Macrophages mediate cardioprotective cellular postconditioning in acute myocardial infarction

Geoffrey de Couto, … , Moshe Arditi, Eduardo Marbán


Ischemic injury in the heart induces an inflammatory cascade that both repairs damage and exacerbates scar tissue formation. Cardiosphere-derived cells (CDCs) are a stem-like population that is derived ex vivo from cardiac biopsies; they confer both cardioprotection and regeneration in acute myocardial infarction (MI). While the regenerative effects of CDCs in chronic settings have been studied extensively, little is known about how CDCs confer the cardioprotective process known as cellular postconditioning. Here, we used an in vivo rat model of ischemia/reperfusion (IR) injury–induced MI and in vitro coculture assays to investigate how CDCs protect stressed cardiomyocytes. Compared with control animals, animals that received CDCs 20 minutes after IR had reduced infarct size when measured at 48 hours. CDCs modified the myocardial leukocyte population after ischemic injury. Specifically, introduction of CDCs reduced the number of CD68+ macrophages, and these CDCs secreted factors that polarized macrophages toward a distinctive cardioprotective phenotype that was not M1 or M2. Systemic depletion of macrophages with clodronate abolished CDC-mediated cardioprotection. Using both in vitro coculture assays and a rat model of adoptive transfer after IR, we determined that CDC-conditioned macrophages attenuated cardiomyocyte apoptosis and reduced infarct size, thereby recapitulating the beneficial effects of CDC therapy. Together, our data indicate that CDCs limit acute injury by polarizing an effector macrophage population within the heart.

Find the latest version:

http://jci.me/81321-pdf
Ischemic injury in the heart induces an inflammatory cascade that both repairs damage and exacerbates scar tissue formation. Cardiosphere-derived cells (CDCs) are a stem-like population that is derived ex vivo from cardiac biopsies; they confer both cardioprotection and regeneration in acute myocardial infarction (MI). While the regenerative effects of CDCs in chronic settings have been studied extensively, little is known about how CDCs confer the cardioprotective process known as cellular postconditioning. Here, we used an in vivo rat model of ischemia/reperfusion (IR) injury–induced MI and in vitro coculture assays to investigate how CDCs protect stressed cardiomyocytes. Compared with control animals, animals that received CDCs 20 minutes after IR had reduced infarct size when measured at 48 hours. CDCs modified the myocardial leukocyte population after ischemic injury. Specifically, introduction of CDCs reduced the number of CD68+ macrophages, and these CDCs secreted factors that polarized macrophages toward a distinctive cardioprotective phenotype that was not M1 or M2. Systemic depletion of macrophages with clodronate abolished CDC-mediated cardioprotection. Using both in vitro coculture assays and a rat model of adoptive transfer after IR, we determined that CDC-conditioned macrophages attenuated cardiomyocyte apoptosis and reduced infarct size, thereby recapitulating the beneficial effects of CDC therapy. Together, our data indicate that CDCs limit acute injury by polarizing an effector macrophage population within the heart.

Introduction

Cardiac ischemic injury recruits a heterogeneous mixture of both protective and cytotoxic cell types. This inflammatory cascade proceeds through a canonical series of events: first, an influx of neutrophils and macrophages clear necrotic debris after inflammation; later, extracellular matrix is deposited and growth factors are released; and finally, the inflammation is resolved, followed by maturation of the scar (1, 2). Thus, inflammation converts necrotic tissue into scar, but the abundance of cytotoxic cells recruited into the myocardium has the potential to exacerbate injury. Despite longstanding appreciation of the detrimental inflammatory consequences of ischemia and reperfusion (3–5), clinically useful interventions targeting this pathway are lacking.

Macrophages are an important, heterogeneous cell population that may be functionally traced to their site of origin (bone marrow [BM] vs. yolk sac) and spatial localization (tissue resident vs. peripheral, monocyte derived). Resident macrophages confer environmental homeostasis (6, 7) and maintain residency through local proliferation (8–10). However, following tissue injury, inflammatory monocytes are recruited to the site of injury, differentiate into macrophages, and proliferate to support repair (11–13). In myocardial infarction (MI) and ischemia/reperfusion (IR) injury, monocytes are recruited from BM and splenic reserves in a biphasic manner (14–16). The early Ly6C+ population, which is associated with the M1 proinflammatory macrophage phenotype, is recruited as a result of increased MCP-1/CCR2 chemokine/monocyte receptor interaction and elevated expression of endothelial adhesion molecules (17). Between days 4 and 7 after MI, a late Ly6C+ population, associated with the M2 “healing” phenotype, infiltrates the myocardium (18). Interestingly, targeted depletion of either population with clodronate liposomes leads to impaired infarct healing (14, 19, 20). Characterization of M1 and M2 subpopulations has been well-established (21–23), and macrophages can assume a multitude of activated states in response to microenvironmental cues (6, 24). In fact, within the adult heart at least 4 distinct resident macrophage subsets exist under steady-state conditions (25). Following MI, endogenous cardiac-derived chemotactic signals and danger-associated molecular patterns are released from the infarcted tissue, promoting the expansion of resident and monocyte-derived macrophages into distinct phenotypes.

Cardiosphere-derived cells (CDCs) (26) are a unique heart-derived cell type that confers functional and structural benefits, including reduction of infarct size, improvement of cardiac function, enhanced angiogenesis, and modulation of the inflammatory response after MI (27–31). Recently, in a porcine model, we have found that CDCs are able to confer acute cardioprotection when administered 30 minutes after IR (a phenomenon called “cellular postconditioning”; ref. 32). While this was the first demonstration that infarct size could be reduced by delayed therapy, the mechanism was not addressed. Here, we confirm in a rat model that administration of CDCs following a delay after IR reduces infarct mass and improves function. These effects are abolished by systemic macrophage depletion and reproduced...
angioplasty to reopen the occluded coronary artery. After flow has been reestablished, the use of adjunctive therapy can be considered without distraction. Adjunctive cell therapy would require thawing of an allogeneic, off-the-shelf product and preparation for administration, which could introduce a delay of up to 20 minutes. Therefore, in our rat model, we subjected rats to 45 minutes of ischemia, followed by 20 minutes of reperfusion. Cells (or vehicle) were then delivered to the coronary circulation (Figure 1A). To examine whether cell administration could by adoptive transfer of CDC-primed macrophages. Distinctive changes in macrophage gene expression and function underlie the cardioprotective effects of CDCs.

**Results**

*Allogeneic CDCs confer cardioprotection within 20 minutes of infarction after IR.* To investigate the role of CDCs in cellular postconditioning, we designed a protocol that would simulate clinical IR injury. As an urgent priority, patients with MI undergo prompt angioplasty to reopen the occluded coronary artery. After flow has been reestablished, the use of adjunctive therapy can be considered without distraction. Adjunctive cell therapy would require thawing of an allogeneic, off-the-shelf product and preparation for administration, which could introduce a delay of up to 20 minutes. Therefore, in our rat model, we subjected rats to 45 minutes of ischemia, followed by 20 minutes of reperfusion. Cells (or vehicle) were then delivered to the coronary circulation (Figure 1A). To examine whether cell administration could...
be delayed further, we compared the results to those from a “delayed infusion” group, in which CDCs were infused 2 hours after IR (Figure 1A). In general, to enable study of the cardioprotective effect in isolation, we quantified endpoints at 48 hours, well before the regenerative mechanisms of cardiomyocyte proliferation and activation of endogenous cardioblasts come into play (on a time scale of weeks; ref. 29, 33, 34).

CDC-treated animals exhibited preserved cardiac function (Figure 1B) and reduced infarct size (Figure 1, C and D), relative to those of animals treated with vehicle (PBS) control or delayed infusion. We measured area at risk (AAR) and found it to be consistent among animals and independent of the cardioprotective effect (Figure 1, E–G). While these benefits occurred during the acute reparative phase, the functional and structural benefits of CDCs persisted for at least 2 weeks (Figure 2). During this chronic repair phase, cardiac function did not deteriorate, as it did in controls, leading to preserved left ventricular (LV) systolic and diastolic dimensions (Figure 2B and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI81321DS1), less thinning of the LV anterior wall (Figure 2, C and D), attenuated fibrosis (Supplemental Figure 1, A and B), and reduced hypertrophy of surviving cardiomyocytes (Figure 2, E and F). Thus, CDCs acutely administered after MI reduce myocardial injury at 48 hours, leading to sustained functional and structural benefits.

Infusion of CDCs reduces cardiac stress, attenuating cardiomyocyte death and proinflammatory cytokine expression. The observed reduction in infarct mass may reflect, at least partially, a reduction in programmed cardiomyocyte death. To test this hypothesis, we probed cell death in the infarct, border, and normal zones at various times (Figure 3A) and observed a reduction in cleaved caspase-3 and RIP1 proteins within the infarct (Figure 3, B and C).

**Figure 2.** The acute cardioprotective effect of CDCs is sustained for at least 2 weeks following IR. (A) Schematic of infusion protocol. Rats underwent 45 minutes of ischemia, followed by 20 minutes of reperfusion before infusion of CDCs or PBS control. Animals were followed for 2 weeks. (B) Representative echocardiography long-axis images of the LV cavity during diastole and systole from PBS- and CDC-treated animals. Scale bar: 2 mm. (D) Pooled data from Masson’s trichrome–stained hearts in C reveal less infarct thinning in CDC-treated animals (n = 4–5 rats per group). (E) Immunohistochemical staining of cardiomyocytes in the contralateral infarct zone. Cell size was determined from cardiomyocytes (α-actinin plus wheat-germ agglutinin [WGA]) with centrally localized nuclei (DAPI). Scale bar: 50 μm. (F) Pooled data from analyses in E depicting a reduction in cardiomyocyte size in CDC-treated animals (n = 5–7 rats per group). Graphs depict mean ± SEM. Statistical significance was determined using Student’s t test and 2-way ANOVA followed by Bonferroni’s multiple comparisons test. *P < 0.05.
CDC-treated hearts showed a reduction in TUNEL+ cardiomyocytes within the infarct region (Figure 3D), which was most dramatic 2 and 6 hours after IR (Figure 3, D and E). No change in vessel density was observed 48 hours after IR (Supplemental Figure 1, C and D). Cytokine protein arrays revealed elevated protein expression of MMP8, which has been associated with wound healing (35) and macrophage inactivation (36), and CXCL7, which is inductibly expressed in monocytes in response to stromal stimulation (ref. 37 and Figure 3F). These were the first hints that macrophages might be involved in the cardioprotective effect of CDCs.

**CDCs reduce the number of CD68+ macrophages within the ischemic heart.** To test the idea that CDCs modulate inflammation following IR, we examined the leukocyte profile of peripheral blood and cardiac tissue (Figure 4A). Delivery of CDCs to the heart altered neither circulating leukocytes (Supplemental Table 2) nor serum expression of proinflammatory MCP-1 or IL-4 (Figure 4E). It did, however, reduce specific leukocyte populations within the heart, notably CD45+CD68+ macrophages (Figure 4B) and CD45+CD11b+CD11c+ dendritic cells (Supplemental Table 3); both are members of the mononuclear phagocyte system. Interestingly, granulocytes (CD45+Gran+), another acute infiltrating inflammatory cell type (2), were unaltered (Figure 4B). These data were validated using immunohistochemistry to detect CD68 within the infarct region of hearts isolated 2, 6, and 48 hours following IR (Figure 4, C and D). While the CD68+ cell number was similar in hearts treated with PBS or CDCs at 2 and 6 hours after IR, the number of CD68+ cells (macrophages) was reduced at 48 hours in the hearts of CDC-treated animals (Figure 4, C and D). Furthermore, we did not observe changes in circulating levels of MCP-1 (CCL2) or IL-4 (Figure 4E), which might have accounted for the altered macrophage influx between treatment groups.

**Systemic depletion of macrophages using clodronate reduces the efficacy of CDC therapy.** Since we observed a decrease in macrophage count within the infarcted myocardium after CDC delivery, we tested whether systemic depletion of macrophages would recapitulate the benefits of CDC therapy. To do so, we adminis-
CDCs shift the cardiac CD68+ macrophage population away from an M1 phenotype in vivo. It is well recognized that macrophages exhibit the capacity to polarize between M1 and M2 phenotypes (6, 24). The M1 population is generally defined by its early infiltration into the myocardium and proinflammatory cytokine expression (e.g., \(\text{Nos2}\), \(\text{Tnf}\), \(\text{Il1b}\), and \(\text{Il6}\)), while the M2 population is associated with resolution of late-phase inflammation and promotion of tissue repair (e.g., \(\text{Arg1}\), \(\text{Il10}\), and \(\text{Pparg}\)).

We therefore asked whether CDCs polarize macrophages toward the M1 or M2 phenotype. To do so, we induced MI by permanently ligating the left anterior coronary artery and randomly allocated rats to receive \(2 \times 10^6\) CDCs or an equivalent volume of vehicle (PBS) through direct injection at 4 sites in the border zone immediately following infarction. Two days later, hearts were harvested and the infarct and surrounding border tissue were digested. The resulting cell suspension was separated using a density gradient to isolate the mononuclear cell fraction, and then cardiac macrophages were purified by attachment on cell culture dishes (Figure 6A). The >85% pure CD68+ populations were then analyzed by qRT-PCR for M1 and M2 gene expression (Figure 6B). Interestingly, M1 markers, \(\text{Nos2}\), \(\text{Tnf}\), and \(\text{Il1b}\), were significantly reduced, but there was no concomitant increase in M2 markers, such as \(\text{Arg1}\), \(\text{Il10}\), \(\text{Il4ra}\), or \(\text{Pparg}\).

Clodronate (dichloromethylene diphosphonate [Cl\(_2\)MDP]) liposomes 24 hours prior to and following IR, using the 20-minute delay protocol from PBS/CDC delivery (Figure 5A). As expected (38), clodronate reduced blood and spleen macrophage populations (Figure 5B). First, we assessed infarct size as an indicator of bioactivity. Clodronate itself did not aggravate IR injury, as we observed no significant difference in infarct mass between the group treated with PBS and the group treated with PBS and Cl\(_2\)MDP. However, clodronate attenuated the benefits of CDCs: infarct mass was greater in the group treated with CDCs and Cl\(_2\)MDP relative to that in the CDC-treated group (Figure 5, C and D). The functional changes mirrored those observed pathologically, in that no significant difference in ejection fraction was observed between the group treated with PBS and that treated with Cl\(_2\)MDP 2 days after IR (Figure 5E). Thus, we rejected the hypothesis that macrophage depletion would recapitulate the benefits of CDC therapy. Instead, the data indicate that macrophages are required for the cardioprotective effects of CDC therapy. Thus, we went on to investigate, in detail, potential effects of CDCs on macrophage phenotype and function. As the CD45+CD68+ macrophage population showed the greatest changes in tissue after MI (Figure 4B), we decided to focus on the role of this subpopulation in cardioprotection.
Thus, CDCs reduce the number of CD68+ macrophages in the infarcted myocardium and polarize macrophages away from the M1 phenotype but not toward a classical M2 state.

CDCs polarize thioglycollate-activated macrophages away from an M1 phenotype in vitro. To test whether CDCs have the capacity to modulate macrophage polarity indirectly, we devised an in vitro Transwell coculture protocol (Supplemental Figure 2A). With limited macrophage yield from cardiac tissue, we used readily available macrophages derived from the peritoneal cavity following thioglycollate stimulation (39). Although these peritoneal
macrophages are partially activated (40, 41), we sought to examine whether CDCs could shift their activation profile away from a proinflammatory state.

A process of peritoneal lavage, red blood cell lysis, and attachment to cell culture plates yielded a highly pure (>90%) CD68+ mononuclear cell population (Supplemental Figure 2B). Peritoneal macrophages were then preincubated with CDCs in a Transwell coculture system or PBS. After 6 hours of incubation, CDC-primed peritoneal macrophages exhibited reduced M1 gene expression (Il1, Il6, Nos2, and Tnf), without any significant changes in Arg1, Vegfa, or Tgfb1 (Supplemental Figure 2C). Although the gene expression profile was consistent with a reduction in cytotoxic, proinflammatory cytokines, we wanted to test the hypothesis that CDC-primed peritoneal macrophages confer cytoprotection upon distressed ventricular cardiomyocytes without direct cell contact. To do so, we exposed neonatal rat ventricular myocytes (NRVMs) to 50 μM H2O2 and then cocultured them in a Transwell system with CDC- or PBS-primed peritoneal macrophages to simulate an in vivo oxidative stress IR-relevant environment (Supplemental Figure 2A). After 6 hours, we observed reductions in phospho-JNK, phospho-p65, and cleaved caspase-3 activity in NRVMs that had been Transwell cocultured with CDC-primed peritoneal macrophages (Supplemental Figure 2, D and E). Gene expression profiling generally corroborated the protein data. Although not all NRVM genes were concordant, the directional change of a large proportion suggested a protective phenotype, including reduced expression of TLR signaling mediators (Traf6, Innk1, and Torf) and proinflammatory cytokines (Crt, Il23a, Il6, Nlrp3, and Tnf) (Supplemental Figure 2F).

CDCs polarize unstimulated BM-derived macrophages toward a phenotype distinct from that of either M1 or M2. Following ischemic insult, monocytes are actively recruited from both splenic and BM reserves and subsequently differentiate into macrophages at the site of injury (42, 43). To reproduce the in vivo recruitment of naive/unprimed monocytes to the site of injury in vitro, we isolated BM cells from femurs, cultured the cells with M-CSF, and then differentiated them into M1 (IFN-γ and LPS), M2 (IL-4 and IL-13), Mϕ (fibroblast Transwell), or CDC-Transwell macrophages (Figure 7A and Supplemental Figure 3A). To examine whether MDC macrophages were similar or distinct from M1, M2, or MFB macrophages, we compared the gene expression profiles for known M1 and M2 genes and general macrophage markers (Figure 7B and Supplemental Figure 3B). As expected, M1 macrophages had elevated Nos2 expression, while M2 macrophages had higher Arg1 and Pparg expression, relative to that of untreated macrophages. MDC macrophages, on the other hand, exhibited reduced Nos2 and Arg1 relative to both M1 and M2 macrophages, indicating that MDC macrophages are polarized to neither a true M1 state nor an M2 state. Of all the genes examined, MDC macrophages expressed the highest level of Il10, which encodes an antiinflammatory cytokine.

Two well-established markers for M1 and M2 macrophage polarity are Nos2 and Arg1, respectively (43). The divergent phenotypes involve a common metabolic pathway that converts l-arginine to either l-citrulline and nitric oxide (Nos2 catalysis) or l-ornithine and urea (Arg1 catalysis) (44). Therefore, we examined the relative ratio of Nos2 and Arg1 protein expression. As expected, M2 macrophages exhibited the largest Arg1/Nos2 ratio as well as Lysve-1 and p50 expression, whereas M1 macrophages had the lowest Arg1/Nos2 ratio as well as elevated phospho-p65 expression (Figure 7, C and D). Interestingly, MDC macrophages have several intermediate protein expression patterns, exhibiting an Arg1/Nos2 ratio between those of M1 and M2; slightly elevated Lysve-1 relative to untreated, low phospho-p65 (similar to M2); and low p50 expression (similar to M1) (Figure 7, C and D). Flow cytometric analyses of MDC macrophages revealed a reduction in cell size as well as distinct expression of surface markers CD68, CD80, CD86, CD11b, and CD45 relative to M1, M2, or unstimulated BM-derived macrophages (BMDMs) (Figure 8, A and B). To further characterize and distinguish the functional phenotype of MDC macrophages, we performed a fluorescent microscopy phagocytosis assay (45). Relative to M1 and M2 macrophages, MDC macrophages exhibited increased cellular uptake of fluorescent beads following overnight culture (89% vs. 74% [M1] or 61% [M2]) (Figure 8, C and D). Thus, MDC macrophages are functionally distinct from M1 and M2 populations, exhibiting higher phagocytic activity.

Together, these data demonstrate that MDC macrophages are distinct from either M1 or M2 macrophages. While clearly differ-
B). The enhanced phagocytosis further distinguishes MCDC macrophages from M1 or M2 macrophages.

MCDC macrophages reduce apoptosis in oxidatively stressed cardiomyocytes in vitro. The recruitment of macrophages to a site of injury results in the phagocytosis of cellular debris and expression of cytokines and chemokines. This process is critical for tissue repair and inflammation resolution.


dent from M1, they are also distinguishable from M2 sub-populations (M2a, M2b, and M2c) (46) based on their low gene expression of Arg1 (in contrast to that of M2a), surface expression of CD86 (in contrast to that of M2b), and gene expression of Tgfb1 (in contrast to that of M2c) (Figure 7, B and C, and Figure 8, A and B). The enhanced phagocytosis further distinguishes M_{CDC} macrophages from M1 or M2 macrophages.

M_{CDC} macrophages reduce apoptosis in oxidatively stressed cardiomyocytes in vitro. The recruitment of macrophages to a site of injury results in the phagocytosis of cellular debris and expression
but rather limit bystander cardiomyocyte apoptosis. We therefore tested whether MCDC macrophages confer cardioprotection in vivo. Adoptive transfer of MCDC macrophages but not M1 or M2 macrophages simulates CDC therapy after IR. We used adoptive transfer to examine whether MCDC macrophages could recapitulate the benefits of CDCs in vivo. To minimize confounding effects of endogenously recruited macrophages, we focused on the time window in which macrophage levels are low in the infarcted myocardium (<6 hours after IR) (Figure 4C). Therefore, we devised a delivery protocol similar to that described earlier in the study but infused vehicle (PBS) or polarized macrophages (M1, M2, or MCDC) rather than CDCs 20 minutes after IR (Figure 10A). All macrophages were labeled with DiI to trace the cells following delivery. After 48 hours of reperfusion, MCDC macrophage-treated animals had reduced infarct mass relative to PBS-, M1 macrophage-, and M2 macrophage-treated animals (Figure 10, B of an array of cytokines. As our in vitro gene expression analyses suggested that MCDC macrophages secrete a unique cytokine profile, we sought to examine whether MCDC macrophages are protective to stressed cardiomyocytes. In in vitro coculture, NRVMs were stressed with 50 μM H₂O₂ prior to the addition of DiO-labeled M1, M2, or MCDC macrophages (Figure 9A). Cells were examined for viability and number following 6 hours of coincubation. Interestingly, the addition of MCDC macrophages to H₂O₂-treated NRVMs significantly reduced TUNEL⁺ cardiomyocytes and preserved viable cardiomyocytes relative to M1 or M2 macrophages (Figure 9, B–D). At the end of the coculture period we observed differences in the remaining number of macrophages. We found a greater number of M1 macrophages relative to M2 or MCDC macrophages and increased macrophage TUNEL positivity in M2 macrophages relative to M1 or MCDC macrophages (Figure 9E). Thus, in an oxidative stress model, MCDC macrophages do not themselves undergo significant apoptosis but rather limit bystander cardiomyocyte apoptosis. We therefore tested whether MCDC macrophages confer cardioprotection in vivo.

Adaptive transfer of MCDC macrophages but not M1 or M2 macrophages simulates CDC therapy after IR. We used adoptive transfer to examine whether MCDC macrophages could recapitulate the benefits of CDCs in vivo. To minimize confounding effects of endogenously recruited macrophages, we focused on the time window in which macrophage levels are low in the infarcted myocardium (<6 hours after IR) (Figure 4C). Therefore, we devised a delivery protocol similar to that described earlier in the study but infused vehicle (PBS) or polarized macrophages (M1, M2, or MCDC) rather than CDCs 20 minutes after IR (Figure 10A). All macrophages were labeled with DiI to trace the cells following delivery. After 48 hours of reperfusion, MCDC macrophage-treated animals had reduced infarct mass relative to PBS-, M1 macrophage-, and M2 macrophage-treated animals (Figure 10, B...
attempts to limit infarct size by manipulating the inflammatory cascade have failed in translation (56). Nevertheless, the benefits seen here are consistent with the immunomodulatory properties recently described for CDCs in other experimental settings (57, 58). Exogenous cell therapy is under development as an adjunctive strategy to treat ischemic heart disease (59–61). The focus, however, has been on subacute or chronic stages of myocardial injury, at which time the opportunity for myocardial preservation has long passed. Here, we have demonstrated that infusion of CDCs into the coronary circulation 20 minutes after IR confers profound cardioprotection to the damaged myocardium (in contrast to the regenerative role of CDCs in established MI; refs. 27, 62–64). This effect is lost, however, if CDC infusion is delayed by 2 hours after IR. These findings are consistent with the observation that reperfusion injury increases over time and in parallel with microvascular obstruction (47, 65), which may physically restrict efficient transport of CDCs through the coronary circulation. Inflammation is a critical but poorly understood facet of IR injury (2). With evidence supporting an immunomodulatory role for CDCs in vitro (58) and in a chronic model of MI (28, 31), we hypothesized that CDCs would modify the local, innate immune response to confer cardioprotection following IR.

Discussion

The gold standard of therapy for acute MI is percutaneous intervention, with the aim of opening the occluded vessel as quickly as possible (47). Nevertheless, reperfusion itself confers some injury to the myocardium (48, 49). Several strategies have been used in efforts to reduce the detrimental effects of IR, including ischemic preconditioning (50), but pretreatment is required, limiting realistic utility in patients with MI. A more clinically tractable strategy includes ischemic postconditioning, whereby brief cycles of ischemia imposed during early reperfusion can reduce infarct size (51, 52); but, without immediate manipulation of flow at the time of reperfusion, benefit is lost (53, 54). The discovery of cellular postconditioning, whereby CDCs work in MI despite having been administered with some delay after reperfusion, is notable: no other cardioprotective modality successfully reduces infarct size without pretreatment and/or immediate intervention upon reopening the affected artery (55). The idea that cell therapy may mitigate ischemic injury by modulating macrophages is also conceptually novel but should be viewed with caution, given that prior attempts to limit infarct size by manipulating the inflammatory cascade have failed in translation (56). Nevertheless, the benefits seen here are consistent with the immunomodulatory properties recently described for CDCs in other experimental settings (57, 58).

Exogenous cell therapy is under development as an adjunctive strategy to treat ischemic heart disease (59–61). The focus, however, has been on subacute or chronic stages of myocardial injury, at which time the opportunity for myocardial preservation has long passed. Here, we have demonstrated that infusion of CDCs into the coronary circulation 20 minutes after IR confers profound cardioprotection to the damaged myocardium (in contrast to the regenerative role of CDCs in established MI; refs. 27, 62–64). This effect is lost, however, if CDC infusion is delayed by 2 hours after IR. These findings are consistent with the observation that reperfusion injury increases over time and in parallel with microvascular obstruction (47, 65), which may physically restrict efficient transport of CDCs through the coronary circulation. Inflammation is a critical but poorly understood facet of IR injury (2). With evidence supporting an immunomodulatory role for CDCs in vitro (58) and in a chronic model of MI (28, 31), we hypothesized that CDCs would modify the local, innate immune response to confer cardioprotection following IR.

and C). These macrophages were localized to the border zone and observed in high frequency (Figure 10D).
Macrophages are a populous and highly plastic immune cell source. During the acute phase of inflammation, these cells are either found endogenously within tissues (e.g., skin, brain, liver, and heart) as resident macrophages or recruited from peripheral BM or splenic reserves, as inflammatory macrophages (6, 42). Within the heart, at least 4 populations exist at steady state (25). During an inflammatory reaction such as ischemia, Ly6hi monocytes are rapidly recruited to the site of injury within the heart and support the replenishment of resident cell subpopulations (25). Here, we demonstrate in macrophages from three distinct sources (cardiac, peritoneal, and BM-derived) that CDCs specifically shift macrophages away from a proinflammatory (M1-like) phenotype. Given the low retention rate following coronary infusion (66, 67), we propose that CDCs secrete factors that foster a cardioprotective microenvironment with extensive crosstalk between resident and infiltrating cell types and direct antiapoptotic effects on distressed cardiomyocytes (32, 33, 62). Our data are consistent with the following hypothesis: cellular postconditioning initially works by improving cardiomyocyte viability and modifying resident macrophages, while sustained benefit is mediated by CDC-induced changes in the polarization state of infiltrating macrophages. Exosomes may mediate the CDC effects on macrophages (62, 68).

Figure 10. Adoptive transfer of M_{CDC} macrophages reduces infarct size when administered 20 minutes following reperfusion. (A) Schematic of infusion protocol. Rats underwent 45 minutes of ischemia, followed by 20 minutes of reperfusion prior to administration of PBS or Dil-labeled M1, M2, or M_{CDC} macrophages. Analyses were performed 48 hours after IR injury. (B) Representative images of TTC-stained hearts treated with PBS or M1, M2, or M_{CDC} macrophages. (C) Pooled data demonstrating percentage of infarct mass and LV viable mass, as assessed from TTC-stained hearts (n = 4–8 rats per group). (D) Representative image of the localization of Dil-labeled macrophages within the infarct border zone; no Dil-labeled macrophages were observed in the noninfarcted region. Scale bar: 50 μm. Graphs depict mean ± SEM. Statistical significance was determined using 1-way ANOVA followed by Tukey’s multiple comparisons test. *P < 0.05.
phages is consistent with such a conjecture, but definitive testing will require new lines of experimentation.

Tissue microenvironments have been well described and studied over the past several decades, most notably within the BM and tumors (70, 71). These distinct niches support not only normal stem cell function and therapeutic activation but also malignancy (72). Inflammatory cells, and most specifically macrophages that exist in close proximity to stromal cells and resident stem cells, are essential in maintaining the hematopoietic stem cell niche (73). It is likely that macrophages and stromal cells bidirectionally communicate to support repair in several different tissue microenvironments. For instance, macrophages are necessary for the formation of niches required for limb regeneration in salamanders (74), for skeletal muscle regeneration following toxin-mediated injury (75), and for cardiac regeneration in neonatal mice after MI (38). With a growing appreciation for macrophage heterogeneity, it will be important to delineate the factors governing the polarization (denoted by transcriptional regulation and expression of surface markers) of macrophages during ischemic injury (24, 25, 43). Since resident and inflammatory macrophages derive from distinct progenitor sources (yolk sac vs. BM) (7, 9, 25), it is likely that each population is intrinsically distinct but endowed with a capacity to serve functionally redundant roles, as demonstrated through the repopulation of resident cardiac macrophages with Ly6C<sup>hi</sup>-monocytes following ischemic injury (25).

We believe that our results provide novel insight into the mechanism of cellular therapy following ischemic injury. As a result of CDC-induced macrophage priming, macrophages express genes in a pattern that differs from either M1 or M2 (including M2a, M2b, and M2c) states (46). Specifically, we demonstrate not only the capacity to reverse the preactivated, thioglycollate-stimulated peritoneal macrophages away from a cytotoxic phenotype (simulating activated macrophages within the myocardium), but also the directed transition from a more naive state, as observed in our BMDM experiment (simulating recruited macrophages within the myocardium), toward a cytoprotective phenotype. Macrophages primed by CDCs exhibit a distinctive polarization state characterized by the expression of specific genes, cytokines, and membrane markers as well as elevated phagocytic capacity, which together confer cytoprotective properties. M<sub>CD<sup>+</sup></sub> macrophages infused after IR have the endogenous capacity to home to the ischemic border zone, where they reduce infarct size. While our data include endpoints at 2 weeks after IR injury, it would be worthwhile to characterize the long-term cardioprotective effects of CDC therapy to discern whether dysrhythmias and longer-term LV remodeling are affected.

The finding of a macrophage-mediated mechanism of cardioprotection highlights the protean effects of heterogeneous macrophage populations. By favoring one particular polarized macrophage state, CDCs confer therapeutic efficacy when administered after reperfusion, a setting previously believed to be refractory to medical intervention beyond the narrow time window of ischemic postconditioning. The present work defines a promising intervention, targeted at the inflammatory cascade, which can limit myocardial injury. We not only pinpoint macrophages as the key effectors of cellular postconditioning, but also find that macrophages themselves, when appropriately primed, can have therapeutic utility. The fact that functional and structural benefits can be recruited 20 minutes after reperfusion, a time when previous work would suggest that the cascade of death is set in stone (47, 76), is noteworthy.

**Methods**

**Experimental protocols, animals, and surgical procedures**

Seven- to ten-week-old female Wistar-Kyoto (WKY) rats (Charles River Labs) were used for all in vivo experimental protocols. To induce IR injury, rats were provided general anesthesia and then a thoracotomy was performed at the fourth intercostal space to expose the heart and left anterior descending coronary artery (LAD). A 7-0 silk suture was then used to ligate the LAD, which was subsequently removed after 45 minutes to allow for reperfusion. Twenty minutes (or two hours) later, cells (or PBS control) were injected into the LV cavity with an aortic cross-clamp, over a period of 20 seconds. To induce MI, the LAD was permanently ligated and cells (or PBS control) were injected into 4 regions within ischemic border zone.

For our macrophage depletion studies, WKY rats were intravenously injected with 1 ml (5 mg/ml) clodronate liposomes (Clodrosome, Encapsula NanoSciences) 1 day prior to and 1 day following IR injury.

**Rat CDC isolation**

Allogeneic CDCs were derived as previously described (26). Briefly, heart tissue from Sprague-Dawley rats (Charles River Labs) was isolated, minced, enzymatically digested, and then plated to allow cardiac explant cell growth. After 7 to 10 days, cells were harvested and plated into a nonadherent cell culture dish to support cardiosphere formation. After 2 days, cardiospheres were isolated and then plated on an adherent dish to allow CDC growth. Cells were subsequently expanded to passage 4 to 6 and used for all experimental work. Based on previously established in vivo dosing studies (67), we used 5 × 10<sup>6</sup> CDCs resuspended in 100 μl PBS (5% heparin, 1% nitroglycerin) for treatment after IR and 2 × 10<sup>6</sup> CDCs resuspended in 120 μl PBS after MI.

**Macrophage cell isolation and differentiation**

*Cardiac macrophages.* WKY rats underwent MI and then were randomly allocated to receive either PBS or CDCs, as described above. After 48 hours, hearts were harvested following perfusion with PBS. The infarct and infarct border zones were isolated, minced, enzymatically digested (Liberase Enzyme, Roche), and then filtered through a 70-μm mesh. Mononuclear cells were isolated using a density gradient (Histopaque 1083, Sigma-Aldrich), washed, resuspended in RPMI (supplemented with 1% FBS), and then plated. Following a 2-hour incubation at 37°C, 5% CO<sub>2</sub>, the attached cardiac macrophage cells were washed with PBS and then incubated with RPMI for downstream analyses.

*Peritoneal macrophages.* Brewer’s Thioglycollate solution (3% in PBS; Sigma-Aldrich) was injected into the peritoneal cavity of WKY rats. Three days later, macrophage cells were harvested following intraperitoneal lavage with PBS. Cells were filtered through a 70-μm mesh, lysed with ACK buffer (Invitrogen), and then resuspended and incubated with RPMI. Following a 2-hour incubation at 37°C, 5% CO<sub>2</sub>, the attached peritoneal macrophage cells were washed with PBS and then incubated with RPMI for further analyses.

*BMDMs.* Femurs were isolated from 7- to 10-week-old WKY rats. BM was isolated, flushed with PBS (containing 1% FBS, 2 mM EDTA),
filtered through a 70-μm mesh. The resulting cell suspension was used for 15 minutes. The buffy coat layer was isolated and resuspended in invasion, 1 × 10^6 BMDMs were labeled with DiI (Vybrant Cell-Labeling microsphere uptake in each group. nohistochemistry for quantitation and qualitative assessment of (Invitrogen) to examine all BM-derived cells and exclude dead cells, and then stained with CD45-PE-Cy7 (BD Biosciences) and DAPI. Only cardiomyocytes with centrally located nuclei were stained according to the manufacturer’s protocol (Sigma-Aldrich) and stained with Alexa Fluor 488–conjugated wheat-germ agglutinin (Invitrogen Life Technologies) stain in conjunction with DAPI. For analyses of cardiomyocyte size and size measurements were obtained from the midpapillary level of the infarcted heart. Infarct fibrosis measurements were obtained at the base, midpapillary, and apical regions of the infarct heart.

Immunohistochemistry. For analyses of cardiomyocyte size and inflammatory cell distribution, OCT-embedded tissue sections were fixed with 4% PFA and stained with the following primary antibodies for confocal microscopy: mouse anti-rat α-actinin (Sigma-Aldrich), mouse anti-rat CD68 (AbD Serotec), and mouse anti-rat-actinin (Sigma-Aldrich). For analyses of cardiomyocyte size and inflammatory cell distribution, OCT-embedded tissue sections were fixed with 4% PFA and stained with the following primary antibodies for confocal microscopy: mouse anti-rat α-actinin (Sigma-Aldrich), mouse anti-rat CD68 (AbD Serotec), and mouse anti-rat CD45 (BD Pharmingen). The appropriate fluorescently conjugated secondary antibodies (Invitrogen) were applied prior to mounting using Fluoroshield with DAPI (Sigma-Aldrich). To detect apoptotic cardiomyocytes, we performed a TdT UDP Nick-End Labeling Assay (TUNEL, Roche) according to the manufacturer’s protocol and stained with α-actinin and DAPI. To determine cardiomyocyte size, we used an Alexa Fluor 488–conjugated wheat-germ agglutinin (Invitrogen Life Technologies) stain in conjunction with α-actinin and DAPI. Only cardiomyocytes with centrally located nuclei were used for cell size determination.
**Immunocytochemistry.** Peritoneal and cardiac macrophage cells were cultured on fibronectin-coated slides, fixed with 4% PFA, and stained with mouse anti-rat CD68 (AbD Serotec). The appropriate fluorescently conjugated antibody was added, and then cells were counterstained with DAPI. For live cultured BMDM cells, Hoechst 33342 (Sigma-Aldrich 14533) was used to distinguish nucleated/multinucleated cells.

**Protein and RNA isolation**

**Protein.** At the appropriate time point following surgery, the heart was harvested and rinsed in PBS. The border, infarct, and normal zones were dissected; placed in Allprotect Tissue Reagent (QIAGEN); and stored at −80°C until use. Tissues were minced, suspended in T-PER (with HALT protease and phosphatase inhibitors, Thermo Scientific), and homogenized with a bead rotor. For cell culture experiments, cells were lysed with RIPA (with HALT protease and phosphatase inhibitors, Thermo Scientific), scraped off culture plates, and sonicated for 3 cycles of 10-second bursts (Active Motif) on ice. The resulting suspensions were centrifuged at 10,000 g for 15 minutes at 4°C, and the protein supernatant was collected. Protein concentrations were measured using a BCA assay (Thermo Scientific).

**RNA.** At the appropriate time points, cells were washed and collected for RNA isolation using an RNeasy Mini Kit (QIAGEN) according to the manufacturer’s protocol. RNA concentration and purity were determined using a NanoDrop Spectrophotometer (Thermo Scientific).

**Quantitative RT-PCR**

To compare the gene expression levels among cells at rest, following coculture or after stimulation, we used both SYBR Green and TaqMan technologies (Applied Biosystems).

**SYBR Green.** To assess gene expression, cDNA was synthesized from mRNA using a RT² First Strand Synthesis Kit (QIAGEN) according to the manufacturer’s protocol. The resulting cDNA was standardized across samples and loaded into the predesigned RT² Profiler PCR Array (QIAGEN) plates. Gene expression was then amplified over the course of 40 cycles and analyzed by ddCt.

**TaqMan.** To assess gene expression, cDNA was synthesized from mRNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol. The resulting cDNA was standardized across samples and then mixed with master mix and designated primer sets (Life Technologies, Invitrogen). Predesigned TaqMan primer sets for the following genes were purchased from Life Technologies: Arg1, Tnf, Nos2, Tgb1, Ila, Illb, Il6, Il10, Il4ra, Ccl3, Ccl5, Pparg, Nfkb1, Vegfa, Pod2, and Tlr9.

**Western blot analysis and protein array**

Protein samples were prepared for gel electrophoresis (NuPAGE 4%-12% Bis-Tris, Invitrogen) according to the manufacturer’s protocol. For all experiments, a normalized final loading concentration of between 10 and 30 μg per well was used prior to separation. Proteins were then transferred to a polyvinylidene fluoride membrane (Bio-Rad) for immunoblotting with designated antibodies. Bands were visualized following activation with ECL (Thermo Scientific) and exposure on film (Kodak Carestream Biomax, Sigma-Aldrich).

Rat cytokines were analyzed on a protein array (RayBiotech) according to the manufacturer’s protocol. Briefly, tissue lysates were incubated with the antibody array, membranes washed, and then a secondary biotinylated antibody was introduced. Incubation with streptavidin and subsequent exposure with a detection buffer allowed for visualization of dots on film (Kodak Carestream Biomax, Sigma-Aldrich).

**Cytokine bead array for serum cytokine analysis**

Serum levels of cytokines were analyzed with a FlowCytomix Multi-plex bead array (eBioscience) according to the manufacturer’s protocol. Briefly, blood was collected from rats after 48 hours after IR. Sera were then separated by centrifugation and incubated with antibody-coated beads (MCP-1, IFN-γ, IL-1α, IL-4, TNF-α). After the appropriate labeling, beads were resuspended with buffer then analyzed using a CyAn ADP (Beckman Coulter) flow cytometer.

**NRVM isolation and in vitro assay**

NRVMs were cultured as previously described (77). Briefly, hearts were harvested from 2-day-old Sprague-Dawley rats. Ventricles were isolated, minced, and then enzymatically digested in a solution of trypsin and collagenase overnight. Cells were then resuspended in m199 media (10% FBS, glucose, penicillin, vitamin B₁₂, HEPES, and MEM nonessential amino acids (Gibco)) and preplated to allow non-cardiomyocyte cells to attach. The resulting NRVM suspension was collected and counted prior to plating for experimental use.

To induce oxidative stress in NRVMs, 10 M H₂O₂ (Sigma-Aldrich) was diluted in IMDM (Gibco) to a final concentration of 50 μM. Cells were then incubated for 15 minutes at 37°C prior to media exchange. For in vitro NRVM-macrophage coculture, macrophages were dyed with DiO (Vybrant Cell-Labeling Solutions, Invitrogen) for 3 minutes at 37°C, washed with FACS buffer, resuspended in IMDM, and then added to the NRVM culture dish.

**Statistics**

Results are expressed as mean ± SEM. To determine differences between groups at a single time point, data were tested using either 2-tailed, unpaired, Student’s t test or 1-way ANOVA followed by Tukey’s multiple comparisons test. To determine differences between groups at multiple time points, data were tested using 2-way ANOVA followed by Sidak’s or Bonferroni’s multiple comparisons test. All analyses were performed using Prism 5 software (GraphPad), and only differences with a P value of less than 0.05 were considered statistically significant.

**Study approval**

The Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center (Los Angeles, California, USA) approved all animal care and experimental procedures prior to commencement of this study.

**Acknowledgments**

We would like to thank Patricia Lin and Carolina Castillo for assistance with flow cytometry and tissue sectioning, Hee Cheol Cho and Eugenio Cingolani for helpful discussions, P.K. Shah for comments on the manuscript, and Elizabeth Kim, Eli Simolo, and Ryan Middleton for technical assistance. This work was supported by the Board of Governors of the Cedars-Sinai Medical Center. General laboratory support was provided by grants to E. Marbán from the NIH and the Department of Defense.

Address correspondence to: Eduardo Marbán, 8700 Beverly Blvd., Los Angeles, California 90048, USA. Phone: 310.423.7557; E-mail: eduardo.marban@cshs.org.


42. Kin H, et al. Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. Car-


