

# Preserving Prostaglandin E2 Level Prevents Rejection of Implanted Allogeneic Mesenchymal Stem Cells and Restores Postinfarction Ventricular Function

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**Background**—Allogeneic mesenchymal stem cells (MSCs) were immunoprivileged early after cardiac implantation and improved heart function in preclinical and clinical studies. However, long-term preclinical studies demonstrated that allogeneic MSCs lost their immunoprivilege and were rejected in the injured myocardium, resulting in recurrent ventricular dysfunction. This study identifies some of the mechanisms responsible for the immune switch in MSCs and suggests a new treatment to maintain immunoprivilege and preserve heart function.

**Methods and Results**—Rat MSC immunoprivilege was mediated by prostaglandin E2 (PGE2)-induced secretion of 2 critical chemokines, CCL12 and CCL5. These chemokines stimulated the chemoattraction of T cells toward MSCs, suppressed cytotoxic T-cell proliferation, and induced the production of T regulatory cells. MSCs treated with 5-azacytidine for 24 hours differentiated into myogenic cells after 2 weeks, which was associated with decreased PGE2 and chemokine production and the loss of immunoprivilege. Treatment of differentiated MSCs with PGE2 restored chemokine levels and preserved MSC immunoprivilege. In a rat myocardial infarction model, allogeneic MSCs ( $3 \times 10^6$  cells/rat) were injected into the infarct region with or without a biodegradable hydrogel that slowly released PGE2. Five weeks later, the transplanted MSCs expressed myogenic lineage markers and were rejected in the control group, but in the PGE2-treated group, the transplanted cells survived and heart function improved.

**Conclusions**—Allogeneic MSCs maintained immunoprivilege by PGE2-induced secretion of chemokines CCL12 and CCL5. Differentiation of MSCs decreased PGE2 levels, and immunoprivilege was lost. Maintaining PGE2 levels preserved immunoprivilege after differentiation, prevented rejection of implanted MSCs, and restored cardiac function. (*Circulation*. 2013;128[suppl 1]:S69-S78.)

**Key Words:** immunoprivilege ■ myocardial infarction ■ stem cells ■ transplantation

Accumulated evidence suggests that stem cells may regenerate the heart after injury, restore cardiac function, and prevent progressive heart failure. Bone marrow-derived mesenchymal stem cells (MSCs) are the most immediate candidates for clinical application because of their availability and proven effectiveness in preclinical studies.<sup>1</sup> However, the initial clinical trials with autologous MSCs in aging patients suggested only marginal benefits on heart function,<sup>2</sup> which might be because of age-related decreases in the proliferative and regenerative capability of MSCs.<sup>3</sup> Therefore, highly regenerative young donor cells such as allogeneic MSCs may be ideal candidate cells to induce cardiac regeneration after myocardial infarction.

Allogeneic MSCs isolated from young healthy donors have excellent regenerative potential and immediate availability for clinical application.<sup>4,5</sup> These cells are thought to be immunoprivileged and were successful in regenerating the myocardium.<sup>6–8</sup> Early-phase trials of intravenous administration of allogeneic MSCs demonstrated a trend toward improved clinical outcomes.<sup>9</sup> However, the long-term fate of the cells in the recipient heart was not assessed. Poncelet et al<sup>10</sup> reported that allogeneic MSCs were not immunogenic in vitro but elicited immune responses after intracardiac injection in a pig model. We previously reported that differentiation of allogeneic stem cells after implantation in the infarcted myocardium was associated with loss of their immunoprivilege, cell rejection, and

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progressive ventricular dysfunction.<sup>11</sup> Therefore, to prevent the rejection of transplanted allogeneic MSCs and improve their efficacy for cardiac repair, we initiated an extensive investigation of the immune characteristics of allogeneic MSCs and their interaction with the recipient immune system.

The immunoprivilege of MSCs is maintained by several immunosuppressive factors, including prostaglandin E2 (PGE2),<sup>4,7,12</sup> a small lipid molecule that prevents the maturation of dendritic cells and inhibits the proliferation of cytotoxic T cells.<sup>12</sup> In the present study, we demonstrated that MSC PGE2 levels decreased significantly after myogenic differentiation, which was associated with the loss of immunoprivilege. We found that maintaining optimal levels of PGE2 in differentiated MSCs (D-MSCs) preserved their immunoprivilege, prevented their rejection from the myocardium, and improved heart function.

## Methods

Detailed methodology is provided in the online-only Data Supplement. All animal procedures were approved by the Animal Care Committee of 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, revised 1996). Myogenic differentiation of rat MSCs was induced with 10  $\mu\text{mol/L}$  5-azacytidine and identified with  $\alpha$ - and  $\beta$ -myosin heavy chain (MHC) markers. In the in vitro study, peripheral blood leukocytes from Lewis rats were cocultured with MSCs from Wistar rats in a 10:1 ratio. PGE2 at concentrations of 10 and 50 ng/mL was used to treat cells to preserve immunoprivilege. Cytotoxicity (by lactate dehydrogenase assay), leukocyte proliferation (bromodeoxyuridine labeling), and cytokine levels were evaluated after 72 hours of coculture. Chemokines were evaluated by polymerase chain reaction (PCR) array and real-time PCR.

In the in vivo study, myocardial infarction was induced in female Lewis rats by coronary artery ligation. One week later, allogeneic green fluorescent protein positive (GFP+) or GFP- MSCs (undifferentiated

or induced with 5-azacytidine;  $3 \times 10^6$  cells/rat) from male Wistar rats were transplanted into the infarct area. To provide sustained PGE2 delivery to the infarct area, a biodegradable, temperature-sensitive hydrogel was used for slow release of PGE2 at the site of cell transplantation. Host immune response against MSCs was measured after cell implantation by the assessment of CD8+ T-cell infiltration in the myocardium and alloantibody production. Transplanted cell survival was analyzed by quantifying the number of Y chromosomes by real-time PCR and anti-GFP immunostaining. Differentiation of transplanted MSCs was studied by coimmunostaining the myocardial tissue with anti-GFP and anti- $\alpha$ -smooth muscle actin, factor VIII, or MHC antibodies (smooth muscle, endothelial, and myogenic cell markers, respectively). Cardiac function was evaluated using echocardiography.

Data are expressed as mean  $\pm$  SD. Comparisons between 2 groups were made with a 2-tailed Student *t* test. Comparisons among multiple groups were performed with either 1-way or 2-way ANOVA. When *F* values were significant, group differences were specified with Tukey or Bonferroni post-tests. Differences were considered statistically significant when  $P < 0.05$ .

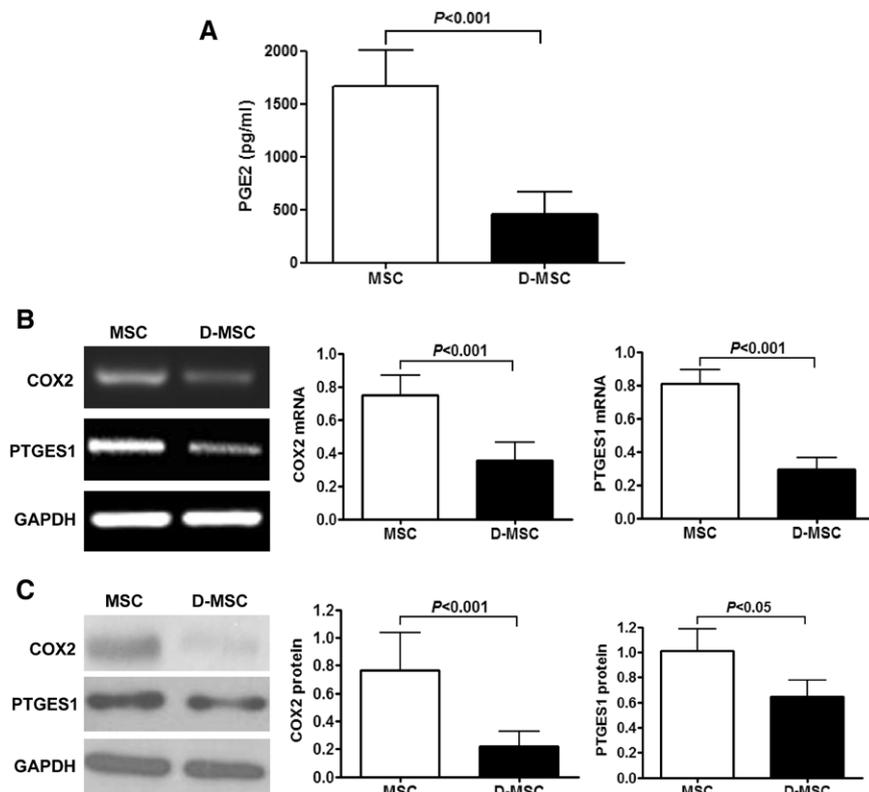
## Results

### In Vitro Model of MSC Differentiation

MSCs treated with 5-azacytidine differentiated into myogenic cells as evidenced by the increase in myogenic-specific  $\alpha$ - and  $\beta$ -MHC mRNA and protein levels (Figure I in the online-only Data Supplement). As we previously reported, 5-azacytidine treatment stimulated MSCs to form myotube-like structures and express the myogenic-specific genes *Nkx2.5* and *MyoD*, suggesting that 5-azacytidine-treated MSCs acquired the characteristics of myogenic cells.<sup>11</sup>

### PGE2 Levels Decreased in MSCs After Differentiation

We measured the levels of several critical soluble factors involved in maintaining MSC immunoprivilege, such as



**Figure 1.** Differentiation of mesenchymal stem cells (MSCs) decreased prostaglandin E2 (PGE2), cyclooxygenase 2 (COX2), and prostaglandin E synthase-1 (PTGES1) levels. MSCs were treated with 5-azacytidine to induce myogenic differentiation. **A**, PGE2 levels were measured by ELISA ( $n=12$ ). **B**, COX2 and PTGES1 mRNA expression was evaluated by reverse transcriptase-polymerase chain reaction ( $n=6$ ). **C**, COX2 and PTGES1 protein expression was assessed by Western blot ( $n=4-6$ ). Data are derived from 2 to 3 independent experiments. D-MSC indicates differentiated MSC.

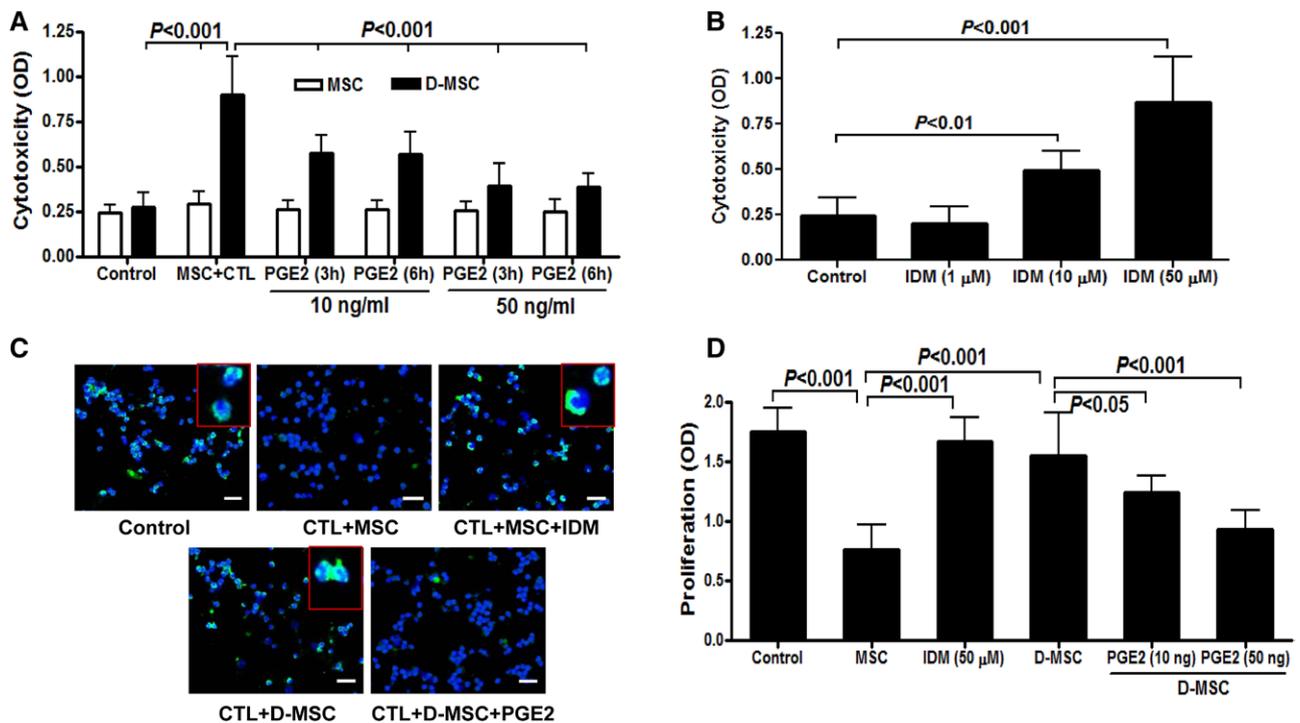
indoleamine 2,3-dioxygenase, PGE2, transforming growth factor- $\beta$ , nitric oxide, and hepatocyte growth factor. Myogenic differentiation did not alter the mRNA or protein expression of indoleamine 2,3-dioxygenase, nitric oxide, transforming growth factor- $\beta$ , or hepatocyte growth factor (Figure II in the online-only Data Supplement). However, PGE2 levels significantly decreased in D-MSCs in comparison with undifferentiated cells (Figure 1A). PGE2 is synthesized in cells after oxidation of arachidonic acid by cyclooxygenase 2 and prostaglandin E synthase-1. To understand the mechanisms responsible for the alteration of PGE2 levels, we measured the expression of cyclooxygenase 2 and prostaglandin E synthase-1 in MSCs and observed a significant decrease in mRNA and protein levels of these enzymes in D-MSCs compared with undifferentiated MSCs (Figure 1B and 1C).

**PGE2 Decrease in MSCs Was Associated With Loss of Immunoprivilege**

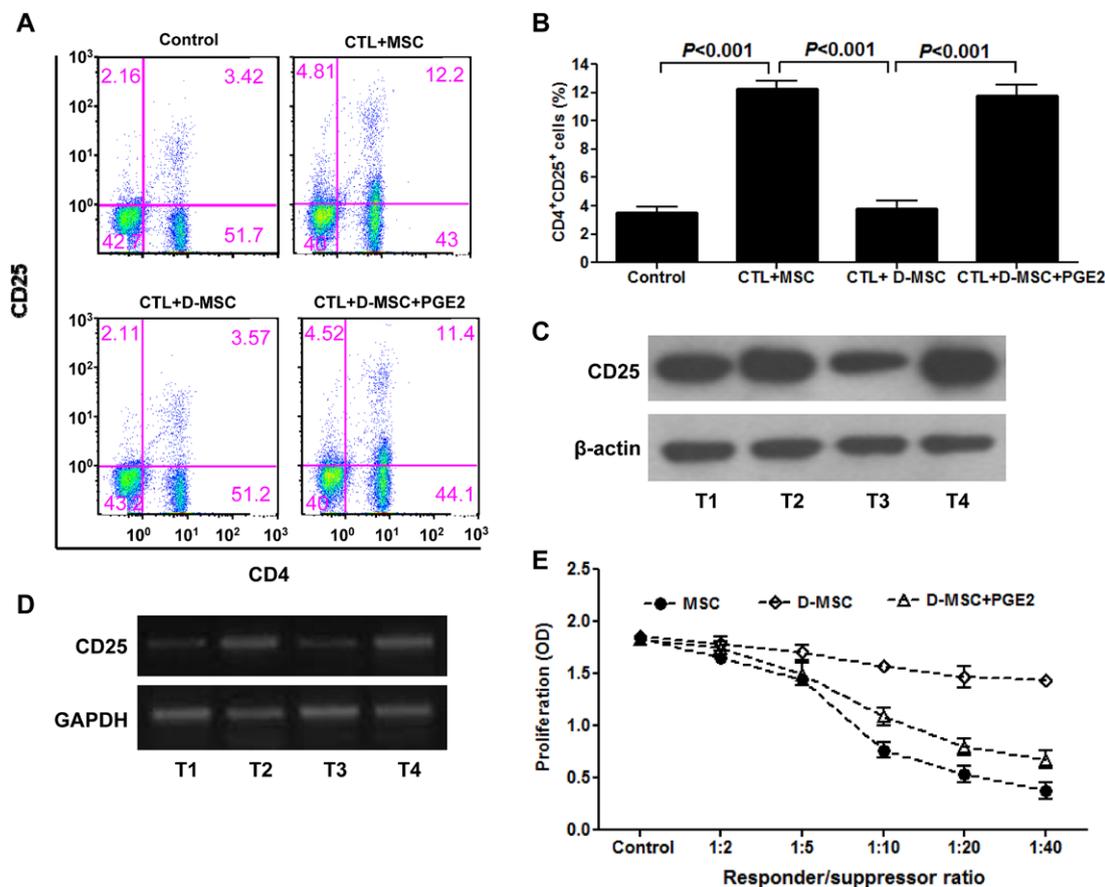
To explore the relationship between the PGE2 decrease in D-MSCs and their loss of immunoprivilege, we cocultured MSCs with allogeneic leukocytes and assessed the extent of MSC injury induced by the cytotoxic leukocytes. The level of cytotoxicity was significantly greater after MSC differentiation (Figure 2A). PGE2 treatment of D-MSCs at 10 and 50 ng/mL (for 3 and 6 hours) decreased the leukocyte-mediated cytotoxicity in a dose-dependent manner (Figure 2A). We also determined whether PGE2 treatment itself produced any cytotoxicity and found that at these doses no toxicity was

detected in these cells (data not shown). Next, we analyzed the effect of PGE2 on MSC differentiation and dedifferentiation. It was observed that PGE2 treatment at 50 ng/mL for 6 hours had no effect on 5-azacytidine-induced myogenic differentiation, and this dose of PGE2 did not cause any dedifferentiation of myogenic cells (Figure IIIA and IIIB in the online-only Data Supplement). These pilot studies demonstrated that this dose and treatment protocol was optimal. Furthermore, when PGE2 biosynthesis in undifferentiated cells was blocked with indomethacin, the level of cytotoxicity associated with leukocyte coculture increased proportionally to the dose of PGE2 blocker (Figure 2B). We also measured the effect of MSCs on leukocyte proliferation after 72 hours of coculture. Undifferentiated MSCs significantly decreased leukocyte proliferation, whereas D-MSCs had no effect on leukocyte proliferation. However, pretreatment of D-MSCs with PGE2 resulted in the suppression of leukocyte proliferation in a dose-dependent manner, and indomethacin pretreatment of undifferentiated MSCs prevented the suppression of leukocyte proliferation (Figure 2C and 2D).

Another mechanism of MSC-mediated immunoprivilege is the induction of host T regulatory (Treg) cells, which suppress immune responses.<sup>8</sup> In the present study, the number of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells increased significantly after coculture with undifferentiated MSCs, whereas coculture with D-MSCs did not induce any increase in the number of Treg cells. Pretreatment of differentiated cells with PGE2 significantly increased the CD4<sup>+</sup>CD25<sup>+</sup> T cell population after coculture



**Figure 2.** Prostaglandin E2 (PGE2) decrease in differentiated mesenchymal stem cells (D-MSCs) was associated with the loss of immunoprivilege. MSCs were cocultured with allogeneic leukocytes (CTL). PGE2 treatment (50 ng/mL) persisted for 6 hours unless otherwise stated. **A**, Leukocyte-mediated cytotoxicity was significantly greater with D-MSCs compared with undifferentiated MSCs, and this effect was reduced with PGE2. Control=MSCs without leukocytes (n=10). **B**, Undifferentiated MSCs were treated with the PGE2 blocker indomethacin (IDM). Increasing doses of IDM resulted in an increase in leukocyte-mediated cytotoxicity. Control=no IDM (n=10). **C** and **D**, Bromodeoxyuridine uptake to assess leukocyte proliferation was detected by fluorescein isothiocyanate-labeled antibody (scale bar, 20  $\mu$ m, n=6) and ELISA (**D**, n=12). Control=leukocytes (CTL) activated with phytohemagglutinin (10  $\mu$ g/mL), without MSCs. Data are derived from 2 to 3 independent experiments.



**Figure 3.** Prostaglandin E2 (PGE2) decrease in differentiated mesenchymal stem cells (D-MSCs) was associated with loss of T regulatory (Treg) cell induction. MSCs were cocultured with allogeneic leukocytes (CTL). PGE2 treatment (50 ng/mL) persisted for 6 hours. The effect of MSCs on Treg cell induction was assessed. **A**, Representative flow cytometry plots of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the mixed leukocyte population. **B**, Percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells. Control=leukocytes (CTL) activated with phytohemagglutinin (PHA; 10 μg/mL), without MSCs. **C** and **D**, CD4<sup>+</sup> T cells isolated from total leukocyte population were cocultured with stem cells. CD25 expression by Western blot (**C**) and reverse transcriptase-polymerase chain reaction (**D**). T1=T cells activated with PHA, T2=T cells cocultured with undifferentiated MSCs, T3=T cells cocultured with D-MSCs, T4=T cells cocultured with PGE2-treated D-MSCs. **E**, Leukocytes after coculture with MSCs were irradiated and cocultured as potential suppressor cells with autologous responder leukocytes (activated with 10 μg/mL PHA) at different responder/suppressor ratios. Control=only responder leukocytes. (n=4 in each experiment). Data are derived from 2 independent experiments.

(Figure 3A and 3B). The CD25 protein and mRNA expression in the purified CD4<sup>+</sup> T-cell population increased significantly after the coculture of CD4<sup>+</sup> T cells with undifferentiated MSCs (Figure 3C and 3D). D-MSCs had no significant effect on CD25 expression, but PGE2 treatment of D-MSCs increased CD25 expression in purified CD4<sup>+</sup> T cells after coculture.

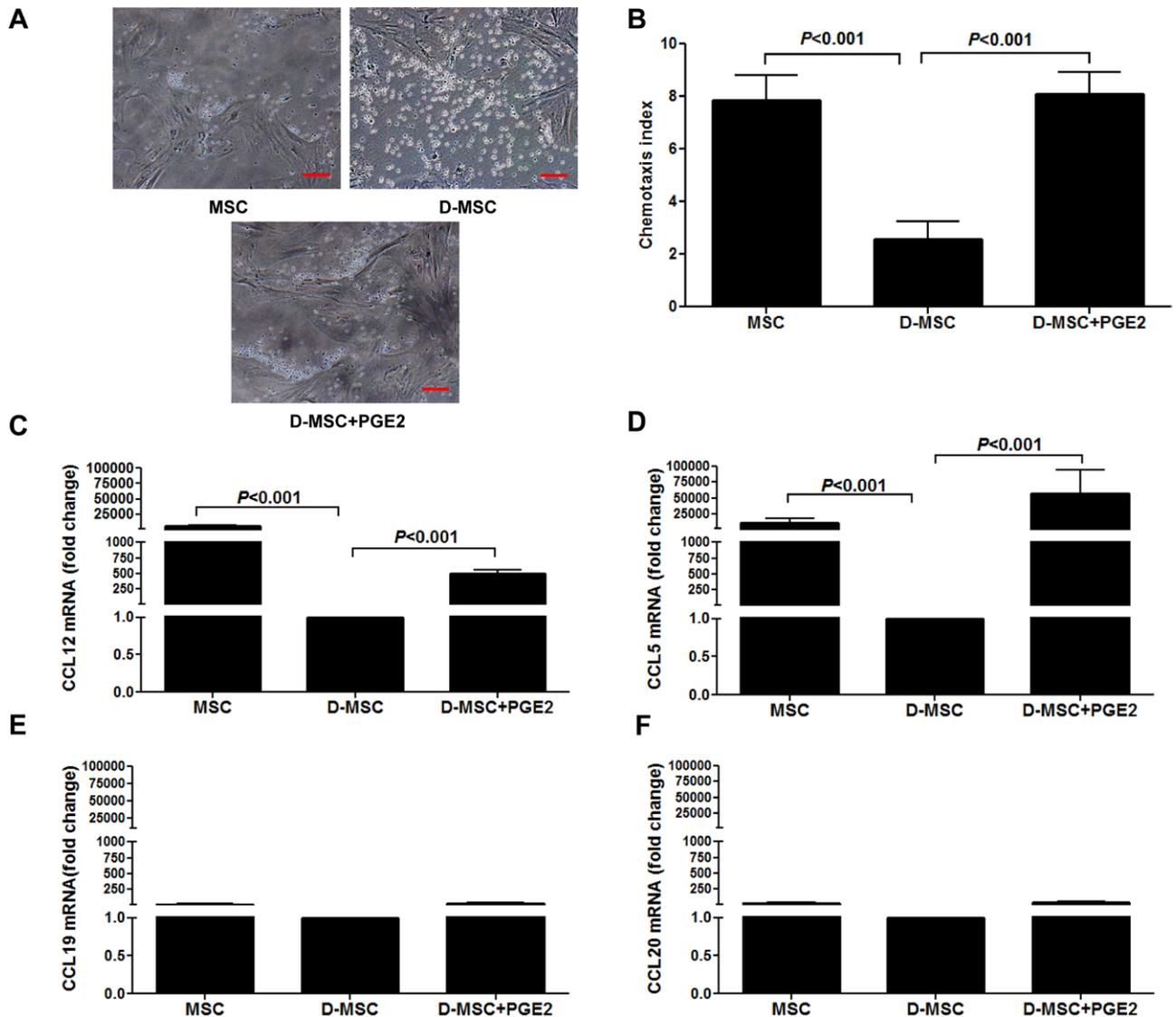
To further confirm that CD4<sup>+</sup> T cells from the mixed leukocyte population acquired the regulatory (immunosuppressive) profile after coculture with MSCs, leukocytes that had been cocultured with MSCs (primary coculture) were irradiated (15 Gy) and then cocultured again, as potential suppressor cells, with preactivated (treated with 10 μg/mL of phytohemagglutinin) autologous leukocytes (responder cells) at different responder/suppressor ratios, and responder cell proliferation was measured. In the secondary coculture, as the number of suppressor cells increased, the proliferation of responders decreased significantly (Figure 3E). Suppressors from the primary coculture with D-MSCs were not able to decrease the proliferation of responder cells at all responder/suppressor ratios. PGE2 treatment of D-MSCs preserved their capacity to change the phenotype of T cells

toward Treg cells, and these leukocytes significantly suppressed the proliferation of responder cells in the secondary coculture (Figure 3E).

### PGE2 Maintained MSC Chemotactic Ability

In the present study, we observed an interesting phenomenon in the coculture experiments—leukocytes actively migrating toward undifferentiated MSCs (Figure 4A), which is illustrated in the appended Movie (Movie I in the online-only Data Supplement). However, with D-MSCs, the leukocytes did not migrate, but were randomly scattered around the culture plates (Figure 4A). PGE2-treated D-MSCs regained the ability to attract leukocytes. In transwell cocultures, the leukocyte chemotaxis index was significantly higher for undifferentiated MSCs compared with D-MSCs. PGE2 supplementation of D-MSCs increased the leukocyte chemotaxis index (Figure 4B). These findings suggest that undifferentiated MSCs secrete potent chemotactic chemokines to attract leukocytes.

To determine which chemokines were involved in the MSC-induced leukocyte chemotaxis, we screened transcripts of 84 chemokines and their receptors in MSCs after coculture



**Figure 4.** Prostaglandin E2 (PGE2) maintained mesenchymal stem cell (MSC) chemotactic ability. MSCs were cocultured with allogeneic leukocytes. PGE2 treatment (50 ng/mL) persisted for 6 hours (MSC+PGE2). **A**, Extent of leukocyte migration was observed microscopically (scale bar, 50  $\mu$ m, n=10). **B**, The chemotaxis index is the ratio of the number of leukocytes migrated in response to MSCs vs the number migrated to the medium alone (n=10). **C–F**, CCL12, CCL5, CCL19, and CCL20 mRNA expression by real-time polymerase chain reaction (n=6). Data are derived from 2 to 3 independent experiments.

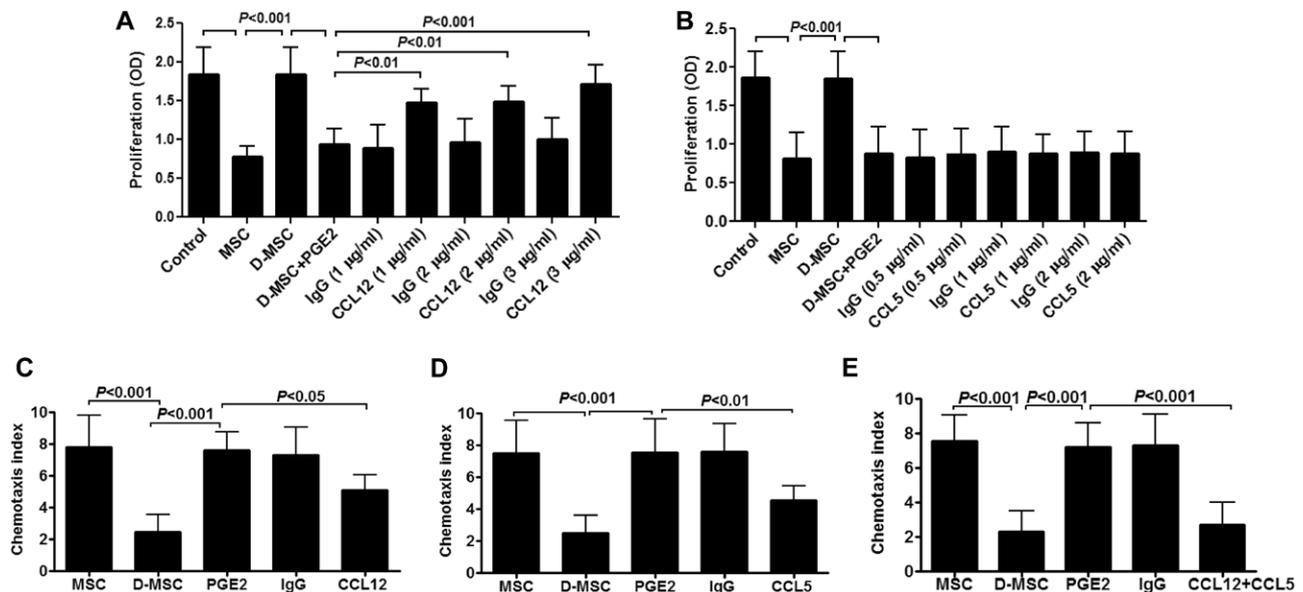
with leukocytes. The PCR array results indicated that the mRNA expression of CCL5, CCL12, CCL19, and CCL20 decreased significantly in D-MSCs compared with undifferentiated MSCs (Table I in the online-only Data Supplement). For confirmation, real-time PCR using TaqMan probes was performed to evaluate the mRNA expression of these 4 chemokines in MSCs (Figure 4C–4F). Interestingly, CCL5 and CCL12 expression decreased significantly in differentiated cells, and PGE2 treatment increased the level of these 2 chemokines in D-MSCs, whereas CCL19 and CCL20 expression did not change in D-MSCs, with or without PGE2 treatment (Figure 4C–4F).

#### PGE2-Mediated Induction of MSC Chemokines Maintained Immunoprivilege

To explore the involvement of CCL5 and CCL12 in PGE2-mediated MSC immunoprivilege, we added neutralizing

antibodies for these 2 chemokines in the MSC and leukocyte cocultures and assessed leukocyte proliferation. The addition of CCL12 neutralizing antibody significantly prevented the PGE2-mediated decrease in leukocyte proliferation by MSCs (Figure 5A). The blocking antibody for CCL5 had no effect on leukocyte proliferation (Figure 5B).

Furthermore, the presence of CCL12 and CCL5 blocking antibodies in the coculture, individually as well as in combination, prevented migration of leukocytes toward MSCs (Figure 5C–5E). Thus, both CCL12 and CCL5 participate in the chemoattraction of leukocytes toward MSCs. However, CCL12 seems to have a direct role in the suppression of leukocyte proliferation. Myogenic differentiation of MSCs leads to a decrease in the production of CCL12 and CCL5 and a loss of the cells' ability to attract leukocytes. PGE2 maintains the levels of these 2 chemokines in MSCs and preserves their ability to attract leukocytes.



**Figure 5.** Chemokines maintained mesenchymal stem cell (MSC) immunoprivilege. **A** and **B**, MSCs were cocultured with allogeneic leukocytes and the effect on leukocyte proliferation was assessed. Control (leukocytes activated with 10 µg/mL phytohemagglutinin [PHA]), MSC (leukocytes+undifferentiated MSC), differentiated MSC (D-MSC; leukocytes+D-MSC), D-MSC+prostaglandin E2 (PGE2; D-MSC treated with PGE2 for 6 hours at 50 ng/mL), and PGE2-treated D-MSCs in the presence of CCL12 (**A**) or CCL5 (**B**) neutralizing antibodies or isotype control (n=8). **C–E**, Leukocyte chemotaxis index. MSC (leukocytes+MSCs), D-MSC (leukocytes+D-MSC), D-MSC+PGE2 (PGE2-treated D-MSC), and PGE2-treated D-MSCs in the presence of CCL12 (2 µg/mL, **C**) or CCL5 (2 µg/mL, **D**) neutralizing antibodies individually or in combination (CCL12+CCL5, **E**) or an isotype control (2 µg/mL) (n=8). Data are derived from 2 independent experiments.

### Maintaining PGE2 Levels in MSCs Alleviated Allogeneic Immune Response and Prevented Rejection of Transplanted Cells In Vivo

Because of the important role of PGE2 in MSC immunoprivilege, we hypothesized that maintaining optimal levels of PGE2 in MSCs during differentiation would mitigate the alloimmune response by the host and prevent the rejection of transplanted allogeneic MSCs in the injured heart. To test this hypothesis, undifferentiated and differentiated (5-azacytidine-treated) allogeneic MSCs were transplanted into infarcted rat hearts, and the host immune response and transplanted cell survival were assessed at 1 and 5 weeks. PGE2 has a short half-life under physiological conditions, so to achieve sustained PGE2 effects, a biodegradable, temperature-sensitive hydrogel was used to deliver PGE2 to the heart at a dose of 300 ng/kg of body weight. This dose of PGE2 was derived based on the in vitro PGE2 release kinetics of different amounts of PGE2 conjugated to the hydrogel (Figure IV in the online-only Data Supplement).

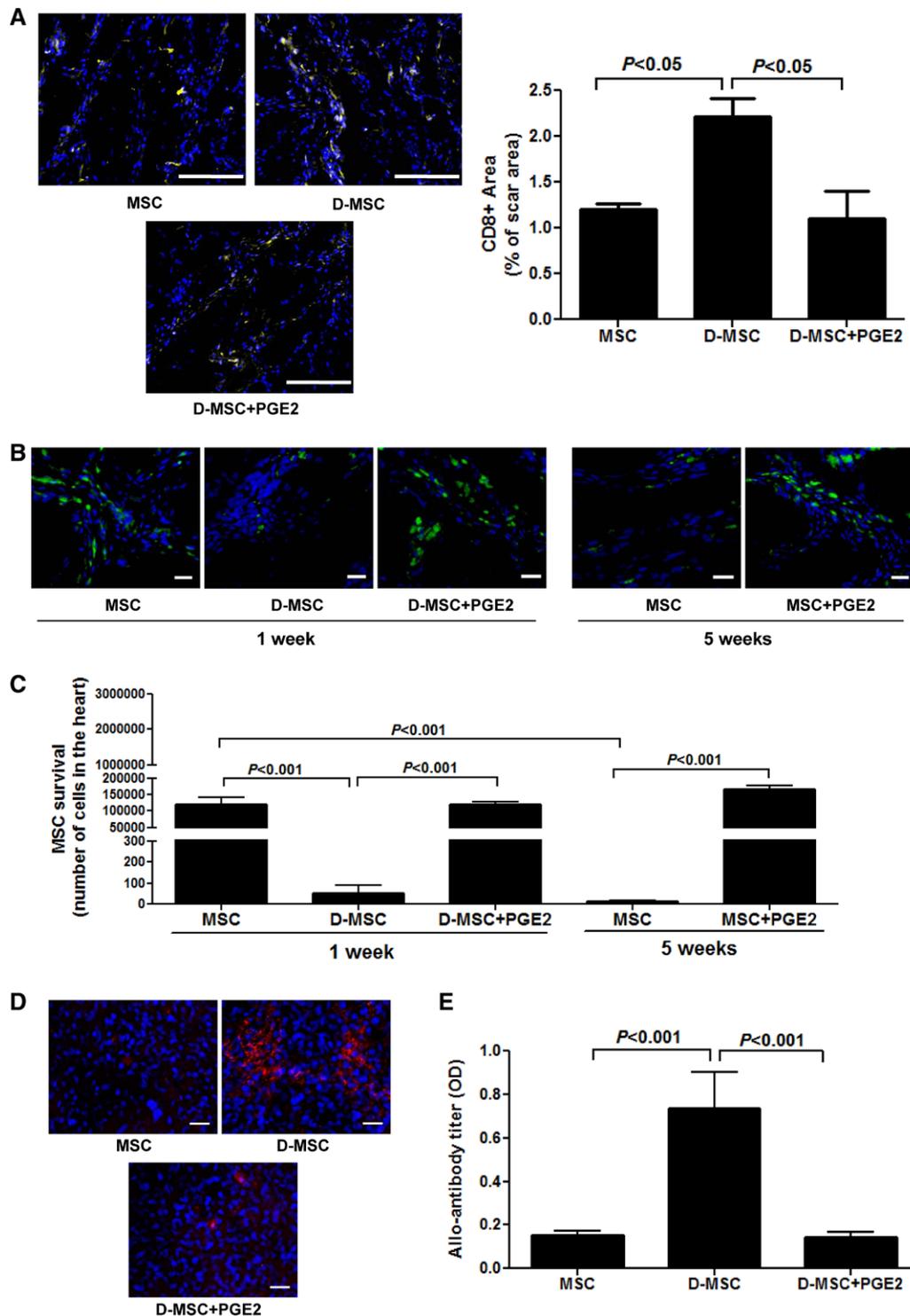
One week after cell transplantation, we observed significantly more cytotoxic CD8<sup>+</sup> T cells infiltrated into the scar area of the myocardium transplanted with D-MSCs in comparison with undifferentiated MSCs (Figure 6A), and most of the differentiated cells were rejected in the heart because transplanted cell survival decreased significantly for differentiated versus undifferentiated MSCs (Figure 6B and 6C). PGE2 treatment of D-MSCs decreased the number of CD8<sup>+</sup> T cells in the myocardium and significantly enhanced the survival of transplanted MSCs. Although a significant number of undifferentiated MSCs survived in the myocardium 1 week after cell transplantation, almost all the cells were rejected after 5 weeks, at a time when MSCs differentiated in our previous study.<sup>11</sup> However, PGE2 treatment

significantly increased the survival of transplanted cells after 5 weeks (Figure 6B and 6C). We also analyzed the phenotype of these surviving cells at 5 weeks. In the PGE2-treated group, 2.74±1.28% of the engrafted MSCs expressed MHC (Figure 7A and 7B), 4.64±2.16% expressed α-smooth muscle actin, and 5.31±1.97% expressed factor VIII (Figures V and VI in the online-only Data Supplement). However, at 1 week after cell implantation, although a significant number of GFP<sup>+</sup> cells were detected in the myocardium, the cells seemed to remain undifferentiated because we could not detect colocalization of GFP and MHC, α-smooth muscle actin, or factor VIII in either the MSC or MSC+PGE2 group (Figure 7A and 7B; online-only Data Supplement Figure V and VI). In both the in vitro and in vivo studies, although the cells expressed the markers of myogenic lineage, we did not observe MSCs differentiating toward mature cardiac cells.

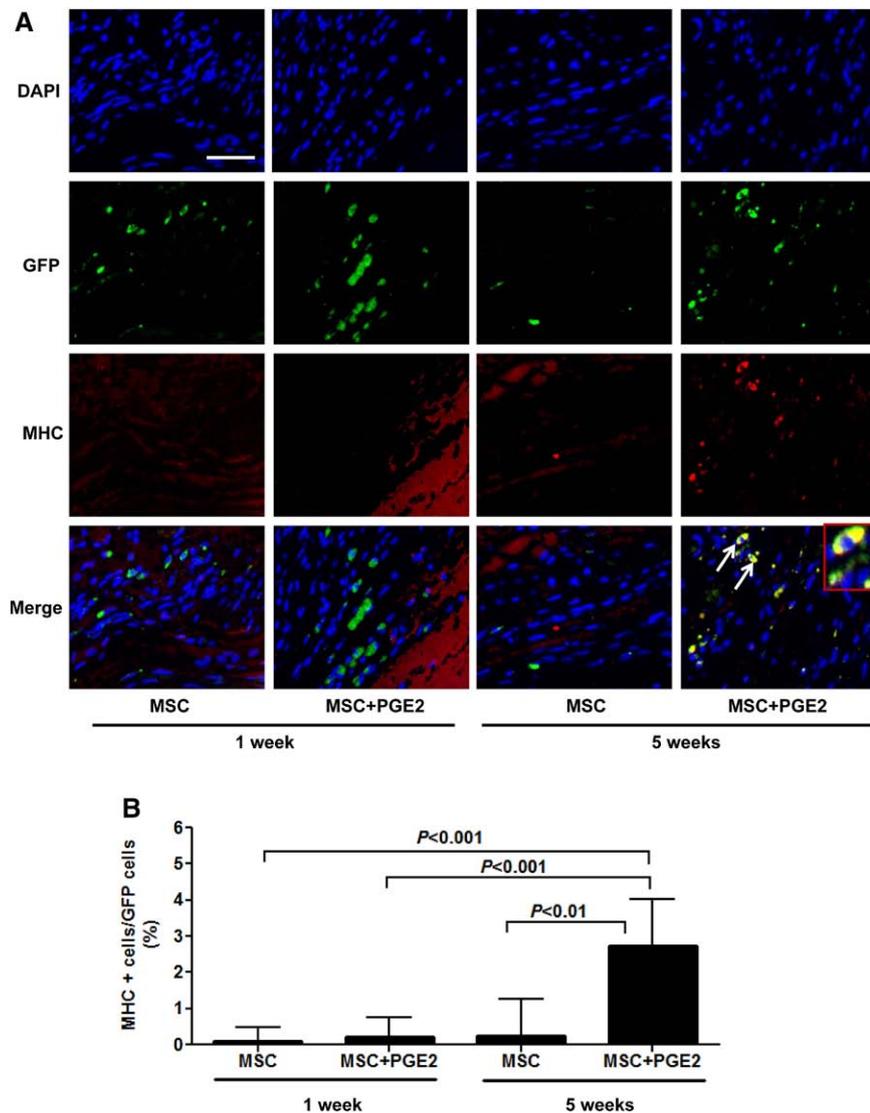
We investigated whether these surviving allogeneic MSCs elicited any long-term adaptive immune response in terms of alloantibody production by the host against the transplanted cells. At 5 weeks after cell transplantation, the blood collected from allogeneic cell recipients contained alloantibodies that reacted with D-MSCs, but not with undifferentiated allogeneic MSCs. The alloantibody titers significantly decreased in cell recipients supplemented with PGE2 (Figure 6D and 6E). Thus, PGE2 secreted by MSCs maintained the immunoprivilege and mitigated both cellular and humoral host immune responses after cell transplantation.

### Maintaining PGE2 Levels in Transplanted MSCs Restored Postinfarction Cardiac Function

Coronary artery ligation produced significant left ventricular dilatation at 7 days after myocardial infarction, with



**Figure 6.** Prostaglandin E2 (PGE2) increased survival of transplanted mesenchymal stem cells (MSCs) in the heart and alleviated the host immune response. Allogeneic GFP<sup>+</sup> or GFP<sup>-</sup> MSCs from male Wistar rats were transplanted into the infarct area 7 days after myocardial infarction in female Lewis rats. MSC=undifferentiated MSCs, D-MSC=differentiated MSCs, MSC+PGE2, D-MSC+PGE2. 4',6-diamidino-2-phenylindole was used as a nuclear stain (blue). **A**, CD8 expression by immunohistochemistry in myocardial sections 7 days after cell transplantation. CD8<sup>+</sup> positive area was calculated (scale bar, 100  $\mu$ m, n=3). **B**, GFP<sup>+</sup> transplanted cells were detected by staining myocardial sections with an anti-GFP antibody (scale bar, 20  $\mu$ m, n=5). **C**, Cell survival by real-time polymerase chain reaction to quantify the number of Y chromosomes in female myocardium (n=5). **D**, At 5 weeks after cell transplantation, recipient serum was incubated with allogeneic MSCs for 2 hours, and the MSCs were stained with phycoerythrin-conjugated antirat IgG. Representative micrographs illustrate IgG expression (red) in MSCs (scale bar, 50  $\mu$ m, n=4). **E**, Allo-antibody titers (IgG levels) were measured in the recipient serum by ELISA (n=6). Data are derived from 2 independent experiments.



**Figure 7.** In vivo differentiation of transplanted mesenchymal stem cells (MSCs). **A**, Representative micrographs illustrate expression of GFP (green, implanted cells) and myosin heavy chain (MHC; red) in the myocardial tissue of recipients at 1 and 5 weeks after cell transplantation. 4',6-diamidino-2-phenylindole (DAPI) was used as a nuclear stain (scale bar, 50  $\mu$ m, n=4). **B**, Quantification of MHC-expressing GFP cells in the myocardial tissue. A total of 4 fields per slide were counted to quantify the number of MHC-expressing GFP<sup>+</sup> cells. After 1 week, GFP<sup>+</sup> cells were visible in both MSC and MSC+PGE2 groups, but colocalization of MHC with GFP was observed only after 5 weeks in the PGE2-treated group.

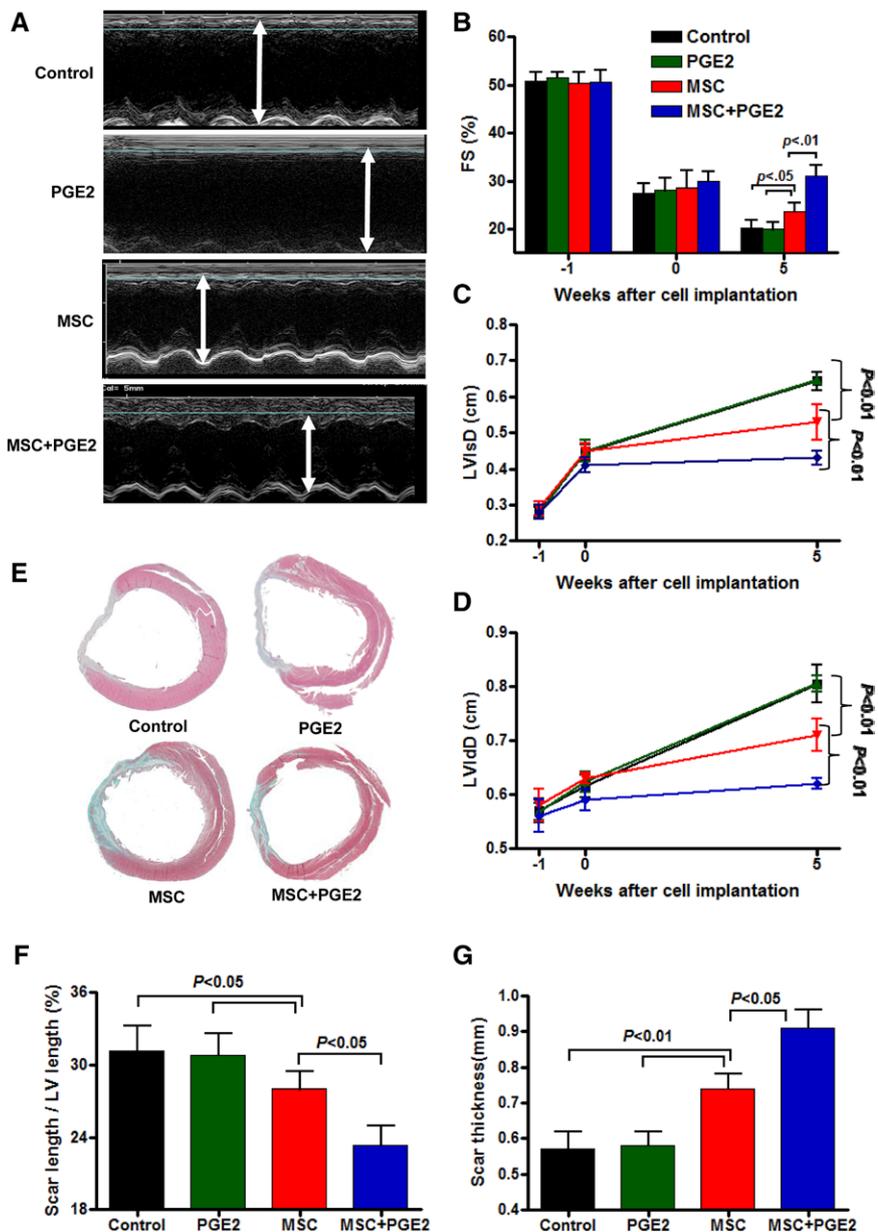
increased left ventricular diameter and a substantial decrease in fractional shortening in all animals, with no significant differences among groups (Figure 8A–8D). However, 5 weeks after cell transplantation, PGE2 treatment demonstrated significantly improved fractional shortening and decreased left ventricular dimensions compared with the medium control, PGE2-only, and MSC-only groups (Figure 8A–8D). PGE2 treatment in the cell-transplanted group also prevented scar thinning and expansion because we observed decreased scar length and increased scar thickness in the MSC+PGE2 group (Figure 8E–8G).

## Discussion

Here, we demonstrated that myogenic differentiation of MSCs resulted in a decrease in PGE2 levels, which was associated with the loss of immunoprivilege and evidence of immune rejection. Experimentally blocking PGE2 biosynthesis in undifferentiated MSCs also resulted in the loss of immunoprivilege, and maintaining optimal levels of PGE2 in D-MSCs preserved their immunoprivileged status. Consistent with the conclusions drawn from our in vitro investigations, we also observed that implantation of allogeneic MSCs into the

injured myocardium was associated with late rejection, but maintaining the optimal levels of PGE2 alleviated the host alloimmune response, increased transplanted MSC survival, and improved heart function. Furthermore, the present study demonstrated that PGE2 preserves the immunoprivilege of MSCs by maintaining the required levels of 2 chemotactic chemokines (CCL5 and CCL12) and downregulating the proliferation of host cytotoxic leukocytes (Figure VII in the online-only Data Supplement).

Allogeneic MSCs in preclinical and clinical studies improved heart function; however, the long-term survival and efficacy of these cells remain to be established. In the present study, undifferentiated MSCs suppressed allogeneic cytotoxic leukocyte proliferation. However, after myogenic differentiation, MSCs lost their immunoprivilege, and leukocyte proliferation and cytotoxicity increased with differentiated compared with undifferentiated MSCs in the coculture. In our in vivo studies, the hearts implanted with D-MSCs had significantly more infiltrating cytotoxic CD8<sup>+</sup> T cells, and the differentiated cells were eliminated from the host myocardium much more readily than undifferentiated cells. Differentiation of MSCs was also associated with a significant decrease in PGE2 levels



**Figure 8.** Maintaining prostaglandin E2 (PGE2) levels in transplanted mesenchymal stem cells (MSCs) preserved heart function. Allogeneic MSCs from male Wistar rats were transplanted into female Lewis rats after coronary artery ligation. Control=medium without MSCs, PGE2=only PGE2 (without MSCs). **A**, Representative M-mode images. **B**, Fractional shortening. **C**, Left ventricular internal end-systolic dimension (LVISDs). **D**, Left ventricular internal end-diastolic dimension (LVIDd). **E**, Representative heart sections stained with Masson's trichrome illustrating the scar area. **F**, Scar length. **G**, Scar thickness (n=4–8 in each experiment). Data are derived from 2 to 3 independent experiments.

and the upstream enzymes cyclooxygenase 2 and prostaglandin E synthase-1. Furthermore, PGE2 treatment of D-MSCs downregulated leukocyte proliferation in vitro, decreased the number of cytotoxic CD8<sup>+</sup> T cells in the myocardium, and prevented the generation of alloantibodies against transplanted D-MSCs, thus preserving the immunoprivilege of MSCs. In addition to the suppression of cytotoxic T-cell proliferation, MSCs also stimulate the induction of Treg cells and promote immune tolerance because Treg cells suppress the alloreactive T-cell responses.<sup>8,13</sup> Our in vitro coculture studies of allogeneic MSCs with CD4<sup>+</sup> T cells revealed that undifferentiated MSCs induced a phenotypic change from cytotoxic T cells toward CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, thus promoting immune tolerance. After differentiation, MSCs lost their ability to induce Treg cells, and PGE2 supplementation of differentiated cells restored that property. Our in vivo studies demonstrated that maintaining PGE2 levels in transplanted allogeneic MSCs enhanced their survival in the injured myocardium. Thus,

both in vitro and in vivo data suggest that preserving PGE2 levels in MSCs after differentiation mitigates host immune responses, prevents the rejection of transplanted allogeneic MSCs, and significantly improves heart function after a myocardial infarction. Although the surviving allogeneic MSCs in the PGE2-treated group expressed the markers of smooth muscle, endothelial, and myogenic lineages, we did not observe the implanted cells differentiating to mature cardiac cells. Thus, the primary benefits of cell therapy for cardiac repair are mainly because of paracrine effects.

MSCs constitutively produce PGE2, which has been reported to inhibit the maturation of antigen-presenting dendritic cells.<sup>4</sup> MSCs transplanted in a mouse model of cecal ligation attenuated septic organ failure via PGE2-dependent reprogramming of host macrophages to increase their anti-inflammatory cytokine production.<sup>12</sup> Previous reports suggested that transplanted MSCs attract host leukocytes to the site of antigenic challenge, where their activity is inhibited

by MSCs.<sup>7</sup> In the present study, leukocyte migration toward undifferentiated MSCs was guided by CCL12 and CCL5 chemotactic chemokines. Furthermore, the CCL12 neutralizing antibody prevented PGE2-mediated suppression of leukocyte proliferation. CCL12 neutralization was recently reported to augment cancer immunotherapy by increasing intratumoral CD8<sup>+</sup> T-cell proliferation and reducing the Treg cell population.<sup>14</sup> In our studies, D-MSCs produced lower levels of these chemokines and lost their ability to attract and suppress proliferation of allogeneic leukocytes. PGE2 treatment maintained CCL12 and CCL5 production and preserved their chemotactic capability. These observations provide unique insight into the mechanisms responsible for the loss of MSC immunoprivilege after differentiation. Maintaining the optimal level of PGE2 in MSCs during differentiation preserves their immunoprivilege by maintaining the required levels of CCL5 and CCL12, prevents rejection of the cells in the injured myocardium, and improves heart function. More significantly, our biodegradable temperature-sensitive hydrogel permits controlled release of PGE2 in the infarct area and may offer future therapeutic potential for the clinical translation of allogeneic MSC-based cardiac repair and enhance the benefits achieved by transplanting young, healthy, allogeneic stem cells into old, debilitated cardiac patients.

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### Disclosures

None.

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