Natural killer cell crosstalk with allogeneic human cardiac-derived stem/progenitor cells controls persistence

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Aims
Allogeneic human cardiac-derived stem/progenitor cells (hCPC) are promising candidates for cardiac repair. They interact with T cells, major effectors of the adaptive immune response, inducing 'paracrine' anti-inflammatory effects that could sustain tissue repair/regeneration. Natural killer (NK) cells are major effectors of the innate immune system that might influence the persistence of therapeutic stem/progenitor cells. Therefore, to get through successful clinical translation and anticipate allogeneic hCPC persistence, we defined their crosstalk with NK cells under steady state and inflammatory conditions.

Methods and results
By using an experimental model of allogeneic hCPC/NK cell interaction, we demonstrate that hCPC moderately trigger cytokine-activated, but not resting, NK cell killing that occurs through formation of lytic immunological synapse and NK cell natural cytotoxicity. Yet, inflammatory context substantially decreases their capacity to set cytokine-activated NK cell functions towards NK cell-cytotoxicity and protects hCPC from NK cell killing. Allogeneic hCPC also restrain NK cell-cytotoxicity against conventional targets and inflammatory cytokine secretion biasing the latter towards anti-inflammatory cytokines. Thus, hCPC are unprivileged targets for allogeneic NK cells and can restrain NK cell functions in allogeneic setting.

Conclusion
Collectively, our data suggest that allogeneic hCPC/innate NK cells crosstalk within injured inflamed myocardium would permit their retention and might contribute to attenuating inflammation and to preventing adverse cardiac remodelling.

Keywords
Human cardiac stem/progenitor cells • Myocardial infarction • Allogenicity • NK cells

1. Introduction
Cardiac stem/progenitor cells including human cardiac-derived progenitor cells (hCPC) are intensively investigated as therapeutics for heart failure (HF) post-myocardial infarction (MI).1–3 Pre-clinical studies with allogeneic cardiosphere-derived cells4 and cardiospheres5 showed the efficiency of these cells in improving ventricular function in post-MI HF settings, without eliciting deleterious immune reactions. This has made the use of these cells a pragmatic proposition for cardiac repair. Our recent immunological studies of hCPC further reinforced this notion.3

The c-kit-immunoselected hCPC are cardiac-derived progenitors with a mixed stem-cell phenotype.3 They express SSEA-1, SSEA-4, CD90, CD73, CD105, and CD166 stem/progenitor markers and low levels of c-Kit. They also express OCT4, SOX2, and NANOG pluripotency transcription factors and are negative for haematopoietic and endothelial markers. hCPC express the cardiac lineage commitment factors Nkx2.5, GATA-4, Islet-1, and MEF2C.5 Immunologically, they express Human Leukocyte Antigens (HLA) class I (HLA-I) molecules but negligible levels of HLA class II (HLA-II), are negative for co-stimulatory molecules CD40, CD80, CD86, but express PD-L1 (CD274). The presence of
hCPC within an inflammatory environment, without modifying their stem/progenitor phenotype changes their immunological profile notably upregulates expression of HLA molecules.\(^3\) These cells differentiate into the three principal cardiac lineages in vitro and promote cardiac repair/ regeneration in experimental rat models.\(^3\) In a predictable pre-transplantation in vitro assay for allogeneic grafts, hCPC trigger activation and expansion of regulatory T cells and promote a contact-dependent allogeneic-driven immunomodulation.\(^3\) Thus in regard of adaptive immune response, the inherent immune features of hCPC would promote their eventual clinical translation.

MI causes inflammation typified by recruitment and activation not only of the adaptive but also the innate immune cells.\(^6\) Allogeneic immune reactions were believed to be only an adaptive immune T cell-mediated event that determines the outcome of organ or cell transplantation. Yet, the complexity in reaching allogeneic transplant tolerance stressed the importance of Natural killer (NK) cells, major effectors of the innate immune system, in allogeneic transplantation.\(^7\) In animal models, steady-state NK cells are sufficient to kill allogeneic cells in the absence of adaptive immunity.\(^6\) In humans, NK cells are involved in antibody-mediated graft rejection\(^9\) but might also control leukaemia relapse and eliminate rejection of haematopoietic transplants.\(^10\) Therefore, besides T cell-mediated events, understanding the crosstalk between allogeneic cardiac-derived stem/progenitor cells and NK cells is important for rational therapies to HF post-MI.

NK cells use an array of activating and inhibitory receptors to sense their environment and respond. No single NK activating receptor dominates; instead synergistic signals from combinations of receptors are integrated to activate NK cell-cytotoxicity and -cytokine secretion.\(^11\) The NK cell inhibitory receptors are specific for HLA class I molecules and are necessary for the maintenance of self-tolerance.\(^11\) In allogeneic transplant models alloreactive NK cells are fully responsive and the net balance of signals emanating from their activating and inhibitory receptors would determine the outcome of their effector functions. In addition, a large panel of cytokines regulates NK cell effector functions. Inflammatory cytokines including IL15 and IL18 promote their cytotoxicity and cytokine secretion, whereas TGF\(\beta\) and IL10 down-regulate these effector functions.\(^12\)

Aiming at hCPC therapeutics, we investigated the interaction of allogeneic hCPC with NK cells. We found that hCPC are modestly susceptible for allogeneic NK cell-cytotoxicity and are significantly protected from this cytotoxicity within an inflammatory context. hCPC also down-regulate NK cell-cytotoxicity against conventional targets and bias their cytokine secretion function towards anti-inflammatory cytokine TNF\(\alpha\) IL10, instead of IFN\(\gamma\) and TNF\(\alpha\) inflammatory cytokines. These data suggest that the hCPC crosstalk with NK cells in allogeneic settings would permit hCPC retention within an injured heart and as such might contribute to their therapeutic efficiency.

2. **Methods**

Refer to Supplementary Material online for detailed experimental methods.

2.1 **Experimental model**

Clinical settings for cardiac repair would administrate human cardiac stem/progenitor cells (hCPC) in a post-MI inflammatory context. The in vivo micro-environment of stem cells is characterized by low oxygen concentration (1–7% depending on the tissue).\(^3\) Thus, all hCPC experiments were set up at low oxygen tension (3% \(O_2\)) under steady-state and inflammatory conditions as described previously.\(^3\) Inflammatory conditions were mimicked by maintaining hCPC in medium containing IFN\(\gamma\) (IFN\(\gamma\)-hCPC); a cytokine model of inflammation impacting immunogenicity without affecting the stem/progenitor properties.\(^3\) NK cells were isolated from peripheral blood mononuclear cells using immunomagnetic beads and stimulated with recombinant human cytokines in RPMI-10% foetal bovine serum medium. All cells were genotyped for HLA (see Supplementary material online, Table S1). The Ethical Committees of ‘Hospital 12 de Octubre’ and ‘Fundación Jimenez Díaz’, Madrid–Spain, and ‘Saint Louis Hospital’, Paris–France, have approved the project and the study was performed according to the Declaration of Helsinki.

2.2 **NK cell-cytotoxicity**

Specific lysis of CFSE-labelled target cells was evaluated by CFSE/7AAD staining and calculated according to: \(100 \times [(\text{observed lysis-spontaneous lysis})/(100-\text{spontaneous lysis})].\(^14\)

2.3 **NK cell-degranulation**

The expression of CD107a (see Supplementary material online, Table S2) was analysed on CD3\(^+\)CD56\(^+\) NK cells, cultured alone or in the presence of different target cells by flow cytometry.

2.4 **Conjugates detection**

CFSE-labelled NK cells were mixed with CMRA-labelled-hCPC or CMRA-labelled-IFN\(\gamma\)-hCPC and the number of CFSE/CMRA double positive events in CFSE-positive NK cells was determined by flow cytometry.

2.5 **Immunological synapse**

NK cells were incubated with hCPC or IFN\(\gamma\)-hCPC and stained with mouse anti-perforin (see Supplementary material online, Table S2) and phalloidin-Alexa568 (to detect actin). Immunological synapses were visualized by Axiovert-200 microscope. Synapses were considered polarized when the perforin granules and actin network of NK cells were oriented towards hCPC.

2.6 **NK and hCPC phenotyping**

Expression of NK cell activating and inhibitory receptors and their ligands on hCPC was analyzed by flow cytometry using specific antibodies (see Supplementary material online, Table S2), and is presented as percentage of positive cells and geometric mean of fluorescence intensity.

2.7 **Immunomodulation assays**

NK cells were cultured alone or in the presence of hCPC or IFN\(\gamma\)-hCPC then re-cultured with conventional targets and their cytotoxicity was determined. For effect of hCPC on NK cell proliferation, CFSE-labelled NK cells were stimulated with cytokines in the absence or presence of hCPC or IFN\(\gamma\)-hCPC, and their proliferation was estimated by the decrease of CFSE staining on CD3\(^+\)CD56\(^+\) NK cells.

2.8 **Cytokine assays**

The level of cytokines was measured in the supernatants of cultures by ELISA specific kits or premixed Multiplex kits.

2.9 **Statistical analyses**

Statistical analyses were performed using the GraphPad InStat3 software. Statistical significance (P-values) was calculated using One-Way Analysis of Variance (ANOVA)-Student Newman Keuls Multiple Comparisons Test. A P-value < 0.05 was considered statistically significant.
3 Results

3.1 hCPC display moderate susceptibility to allogeneic cytokine-activated NK cells

We investigated the susceptibility of hCPC to NK cell-mediated killing under steady-state and inflammatory conditions. hCPC and IFNγ-hCPC were co-cultured in allogeneic settings with freshly isolated or IL15-activated NK cells and their cytosis was measured. The prototype cell lines for NK cell-susceptibility and -resistance, HLA-I-negative NK-sensitive leukemic cell line K562 and highly HLA-I-positive NK-resistant B-cell lymphoma Raji cells were used as positive and negative control targets, respectively. Allogeneic freshly isolated NK cells neither killed hCPC nor IFNγ-hCPC nor Raji cells but did kill K562 cells (Figure 1A, left panel). However, when hCPC were cultured with IL15-activated NK cells they were killed although to lesser extent than conventional NK cell target (P < 0.001). Specific lysis of hCPC was almost 50% compared with 95% for K562 cells (Figure 1A, right panel). The IL15-activated NK cell killing towards IFNγ-hCPC was decreased by 50% compared with hCPC (Figure 1A, right panel) (P < 0.001). Blocking TRAIL and/or FasL in co-cultures of hCPC with activated-NK cells did not reduce their cytotoxicity towards hCPC and had negligible effect on the cytotoxicity against IFNγ-hCPC (data not shown). This signifies that lysis of hCPC is independent of the death receptors pathway and it probably occurs through NK cell-based natural cytotoxicity. This pathway is reflected by the membrane expression of NK degranulation marker CD107a on effector NK cells. In our settings, we found that 4.5% of freshly isolated NK cells and nearly 40% of IL15-activated NK express CD107a upon their recognition of hCPC. Only 2.5% of freshly isolated NK cells and 19.5% of IL15-activated NK cells express CD107a upon recognition of IFNγ-hCPC (Figure 1B). Varying co-cultures time periods increased neither the freshly isolated nor IL15-activated NK cell-mediated hCPC nor IFNγ-hCPC killing (see Supplementary material online, Figure S1). Similar results were obtained with IL2- or IL12/IL18-activated NK cells (see Supplementary material online, Figure S2). Thus, although to a much lesser extent than conventional NK cell target, allogeneic NK cells when activated by inflammatory cytokines can kill hCPC yet IFNγ protects stem/progenitor cells from this killing.

3.2 hCPC are more potent than IFNγ-hCPC to set the NK cell-function towards killing

NK cell recognition of target cells and conjugate formation leads to lytic immunological synapse (IS) formation. At the IS, cortical filamentous actin remodelling and microtubule-organizing centre (MTOC) polarization allow perforin-containing lytic granules traffic to the plasma membrane. To provide insights into the mechanisms of NK cell-mediated killing of hCPC, we investigated IS formation upon interaction of hCPC with allogeneic NK cells. CFSE-labelled freshly isolated and IL15-activated allogeneic NK cells were co-cultured with CFSE-labelled hCPC or IFNγ-hCPC, and conjugate formation (double-positive events, see Supplementary material online, Figure S3) was evaluated as a primer indicator of IS formation. Nearly 20% of freshly isolated NK cells formed conjugates with both hCPC and IFNγ-hCPC. Up to 40% of IL15-activated NK cells were able to interact with hCPC but only 21% of activated NK cells interacted with IFNγ-hCPC (Figure 2A). Conjugates formation can result in either cytolytic (polarized synapse) or neutral (non-polarized) events. Therefore, we determined the percentage of polarized synapses for each interaction. Using fluorescent microscopy, we monitored F-actin remodelling (Figure 2B, left panel) or MTOC polarization (see Supplementary material online, Figure S4) and orientation of perforin containing granules. Nearly 50% of conjugates formed with hCPC and either resting or IL15-activated NK cells were polarized while only 30% of conjugates formed with IFNγ-hCPC were polarized (Figure 2B right panel). Thus, cytokine-activated NK cells are more prone to form IS with hCPC than freshly isolated NK cells (P < 0.001). However, both resting and activated NK cells have the same capacity to polarize their cytotoxic granules to the IS while they have lower capacity (P < 0.01) to form IS with IFNγ-hCPC.

Hence, hCPC have higher capacity than IFNγ-hCPC to set the function of allogeneic cytokine-activated NK cells towards killing. This suggests that inflammatory context although may well activate NK cells favouring their recognition and killing of hCPC, it would mutually provide protection.

3.3 HLA-I protects IFNγ-hCPC from NK cell-mediated killing

NK cell-based natural cytotoxicity is dependent on the net balance between activating and inhibitory signals they receive from target cells. The expression level of ligands for both activating and inhibitory receptors also manages NK cell responsiveness. Treatment of hCPC with IFNγ does not significantly alter the expression of NK cell-activating receptor ligands (see Supplementary material online, Figure S5), but increases HLA-I (HLA-A, B, C) and non-classical HLA-E molecules, which are ligands for killer-cell immunoglobulin-like receptors (KIRs) and the NKG2A inhibitory NK cell receptor, respectively (Figure 3A). The non-classical HLA-G, which is also a ligand for KIRs, was neither expressed by hCPC nor induced on IFNγ-hCPC (Figure 3A). Therefore, we investigated whether neutralization of HLA I or HLA-E would increase NK cell-specific lysis. Neutralizing HLA-I, but not HLA-E, with specific blocking antibodies increased IL15-activated NK cell-cytotoxicity (P < 0.01) against IFNγ-hCPC but did not affect hCPC specific lysis (Figure 3B). Thus, the increase of HLA-I expression by hCPC under inflammatory conditions is one mechanism responsible for shifting the balance of signals received by NK cells from hCPC towards inhibition.

3.4 hCPC impair NK cell-cytotoxic activity and inflammatory cytokine-induced proliferation

hCPC have the capacity to down-modulate inflammatory adaptive T cell response, we therefore investigated whether hCPC can modulate NK cell-cytotoxicity. IL15-activated NK cells were cultured with hCPC or IFNγ-hCPC then their degranulation was tested against conventional targets HLA-I-negative K562 and HLA-I-positive THP-1. Upon their interaction with K562 cells, IL15-activated NK cells primed with hCPC or IFNγ-hCPC showed lower cytolytic degranulation compared with unprimed cells (Figure 4A, upper panel). In average, 30 and 38% of IL15-activated NK cells primed with hCPC or IFNγ-hCPC, respectively, expressed CD107a vs. 70% for unprimed cells (P < 0.001) (Figure 4A, lower panel). The priming effect was contact-dependent since no difference in the degranulation was observed in Transwell settings (Figure 4A). IFNγ-hCPC were less potent than hCPC in down-regulating the lytic potential of IL15-activated NK cells (P < 0.01), which is in line with lower capacity to form functional IS with cytokine-activated NK cells compared with hCPC (Figure 2), IL15-activated NK cells primed with hCPC or IFNγ-hCPC compared with unprimed cells also displayed decreased cytotoxicity. hCPC- or IFNγ-hCPC-primed IL15-activated NK cells resulted in 35% cytotoxicity towards K562 cells compared with 85% for unprimed cells (see Supplementary material online,
In inflammatory environment, NK cell-proliferation can be induced by various inflammatory cytokines. We investigated whether the presence of hCPC or IFNγ-hCPC would affect cytokine-induced proliferation of NK cells. Freshly isolated NK cells were stimulated with IL-15 in the presence or absence of allogeneic hCPC or IFNγ-hCPC then their proliferation was evaluated. The presence of both hCPC
and IFNγ-hCPC inhibited the IL15-induced proliferation [by 30 and 42% (P < 0.01 and < 0.001), respectively] (Figure 4B). Although not reaching significance, IFNγ-hCPC were more prone than hCPC to modulate IL15-induced NK cell proliferation. The reduced proliferation of NK cells was not due to cell death since both hCPC and IFNγ-hCPC NK cells showed lower cell death than cells cultured in medium alone (see Supplementary material online, Figure S7A). hCPC and IFNγ-hCPC also decreased IL2- and IL12/IL18-induced allogeneic NK cells proliferation (see Supplementary material online, Figure S7B).

Thus, hCPC impair not only allogeneic NK cell-cytotoxicity but also their cytokine-induced proliferation, which is further strengthened by inflammatory conditions.

### 3.5 Interaction of allogeneic NK cells with hCPC down-modulates expression of their activating receptors

To provide insights into how hCPC can modulate NK cell activities, we investigated whether interaction of hCPC or IFNγ-hCPC with...
allogeneic NK cells could affect the surface expression of NK cell activating and/or inhibitory receptors.

Freshly isolated or IL15-activated NK cells were cultured alone or in the presence of hCPC or IFNγ-hCPC and their expression levels of activating (NKp30, 44, and 46, NKG2D, DNAM-1) and inhibitory (KIRs, NKG2A) receptors as well as the activation marker CD69 were determined. Both resting and activated NK cells expressed the activating and inhibitory receptors. IL15-activated NK cells showed increased expression of CD69 and NKG2D, DNAM-1, and NKp44 receptors. Only minor changes were observed for NKp30, NKp46, KIRs, and NKG2A receptors (see Supplementary material online, Figure S8A and B). When co-cultured with hCPC or IFNγ-hCPC in allogeneic settings, IL15-activated NK cells showed increased expression of CD69 and NKG2D, DNAM-1, and NKp44 receptors. Only minor changes were observed for NKp30, NKp46, KIRs, and NKG2A receptors (see Supplementary material online, Figure S8A and B). When co-cultured with hCPC or IFNγ-hCPC in allogeneic settings, IL15-activated NK cells showed increased expression of CD69 and NKG2D, DNAM-1, and NKp44 receptors. Only minor changes were observed for NKp30, NKp46, KIRs, and NKG2A receptors (see Supplementary material online, Figure S8A and B). When co-cultured with hCPC or IFNγ-hCPC in allogeneic settings.

Figure 3  HLA-I protects IFNγ-hCPC from NK cell-killing. (A) Representative expression of ligands for inhibitory NK cell receptors (KIRs and NKG2A) by hCPC (black histograms) or IFNγ-hCPC (red histograms) against respective isotypes (grey-filled histograms). Geometric means are indicated. (B) Specific lysis of CFSE-labelled hCPC and IFNγ-hCPC by IL15-activated NK cells at E:T ratio of 10:1 in the presence or absence of specific blocking antibodies for HLA-I or HLA-E or their respective isotypes. Specific lysis for all conditions were evaluated by staining with 7-AAD and results are presented as mean values ± SD of three independent experiments performed in triplicates. **P < 0.01 compared with isotype.

3.6 NK cells produce IL-10 rather than IFNγ or TNFα upon their interaction with hCPC or IFNγ-hCPC

NK cells produce a plethora of pro-inflammatory type-I (IFNγ, TNFα) and anti-inflammatory/regulatory type-II (IL4, IL5, IL13, IL10) cytokines. Therefore, we investigated the cytokines secreted by NK cells upon their interaction with hCPC.
**Figure 4** hCPC down-regulate NK cell-activities. (A) IL15-activated NK cells were primed with hCPC or IFNγ-hCPC at E:T ratio of 1:1 using direct contact or transwell, or cultured alone for 2 days. Cells were then harvested and cultured with K562 target at E:T ratio of 1:1. Representative dot plots show percentages of CD107a surface expression on CD3⁻CD56⁺ NK cells under different priming conditions (upper panel). CD107a surface expression on NK cells is also presented as mean values ± SD of three independent experiments performed in triplicates (lower panel). Similar results were obtained when priming was conducted for 4 days (data not shown). (B) Freshly isolated CFSE-labelled NK cells were cultured alone or in the presence of hCPC or IFNγ-hCPC at E:T ratio 4:1 and were simultaneously activated with IL15. Representative dot plots indicate the percentages of proliferating CD3⁻CD56⁺ NK cells that have decreased CFSE under each condition (upper panel). Results are also presented as mean values ± SD of three independent experiments performed in triplicates (lower panel). **p < 0.01, ***p < 0.001 compared with medium. P-values between hCPC and IFNγ-hCPC are indicated.
hCPC and IFNγ-hCPC increased IL15-activated NK cell secretion of IFNγ (P < 0.001) but did not significantly increase the production of TNFα (Figure 6A). The interaction of hCPC with allogeneic IL15-activated NK also led to the secretion of IL13 and IL10 (P < 0.05) (Figure 6B) but no effects were observed for IL4 or IL5 (data not shown). Compared with K562 cells, hCPC and IFNγ-hCPC triggered lower levels of type-I
pro-inflammatory cytokines while they induced higher amounts of type-II anti-inflammatory cytokines \( (P < 0.001) \). Interestingly, IFNγ-hCPC compared with hCPC triggered lower \( (P < 0.01) \) secretion of IFNγ and TNFα but higher \( (P < 0.05) \) secretion of IL10 by activated-NK cells. Again both IL10/TNFα and IL10/IFNγ ratios were higher in the presence of IFNγ-hCPC than hCPC \( (P < 0.001 \text{ and } P < 0.01, \text{ respectively}) \) (Figure 6C). The level of analysed cytokines was higher in supernatants from long-term hCPC or IFNγ-hCPC co-cultures, but again the levels of type-I cytokines were lower while those of type-II cytokines were much higher leading to higher ratios of IL10/IFNγ or IL10/TNFα when compared with K562 cells co-cultures or to IL15-activated NK cells cultured alone (see Supplementary material online, Figure S9). Of note, neither the hCPC nor the IFNγ-hCPC produced any of the tested cytokines (data not shown).

We next analysed whether priming of NK cells with hCPC and IFNγ-hCPC could modulate their cytokine secretion. When co-cultured...
with K562 target, IL15-activated NK cells primed with hCPC or IFNγ-hCPC secrete less IFNγ and TNFα compared with non-primed NK cells (\( P < 0.001 \) and \( P < 0.01 \), respectively) (Figure 6D). Again, IFNγ-hCPC were less potent in decreasing NK cell-cytokine secretion (\( P < 0.01 \)). Furthermore, priming of freshly isolated NK cells with hCPC or IFNγ-hCPC also decreased their capacity to secrete IFNγ and TNFα upon recognition of K562 (data not shown).

Together, these results indicate that NK cells interaction with hCPC not only bias their cytokine secretion towards anti-inflammatory/regulatory type-II cytokine, which may well be accentuated within an inflammatory context, but also down-modulates their capacity to secrete inflammatory cytokines.

4. Discussion

Allogeneic cardiac stem/progenitor cells are promising candidates for cardiac repair.\(^{1,2}\) Yet, to comfort the proof of concept, their eventual behaviour within clinical settings should be scrutinized. In the context of transplantation, whether organs, tissues, or haematopoietic stem cells transplantation (HSCT), various in vitro strategies have been developed over the years, and optimized in vivo assays have been validated as pre-transplantation tests to predict the immunological behaviour of transplanted allogeneic cells or tissues. Even if these assays could not fully predict their in vivo behaviour, they contributed great deal to the success of allogeneic transplantation and HSCT fields. Therefore, we undertook similar approaches to examine the interaction of allogeneic hCPC with innate immune NK cells. Using tailored pre-transplantation assays; we demonstrate that hCPC interaction with NK cells in allogeneic settings would favour their retention within injured heart rather than provoke their immediate elimination and thus contribute to their therapeutic efficiency by modulating inflammatory immune responses.

Although expressing various ligands for NK cell activating receptors, allogeneic hCPC are resistant to freshly isolated resting NK cell lysis and they trigger modest degranulation and cytotoxicity of inflammatory cytokine-activated NK cells, which express higher levels of activating receptors compared with resting cells. Lysis occurs when combination of activation signals overcomes inhibitory signals provided by ligands on target cells. The susceptibility of targets to NK cell-lysis is often related to their degree of HLA-I expression and in particular to the HLA-C1 and HLA-C2, two mutually exclusive HLA-C allotypes.\(^{17}\) Our panel of hCPC expresses substantial level of HLA-I and both HLA-C allotypes, except for one homozygote for C2. Thus, lack of hCPC susceptibility to resting NK-mediated lysis could be attributed to the expression of inhibitory KIRs ligands, which upon ligation to their receptors provide inhibitory signals and inhibit NK cell-cytotoxicity. Although we did not observe significant differences in the susceptibility to NK cell-lysis of homozygote C2/C2-hCPC, caution should be taken concerning HLA-C allotype of banked hCPC. Moreover, the modest susceptibility of hCPC to lysis by cytokine-activated NK cells implies that hCPC express sufficient levels of activating ligands to overcome inhibitory signals emanating from the ligation of HLA-I to inhibitory KIR.

In vivo, NK cells are exposed to combinations of cytokines that influence their maturation and effector functions.\(^{12}\) In allograft rejection models, IL15-activated NK cells are potent alloreactive cells that mediate acute rejection of skin allograft and allogeneic cells engraftment in the absence of any adaptive immune cells.\(^{6}\) Inflammation markers, including cytokine secretion and HLA-I and HLA-II molecule expression, are up-regulated in cardiac microvessels and cardiomyocytes biopsies from patients with cardiovascular disease.\(^{18}\) Increased expression of HLA-I in cardiomyocytes has also been reported for acute myocarditis, dilatative cardiomyopathy, and arteriosclerosis.\(^{17}\) Moreover, MI is associated with an inflammatory reaction characterized by early infiltration of immune cells and enhanced production of various cytokines including TNFα, and IFNγ.\(^{6,20}\) These cytokines are known regulators of HLA-I and HLA-II in vivo in patients suffering from acute or chronic inflammation but also in vitro in all nucleated cells including stem/progenitor cells.\(^{21-23}\) This denotes that the cytokine milieu present during MI inflammation is likely to influence NK cell responsiveness and hCPC susceptibility to lysis in situ.\(^{6,24}\) Treatment of hCPC with IFNγ, which is the most in vitro common model of inflammation, endorses their protection from NK cell-cytotoxicity. This was mainly due to increasing their HLA-I expression as ligand for inhibitory receptors rather than modulating the expression of activating NK receptor ligands. Increased HLA-I would probably counterbalance the higher expression of activating NK cell receptors within inflammatory environment. The direct in vivo evidence for enhanced HLA-I expression by hCPC post-MI or during heart inflammation is lacking. However, hCPC constitutively express HLA-I and respond to inflammatory context by upregulating both HLA-I and HLA-II without any pre-treatment with epigenetic agents.\(^{3}\) This indicates that the HLA loci in hCPC are probably unmethylated and it is highly likely that within MI inflammatory microenvironment in vivo they would upregulate these molecules. IFNγ-induced expression of HLA-I on embryonic stem cells (ESC)-derived progenitors is associated with attenuated host NK cell attack, enhanced graft survival, and further differentiation of transplanted cells.\(^{25}\) This suggests that upon their administration in vivo and despite the possible attack by cytokine-activated NK cells, a substantial proportion of hCPC would be protected by the inflammatory MI microenvironment, and would likely persist for a period of time sufficient to exert beneficial reparative activities.

NK cell killing of hCPC occurs essentially through natural cytotoxicity and the perforin/granzyme pathway, which is in line with the expression of activating NK cell receptor ligands by hCPC. The formation of polarized IS further confirms this pathway as a dominant mechanism in NK cell-cytotoxicity towards hCPC. IFNγ-hCPC formed less conjugates with NK cells, which resulted in fewer functional IS correlating with the inhibitory effect of HLA-I increase. This further confirms the protection of hCPC that is provided by the MI inflammatory microenvironment. NK cell-mediated killing of multipotent foetal mesenchymal stem cells involves TRAIL receptor pathway.\(^{25}\) Lysis of hCPC was independent of death receptors (Fas and TRAIL). This could be due to the inherent resistance of hCPC to TRAIL/Fas cell death and their modest sensitization to these pathways by IFNγ (data not shown). Multipotent cells are likely early precursors to hCPC, and TRAIL/FasL have developmental roles that are consistent with these differences. In this study, we did not identify activating NK cell receptor(s) that triggers perforin/granzyme pathway in order to kill hCPC and therefore, we do not rule out any other unidentified activators of cell death that might contribute to cytokine-activated NK cell-killing of hCPC.

We provide evidence illustrating the capacity of hCPC to exert inhibitory/modulatory retrocontrol of NK cell-function in allogeneic settings. Interaction of both resting and cytokine-activated NK cells with hCPC and IFNγ-hCPC down-regulates their cytotoxicity and pro-inflammatory cytokine production, but also their cytokine-induced proliferation. Down-regulation is documented by degranulation and cytotoxicity against both HLA I-negative and -positive targets expressing multiple ligands for different NK activating receptors and is thoroughly cell–cell contact dependent. NK cell-cytotoxicity and cytokine production are regulated by a series of surface receptors that transduce either
activating or inhibitory signals. The observed decrease in NK cell effector functions is likely related to the significant decrease in the expression level of several activating receptors that are involved in NK-activation and target cell-killing. Despite the fact that they express high level of inhibitory receptor ligands and have similar capacity to down-regulate activating receptors, IFNγ-hCPC were less effective than hCPC in decreasing NK cell functions. The lower capacity of IFNγ-hCPC to form conjugates with NK cells could probably give details to their effect in downmodulating NK cell functions. Yet, IFNγ-hCPC are more potent than hCPC in down-regulating cytokine-induced NK cell-proliferation indicating that mechanism(s) other than those involved in the down-regulation of NK cell functions control cytokine-induced NK cell proliferation. These mechanisms may include soluble factors derived from hCPC and/or produced upon recognition of hCPC by NK cells in allogeneic settings, and might be increased within an inflammatory microenvironment. Several soluble factors including Indoleamine 2,3-dioxygenase, prostaglandin E2, and soluble HLA-G have a role in various stem cell-provoked inhibition of cytokine-induced NK cells activities. Similar factors might operate in our model. Then again, HLA-I/KIR interactions influence human NK cell-proliferation and generate a population of ‘safe’ alloreactive NK cells, which proliferate less when stimulated with IL2.

Upon interaction with hCPC, activated NK cells produce moderate levels of pro-inflammatory IFNγ and TNFα but high level of anti-inflammatory IL10 cytokines. The level of IL10 production is higher when NK cells interact with IFNγ-hCPC, which may support an eventual role for this cytokine in down-regulating cytokine-induced NK cell-proliferation by hCPC. Upon interactions with hCPC, NK cells also produce IL13 at levels similar to those of NK cells that are in contact with conventional target, but they become higher after prolonged interactions. Even if we did not characterize the type of NK cells triggered by hCPC, this cytokinic profile is in line with generation of NK cells with regulatory functions. Such subset might also participate in the regulation of NK cell-proliferation and functions.

Figure 7 Model of hCPC interactions with adaptive and innate immune cells. Schematic representation of how allogeneic hCPC crosstalk with NK cells and adaptive (CD4+) immune effectors might contribute to hCPC longevity and cardiac tissue repair: NK cell expressing high levels of activating receptors (NKR) form IS with hCPC, secrete cytokines (IFNγ and TNFα), and cytolytic granules. But, inflammatory context increases MHC-I expression and protects hCPC from NK cell-lysis. The shift in NKR balance, the expression of inhibitory KIR (KIR-L), and secretion of IL10 might bias towards Treg-phenotype. Vis-versa CD4+ T cell polarization and IL10-producing Treg expansion might contribute to NK cell disarming. How increased MHC expression would further contribute to the tolerogenic phenotype remains an open question.
Acute MI represents a condition of acute inflammation that includes tissue damage and the presence of NK cell activity. Early studies in patients after MI showed that peripheral NK cells display defective cytotoxicity and studies in animal models indicated that NK cells are involved in cardiac repair following MI probably through direct and indirect mechanisms. Our results on NK cell interactions with hCPC enlighten these findings. hCPC induced a switch in NK cell cytotoxic/pro-inflammatory cytokines secretion towards a modulatory/anti-inflammatory cytokines secreting profile. Indeed, interaction of allogeneic hCPC with NK cells might eliminate some of them but also possibly select those with a greater potential for tissue repair. In support, animal studies suggest that host regulatory immune response and IL10 production could be beneficial in attenuating adverse remodelling and cardiac repair. Although the use of humanized immunodeficient mice would be of high value, today it is highly unlikely that such model would predict the in vivo situation in humans given the differences between species. Particularly in regard of the current study given the existence of substantial differences in NK cell receptors and pathways that regulate cell-activation between these two species. Moreover, the reconstitution of NK and B cells compartments is only partial in humanized mouse models, which further limits their utilities. Nevertheless, given that we used a tailored predictable in vitro pre-transplantation assay for clinical translation, it is conceivable to suggest that administration of allogeneic hCPC to injured inflamed myocardium and their interaction with NK cells might contribute to cardiac repair. Collectively, and despite the still lacking in vivo studies, interactions of hCPC with the adaptive and innate immune systems, presented in this work, propose a model for their eventual behaviour upon administration to injured inflamed myocardium. We speculate that in vivo application of hCPC in an allogeneic immuno-competent recipient post-MI (typically 5–7 day after) would not provoke rapid elimination by either CD8+ T or NK cells. This would allow hCPC enough time to exert their beneficial reparative effects by stimulating endogenous stem/progenitor cells, paracrine effects, eventual trans-differentiation or a combination of all (Figure 7). Besides triggering and expanding regulatory anti-inflammatory rather than inflammatory immune cells, hCPC administration might engender a ‘crosstalk’ between various actors of the immune response, whether cellular or humoral, which would ultimately emphasize an allogeneic beneficial over detrimental effect reinforcing cardiac repair. The mechanisms of action of cardiac-derived stem/progenitor cells are partially understood. However, growing evidence indicates that improvement of cardiac function might involve cardiac self-repair as principal target of these heart-derived cells. Their contribution includes new myocytes formation following infarction, injury-repair through cell–cell contact, and paracrine effects. Our results concord with this notion and suggest incorporating the interactions of allogeneic hCPC with immune effectors among anti-inflammatory paracrine effects.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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