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Original article

Mesenchymal stem cells stimulate protective genetic reprogramming of injured cardiac ventricular myocytes

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ABSTRACT

Since massive irreversible loss of cardiac myocytes occurs following myocardial injury, injection of human mesenchymal stem cells (hMSCs) has emerged as a promising therapeutic intervention. Despite the growing enthusiasm for this approach, the understanding of how hMSCs evoke cardiac improvement is ever more controversial. The present study critically tests hypothesis that hMSCs provide specific benefit directly to damaged ventricular myocytes. Cultures of neonatal mouse ventricular cardiac myocytes (nMCM) were subjected to two distinct acute stress protocols; incubations with either endotoxin, lipopolysaccharide (LPS) or toxic cytokine, IL-1β. Myocyte injury was assessed in intracellular Ca^{2+} signaling assays in fluo-3-loaded nMCMs that were imaged with high temporal resolution by fluorescent microscopy. Following LPS or IL-1β treatment there was profound myocyte injury, manifest by chaotic $[Ca^{2+}]_i$ handling, quantified as a 3- to 5-fold increase in spontaneous $[Ca^{2+}]_i$ transients. Antibody neutralization experiments reveal such damage is mediated in part by interleukin-18 and not by tumor necrosis factor-α (TNF-α). Importantly, normal $[Ca^{2+}]_i$ signaling was preserved when cardiomyocytes were co-cultured with hMSCs. Since normal $[Ca^{2+}]_i$ handling was maintained in transwell cultures, where nMCMs and hMSCs were separated by a permeable membrane, a protective paracrine signaling cascade is operable. hMSCs provoke a genetic reprogramming of cardiomyocytes. LPS provokes release of TNFα from nMCMs which is blocked by hMSCs grown in co- or transwell cultures. Consistent with cytokine release, flow cytometry analyses reveal that hMSCs also block the LPS- and IL-1β-dependent activation of cardiac transcription factor, NF-κB. Importantly, hMSC-conditioned medium restores normal Ca^{2+} signaling in LPS- and IL-1β-damaged nMCMs. These results reveal new evidence that hMSCs elicit protective and reparative effects on cardiac tissue through molecular reprogramming of the cardiac myocytes themselves. Thus these studies provide novel new insight into the cellular and molecular mechanisms that underlie the therapeutic benefit of hMSCs in the setting of heart failure. This article is part of a special issue entitled, "Cardiovascular Stem Cells Revisited".

1. Introduction

Acute myocardial infarction (AMI) and other damaging stimuli such as sepsis and inflammation lead to a massive and irreversible loss of cardiac myocytes. Since current therapies do not directly address this loss in cardiac cells, patients continue to experience multiple hospitalizations and premature death. In theory stem cells offer great promise in regenerative therapies to restore damaged cardiac tissue. Within the field of cardiac stem cell therapy, bone marrow-derived human mesenchymal stem cells (hMSCs) have emerged as a viable approach [1,2]. Cell and animal studies have prompted early clinical trials which reveal that perfusion of hMSCs can improve cardiac function and contractile performance in patients with AMI or chronic ischemia, although the benefits are minimal and variable [3,4]. Clearly there is a need to understand the cellular and molecular mechanisms of such interventions so that robust and reproducible therapeutic responses can be achieved.

The multipotent self-renewing bone-marrow-derived stem cells, termed mesenchymal stem cells, hold great promise in promoting cardiac tissue repair [5,6]. While stem cell transdifferentiation was an appealing early model for hMSC action, a collection of more recent studies reveal the beneficial effects of such cells is derived in part from their secretion of multiple soluble factors that seem to function in a paracrine fashion [7–12]. Such soluble factors may act through a reduction in infiltration of inflammatory neutrophils, inactivation of fibrogenic cells and scarring, stimulation of angiogenesis and

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vascularization, or recruitment and activation of resident cardiac stem cells. However these models may not sufficiently explain the mechanisms of hMSC action. For example, in doxorubicin-induced rabbit heart failure BM-derived stem cells limited the downregulation of cardiac β-adrenergic receptors normally seen while contractility was in fact increased [13]. This broadens the scope of the biology of MSC–cardiac myocyte interactions to include the notion of direct action of stem cells on damaged cardiac myocytes themselves.

The present study critically tests the hypothesis that hMSCs have direct protective and reparative effects on stressed cardiac myocytes. We have exploited cultured mouse myocytes exposed to established mediators of cardiac stress and inflammation, bacterial endotoxin, LPS, or proinflammatory cytokine, IL-1β [14–16]. This model system excludes confounding contributions from angiogenesis, recruitment of resident cardiac stem cells, or reduction in inflammatory cells. In this defined cellular setting experiments focused on the alterations in molecular and genetic properties of the cardiac myocytes. We report that hMSCs can protect cardiac myocytes from stress and can restore normal intracellular Ca^{2+} signaling to damaged myocytes. These direct beneficial effects are mediated though a paracrine signaling cascade that includes genetic reprogramming of the cardiac cells. This study provides new molecular insight into reparative pathways that underlie cardiac stem cell therapies.

2. Methods

2.1. Experimental materials

Human mesenchymal stem cells (hMSCs) were obtained from Lonza, Basel Switzerland. According to manufacturer cells are tested for purity by flow cytometry and are positive for CD105, CD166, CD29, and CD44 and test negative for CD14, CD34 and CD45. hMSCs were maintained in culture and used in experiments passage numbers 3 to 8. Two control cell lines were used; human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and human endothelial fibroblasts were obtained from ATCC (CRL-2522). Lipopolysaccharide (LPS) (Sigma-Aldrich, L2630) was added to a well at a concentration of 1 μg/ml and incubated for 3 h at 37 °C. Interleukin-1β (IL-1β) (Sigma-Aldrich, I5271) was added to cultures at a concentration of 100 ng/ml and incubated for 3 h at 37 °C. For transwell experiments, both LPS and IL-1β were added at the same concentration to both the upper and lower chambers. Anti-mouse TNF-α/TNFSF1A neutralizing antibody (R&D Systems, AB-410-NA), neutralizing anti-mouse IL-18 (MBL, D048-3), or control rat IgG (R&D Systems, MAB005) were added to cultures along with LPS at 1 μg/ml and incubated for 3 h at 37 °C.

2.2. Preparation of cultured neonatal mouse heart myocytes

Cardiac myocytes were isolated from hearts of 1-day-old CD1 mice by enzymatic digestion according to the methods described by Wright et al. [16] The isolated cells were resuspended in DMEM-F12, 10% FBS, 1% penicillin–streptomycin and irradiated with 3500 rads in a Gamma-cell 3000 Elan. The cells were plated on fibronectin-coated 25 mm glass cover slips (VWR, #16004-310) at a density of 3×10^5 cells per cover slip. After 24 h, the cultures were washed once with DMEM-F12 and changed to a maintenance medium consisting of DMEM-F12, 1% insulin–transferrin–selenium, 1% penicillin streptomycin.

2.3. Human mesenchymal stem cells (hMSC) and co-culturing

Fibronectin-coated culturing of hMSC (Lonza) was carried out according to Lonza protocols. Contact co-culturing of nMCMs and hMSCs was initiated on day-3 following myocyte plating. hMSCs were resuspended in maintenance medium and added directly to the myocyte cover slips at a plating density 7-fold less than that of the myocytes. A total volume of 2 ml was maintained. Transwell culturing was carried out in Costar (#3414) transwell permeable supports. The myocytes were initially plated on fibronectin-coated cover slips placed in the bottom of the 6-well plate. On day-3 in culture, inserts seeded with hMSCs at a density of 2-fold less than that of the myocytes, were placed in each well over the cover slips. Maintenance medium was used, maintaining a 2.5 ml volume in the lower chamber and a 1 ml volume in the insert.

2.4. Fibroblasts and co-culturing

Culturing of CRL-2522 fibroblasts was carried out according to ATCC protocols. Contact culturing of nMCM and fibroblasts was initiated on day-3 following myocyte plating. Fibroblasts were resuspended in maintenance medium and added directly to the myocyte cover slips at a plating density of 5-fold less than that of the myocytes. A total volume of 2 ml was maintained. Transwell culturing was carried out in Costar transwell permeable supports. The myocytes were initially plated on fibronectin coated cover slips placed in the bottom of the 6-well plate. On day-3 in culture, inserts plated with fibroblasts at a density of 5-fold less than that of the myocytes were placed in each well over the cover slips. Maintenance medium was used, with a 2.5 ml volume in the well and a 1 ml volume in the support insert.

2.5. Repair experiments

nMCMs were grown in culture for 3 days on fibronectin-coated cover slips. On day-3, LPS was added to the well and incubated overnight. On day 4, the maintenance medium was removed from the well and replaced with 2 ml of hMSC medium removed from an 80% to 90% confluent 75 cm^2 flask of hMSC. The original volume of hMSC medium in the flask was 15 ml. This was incubated for 3 h at 37 °C.

2.6. Measurements of $[Ca^{2+}]_i$ transients

The IonOptix detection system was used to measure $[Ca^{2+}]_i$ transients (F/F_0) of intact nMCMs. Cultured cells were washed with Ringer's potassium bicarbonate (KRB, 100 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM $MgsSO_4$–$7H_2O$, 1 mM Na_2HPO_4, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM Glucose, pH 7.35) and loaded with the fluorescent Ca^{2+} indicator fluo 3-AM (4–5 μM; Molecular Probes) for 20 min. Cells were transferred to KRB for 5 min before being placed in an experimental chamber with buffer. Field stimulation (80 V) was applied to the cells at 1 Hz though platinum wires mounted in the bottom of the chamber. All measurements were collected using a Nikon Eclipse (TS100) inverted microscope equipped with an IonOptix PMT for fluorescent detection. A selected region within a single neonatal ventricular myocyte was chosen for recording. Field stimulation protocol consisted of two sets of 10 electrical stimuli at 1 Hz with a 10 s rest interval in between the sets.

2.7. Data analysis

Unstimulated Ca^{2+} transients were counted during the rest interval as a metric of cardiac dysfunction. Only spontaneous peaks of at least 25% the fluorescence of stimulated transients (F/F_0) were included in results. Results represent the means ± SEM of repeated experiments from several independent culture preparations. Statistical analysis of the differences was assessed using one factor analysis of variance (ANOVA) with Newman–Keuls Test and the Student's *t*-test (2-tailed, separate variances) applying the Bonferroni Adjustment. $p<0.05$ was considered statistically significant.

2.8. nMCM cytokine isolation

Cultured cells were incubated in maintenance medium, maintenance medium with LPS (1 μg/ml), or maintenance medium with IL-1β (100 ng/ml) for 5 h. After incubation, the supernatants were removed

from each culture and flash frozen on dry ice. Samples were stored at −80 °C until cytokine concentration analysis was performed at the University of Maryland Medical School Cytokine Core Facility using ELISA techniques.

2.9. Flow cytometry based analysis of cell signaling

Purified nMCMs that were co-cultured with, or separated in culture by transwell inserts, with hMSCs and fibroblasts, were harvested 48 h after co-culture, 24 h after serum starvation. Cells were detached with 0.05% trypsin-EDTA (Invitrogen Corp., Carlsbad, CA) and counted. Flow cytometry based signaling was performed as we have previously described [17]. Briefly, cells were prepared for intracellular staining by fixation in 2% formaldehyde for 10 min at 37 °C, followed by permeabilization in 90% ice-cold methanol for 30 min. 0.5×10^6 aliquots of cells were washed twice in PBS + 1% BSA. Antibodies to phospho-NF-κB p65 (Ser536, Cell Signaling Technology, Danvers, MA) were aliquoted into each tube in 100 μl PBS + 1% BSA and incubated at room temperature for 30 min. Secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen Corp.) were used for the detection of this unconjugated primary antibody. Co-cultured cells were also stained with PE-conjugated CD44 antibody (BD Biosciences, San Jose, CA) to gate out hMSCs in co-culture experiments. Cells were washed twice in PBS + 1% BSA, and the samples were subsequently analyzed using a flow cytometer (LSR II, FACSDiva Software, BD Biosciences). Appropriate isotype controls were used as a control for nonspecific antibody binding. FlowJo software (Tree Star Inc., Ashland, OR) was used to analyze the raw FACS data and plot histograms. Data shown are representative of at least three independent experiments.

2.10. Immunostaining

Co-cultures of nMCM and hMSC were fixed with 2% paraformaldehyde for 10 min at room temperature. Cells were washed 4 × 15 min with the blocking/wash buffer (3% BSA, 1% Normal Goat Serum, 1 mg/ml Saponin). The primary antibodies (IL-18 polyclonal from BioVision and α-actinin monoclonal from Sigma) were added at a concentration of 1:50 and incubated overnight in the cold room. Cells were washed again with the above buffer 4 × 15 min. The secondary antibodies (Alexa-488 goat anti-mouse and Alexa 633 goat anti-rabbit—Invitrogen Corp.) were added at a concentration of 1:200 for 2 h at room temperature. Cells were washed a final time and mounted on slides using Slow Fade Light (Invitrogen Corp.). Fluorescence was measured on a Zeiss 510 Laser Scanning Confocal Microscope using the emission wavelengths 488 and 633.

3. Results

3.1. Co-cultures of cardiomyocytes and hMSCs

While it is clear that locally applied hMSCs provide benefit to cardiac myocytes in the setting of ischemia–reperfusion injury, it is uncertain which molecular signaling cascades underlie these effects. In order to assess the notion that such stem cells minimize the responses of cardiomyocytes to stress, a co-culture system was established in which hMSCs were seeded onto a layer of primary neonatal mouse ventricular myocytes (nMCM) in a ratio of 1:7 (hMSCs/nMCM). As shown in immunofluorescent confocal photomicrograph of live cells in Fig. 1A, low passage number hMSCs could be imaged with surface marker, CD44+, while nMCMs were seen as CD44− cells that had a strong $[Ca^{2+}]_i$ signal when loaded with Ca^{2+} indicator dye, Fluo-4 (in green). The merged image in Fig. 1A reveals that hMSCs grow in close proximity to the nMCMs. As a control for human cells, human fibroblast cells (CRL 2522), seen as CD44+, were co-cultured with nMCMs depicted in Fig. 1B. It is important to note that there were no CD44+ cells seen in cultures of nMCMs alone, revealing that mouse cardiac fibroblasts were not labeled by this marker (data not shown). For all of the co-culture experiments described below the cells were grown in ratio of hMSC/nMCM 1:7.

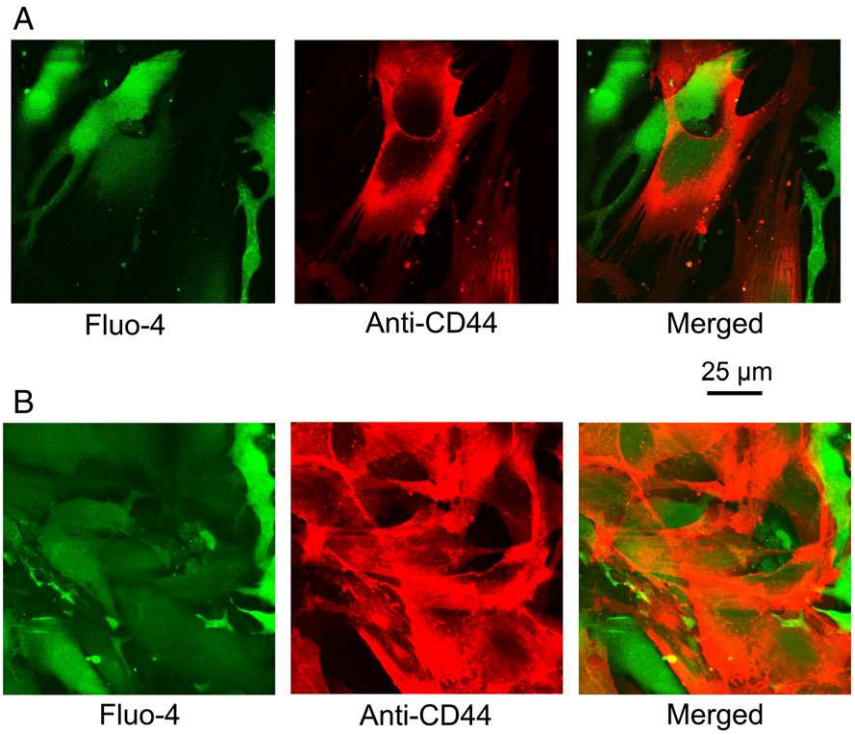

Fig. 1. Imaging of co-cultures of neonatal mouse cardiac myocyte (nMCMs) and human mesenchymal stem cells (hMSCs). nMCMs were isolated and cultured for 48 h as described in Methods at which time hMSCs were seeded into cultures at a density of 1:7. The co-cultures were incubated for 24 h at which time live cells were surface labeled with anti-CD44 and loaded with Ca^{2+} indicator dye, fluo-3AM. (A) A set of typical confocal images is shown with nMCMs imaged as $CD44^-$, Fluo-4 labeled cells in green. The hMSCs were $CD44^+$ cells shown in red. (B) Parallel co-cultures were prepared in identical manner except that after 48 h nMCMs cultures were seeded with human epithelial fibroblasts, CD 2533, which were imaged as $CD44^+$ cells in red.

3.2. hMSCs protect nMCMs from damage provoked by inflammatory agents

Since damage following myocardial infarction is associated with inflammatory signaling cascades several established proinflammatory agents, the bacterial endotoxin, lipopolysaccharide (LPS), and the cytokine, IL-1β, were exploited in stress-inducing protocols [14,15]. Stimulus-dependent $[Ca^{2+}]_i$ signaling was used as a sensitive measure of cardiac myocyte function in the mixed co-cultures as depicted in Fig. 2. Panel A shows nMCMs, cultured alone, responded normally to a train of electrical depolarizations with $[Ca^{2+}]_i$ transients seen only when depolarizations occurred as indicated by red arrows. When myocytes were incubated with LPS (3 h), or IL-β (3 h), chaotic $[Ca^{2+}]_i$ signaling was seen. This is evidenced by a failure of $[Ca^{2+}]_i$ transients to precisely follow the electrical pulse protocol followed by appearance of many spontaneous $[Ca^{2+}]_i$ transients between trains of pulses as indicated in tracing as "abnormal Ca^{2+} activity" (Fig. 2A). When nMCMs were co-cultured with hMSCs, Ca^{2+} signaling responses were quite different. Although depolarization-evoked Ca^{2+} signaling was normal in myocytes in the presence of these stem cells (Fig. 2B), a critical finding was that such myocytes were protected from the damaging effects of either LPS or IL-1β. Thus nMCMs behaved normally following exposure to either IL-1β or LPS under conditions identical to those in Fig. 2A. This dysfunction was quantified as the number of spontaneous $[Ca^{2+}]_i$ transients in the 10 s interval between the two trains of 10 stimuli (see Fig. 2). In this analysis, "abnormal" was assigned to any spontaneous $[Ca^{2+}]_i$ transient with peak F/F_o equal to or greater than 25% of peak F/F_omax as seen in normally stimulated $[Ca^{2+}]_i$ transients. As shown in Fig. 3A, LPS evoked a 2-fold increase in spontaneous $[Ca^{2+}]_i$ transients in nMCM monocultures. In contrast, in the hMSC co-cultures there was no increase in abnormal $[Ca^{2+}]_i$ signaling following LPS treatment. It is interesting to note that basal $[Ca^{2+}]_i$ transients were reduced by 50% in

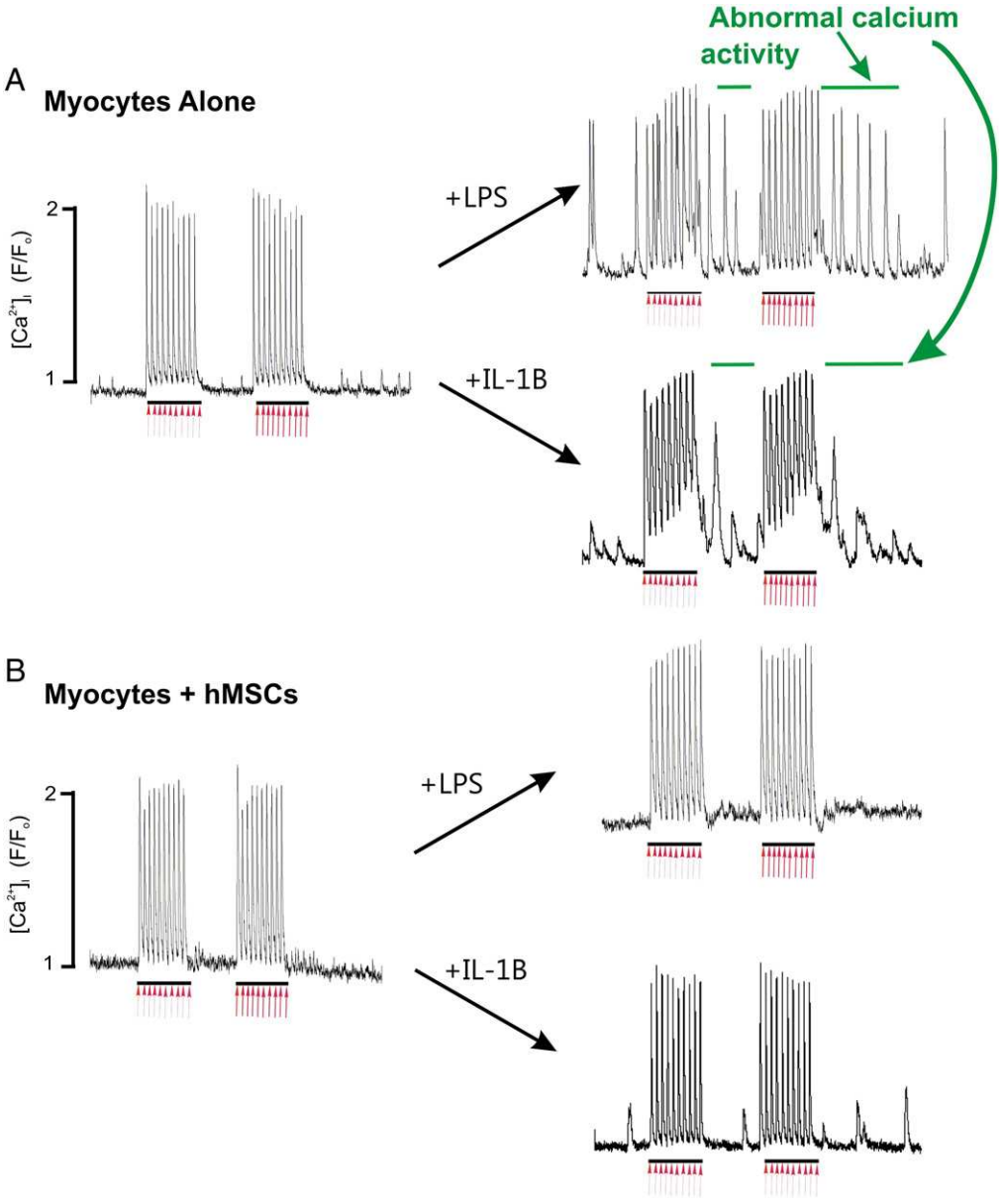

Fig. 2. Human mesenchymal stem cells protect neonatal myocytes from stress injury. The cardiac $[Ca^{2+}]_i$ transient was stimulated in Fluo-3 loaded cultured neonatal mouse ventricular myocytes by 40 to 80 V field stimulations (5 mm wide field) at 1 Hz and were imaged at high temporal resolution using a fluorescent microscope. The red arrows below the $[Ca^{2+}]_i$ transients indicate when the field stimulations occurred. A. Control nMCMs (no LPS, no hMSCs) responded to all electrical stimulations normally (with $[Ca^{2+}]_i$ transients and with contractions). When either LPS (1 mg/ml, 4 h) or IL-1b (100 ng/ml, 3 h) were added abnormal $[Ca^{2+}]_i$ transients (spontaneous activity) were seen as indicated by green bars. B. When nMCMs were grown in co-culture with hMSCs (see Fig. 1) the heart cells were protected from injury by treatment with LPS or IL-1β (right panel).

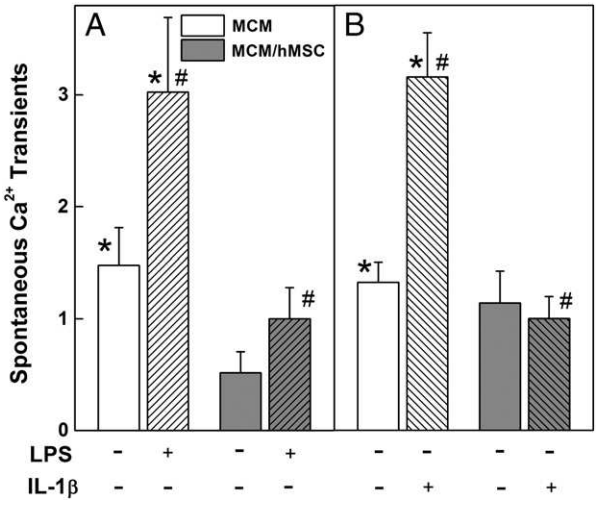

Fig. 3. hMSCs prevent spontaneous Ca^{2+} signaling evoked by stress stimuli. Summary data is presented from experiments depicted in Fig. 2. The numbers of spontaneous $[Ca^{2+}]_i$ transients were measured during the 10 s interval between the trains of 10 electrical stimuli. A. Shown are the results following incubation with LPS as in Fig. 2. The number of spontaneous $[Ca^{2+}]_i$ transients is shown for control nMCM cultures before (open bar) and following LPS (hatched bar). Parallel Ca^{2+} signaling experiments were conducted with myocytes co-cultured with hMSCs (nMCM/hMSC) for 24 h without and with LPS incubations as shown in gray bars. These results are the means ± SEM (n = 25–45 separate cells, $^*p = 0.04$, $\#p = 0.02$). B. Identical experiments were done on cultures except that cells were stimulated with IL-1β for 3 h as depicted in Fig. 2. Shown are the summary data of spontaneous $[Ca^{2+}]_i$ events in 10 s intervals in control nMCM cultures and nMCM/hMSC co-cultures as indicated. The bars are the means ±SEM (n = 25–45 cells, $^*p = 0.00008$, $\#p = 0.0018$).

these co-cultures compared to the nMCM monocultures (Fig. 3A). Fig. 3B shows that hMSCs also protect nMCMs from the chaotic $[Ca^{2+}]_i$ signaling evoked by IL-1β. In this case the 2.2-fold increase in spontaneous $[Ca^{2+}]_i$ transients is completely blocked when hMSCs are present in co-culture for 24 h prior to stimulation. It is important to note that functional experiments in co-cultures with control human cell lines, including CRL 2522 fibroblasts and human umbilical vein endothelial cells (HUVECS), could not be performed since these cells themselves provoked considerable spontaneous myocardial $[Ca^{2+}]_i$ signaling in the absence of stress reagents (data not shown). However such cells can be exploited as controls in transwell cultures as described below.

3.3. hMSCs alter pattern of myocardial cytokine release

It is well known that inflammatory agents, such as LPS, evoke the release of pro-inflammatory cytokines from cardiac myocytes, including tumor necrosis factor-α (TNF-α) [14,16]. Thus, it is possible that hMSCs protect cardiac myocytes through *inhibition of harmful cytokine release*. We exploited the cross-species nature of the co-cultures to measure cardiac-specific cytokine release though a panel of mouse-specific ELISAs. As shown in Fig. 4A LPS stimulates the release of TNF-α about 70-fold in monocultures of nMCMs. In contrast, this response is completely blocked in the hMSC co-cultures. As a control, myocytes were cultured with another human cell line, HUVECs. In that case there was still a 40-fold increase in LPS-evoked TNFα release into the culture medium (Fig. 4A, left panel). Potential IL-1β-evoked TNFα release was also analyzed. This cytokine failed to stimulate TNFα release from nMCM monocultures (Fig. 4A, right panel). Further there was no IL-1β-stimulated release in either hMSC or HUVEC co-cultures (Fig 4A right panel). Since both LPS and IL-1β increase NF-κB signaling in cardiac cells it may seem surprising that IL-1β failed to evoke TNFα release. See Discussion for rationale of these results.

The specificity of the hMSC impact on myocardial cytokine release was critically examined. As shown in Fig. 4B, both LPS and IL-1β stimulated the release of cardiac IL-6 in a nearly identical manner in control nMCM monocultures. Similar results were seen in nMCM/HUVEC co-cultures (Fig 4B, left and right panels). In contrast presence of cultured hMSCs provoked basal release of cardiac IL-6 without any further increase noted following stimulation with either LPS or IL-1β (Fig. 4B). These results were broadened through an analysis of a panel of cardiac (mouse) cytokines which revealed that the action of hMSCs on cytokine production was limited to TNFα and IL-6. Basal release of the beneficial cytokine, IL-10, was 9.6 ± 0.1 pg/ml and was not increased by LPS or IL-1β. Notably IL-10 release was maintained in hMSC/nMCM co-cultures. These analyses were extended to IL-2, IL-4, IL-10, IL-12, interferon-γ, and MCP-1. Mouse ELISA assays detected basal release of all of these agents in nMCM cultures, values unchanged under any experimental conditions, including *hMSCs* LPS or IL-1β (data not shown). These results reveal that hMSCs provoke a reprogramming the cardiac cytokine production in a specific manner. Further, these analyses suggest that TNFα is a likely mediator of damaging effects of LPS and IL-1β on cardiac $[Ca^{2+}]_i$ signaling.

3.4. Neutralizing anti-IL18 disrupts damaging action of LPS and IL-1β

It is possible that LPS disrupts cardiac $[Ca^{2+}]_i$ signaling though production and release of TNFα. This notion was based on observations that this proinflammatory cytokine is released from nMCMs, blocked by protective hMSCs (Fig. 4), and linked to cardiac damage and dysfunction in several model systems [18,19]. To test this hypothesis, 2 μg/ml neutralizing anti-TNFα was added along with LPS in incubations of cardiac myocyte monocultures. This is a concentration more than sufficient to neutralize as much as 250 pg/ml mouse TNFα (see Methods). Fig. 5A shows the unexpected result that the LPS response, a 5.4-fold increase in spontaneous $[Ca^{2+}]_i$ transients, was not inhibited in presence of anti-TNFα. We were motivated by observations from several groups that LPS-evoked cardiac damage in intact animal models may be linked to IL-18 [20,21]. Thus, a series of parallel experiments were designed to include neutralizing anti-IL-18 along with LPS for 3 h. As shown in Fig. 5B, when nMCMs were incubated with control non-immune rat IgG, LPS evoked the typical abnormal, spontaneous $[Ca^{2+}]_i$ signaling. In contrast when anti-IL-18 was included in LPS incubations, at a dose sufficient to neutralize 30,000 pg/ml of mouse IL-18, normal $[Ca^{2+}]_i$ signaling is preserved (Fig. 5B). This protection was not restricted to LPS. As shown in Fig. 5C, while control IgG afforded no protection of myocytes from the action of IL-1β, anti-IL-18 markedly abrogated the effects of this cytokine on myocardial Ca^{2+} signaling.

This unexpected finding that IL-18 rather than TNF-α mediates LPS-evoked myocyte dysfunction in these cultured cells motivated a series of additional IL-18 studies. Available ELISA assays were not sufficiently sensitive to measure any basal or LPS-evoked release of this cytokine. Thus, an immunofluorescent confocal imaging approach was used to determine if cardiac myocytes in culture express IL-18. As shown in the double-labeling fluorescent photomicrographs in Fig. 5D, nMCMs, labeled with anti-α-actinin (green, striated) harbor IL-18 as imaged with anti-IL-18 (red). The control incubations with secondary antibody alone are shown in Fig. 5E and confirm the specificity of the IL-18 and α-actinin immunolabeling. These results are consistent with the view that IL-18 is involved in dysfunction associated with LPS and IL-1β incubations.

3.5. hMSCs act though paracrine pathway

In all of the functional experiments above where cardiac protection is seen, the stem cells are co-mingled with the myocytes as depicted in Fig. 1. The close proximity of the cells suggests that stem cell–myocyte direct contact is critical in this protective process. In order to test this hypothesis, hMSCs were cultured in the upper chamber of a transwell culture dish which segregates the stem

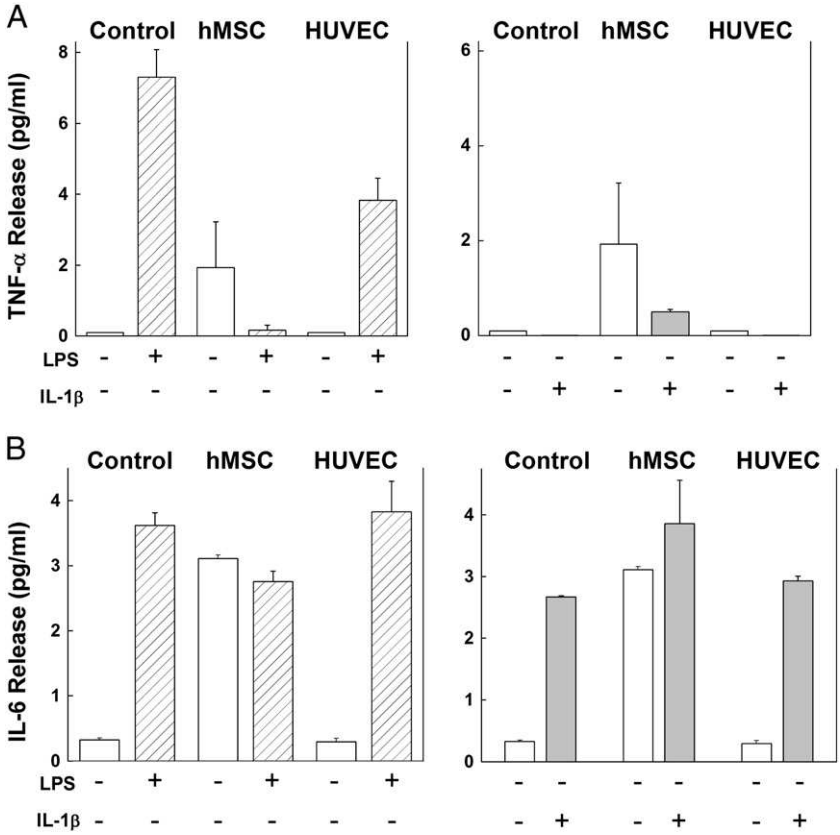

Fig. 4. Effects of hMSCs on myocardial cytokine production in response to stress evoked by LPS or IL-1β. nMCMs were cultured alone (Control) or co-cultured with hMSCs (1:7 ratio) (+MSC) or human umbilical vein endothelial cells (+HUVEC) for 24 h. The cultures were then subjected to stress though incubation with LPS (1 μg/ml) or IL-1β (100 ng/ml) for 5 h. Supernatants were collected and cytokine release quantified by mouse-specific ELISAs. (A). Levels of TNFα release under unstimulated conditions (white bars) or following stimulation by LPS (hatched bars) is shown in histogram at left. The histogram on the right shows summary data from a series of parallel experiments with IL-1β as stimulating agent. Shown are results of basal TNFα release (open bars) and release of cytokine following IL-1β stimulation (gray bars). (B) Levels of release of another cytokine, IL-6, in response to LPS is displayed. Summary data are shown for basal release (open bars) and levels following LPS stimulation (hatched bars). The panel at right displays results from a series of parallel IL-6 release experiments with IL-1β as stimulating agent. Shown are results of basal IL-6 release (open bars) and release of cytokine following IL-1β stimulation (gray bars). The data are the means ± SEM from three separate experiments ($n=3$).

cells from the myocytes in the lower chamber with a permeable membrane. As shown in Fig. 6A, LPS evoked the typical increase in spontaneous $[Ca^{2+}]_i$ transients when nMCMs were cultured alone. Importantly, this LPS response is completely blocked when hMSCs are cultured in the upper compartment for 24 h (middle bars). In transwell control experiments with human fibroblasts LPS-evoked cardiac damage is not attenuated (Fig. 6A). The hMSC protective effect was still evident when the proinflammatory cytokine, IL-1β was used as stressor agent. As shown in Fig. 6B, the 5-fold increase in spontaneous $[Ca^{2+}]_i$ transients seen with IL-1β was completely blocked when hMSCs were cultured in upper chamber. Although hMSCs express proteins promoting cell–cell adhesion and in fact form gap junctions with cardiac myocytes [22–24], these results demonstrate the stem cells act though a paracrine mechanism to protect cardiac myocytes from damage.

3.6. hMSCs abrogate activation of cardiac NF-κB

The marked shift in the pattern of cardiac-specific cytokine release evoked by hMSCs suggests these stem cells stimulate a beneficial reprogramming of myocardial gene expression. Since it is well known that both LPS and IL-1β activate the NF-κB transcription factor signaling cascade in cardiac myocytes, we focused on potential increases in phospho-p65 of this complex, an established marker for NF-κB activation *in situ* [15,25]. We used flow cytometry approach to quantify the specific phospho-p65 response in the cardiac myocytes isolated from co-cultures. This strategy was validated in our previous study where signals from the myocyte population can be gated from the CD44+ hMSCs [17]. As shown in Fig. 7A, there is a time-dependent increase in cardiac phospho-p65 when IL-1β is incubated over 30 min with nMCM cultures. Fig. 7B shows that the IL-1β evoked increase in phospho-p65 (30 min) in the left panel is completely blocked when hMSCs are grown in co-culture. In a set of parallel experiments LPS (30 min) also increases cardiac phospho-p65 as expected (Fig. 7C, left panel), a response completely blocked in the hMSC co-cultures (Fig. 7C, right panel). These results support the conclusion that hMSCs block a cardiac cell transcriptional cascade critical for inflammatory responses in heart [15].

If the inhibition of NF-κB signaling seen in Fig. 7 is linked to hMSC functional protection then the blockade of p65 phosphorylation should be seen in hMSCs grown in the transwell configuration. When the nMCMs were grown alone in the lower chamber, LPS evoked the expected increases in phospho-p65 (Fig. 8A). Fig. 8B shows that LPS-evoked p-65 phosphorylation is completely blocked in hMSC transwell cultures. These results further support the notion that hMSCs provide protection though a reprogramming of gene expression in myocardial cells.

3.7. hMSCs restore normal $[Ca^{2+}]_i$ signaling to damaged cardiomyocytes

All of the functional experiments above focus on the protective benefits of hMSCs. A series of experiments were designed to address hypothesis that hMSCs can repair damaged heart cells as well. We established two distinct and complementary stress protocols that would determine if rapid and more long-term hMSC-mediated repairs are possible. The timelines for these protocols are depicted in the

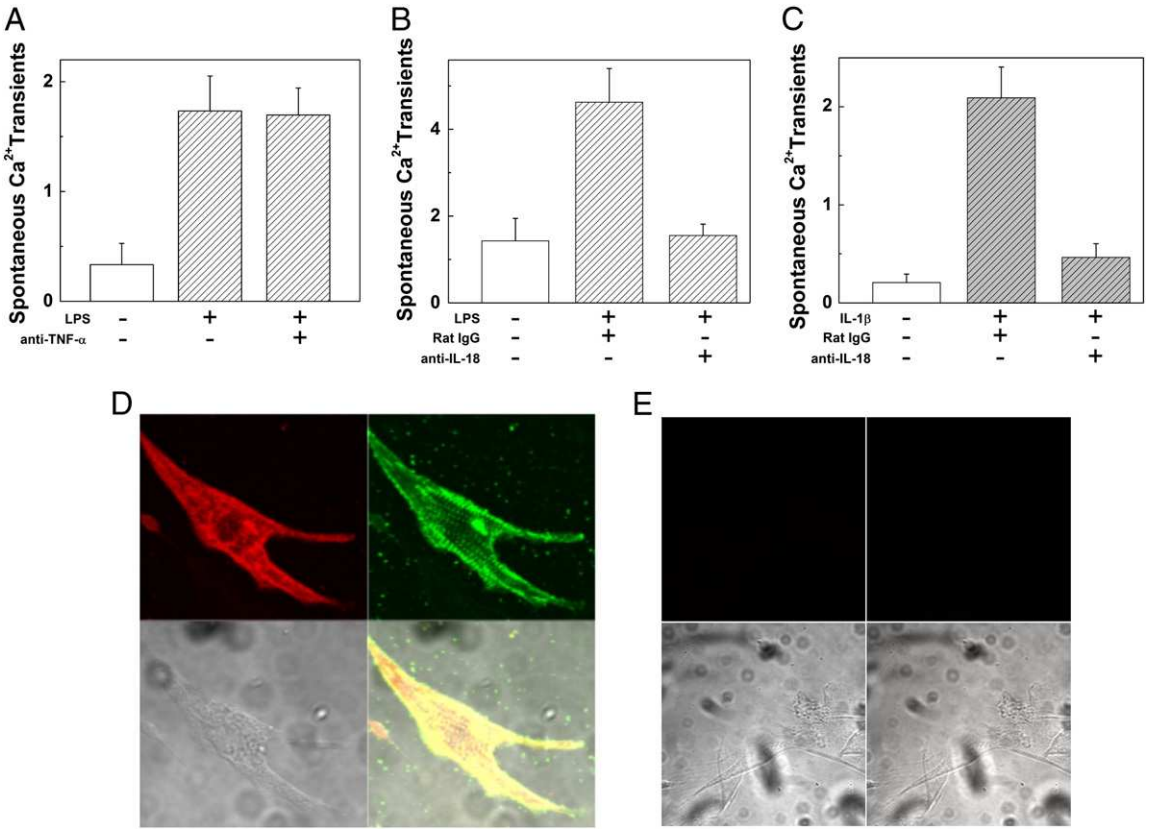

Fig. 5. The role of IL-18 in LPS- and IL-1β-evoked abnormal Ca^{2+} signaling in cultured nMCMs. nMCMs were isolated and cultured for 3 days as described in Methods. Cells were incubated with LPS or IL-1β for 3 h as described in Fig. 2. (A) LPS was added to cultures as indicated and spontaneous $[Ca^{2+}]_i$ transients were quantified at 3 h. In parallel experiments anti-TNFα (2 μg/ml) was added at the same time as LPS as indicated. (B) Cultures were incubated for 3 h with LPS along with either 1 μg/ml non-immune rat IgG or 1 μg/ml anti-IL-18 prior to spontaneous $[Ca^{2+}]_i$ transient measurements. Values are means ± SEM ($n = 136$) (C) Antibody experiments were identical to those in Panel B except that IL-1β was added to cultures instead of LPS as indicated. Values are the means ± SEM ($n = 33$). (D) Cultured nMCMs were fixed and labeled with anti-α-actinin (green) and anti-IL-18 (red) and imaged by confocal laser scanning immunofluorescent microscopy as described in Methods. Lower panels show transmitted light image and merged image of same field. (E) Control experiments were performed as in Panel D except fixed cells were labeled with secondary antibodies only and imaged at same intensity and contrast as used in Panel D. Upper panels show immunofluorescent images while lower panels show transmitted light photomicrograph.

scheme in Fig. 9A. In the first rapid action protocol ((1) in Fig. 9A) nMCMs were incubated with LPS for 24 h. Since hMSC action is expected to be a paracrine one, the repair phase was initiated by changing media to hMSC-conditioned medium or to naive medium alone. $[Ca^{2+}]_i$ signaling assays were performed after three additional hours. As shown in Fig. 9B, the increased spontaneous $[Ca^{2+}]_i$ transients that were seen in LPS-treated cells were reversed by a 3 h incubation in conditioned medium. Cells that were incubated in non-conditioned medium showed the typical abnormal $[Ca^{2+}]_i$ signaling at the end of 3 h (Fig. 9B). In the second long-term action repair protocol

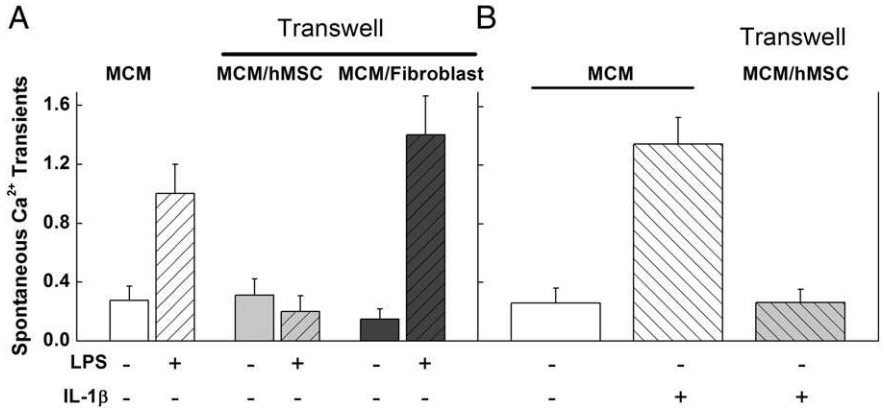

Fig. 6. hMSC protective effects are seen in transwell cultures. nMCMs were cultured in lower chamber of transwell culture dish. After 48 h hMSCs were seeded onto surface of upper chamber of the transwell culture dish at a density of 1:2. Summary data represent the number of spontaneous $[Ca^{2+}]_i$ transients measured during the 10 s interval between the trains of 10 electrical stimuli. (A) When nMCMs are cultured alone, LPS (3 h, 1 μg/ml) gives normal increase in spontaneous $[Ca^{2+}]_i$ signaling (open bars). When hMSCs are cultured in upper chamber, complete protection from LPS is seen (gray bars). When human fibroblast cells are included in upper chamber LPS still evokes a stress response (black bars). (B) Parallel experiments were conducted which were identical to those in A except cells were stimulated with IL-1β (3 h, 100 ng/ml). In nMCM cultures, IL-1β provokes a large increase in $[Ca^{2+}]_i$ signaling response (striped bar) which is completely blocked when hMSCs are cultured in upper chamber (striped gray bar). All values reported as means ± SEM ($n = 22$ to 33).

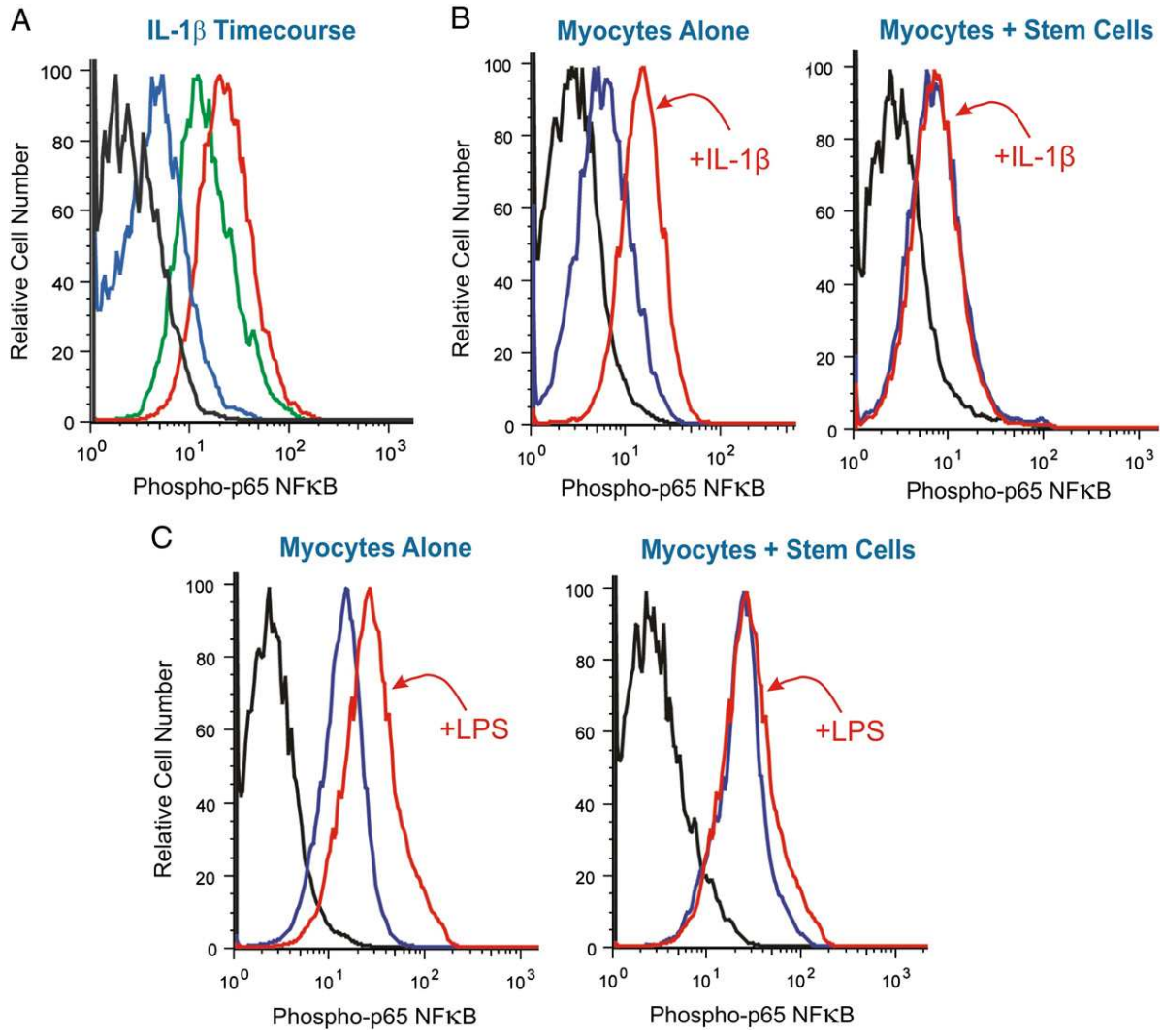

Fig. 7. Human mesenchymal stem cells block activation of NF-κB transcriptional pathway in neonatal myocytes. nMCMs were cultured for 48 h and were cultured for another 24 h with or without hMSCs in co-culture configuration. The cells were then fixed and labeled with fluorescent anti-phospho NF-κB p65(Ser536) and analyzed by flow cytometry. The myocytes and CD44− cells were gated as described in Methods. (A) Cultures of nMCMs were incubated with IL-1β (2 μg/ml) over time and analyzed for NF-κB phosphorylation. Shown are levels of phospho-protein at time 0 min (blue), 10 min (green) and 30 min (red). The black plot is the isotype control for these experiments. (B) Flow cytometry profiles for phospho-p65 levels in cardiac cells at 0 min (blue) or 30 min (red) following IL-1β addition. The left figure shows phospho-p65 response in nMCMs when cultured alone while the panel at right shows the cardiac response in the nMCM/hMSC co-cultures. (C) When myocytes were cultured alone, LPS (3 h, 1 μg/ml) provokes increase in phospho-p65 signal (red plot, left graph). While LPS stimulated p-65 phosphorylation in neonatal mouse cardiac myocyte monocultures, hMSCs clearly prevented LPS-evoked activation of cardiac p-65 NFkB when grown in co-culture (right panel).

depicted in (2) in Fig. 9A, nMCMs were treated for 3 h with LPS to evoke damage as seen in Fig. 3, followed by a 24 h incubation with conditioned medium or naive medium as a control. As shown in Fig. 9C the $[Ca^{2+}]_i$ signaling dysfunction was still evident in nMCMs after 24 h incubation in control medium, while the hMSC-conditioned medium restored and maintained normal Ca^{2+} signaling function. Taken together these results broaden the significance of the beneficial action of hMSCs as they can restore normal function to damaged or stressed cardiac cells.

4. Discussion

Cellular therapy for cardiac disease has rapidly evolved from an intriguing promise into ongoing clinical trials. While perfused adult bone marrow-derived mesenchymal stem cells (hMSCs) are now being used in numerous human studies there remains uncertainty about how such cells act in restoring damaged heart tissue [1]. While stem cell transdifferentiation was an appealing early model for hMSC action, a collection of more recent studies reveal the beneficial effects of such cells is derived in part from their secretion of multiple soluble factors reported to act in a paracrine fashion [7–12]. Such soluble factors may act though a reduction in infiltration of inflammatory neutrophils, inactivation of fibrogenic cells and scarring, stimulation of angiogenesis and vascularization, or recruitment and activation of resident cardiac stem cells [12]. While these proposed mechanisms raise many intriguing hints of the biology of cardioprotection, it is possible that perfused hMSCs provide direct benefit to the damaged cardiac myocytes themselves. In this study we have critically examined this largely unexplored hypothesis through studies that capitalize on a mixed co-culture of hMSCs and mouse cardiac myocytes exposed to agents that recapitulate stress and injury. The main findings are that (i) inflammatory-like cardiac damage, evoked by LPS and IL-1β in this cell setting, is mediated by signaling evoked by the proinflammatory cytokine, IL-18. (ii) hMSCs are powerful benefactors of cardiac myocytes as normal Ca^{2+} signaling is maintained in the presence of these damaging stimuli. (iii) hMSC-derived factors act in a reparative fashion, restoring normal function to damaged cardiac myocytes. (iv) hMSCs provoke a genetic

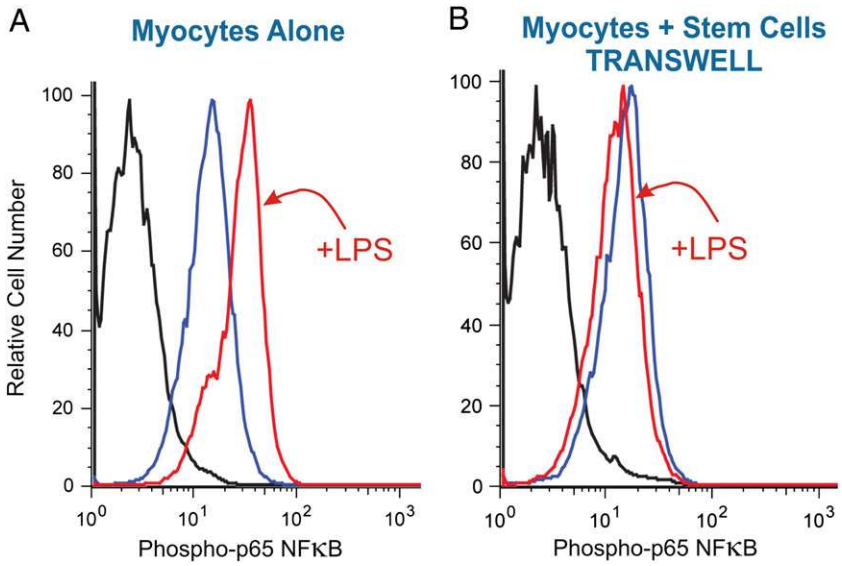

Fig. 8. Human mesenchymal stem cells block activation of cardiac stress-induced transcriptional pathway though a paracrine mechanism. Cultures were grown in transwell configuration as in Fig. 6. This method prevents direct cell to cell contact but allows released factors to freely diffuse though the membrane to myocytes. Cultures were treated with LPS for 30 min, then fixed and labeled with anti-phospho-p65 and analyzed by flow cytometry as described in Fig. 7 and Methods. (A) Levels of phospho-p65 were compared between control myocytes and those incubated with LPS as indicated. (B) Levels of phospho-p-65 were not increased by LPS when hMSCs were included in transwell configuration. These results are representative of three separate experiments.

reprogramming of cardiac myocytes manifest by alterations in the pattern of cytokine release and attenuation of the activation of proinflammatory transcription factor, NF-κB. A scheme that is consistent with these findings is depicted in Fig. 10.

4.1. Modeling of inflammation in cultured cardiac myocytes

In this study we have exploited the endotoxin, LPS, and a downstream signaling proinflammatory cytokine, IL-1β, as stressors since these agents have been implicated in cardiac damage associated with sepsis and ischemia/reperfusion [15,25,26]. While prevention of cardiomyocyte apoptosis may be an important protective outcome [10,27] we have monitored and quantified dysregulation of intracellular $[Ca^{2+}]_i$ signaling as a sensitive readout for early-stage myocyte stress and damage. Accordingly, we measured abnormal spontaneous $[Ca^{2+}]_i$ transients that arose in the context of an otherwise orderly train of depolarizing stimuli as a measure of $[Ca^{2+}]_i$ handling dysfunction. This metric for cardiac damage was seen in both acute (3 h) and long term (24 h) exposures to either LPS or IL-1β. The mechanisms that underlie this abnormal behavior may be due to multiple factors such a cell depolarization, changes in repolarizing currents, mitochondrial dysfunction, or Ca^{2+} overload of the sarcoplasmic reticulum. The networked nature of these neonatal cultures and nMCM/hMSC co-cultures precluded detailed single-cell whole-cell voltage clamp studies which could unravel some of these alternatives.

It was important to identify the agents that mediate LPS and IL-1β damage if the mechanisms of hMSCs are to be illuminated. Thus LPS and IL-1β may act directly on cardiac myocytes through stimulation of their cognate myocardial receptors, TLR4 and ILR respectively [25,26]. Alternatively, proinflammatory cytokines that are downstream products of the receptor-stimulated NFκB signaling cascade, such as TNFα, could be the ultimate harmful agents [15,18,19]. This hypothesis was tested initially in functional studies through the addition of neutralizing anti-TNFα since LPS-provoked TNFα secretion was seen here and in other reports [15]. Although it was surprising that this antibody had no protective effect, neutralizing anti-IL-18 did completely block LPS- and IL-1β-evoked Ca^{2+} signaling dysfunction. This intriguing finding supports the notion that cardiomyocyte damage in this model system was mediated by the proinflammatory cytokine, IL-18 rather than TNFα. This conclusion is incorporated into a model depicted in Fig. 10. While currently available ELISAs were not of sufficient sensitivity to measure basal or stimulated mouse IL-18 in these cardiac cultures, complementary confocal immunofluorescent studies revealed that IL-18 is indeed expressed in these cultured mouse ventricular cardiac myocytes. It is important to note that IL-18 expression is increased in experimental ischemia/reperfusion studies and IL-18 neutralization reduces LPS evoked damage in intact mouse hearts [20,28]. Clinically, elevated IL-18 has been observed in congestive heart failure and myocardial ischemia while a common IL-18 gene haplotype is associated with coronary heart disease [29,30]. Thus, there is compelling evidence to support a critical role for IL-18 as a damaging cytokine in heart disease in general. It will be important in future studies to identify the myocardial IL-18 receptor and its downstream targets.

In apparent contradiction, while both LPS and IL-1β activate NF-kB in myocytes [15] only LPS induced TNF-α protein expression. This pattern of expression is gene-specific as both LPS and IL-1β were potent inducers of myocyte IL-6 release, as shown here. Although the receptors for LPS (TLR4) and IL-1β (ILR) share many common downstream signaling elements including the MyD88/TRAF6/TAK1/IKKβ signaling pathway [26], it is possible that IL-1β activates a repressor by an independent cascade. Consistent with this notion and in contrast to LPS, we have seen that IL-1β fails to stimulate TNF-α mRNA expression in cardiac myocytes (data not shown). It will be important to identify such novel mechanisms that regulate gene repression which are related to maladaptive responses of cardiac myocytes.

4.2. hMSCs have direct protective effects on stressed cardiac myocytes

Since hMSCs hold great promise for cardiac therapies there has been much interest in elucidating the fundamental mechanisms through which such stem cells act in the setting of cardiac damage and subsequent repair [2,7]. This study initially focused on the hypothesis that acute protection of the myocytes themselves is a critical mechanism. Accordingly, studies assessed the impact of hMSCs on the stress-evoked dysfunctional Ca^{2+} signaling in cardiac myocytes in a cell culture system independent of potentially confounding contributions from angiogenesis or recruitment of inflammatory and resident stem cells. Initial findings revealed, unlike endothelial fibroblasts, which themselves evoked many spontaneous $[Ca^{2+}]_i$ transients,

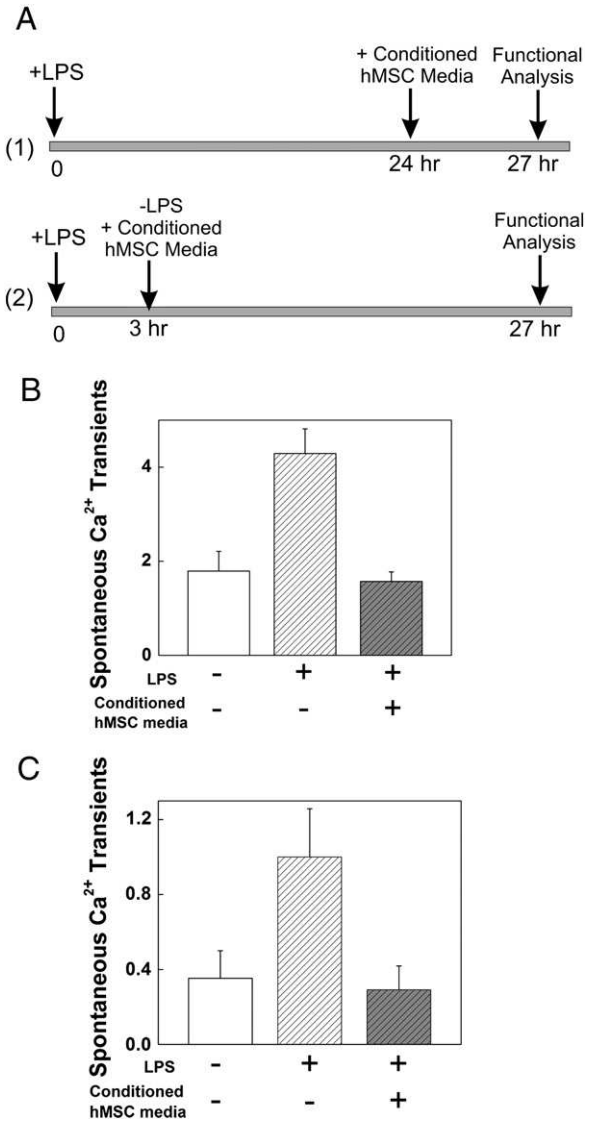

Fig. 9. hMSC-conditioned media rescues cardiac myocytes from stress induced by LPS. (A) The timeline for two rescue protocols is depicted. In (1) nMCMs were treated with LPS for 24 h and were subjected to hMSC-conditioned media for 3 h prior to functional analysis. In timeline (2) nMCMs were incubated with LPS for 3 h followed by incubation with conditioned medium for 24 h prior to functional analysis. (B) Summary results from experiments are shown from protocol (1) above. The results show spontaneous Ca^{2+} signaling events for control nMCMs at 27 h (open bar), nMCMs treated with LPS for 24 h followed by control medium (hatched bar), or LPS for 24 h followed by hMSC conditioned medium (gray hatched bar). The results are the means ± SEM ($n = 50$ to 70) (C) Summary results from experiments are shown from protocol (2) above. The results show spontaneous Ca^{2+} signaling events for control nMCMs at 27 h (open bar), nMCMs treated with LPS for 3 h followed by control media (hatched bar), or LPS for 3 h followed by hMSC conditioned media for 24 h (gray hatched bar). The results are the means ± SEM ($n = 15$ to 24).

hMSCs in co-culture actually stabilized basal Ca^{2+} signaling in cardiac myocytes, with reductions in spontaneous $[Ca^{2+}]_i$ transients frequently observed (Fig. 3A). Since this basal stabilization is not seen in transwell cultures (Fig. 6, for example) cell–cell contact may be important in this effect. Multiple studies reveal that hMSCs can form stable gap junctions with cardiac myocytes in culture and in situ [22,23,31]. It will be interesting to determine if gap junction formation plays a role in this hMSC effect on Ca^{2+} signaling. Regardless, the critical finding was that cardiac myocyte function was protected from the damaging effects of LPS or IL-1β when hMSCs were grown in co-culture or under conditions where cell–cell contact was prevented, confirming the paracrine mechanism of

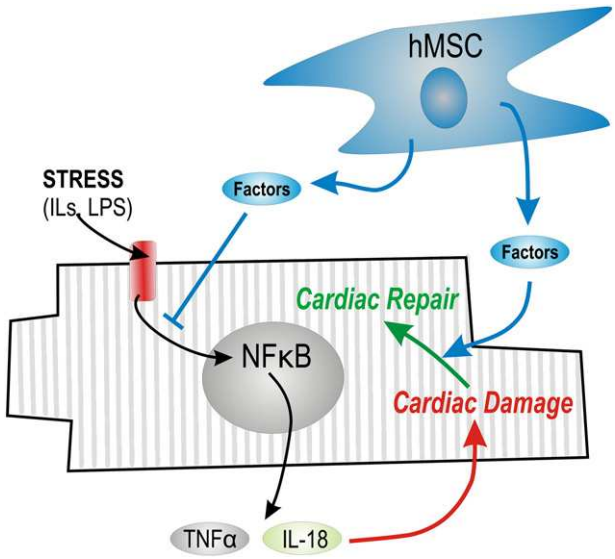

Fig. 10. Schematic model of hMSC-stressed cardiac myocyte interactions. In this model the cascade of cardiac cell stress and damage provoked by LPS or interleukins begins with cardiac receptor-mediated stimulation of NF-κB followed by production of myocardial cytokines such as TNFα and IL-18. Based on results presented here the potential damaging action of IL-18 is depicted. hMSCs release soluble factors that provide protection by preventing activation of cardiac NF-κB. The same or other hMSC-based factors stimulate repair of damaged cardiac myocytes by mechanisms that remain to be identified in future studies.

this protective effect. Although it has been reported that stress stimulates the secretion of a variety of growth factors and cytokines from hMSCs [12,32], in our experiments conditioned medium from non-stimulated hMSCs was sufficient to provide beneficial effects.

The hMSC-mediated stabilization of contractile frequency seen here resonates well with several studies where a reduction in arrhythmias is observed following application of mesenchymal stem cells in animal models and randomized clinical trials [33,34]. It is interesting to note that early clinical trials with skeletal muscle myoblasts report an increased incidence of ventricular arrhythmias, which is now appreciated to represent a serious clinical risk factor [35]. The differences in outcomes may be related to the ability of hMSCs, and not skeletal myoblasts, to form connexin-based gap junctions with cardiac myocytes. These uncertainties may be resolved with the model system exploited here which should reveal new insights into the cellular and molecular basis for the reduction in ventricular arrhythmias.

4.3. hMSCs provoke protective genetic reprogramming of cultured cardiac myocytes

An important conclusion from this study is that beneficial effects of hMSCs on cardiac myocyte Ca^{2+} signaling is accompanied by a genetic reprogramming of these muscle cells. Several results support this conclusion. First, the pattern of myocardial cytokine release was markedly changed by hMSC. The well characterized LPS-evoked release of TNF-α [14–16] was completely blunted in co-cultures while the spontaneous release of a beneficial cytokine, IL-10 [9], was unaltered. Second, consistent with the inhibition of TNF-α production, hMSCs blocked the activation of NF-κB signaling cascade in cardiac myocytes. The conclusion was supported by flow cytometry experiments where increases in cardiac phospho-p65 subunit of NF-κB was used as a direct measure of myocardial activation from co-cultures or transwell cultures [14–16]. As depicted in the model in Fig. 10, these results show for the first time that hMSCs have direct action on cardiac myocyte responses to stress at the gene transcriptional level that are consistent with their beneficial effects on function.

4.4. hMSCs repair cardiac myocytes damaged by stress stimuli

The optimal timing of the application of stem cell therapy is a crucial clinical issue in the treatment of heart disease. Accordingly, further experiments tested the notion that hMSCs could also repair cardiac myocytes after damage had occurred. Rescue protocols included addition of conditioned medium to damaged cells to test the role of paracrine mechanisms in hMSC-mediated repair. Different protocols revealed that conditioned medium could repair stress-induced abnormal Ca^{2+} signaling both in long term (24 h) and acute (3 h) incubations. The doses of LPS and IL-1β were sufficient to provoke damage that did not spontaneously revert over a 24 h incubation, while cell death was minimal over the same time interval. This allowed for quantification of the repair of a broad population of cells to normal Ca^{2+} handling activity. Further experiments will be required to determine if these soluble factors are identical to or distinct from those that mediate cardiac protection. This notion is incorporated into the scheme in Fig. 10.

The paracrine signaling action of hMSCs on isolated cardiac myocytes reported here is consistent with whole-organ animal studies by others [11,36,37]. Although there is a long list of potential soluble factors underlying beneficial action of MSCs [12], evidence has emerged to implicate several agents including stromal derived factor-1α, secreted frizzled-related protein 2 (sfrp-2), IL-10, TNFα induced protein 6 (TSG-6), and VEGF [9,10,38–40]. This model cell system will provide an excellent experimental scheme to further study these releasable factors and determine their distinct roles in cardiac protection and/or repair.

In summary, several studies in whole animal myocardial disease models and early human clinical trials demonstrate that hMSCs can improve cardiac performance following injury. While many potential mechanisms are proposed, this focused study reveals cardiac myocytes themselves are a likely target for the beneficial effects of stem cell therapy. Thus, these signaling mechanisms that underlie cardiac cell repair are likely to be operative in bone-marrow derived stem cell therapy in heart disease currently in clinical trials.

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