Background—Cardiac cell therapy for older patients who experience a myocardial infarction may require highly regenerative cells from young, healthy (allogeneic) donors. Bone marrow mesenchymal stem cells (MSCs) are currently under clinical investigation because they can induce cardiac repair and may also be immunoprivileged (suitable for allogeneic applications). However, it is unclear whether allogeneic MSCs retain their immunoprivilege or functional efficacy late after myocardial implantation. We evaluated the effects of MSC differentiation on the immune characteristics of cells in vitro and in vivo and monitored cardiac function for 6 months after post-myocardial infarction MSC therapy.

Methods and Results—In the in vitro experiments, inducing MSCs to acquire myogenic, endothelial, or smooth muscle characteristics (via 5-azacytidine or cytokine treatment) increased major histocompatibility complex-Ia and -II (immunogenic) expression and reduced major histocompatibility complex-Ib (immunosuppressive) expression, in association with increased cytotoxicity in coculture with allogeneic leukocytes. In the in vivo experiments, we implanted allogeneic or syngeneic MSCs into infarcted rat myocardia. We measured cell differentiation and survival (immuno-histochemistry, real-time polymerase chain reaction) and cardiac function (echocardiography, pressure-volume catheter) for 6 months. MSCs (versus media) significantly improved ventricular function for at least 3 months after implantation. Allogeneic (but not syngeneic) cells were eliminated from the heart by 5 weeks after implantation, and their functional benefits were lost within 5 months.

Conclusions—The long-term ability of allogeneic MSCs to preserve function in the infarcted heart is limited by a biphasic immune response whereby they transition from an immunoprivileged to an immunogenic state after differentiation, which is associated with an alteration in major histocompatibility complex–immune antigen profile. (Circulation. 2010;122:2419-2429.)

Key Words: stem cells ■ immune system ■ myocardial infarction ■ transplantation

Bone marrow mesenchymal stem cells (MSCs) have been widely investigated for their potential to prevent cardiac dysfunction after a myocardial infarction (MI). In preclinical studies conducted with young animals, implanted MSCs effectively restored ventricular function after acute or chronic MI.1,2 The early clinical trials with aging patients demonstrated statistically significant, but comparatively limited, beneficial effects on ventricular volumes and ejection fraction when the patients received autologous MSCs.3 This muted response was due largely to an age-related decrease in the regenerative capacity of the patients’ cells, as demonstrated in studies that examined age-related changes in autologous progenitor cells.4,5 A source of highly regenerative donor cells would thus dramatically advance the prevention of congestive heart failure in aged patients who have multiple comorbidities.

Clinical Perspective on p 2429

Allogeneic MSCs isolated from healthy, young donors are promising candidate cells because MSCs have low cell surface expression of immunogenic proteins from the major histocompatibility complex (MHC) and they secrete immunosuppressive cytokines after interaction with a host.6,7 Indeed, allogeneic MSCs can restore cardiac function early after an MI in animals, and results from the initial clinical trials are promising.8,9 However, most studies measured functional improvements within 3 months of MSC implantation, and so it remains unclear whether these cells would retain their unique immune characteristics in the infarcted myocardium after prolonged engraftment. Some groups reported that allogeneic MSCs were immunosuppressive in vivo or improved cardiac function after implantation into the...
infarcted myocardium,10–12 but others found that the cells were recognized by the host immune system, elicited cellular and humoral immune responses, and were immune rejected.13,14 The reason for this inconsistency and the ultimate fate of MSCs from allogeneic donors need to be determined to aid the design of the next series of clinical investigations (see the article by Kinkaid et al15).

Engrafted MSCs can differentiate into multiple cell types in the infarcted heart, including myogenic, endothelial, and smooth muscle cells.12 However, the effect of differentiation on the expression profile of MHC proteins in allogeneic MSCs is largely unknown. We hypothesized that differentiation of MSCs leads to loss of immunoprivilege. In the context of cell therapy, this could promote a delayed immune rejection of allogeneic MSCs (after differentiation) and the loss (over time) of the ability of the cells to preserve ventricular function. To test this theory, we identified the effects of multiple-lineage MSC differentiation (in vitro or in vivo) on cellular antigen profile and leukocyte toxicity and measured host immune responses and long-term functional outcomes after allogeneic MSCs were implanted into the infarcted myocardium.

Methods
Detailed methodology is provided in the online-only Data Supplement.

Experimental Animals
We used male Wistar and Lewis rats (allogeneic and syngeneic cell donors, respectively) and female inbred Lewis rats (cell recipients; Charles River Canada, Senneville, Quebec, Canada). To study MSC differentiation and immune antigen expression in vivo, we obtained MSCs from male, green fluorescent protein–positive (GFP+) Wistar rats (YS Institute, Inc, Utsunomiya, Tochigi, Japan). Sample sizes for each in vivo experiment are listed in Table I in the online-only Data Supplement. All animal procedures were approved by the Animal Care Committee of the University Health Network, and all animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, No. 85-23, revised 1996).

MSC Preparation and Differentiation In Vitro
MSCs were isolated from donor femurs and tibias and then cultured with 5-azacytidine to induce myogenic differentiation. Differentiated cells were assessed for the expression of myogenic genes or proteins. MSCs with myogenic characteristics were counted with flow cytometry to detect cells labeled with an anti–β-myosin heavy chain (β-mhc) antibody (n=6).

To induce differentiation toward endothelial or smooth muscle cells, MSCs were cultured in media containing FBS with either vascular endothelial growth factor (VEGF) or transforming growth factor-β (TGF-β), respectively. Differentiation along the appropriate lineage was confirmed with immunostaining for factor VIII (endothelial) or α-smooth muscle actin (α-SMA) (n=6).

Immune Antigen Expression in MSCs Differentiated In Vitro
Expression of MHC class I (MHC-Ia and MHC-Ib), MHC class II (MHC-II), and CD86 genes was evaluated in differentiated and undifferentiated MSCs by the use of reverse-transcription polymerase chain reaction (RT-PCR). MHC proteins were detected through the use of immunohistochemical staining with antibodies against MHC-I and -II. The number of cells expressing MHC-I or -II proteins was quantified with flow cytometry (n=6 to 7).

Leukocyte-Mediated Cytotoxicity and Leukocyte Proliferation
Mixed peripheral blood leukocytes (PBLs; 5×10⁶) were isolated and cocultured with differentiated (5-azacytidine–treated) or undifferentiated allogeneic or syngeneic MSCs (5×10⁶) in 24-well plates. After 3 days, leukocyte-mediated cytotoxicity was estimated by measuring lactate dehydrogenase (LDH) released from damaged cells. Leukocyte proliferation was evaluated in 2 different ways: flow cytometric assessment of carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled PBLs in coculture with irradiated allogeneic MSCs (n=4 experiments) or immunohistochemical assessment of BrdU uptake by PBLs in coculture with allogeneic MSCs (n=5 to 6).

MSC Differentiation and Immune Antigen Expression In Vivo
Female Lewis rats underwent left coronary artery ligation (MI). Three weeks later, allogeneic GFP+ MSCs isolated from male GFP+Wistar rats (3×10⁶ per rat) were injected into the infarct. No immunosuppressive agents were administered during the course of the study. Immunohistochemical staining identified GFP (identify implanted cells), α-SMA, factor VIII, or β-mhc (smooth muscle, endothelial, or myogenic cell markers, respectively) and either MHC-Ia or MHC-II (immune antigens) in tissue obtained at days 3, 7, and 14 after cell implantation (n=6 rats per group).

Postimplantation Immune Rejection of Differentiated MSCs
Three weeks after MI in Lewis rats, allogeneic MSCs isolated from Wistar rats (undifferentiated or induced to differentiate with 5-azacytidine; 3×10⁶ per rat) were prelabeled with DiI (Molecular Probes, Eugene, Ore) and then injected into the infarct. We labeled the MSCs with DiI rather than GFP to avoid the potential for a confounding immune response triggered by the GFP transgene, which can be immunogenic and can affect the contractility of cardiomyocytes.16 The hearts were harvested and frozen at days 3 and 7 after cell implantation, and sections containing the scar were stained with DAPI (n=6 rats per group). DiI fluorescence intensity was measured in 5 randomly selected fields (under the ×20 objective) per section with a Nikon ECLIPSE Ti microscope.

Long-Term Fate and Functional Effects of Undifferentiated MSCs Implanted Into the Infarcted Myocardium
Female Lewis rats underwent MI. Three weeks later, allogeneic or syngeneic MSCs (non-GFP, as described above for the in vivo immune rejection experiments) isolated from male Wistar or Lewis rats, respectively (3×10⁶ per rat), or media (control) was injected into the infarct. No immunosuppressive reagents were administrated during the course of the study.

Host Immune Responses and Cell Survival
We evaluated cytokine expression in the recipient hearts at 1 and 7 days after cell implantation (n=6), leukocyte infiltration in the implanted area at 1 week (n=6), and expression of an alloantibody against the donor MSCs in the recipient circulation at 1 and 5 weeks (n=12). To estimate the number of MSCs remaining in the recipient hearts at 1, 7, or 35 days after cell implantation, we used real-time PCR to determine the number of Y chromosomes in the implanted area (n=7).

Cardiac Function and Morphometry
Echocardiography was performed before and 3 weeks after MI (before cell implantation) and at the following time points after implantation: 1, 5, 8, 12, 16, 20, and 24 weeks. Measurements were taken with a pressure-volume catheter at 5 weeks (n=5 for media group; n=15 to 16 for cell groups) and 24 weeks (6 months; n=5 to 7) after cell implantation. After functional analysis at 5 or 24 weeks after cell implantation, hearts were either immediately frozen in liquid nitrogen (for genomic DNA/RNA extraction; n=6 to 7) or fixed for

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morphometric assessment of scar length and thickness (n=6 to 7) or immunohistochemical assessment of blood vessel density.

**Statistical Analyses**

Cell implantation and cardiac functional measurements were carried out in a blinded fashion. All data were analyzed with GraphPad software and are expressed as mean±SD unless otherwise indicated. Comparisons between 2 groups were made with a 2-tailed Student t tests (The Welch correction for the Student t test was applied when unequal variances were identified). Comparisons among multiple groups were made with 1-way ANOVAs (except those involving the cardiac functional data measured using echocardiography, which were analyzed with a repeated-measures ANOVA). When F values were significant, group differences were specified with the Tukey multiple-comparison posttests (or Bonferroni posttests for the repeated-measures ANOVA). Differences were considered statistically significant when P<0.05.

**Results**

**MSC Differentiation Alters Cellular Immunogenicity In Vitro and In Vivo**

**MSC Differentiation and Immune Antigen Expression In Vitro**

MSCs cultured with 5-azacytidine (to induce myogenic differentiation) formed myotube-like structures after 2 weeks (Figure 1A). Compared with untreated MSCs, 5-azacytidine–treated MSCs exhibited upregulated expression of the myogenic-specific genes Nkx2.5, MyoD, and β-mhc (Figure 1B) and contractile proteins β-mhc, α-sarcomeric actinin, and troponin I (arrows in C, by immunostaining). GAPDH is the housekeeping gene; DAPI, the nuclear stain. D and E, By flow cytometry, significantly more dMSCs (>50%) than MSCs expressed β-mhc.

![Figure 1. Evidence for myogenic differentiation.](image-url)
differentiation was associated with a $\approx 30\%$ decrease in the percentage of cells expressing MHC-Ib molecules ($P<0.001$; Figure 2B, 2C, 2E, and 2F).

We confirmed these results in MSCs induced to differentiate using cytokines (TGF-$\beta$ or VEGF) rather than 5-azacytidine. Compared with undifferentiated MSCs, those with characteristics of smooth muscle cells (alpha-SMA$^+$ cells) or endothelial cells (factor VIII$^+$ cells) had increased expression of MHC-Ia and reduced expression of MHC-Ib molecules. The differentiated MSCs also expressed MHC-II (Figure I in the online-only Data Supplement).
MSC Differentiation and Immune Antigen Expression in the Infarcted Myocardium

Allogeneic GFP⁺ MSCs were implanted into the infarcted myocardium 3 weeks after MI in rats. Immunostaining was used to identify the implanted cells (GFP), MSCs that expressed markers of differentiated cells (smooth muscle cells [α-SMA], endothelial cells [factor VIII], or myogenic cells [β-mhc]), and immune antigens (MHC-Ia, MHC-II) at various time points after cell implantation. At days 3 and 7 after implantation, the cells appeared to remain undifferentiated (no α-SMA expression) and expressed low levels of MHC-Ia. By 14 days after implantation, the MSCs expressed both α-SMA (smooth muscle cell marker) and high levels of MHC-Ia (Figure 3A). Some of the implanted MSCs coexpressed factor VIII or β-mhc (endothelial or myogenic markers, respectively) and high levels of MHC-Ia (Figure 3B) at this time point, and differentiated cells of all 3 lineages also expressed MHC-II (Figure 3C).

Differentiated MSCs Initiate Leukocyte Cytotoxicity In Vitro and In Vivo

To examine the relationship between immune antigen upregulation in the differentiated MSCs and immune rejection, we measured leukocyte proliferation and cytotoxicity in cocultures of PBLs and differentiated (5-azacytidine–treated) or undifferentiated MSCs (allogeneic or syngeneic). No cell toxicity (LDH release) was observed in the cocultures containing syngeneic cells or undifferentiated allogeneic MSCs. However,
we measured significant cytotoxicity in the cocultures that contained differentiated allogeneic MSCs (P<0.001; Figure 4A). Corresponding to these results were increases in leukocyte proliferation (identified by BrdU uptake and the division of CFSE-labeled leukocytes) and the activation of CD3⁺, CD4⁺, and CD8α⁺ cells after leukocytes were cocultured with myogenic differentiated allogeneic MSCs (versus undifferentiated MSCs) (Figure 4B through 4D).

Findings were similar when cytokine-differentiated MSCs were evaluated with this system. In cocultures of PBLs and allogeneic MSCs treated with TGF-β or VEGF, we documented increased cytotoxicity (versus cocultures with undifferentiated allogeneic MSCs; P<0.001; Figure 4E) and the presence of CD4⁺ and CD8α⁺ cells (Figure 4F).

To evaluate immune rejection in vivo, we implanted DiI-labeled undifferentiated or myogenic differentiated allogeneic MSCs into a myocardial infarct. Three days later, many more undifferentiated than differentiated cells were visible within the implanted area (data not shown). By day 7, the number of engrafted MSCs was ≈70% smaller (P<0.001; Figure 5A through 5C) and CD4⁺ and CD8α⁺ leukocytes were more numerous (Figure 5D) in the hearts implanted with differentiated (versus undifferentiated) cells.

Long-Term Fate and Functional Effects of Undifferentiated MSCs Implanted Into the Infarcted Myocardium

Allogeneic MSCs Are Immunoprivileged at 1 Week, but Not 5 Weeks, After Cardiac Implantation

Undifferentiated MSCs, whether allogeneic or syngeneic, did not initiate a significant immune reaction early after implantation. At 1 and 7 days after cell implantation, cytokine (TGF-β, interleukin-10, interferon-γ) gene expression in the recipient hearts (RT-PCR) was similar whether the MSCs were allogeneic or syngeneic (Figure 6A through C). On day 7, immunohistochemical staining demonstrated no differences between the 2 groups in the numbers of total leukocytes (CD45RA⁺ cells) or CD3⁺ cells in the implanted area (Figure 6D and 6E).

Figure 4. Leukocyte-mediated cytotoxicity and leukocyte proliferation in vitro. A, Peripheral leukocytes were cocultured for 3 days with either undifferentiated (untreated; MSCs) or myogenic differentiated (5-azacytidine–treated; dMSCs) MSCs from either allogeneic (Allo) or syngeneic (Syn) donors. As illustrated in the graph, significant cytotoxicity (LDH level) was observed only in the cocultures containing allo-dMSCs. B, i, ii, Leukocyte proliferation. BrdU was added to the cocultures at day 5. At day 6, BrdU⁺ leukocytes (arrow, inset) were observed in the cocultures containing allo-dMSCs but not in those containing MSCs. iii, iv, Many new leukocytes were CD3⁺ T cells. v through viii, Specific T-cell activation. Representative micrographs illustrate CD4⁺ and CD8α⁺ T cells in cocultures containing allo-dMSCs but not MSCs. C and D, Leukocyte division was evaluated in CFSE-labeled leukocytes cultured with media only (unstimulated) or cocultured with irradiated allo-MSCs or allo-dMSCs. Histograms illustrate CFSE fluorescence intensity in the 3 groups (C). Unstimulated leukocytes did not divide (fluorescence intensity was similar to that of the original population), but significant division was evident in the cocultures containing dMSCs (P<0.001 vs unstimulated and MSCs; D). E and F, Cytotoxicity and T-cell activation were assessed in cocultures of leukocytes and allo-dMSCs induced with TGF-β or VEGF (to promote smooth muscle or endothelial characteristics, respectively). Cytotoxicity was increased in both groups relative to cocultures with allo-MSCs (E); CD4⁺ and CD8α⁺ T cells were observed in both groups (F).
To investigate the possibility that allogeneic MSCs elicit an adaptive immune reaction later after implantation, we compared immune reactive antibodies in the serum collected from recipients of allogeneic or syngeneic MSCs at 1 and 5 weeks after implantation. At 5 weeks, the serum of allogeneic MSC recipients contained a specific anti-donor alloantibody (IgG1) that reacted with differentiated, but not undifferentiated, allogeneic MSCs (Figure 6F). This antibody was not detectable earlier (at 1 week) after cell implantation (data not shown), and no anti-donor antibodies were produced in the serum of syngeneic MSC recipients at either time point (Figure 6G).

We measured the survival of implanted allogeneic and syngeneic MSCs by determining the number of Y chromosomes in the female recipient hearts (real-time PCR) at different time points after implantation. Survival of allogeneic and syngeneic MSCs was similar early (1 and 7 days) after MSC implantation. However, by 35 days (5 weeks), only syngeneic cells were detected in the recipient hearts ($P<0.001$; Figure 7A). At 6 months after implantation, Y chromosome staining identified positive cells ($\approx$2 to 3 per field) in the hearts of syngeneic, but not allogeneic, MSC recipients (Figure 7B).

Allogeneic MSCs Restore Cardiac Function as Effectively as Syngeneic MSCs for 3 Months, but Not 6 Months, After Implantation

To determine the long-term effects of implanted allogeneic MSCs on cardiac function, we measured function before and over 24 weeks (6 months) after an MI followed by the implantation of undifferentiated allogeneic or syngeneic MSCs, or media. A repeated-measures ANOVA revealed significant main and interaction effects of time (after implantation) and treatment group (time: $F=11.32, P<0.01$; group: $F=7.092, P<0.01$; time$\times$group: $F=2.30, P=0.01$). A posthoc examination using Bonferroni multiple comparison posttests specified the differences illustrated in Figure 8A. By 3 weeks after MI (before cell implantation), there was a sharp and similar decrease (versus pre-MI levels) in fractional shortening in all 3 groups. Implantation of either allogeneic or syngeneic MSCs prevented the progressive deterioration in ventricular function exhibited by the media control group for 12 weeks (3 months) after implantation. Between 3 and 6 months, fractional shortening remained unchanged in the syngeneic group but began to decrease in the allogeneic group.
(P<0.05 versus syngeneic at 5 months), indicating progressive ventricular dysfunction. At 6 months, fractional shorting did not differ between the allogeneic and media control groups and remained significantly improved in the syngeneic group (P<0.05 versus allogeneic; Figure 8A).

Cardiac function in the 3 groups was further assessed with pressure-volume analyses at 5 and 24 weeks (6 months) after cell implantation. Consistent with the results from echocardiography, left ventricular contractility was improved similarly in response to either allogeneic or syngeneic MSCs at the early time point (Figure II in the online-only Data Supplement), whereas at 6 months, load-dependent (ejection fraction, \( \tau \)) and -independent (end-systolic pressure-volume relationship, preload recruitable stroke work) indexes of ventricular function were significantly improved (P<0.05) in those that received syngeneic MSCs compared with allogeneic MSCs or media (Figure 8B). End-systolic and end-diastolic volumes did not differ in the allogeneic and control groups but were significantly smaller in the syngeneic group (P<0.05 versus allogeneic and control). These results were supported by data on ventricular morphometry (Masson trichrome staining) and blood vessel density (\( \alpha \)-SMA and factor VIII immunostaining). At 6 months after implantation, syngeneic MSCs, but not allogeneic MSCs, prevented scar thinning and expansion (P<0.05 versus allogeneic and control; Figure 8C through 8E) and increased vascular density in the heart (P<0.001 versus allogeneic and control; Figure III in the online-only Data Supplement).

**Discussion**

This study reconciles inconsistent results from studies of cardiac cell therapy with allogeneic MSCs by describing the previously obscure biphasic immune response to these cells and identifying their long-term fate and functional efficacy in the infarcted myocardium. Because highly regenerative cells such as embryonic or induced pluripotent stem cells are not...
yet ready for clinical application, allogeneic MSCs isolated from healthy, young donors are the best immediate candidates for clinical cell therapy in aging patients. The present study establishes the biphasic immune character of MSCs in cardiac cell therapy. Donor MSCs are immunoprivileged in the allogeneic heart in their undifferentiated state, but a switch to an immune reactive phenotype is triggered after differentiation by a shift in the expression of immune antigens that renders them susceptible to immune rejection by the host.

We showed that undifferentiated MSCs expressed very low levels of immunogenic class Ia and II molecules from the MHC, along with high levels of nonclassic MHC-Ib. These data agree with previous reports that MSCs lack immunogenic antigens or that MHC-Ib expression is immunomodulatory and protects allogeneic organs from the host immune system. After MSCs differentiated along myogenic, smooth muscle, or endothelial lineages, the cellular expression of MHC-Ia and MHC-II increased significantly and MHC-Ib expression decreased. We propose that differentiation initiates an immune “switch” that alters the immune characteristics of MSCs.

Our in vitro and in vivo data further showed that the immune “switch” induced by cellular differentiation caused the MSCs to transition from an immunoprivileged to an immunogenic phenotype that triggered cellular cytotoxicity or immune rejection. For example, high levels of LDH and activation of CD3+, CD4+, and CD8α+ T cells were documented in cocultures of differentiated, but not undifferentiated, MSCs cocultured with allogeneic leukocytes. T-cell activation was also increased in hearts implanted with differentiated allogeneic MSCs, and the differentiated cells were eliminated from the host tissue much more readily than undifferentiated cells. The increased susceptibility of differentiated MSCs to T-cell–mediated cytolysis was likely a direct result of the shift in MHC antigen profile. Specifically, cells expressing MHC-Ia would be eliminated by cytolysis, and the loss of MHC–Ib, which has been reported to suppress CD4+ T-cell responses via CD94/NKG2A or CD94/NKG2C receptors, may have reduced their immunosuppressive status. The human form of MHC-Ib, HLA-G, is overexpressed in the placenta, where it protects the fetal allograft during pregnancy. In human MSCs, HLA-G contributes to the immunosuppressive properties that allow MSCs to block alloimmune reactions. The molecule may not be sufficient to prevent immune rejection, however, because cardiac transplantation studies have found that human MSCs were lost from the infarcted rat heart within 6 weeks of implantation.

It should be noted that 5-azacytidine is a demethylating agent, and as such, it could induce phenotypic changes that trigger the immune system even without cell differentiation. However, given that we documented similar immune responses whether the MSCs were treated in culture with 5-azacytidine or cytokines or allowed to differentiate naturally in vivo, we believe that the changes in MHC antigen expression were associated with the differentiation of these cells. Because MSCs are well known to differentiate after myocardial implantation, the clinical success of cardiac cell therapies using allogeneic MSCs will depend on whether the biphasic immune character of the cells affects their survival (and thus their net regenerative potential) after they undergo differentiation. From the present data, we conclude that undifferentiated allogeneic MSCs implanted into the infarcted myocardium restored cardiac function as effectively as syngeneic MSCs early (for at least 3 months), but not later (5 or 6 months), after implantation.

Like syngeneic MSCs that sustained functional effects for 6 months, allogeneic MSCs that survived early after implantation (for at least 1 week) likely improved function via paracrine secretion of factors that induced angiogenesis and homing of host cells. However, the implanted cells differentiated by about 2 weeks after implantation, and at 5 weeks, antibodies against differentiated, but not undifferentiated, allogeneic MSCs were detected in the circulation of recipients; these antibodies were not observed earlier and were not found in the circulation of control recipients that received syngeneic MSCs. Thus, along with T-cell and B-cell activation, the immune switch (change in MHC profile) triggered by differentiation of the implanted MSCs may have stimulated the humoral immune system to produce antibodies against the allogeneic cells. Both cellular and humoral reactions initiated by the differentiated allogeneic MSCs could result in cellular rejection. Indeed, at 5 weeks after implantation, allogeneic MSCs were not detected at the implanted area, whereas some syngeneic cells survived.

Interestingly, in those that received allogeneic MSCs, the decay in cardiac function was not significant until 5 months after cell therapy. We did not definitively establish the sequence of events that produced this delay in functional deterioration. One possibility is that, during the early phase of immune rejection, cytokines continued to be released by both implanted and recruited cells even as the implanted cells...

Figure 7. Implanted cell survival. A, Graph illustrating the number of cells surviving in the left ventricle (LV) at 1, 7, and 35 days after implantation of allogeneic (Allo) or syngeneic (Syn) MSCs. There were no significant differences between the groups at days 1 or 7. At day 35, Syn-MSCs, but not Allo-MSCs, were detected in the LV. The number of surviving Syn-MSCs did not change significantly between days 7 and 35. B, Representative micrographs illustrating Y chromosome staining (arrows and enlarged in inset) to identify Allo-MSCs or Syn-MSCs in recipient heart sections at 24 weeks (6 months) after implantation.

MSC Survival (# of cells in LV)

0 100000 200000 300000 400000 500000 600000
1 7 35

Allo-MSCs Syn-MSCs

P<0.001

A

B

Allo-MSCs Syn-MSCs
began to die. Thus, whereas the initial host response to the allogeneic MSCs stimulated regional tissue repair and maintained cardiac function for days to weeks after cell loss, the removal of continued paracrine support caused the ventricle to slowly thin and dilate (remodel).

This study determined that the efficacy of cardiac cell therapy with naive immunoprivileged MSCs is limited to the first 3 to 4 months after cell implantation because allogeneic MSCs transition to an immunogenic phenotype in the myocardium. We also identified an important contributing mechanism: Differentiation triggers a switch in MSC antigen composition that renders the allogeneic cells susceptible to both humoral and cell-mediated cytotoxicity. One limitation of these data is that they do not exclude the contribution of immunosuppressive soluble factors that may modify the rejection of the differentiated allogeneic MSCs. In addition, although we maintained both 5-azacytidine–treated and untreated MSCs in culture for 2 weeks, the prolonged cell culture conditions might have introduced factors that confounded the interpretation of MSC gene expression before and after differentiation. Finally, because we used unpurified PBLs for the leukocyte coculture experiments, we do not know whether T-cell activation was direct (if the differentiated MSCs acted as antigen-presenting cells themselves) or indirect (via professional antigen-presenting cells). Still, our results suggest that the successful development of allogeneic cell therapy for aged patients who have suffered an MI will require new approaches to reduce the immunologic responses that follow the differentiation of engrafted allogeneic MSCs.

Figure 8. Allogeneic MSCs restore cardiac function as effectively as syngeneic MSCs for 3 months, but not 6 months, after implantation. A, Cardiac function (fractional shortening; %FS) was evaluated by echocardiography before MI (−3 weeks), immediately before implantation (Tx) of undifferentiated allogeneic (Allo) or syngeneic (Syn) MSCs or media (control) (0 weeks), and 1, 5, 8, 12, 16, 20, and 24 weeks after Tx. Both Allo-MSCs and Syn-MSCs significantly prevented the decline in cardiac function seen in the control group until 12 weeks after Tx, with no significant differences between cell groups. After 12 weeks, function began to decline in Allo-MSCs. By 20 weeks, %FS in Allo-MSCs was statistically lower than in Syn-MSCs and similar to control. *P<0.05 for Syn-MSCs vs Allo-MSCs and control. m Indicates months after Tx. B, Left ventricular (LV) pressure-volume relationships were measured at 6 months after Tx. Pressure-volume loops are presented, along with end-systolic pressure-volume relation and preload recruitable stroke work (slope of stroke work−end-diastolic volume relation). Load-dependent and −independent indexes of ventricular contractility (end-systolic elastance [Ees], left ventricular end-diastolic volume [EDV], left ventricular end-systolic volume [ESV], percent ejection fraction [EF], d) were significantly improved in Syn-MSCs compared with Allo-MSCs and control. *P<0.05. C, Representative heart slices obtained from all 3 groups at 6 months after Tx illustrating the infarct (scar, stained with Masson trichrome; arrows). Scar thickness (D) and scar length (E) were significantly reduced in Syn-MSCs compared with Allo-MSCs and control. *P<0.05, **P<0.01.
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Disclosures
None.

References

CLINICAL PERSPECTIVE
Heart failure after a myocardial infarction remains a significant cause of mortality. Many have advocated stem cell therapy to prevent the progression of adverse events, but initial clinical trials did not reproduce the extensive benefits reported in preclinical animal trials. Compared with stem cells from young, healthy individuals, cells derived from elderly patients were found to have a limited ability to restore cardiac function in nonreactive animals. This diminished regenerative capacity of the older patients’ stem cells might partially explain the limited benefits of stem cell therapy. Allogeneic mesenchymal stromal cells (MSCs) are an enriched population of cells with advanced therapeutic properties. Allogeneic MSCs offer a source of young, healthy, highly regenerative stem cells for implantation into the postinfarct myocardium. However, it is unclear whether these cells can avoid immune surveillance and engraft in the heart, in part because most preclinical assessments of allogeneic MSC therapy were restricted to the first 8 to 12 weeks after cell implantation. This study included a 6-month-long evaluation of outcomes after allogeneic MSC therapy. We found that, although MSCs are immunoprivileged and can engraft in the heart and improve cardiac function early after implantation, they acquire an immunogenic phenotype and are immune rejected later, after they differentiate into specialized cells. Our findings provide an explanation for the diverse responses to allogeneic MSCs reported in previous studies and caution that these cells appear to have only short-term clinical benefits for the heart. Modifying the late immunogenic phenotype of MSCs may produce prolonged benefits after implantation.
Differentiation of Allogeneic Mesenchymal Stem Cells Induces Immunogenicity and Limits Their Long-Term Benefits for Myocardial Repair

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Supplemental Methods

MSC isolation and culture

Bone marrow cells were flushed from the cavities of tibias and femurs from male Wistar or Lewis rats (all 200-250 g) as described previously. The cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco) with 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin and maintained for 3–5 days in a humidified incubator at 37°C with 5% CO₂. The non-adherent hematopoietic cells were washed off and the adherent MSCs were expanded for 4-6 passages in preparation for transplantation. MSCs were characterized using FACS with antibodies against CD45, CD34, CD90.1, and CD29. The cells were CD45⁻ and CD34⁻, but 95.6±3.4% expressed CD90.1⁺ and 95.7±2.0% expressed CD29⁺. On the day of transplantation, the cells were detached from the culture dishes using 0.05% trypsin, suspended in media, and then centrifuged. The cell pellet was resuspended in FBS free medium and the number of cells was counted. The cell suspension (3 x 10⁶ cells) was injected into the infarct at a volume of 50 μL FBS free medium per animal.

Antibodies

All antibodies used for FACS or immunohistochemical analyses were obtained from BD Biosciences (Ontario, Canada), with the following exceptions: Anti-β-mhc and MHC-I (2G5) was from Santa Cruz Biotechnology (California, USA), anti-α-sarcomeric actinin was from Sigma (Ontario, Canada), anti-MHC-II was from eBioscience (California, USA), anti-CD3 was from Dako (California, USA), and anti-GFP was from Molecular Probes (Invitrogen, California, USA).

MSC preparation and differentiation in vitro
MSCs (CD45/CD34/CD90.1+/CD29+ by FACS analysis) were isolated from donor femurs and tibias. To induce myogenic differentiation, MSCs were cultured with 5-azacytidine (5-aza, 10μM) for 24 h, and then maintained in normal growth media for 2 weeks as described previously\(^2\). Differentiated cells were harvested and assessed for the expression of myogenic genes [including Nkx2.5, MyoD, β-myosin heavy chain (β-mhc); RT-PCR primers listed in Supplemental Table 2] or proteins (immunostaining with antibodies against α-sarcomeric actinin, β-mhc, troponin I, or FACS analysis with anti-β-mhc antibody). Undifferentiated MSCs were used as controls. PCR reactions were conducted using 1 μg cDNA and 30 cycles, except in the case of β-mhc (2 μg cDNA and 32 cycles).

To induce differentiation towards endothelial or smooth muscle cells, MSCs were cultured for 2 weeks in media containing 2% FBS with either VEGF (50 ng/ml; for endothelial characteristics) or TGF-β (15 ng/ml; for smooth muscle characteristics). Differentiation along the appropriate lineage was confirmed using immunostaining for Factor VIII or α-smooth muscle actin (α-SM), respectively.

**Immune antigen expression in MSCs differentiated in vitro**

Expression of MHC class I (MHC-Ia and MHC-Ib), MHC class II (MHC-II), and CD86 genes was evaluated in differentiated and undifferentiated MSCs by using RT-PCR (primers listed in Supplemental Table 2). MHC proteins were detected using immunohistochemical staining with antibodies against MHC-I and –II. Unless otherwise indicated, secondary antibodies were conjugated with either PE (red) or FITC (green). The number of cells expressing MHC-I or –II proteins was quantified using FACS. Anti-MHC-Ia antibody 2G5 identified MHC-Ia molecules, while MHC-Ib was identified using an indirect immunostaining method as described previously\(^3\).
Briefly, anti-pan MHC-I antibody OX18, which recognizes both MHC-Ia and -Ib molecules, was used in combination with a specific anti-MHC-Ia antibody.

**FACS analysis of MSC immune antigen expression**

Differentiated and undifferentiated MSCs were characterized. Adherent cells were detached using a cell dissociation solution according to the manufacturer’s instructions (Sigma). Next, 1 × 10^6 cells were incubated with antibodies [anti-β-mhc (after pretreatment with 0.1% TX-100), anti-MHC-I (2G5), anti-pan MHC-I (Ia + Ib, OX-18), FITC-conjugated anti-MHC-II, PE-conjugated anti-CD86] for 30 min at 4°C in the dark. FITC-conjugated anti-mouse IgG1 antibody was used to detect anti-β-mhc, anti-MHC-I (2G5), and anti pan-MHC-I (OX18). Isotype-identical antibodies (BD Pharmingen) served as controls. Cells were analyzed using a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software. The fluorescence intensity was quantified in 10,000 cells per sample.

**Leukocyte-mediated cytotoxicity**

Mixed peripheral blood leukocytes (PBLs) were isolated from the blood of Lewis rats using gradient centrifugation (Sigma, Canada) according to the manufacturers’ protocol. PBLs (5x10^5) were co-cultured with myogenic differentiated (5-aza-treated) or undifferentiated allogeneic or syngeneic MSCs (5x10^4) in 24 well plates. After 3 days, leukocyte-mediated cytotoxicity was assessed in the collected supernatant using a cytotoxicity detection kit (Roche Applied Science), which measures lactate dehydrogenase (LDH) released from damaged cells. Cytotoxicity (percent lysis) was calculated using the formula: 100 x (E-M)/(T-M), where E is experimental release, M is spontaneous release in the presence of media alone, and T is maximum release in the presence of 5% Triton X-100.

**Leukocyte proliferation**
**CFSE-labeled leukocytes:** PBLs were isolated as described for “Leukocyte-mediated cytotoxicity”, and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) according to the manufacturer’s protocol. Briefly, a PBL pellet was suspended in pre-warmed PBS containing 5 μM of CFSE for a final cell concentration of 10x10^6 cells/ml. The samples were incubated at 37°C for 15 minutes, then centrifuged and washed with pre-warmed culture media (RPMI-1640, 2 mM glutamine, 10% FBS), and then incubated at 37°C for 30 minutes to ensure complete probe modification. Finally, the cells were re-suspended in the media at a concentration of 6x10^4 cells/ml. To assess proliferation, the cell suspension was cultured (24-well plates; 1 ml per well) alone (unstimulated) or co-cultured with irradiated (20G) allogeneic MSCs (6x10^4) either myogenic differentiated (5-aza-treated) or undifferentiated. After 6 days, PBLs were collected and proliferation was evaluated using flow cytometry.

**BrdU uptake:** BrdU (10μM) was spiked in the PBL-MSC co-cultures for 24 hours on day 5 of the “Leukocyte-mediated cytotoxicity” experiment. On day 6, PBLs were collected. PBL proliferation was evaluated using immunostaining for BrdU, and specific T cell activation was characterized using antibodies against CD3, CD4, and CD8α.

**Experimental MI: coronary artery ligation**

Adult rats were pre-medicated with 3-4% Isoflurane and then intubated and ventilated with a mixture of room air, oxygen and 1-3% isoflurane. The heart was exposed through a left thoracotomy, and the left anterior descending artery on the left ventricle free wall was ligated with a 7-0 polypropylene suture. The chest incision was closed in layers using 3-0 Vicryl sutures. Animals were treated with antibiotics (150,000 IU/Kg Penlong XL) and analgesics (0.03 mg/Kg Buprenorphine).

**MSC differentiation and immune antigen expression in vivo**
Female Lewis rats underwent left coronary artery ligation (MI). Three weeks later, allogeneic GFP+ MSCs isolated from male GFP+ Wistar rats (3x10^6/rat) were injected into the infarct. No immunosuppressive agents were administrated during the course of the study. At days 3, 7, and 14 after cell implantation, hearts were arrested and frozen tissue blocks were embedded in Tissue-Tek® OCT (Sakura Finetek USA, Inc., Torrance, CA). Each sample was cut into 5 μm-thick sections and co-immunostained with antibodies against GFP (to identify implanted cells), α-SM, FVIII, or β-mhc (smooth muscle, endothelial, or myogenic cell markers, respectively), and either MHC-Ia or MHC-II (immune antigens). Samples were analyzed using confocal microscopy.

**Immunohistochemical staining and confocal microscopy**

Frozen tissue sections were fixed in 4% paraformaldehyde and permeabilized with 0.5% triton X-100. Fixed sections were incubated with anti-GFP antibody (1:300) either alone, or with one of anti-α-SM-Cy3 (1:300), anti-FVIII (1:300), or anti-β-mhc (1:200), or with α-SM-Cy3, anti-FVIII, or anti-β-mhc plus one of MHC-Ia (1:200) or MHC-II (1:200) (double or triple labeling). All sections were incubated with an appropriate secondary antibody. Nuclei were identified using DAPI staining. Detector gains and voltage were set before scanning using sections incubated in secondary antibody. An Olympus Fluoview 2000 laser scanning confocal microscope was used to acquire 5 random fields of view from the infarct region.

**Long-term fate and functional effects of undifferentiated MSCs implanted into the infarcted myocardium**

**Host immune responses:**

*Cytokine expression:* Hearts were collected at 1 and 7 days after cell implantation. RNA was isolated as described previously^4^. Cytokine (TGF-β, IL-10, INF-γ) gene expression was
identified by using RT-PCR (primers listed in Supplemental Table 2). Measures from the housekeeping gene encoding GAPDH were used to normalize gene levels.

**Leukocyte infiltration:** At 1 week after cell implantation, hearts were fixed and leukocytes in the implanted area were identified immunohistochemically, using antibodies against CD3 (total T cells) or CD45RA (total leukocytes), and then counted in 5 randomly selected fields (under the 40x objective) per section. Counts were expressed as the percentage of positive cells/0.2 mm$^2$.

**Allogeneic antibody production:** At 1 and 5 weeks after cell implantation, blood was collected from recipients, clotted and centrifuged. The serum was collected. Cultured myogenic differentiated (5-aza-treated) or undifferentiated allogeneic MSCs were fixed, and then incubated with the serum for 2 h at room temperature. The cells were carefully washed, and then immunostained using a PE-conjugated antibody against rat IgG1 (to reveal expression of an allo-antibody against the donor MSCs in the recipient circulation).

**Implanted cell survival:** The number of Y-chromosomes (representing implanted cells) remaining in the female recipient heart tissue at 1, 7, or 35 days after cell implantation was determined using real-time PCR as described previously$^5$. The hearts were quickly removed and frozen in liquid nitrogen. Myocardial DNA was extracted using a kit (Qiagen, Mississauga, Canada), and Y-chromosomes were detected using the TaqMan method. We used a Cy3 Y-chromosome probe kit to visualize Y-chromosomes that persisted in recipient hearts at 24 weeks (6 months) after cell implantation via fluorescence in situ hybridization.

**Fluorescence in situ hybridization: Y-chromosomes:** We used a Cy3 Y-chromosome probe kit (Cambio, Cambridge, UK; Cat. # CA-1631) to visualize Y-chromosomes that persisted in female recipient hearts at 24 weeks (6 months) after cell implantation. Briefly, tissue sections were fixed
with 2% paraformaldehyde, then digested with pepsin solution for 10 min at 37°C, and then dehydrated using a graded series of alcohols. Next, the Cy3 Y-chromosome probe was placed directly onto the sections, and the samples were denatured for 10 min at 60°C. The sealed slides were placed horizontally into a humid chamber and hybridized overnight at 37°C. Finally, the probe was rinsed away by washing in 50% formamide/2X SSC and then 2X SSC for 5 min at 37°C. The nuclei were counterstained with DAPI.

Cardiac functional assessment:

**Echocardiography:** Assessments were performed using an ACUSON SEQUOIA C256 System (SIEMENS Medical Solutions USA, Inc; California, USA) with a 15L8 transducer. Depth and frequency were set at 2 cm and 13 MHz, respectively. Short-axis views were obtained from the parasternal approach. Left ventricular dimensions [left ventricular end-diastolic diameter (LVEDd) and left ventricular end-systolic diameter (LVEDs)] and areas [left ventricular end-diastolic area (LVEAd) and left ventricular end-systolic area (LVEAs)] were measured from M-mode short axis views of the mid-level left ventricle. Fractional shortening (%) was calculated using the equation: \( \frac{(LVEDd - LVEDs)}{LVEDd} \times 100 \).

**Pressure-volume analysis:** Under general anesthesia, the carotid artery was exposed and a calibrated Millar and conductance pressure-volume catheter (Millar Instruments, USA) was inserted into the left ventricular cavity through the artery. Pressure and volume data were collected using Millar P-V software. When stable traces of ventricular pressure and volume were obtained on the computer, the catheters were secured, and the baseline and occlusion curves were recorded. To establish occlusion measurements, the inferior vena cava was occluded with a pre-positioned surgical tourniquet until the left ventricle emptied as indicated on the volume curve. The occlusion was then released. Parallel conductance was evaluated after hypertonic saline
solution was injected into the right jugular vein; volume measurements were corrected for the parallel conductance. The left ventricular pressure-volume relationship was analyzed, and real-time pressure-volume loops constructed. Left ventricular end-diastolic and end-systolic volumes were measured; end-systolic pressure-volume relationship, preload recruitable stroke work (load independent), percent ejection fraction, and Tau (load dependent) were calculated.

**Tissue preparation and cardiac morphometry:** Hearts were arrested with KCL, rapidly excised, and fixed in 10% formaldehyde for 7-14 days. Formalin-fixed hearts were cut into 2 mm thick slices and stained with Masson’s trichrome, and then both apical and basal sections were digitally photographed (Coolpix, Nikon; Tokyo, Japan). Image J software was used for morphometric analysis. Scar length was measured along the middle of the scar between the edges of the border zone (identifiable by blue staining), and then expressed as the average of lengths for 3-4 slices per heart. Scar thickness was expressed as the average of wall thicknesses measured at the middle of the scar and each edge (on the slice in which the scar was thinnest). Immunohistochemical assessment of blood vessel density in fixed tissue sections was performed as described previously⁶,⁷.
Supplemental Tables
**Supplemental Table 1.** *In vivo* experimental timeline and sample sizes.

<table>
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<th>Nature of <em>in vivo</em> study</th>
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MI=coronary artery ligation; Tx=MSC implantation; d=day; wk=week; mo=month
Supplemental Table 2. Primers used for RT-PCR.

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Supplemental Figure
Supplemental Figure 1
Supplemental Figure 2
Supplemental Figure 3
Supplemental Figure Legends

Supplemental Figure 1: Immune antigen expression in vitro: smooth muscle and endothelial differentiation. (A) MSCs were cultured in media containing 2% FBS with either TGF-β or VEGF to induce differentiation to smooth muscle or endothelial cells, respectively. Representative micrographs show up regulation of smooth muscle cell marker α-smooth muscle actin (α-SM) or endothelial marker FactorVIII (FVIII) in the differentiated MSCs (dMSCs). (B) Undifferentiated MSCs expressed low levels of major histocompatibility complex class Ia (MHC-Ia), but high levels of MHC class Ib (MHC-Ib). After cytokine treatment, dMSCs (induced with TGF-β or VEGF) exhibited significant relative increases in MHC-Ia expression and decreases in MHC-Ib expression. (C) dMSCs co-expressed MHC class II (MHC-II) and either α-SM (in TGF-β-differentiated smooth muscle cells) or FVIII (in VEGF-differentiated endothelial cells). Arrows identify dMSCs co-localizing both proteins. DAPI=nuclear stain. In TGF-β-differentiated dMSCs, α-SM was identified using a Cy5.5-conjugated secondary antibody.

Supplemental Figure 2: Cardiac function and contractility by pressure-volume analysis at 5 weeks after cell implantation. Left ventricular (LV) pressure-volume relationships were measured at 5 weeks after implantation of undifferentiated allogeneic (Allo) or syngeneic (Syn) MSCs, or media (Control). (A) Graphs illustrating end-systolic pressure-volume relationship and preload recruitable stroke work (slope of stroke work - end-diastolic volume relation). (B-E) Graphs illustrating load dependent and independent indices of ventricular contractility in the 3 groups: percent ejection fraction (EF; B), Tau (C), LV end-systolic volume (ESV; D), LV end-diastolic volume (EDV; E).

Supplemental Figure 3: Blood vessel density at 6 months after cell implantation. (A,B) Representative heart sections obtained at 6 months after implantation of undifferentiated
allogeneic (Allo) or syngeneic (Syn) MSCs, or media (Control), illustrating expression of \( \alpha \)-smooth muscle actin (\( \alpha \)-SM; A) or factor VIII (FVIII; B). Graphs show that the densities of both \( \alpha \)-SM\(^+\) (A) and FVIII\(^+\) (B) blood vessels were significantly increased in Syn-MSCs compared to Allo-MSCs and Control.
Supplemental References


