Allogenicity of Human Cardiac Stem/Progenitor Cells Orchestrated by Programmed Death Ligand 1

Laura Lauden,* Wahid Boukouaci,† Luis R. Borlado, Itziar Palacios López, Pilar Sepúlveda, Ryad Tamouza, Dominique Charron,‡ Reem Al-Daccak†

Rationale: Transplantation of allogeneic cardiac stem/progenitor cells (CPC) in experimental myocardial infarction promoted cardiac regeneration and improved heart function. Although this has enhanced prospects of using allogeneic CPC for cardiac repair, the mechanisms regulating the behavior of these allogeneic cells, which are central to clinical applications, remain poorly understood.

Objective: T cells orchestrate the allogeneic adaptive immune response. Therefore, to provide insight into the mechanisms regulating the immunologic behavior of human CPC (hCPC), we investigated the allogeneic T-cell response elicited by cryopreserved c-kit–selected hCPC.

Methods and Results: By using an experimental model of allogeneic stimulation, we demonstrate that, whether under inflammatory conditions or not, hCPC do not trigger conventional allogeneic Th1 or Th2 type responses but instead induce proliferation and selective expansion of suppressive CD25highCD127low human leukocyte antigen-DR+FoxP3high effector regulatory T cells. The regulatory T-cell proliferation and amplification were dependent on the interaction with the B7 family member programmed death ligand 1 (PD-L1), which is substantially expressed on hCPC and increased under inflammatory conditions. Thus, hCPC in allogeneic settings acquire the capacity to downregulate an ongoing immune response, which was dependent on PD-L1.

Conclusions: Collectively, these data reveal that hCPC in allogeneic settings have a tolerogenic immune behavior, promoting a contact PD-L1–dependent regulatory response and a PD-L1–dependent allogeneic-driven immunomodulation. Our study attributes an important role for PD-L1 in the immune behavior of allogeneic hCPC and raises the possibility of using PD-L1 expression as a marker to identify and select low-risk high-benefit allogeneic cardiac repair cells. (Circ Res. 2013;112:451-464.)

Key Words: allogenicity ■ cell transplantation ■ human cardiac stem/progenitor cells ■ myocardial infarction ■ PD-L1 ■ regulatory T cells

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stem cell–based strategies to address the major cause of incurable heart failure, the myocyte deficiency, have raised hopes for novel therapeutic approaches.1–4 Transplantation of cardiac stem/progenitor cells5 (CPC) into animal models of postmyocardial infarction (MI) heart failure attenuated left ventricular remodeling and improved ventricular function in the settings of acute and chronic MI.5,6 Currently, several different populations of CPC are being put forward as potential cell therapies to achieve cardiac repair/regeneration.8 Clinical applications of autologous human CPC from the Stem Cell Infusion in Patients with Cardiomyopathy (SCIPIO) trial, which uses autologous c-kit–positive cells, and from the Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction (CADUCEUS) trial, which uses autologous cardiosphere-derived cells, provide evidence of feasibility and early hints of efficacy in a clinically meaningful setting.2,9 However, autologous approaches have their limitations, including a lack of therapeutic activity of CPC from the elderly and then typically disease-affected patient population, as well as logistical, economic, and time constraints.10 Recent studies have shown in a rat MI model that transplantation of allogeneic cardiosphere-derived cells promotes cardiac regeneration and improves heart function.11 Although the mechanisms that regulate the immunologic behavior of these allogeneic cells remain poorly understood, these studies provide a proof of concept for using allogeneic human CPC in clinical setting.

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*These authors contributed equally to this study.
†These authors are co-senior authors.

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Correspondence to Reem Al-Daccak, UMRS U940, Hôpital Saint Louis, Batiment Bazin, 1 Ave Claude Vellefaux, 75010 Paris, France. E-mail reem.al-daccak@inserm.fr

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Whether immunologic behaviors of allogeneic CPC are linked to their therapeutic effects is unknown but is central to clinical application. The allogeneic adaptive immune responses are orchestrated by T cells and are initiated against donor antigens presented by the molecules of major histocompatibility complex (human leukocytes antigens or HLA in humans) on stimulating cells or tissues.\textsuperscript{13} This first activating signal to T cells is promoted by a second critical signal delivered by the interaction of B7 family costimulatory molecules, CD80/CD86, on stimulating cells with their binding partner CD28 on T cells, thus controlling alloantigen-specific T-cell proliferation and production of cytokine.\textsuperscript{13} Other B7 members, including inducible costimulator ligand and programmed death ligand 1 (PD-L1), which is expressed in various types of cells, are also central regulators of T-cell–mediated immune responses.\textsuperscript{13,14} The alloimmune responses leading to graft rejection are predominantly mediated by CD4\textsuperscript{+} effector cells of the Th1 and Th2 proinflammatory phenotypes,\textsuperscript{15–17} whereas CD4\textsuperscript{+}FoxP3\textsuperscript{+} regulatory T cells (Treg) limit alloimmunity.\textsuperscript{18} Differentiation plasticity of proinflammatory Th1/Th2 and tolerogenic Treg subsets has been documented in murine models of transplantation.\textsuperscript{19} Whether this equilibrium between Treg and proinflammatory Th1/Th2 subsets occurs in allogeneic CPC transplantation or it is skewed toward one direction is unknown.

Heart-derived progenitor cells are relative newcomers to regenerative cardiology, and understanding their immunologic behavior is critical for their establishment as candidates for regenerative/reparative therapies. Therefore, in this study we investigated the mechanisms regulating the immunologic behavior of human c-kit–selected CPC (human CPC [hCPC]) by analyzing the allogeneic T-cell response elicited by cryopreserved cells. We provide the first characterization of human allogeneic T-cell responses, in terms of proliferation and cytokine production, to allogeneic hCPC under physiological low-oxygen inflammatory conditions or not. We demonstrate the capacity of allogeneic hCPC to activate and expand Treg and to modulate ongoing immune responses. In addition, we demonstrate the involvement of the PD-L1/programmed cell death-1 (PD-1) pathway in the immunomodulatory capacities of these cells. Together, our data reveal that hCPC have a tolerogenic immune behavior controlled by PD-L1 molecule.

**Methods**

**Cell Culture**

Human CPC, referred to throughout the text as hCPC, were purified and expanded from 5 human myocardial samples by c-kit immunoselection as described,\textsuperscript{20} fully characterized (Online Figures I–III), and subsequently cryopreserved. After thawing, the cells were cultured as described under Detailed Methods in Online Data Supplement and grown at 37°C in a 3% O\textsubscript{2} atmosphere, thereby facilitating proper functioning and mimicking physio/pathological conditions.\textsuperscript{21} Given their well-established immune behavior,\textsuperscript{22} cryopreserved human bone marrow–derived mesenchymal stem cells (hMSC; Online Figure IV), kindly provided by Dr J. Larghero (Cellular Therapy, Saint Louis Hospital, Paris, France), were cultured as described under Detailed Methods in Online Data Supplement and used as reference control cells. All experiments were performed with cells that had undergone no more than 8 passages and in 3% O\textsubscript{2} atmosphere. Peripheral blood mononuclear cells (PBMC) were prepared from the blood samples of 6 healthy donors and cryopreserved. The hCPC, hMSC, and PBMC from all the donors were genotyped for HLA using routine standard techniques at the Laboratory of Immunology and Histocompatibility, Saint Louis Hospital, Paris, France (Online Table I).

**Flow Cytometry**

The expression of hCPC surface markers was analyzed by flow cytometry using specific antibodies (Online Table II). Cells were acquired using the Canto II flow cytometer (BD Biosciences, le Pont-de-Clair, France) and analyzed using either the BD FACS Diva or the FlowJo software (Celesa, Olten, Switzerland). The expression of an antigen is presented as percentage of positive cells and as geometric mean of fluorescence intensity (MFI). Relative geometric MFI is calculated by dividing the geometric MFI of each test antibody by the geometric MFI of its isotype-matched control antibody as described.\textsuperscript{23}

**Western Blot**

Proteins were separated from total cell extracts by SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, Aulnay-sous-Bois, France) and treated as described under Methods in Online Data Supplement.

**Immunofluorescence Detection**

The hCPC were grown on glass chamber slides (BD Biosciences). At subconfluence, the cells were fixed, permeabilized, neutralized, and then stained with appropriate antibodies as described under Detailed Methods in Online Data Supplement.

**Allogeneic Immune Responses**

Tailored mixed lymphocyte reactions to determine T-cell allogeneic immune responses were performed as previously described by us.\textsuperscript{24} Briefly, responding carboxyfluorescein succinimidyl ester (CFSE)–labeled PBMC were cocultured with HLA-mismatched mitomycin C–treated stimulatory PBMC, hCPC, or hMSC. Staining with anti–CD3-PE-Cy7, anti–CD4-APC, anti–CD8-APC-H7, anti–CD25-PE, and 7-aminoactinomycin D (BD Biosciences) and flow cytometry were used to monitor the activation, proliferation, and cell death of different T-cell subsets. Treg were identified using anti–CD4-viole, anti–CD25-PE, anti–CD127-PE-Cy7, and anti–FoxP3-APC antibody staining, and their phenotypes were determined using anti–HLA-DR-PerCP, anti–PD-1(CD279)–fluorescein isothiocyanate (FITC), and anti CD45RA–fluorescein isothiocyanate antibodies and flow cytometry. Regulatory CD4\textsuperscript{+}CD25\textsuperscript{hi}CD127\textsuperscript{lo/−}–T cells were sorted from PMBC using the anti–CD4-APC, anti–CD25-PE, and anti–CD127-PE-Cy7 antibodies and FACSsAria cytometer. Sorted cells were then labeled...
with CFSE and expanded by coculturing with mitomycin C–treated hCPC in the presence of interleukin (IL)-2 (50 U/mL) for 7 days.

**Immunomodulation and Suppressive Assays**

For immunomodulation, HLA-mismatched CFSE-labeled PBMC were stimulated with phytohemagglutinin (PHA) 1 µg/mL (Sigma-Aldrich, Saint-Quentin-Fallavier, France) in the absence or presence of mitomycin C–treated hCPC or hMSC. Some cocultures were set up in the transwell system or in the presence of blocking anti–PD-L1 (29E.2A3; 5 µg/mL) or its isotype control IgG2b (Biolegend, Saint Quentin en Yvelines, France) as indicated. Activation, proliferation, and cell death of different T-cell subsets were monitored as above. To test the suppressive capacity of hCPC–activated CD4+CD25highCD127low T cells, we sorted the cells from 7-day cocultures between HLA-mismatched CD4+ T cells and mitomycin C–treated hCPC, and then cocultured them with CFSE-labeled autologous PBMC in the presence of PHA (1 µg/mL). The proliferation of autologous CFSE-labeled PBMC was evaluated by flow cytometry after 5 days of coculture. A division index was calculated using the FlowJo software.

**Cytokine Assays**

The levels of interferon-γ (IFN-γ), IL-2, IL-10, and IL-4 in the supernatants of various cocultures were determined at the indicated time points by ELISA using specific kits (BD Biosciences) following the manufacturers’ instructions.

**RNA Silencing**

To knockdown the surface expression of PD-L1, the hCPC were transiently transfected with 3 different PD-L1–specific stealth RNAi species ([1] CD274HSS120931, [2] CD274HSS120932, and [3] CD274HSS120933; Invitrogen Ltd, Paisley, UK) following standard procedures and as described under Detailed Methods in Online Data Supplement.

**Statistical Analyses**

Statistical analyses were performed using the GraphPad InStat3 software. Statistical significance (P values) was calculated using 1-way ANOVA, Student-Newman-Keuls multiple comparisons test. P<0.05 was considered statistically significant.

**Results**

**Cellular Phenotype of Cryopreserved hCPC**

hCPC were purified by c-kit immunoselection, as described, from 5 different donors. Their phenotype, clonogenicity, genomic stability, capacity to form spheres and to differentiate to smooth muscle, endothelial cells, and cardiomyocytes, as well as their in vivo potency, were confirmed before cryopreservation (Online Figures I–III). Further flow cytometry and immunostaining analyses of cryopreserved cells confirmed that these cells are negative for hematopoietic CD34 and CD45, and endothelial CD133 markers and display low levels of...
c-kit (2%–5%), but express SSEA-1, SSEA-4, CD90, CD73, CD105, and CD166 stem and progenitor markers (Figure 1A) and the cardiac lineage commitment factors Nkx2.5, GATA-4, Islet-1, and MEF2C (Figure 1B). These cells like the control hMSC also express the pluripotency transcription factors OCT4, SOX2, and NANOG, which were not detected in PBMC used as a negative control (Figure 1C). In contrast, the control hMSC do not express cardiac progenitor markers, such as GATA-4 and Islet-1 (Online Figure IVB). In accordance, heat-map analysis shows that cardiac markers (FLK-1, TBX5) are expressed by these cardiac-derived progenitors but not by hMSC (Online Figure IC and ID), indicating that they have a phenotype different from that of hMSC. They are also unlikely to be related to the recently described adult cardiac-resident MSC-like stem cells (cardiac colony forming unit-fibroblast).25,26 These cells, in contrast to the cardiac-derived progenitors under investigation, do not express the cardiac stem/progenitor markers Nkx2.5, GATA-4, MEF2C, or SOX2 and NANOG pluripotency factors. These results indicate that both the initially (Online Figure I) and the cryopreserved investigated cells exhibit phenotypic traits typified by multiple types of previously reported human heart–derived stem/progenitor cell populations.8,27 Thus, they present a population of CPC with a mixed stem cell phenotype and will be referred to as hCPC throughout the text.

**hCPC Display an Immune Phenotype Suitable for Allogeneic Applications**

To determine the capacity of hCPC to induce an allogeneic immune response, we first characterized their expression of immune-relevant molecules, the major histocompatibility complex HLA class I and class II and costimulatory molecules. The hCPC from different donors (n=5) had similar immune phenotypes in that at baseline they expressed HLA class I, very low or negligible levels of HLA class II molecules, were negative for costimulatory molecules CD40, CD80, CD86, and inducible costimulator ligand (CD275), but expressed PD-L1 (CD274) (Figure 2A). During inflammation,
such as after myocardial injury, the presence of proinflammatory cytokines alters the phenotype and activity of various cell types. The cytokine IFN\(\gamma\) constitutes one of the most potent proinflammatory cytokines and upregulates/induces the expression of immune-relevant molecules. Treatment with IFN\(\gamma\) (100 U/mL) for 72 hours upregulated the expression of HLA class I and class II molecules, as well as of costimulatory PD-L1 molecule in hCPC as shown in Figure 2A by the percentage of positive cells and in Figure 2B by the relative geometric MFI (\(P<0.01\)). However, IFN\(\gamma\)-treated hCPC (IFN\(\gamma\)-hCPC) did not show increased levels of the other costimulatory molecules (Figure 2A and 2B). Treatment with IFN\(\gamma\) did not induce noticeable morphological changes in hCPC (Figure 2C, left) and did not modulate the expression levels of OCT4, SOX2, and NANOG (Figure 2C, right) or of cell surface molecules, including adhesion molecules (Online Table III). Together, these results indicate that the baseline immunophenotype of hCPC represents a weak immunogenic profile, which supports their clinical application in allogeneic settings. But their presence within an inflammatory environment, without modifying their progenitor phenotype, might enhance their immunogenic capacity given the potential expression of HLA molecules, which could facilitate their recognition by allogeneic T cells.\(^{28}\)

**hCPC Elicit Weak Allogeneic T-Cell Response In Vitro**

We then determined whether hCPC or IFN\(\gamma\)-hCPC could induce an allogeneic response using tailored 1-way mixed lymphocyte cultures. We investigated the responses of unfractionated PBMC from 6 HLA-mismatched healthy donors to the hCPC from 5 different donors. hMSC are

![Figure 3. Human cardiac-derived progenitor cells (hCPC) induce low allogeneic T-cell response.](http://circres.ahajournals.org/)

**A.** Carboxyfluorescein succinimidyl ester (CFSE)-labeled peripheral blood mononuclear cell (PBMC) were cultured alone (medium) or with human leukocyte antigen-mismatched mitomycin C–treated PBMC (allo-PBMC), hCPC, interferon-\(\gamma\) (IFN\(\gamma\))-hCPC, or human mesenchymal stem cell (hMSC) as reference control cells. The levels of CD4\(^+\) and CD8\(^+\) T-cell proliferation were determined by loss in CFSE labeling as shown in representative dot plots (top) and are presented as the percentages of proliferating cells (bottom). **B.** IFN\(\gamma\) (left) and interleukin (IL)-2 (right) levels and (C) IL-10 (left) levels and IL-10 to IFN\(\gamma\) ratio (right) in the supernatants of the allogeneic cocultures described in **A** at the indicated time point. Results are presented as mean±SD from 5 independent experiments conducted with allogeneic PBMC from 5 different donors against hCPC from the same donor. \(^*P<0.01\) compared with medium; \(\dagger P<0.001\) compared with conventional allo-PBMC. \(P\) values between hCPC and IFN\(\gamma\)-hCPC–induced IL-10 production and IL-10 to IFN\(\gamma\) ratios are indicated.
low-immunogenic immunoregulatory cells that have been extensively studied\textsuperscript{22} and, therefore, were used as reference control cells in our experiments. The hCPC from all the donors were able to elicit proliferation of CD4\textsuperscript{+} T cells (P<0.01 versus medium) but not CD8\textsuperscript{+} (Figure 3A). The hCPC-induced response was significantly lower than conventional allogeneic PBMC response (P<0.001) but comparable with that induced by hMSC (Figure 3A). Treatment of hCPC with IFN\textgamma before coculturing with HLA-mismatched PBMC did not enhance their capacity to induce CD4\textsuperscript{+} or CD8\textsuperscript{+} T-cell proliferation (Figure 3A). Coculturing with higher titers of the hCPC did not enhance the allogeneic proliferative responses (data not shown), and annexin V/7-aminoactinomycin D staining and flow cytometry analysis indicated that the weak proliferation observed with hCPC was not because of a higher rate of PBMC death (Online Figure VA). Similar CD4\textsuperscript{+} proliferation, but not CD8\textsuperscript{+}, was observed when purified HLA-mismatched CD3\textsuperscript{+} T cells were used as responders to hCPC and IFN\textgamma-hCPC, but we did not observe significant proliferation in the presence of hMSC (Online Figure VB).

In line with the low proliferative response, the levels of IFN\textgamma in the supernatants of both hCPC and IFN\textgamma-hCPC cocultures were weak and similar to those in the supernatants of T cells cultured in medium (P<0.05 versus medium; Figure 3B, left). However, low levels of IL-2 in the supernatants of both hCPC and IFN\textgamma-hCPC cocultures were detected (Figure 3B, right), and these levels are higher than those in the supernatants of T cells cultured with medium alone (P<0.001). No IL-4 was detected (data not shown), but substantial levels of IL-10 were found in hCPC cocultures (P<0.001 versus medium) and were higher when IFN\textgamma-hCPC were used as stimulators (P<0.001 versus hCPC) (Figure 3C, left). The IL-10 to IFN\textgamma ratio was also significantly increased in both the hCPC and IFN\textgamma-hCPC cocultures, compared with medium alone or with the allogeneic PBMC (P<0.001; Figure 3C, right). Of note, neither the hCPC nor the IFN\textgamma-hCPC produced any of the tested cytokines (Online Figure VC). The supernatants of the control hMSC cocultures showed significant levels of IFN\textgamma and IL-2 compared with medium (P<0.001), but lower than conventional allogeneic PBMC response (P<0.001; Figure 3B). Both the IL-10 and IL-10 to IFN\textgamma ratio in the presence of hMSC were lower than those observed with hCPC (P<0.001) and similar to that in the medium or within conventional allogeneic response (Figure 3C). Thus, although comparable with the control hMSC-induced response (cell proliferation), the hCPC-induced T-cell response in allogeneic settings has its particularities. hCPC do not induce a substantial Th1 (IFN\textgamma production) or Th2 (IL-4 production) response but rather elicit the proliferation of a specific subpopulation of IL-10–producing CD4\textsuperscript{+} T cells.

**hCPC Have an Immunomodulatory Capacity**

Then we investigated the capacities of hCPC to modulate an ongoing immune response in an allogeneic setting. HLA-mismatched PBMC were stimulated with the polyclonal activator of T-cell PHA in the absence or presence of mitomycin C–treated hCPC, IFN\textgamma-hCPC, or control hMSC. The presence of hCPC or IFN\textgamma-hCPC efficiently downmodulated the PHA-induced proliferation of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. The division index of the PHA-activated CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells decreased by ≈75% (Figure 4A, top), whereas the percentage of proliferating cells decreased by ≈50% (P<0.001 versus medium; Figure 4A, bottom). Coculturing hCPC or IFN\textgamma-hCPC in transwell settings almost completely blocked their inhibitory effect on PHA-induced CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell proliferation (P<0.001), indicating that these cells exert their modulatory effects mainly through cell–cell contact (Figure 4A). hMSC also decreased PHA-induced proliferation of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells by ≈65% (P<0.001 versus medium). But when cultured in transwell settings, inhibition of proliferation was only partially affected (Figure 4A, bottom), which is in line with previous studies demonstrating that hMSC immunomodulatory function requires soluble factors with limited diffusion distance.\textsuperscript{22,29} The presence of hCPC, similar to hMSC, also downregulated the production of IFN\textgamma and IL-2 by PHA-activated T cells by 80% to 85% (P<0.001 versus medium; Figure 4B). However, both hCPC and hMSC upregulated the production of IL-10 and increased the IL-10 to IFN\textgamma ratios (P<0.001 versus medium; Figure 4C). Interestingly, IFN\textgamma-hCPC were more efficient than untreated hCPC in downregulating the production of IL-2 (P<0.01) and in increasing IL-10 production and IL-10 to IFN\textgamma ratios (P<0.001). Taken together, our results indicate that hCPC are endowed with immunoregulatory function(s), which is likely to be maintained in an inflammatory environment, as IFN\textgamma-hCPC were also efficient suppressors of an ongoing immune response.

**hCPC Activate Treg in Allogeneic Settings**

hCPC in allogeneic settings induce the proliferation of a CD4\textsuperscript{+} T-cell subpopulation and downmodulate an ongoing immune response in a cell–cell contact manner. Therefore, we investigated whether this subpopulation has a regulatory/suppressive capacity and could be implicated in the hCPC immunomodulatory effect. HLA-mismatched CD4\textsuperscript{+} T cells were cultured alone or cocultured with mitomycin C–treated hCPC, IFN\textgamma-hCPC, or control hMSC, and the phenotype of proliferating cells was determined. Coculturing with hCPC or IFN\textgamma-hCPC induced the proliferation of a CD4\textsuperscript{+} T-cell subset that express high levels of CD25 and FoxP3 but low levels of CD127, which is indicative of a T-regulatory phenotype (Figure 5A, top). Quantification analysis indicates that hCPC and IFN\textgamma-hCPC induce almost a 6-fold increase in the percentage of CD4\textsuperscript{+}CD25\textsuperscript{high}CD127\textsuperscript{lo}FoxP3\textsuperscript{high} cells (Figure 5A, left histogram; P<0.001 versus medium). In addition, at least 80% of these cells were proliferative as determined by CFSE labeling compared with only 20% of cells in control conditions (medium; P<0.001; Figure 5A, right histogram). As a reference control, we found that hMSC have the same capacity as hCPC in inducing this Treg phenotypic cell population (Figure 5A).

We also checked for the expansion of these cells in cocultures of PHA-stimulated HLA-mismatched PBMC and mitomycin C–treated hCPC, IFN\textgamma-hCPC, or control hMSC. Interestingly, we observed a significant increase in the number of CD4\textsuperscript{+}CD25\textsuperscript{high}CD127\textsuperscript{lo}FoxP3\textsuperscript{high} T cells in the presence of hCPC or IFN\textgamma-hCPC, compared with PHA stimulation alone, and this was dependent on the PBMC to hCPC ratio (Figure 5B), which strongly supports their implication in hCPC-induced immunomodulation.

Then, we investigated the capacity of the T-cell subset activated by hCPC in allogeneic settings to suppress T-cell activation.
We sorted the hCPC-induced CD4+CD25hiCD127lo/− and CD4+CD25hiCD127lo/− T cells and the hMSC-induced CD4+CD25hiCD127lo/− from allogeneic cocultures, verified the expression of FoxP3, and then recultured them with autologous PBMC in the presence of polyclonal activator PHA. The presence of hCPC-induced CD4+CD25hiCD127lo/−FoxP3hi T cells, similar to hMSC-induced CD4+CD25hiCD127lo/− T cells, reduced the PHA-induced activation of autologous CD3+ T cells (division index decreased by 81% and 69%, respectively). The presence of hCPC-induced CD4+CD25hiCD127lo/−FoxP3lo/− T cells had only minor effect, which was significantly different from that of hCPC-induced and hMSC-induced CD4+CD25hiCD127lo/− T cells (P<0.001; Figure 5C). These results indicate that the subpopulation of CD4+ T cells that is activated by hCPC in our allogeneic model system has the phenotypic and functional characteristics of suppressive Treg.

**Characterization of hCPC-Activated Treg**

We then focused on characterizing the hCPC-activated CD4+CD25hiCD127lo/−FoxP3hi Treg. We found that ≈90% of these cells are HLA-DR-positive, whereas ≈77% were found to be CD45RA-negative (Figure 6A). hCPC express the costimulatory molecule PD-L1. We, therefore, examined whether hCPC-induced CD4+CD25hiCD127lo/−FoxP3hi HLA-DR+ cells also express PD-L1, a binding partner of PD-L1,30 and found that ≈70% were also positive for PD-L1 (Figure 6A, top). This is in contrast to CD4+CD25hiCD127FoxP3lo/− cells (nonregulatory phenotype), which poorly express PD-L1 and
HLA-DR, but which are positive for CD45RA. This indicates that the hCPC-induced CD4+CD25^{high}CD127^{low/-} Treg have an activated (expression of HLA-DR and PD-1) effector (loss of CD45RA) phenotype according to that described by Sakaguchi et al.18 These cells have been described as potent downmodulators of the immune and inflammatory responses in normal and pathological context, as well as in allogeneic transplantation.18

We then confirmed the capacity of the hCPC to activate and expand effector allogeneic Treg. We sorted CD4+CD25^{high}CD127^{low/-} Treg from HLA-mismatched PBMC, verified that they express FoxP3, and then cocultured them with hCPC for 7 days. As depicted in Figure 6B (top), the hCPC elicited significant proliferation of allogeneic Treg as determined by CFSE labeling, and many of these proliferating cells were strongly positive for HLA-DR. Approximately 45% of the purified CD4+CD25^{high}CD127^{low/-} Treg proliferated in response to allogeneic hCPC, and nearly all of the HLA-DR^+ cells were proliferating (P<0.001 versus medium; Figure 6B, bottom). These results indicate that, in allogeneic settings, hCPC have the capacity to expand and activate Treg toward activated effector phenotype.

We then determined the mode of action of hCPC-induced CD4+CD25^{high}CD127^{low/-} regulatory cells. CD4+CD25^{high}...
CD127low/− cells from allogeneic cocultures were recultured with autologous PBMC in the presence of PHA. The presence of hCPC-induced CD4+CD25+CD127low/− T cells reduced the PHA-induced proliferation of autologous CD4+ and CD8+ T cells (by 60% and 70%, respectively; Figure 6C), whereas the presence of hCPC-induced CD4+CD25+CD127+ T cells used as a control did not have a significant effect. Culturing in transwell settings almost abolished the observed downregulation of PHA-induced T-cell activation by CD4+CD25+CD127low/− T cells (P<0.001; Figure 6C), demonstrating that the suppressive capacity of these cells occurs mainly through cell–cell contacts (Figure 6C).

PD-L1/PD-1 Orchestrates the Immunologic Properties of hCPC

We found that hCPC preferentially activate the CD4+CD25+CD127low/−FoxP3high Treg subset that displays an effector regulatory phenotype (HLA-DR+) and expresses PD-1 (Figure 6A). In addition, hCPC express PD-L1 (Figure 2), and we showed that hCPC-induced immune regulation is mainly through cell–cell contacts (Figure 4A). Therefore, we looked at the possible involvement of the PD-L1/PD-1 system in the immunoregulatory behavior of hCPC. We first investigated the formation and proliferation of the CD4+CD25+CD127low/−FoxP3high Treg subset on stimulation of HLA-mismatched PBMC by hCPC and IFNγ-hCPC in the presence or absence of a blocking anti–PD-L1 antibody, known to disrupt the PD-L1/PD-1 interactions.31 As depicted in Figure 7A, the presence of the blocking anti–PD-L1, but not its isotype control, almost completely abolished the formation and proliferation of CD4+CD25+CD127low/−FoxP3high Treg; only 0.5% of CD4+CD25+CD127low/−FoxP3high T cells were observed in the presence of anti–PD-L1 versus 4.5% in the isotype control (P<0.001), and none of these cells were proliferating (P<0.001). We also examined the effect of the blocking anti–PD-L1 antibody on the capacities of the hCPC to induce the proliferation and expansion of freshly isolated CD4+CD25+FoxP3high Treg. The percentage of proliferating cells decreased by ≈69%, and only 4% to 5% of these proliferating cells were HLA-DR–positive (activated/effector) in the presence of anti–PD-L1. Then we examined the effect of blocking anti–PD-L1 on hCPC-mediated immune modulation. We found that PHA-induced proliferation of CD4+ and CD8+ T cells was restored by 76% and 92%, respectively, in the presence of blocking anti–PD-L1 but not its isotype control (P<0.001; Figure 7C).

We then knocking down PD-L1 in the hCPC by RNA interference and tested the capacity of these cells to downregulate PHA-induced T-cell activation. Transfection of hCPC with 3 different PD-L1–specific small interfering RNA (siRNA) sequences (siRNA 1, 2, and 3) remarkably reduced the cell surface expression of PD-L1 (average reduction of 85%), whereas transfection with 2 different control siRNA had no effect (Figure 8A). The presence of allogeneic hCPC and control siRNA–transfected hCPC decreased the division index of HLA-mismatched PHA-stimulated T cells by 66% and 51%, respectively, but PD-L1 siRNA–transfected cells

Figure 6. Characteristics of human cardiac-derived progenitor cell (hCPC)–activated regulatory T cells (Treg). A, Representative expression of CD45RA and programmed cell death-1 (PD-1) vs labeled human leukocyte antigen (HLA)-DR by hCPC-induced CD4+CD25+CD127low/−FoxP3high or CD4+CD25+CD127+ T cells used as a control. The percentage of positive cells (%) is indicated. B, Treg were sorted from HLA-mismatched peripheral blood mononuclear cell (PBMC) and then cultured with interleukin (IL)-2 (50 U/mL) in the absence (medium) or presence of hCPC. Top, Representative dot-plot of Treg proliferation, using carboxyfluorescein succinimidyl ester (CFSE) labeling, and activation, monitored by HLA-DR staining. Bottom, Percentages of proliferating (left) and percentages of HLA-DR+ (right) Treg. C, Percentages of phytohemagglutinin (PHA)-induced proliferating autologous CD4+ and CD8+ T cells in the presence of hCPC-induced CD4+CD25+CD127+ (control cells) or CD4+CD25+CD127low/−FoxP3high T cells set up or not in the transwell system. Results are presented as mean±SD from 3 independent experiments. *P<0.01 compared with medium; †P<0.001 between samples in transwell settings or not.
lost this regulatory capacity (Figure 8B). The decrease in the percentage of proliferating CD4+ and CD8+ observed in the presence of allogeneic hCPC or control siRNA–transfected cells was also almost completely abrogated by the presence of PD-L1 siRNA–transfected cells (P<0.01; Figure 8C). Furthermore, we found that the level of IL-10 detected in the PD-L1 siRNA–transfected hCPC cocultures was significantly decreased compared with that detected in the allogeneic hCPC or control siRNA–transfected cocultures (P<0.01; Figure 8D). This suggests that the PD-L1/PD-1 system is implicated in the production of IL-10 that we detected in all the hCPC/HLA-mismatched PBMC cocultures. Our results indicate that in allogeneic settings, PD-L1 is involved in both the capacities of hCPC to activate and expand effector Treg and in their capacity to downregulate an ongoing immune response.

**Discussion**

Preclinical experiments with allogeneic cardiosphere-derived cells have opened up a new treatment paradigm and have made the use of allogeneic hCPC via cell banks a more realistic proposition, assuming that cryopreserved cells retain their original characteristics and are immunologically safe. In the present study, we provide the first detailed description of T-cell responses to cryopreserved allogeneic hCPC. We show that cryopreserved hCPC retain their primitive pluripotent and early cardiac lineage–committed phenotype. Tailored immune assays showed that these cells are hypoimmunogenic because, whether under inflammatory conditions or not, they lack the costimulatory molecules CD80/CD86 required for conventional Th1 or Th2 type T-cell responses. In contrast, the hCPC express the costimulatory molecule PD-L1, which endows them with the capacity to...
drive significant allogeneic Treg responses and to attenuate an ongoing immune response.

Patients who experience heart failure after MI have reduced numbers of circulating Treg.32 We demonstrate that under conditions mimicking the physio/pathological environment of the injured myocardium, allogeneic hCPC mainly activate and expand Treg rather than proinflammatory Th1 cells, which are typically activated in allogeneic reactions. Recent studies have attributed a critical role to Treg in cardiac repair after MI. Insufficient recruitment of Treg worsens ventricular remodeling,33 whereas adoptive transfer of Treg in a rat model of MI prevents adverse cardiac remodeling at the infarcted site, reduces macrophage and T-cell infiltration to the site, and protects the resident cardiomyocytes against apoptosis.34 Our results strongly suggest that the administration of allogeneic hCPC would, through activation and expansion of Treg, provide similar protective/reparative effects. The mechanisms by which stem cells promote cardiac repair are not yet fully understood. Initially, it was established that the transplanted cells differentiate into cardiac cells and blood vessels and replace damaged cells. Growing evidence also indicated that stem cells, including hMSC and hCPC, perform the following: (1) release growth factors and molecules that promote angiogenesis; (2) attenuate the postinfarct adverse remodeling; (3) reduce myocardial apoptosis; and (4) stimulate resident CPC to repair damage. Collectively, this can be summarized as the paracrine effect. Although animal studies clearly indicated that the engraftment and differentiation specify the regenerative mechanism,35,36 the paracrine effect is today also recognized as part of the overall regenerative process.2,37–42 In this context, our present study underscores the capacity of allogeneic hCPC to elicit Treg response as an indirect paracrine effect that would promote cardiac repair and suggests that the presumed functional benefit of allogeneic CPC could be also linked to their inherent immune properties.
This notion is reinforced by our results showing that in allo-
genetic settings hCPC can inhibit an ongoing inflammatory T-cell response. This is the first demonstration of the immunomodula-
tory capacity of hCPC in vitro. Our results indicate that hCPC can
downregulate a T-cell response elicited by mismatched major his-
tocompatibility complex molecules in inflammatory conditions,
which mimics the post-MI inflammatory response. Indeed, main-
taining the hCPC in the presence of the proinflammatory cytokine
IFNγ does not alter the immunomodulatory capacity of hCPC or
favor activation of a Th1 type response over a regulatory immune
response. These data suggest that even after their introduction
into an inflammatory environment, the hCPC would still have the
same or better immunomodulatory potential. Thus, transplanta-
tion of allogeneic hCPC would not aggravate MI inflammation
but would rather participate in its resolution. This is likely because
of the fact that IFNγ, although it considerably upregulated HLA
class II molecules on hCPC promoting the first T-cell activation
signals, did not induce the promoters of conventional Th1 or Th2
responses, CD80/CD86, but rather increased the expression of im-
mune regulator PD-L1 costimulatory molecule.

Allogeneic effector T-cell responses are susceptible to PD-1
pathway modulation as evidenced in models of graft-versus-host
disease43 and allogeneic organ transplantation.44 We showed that
hCPC in allogeneic settings activate and expand functional Treg,
and the presence of anti–PD-L1 blocking antibody or PD-L1
knockdown almost completely abolishes hCPC-induced activation
and proliferation of Treg and the immunomodulatory actions of
the hCPC. Our data do not dismiss that soluble factors in
conjunction with cell–cell contact could be implicated in hCPC
immunomodulatory capacity. In hMSC immunomodulation, a
role for the soluble HLA-G5 in a cell contact–dependent manner,
for example, has been identified.22,45 Similar scenarios might
occur with hCPC. Reverse signaling through PD-L1 interaction
with PD-1 delivers signals to PD-L1–expressing cells,14 which
might upregulate the production or release of soluble factor(s)
by hCPC, contributing to the suppression of allogeneic T-cell
proliferation. Signaling through PD-L1 in hCPC is not known,
and future studies will investigate this possibility.

Our results assign a critical role to PD-L1 in orchestrating the
immune behavior of hCPC. PD-L1 is expressed constitutively
on both hematopoietic (resting T cells, B cells, dendritic cells,
macrophages, and Treg) and nonhematopoietic cells (pa-
renchymal and endothelial cells).30 Resting T cells express low
levels of PD-1 receptor, the expression of which is inducible
on CD4+ and CD8+ T cells, natural killer cells, activated mono-
cytes, and B cells.30 We found that hCPC constitutively express
a substantial level of PD-L1, and hCPC-induced allogeneic
Treg express substantial amounts of PD-1. This pathway has
been shown to regulate T-cell responses and inflammation in
various disease settings, including atherosclerosis,46 allograft
vascular disease,47 and, more recently, myocarditis.48 PD-
L1 regulates the development, maintenance, and function of
Treg.49 Murine PD-L1−/− antigen-presenting cells are profoundly
defective in terms of the conversion of CD4+CD62L−Foxp3+
T cells to regulatory cells, and PD-L1 alone is sufficient to
induce the conversion of naive T cells to functional Foxp3high
Treg.49 The constitutive expression of PD-L1 endows the hCPC
with a dominant pathway that in allogeneic settings polarizes
CD4+ T cells toward a Treg phenotype.

We did not investigate the mechanisms by which PD-L1 on
hCPC regulates the development of allogeneic Treg. However,
previous studies have shown the implication of PD-L1/PD-1
axis in converting effector CD4+ Th1 cells into Treg. PD-1
activation and subsequent activation of SHP1/SHP2 signaling
on binding of PD-L1 induce Th1 cell plasticity by reducing
STAT1 activation, which is critical for maintaining Th1 phe-
notype.19 The PD-L1/PD-1 pathway can also flip the molecu-
lar switch in a naive CD4+ T cell toward Treg development by
inhibiting the Akt/mTOR signaling cascade, probably through
the upregulation of the phosphoinositide 3-kinase signaling
antagonist phosphatase and tensin homolog.49 PD-L1 also
promotes and maintains the induced Treg by sustaining and
enhancing Foxp3 expression.49 In our model, similar scenarios
could be implicated in the development of allogeneic Treg by
hCPC, although this warrants further investigation.

The PD-L1/PD-1 interaction delivers cosignals to T cells,
thereby promoting their proliferation and secretion of IL-10,
naturally when the antigen-presenting cells lack expression of
CD80/CD86 and the CD28 cosignaling pathway is not op-
erating.30 We demonstrated the following: (1) hCPC express
neither CD80 nor CD86; (2) hCPC activate a Treg response
(IL-10) rather than a Th1 (IFNγ) or Th2 (IL-4) type response
in a PD-L1–dependent manner; (3) hCPC immunomodulate
the PHA-induced response in a manner that is dependent on
their activation of regulatory cells and PD-L1; (4) IFNγ-hCPC
compared with hCPC induce higher production of IL-10 and
have higher expression of PD-L1; and (5) knocking down PD-
L1 significantly decreases the secretion of IL-10 in immuno-
modulation assays. Taken together, these findings suggest that
IL-10 in our allogeneic model system is related to PD-L1/PD-1
cosignaling to T cells. Interestingly, a similar correlation might
also exist in the allogeneic hMSC model. hMSC do not consti-
tutively express PD-L1, but we found that the significant pro-
duction of IL-10 in hMSC/PHA-activated HLA-mismatched
PBMC cocultures coincides with the significant upregulation
of PD-L1 expression on hMSC (Online Figure VI). IL-10 has
been implicated in the regulation of the postinfarction inflam-
matory response.51 Although the results from loss-of-function
studies in mouse models have been somewhat contradic-
tory,52,53 administration of exogenous IL-10 significantly reduces
inflammation, improves cardiac function, and attenuates ven-
tricular remodeling after MI in mice.34 The role of the PD-L1/PD-
1 pathway in promoting transplantation tolerance has been
demonstrated in various murine models of transplantation, no-
tably heart allografts.30 Given its implication in IL-10 secretion
and Treg generation after an interaction between T cells and
allogeneic hCPC, we suggest a previously undemonstrated role
in cardiac cell therapy for the PD-L1/PD-1 pathway, that is, in
promoting the hCPC paracrine effect. This idea draws together
current evidence indicating a pivotal role for PD-L1/PD-1 in
controlling and managing allogeneic but also xenogeneic re-
actions via Treg-dependent and T-cell–independent mecha-
nisms19,55,56 and the critical role of myocardial PD-L1/PD-1 in
the control of immune-mediated cardiac injury.48,57

The balance between the positive (activating Th1 inflammat-
ory response) and negative (activating regulatory response) signals
that an antigen-presenting cell delivers to a T cell on encounter
determines the outcome of the alloimmune response. Regardless
of the underlying mechanisms, the present work demonstrates that the inherent immune features of hCPC shift their signaling capacities within the allogeneic setting toward delivery of signals that promote the development, maintenance, and functioning of an anti-inflammatory immunoregulatory response. This is critical to decrease allogeneic rejection risk and to allow the persistence of repair cells so as to favor cardiac repair/regeneration. Our work with hCPC emphasizes the paradigm proposed by the Marban group of using allogeneic human cardiac repair cells from cell banks as off-the-shelf products for cellular cardiac repair13 and provides the first evidence for a potential allogeneic-driven benefit of human CPC. The herein demonstrated PD-L1–dependent allogeneic-driven immunomodulatory capacity of hCPC promotes their clinical translation and paves the way for using the expression of PD-L1 as a biomarker to identify and select low-risk, high-benefit allogeneic cardiac repair cells.

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Disclosures

None.

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The B7 family member programmed death ligand 1 (PD-L1) regulates human cardiac-derived progenitor cells with a mixed stem cell function. Engraftment and differentiation are integral to cardiac progenitor cell behavior. Proof of concept using allogeneic cells has been provided in a rat model of off-the-shelf allogeneic cells. However, limitations have prompted studies to determine the efficacy and immune behavior of hCPC. This review summarizes our current understanding of the immunologic behavior of allogeneic CPC influences therapeutic potential and mechanisms. Stem Cell Rev. 2012


Allogenicity of Human Cardiac Stem/Progenitor Cells Orchestrated by Programmed Death Ligand 1
Laura Lauden, Wahid Boukouaci, Luis R. Borlado, Itziar Palacios López, Pilar Sepúlveda, Ryad Tamouza, Dominique Charron and Reem Al-Daccak

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Detailed Methods

Human Cardiac stem/progenitor cells (hCPC) isolation and culture
Human cardiac biopsies were obtained from patients (n=5) suffering from an open-chest surgery, usually for valve replacement, after signed informed consent. The project has been approved by the ethical committees of “Hospital 12 de Octubre” and “Fundación Jiménez Díaz” Madrid, Spain. Starting material was obtained from the right atria appendage, which is routinely removed in order to place the cannulae for the extracorporeal circulation. Tissue samples were minced into small pieces (<1 mm3) and treated with collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ, USA) for 3 cycles of 30 min each to obtain a cellular suspension. Cardiomyocytes were removed by centrifugation and filtration using 40 µm cell strainers. Cardiac stem/progenitor cells were obtained after immunodepletion of CD45-positive cells and immunoselection of CD117 (c-kit)-positive cells, using specific microbeads (Miltenyi Biotech, Bergish Gladbach, Germany) and following manufacturer recommendations. After isolation, cells were seeded in Matrigel (BD Biosciences, Madrid, Spain) coated plates in isolation medium (DMEM/F12 medium supplemented with 10% fetal bovine serum embryonic stem cell qualified (FBS ESCq), L-Glutamine (2 mM), Penicillin-Streptomycin (100 U/mL and 100 µg/mL), bFGF (10ng/mL) and ITS (Invitrogen, Madrid, Spain and Saint-Aubin, France), IGF-II (30ng/mL) and EGF (20ng/mL) (Peprotech, Neuilly-sur-Seine, France) and hEPO (Sigma-Aldrich, Madrid, spain) (Online Figure IA), and were grown at 37°C in 3% O2 atmosphere, thereby facilitating proper functioning and mimicking physiologic/pathologic conditions. One week after cell seeding, growing medium, which is a combination of DMEM/F12 and Neurobasal medium (1:1) supplemented with 10% FBS ESCq, L-Glutamine, Penicillin-Streptomycin, B27 (1X), N2 (1X), β-mercaptoethanol (50µM), ITS and growth factors (bFGF, IGF-II, EGF) replaced the isolation medium and cells were thereafter grown in this medium at 3% O2 atmosphere. hCPC were negative for CD34 and CD45 but expressed CD166, CD90, SSEA1 and SSEA4 (Online Figure IB). Transcriptional analysis of hCPC in comparison with human mesenchymal stem cells (MSC) as reference cell line was conducted. hCPC and hMSC were harvested and total RNA isolated using TRI-Reagent (Sigma-Aldrich) according to manufacturing indications. RNA concentration was determined by photometric measurement. The SuperScript® III First Strand Synthesis System for RT-PCR (Invitrogen) was used to synthesize cDNA of 2 µg RNA following manufacturer's recommendations. The synthesized cDNA was diluted 1:10 and 50-200ng of cDNA subjected to quantitative real-time PCR (qPCR) using human-specific probes that are listed in Online Table IV. qPCR reactions were performed in triplicates using TaqMan Universal PCR Master Mix (Applied Biosystems). PCR reactions were run on a StepOnePlus (Applied Biosystems) machine and StepOne Software v2.2.2 and Data Assist v3.0 software were used to analyze results. Expression of mRNAs was normalized to expression of beta Glucoronidase. For RQ calculation (2^{-ΔΔCT}) hMSC were used as reference cell line. The Heat map analysis of transcriptional expression levels of cardiac markers Tbx5, Flk1, and GATA4 and of stem cells marker Nestin in hCPC from two different donors (hCPC1 and hCPC2) indicated that these markers are expressed in hCPC but not in hMSC (Online Figure IC&D). hCPC were then expanded and cryopreserved until passage 7. hCPC were approximately 12 µm as determined by the CellCountess machine (Invitrogen) at passage 5.
Clonogenicity and Genomic stability
The clonogenicity of the hCPC was analyzed at passage 2 (P2) and passage 7 (P7) by seeding single cells in 96-well plates (Online Figure IIA). To determine the genomic stability of isolated hCPC, oligo array-CGH analysis was performed using Human Genome CGH 44k microarrays (Agilent Technologies, Santa Clara, CA, USA). A total of 1 µg of genomic DNA from the cells and a reference healthy genomic DNA (Promega, Madison, WI, USA), were differentially labeled by random priming with Cy5-dCTP and Cy3-dUTP. The hybridization was carried out according to the manufacturer's protocol. Copy number altered regions were detected using ADM-2 (set as 6) statistics provided by AGW, with a minimum number of five consecutive probes; therefore, we were able to detect aberrant regions of at least 200 Kb (Online Figure IIB). G1 cell cycle checkpoint was also studied in isolated hCPC. In normal cycling cells, the expression level of p53 is low. p53 stabilization and G1 cell cycle checkpoint activation is induced after DNA damage in primary cells. Most of the transformed human cells have alterations in the p53 pathway preventing cell cycle arrest induction. Therefore, we undertook the expression of p53 in cells treated or not with doxorubicin or paclitaxel as readout of cells normal status. Cells were either kept untreated or treated for one hour pulse with 50 mM doxorubicin (Sigma-Aldrich) and collected after overnight culture, or treated overnight with 1 mM of paclitaxel (Sigma-Aldrich), then were lysed in RIPA buffer (50 mM Tris pH 8; 150 mM NaCl; 1% NP40; 0.5% DOC; 0.1% SDS). Fifty micrograms of lysate was separated on 12% SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Madrid, Spain). Western blots were probed with anti-p53 (DO-1) antibody (Invitrogen) and re-probed with anti-β-actin (Sigma-Aldrich) to ensure equal loading (Online Figure IIC).

Cardiovascular differentiation assay
Human CPC were seeded at 5000 cells/cm² on 0.1% gelatin coated 6 wells plates and incubated in DMEM/F12 and Neurobasal (1:1) medium supplemented with 10% FBS ESCq, plus growth factors for 24h. Cells were treated with 100nM Oxytocin (Sigma-Aldrich) for three days and then trypsinized and seeded in p24 ultra-low adherent wells (Costar) (2000 cells/well). Seven days later, cardiospheres were harvested and distributed in 24 well plates, with laminin (Sigma-Aldrich) pre-coated glass slides (10 µg/ml), in differentiation media: α-MEM, FBS 2%, dexamethasone (1 µM) (Sigma-Aldrich), beta-glycerolphosphate (10 mM) (Sigma-Aldrich), ascorbic acid (50 µg/ml) (Sigma-Aldrich). During the first 4 days media was supplemented with TGF-β1 (5 ng/ml) (Peprotech), BMP-2 (10 ng/ml) (R&D systems, Madrid, Spain) and BMP-4 (10 ng/ml) (R&D systems) and then this supplement was replaced by DKK-1 (0.15 µg/ml) (Peprotech). At the end of the differentiation protocol (day 30) cells were analyzed by immunofluorescence (Online Figure IIIA).

In vivo rat model
All procedures with animals were approved by the “Instituto de Salud Carlos III” and institutional ethical and animal care committees. Nude rats of 200 to 250 g (HIH-Foxn1 rnu, Charles River Laboratories, Inc., Wilmington, Massachusetts) were used. The initial number of animals included in the study was 30. Mortality in all groups due to surgical procedures was approximately 30%.

MI and cell transplantation. Permanent ligation of the left coronary artery was performed as previously described[2]. Once the affected tissue acquired a pale colour the intramyocardical transplantation was done (saline or 0.5x10⁶ CPC) at 2 points of the infarct border zone with a Hamilton syringe.
Histochemistry. Two months after implantation, animals were killed, hearts removed, washed with phosphate-buffered saline, fixed in 4% paraformaldehyde for 24h and stored in 70% ethanol till further processing.

Before inclusion, heart atriums were removed and the heart cut in three sections of similar size: apex region (A zone), medium region (B zone) where the ligation was done, and the region close to the valves (C zone). Every region was included in paraffin and three sections of 5 μm, spaced 75 μm from selected hearts (showed below), were dyed using Masson’s Thrichrome stain technique. Histological analyses were performed and scar size with respect to the total diameter of the left ventricle was calculated (Online Figure III B). The percentage of muscular fibres with respect to the total wall thickness was calculated in a representative infarcted area (Online Figure III C). The Cell® Olympus software was used to obtain histomorphometric measurements. Student’s t test was used to analyze the significance of the data obtained by echocardiography or histological analysis. Histological studies were performed at Anapath (Granada, Spain).

Mesenchymal stem cells culture

Cryopreserved human bone marrow-derived mesenchymal stem cells (hMSC), which are used in the present study, were provided by Dr J. Larghero (Cellular Therapy, Saint Louis Hospital, Paris, France). Upon thawing cells were cultured at 37°C in 5% CO₂ atmosphere in α-MEM supplemented with 10% FBS, L-Glutamine (2 mM), Penicilline-Streptomycine (100 U/mL and 100 µg/mL) and bFGF (1 ng/mL), and their phenotype and immunophenotype were determined by flow cytometry and immunofluorescence staining (Online Figure IV).

Flow cytometry

The expression of cell surface markers on the original and cryopreserved hCPC as well as hMSC was analyzed by flow cytometry. The current study used the following antibodies: anti-MHC I (W6/32), anti-MHC II HLA-DR (L243), HLA-DP (B7/21), HLA-DQ (33.1), anti-CD40 (G28.5) were affinity-purified in the laboratory from ascites using a protein A-Sepharose column (Amersham Pharmacia Biotech, Orsay, France), anti-SSEA-4 (MC813) (Abcam, Cambridge, UK), SSEA-1 (MC-480) (Millipore, Molsheim, France), APC-conjugated anti-CD29 (MAR4), FITC-conjugated anti-CD34 (581), Pecy7-conjugated anti-CD45 (HI30), PE-conjugated anti-CD49a (SR84), anti-CD49b (12F1), anti-CD49d (9F10), anti-CD49e (IIA1), anti-CD80 (L307.4), anti-CD86 (FUN-1), anti-CD44 (515), anti-CD274 (MIH1), anti-CD275 (2D3), anti-CD166 (3A6), anti-CD90 (5E10), anti-CD73 (AD2), anti-CD105 (266), anti-CD54 (HA58) (BD Biosciences, Le Pont de Claix, France), anti-c-kit (104D2) (Santa Cruz Biotechnologies, Heidelberg), and anti-CD133/2 (293C3) (Miltenyi Biotech) Un-conjugated primary antibodies were detected with PE-conjugated goat anti-mouse IgG (H+L) antibody (BD Biosciences). Cells were acquired using the Canto II flow cytometer (BD Biosciences) and analyzed using either the BD FACS Diva or FlowJo software (Celeza, Olten, Switzerland).

Immunofluorescence detection

The hCPC were grown in 4-well glass chamber slides (BD Biosciences) in their complete medium. At subconfluence, cells were fixed with 4% paraformaldehyde then permeabilized with 0.1% Triton X-100 and neutralized with Glycine (0.1 M). Slides were blocked by 1% BSA and then stained with un-conjugated anti-sarcomeric actinin (Sigma-Aldrich), anti-Smooth muscle actin (Sigma-Aldrich), anti-cardiac Troponin I (Santa Cruz), anti-von Willebrand factor (Dako, Barcelone, Spain), anti-Nkx2.5, anti-GATA-4, anti-Islet-1, anti-MEF2C, anti-OCT4 or anti-SSEA-4 specific antibodies (Abcam) or with isotype control antibodies. Fluorochrome-conjugated secondary antibodies were then used to
reveal the presence of the proteins. Nuclei were stained with DAPI and the slides were mounted in Vectashield. Images were acquired by immunofluorescence microscopy using the Zeiss Axiovert 200M microscope (Zeiss, Oberkochen, Germany) at 40x magnification and the Axiovision v4.5.0.0 software (Zeiss). Alternatively, slides were analyzed with a confocal microscope (Leica).

Peripheral Blood Mononuclear Cells
Peripheral blood mononuclear cells (PBMC) were prepared from the blood samples of six different healthy donors by centrifugation on a Ficoll-Hypeaque density gradient and cryopreserved for use in different experiments. Donors signed an informed consent following human ethics committee “Comité consultatif pour la protection des personnes dans les recherches biomédicales” - Saint Louis Hospital, Paris, France), and the study has been approved by the institution.

Western blotting
Expression of pluripotency transcription factors OCT4, NANOG and SOX2 was analyzed in total cell extracts (50 µg) separated by 10% SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare Life Sciences, Aulnay-sous-bois, France). The membranes were washed in Tris-buffered saline with Tween (10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% Tween 20), blocked 1h at room temperature with 5% nonfat milk or 1% BSA in Tris-buffered saline with Tween, then probed 1h at room temperature with anti-OCT4 (Abcam), anti-NANOG (Abcam), or anti-SOX2 (Santa Cruz Biotechnologies, Heidelberg, Germany) antibodies. Equal loading was ensured by reprobing with anti-β-actin antibody (Sigma-Aldrich, Saint-Quentin Fallavier, France). Immunoblots were treated with an HRP-conjugated secondary antibody (Santa Cruz Biotechnologies) followed by enhanced chemiluminescence detection and phosphorimaging (Amersham Biosciences).

Allogenic immune responses and immunomodulation assays
Tailored mixed lymphocyte reactions were performed as we previously described. Briefly, responding PBMC (1x10^5) labeled with CFSE (2.5 µM for 10 min, Invitrogen) were co-cultured in RPMI-10% FBS in U-bottom 96-wells plates with either HLA-mismatched mitomycin-C-treated stimulatory PBMC (1x10^5) or HLA-mismatched mitomycin-C-treated hCPC or IFNγ-treated hCPC (1x10^4), or mitomycin-C-treated hMSC (1x10^4). At the end of co-cultures staining with anti-CD3-Pe-cy7, anti-CD4-APC, anti-CD8-APC-H7, anti-CD25-PE and 7AAD (BD Biosciences) and flow cytometry analyses were used to monitor the activation, proliferation, and cell death of different T-cell subsets. Regulatory T cells were identified using anti-CD4-vioblue (Miltenyi Biotech), anti-CD25-PE, anti-CD127-Pecy7 and anti-FoxP3-APC antibody staining (e-Biosciences, Paris, France), and the phenotypes of these regulatory T cells were determined using anti-HLA-DR-PerCP, anti-PD1-FITC, and anti-CD45RA-FITC (BD Biosciences) antibodies and flow cytometry.

Regulatory CD4⁺CD25^high^CD127^low/- T cells were also sorted from PMBC using anti-CD4-APC, anti-CD25-PE and anti-CD127-PE-cy7 antibodies and the FACSAria cytometer. FoxP3 expression was then verified using specific antibodies. Sorted Treg cells were then labeled with CFSE and co-cultured with mitomycin-C-treated hCPC or IFNγ-treated hCPC at a ratio of (5:1) in the presence of IL-2 (50U/mL) for 7 days. In assays determining hCPC immunomodulatory effect, HLA-mismatched CFSE-labeled PBMC (1x10⁵) were stimulated with phytohemagglutinin (PHA, 1 µg/mL, Sigma-Aldrich) in the absence or the presence of mitomycin-C-treated hCPC or IFNγ-treated hCPC, or mitomycin-C-treated hMSC (1x10^⁴) for 5 days. Co-cultures were set up in the Transwell
system (Sigma-Aldrich) and blocking anti-PD-L1 (29E.2A3) (5 µg/ml) or its isotype control IgG2b (Biolegend, Saint Quentin en Yvelines, France) were used in some experiments as indicated. At the end of co-cultures, the activation, proliferation, and cell death of different T-cell subset was monitored as above.

**Cytokine assays**
The levels of IFNγ, IL-2, IL-10 and IL-4 in the supernatants of various co-cultures were determined at the indicated time-points by ELISA using specific kits (BD biosciences), following manufacturers’ instructions.

**Suppressive assays**
Purified CD4+ T cells (1x10^6 cells) were co-cultured in 6-well plates with mitomycin-C-treated hCPC (2x10^5 cells) for 7 days. hCPC-induced CD4^+CD25^{high}CD127^{low} and CD4^+CD25^{low}CD127^{+} T cells were then sorted in the FACSARia cytometer, verified for the expression of FoxP3, then co-cultured with CFSE-labeled autologous PBMC stimulated with PHA. The proliferation of autologous CFSE-labeled PBMC was evaluated by flow cytometry after five days.

**Silencer RNA**
To knockdown the surface expression of PD-L1, the hCPC were transiently transfected with three different PD-L1-specific stealth RNAi species from Invitrogen limited (Paisley, UK).

**CD274HSS120931:**
- 201946 C09 sense 5’GAGGAAGACCUGAAGGUUCAGCAUA3’
- 201946 C10 anti-sense 5’UAUGCUGAACCUUCAGGUCUCCUC3’

**CD274HSS120932:**
- 201946 C11 sense 5’CCUACUGGCAUUUGCUAGCAUCU3’
- 201946 C12 anti-sense 5’AAUGCGUUCAGCAUAAUGGAGUAGG3’

**CD274HSS120933:**
- 201946 D01 sense 5’UGAUACACAUUUUGGAGGACAGUA3’
- 201946 D02 anti-sense 5’UUACGUCUCCUCUAAUGUUAUCA5’

Two different stealth RNAi negative control duplexes were used, a low GC duplex (for CD274HSS120931 RNAi) and a medium GC duplex (for CD274HSS120932 and CD274HSS120933 RNAis). Knockdown was carried out following standard procedures. Briefly, cells were grown in their complete medium in 24-well plate at 4x10^4 cells/well for 24h to reach a maximum of 50% confluence. The medium was then replaced by OptiMEM medium without antibiotics (Invitrogen). The stealth RNAi (100nM) or negative controls duplexes (low and medium GC) (Invitrogen) was then mixed with Lipofectamine in OptiMEM medium and incubated at room temperature for 20min. The oligomer-Lipofectamine complexes thus formed were incubated with the cells for an overnight. The culture medium was then changed, and cells were allowed to grow for an additional 48 h before testing for surface expression of PD-L1 using specific antibodies and flow cytometry.
Online Figure I: Isolation of hCPC and initial characterization: A) Human biopsies from atrial appendage were minced up and digested with collagenase type 2. Cell suspensions of CD45-CD117\(^{+}\)(c-Kit) cells were selected as described under “Detailed Methods” then seeded in Matrigel-coated plates and expanded until passage 7. B) Representative expression of SSEA-1 and -4, CD166 and CD90 by primary hCPC. The number of positive cells is indicated (%). C) Comparative transcriptional analysis of hCPC and hMSC. Heat map analysis of transcriptional expression levels of Tbx5, Flk1, GATA4 and Nestin in hCPC from two different donors (hCPC1 and hCPC2) and hMSC. mRNA expression was normalized to expression of beta glucoronidase mRNA. D) RQ values \(2^{\Delta\Delta CT}\) using beta glucuronidase as internal control and hMSC as control reference cells.
Online Figure II: Clonogenicity and genetic stability of hCPC: A) hCPC clonogenicity was analyzed at passage 2 (P2) and passage 7 (P7). Results presented as the percentage of single seeded cells able to grow till full confluence is reached in a 96-well plate (clonogenic cells). B) Genetic stability of hCPC expanded in vitro till passage 6, was tested by CGH as described under “Detailed Methods. No genetic alteration was observed. C) G1 cell cycle checkpoint was studied in hCPC and hMSC. Expression of p53 in untreated cells (C), treated with doxorubicin (D) or treated with paclitaxel (T) was analyzed by western blot. hCPC and hMSC had undetectable p53 at basal levels which increased significantly after DNA damage (cells treated with doxorubicin). As expected, no up-regulation of p53 protein was detected when cell cycle arrest was produced by mitosis inhibition after paclitaxel addition.
Online Figure III: Human CPC in vitro and in vivo potential. A) hCPC seeded in low adherent plates formed cardiospheres after ten days of culture (a). Cardiospheres seeded in differentiation medium (b) start their differentiation (c). hCPC were stained with anti-smooth muscle actin (d) and with anti-von Willebrand factor (e) or with anti-cardiac Troponin I labelled antibody and with anti-sarcomeric actin antibody (f). In all preparations cell nuclei were stained with DAPI. B) hCPC promote cardiac regeneration. Representative heart sections from infarcted nude rats receiving PBS (a) or hCPC (b) stained with Masson’s Trichrome. Heart sections were obtained from comparable levels using apex as reference. (c) Percentage of muscular fibers in the infarcted area referred to the total thickness of the ventricle wall and calculated in stained sections. *p <0.05
Online Figure IV: Phenotype of bone marrow-derived human mesenchymal stem cells.  A) Representative expression of stem cells markers by hMSC. The number of positive cells (%) and geometric mean fluorescence intensity (MFI) values are indicated. B) Representative expression of cardiac lineage markers and pluripotency transcription factors by hMSC. Nuclei were stained with DAPI and images were captured under the 40x objective. C) Expression of adhesion molecules by hMSC. Percentage of positive cells (%) and geometric mean fluorescence intensity (MFI) are indicated. D) Representative expression of immune relevant molecules by hMSC (black histograms) against isotype control (gray filled histograms). The numbers in the histograms denote the percentage of positive cells (%) and the geometric mean fluorescence intensity.
Online Table I: HLA class I and class II genotype. Responder PBMC and stimulator hCPC or hMSC were genotyped for HLA class I (-A, -B and -C) and HLA class II (-DRB1) to monitor allogenicity in mixed lymphocytes reaction.

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Online Table II: List of Antibodies

*Antibodies were purified from ascites using protein A-Sepharose column (Amersham Pharmacia Biotech, Orsay, France.)
Online Table III: Expression of stem cells markers and adhesion molecules by hCPC and IFNγ-hCPC. FACS analysis of the expression of stem cells markers and adhesion molecules in hCPC and in hCPC treated with IFNγ. The number of positive cells (%) and geometric mean fluorescence intensity (MFI) values are indicated.
Online Table IV: TaqMan gene probes. Accessions are derived from http://www.ncbi.nlm.nih.gov/

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Figure V: Co-culture of hCPC with allogeneic T lymphocytes. A) hCPC did not induce T lymphocytes death in allogeneic assays. CFSE-labeled PBMC were cultured with medium alone, HLA-mismatched mitomycin-C-treated PBMC (allo-PBMC), hCPC, IFNγ-hCPC, or hMSC and CD3⁺ T cells death was determined by 7-AAD staining and flow cytometry analysis and presented as percentage of dead cells. B) hCPC induces low allogeneic response of purified T cells. CFSE-labeled CD3⁺ T cells were cultured with medium alone, HLA-mismatched mitomycin C-treated PBMC (allo-PBMC), hCPC, IFNγ-hCPC, or hMSC and CD4⁺ and CD8⁺ T cells proliferation was determined by flow cytometry analysis and presented as percentage of proliferating cells. C) hCPC do not secrete IL-2, IL-10 or IFNγ. Cytokine levels were determined by ELISA in supernatants of hCPC cultured in RPMI medium or PHA-activated-PBMC as a control. Results are presented as mean values ± SD from triplicates. *p≤0.05; †p<0.001.
Figure VI: Expression of PD-L1 by hMSC and hCPC under different culture conditions. Representative experiment of PD-L1 expression by hMSC and hCPC. FACS analysis confirms the absence of constitutive expression of PD-L1 by hMSC and its substantial expression by hCPC (medium). Nearly 60% of hMSC expressed PD-L1 (geometric MFI 212) when co-cultured with PHA-stimulated allogenic PBMC but not when cultured with PHA or allogenic PBMC alone. In contrast, the expression of PD-L1 by hCPC remained stable under different culture conditions. Accordingly, the expression of PD-L1 may underlay and coincide with the substantial IL-10 production when hMSC modulate PHA-induced response. The number of positive cells and geometric mean fluorescence intensity values are indicated.
References

