heart tissue in post-myocardial infarction (MI) patients in the CADUCEUS (Cardiosphere-Derived Cells for Heart Regeneration After Myocardial Infarction) trial (6). The CDCs, as their name implies, are created from self-assembling heart-derived multicellular clusters named "cardiospheres," which are unique in being preformed microtissues (7,8). Cardiospheres themselves are effective in tissue regeneration, but their size (~50 to 100 μm diameter) complicates intracoronary administration (8–11). Direct intramyocardial injection is a viable alternative for delivery of cardiospheres (12,13). Despite the greater ease (and safety) of intracoronary delivery, intramyocardial administration merits further exploration given compelling evidence that cardiospheres

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may surpass CDCs in terms of functional benefit and regenerative potency (12–14). Thus, the disadvantages of intramyocardial delivery may be offset by greater efficacy. Here, we investigate the safety and efficacy of intramyocardial allogeneic cardiosphere administration in a rat model of acute MI.

In the emerging field of cell therapy, it is generally assumed that transplanted cells will behave immunologically like adult organs or bone marrow and elicit strong immune reactions when transplanted into histocompatibility mismatched recipients (15,16). Indeed, the widespread use of autologous cells is motivated by avoidance of immune rejection. However, harvesting cells from individual patients poses considerable logistic, economic and timing constraints, as well as exposure to potential failures in cell manufacturing and inescapable medical comorbidities. Allogeneic cardiospheres could circumvent these limitations and serve as a potential off-the-shelf product in preventing or reversing post-ischemic left ventricular remodeling and dysfunction. The rationale for allogeneic cell products is further strengthened by the emerging realization that long-term survival of transplanted cells is not a prerequisite for durable therapeutic benefit (17). Therefore, the objectives of our study were twofold: first, to characterize the immunologic profile of cardiospheres and the potential immune responses to transplanted allogeneic cardiospheres, and second, to compare the effects of syngeneic and allogeneic cardiospheres on post-ischemic cardiac structure and function.

**Abbreviations and Acronyms**

**CDC** = cardiosphere-derived cell
**DNA** = deoxyribonucleic acid
**HGF** = hepatocyte growth factor
**IFN** = interferon
**IGF** = insulin-like growth factor
**IL** = interleukin
**LV** = left ventricular
**LVEDD** = left ventricular end-diastolic diameter
**LVESD** = left ventricular end-systolic diameter
**MHC** = major histocompatibility complex
**MI** = myocardial infarction
**PCR** = polymerase chain reaction
**RNA** = ribonucleic acid
**TNF** = tumor necrosis factor
**VEGF** = vascular endothelial growth factor

## Methods

**Isolation of secondary cardiospheres.** Cardiospheres were created and expanded as described (7,8). Hearts from male Wistar Kyoto rats (4 to 6 weeks old; Charles River Laboratories, Reno, Nevada) were processed, and cardiospheres were formed by replating CDCs in poly-d-lysine-coated dishes (Invitrogen, Carlsbad, California) to yield "secondary cardiospheres." Such secondary cardiospheres have been shown to be equally effective to primary cardiospheres formed directly from myocardial outgrowth cells (9). Cardiospheres, formed from 2 million initially plated CDCs at passage 1 or 2, were resuspended in 120 μl of cold phosphate-buffered saline (PBS [Invitrogen]) and kept on ice until injection in each animal (9). For the xenogeneic group, secondary cardiospheres were derived from percutaneous endomyocardial human heart biopsies, from 5 different patients undergoing clinically-indicated procedures after informed consent.

**Flow cytometry.** Surface alloantigens major histocompatibility complex (MHC) I and MHC II and the costimulatory factors CD80 and CD86 were quantified by flow cytometry (FACS Calibur with CellQuest software, BD Biosciences, San Jose, California). Singly-passaged CDCs were stained in parallel and used for gating the cardiospheres according to object size and optical density. The following polyclonal conjugated antibodies and isotype matched controls were used: MHCI-PE, MHCII-FITC, CD80-APC, and CD86-APC (BD Biosciences).

**Experimental protocol and animal surgery.** The Wistar Kyoto and Brown Norway rat strains are highly inbred and immunologically divergent (18). Left coronary artery occlusion was performed in female rats of either strain (6 to 7 weeks old [Charles River Laboratories]) under mechanical ventilation as described, with small modifications (7,19). Briefly, a left thoracotomy and pericardiotomy were followed by the induction of a left ventricular anterior transmural MI by permanent ligation of the left anterior descending coronary artery with a 7–0 silk suture. The presence of infarction was verified by pallor at the apex. Shortly afterwards, cardiospheres resuspended as described in the preceding text were injected in equally divided doses into 4 peri-infarct sites in each animal. For the control group, 120 μl PBS was similarly injected. Five experimental groups were used: Wistar Kyoto female rats plus MI plus cardiospheres (syngeneic group, n = 28), Brown Norway female rats plus MI plus cardiospheres (allogeneic group, n = 29), Wistar Kyoto female rats plus MI plus PBS (control, n = 27), Brown Norway female plus MI plus PBS (control, n = 10), and Brown Norway female plus MI plus human cardiospheres (xenogeneic, n = 16).

**Quantitative polymerase chain reaction for cardiosphere engraftment.** To measure engraftment of injected cardiospheres, polymerase chain reaction (PCR) analysis was performed from left ventricular tissue 1 week and 3 weeks after cell transplantation. Tissue samples (n = 5 per group per time point) were excised and maintained in ribonucleic acid (RNA) stabilization reagent (Qiagen, Germantown, Maryland) until processing. The left ventricle was homogenized, and total deoxyribonucleic acid (DNA) extracted using DNAeasy kit (Qiagen). For the standard curve, genomic DNA extracted from male heart tissue was used. Quantitative real-time PCR was performed using a Prism 7900 sequence-detection system (Applied Biosystems, Foster City, California) for 40 total cycles.

**Functional evaluation of the left ventricle.** Transthoracic echocardiography was performed with the animals anesthetized by 2% isoflurane. Two-dimensional short- and long-axis images of the left ventricle were obtained at the papillary muscle level (Vevo 770, Visual Sonics, Toronto, Ontario). The following parameters were measured: left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD), ejection fraction,

and fractional shortening. Three consecutive cardiac cycles were analyzed, and the average was used for data analysis. Four different time points were selected for echocardiographic studies: baseline (15 to 18 h after the surgical procedure), 1 week, 3 weeks, 3 months, and finally, 6 months post-MI.

**Tissue collection.** For pathology analysis, animals were euthanized at 1 of 2 time points: 1 week or 3 weeks. Hearts were arrested in diastole by intraventricular injection of KCl (10%), excised, embedded in Tissue-Tek OCT compound (Sakura, Torrance, California) and stored at −80°C until sectioning. Slices 8-$\mu$m thick taken every 0.2 mm were stained with hematoxylin and eosin for qualitative assessment of inflammation. Such analysis was performed both in the peri-infarct zone where the cardiospheres had been injected, and in the contralateral noninfarcted myocardium by a cardiac pathologist (D.L.) blinded as to experimental group assignment.

**Infarct size, morphometry analysis.** Scar size was measured as infarct mass divided by total LV mass as described (20). The left ventricle was weighed and then immersed in OCT compound. Sections from tissue collected 1 week, 3 weeks, and 6 months after cell transplantation were stained with Masson's trichrome. Morphometric evaluation included quantification of infarct wall thickness and noninfarcted remote wall thickness. Six to 10 sections per heart, from 5 hearts in each group, were analyzed and averaged.

**Collagen content quantification.** Tissue sections collected at 3 weeks were stained with 0.1% picrosirius red (Sigma Aldrich, St. Louis, Missouri) for collagen content evaluation within the infarct zone (21). Ten to 15 images from the infarcted region of each heart were obtained with a ×40 objective lens under polarized light microscope. Three hearts per group were included, and images were analyzed using Image J software. The collagen content was calculated as a percentage of the area of each image.

**Immunostaining and immunohistochemistry.** Secondary cardiospheres were fixed in a 50% ethanol/50% acetone solution, blocked, permeabilized, and stained with the following antibodies for confocal fluorescence imaging: rabbit anti-rat c-kit (Santa Cruz, Santa Cruz, California), rabbit anti-rat CD 105 (Abcam, Cambridge, Massachusetts), rabbit anti-rat Cx 43 (Abcam), mouse anti-rat CD 45 (Bd Biosciences), PE-conjugated anti-MHCI (Bd Biosciences, San Jose, California), FITC-conjugated anti-MHCII (Bd Biosciences), APC-conjugated anti-CD80 (Bd Biosciences), and APC-conjugated anti-CD86 (Bd Biosciences). Matched isotype controls were used in each case.

For characterization and quantification of the inflammatory cell population in the myocardium, fresh-frozen sections (8 $\mu$m) were fixed and stained with the following primary antibodies: rabbit anti-rat CD45RA for B monocytes (Serotec), mouse anti-rat CD3 for lymphocytes (Abcam), and mouse anti-rat CD 68 for macrophages (Serotec). Species-appropriate secondary antibodies were also applied, followed by slide mounting. For each section, cells positive for each antigen were counted in the peri-infarct area in 7 to 10 random high-power fields and expressed as number per mm$^2$.

**One-way mixed lymphocyte reactions and secreted cytokines.** Lymphocytes isolated from the spleens of each experimental group using Ficoll-Hypaque (GE Healthcare, Pasadena, California) density gradient centrifugation (22) had a viability of >95% assessed by Trypan blue (Invitrogen) staining. Cardiospheres were pretreated with mitomycin C (25 $\mu$g/ml) to prevent proliferation and added to lymphocytes pooled from 7 pairs of animal donors at day 7, and 5 at day 21, in a ratio of 1:10 cardiospheres:lymphocytes and cocultured for 5 days in the presence of 10% FBS RPMI 1640 medium (Invitrogen). Lymphocyte proliferation was determined by BrdU (10 $\mu$M) incorporation, which was added for the last 24 h of coculture; optical density was evaluated with the Amersham Cell proliferation Elisa Biotrak System (GE Healthcare) according to the manufacturer's instructions. Proinflammatory cytokines interferon (IFN)-$\gamma$, interleukin (IL)-1$\beta$, IL-2, IL-6, and tumor necrosis factor (TNF)-$\alpha$ were measured in supernatant collected on the fifth day (with day 7 post-MI lymphocytes) by an array assay (Ray Biotech, Norcross, Georgia).

**Cytokine expression in tissue.** The cited cytokines were also measured (Ray Biotech) in extracts of mildly homogenized peri-infarct tissue (17) at days 1 and 7 post-MI. Protein concentration was quantified by the BCA kit (Thermo Scientific, Rockford, Illinois), and a total of 200 $\mu$g was used in each sample (n = 3 in each group). Results were analyzed by Image J and expressed as optical density units.

**Paracrine effect, growth factors.** Tissue expression of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF) was evaluated by Western blots at days 1 and 7 post-MI in both peri-infarct and remote zones. A total of 100 $\mu$g protein (n = 3 in each group) was processed, and the following primary antibodies were used: rabbit anti-rat HGF (Abcam), mouse anti-rat VEGF (Abcam), and mouse anti-rat IGF (Abcam). Anti-rabbit and anti-mouse horseradish peroxidase conjugated secondary antibodies (Cell Signaling, Danvers, Massachusetts) were used for detection. ImageJ software was used for quantitation, and each factor's expression was normalized by that of $\beta$-actin.

**Statistical analysis.** All results are presented as mean ± SD unless specified. Student's $t$ test was used for comparisons between 2 independent groups. Paired data were compared using paired $t$ test. Multiple groups were compared using 1-way analysis of variance, and the Bonferroni test for between-group comparisons. Statistical significance level was set at $p < 0.05$. Data were analyzed using Prism software (version 4.00, GraphPad, San Diego, California).

Although the experimental design included 2 different control groups (Brown Norway and Wistar Kyoto), there were no differences between them in any of the parameters studied. As a result, the 2 control groups were pooled into a single control group for final analysis.

## Results

**Cardiosphere in vitro characteristics.** Cardiospheres had a mean diameter of 47.4 ± 9.75 μm, and each contained 54 ± 17 nuclei as assessed by Cellometer (Nexcelom Bioscience, Lawrence, Massachusetts). The passage through the 30G needle used affected neither their integrity nor their viability (Online Fig. 1). Given the overall cardiosphere-forming cell number of 2 ×$10^6$, which was used to quantify dosage as previously described (9), approximately 40,000 individual cardiospheres ended up being injected in each animal. Consistent with previous findings (9,23), rat cardiospheres are multicellular microtissues that express c-kit as well as mesenchymal/endothelial cell markers (CD 105), but no detectable hematopoietic markers (CD 45) (Figs. 1A to 1D). Immunologically, cardiospheres created from first- or second-passage CDCs comprised a single phenotypic population by flow cytometric and immunohistochemical analysis of surface alloantigens (Figs. 1E to 1H). Cardiospheres under normal culture conditions expressed intermediate levels of the major histocompatibility complex I (MHC I 78 ± 2.8%) on their outer layer, but no detectable MHC II or costimulatory molecules CD 80 and CD 86 (Fig. 1E). Similar results were observed with human cardiospheres (Online Fig. 2). Only after stimulation with interferon γ (100 IU) over 7 days was MHC II antigen expressed, reaching 87 ± 2.5% on the last day of treatment (Figs. 1H and 1I).

**Left ventricular function.** One week after MI, both the syngeneic and allogeneic groups exhibited increases in ejection fraction and fractional shortening compared to control (Figs. 2A to 2F and 3A to 3C). The improvement in LV function was sustained at 3 weeks, 3 months, and 6 months of follow-up in both syngeneic and allogeneic

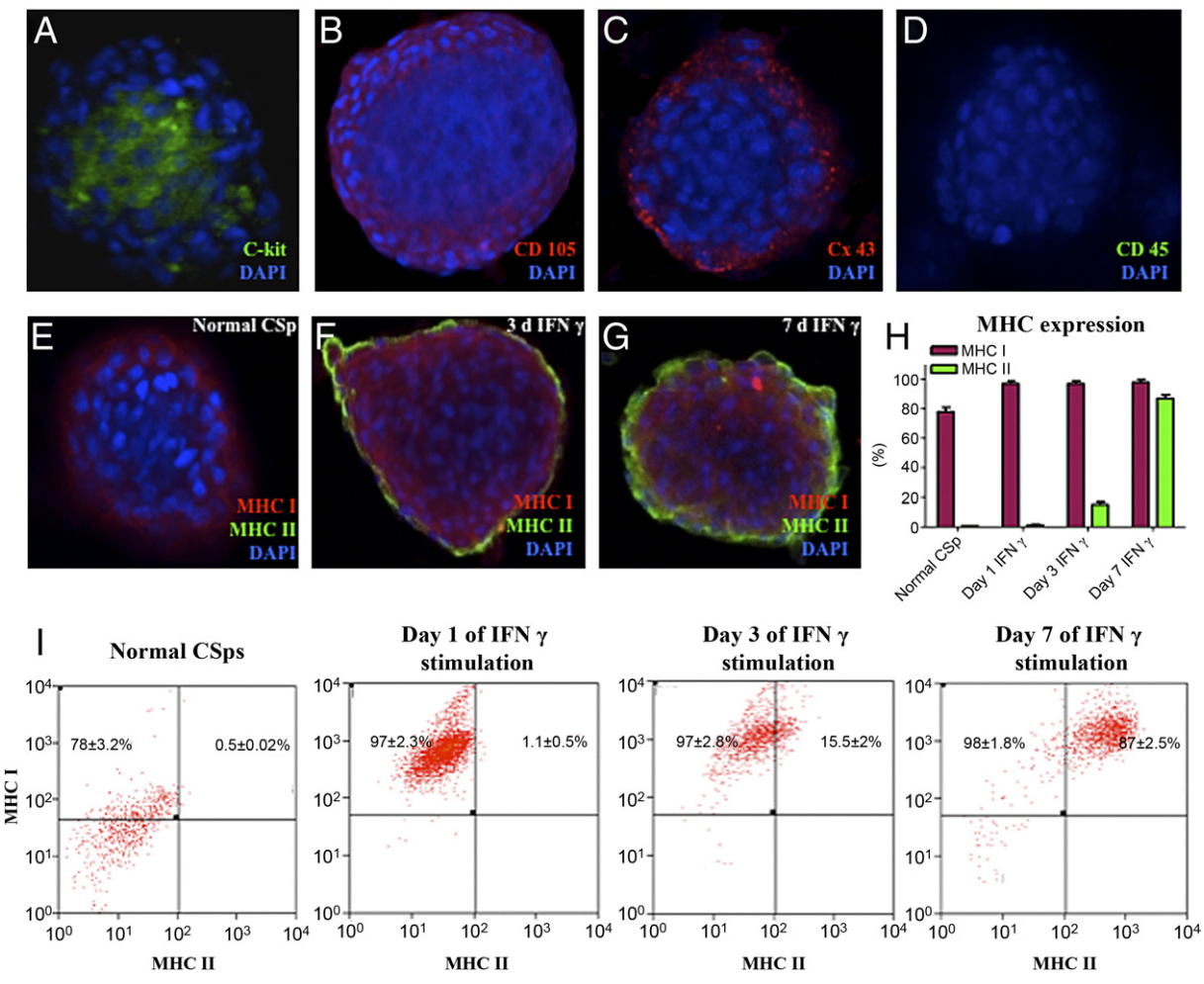


**Figure 1** Characterization of Cardiospheres In Vitro

Immunostaining of selected antigens reveals **(A)** c-kit preferentially localized in the center of the cardiosphere, **(B)** CD 105 and **(C)** Cx 43 on the outer layer, **(D)** the absence of CD 45, **(E)** expression of major histocompatibility complex (MHC) I and MHC II under normal conditions, and after **(F)** 3 days or **(G)** 7 days of stimulation with interferon (IFN)-γ. **(H, I)** Flow cytometry plots of the surface antigens MHC I and MHC II before (Normal CSp) and at various times after stimulation with IFN-γ. DAPI = 4',6-diamidino-2-phenylindole.

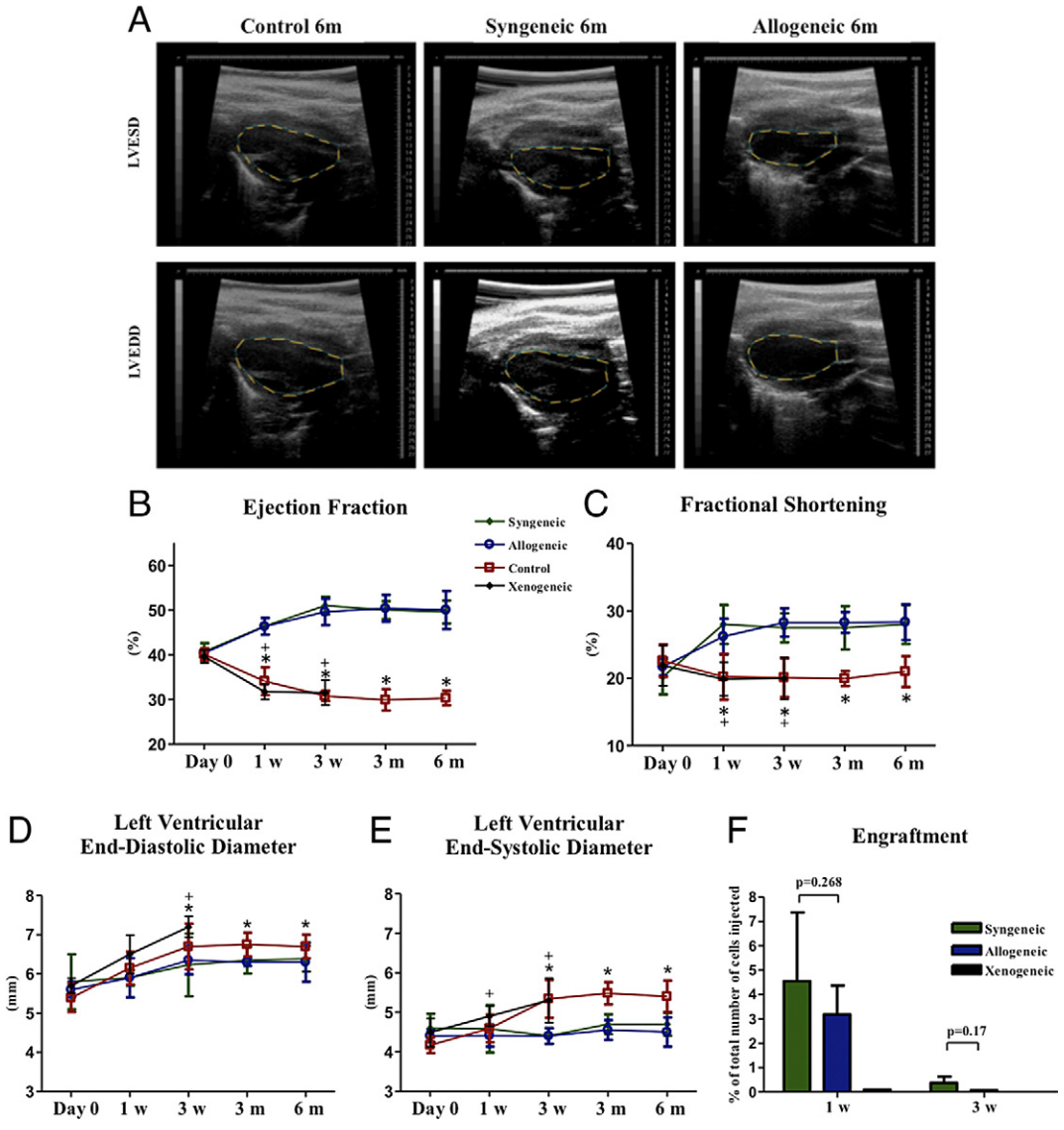


**Figure 2** Cardiac Function and Cell Engraftment

Left ventricular function and remodeling in controls (vehicle injection), and in groups injected with syngeneic, allogeneic, or xenogeneic cardiospheres. **(A)** Representative long-axis echocardiographic images at 6 months from the control, syngeneic, and allogeneic groups. **(B)** Ejection fraction calculated from long-axis views at the level of the papillary muscle shows equivalent improvement in both syngeneic group **(green line)** and allogeneic group **(blue line)** at 1 week, 3 weeks, 3 months, and 6 months of follow up, relative to controls **(red line)** and to xenogeneic group **(black line)**; the latter was followed up for only 3 weeks. **(C)** Fractional shortening calculated from m-mode scans support the findings in panel B. **(D)** Left ventricular end-diastolic diameter (LVEDD) and **(E)** left ventricular end-systolic diameter (LVESD) reveal attenuation of left ventricular remodeling in syngeneic and allogeneic groups. **(F)** Percentage engraftment of cardiospheres at 1 week and 3 weeks post-injection reveal minimal to no survival at 3 weeks in syngeneic group **(green bars)** and allogeneic group **(blue bars)**. All data are mean ± SD. *$p < 0.05$ treated versus control. +$p < 0.05$ treated versus xenogeneic.

groups (Fig. 2B and 2C). In addition, adverse remodeling (as manifested by increases in LVEDD and LVESD) was attenuated in both the syngeneic and allogeneic groups compared to controls. Interestingly, LVEDD and LVESD were increased in the control group by 3 weeks but not in the syngeneic or allogeneic groups (Figs. 2D and 2E). This attenuation of enlargement in both treated groups continued up to 6 months (Figs. 2D and 2E).

**Engraftment.** Consistent with previous observations (9), percentage engraftment at 1 week was in the single digits. This was the case both for syngeneic and allogeneic hearts (Fig. 2F). By 3 weeks, <1% transplanted cell survival was evident in either treated group. In fact, no surviving cells were detectable in any of the 5 allogeneic hearts examined at this time. However, there were no statistically significant differences in engraftment, when comparing syngeneic and allogeneic hearts. In the xenogeneic group though, no live cells could be identified even at day 7. Our observation that no allogeneic CSps survive at 21 days dissociates this measure of engraftment from long-term functional outcomes.

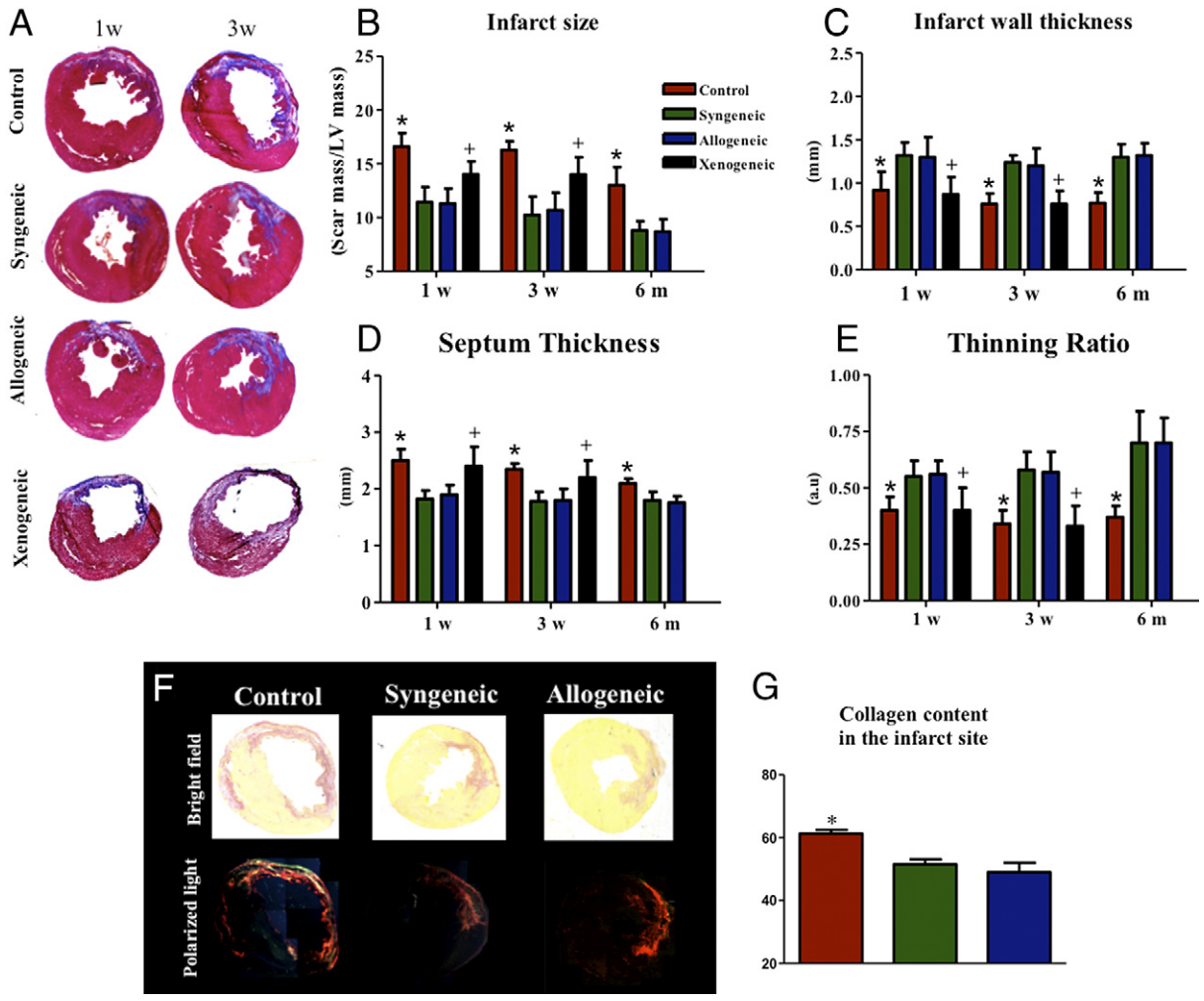


**Figure 3** Infarct Size and Left Ventricular Morphometry

**(A)** Representative Masson's trichrome-stained heart tissue cross sections at 1 and 3 weeks. Scar tissue and viable mass appear **gray-blue** and **red**, respectively. Quantitative analysis of **(B)** infarct size, 1 week ($p < 0.01$ for both treated groups vs. control **[red bars]**), 3 weeks ($p = 0.01$ allogeneic **[blue bars]** vs. control; $p < 0.001$ syngeneic **[green bars]** vs. control), and 6 months ($p < 0.05$ for both treated groups vs. control); **black bars** indicate xenogeneic; **(C)** infarcted wall thickness; **(D)** septum thickness ($p < 0.01$ for both treated groups vs. control); and **(E)** thinning ratio ($p < 0.01$ for both treated groups vs. control). **(F)** Sirius red-stained tissue sections of control, syngeneic, and allogeneic groups. Images represent stained slides visualized under bright field (**red color** correlates with collagen, and **yellow** with normal myocardium) and under polarized light (**black** is the normal myocardium, and **orange-red** the collagen). **(G)** Quantification of total collagen content in the infarct site by ImageJ. Data are mean ± SD. *$p < 0.05$ treated versus control. +$p < 0.05$ treated versus xenogeneic.

**Changes in infarct size and left ventricle morphometry.** At 1 week or 3 weeks after cell transplantation, scar size was reduced in both syngeneic and allogeneic groups compared to the control and xenogeneic groups; the difference was maintained up to 6 months (Figs. 3A and 3B). In addition, infarct wall thickness was greater in the syngeneic and allogeneic cardiosphere-treated groups. Areas of viable myocardium were regularly evident within the infarct region, in contrast to the thin, transmural scar tissue that prevailed in the control and xenogeneic groups (Fig. 3C). We also quantified thinning ratio, which is defined as the ratio of the infarct wall thickness to contralateral noninfarcted wall thickness (24). Both syngeneic and allogeneic cardiosphere injections maintained a higher ratio, reflecting relatively preserved cardiac architecture compared to the remodeled vehicle-treated group (Figs. 3D and 3E).

**Collagen content in the infarct region.** Given the reductions of infarct size with cardiospheres (Fig. 3B), we looked for a potential antifibrotic effect as part of the mechanism of action. Three weeks post-MI, collagen content within the infarct region was higher in the control group than in either treated group, reflecting the presence of more viable tissue (Figs. 3F and 3G). Specifically, the total amount of collagen within the infarct area was reduced by 15% in the syngeneic and 17% in the allogeneic group relative to control (Fig. 3G). These results support the hypothesis that both limitation of collagen accumulation in the scar, as well as myocyte

regeneration and/or preservation, underlie the functional improvement due to cardiosphere injection.

**Lymphocyte proliferative response.** The coculture of cardiospheres with lymphocytes caused modest lymphocyte proliferation, which did not differ between syngeneic and allogeneic groups at 1 week ($p = 0.096$) or 3 weeks ($p = 0.7$) (Fig. 4A). The stimulation index, defined as the ratio of allogeneic to syngeneic proliferation (25), was $1.23 \pm 0.1$ at 1 week and $1.1 \pm 0.038$ at 3 weeks. Although there seemed to be a reduction in lymphocyte proliferation from 1 week to 3 weeks, the difference was not significant. In the xenogeneic group, which was included as a positive control, the proliferation ratio ranged from $5.2 \pm 0.86$ at week 1, to $4 \pm 0.14$ at 3 weeks (Fig. 4A), verifying the sensitivity of the mixed lymphocyte reaction assay.

**Reduction of proinflammatory cytokines in the coculture of syngeneic and allogeneic lymphocytes with cardiospheres.** As shown in Figure 4B, the proinflammatory cytokines IFN-$\gamma$, IL-1$\beta$, IL-2, IL-6, and TNF-$\alpha$ remained at baseline levels when cardiospheres were cocultured with syngeneic or allogeneic lymphocytes, in contrast to the xenogeneic group where these cytokines were elevated.

**Monocyte infiltration.** Hematoxylin and eosin–stained sections did not reveal signs of immune rejection such as edema, hemorrhage, or necrosis in either the syngeneic or allogeneic group. Such was the case in both peri-infarct and remote areas examined 1 and 3 weeks post-MI (Figs. 4C to 4F). Monocyte infiltration actually tended to be diminished in both treated groups at day 21. In contrast, robust cell-mediated rejection (a term adopted for the purposes of this study according to the International Society of Heart and Lung Transplantation criteria [26]) was evident in the xenogeneic group at both time points, with pleomorphic cell infiltration including numerous lymphocytes. Hemosiderin accumulation and coagulative necrosis, sequelae of myocardium broken down by local inflammation, were present only in the xenogeneic group. Quantitative immunohistochemistry 7 days after cardiosphere injection showed that CD 45RA and macrophage infiltration tended to be lower in both syngeneic and allogeneic groups compared to control (allogeneic $45 \pm 12$, syngeneic $30 \pm 10$, and control $60 \pm 17$ for CD 45RA monocytes; and allogeneic $68 \pm 24$, syngeneic $60 \pm 25$, and control $85 \pm 34$ cells/mm$^2$ for macrophages), whereas by day 21, they decreased in number and could barely be identified in the border zone of the infarcted area. Infiltrating lymphocytes exhibited the same pattern: no difference between syngeneic or allogeneic groups and control at day 7, when the peak in their proliferation has been reported (27) (Figs. 4E and 4F) (allogeneic $225 \pm 55$, syngeneic $215 \pm 53$, control $187 \pm 35$ cells/mm$^2$), but a decrease below control levels at day 21, indicating attenuated chronic inflammation after MI in syngeneic and allogeneic hearts. In the xenogeneic group, however, much greater mononuclear and polynuclear cell infiltration was present at days 7 and 21, reflecting cell-mediated rejection and intensified local inflammation (CD 45RA cells $534 \pm 12$, macrophages $708 \pm 20$, lymphocytes $1{,}225 \pm 35$ cells/mm$^2$ ) (Figs. 4E and 4F).

**Reduced proinflammatory cytokines in the border zone.** Cytokines are among the major triggers of the inflammatory response after cardiac tissue damage (28). At day 1 after cell transplantation, there was no significant elevation of TNF-$\alpha$, IFN-$\gamma$, IL-6, and IL-1$\beta$ in the syngeneic or allogeneic groups relative to controls, consistent with the lack of an enhanced inflammatory response due to cell injections ($p > 0.05$ for treated vs. control group) (Fig. 5A). Interestingly, 7 days after treatment, tissue levels of the same cytokines tended to be reduced in both treated groups compared to control evaluated by cytokine array (syngeneic $p = 0.059$, allogeneic $p = 0.1$ vs. control) (Figs. 5B and 5C). The latter finding may reflect an immunomodulatory effect of cardiospheres, attenuating the post-MI inflammatory milieu. Such a conjecture is bolstered by the reduction of tissue inflammation seen with either syngeneic or allogeneic cardiosphere injection.

**Production of growth factors after cardiosphere injection.** Consistent with the previously described secretion of soluble growth factors by cardiospheres (9) or CDCs (17), we identified higher expression of VEGF, IGF, and HGF in the peri-infarct region in both syngeneic and allogeneic groups compared to control. More specifically, at day 1, VEGF and IGF were up-regulated 2-fold, and HGF threefold, compared to control ($p < 0.05$ for treated vs. control), whereas at day 7, only HGF remained elevated (Figs. 6A to 6C). In the xenogeneic group, cytokine expression was evaluated only at day 1, given the evanescence of transplanted xenogeneic cardiosphere survival. The modest elevations in cytokine levels relative to the control group level did not reach significance. We also evaluated the expression of the same growth factors in the remote area (defined here as the septum) (Online Fig. 3). An increase in the expression of VEGF, HGF, and IGF in this region was observed in both treated groups at day 1, a finding that supports the paracrine role of the injected cells and reflects the better-preserved myocardial structure in this region.

As a structural correlate of the observed changes in growth factor secretion, allogeneic cardiospheres performed equally to syngeneic ones with regard to promoting tissue angiogenesis. Capillary density evaluated by immunohistochemistry 21 days post-MI revealed twice the number of capillaries positive for von Willebrand factor in the peri-infarct zone of both syngeneic and allogeneic animals compared to the control (Figs. 6D and 6E). The changes in growth factor secretion may reflect, at least partially, the anti-remodeling effects described earlier and highlight the significant role of capillary genesis in the salutary effects of cardiospheres.

## Discussion

We find that allogeneic cardiospheres exert beneficial functional effects equal to those of syngeneic ones, without

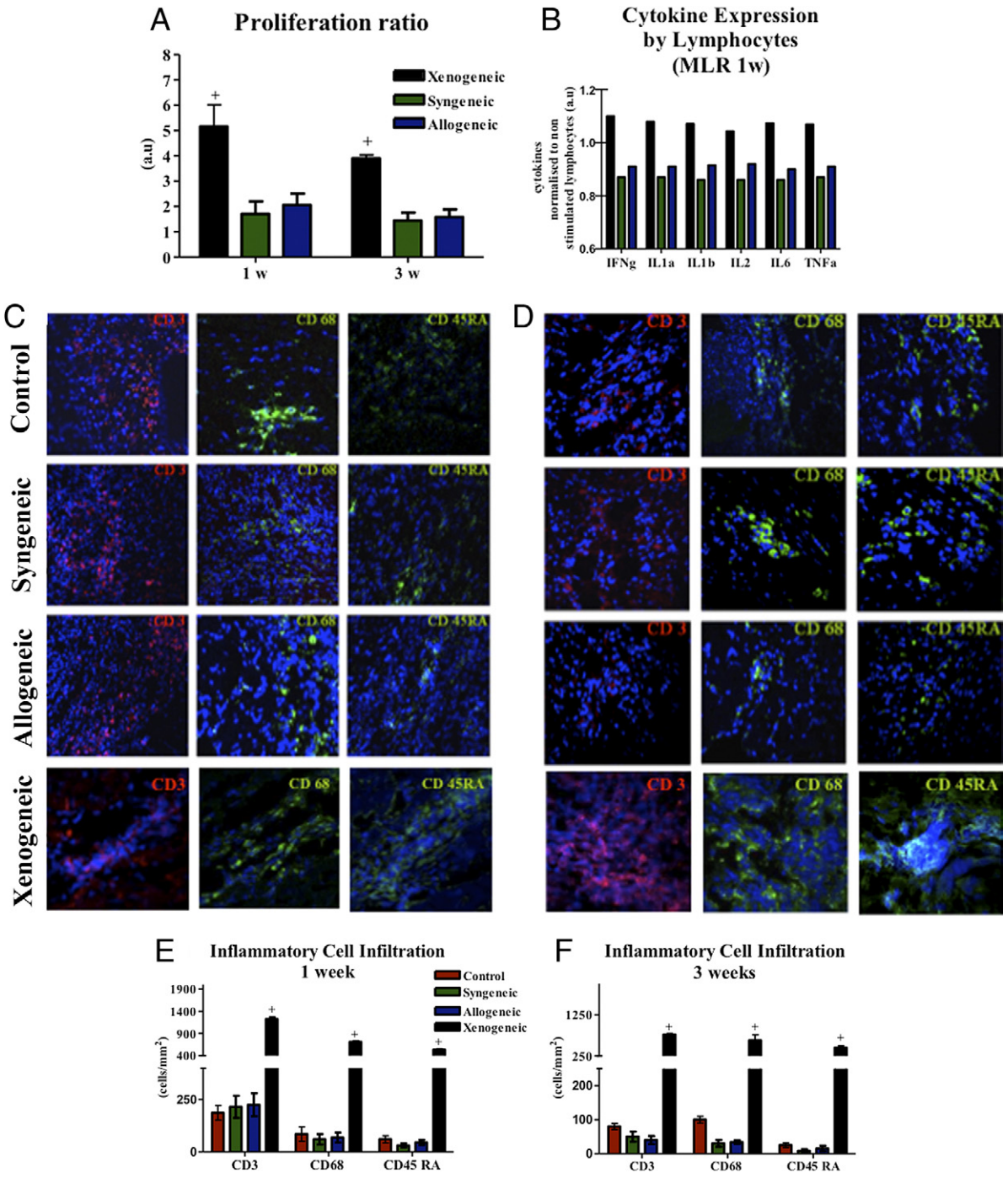


**Figure 4** Lymphocyte Proliferation Assays and Tissue Mononuclear Infiltration

**(A)** Mixed lymphocyte reactions reveal a lack of lymphocyte proliferation in the presence of allogeneic cardiospheres. **Black bars** indicate xenogeneic; **green bars** indicate syngeneic; and **blue bars** indicate allogeneic. Lymphocytes ($1 \times 10^6$ responder cells) were stimulated with $10^5$ mytomycin C-treated cardiospheres for 5 days. The data are expressed as mean ± SD of 7 independent experiments at 1 week and 5 experiments at 3 weeks, each performed in triplicate. **(B)** Proinflammatory cytokines in the supernatant of the mixed lymphocyte reactions (MLR). The levels of the cytokines secreted in coculture are normalized to the levels secreted by nonstimulated lymphocytes. **(C, D)** Confocal immunohistochemistry images of inflammatory cell infiltration at 1 week and 3 weeks, respectively. **(E, F)** Quantitation of CD3 (lymphocytes), CD68 (macrophages), and CD 45RA (B monocytes) 1 week and 3 weeks after cardiosphere injection reveal marked inflammatory reaction in the xenogeneic hearts but no excess inflammation in syngeneic or allogeneic groups relative to control **(red bars)**; indeed, there is a tendency to less inflammation at 3 weeks in both syngeneic and allogeneic groups. Data are mean ± SEM. *$p < 0.05$ treated versus control. +$p < 0.05$ treated versus xenogeneic.

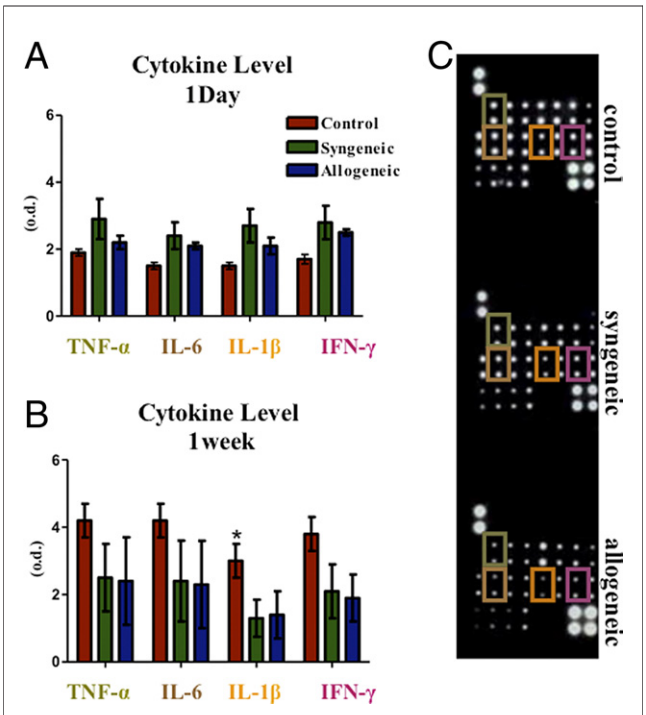


**Figure 5** Cytokines in the Peri-Infarct Area

**(A, B)** Levels of tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-6, IL-1$\beta$, and interferon (IFN)-$\gamma$ at 1 day and 7 days after cardiosphere injection reveal suppression of cytokine levels in the syngeneic group **(green bars)** and allogeneic group **(blue bars)** relative to control **(red bars)**. **(C)** Representative images of the cytokine array at day 7. Colors of rectangular margins match the labels of each of the cytokines evaluated in **A**. Data are expressed as mean ± SD. *$p < 0.05$.

eliciting deleterious immune reactions. Cardiospheres improved global LV function as early as 7 days after transplantation, and attenuated adverse remodeling; these beneficial outcomes were maintained for at least 6 months, far beyond the time when allogeneic cardiospheres had been cleared (by 3 weeks). The sustained benefit, despite the evanescent survival of transplanted cardiospheres, indicates that <3 weeks suffices to set in motion dynamic and durable mechanisms responsible for cardiac regeneration and repair (17), such as the capillary neoangiogenesis and the attenuation of collagen accumulation reported for the first time in our study. Moreover, the initial significant anti-remodeling effect was further enhanced by day 21, despite the disappearance of the allogeneic cardiospheres by that time, further supporting the "role model" hypothesis whereby cardiac-derived cells have been proposed to trigger endogenous repair processes (17).

The salutary effect of allogeneic cardiospheres to blunt adverse ventricular remodeling was accompanied by a 38% reduction in scar size at day 7, and a 32% thicker infarcted wall, than in control (Figs. 3A and 3B). Masson's trichrome and Sirius red-stained tissue sections revealed layers of viable mass within the risk area, in contrast to the thin, more densely fibrotic scar of the control group at day 21, indicating less stiffness in this myocardial territory (29). In addition, the thinning ratio, which indexes both infarct wall thinning as well as compensatory hypertrophy in remote myocardium, was higher in both treated groups compared to control. Thus, cardiospheres lead to enhanced tissue preservation and regeneration in MI, which subsequently minimizes negative remodeling in remote myocardium described in the current study. Meanwhile, growth factors are more plentiful, as are angiogenic factors (30–35), leading to a twofold increase of angiogenesis in peri-infarct areas by 3 weeks (Fig. 6E).

Although injection of allogeneic cardiospheres might logically be expected to elicit an immune response, their immunological phenotype and the histopathological results demonstrate otherwise. The expression of MHC I on the outer layer is important because it protects cardiospheres against the cytotoxic effect of NK cells, while the absence of MHC II gives them the potential to escape recognition by alloreactive CD4 lymphocytes (16,36). In addition to being MHC II negative, cardiospheres did not express the costimulatory molecules CD 80 and CD 86 required for induction of effector lymphocytes, supporting the hypoimmunogenic profile of our preformed microtissues.

Previous in vitro studies from our laboratory (10) have shown that cardiospheres under normal culture conditions, besides their remarkable expression of stemness factors, express cytokines including IL-6, IL-7, IL-8, transforming growth factor-$\beta$, macrophage colony-stimulating factor, and the growth factors HGF and VEGF, which are known to mute local immune reactions (36,37). These cardiosphere-secreted factors may blunt the T lymphocyte response, leading to an evasion of alloreactivity.

Consistent with these findings are our in vitro mixed lymphocyte reaction data. Allogeneic cardiospheres did not elicit greater proliferation to lymphocytes than syngeneic cardiospheres either at 1 week or at 3 weeks after injection. Moreover, the expression of proinflammatory cytokines was suppressed in both syngeneic and allogeneic lymphocyte coculture supernatant. Interestingly, a moderate alleviation of proliferation at 3 weeks was observed, highlighting the hypoimmunogenic phenotype of cardiospheres, which consequently resulted in an immunomodulatory profile in the hostile post-infarct environment.

Indeed, we have found a minimal innate and adaptive alloimmune cellular response after allogeneic transplantation. Macrophages, which are the first responders of innate immunity, were present in a similar proportion in the treated groups and in the control group at day 7, whereas by day 21, they were barely apparent in cardiosphere-transplanted myocardium. In addition, infiltrating CD3 T cells were similar in number in treated and control groups at day 7, with few cells found on day 21. Although we did not examine CD 68 infiltration earlier than 7 days, the tissue cytokine data at day 1 suggest an attenuated inflammatory response which was further suppressed by day 7. The 4 proinflammatory cytokines examined—TNF-$\alpha$, IFN-$\gamma$, IL-

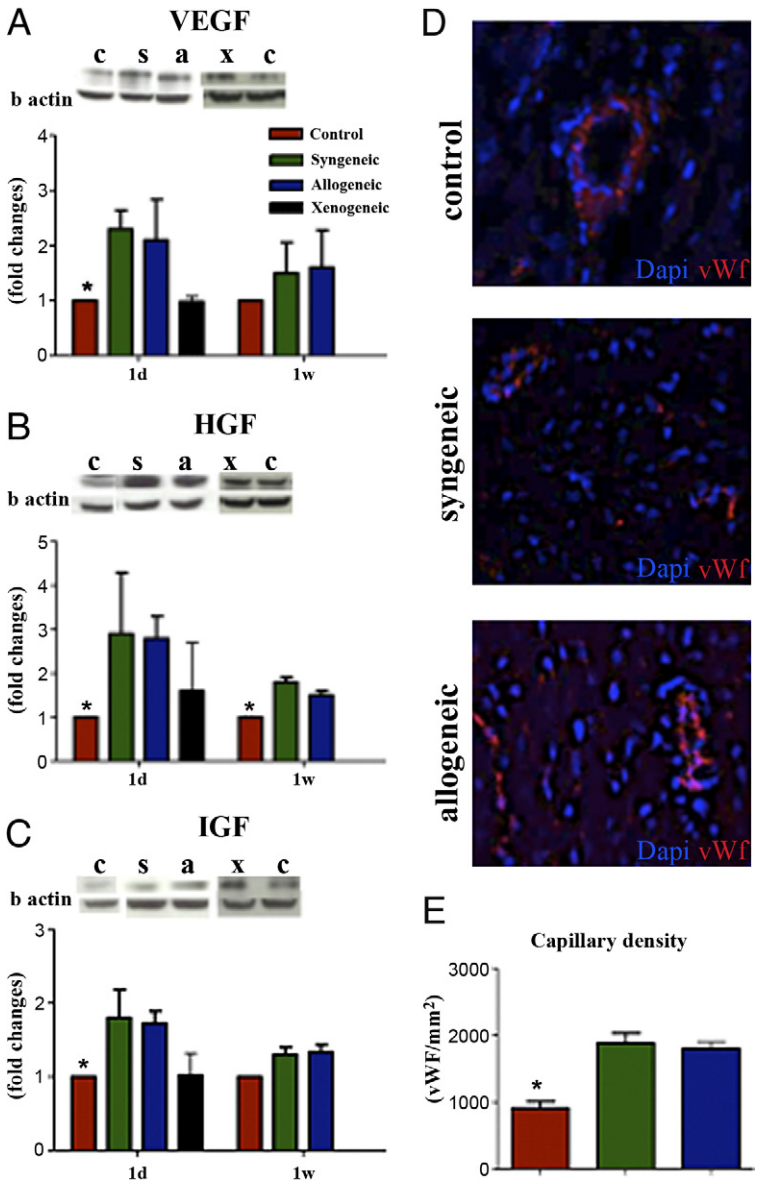


**Figure 6** **Paracrine Factors in the Peri-infarct Myocardium**

Quantification of angiogenic and pro-survival growth factors **(A)** vascular endothelial growth factor (VEGF), **(B)** hepatocyte growth factor (HGF), and **(C)** insulin-like growth factor (IGF) reveal enhanced levels in both the syngeneic group **(green bars)** and the allogeneic group **(blue bars)** 1 day and 7 days after cardiosphere injection. **Black bars** indicate xenogeneic. Data are presented as fold-changes of each factor relative to control. Representative images from the Western blots at day 1 appear above the graphs. **(D)** Confocal images of capillaries in the peri-infarct area 3 weeks after cardiosphere transplantation: **blue** indicates 4',6-diamidino-2-phenylindole (DAPI); **red** indicates von Willebrand factor (vWf). **(E)** Pooled data show increased capillary density in syngeneic and allogeneic groups as compared to control. Data are mean ± SD. *$p < 0.05$ treated versus control.

1$\beta$, and IL-6—are known to be robustly up-regulated within the first few hours of MI, but can be further increased in larger infarcts and in the chronic remodeling phase (28,37). Cardiosphere injection led to a hampered expression of these cytokines 24 h after MI and cell transplantation.

Another in vivo study from our laboratory (38) addressed the immune response to allogeneic CDCs in a similar model. As seen here with cardiospheres, allogeneic CDCs elicited no additional inflammatory response relative to syngeneic cells (38), but the most important immunologic finding of our study is that allogeneic cardiospheres did not trigger an additional inflammatory response even when compared to the vehicle-treated group. Cardiospheres have superior functional and morphological effects as compared to CDCs, when both are delivered by direct intramyocardial injection (9,12), whereas CDCs have been shown to outperform other stem cell types currently in the clinic (14).

The pronounced ability of cardiospheres to secrete immunomodulatory cytokines (9,17) may contribute to their enhanced functional benefits and may be valuable even in the chronic remodeling inflammatory phase, but this conjecture needs to be tested further given the different immune conditions present in the last case (28).

## Conclusions

The large functional and morphological benefits of allogeneic cardiospheres in acute MI highlight the therapeutic potential of these cardiac microtissues. Their hypoimmunogenic phenotype enables cardiospheres to evade alloimmune reactions and to modify the proinflammatory milieu created after MI. A key unanswered question is the relative therapeutic value of cardiospheres and CDCs, especially in light of the significant regenerative potential of the latter in humans revealed in the CADUCEUS trial (6). Potential differences between our 2 cell products in terms of efficacy will have to be weighed against the fact that CDCs can be readily delivered through the coronary route, whereas cardiospheres require more cumbersome intramyocardial injection. More experiments using clinically realistic delivery methods in large-animal models will be required to determine the appropriate development pathway for allogeneic cardiospheres relative to allogeneic CDCs (which are already advancing to phase 2 in the ALLSTAR (Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration) (39).

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### REFERENCES

1. Hughes S. Cardiac stem cells. J Pathol 2002;197:468–78.
2. Lipinski MJ, Biondi-Zoccai GG, Abbate A, et al. Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials. J Am Coll Cardiol 2007;50:1761–7.
3. 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.
4. Malliaras K, Kreke M, Marbán E. The stuttering progress of cell therapy for heart disease. Clin Pharmacol Ther 2011;90:532–41.
5. Gersh BJ, Simari RD, Behfar A, Terzic CM, Terzic A. Cardiac cell repair therapy: a clinical perspective. Mayo Clin Proc 2009;84:876–92.
6. Makkar RR, Smith RR, Cheng K, et al. Intracoronary Cardiosphere-Derived Cells for Heart Regeneration After Myocardial Infarction (CADUCEUS): a prospective, randomised phase 1 trial. Lancet 2012;379:895–904.
7. Smith RR, Barile L, Cho HC, et al. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation 2007;115:896–908.
8. Messina E, De Angelis L, Frati G, et al. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 2004;95:911–21.
9. Li TS, Cheng K, Lee ST, et al. Cardiospheres recapitulate a niche-like microenvironment rich in stemness and cell-matrix interactions, rationalizing their enhanced functional potency for myocardial repair. Stem Cells 2010;28:2088–98.
10. Johnston PV, Sasano T, Mills K, et al. Engraftment, differentiation, and functional benefits of autologous cardiosphere-derived cells in porcine ischemic cardiomyopathy. Circulation 2009;120:1075–83.
11. Davis DR, Kizana E, Terrovitis J, et al. Isolation and expansion of functionally competent cardiac progenitor cells directly from heart biopsies. J Mol Cell Cardiol 2010;49:312–21.
12. Lee ST, White AJ, Matsushita S, et al. Intramyocardial injection of autologous cardiospheres or cardiosphere-derived cells preserves function and minimizes adverse ventricular remodeling in pigs with heart failure post-myocardial infarction. J Am Coll Cardiol 2011;57:455–65.
13. Yee K, Malliaras K, Kanazawa H, et al. Dose-dependent regenerative efficacy and functional improvement in pigs with ischemic cardiomyopathy injected transendocardially with allogeneic cardiospheres. AHA Scientific Sessions 2011 LBS.02.26360.
14. Li TS, Cheng K, Malliaras K, et al. Direct comparison of different stem cell types and subpopulations reveals superior paracrine potency and myocardial repair efficacy with cardiosphere-derived cells. J Am Coll Cardiol 2012;59:942–53.
15. Thomas ED, Blume KG. Historical markers in the development of allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transplant 1999;5:341–6.
16. Buja LM, Vela D. Immunologic and inflammatory reactions to exogenous stem cells implications for experimental studies and clinical trials for myocardial repair. J Am Coll Cardiol 2010;56:1693–700.
17. Chimenti I, Smith RR, Li TS, et al. Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. Circ Res 2010;106:971–80.
18. Stewart R, Butcher G, Herbert J, Roser B. Graft rejection in a congenic panel of rats with defined immune response genes for MHC class I antigens. I. Rejection of and priming to the RT1Aa antigen. Transplantation 1985;40:427–32.
19. Li TS, Hayashi M, Ito H, et al. Regeneration of infarcted myocardium by intramyocardial implantation of ex vivo transforming growth factor-beta preprogrammed bone marrow stem cells. Circulation 2005;111:2438–45.
20. Bersell K, Arab S, Haring B, Kühn B. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. Cell 2009;138:257–70.
21. Junqueira LC, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J 1979;11:447–55.
22. Di Nicola M, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 2002;99:3838–43.
23. Davis DR, Zhang Y, Smith RR, et al. Validation of the cardiosphere method to culture cardiac progenitor cells from myocardial tissue. PLoS One 2009;4:e7195.
24. Jugdutt BI, Joljart MJ, Khan MI. Rate of collagen deposition during healing and ventricular remodeling after myocardial infarction in rat and dog models. Circulation 1996;94:94–101.
25. Talpe S, Oike F, Dehoux JP, et al. Posttransplant lymphoproliferative disorder after liver transplantation in miniature swine. Transplantation 2001;71:1684–8.
26. Patel JK, Kittleson M, Kobashigawa JA. Cardiac allograft rejection. Surgeon 2011;9:160–7.
27. Varda-Bloom N, Leor J, Ohad DG, et al. Cytotoxic T lymphocytes are activated following myocardial infarction and can recognize and kill healthy myocytes in vitro. J Mol Cell Cardiol 2000;32:2141–9.
28. Frangogiannis NG. Regulation of the inflammatory response in cardiac repair. Circ Res 2012;110:159–73.
29. Braunwald E. Biomarkers in heart failure. N Engl J Med 2008;358:2148–59.
30. Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 2008;103:1204–19.
31. Iekushi K, Seeger F, Assmus B, Zeiher AM, Dimmeler S. Regulation of cardiac microRNAs by bone marrow mononuclear cell therapy in myocardial infarction. Circulation 2012;125:1765–73.

32. Angelini A, Castellani C, Ravara B, et al. Stem-cell therapy in an experimental model of pulmonary hypertension and right heart failure: role of paracrine and neurohormonal milieu in the remodeling process. J Heart Lung Transplant 2011;30:1281–93.
33. Urbich C, Aicher A, Heeschen C, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 2005;39:733–42.
34. Cittadini A, Monti MG, Petrillo V, et al. Complementary therapeutic effects of dual delivery of insulin-like growth factor-1 and vascular endothelial growth factor by gelatin microspheres in experimental heart failure. Eur J Heart Fail 2011;13:1264–74.
35. Webber MJ, Tongers J, Newcomb CJ, et al. Supramolecular nanostructures that mimic VEGF as a strategy for ischemic tissue repair. Proc Natl Acad Sci U S A 2011;108:13438–43.
36. Tasso R, Pennesi G. When stem cells meet immunoregulation. Int Immunopharmacol 2009;9:596–8.
37. Nian M, Lee P, Khaper N, Liu P. Inflammatory cytokines and postmyocardial infarction remodeling. Circ Res 2004;94:1543–53.
38. Malliaras K, Li TS, Luthringer D, et al. Safety and efficacy of allogeneic cell therapy in infarcted rats transplanted with mismatched cardiosphere-derived cells. Circulation 2012;125:100–12.
39. ALLSTAR. Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration. Available at: clinicaltrials.gov/nct01458405. Accessed October 20, 2011.

**Key Words:** allogeneic cardiospheres ■ high therapeutic potential ■ safe administration.

**APPENDIX**

**For supplemental figures, please see the online version of this article.**