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Immune response to human embryonic stem cell-derived cardiac progenitors and adipose-derived stromal cells

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Abstract

Transplantation of allogeneic human embryonic stem cell-derived cardiac progenitors triggers an immune response. We assessed whether this response could be modulated by the concomitant use of adipose-derived stromal cells (ADSC). Peripheral blood mononuclear cells were collected from 40 patients with coronary artery disease (CAD) and nine healthy controls. Cardiac progenitors ($CD15^+$ $Mesp1^+$) were generated as already reported from the I6 cell line treated with bone morphogenetic protein (BMP)-2. Adipose-derived stromal cells were obtained from abdominal dermolipectomies. We assessed the proliferative response of peripheral lymphocytes from patients and controls to cardiac progenitors cultured on a monolayer of ADSC, to allogeneic lymphocytes in mixed lymphocyte culture and to the T cell mitogen phytohemaglutin A in presence or absence of ADSC. Cardiac progenitors cultured on a monolayer of ADSC triggered a proliferation of lymphocytes from both patients and controls albeit lower than that induced by allogeneic lymphocytes. When cultured alone, ADSC did not induce any proliferation of allogeneic lymphocytes. When added to cultures of lymphocytes, ADSC significantly inhibited the alloantigen or mitogen-induced proliferative response. Compared to healthy controls, lymphocytes from patients presenting CAD expressed a decreased proliferative capacity, in particular to mitogen-induced stimulation. Adipose-derived stromal cells express an immunomodulatory effect that limits both alloantigen and mitogen-induced lymphocyte responses. Furthermore, lymphocytes from patients with CAD are low responders to conventional stimuli, possibly because of their age and disease-associated treatment regimens. We propose that, in combination, these factors may limit the *in vivo* immunogenicity of cardiac progenitors co-implanted with ADSC in patients with CAD.

Keywords: immunogenicity • cardiac progenitors • embryonic stem cells • alloreactivity • T lymphocytes • mesenchymal stem cells • coronary artery disease

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Introduction

Human embryonic stem cells and their derivatives have been proposed to be less immunogenic than adult cells [1–3]. Nevertheless, compelling evidence has accumulated to suggest that transplantation of undifferentiated [4, 5] embryonic stem cells or of their cardiac-directed progeny [6] into infarcted myocardium triggers an immune response that represents a serious hurdle in the perspective of clinical applications [7]. Although rejection can ultimately be controlled by immunosuppressants, the infectious and tumourigenic risks associated with these drugs justify the search for less aggressive strategies. Because ADSC have been reported to exert mesenchymal stem cell-like immunomodulatory effects [8–10] and may improve graft viability through their trophic effects, we tested whether they could mitigate the response of human lymphocytes to various stimuli including stem cell-derived cardiac progenitors, alloantigens or the T cell mitogen phytohemaglutin A (PHA). To approach as much as possible the clinical situation we compared lymphocytes from patients presenting CAD, potential recipients of cardiac progenitor transplants, with those of healthy controls.

Materials and methods

Cell preparations

Peripheral blood mononuclear cells (PBMCs) were obtained from 9 healthy donors (aged 23–60) and 40 patients (aged 35–83) with documented CAD and planned for surgical bypass after institutional approval and informed consent. Peripheral blood mononuclear cells were collected at the onset of the operation, separated by Ficoll gradient and suspended in complete RPMI medium containing glutamine, human AB serum and antibiotics. Adipose-derived stromal cells were obtained from abdominal dermolipectomies and isolated as previously described [10]. Human bone marrow mesenchymal cells (BM-MSC) were isolated from bone residues obtained during hip surgery as previously described [8]. Cardiac Mesp1$^+$ progenitors were derived from the I6 ESC line challenged with BMP-2 and sorted immunomagnetically (Miltenyi Biotec, Bergisch Gladbach, Germany) on the basis of their positivity for the CD15 antigen, as reported [11–12]. Cardiomyocytes were obtained after a 3-week *in vitro* differentiation treatment of cardiac progenitors co-cultured with cardiac fibroblasts as previously reported [11].

Analysis of the immunophenotype

The expression of cell surface markers was primarily based on flow cytometry using labelled specific antibodies to major histocompatibility complex (MHC) class I (anti-human β_2 microglobulin; Biolegend), MHC class II (anti-HLA-DR), CD80 (all from BD Bioscience, Le Pont de Claix, France) at day 0 following the immunomagnetic sorting and 7 days after co-culture with fibroblast. Activation of allogeneic lymphocytes in response to ESC-derived cardiac progenitors and their combination with ADSC, was assessed by measuring the expression of CD3, CD4, CD8, CD69, CD25 and CD DR by flow cytometry (all from BD Bioscience).

Analysis was performed with an FACS Calibur flow cytometer (BD, Le Pont de Claix, France) and the Cell Quest software (BD).

In a separate set of experiments, hESC from the I6 line were differentiated *in vitro* into embryoid bodies (EBs) using the hanging drop method [13]. Briefly, undifferentiated ESC were allowed to spontaneously aggregate as EBs in a defined culture medium that consisted of KnockOut DMEM (Gibco, Life Technologies, Villebon sur Yvette, France), 20% KnockOut SR (a serum-free formulation; Gibco), 1 mM glutamine (Gibco), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, Lyon, France) and 1% non-essential amino acids (Gibco). After approximately 10 days, these bodies started to beat. Three-week embryoid bodies were fixed and stained for the expression of β_2 microglobulin (1/100) and HLA-DR (1/100). Expression of both MHC molecules and of two cardiac markers [sarcomeric actinin (Sigma-Aldrich) and troponin T (Abcam, Paris, France)] was also assessed by confocal microscopy on cardiomyocytes differentiated following co-culture of the CD15$^+$ cardiac progenitors with fibroblasts.

Lymphocyte proliferation assays

Peripheral blood mononuclear cells (4×10^4 cells in 100 μl) were seeded onto 96-well flat-bottomed microtitre plates in the presence of 2.5 μg/ml of PHA. T cell proliferation was assessed after 72 hrs incubation at 37°C in 5% CO_2-containing atmosphere using ^{3}H-thymidine incorporation (1 μCi per well for 24 hrs). Results were expressed in counts per minute (cpm). For carboxy-fluorescein diacetate succinimidyl ester (CFSE) proliferation assays, staining was performed as previously described [14–15] and T cell subsets were quantified by FACS using specific antibodies.

Analysis of CFSE data was performed by calculating the number of mitotic events as the sum of individual mitotic events under each peak, which equals the number of daughter T cells minus the number of T cell precursors. The number of T cell precursors was calculated by dividing the number of daughter cells events by 2 raised to the power of the given round division. When needed, irradiated ADSC were first plated onto 96-well flat-bottomed plates; cardiac progenitors were seeded 24 hrs later at a 1:1 ratio and cultured for 4 days. Allogeneic PBMCs (1.5×10^5 cells) were then added to wells at a 2:1 (lymphocytes: cardiac progenitors) ratio, cultured for five additional days and pulsed with ^{3}H-thymidine to assess proliferation. Peripheral blood mononuclear cells stimulated with ADSC alone were used as negative controls. To try to analyse the effects of cardiac progenitors alone, in absence of feeder ADSC, progenitors were cultured on plastic and gelatin-coated culture dishes as well as on a collagen-based matrix (Gelfoam®, Pharmacia, Uppsala, Sweden). Their viability was then assessed at time 0 and after 40 hrs of culture using double staining with Annexin V/propidium iodide (PI).

For mixed lymphocyte reactions (MLR) BM-MSCs or ADSC were irradiated at 75 Gy and plated onto flat-bottomed microtitre plates (96 wells) at different concentrations. Peripheral blood mononuclear cells (1.5×10^5 cells) and an equal number of third party irradiated (35 Gy) PBMCs were added to wells. Cultures were incubated for 5 days and pulsed with ^{3}H-thymidine. Unstimulated PBMCs cultured with and without BM-MSCs or ADSC were used as negative controls.

Statistical analysis

Significant differences between groups were assessed using Tukey's multiple comparison test with statistical significance set at $P < 0.05$. Statistical analysis was performed with STATISTICA analysis software Version 8 (Tulsa, OK, USA).

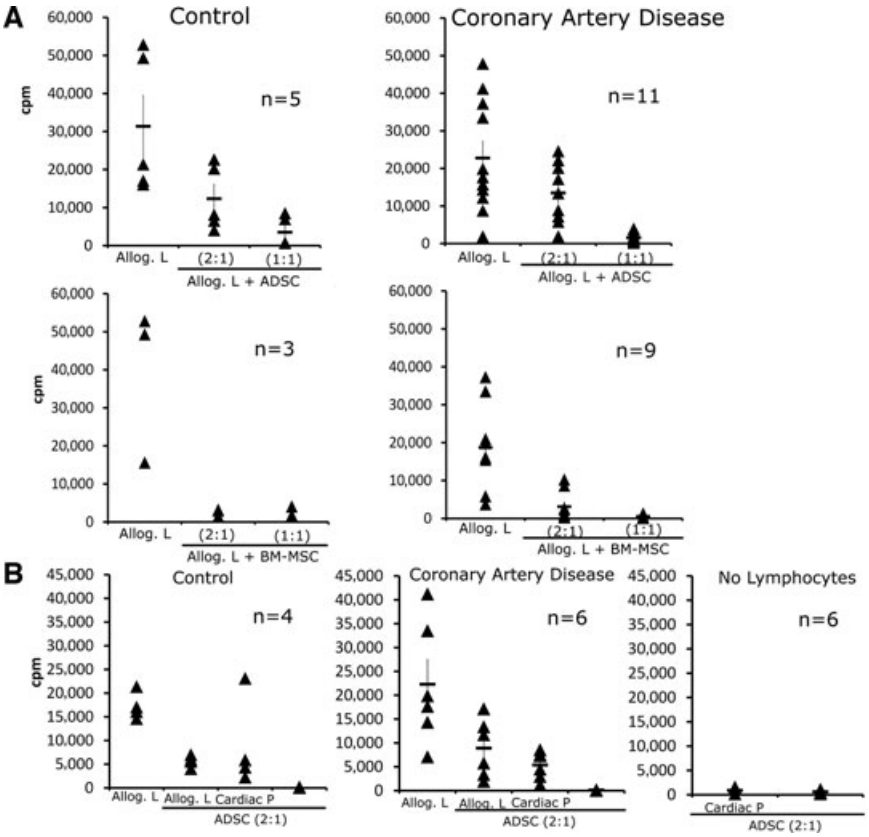

Fig. 1 Allogeneic responses of lymphocytes from controls and patients to lymphocytes or cardiac progenitors co-cultured with ADSC. (**A**) Allogeneic responses of lymphocytes are inhibited by using ADSC or BM-MSCs on MLR. PBMCs from controls (left panel) and patients (right panel) were stimulated with allogeneic lymphocytes in presence or absence of ADSC and BM-MSC. Immunomodulatory properties of ADSC and BM-MSC were not statistically different. Median inhibition for controls PBMCs was 68.4% (54.9–71.8) at a 2:1 lymphocyte/ADSC ratio and 90.8% (83.9–96.1) at a 1:1 ratio *versus* 40.9% (28.0–88.8) and 90.7% (66.6–98.6), respectively, in patients. Stimulation with ADSC or BM-MSC alone showed no lymphocyte proliferation. Horizontal bars indicate the mean ± S.E.M. (**B**) Alloreactive responses induced by cardiac progenitors co-cultured with ADSC represent 30% of that observed following conventional allogeneic stimulation. Proliferative response of peripheral lymphocytes to allogeneic lymphocytes, cardiac progenitors cultured on a monolayer of ADSC, or to ADSC was assessed. Response to cardiac progenitors co-cultured with ADSC is not different from that observed when lymphocytes are stimulated with allogeneic lymphocytes in presence of ADSC. Differences between lymphocytes from controls (left panel) and patients (middle panel) were not statistically different. Cardiac progenitors co-cultured with ADSC or ADSC alone, without lymphocytes (right panel) showed no proliferation of the stem cells in the culture. Horizontal bars indicate the mean ± S.E.M.

Results

Lymphocyte response to alloantigens in mixed lymphocyte culture is inhibited by addition of ADSC

Patients presenting CAD showed a tendency towards decreased lymphocyte responses to alloantigens compared to controls (Fig. 1A). In patients and controls, addition of ADSC induced a dose-dependent inhibition of lymphocyte proliferation. The median inhibition for PBMCs from controls was 68.4% (54.9–71.8) at a 2:1 lymphocyte/ADSC ratio and 90.8% (83.9–96.1) at a 1:1 ratio *versus* 40.9% (28.0–88.8) and 90.7% (66.6–98.6), respectively, in patients (Fig. 1A).

Bone marrow–derived mesenchymal stem cells (BM-MSC) were also analysed: a significant inhibitory effect was similarly observed with a median 93.4% and 91.7% inhibition of control lymphocyte response at 2:1 and 1:1 ratios *versus* 87.7% and 92.5% in patients, respectively (Fig. 1A).

Lymphocyte response to stem cell-derived cardiac progenitors cultured on a monolayer of ADSC is lower than that induced by a conventional allogeneic stimulation

As shown in Figure 1B, when cardiac progenitors were cultured on monolayers of ADSC, both controls and patients with CAD exhibited a detectable lymphocyte proliferative response [median 5.534 cpm (1972–17,184)]. This response represented 29–33% of that observed with the same lymphocytes following conventional allogeneic stimulation in healthy controls and patients, respectively. However, this response to cardiac progenitors + ADSC was fully superimposable and not statistically different ($P = 0.889$) from that observed when the same lymphocytes were stimulated with allogeneic cells in presence of ADSC alone at a 2:1 ratio (Fig. 1B).

To try to separate the intrinsic immune effects of the $CD15^+$ cardiac progenitors from the mix cardiac progenitors-ADSC, the former were cultured alone on different substrates. We observed that under these conditions, cells were unable to adhere to any of

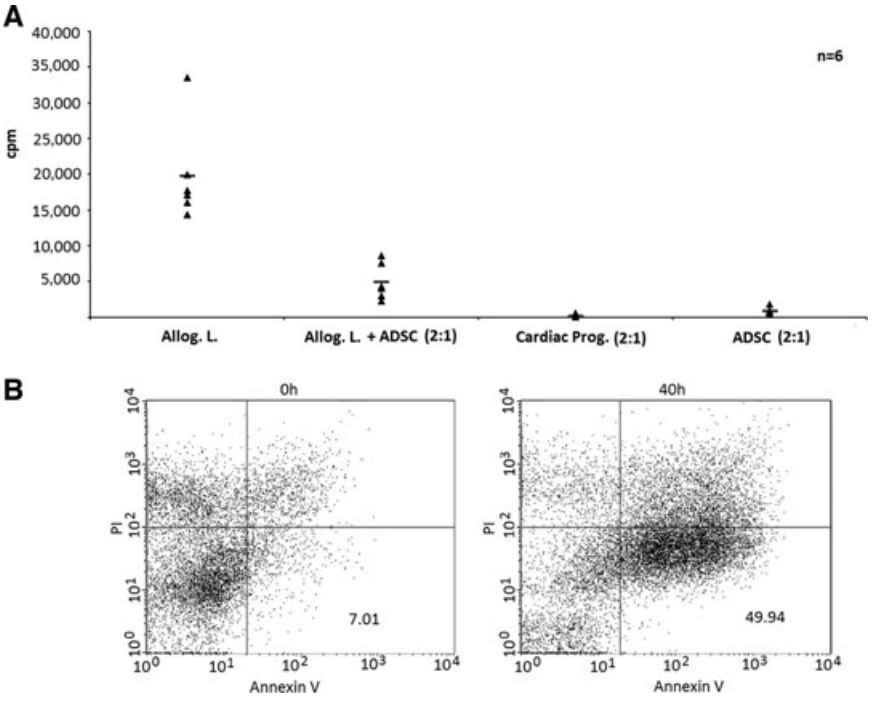

Fig. 2 Immunogenicity and survival of cardiac progenitors cultured in a feeder-free condition. (**A**) Alloreactive response induced by cardiac progenitors without ADSC. Proliferative response of peripheral lymphocytes to allogeneic lymphocytes, or cardiac progenitors was assessed. No proliferation was observed when peripheral lymphocytes were stimulated with allogeneic ADSC or cardiac progenitors in a feeder-free condition. Stimulation with allogeneic lymphocytes was used as positive control. Horizontal bars indicate the mean ± S.E.M. (**B**) Survival of cardiac progenitors without ADSC. To analyse viability, apoptosis and death of cardiac progenitors cultured in a feeder-free condition, double staining with annexin V and propidium iodide was assessed at day 0 and after 40 hrs of culture. At day 0, only 7% of the cells were apoptotic and 20% of the cells were dead (left panel). After only 40 hrs (right panel), annexin V/PI double staining show a high level of cell death, where only 18.96% of the cells were not stained (annexin V−/PI−), 49.94% were apoptotic cells (annexin V+/PI−), 22.70% were apoptotic necrotic cells with loss of membrane integrity (annexin V+/PI+), and 8.40% were late dead cells (annexin V−/PI+).

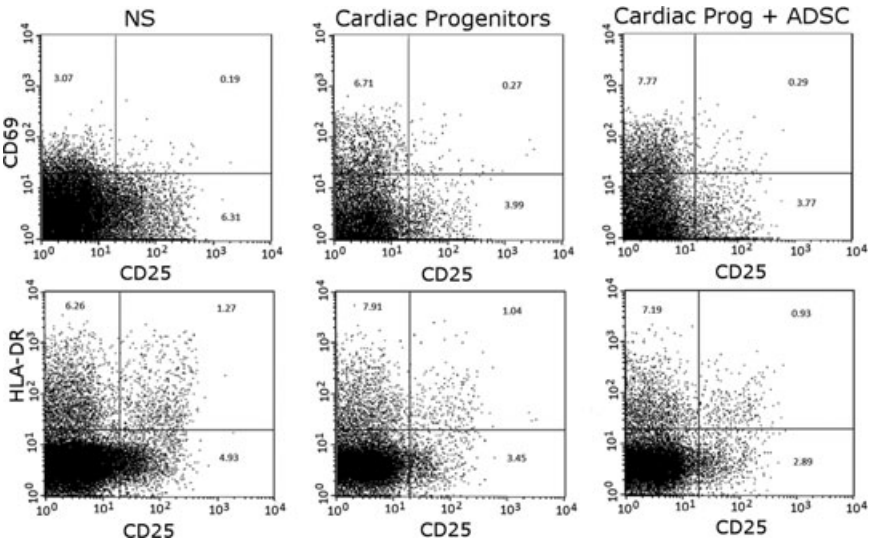

Fig. 3 Lymphocyte activation after 24 hrs of culture with allogeneic cardiac progenitors. Activation of allogeneic lymphocytes in response to ESC-derived cardiac progenitors was assessed by measuring the expression of CD69, CD25 and CD DR molecules at day 0 (left panel) and after 24 hrs (right panel) of culture. T cells were found to up-regulate cell surface expression of the early activation marker CD69. No up-regulation of CD25 and CD DR after antigenic stimulation was observed. In the presence of ADSC no decrease of lymphocyte activation was observed. Results are representative of two independent experiments.

the surfaces that were tested. Instead they remained in suspension without forming aggregates during the 5 days of culture. After 5 days, the progenitors failed to induce an allogeneic lymphocyte stimulation (Fig. 2A). It would be erroneous, however, to conclude that this unresponsiveness was due to their intrinsically low immunogenicity because after 40 hrs of culture under these feeder-free conditions, double staining by annexin V-propidium iodide showed that 80% of the cells had died (Fig. 2B). We thus assessed lymphocyte activation in response to $CD15^{+}$ cardiac progenitors at an earlier time frame (24 hrs) because most of them were still alive at this time point. An up-regulation of CD69 was then observed, reflecting the potential of the cardiac progenitors to induce early lymphocyte activation (Fig. 3). Addition of ADSC failed to decrease this lymphocyte activation.

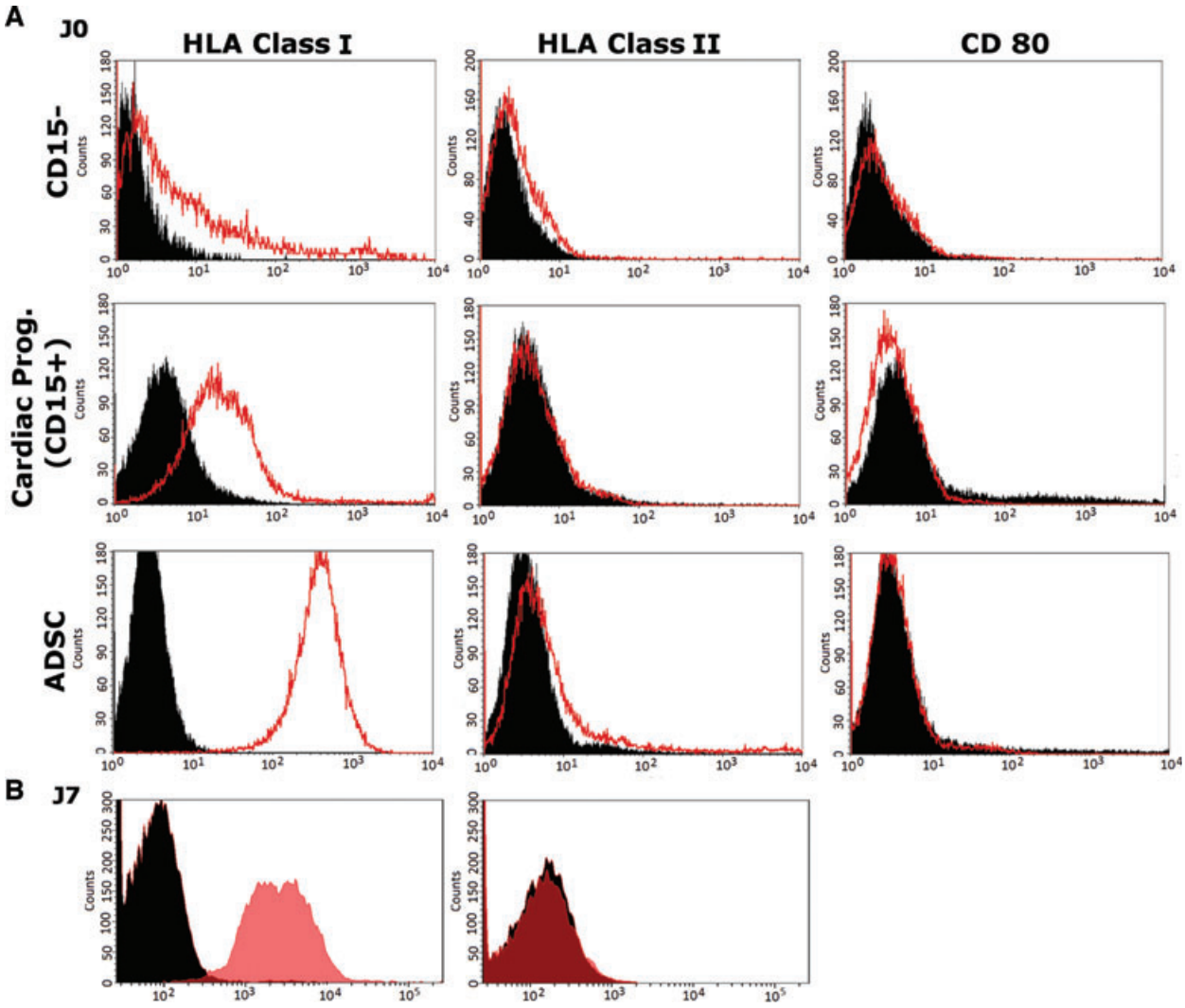

Fig. 4 Immunophenotype of cardiac progenitors and ADSC. Surface expression of MHC class I, MHC class II and co-stimulatory CD80 molecules by cardiac progenitors and ADSC was assessed (**A**) at day 0 before culture or (**B**) after 7 days of culture. Immunofluorescence staining was assessed using β_2 microglobulin antibody labelled with FITC, CD DR antibody labelled with texas Red and CD80 antibody labelled with PE. Shaded black histograms denote background staining using control isotype-matched antibodies. A. Median fluorescence intensity (MFI) of MHC class I molecules expression in $CD15^-$ cells was 3.1, compared with 19.3 in $CD15^+$ cells (hESC-derived cardiac progenitors). No MHC class II molecules or CD80 co-stimulatory molecules were detected on day 0. ADSC expressed high levels of MHC class I but no MHC class II or CD80. (**B**) After 7 days of co-culture, cardiac progenitors showed high expression of β_2 microglobulin, but no expression of the HLA-DR molecule.

hESC-derived cardiac derivatives express low levels of immunostimulatory molecules, which increase upon differentiation

We analysed the expression of β_2 microglobulin and HLA-DR on hESC-derived cardiac progenitors and on ADSC at day 0 (before culture). As previously shown [16] ADSC expressed high levels of β_2 microglobulin but no HLA-DR antigens. Low levels of MHC class I expression were detected on the ESC-derived $CD15^-$ fraction collected after sorting and they only slightly increased on the derived $CD15^+$ cardiac progenitors. No MHC class II molecules or CD80 co-stimulatory molecules were detected at day 0 (Fig. 4A). After 7 days of co-culture, cardiac progenitors strongly up-regulated the expression of MHC class I molecules, without changes in the expression of the HLA-DR molecule.

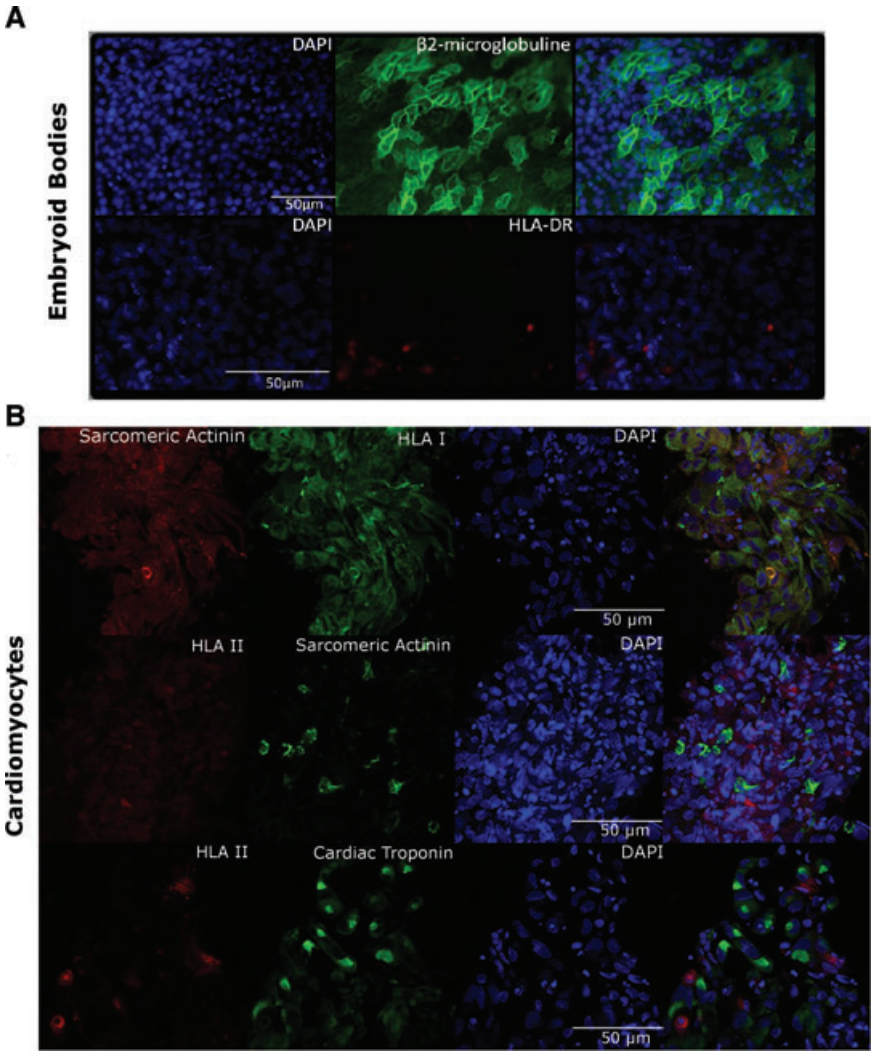

Fig. 5 Expression of MHC class I and class II in 3-week embryoid bodies and cardiomyocytes. (**A**) Embryoid bodies showed high expression of MHC class I molecules but no expression of MHC class II. (**B**) Cardiomyocytes differentiated from the $CD15^+$ progenitors expressed sarcomeric actinin and cardiac troponin T molecules. Co-localization of these staining with MHC class I and MHC class II showed that 3 weeks cardiomyocytes expressed MHC class I but no MHC class II molecules. However, in the cultures some cells negative for the cardiac lineage markers, were found to express HLA-DR.

MHC molecules were also studied in terminally differentiated cardiac cells generated *in vitro* from embryonic bodies of the same cell line from which the cardiac progenitors were derived. Immunohistological staining showed high expression of MHC class I in the vast majority of the cells of the embryoid bodies but no expression of MHC class II molecules (Fig. 5A). A more accurate phenotypic identification of these MHC class I-positive cells was based on the observation of cardiomyocytes differentiated from the $CD15^+$ progenitors; the finding of a co-localization of the staining for both MHC class I and cardiac markers (sarcomeric actinin and troponin T) confirmed that the immunogenicity of ESC-derived cardiac derivatives proceeded in parallel with the maturation of the progenitors towards a fully mature phenotype (Fig. 5B). At this stage, some cells negative for the cardiac lineage markers, were found to express HLA-DR.

Mitogenic T lymphocyte response is inhibited by addition of ADSC

The proliferative response, assessed by thymidine incorporation, of lymphocytes from controls to the T cell mitogen PHA was inhibited by 75.5% [51.0–81.9] upon addition of ADSC. Proliferation was also assessed using CFSE staining with similar results [69.6% (62.4–83.9) inhibition]. Significantly reduced numbers of mitotic events were found from the 4^{th} ($P = 0.005$) and 5^{th} ($P = 0.009$) division cycle, compared to PHA-challenged lymphocytes in absence of ADSC.

Further analysis showed that both $CD4^+$ and $CD8^+$ were inhibited in a dose-dependent fashion.

Although lymphocytes from patients with CAD displayed an immune response to allogenic lymphocytes equivalent to that of

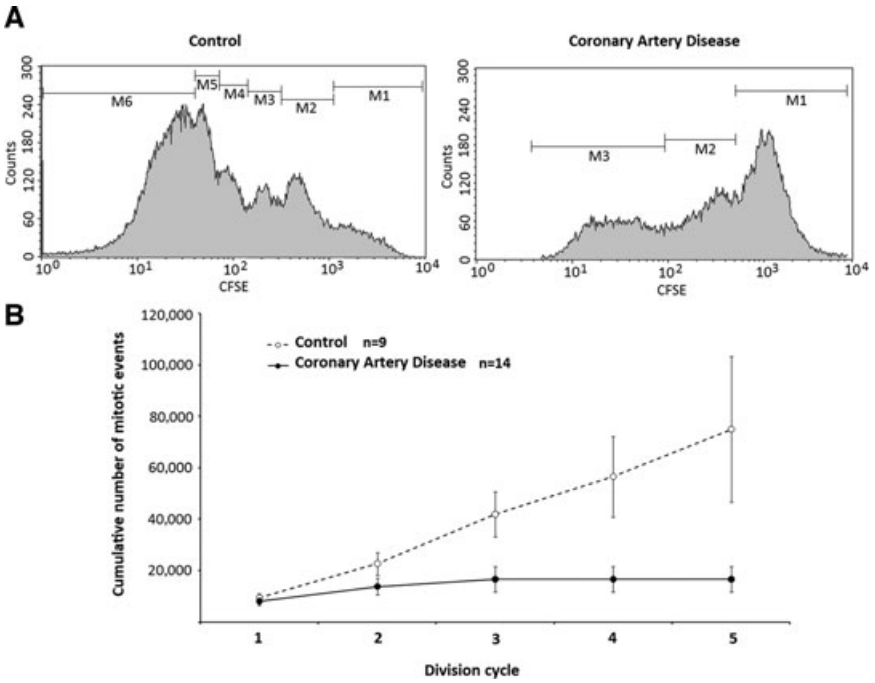

Fig. 6 Patients with coronary artery disease showed decrease lymphocyte proliferation to mitogens compared to controls. Proliferation of CFSE-labelled T cell from controls (left panel) or patients (right panel) stimulated for 72 hrs with mitogen PHA was assessed. (**A**) Histogram shows the parental generation (M1) and each division in a population of proliferating T cells of controls or patients with coronary artery disease (M2–M6). (**B**) Graphic representation of cumulative number of mitotic events in each cycle division for 9 healthy controls and 14 CAD patients. Patients with coronary artery disease showed a significantly decreased proliferative response to mitogen compared with controls.

controls, they showed a significantly reduced ($P = 0.001$) response to PHA as assessed by CFSE staining, 15,591 (1591–47,582) mitotic events in patients *versus* 43,825 (10,420–188,636) in healthy controls ($n = 9$; Fig. 6). Lymphocytes from controls underwent five division cycles over the 72 hrs of culture as compared to patients' lymphocytes that only achieved two division cycles.

Discussion

Here we report three main findings. Firstly, early cardiac progenitors feature the capacity to induce allogeneic lymphocyte activation, as demonstrated by the up-regulation of CD69, an early T cell activation marker, suggesting the recognition of alloantigens present on the cardiac progenitors.

These results validate previously reported *in vivo* findings showing that in immunocompetent rodents and non-human primates, the survival of ESC-derived cardiomyocytes implanted into myocardial infarction sites is limited in absence of immunosuppressants [4–6, 11, 17]. The mechanism by which these cells become immunogenic *in vivo* is unclear. Reports indicate that at the time of implantation ESC express very low levels of surface MHC I molecules but following implantation they differentiate and up-modulate MHC expression thus triggering effective immune responses [1–3, 5, 7]. Furthermore, it is possible that in an inflammatory environment, such as that resulting from myocardial infarction and transplantation-associated tissue injury, cytokines may stimulate ESC-derived cardiac progenitors thus leading to an up-regulation of MHC alloantigens and co-stimulatory molecules. This fits with the increased MHC class I expression we observed at the surface of early cardiac progenitors as compared to $CD15^-$ cells which are assumed to have retained some pluripotency.

Secondly, our results confirm and extend previous reports [8] showing that ADSC possess mesenchymal stem cell-like immunomodulatory properties and significantly decrease $CD4^+$ and $CD8^+$ T cell responses to mitogen and alloantigens. There is compelling evidence suggesting that the mode of action of BM-MSCs depends on the T cell stimuli [18–19], and that immunomodulatory properties appear to be mediated by cell-released paracrine factors such as IL-10 [18, 20–21], although a role for cell-to-cell contact cannot be ruled out. Recent studies have also demonstrated that co-cultures of PBMC with MSC can induce the generation of regulatory $CD4^+$ or $CD8^+$ lymphocytes [22]. In our MLR experiments, the combination of ADSC with cardiac progenitors resulted in an allostimulation of lymphocytes, which did not differ from that seen in the presence of allogeneic lymphocytes with ADSC. However, these results do not allow to conclusively establish that ADSC truly mitigated the intrinsic immunogenicity of $CD15^+$ progenitors because (1) the viability to culture $CD15^+$ progenitors under feeder-free conditions prevented to assess their specific immune response, and (2) ADSC may not be the most suitable feeders for cardiac progenitors. This may have led after a 5-day culture to such a decrease in the number of viable progenitors that not enough of them were left to trigger a detectable immune reaction. At the opposite, it might be equally erroneous to conclude that ADSC are unable to modulate

the immune response to ESC-derived cardiac progenitors on the sole basis that addition of ADSC to progenitors failed to abrogate the CD69 up-regulation triggered by these progenitors. These apparent conflicting results can possibly be reconciliated if one considers, firstly, that lymphocyte activation is not synonymous to lymphocyte proliferation and, secondly, that the immune response manifest at an early time point is not necessarily predictive of events occurring later. Namely, the immunomodulatory effect of ADSC was assessed after only 24 hrs of culture, which could be not sufficient for ADSC to produce the soluble factors that are likely to induce T cell inhibition. This hypothesis is supported by the findings of Ramasamy *et al.*, showing that bone marrow mesenchymal stem cells are unable to prevent the priming of T cell up to 72 hrs [23].

Our immunophenotypic analysis of cardiac progenitors over time demonstrated that they expressed MHC class I molecules but not MHC class II after 7 days of culture in non-inflammatory conditions. This expression of MHC class I co-localized with that of cardiac-specific markers (sarcomeric actinin and toponin T) in mature cardiomyocytes collected following a 3-week period of cardiac differentiation. Put together, these data suggest that allogeneic $CD15^+$ cardiac progenitors may elicit a MHC I- and MHC II-mediated immune response as they further differentiate and that their transplantation (even in the presence of ADSC), without an immunosuppressive or immunomodulatory treatment would be futile, as immune rejection by the host would prevent their persistence for an sustained beneficial effect.

The third finding was that lymphocytes from patients with CAD exhibited significantly decreased lymphocyte responses as compared to control lymphocytes. This effect may be due to age [24] and factors linked to the disease and/or to associated medications, that is pharmacological effect of β-blockers [25] and/or statins [26–29]. Statins are now becoming recognized as powerful anti-inflammatory agents that reduce plasma markers of inflammation, T cell activation, macrophage and monocyte infiltration in vessels and expression of soluble CD40 ligand [27–29]; while β-blockers can decrease circulating levels of pro-inflammatory cytokines such as tumour necrosis factor-α [30], affecting T cell response.

This particular immune status of patients with CAD along with the immunomodulatory effect of ADSC which would be co-implanted with cardiac progenitors might allow decreasing the number and amount of immunosuppressive medications required for long-term cardiac progenitor cell allograft acceptance, thereby shifting the risk–benefit ratio of immunosuppression towards a more favourable direction. This view is supported by the previous findings that mesenchymal stem cells can act synergistically with immunosuppressive drugs to mitigate alloimmune responses [31].

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

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