Differentiation of Allogeneic Mesenchymal Stem Cells Induces Immunogenicity and Limits Their Long-Term Benefits for Myocardial Repair

Xi-Ping Huang, PhD; Zhuo Sun, MD; Yasuo Miyagi, MD; Heather McDonald Kinkaid, MSc; Li Zhang, MD, PhD; Richard D. Weisel, MD; Ren-Ke Li, MD, PhD

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 (immunohistochemistry, 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

Received March 23, 2010; accepted September 20, 2010.

From the Department of Surgery, Division of Cardiovascular Surgery (X.P.H., Z.S., YM., H.M.K., R.D.W., R.K.-L.) and Department of Laboratory Medicine and Pathobiology, Multi-Organ Transplant Program (L.Z.), University of Toronto and University Health Network, Toronto, Ontario, Canada.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.110.955971/DC1.

Correspondence to Ren-Ke Li, MD, PhD, MaRS Centre, Toronto Medical Discovery Tower, Room 3-702, 101 College St, Toronto, Ontario, Canada M5G 1L7. E-mail renkeli@uhnres.utoronto.ca

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 al[15]).

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; 5×10^5) were isolated and cocultured with differentiated (5-azacytidine–treated) or undifferentiated allogeneic or syngeneic MSCs (5×10^4) 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 (3×10^6 per rat) were injected into the infarct. No immunosuppressive agents were administrated 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; 3×10^6 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 $\times20$ 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 (3×10^6 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

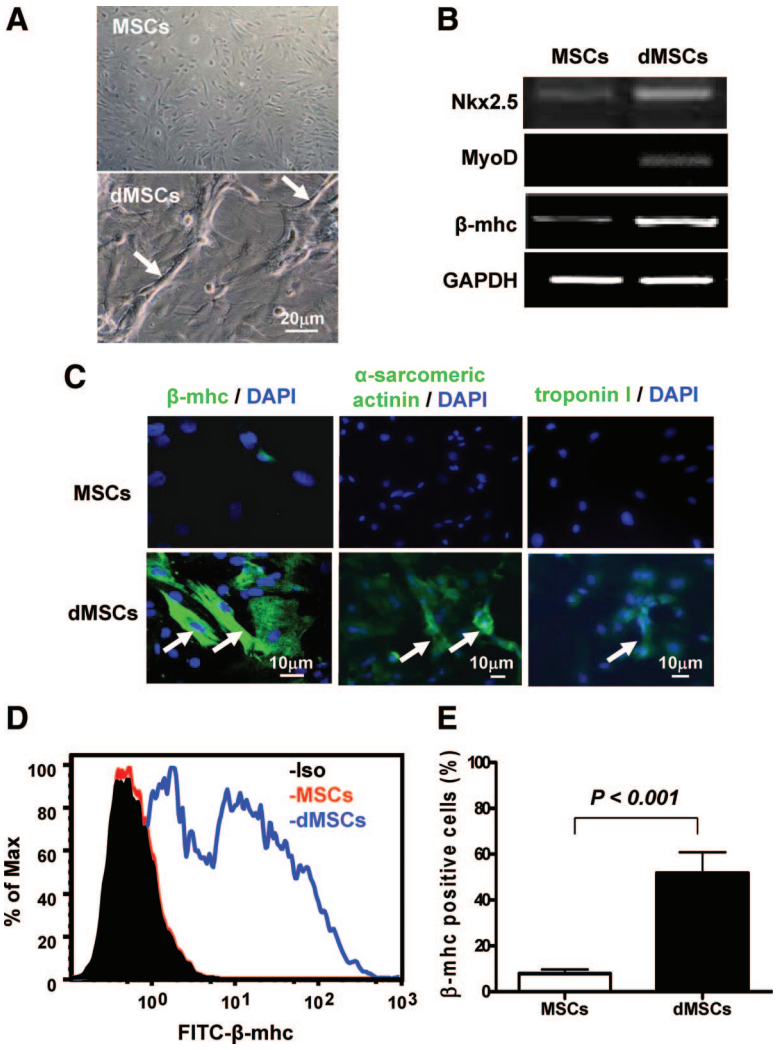

Figure 1. Evidence for myogenic differentiation. MSCs were treated with 5-azacytidine for 24 hours and then cultured for 2 weeks to induce myogenic differentiation. A, Myotube formation (arrow) was observed in differentiated (dMSCs), but not undifferentiated (MSC), MSC cultures. B and C, Coincident with myotube formation in the dMSCs was an upregulation in levels of myogenic genes *Nkx2.5, MyoD,* and *β-mhc* (B, by RT-PCR) and 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.

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 (Figure 1C), suggesting that the 5-azacytidine–treated MSCs acquired characteristics of myogenic cells. Flow cytometric analysis revealed that β-mhc was expressed by 51.8±9.1% of differentiated MSCs but only 7.9±1.4% of undifferentiated MSCs ($P<0.01$; Figure 1D and 1E).

Compared with undifferentiated MSCs, 5-azacytidine–differentiated MSCs exhibited increased mRNA expression of immune antigens MHC-Ia, MHC-II, and CD86 (Figure 2A). Immunostaining revealed that the expression of immunosuppressive MHC-Ib protein, highly expressed by undifferentiated MSCs, was reduced in myogenic differentiated MSCs (Figure 2B), whereas the expression of immunogenic MHC-Ia, MHC-II, and CD86 proteins was strongly increased in the differentiated cells (Figure 2C and 2D), some of which coexpressed MHC-II and CD86. Quantitative analysis using flow cytometry confirmed that differentiation induced a >30% increase in the percentage of MSCs that expressed MHC-Ia ($P<0.001$) and ≈3% to 6% increases in CD86 ($P<0.001$) and MHC-II ($P<0.05$) expression. In contrast,

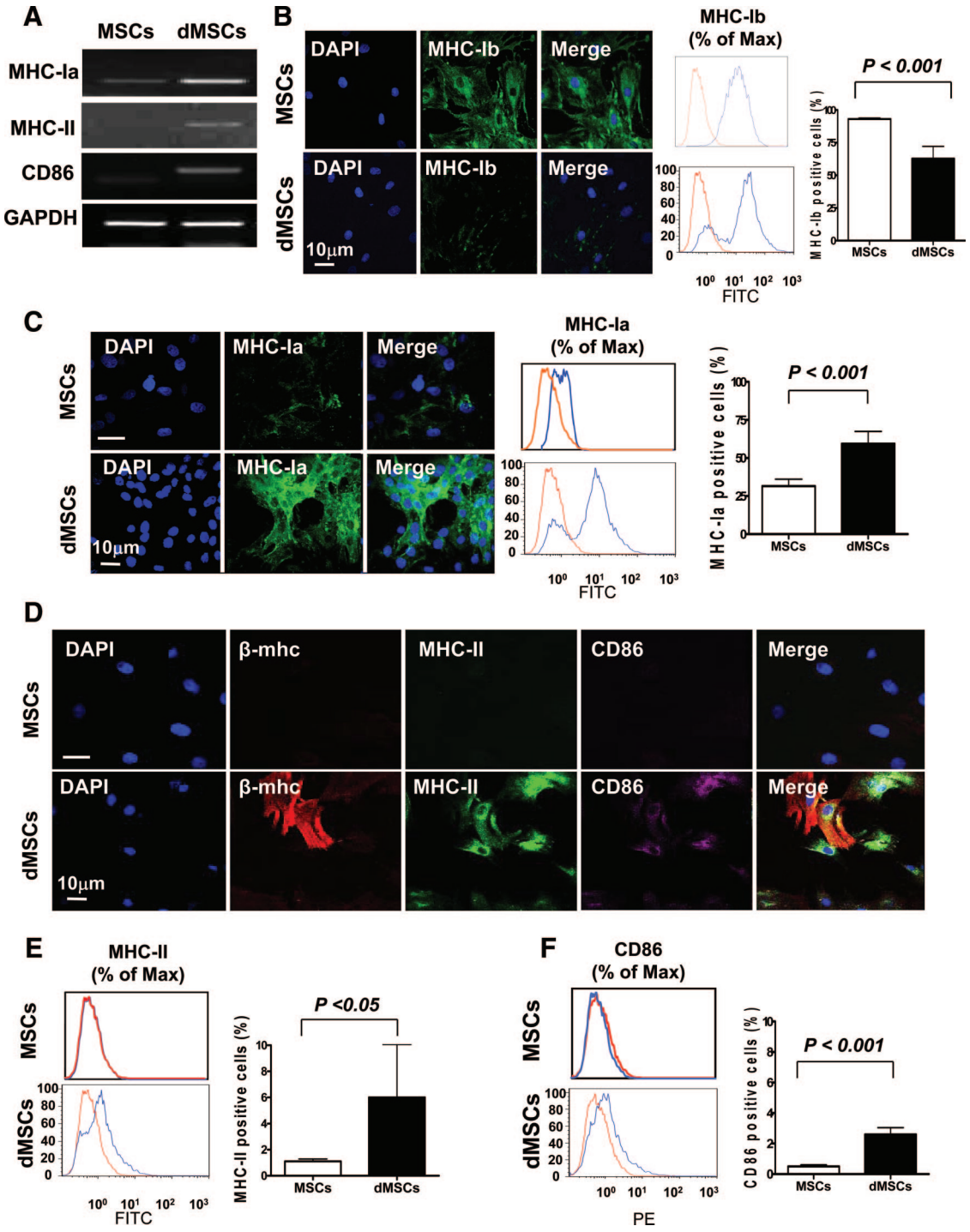

Figure 2. Immune antigen expression in vitro: myogenic differentiation. A, The gene expression of immune antigens (MHC-Ia, MHC-II, and CD86) was significantly increased in differentiated (5-azacytidine–treated) MSCs (dMSCs) compared with untreated (undifferentiated) MSCs (MSCs; by RT-PCR; GAPDH is the housekeeping gene). B through F, Immunostaining and flow cytometry. Compared with MSCs, dMSCs expressed significantly lower levels of MHC-Ib (B) and higher levels of MHC-Ia (C). MHC-II and CD86 proteins were coexpressed by dMSCs [(β-mhc)$^+$] but not MSCs (D through F). DAPI is the nuclear stain. Flow cytometry: red shows isotype control; blue, corresponding antibody. Graphs in B, C, E, and F illustrate the percentage of cells that expressed each molecule.

differentiation was associated with a ≈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-β or VEGF) rather than 5-azacytidine. Compared with undifferentiated MSCs, those with characteristics of smooth muscle cells (α-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).

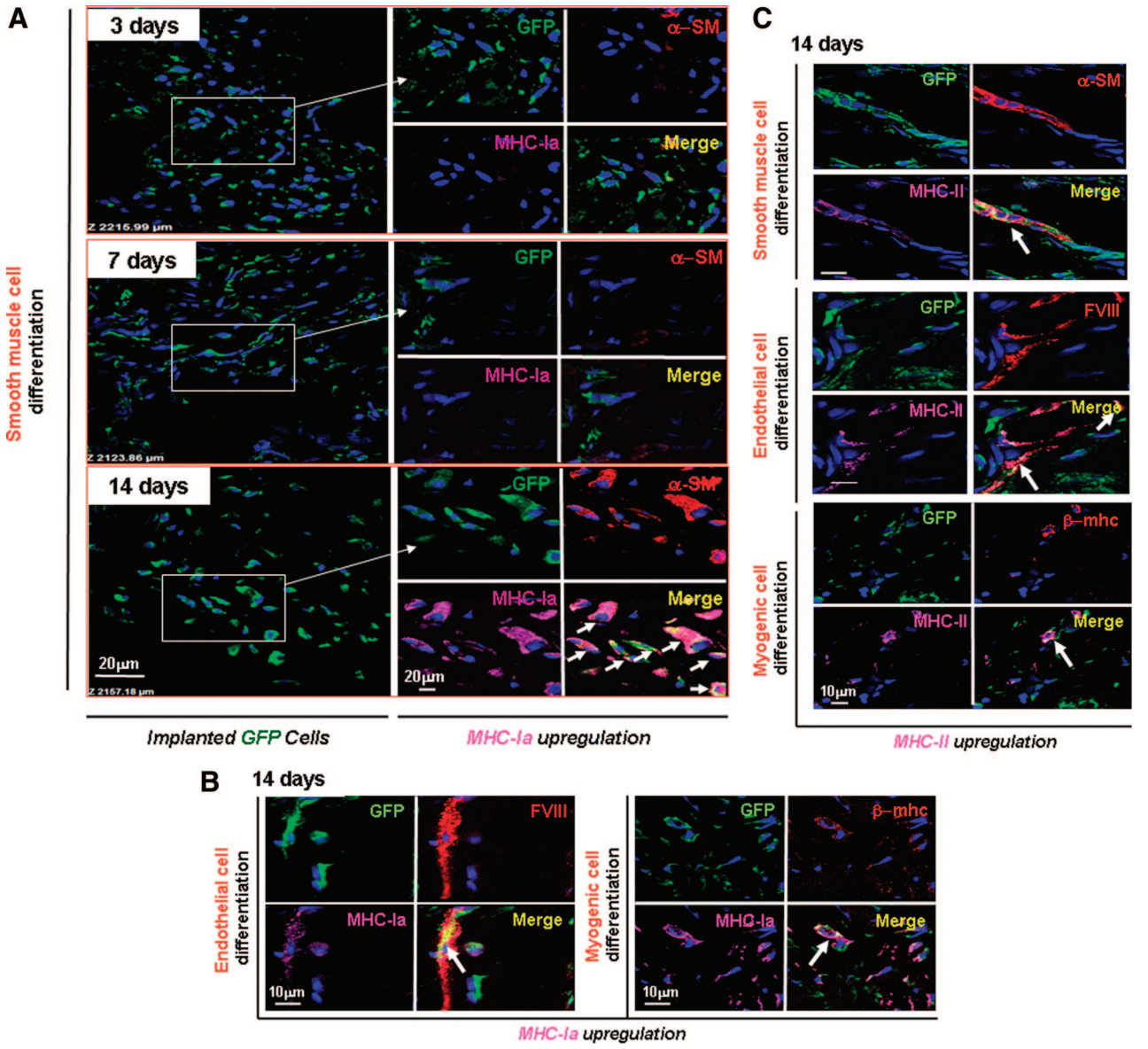

Figure 3. Immune antigen expression in vivo. A through C, Allogeneic GFP$^+$ MSCs were implanted at 3 weeks after MI. Animals were euthanized at 3, 7, and 14 days after implantation. Representative micrographs illustrate expression of GFP (green, implanted cells), differentiation markers (red) (α-SMA [smooth muscle cells], factor VIII [FVIII, endothelial cells], and mhc [β-mhc, myogenic cells]), and MHC proteins (purple; MHC-Ia or MHC-II) in the myocardial tissue of recipients. GFP$^+$ MSCs were visible in all samples, but expression of differentiation markers was visible only at 14 days after cell implantation. By the 14-day time point, the implanted cells expressed characteristics of smooth muscle cells (A), endothelial cells (B, left), or myogenic cells (B, right); all lineages expressed MHC-Ia (A, B) or MHC-II (C) molecules. Blue shows the DAPI nuclear stain.

MSC Differentiation and Immune Antigen Expression in the Infarcted Myocardium

Allogeneic GFP$^+$ MSCs were implanted into the infarcted myocardium 3 weeks after an MI in rats. Coimmunostaining 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,

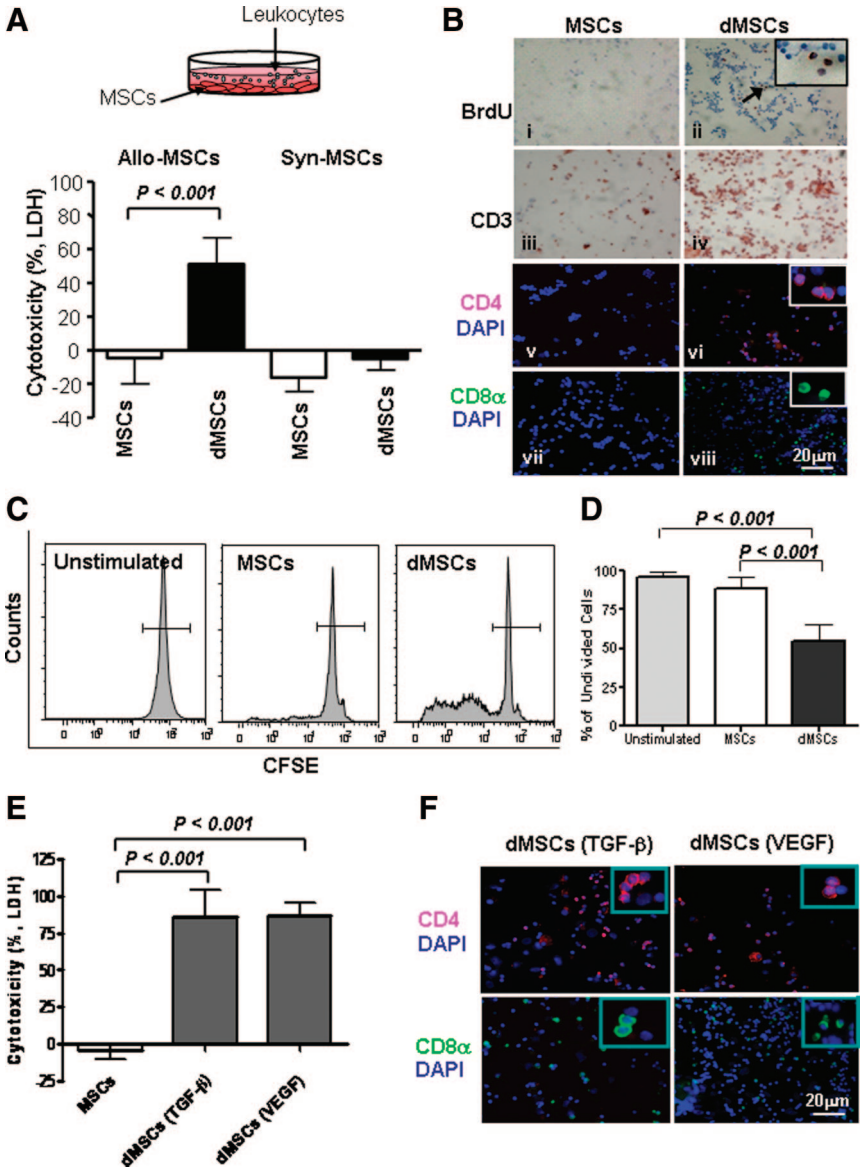

Figure 4. Leukocyte-mediated cytotoxicity and leukocyte proliferation in vitro. A, Peripheral leukocytes were cocultured for 3 days with either undifferentiated (untreated; MSCs) or myogenic differentiated (5-azacytidine–treated; dMSCs) MSCs from either allogeneic (Allo) or syngeneic (Syn) donors. As 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 TFG-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).

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).

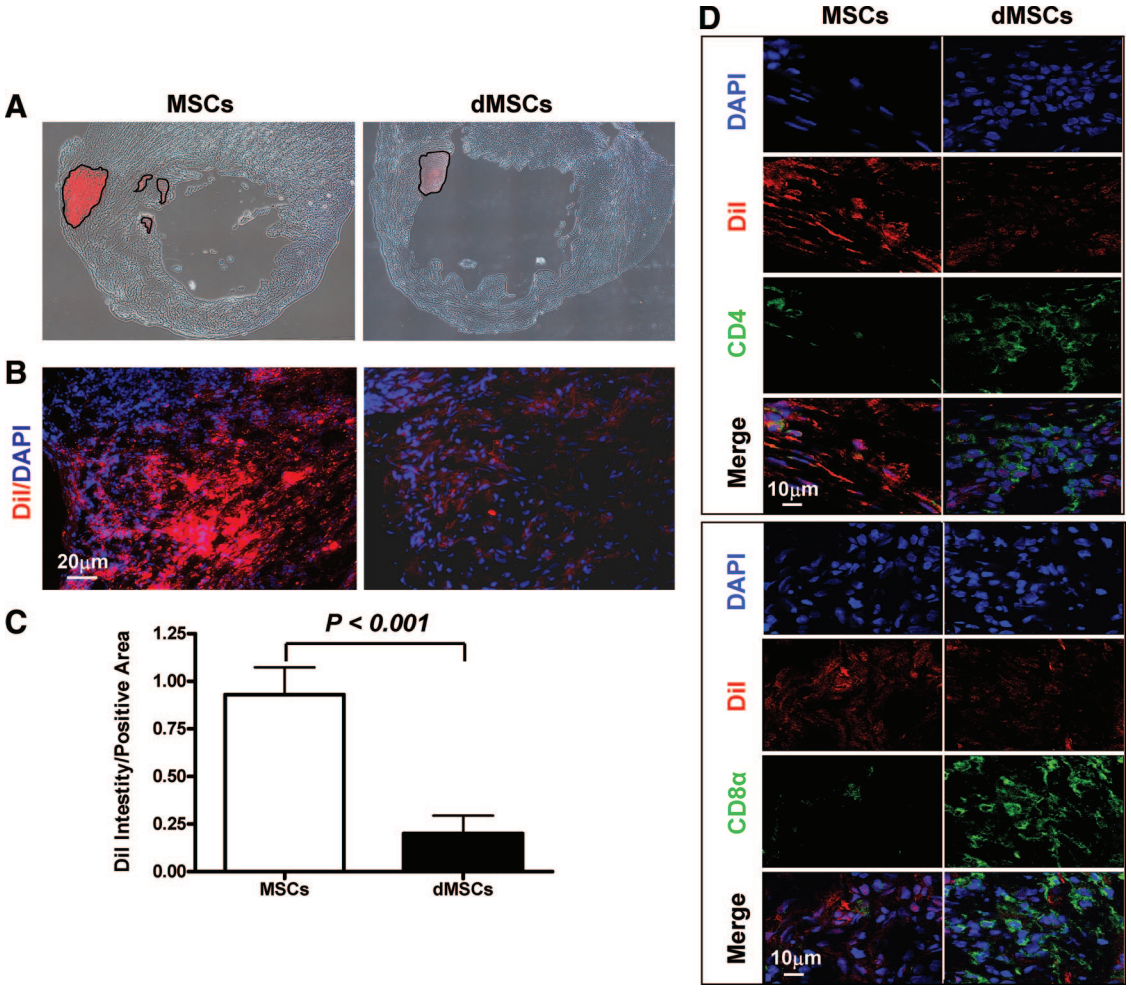

Figure 5. Immune rejection in vivo. A and B, Representative micrographs illustrating DiI label (surviving MSCs) and DAPI (nuclear stain) in heart sections at 7 days after DiI-labeled, undifferentiated or myogenic differentiated (5-azacytidine–treated) allogeneic MSCs (MSCs or dMSCs, respectively) were implanted into the infarcted myocardium. C, Graph illustrating that significantly more implanted MSCs than dMSCs remained at day 7. D, Representative micrographs illustrating DAPI (nuclear stain), DiI label, and expression of CD4 (top) or CD8α (bottom) in day 7 myocardial sections from recipients of allogeneic MSCs or dMSCs.

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 shorting 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 shorting remained unchanged in the syngeneic group but began to decrease in the allogeneic group

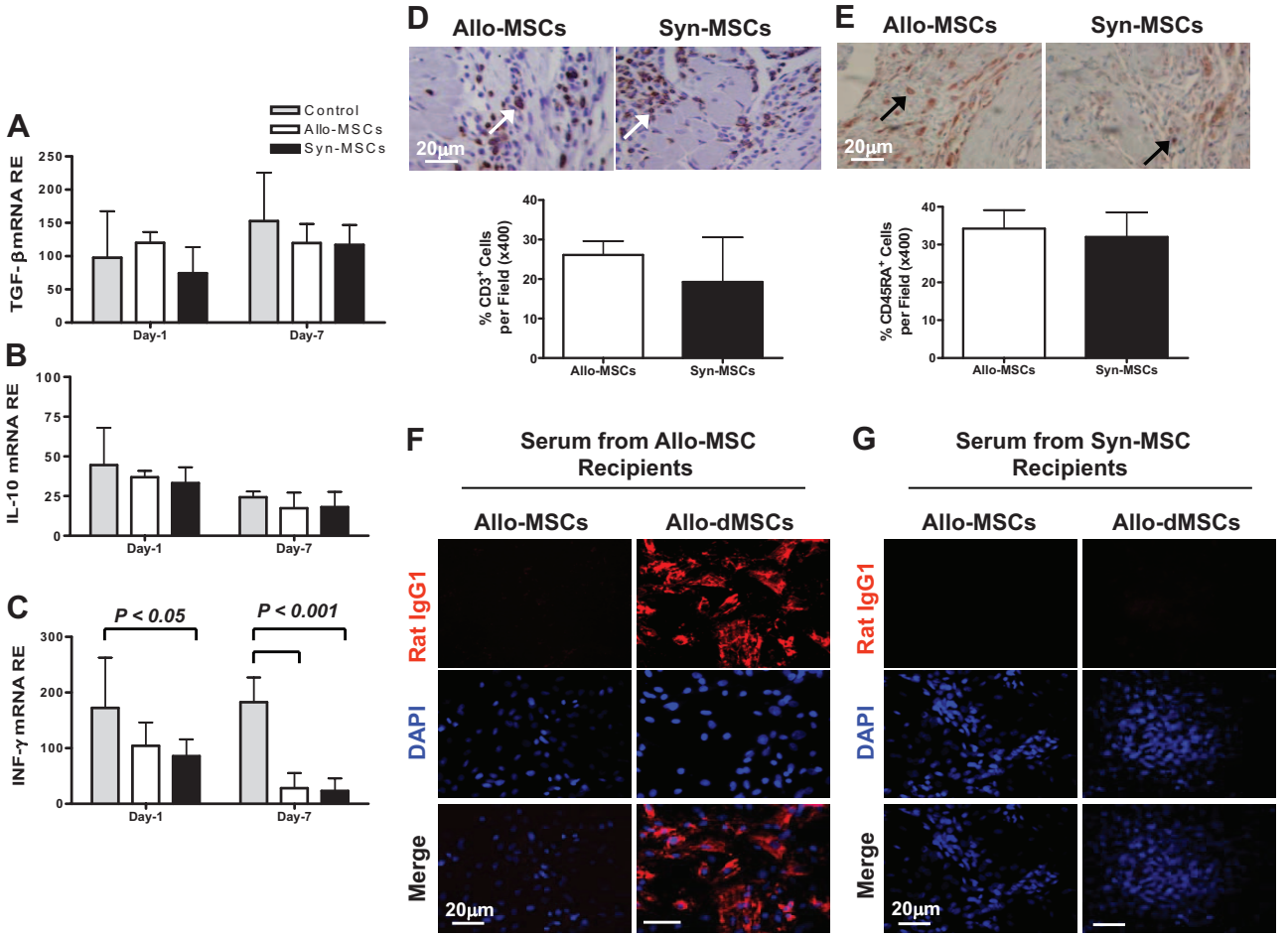

Figure 6. Allogeneic MSCs are immunoprivileged at 1 week, but not 5 weeks, after cardiac implantation. A through C, Cardiac gene expression of immune cytokines (TGF-β [A], interleukin-10 [IL-10; B], interferon-gamma [INF-γ; C]) at days 1 and 7 after implantation of media (control), allogeneic MSCs (Allo-MSCs), or syngeneic MSCs (Syn-MSCs). RE indicates expression level relative to that of housekeeping gene GAPDH. D and E, Representative micrographs illustrating anti-CD3 (T cells, arrows; D) and anti-CD45RA (leukocytes, arrows; E) staining in heart sections obtained 7 days after implantation of Allo-MSCs or Syn-MSCs. Histograms quantify $CD3^+$ and $CD45RA^+$ cells (expressed as percent of total cells) in the infarcted myocardium. There were no significant differences between the groups. F and G, Peripheral blood serum was collected from recipients at 5 weeks after implantation of Allo-MSCs (F) or Syn-MSCs (G) and then incubated with allogeneic MSCs either undifferentiated (Allo-MSCs) or differentiated with 5-azacytidine treatment (Allo-dMSCs). Incubated samples were stained with a phycoerythrin-conjugated secondary antibody against rat IgG1 (red). Representative micrographs illustrate IgG1 expression only in the sample containing Allo-dMSCs mixed with serum from Allo-MSC recipients. Blue indicates DAPI nuclear stain.

($P<0.05$ 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, τ) 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 (α-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

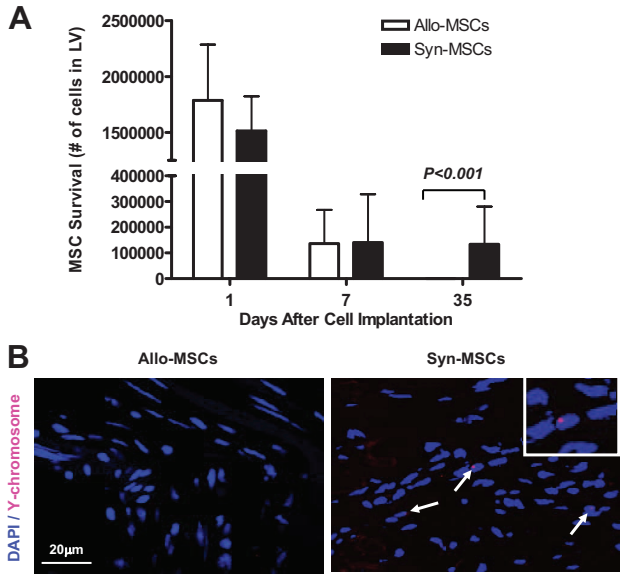

Figure 7. Implanted cell survival. A, Graph illustrating the number of cells surviving in the left ventricle (LV) at 1, 7, and 35 days after implantation of allogeneic (Allo) or syngeneic (Syn) MSCs. There were no significant differences between the groups at days 1 or 7. At day 35, Syn-MSCs, but not 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.

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[17,18] or that MHC-Ib expression is immunomodulatory and protects allogeneic organs from the host immune system.[19] 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 $CD8\alpha^+$ 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 cytotoxicity 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,[20] 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.[21] In human MSCs, HLA-G contributes to the immunosuppressive properties that allow MSCs to block alloimmune reactions.[22] 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.[23,24]

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 also 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

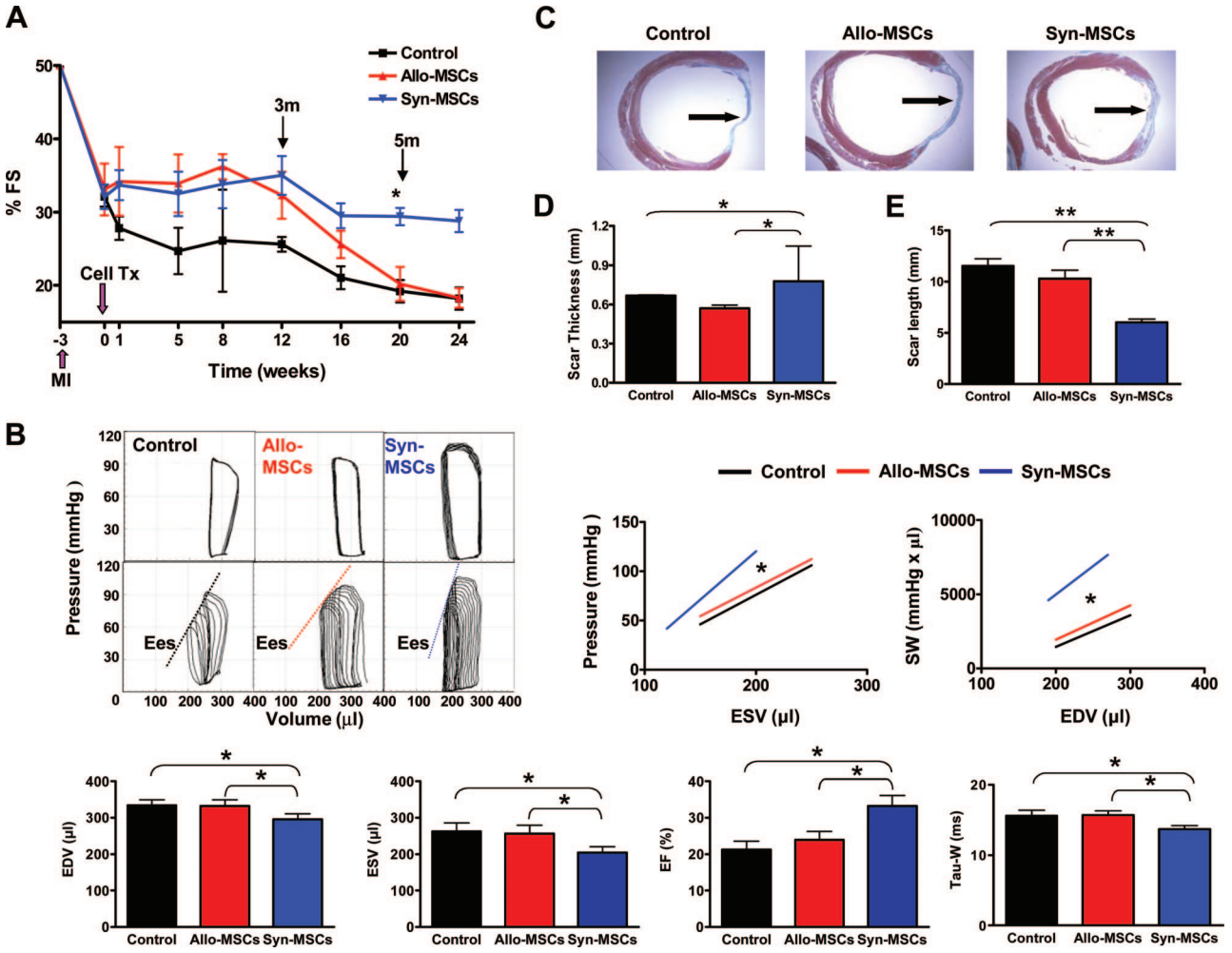

Figure 8. Allogeneic MSCs restore cardiac function as effectively as syngeneic MSCs for 3 months, but not 6 months, after implantation. A, Cardiac function (fractional shortening; %FS) was evaluated by echocardiography before MI (−3 weeks), immediately before implantation (Tx) of undifferentiated allogeneic (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], τ) 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. *$P<0.05$, **$P<0.01$.

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.

Acknowledgments

Dr Li is a Career Investigator of the Heart and Stroke Foundation of Canada and holds a Canada Research Chair in cardiac regeneration. Dr Huang is a Heart and Stroke Foundation of Canada research fellow. We thank Shuhong Li for technical support.

Sources of Funding

This work was supported by the Heart and Stroke Foundation of Ontario (T6604 to Dr Li) and the Canadian Institutes of Health Research (MOP14795 and MOP102535 to Dr Li; team grant RMF82498).

Disclosures

None.

References

1. Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest*. 2005;115:572–583.
2. Tomita S, Mickle DA, Weisel RD, Jia ZQ, Tumiati LC, Allidina Y, Liu P, Li RK. Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. *J Thorac Cardiovasc Surg*. 2002;123:1132–1140.
3. Menasche P. Current status and future prospects for cell transplantation to prevent congestive heart failure. *Semin Thorac Cardiovasc Surg*. 2008; 20:131–137.
4. Heiss C, Keymel S, Niesler U, Ziemann J, Kelm M, Kalka C. Impaired progenitor cell activity in age-related endothelial dysfunction. *J Am Coll Cardiol*. 2005;45:1441–1448.
5. Rauscher FM, Goldschmidt-Clermont PJ, Davis BH, Wang T, Gregg D, Ramaswami P, Pippen AM, Annex BH, Dong C, Taylor DA. Aging, progenitor cell exhaustion, and atherosclerosis. *Circulation*. 2003;108:457–463.
6. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood*. 2005;105:1815–1822.
7. Atoui R, Shum-Tim D, Chiu RC. Myocardial regenerative therapy: immunologic basis for the potential "universal donor cells." *Ann Thorac Surg*. 2008;86:327–334.
8. Hare JM, Traverse JH, Henry TD, Dib N, Strumpf RK, Schulman SP, Gerstenblith G, DeMaria AN, Denktas AE, Gammon RS, Hermiller JB Jr, Reisman MA, Schaer GL, Sherman W. A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. *J Am Coll Cardiol*. 2009;54:2277–2286.
9. Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res*. 2004;95:9–20.
10. Amado LC, Saliaris AP, Schuleri KH, St JM, Xie JS, Cattaneo S, Durand DJ, Fitton T, Kuang JQ, Stewart G, Lehrke S, Baumgartner WW, Martin BJ, Heldman AW, Hare JM. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. *Proc Natl Acad Sci U S A*. 2005;102:11474–11479.
11. Makkar RR, Price M, Lill M. Mutilineage differentiation of transplanted allogeneic mesenchymal stem cells in a porcine model of recent myocardial infarction improves left ventricular function. *Circulation*. 2002; 106(suppl):II-34. Abstract.
12. Quevedo HC, Hatzistergos KE, Oskouei BN, Feigenbaum GS, Rodriguez JE, Valdes D, Pattany PM, Zambrano JP, Hu Q, McNiece I, Heldman AW, Hare JM. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. *Proc Natl Acad Sci U S A*. 2009;106:14022–14027.
13. Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. *Blood*. 2006;108:2114–2120.
14. Poncelet AJ, Vercruysse J, Saliez A, Gianello P. Although pig allogeneic mesenchymal stem cells are not immunogenic in vitro, intracardiac injection elicits an immune response in vivo. *Transplantation*. 2007;83:783–790.
15. Kinkaid HY, Huang XP, Li RK, Weisel RD. What's new in cardiac cell therapy? *J Card Surg*. 2010;25:359–366.
16. Agbulut O, Coirault C, Niederlander N, Huet A, Vicart P, Hagege A, Puceat M, Menasche P. GFP expression in muscle cells impairs actin-myosin interactions: implications for cell therapy. *Nat Methods*. 2006;3:331.
17. Di NM, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood*. 2002;99:3838–3843.
18. Inoue S, Popp FC, Koehl GE, Piso P, Schlitt HJ, Geissler EK, Dahlke MH. Immunomodulatory effects of mesenchymal stem cells in a rat organ transplant model. *Transplantation*. 2006;81:1589–1595.
19. Rodgers JR, Cook RG. MHC class Ib molecules bridge innate and acquired immunity. *Nat Rev Immunol*. 2005;5:459–471.
20. Bainbridge DR, Ellis SA, Sargent IL. HLA-G suppresses proliferation of CD4(+) T-lymphocytes. *J Reprod Immunol*. 2000;48:17–26.
21. Le Bouteiller P, Blaschitz A. The functionality of HLA-G is emerging. *Immunol Rev*. 1999;167:233–244.
22. Nasef A, Mathieu N, Chapel A, Frick J, Francois S, Mazurier C, Boutarfa A, Bouchet S, Gorin NC, Thierry D, Fouillard L. Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G. *Transplantation*. 2007;84: 231–237.
23. Grinnemo KH, Mansson A, Dellgren G, Klingberg D, Wardell E, Drvota V, Tammik C, Holgersson J, Ringden O, Sylven C, Le Blanc K. Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium. *J Thorac Cardiovasc Surg*. 2004;127:1293–1300.
24. Grinnemo KH, Mansson-Broberg A, Leblanc K, Corbascio M, Wardell E, Siddiqui AJ, Hao X, Sylven C, Dellgren G. Human mesenchymal stem cells do not differentiate into cardiomyocytes in a cardiac ischemic xenomodel. *Ann Med*. 2006;38:144–153.

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.