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Ischemia Induces P-Selectin-Mediated Selective Progenitor Cell Engraftment in the Isolated-Perfused Heart

Abstract

Clinical trials infusing Bone Marrow Cells (BMCs) into injured hearts have produced measurable improvements in cardiac performance, but were insufficient to improve patient outcomes. Low engraftment rates are cited as probable contributor to limited improvements. To understand the mechanisms that control myocardial engraftment of BMCs following ischemia-reperfusion injury, in isolated-perfused mouse hearts, stop-flow ischemia was followed by variable-duration reperfusion (0–60 min) before addition of labeled syngenic BMCs to the perfusate. After a buffer-only wash, the heart was disaggregated. Retained BMCs (digest) and infused BMCs (aliquot) were compared by flow cytometry for c-kit and CD45 expression to determine the proportion of cell subtypes engrafted versus delivered (selectivity ratio). In these studies, a time-dependent selective retention of c-kit⁺ cells was apparent starting at 30 min of reperfusion, at which time c-kit⁺/CD45⁺ BMCs showed a selectivity ratio of 18 ± 2 (versus 2 ± 1 in sham-ischemic controls). To study the underlying mechanism for this selective retention, neutralizing antibodies for P-selectin or L-selectin were infused into the heart preparation and incubated with BMCs prior to BMC infusion. Blocking P-selectin in ischemic hearts ablated selectivity for c-kit⁺/CD45⁺ BMCs at 30 min reperfusion (selectivity ratio of 3 ± 1) while selectivity persisted in the presence of L-selectin neutralization (selectivity ratio of 17 ± 2). To corroborate this finding, a parallel plate flow chamber was used to study capture and rolling dynamics of purified c-kit⁺ versus c-kit⁻ BMCs on various selectin molecules. C-kit⁺ BMCs interacted weakly with L-selectin substrates ($0.03 \pm 0.01\%$ adhered) but adhered strongly to P-selectin ($0.28 \pm 0.04\%$ adhered). C-kit⁻ BMCs showed intermediate binding regardless of substrate ($0.18 \pm 0.04\%$ adhered on L-selectin versus $0.17 \pm 0.04\%$ adhered on P-selectin). Myocardial ischemia-reperfusion stress induces selective engraftment of c-kit⁺ bone marrow progenitor cells via P-selectin activation.

Keywords

Ischemia-reperfusion injury, Stem cell therapy, Myocardial infarction, Cell engraftment, CD117, c-kit, CD45, P-selectin, L-selectin

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Ischemia Induces P-Selectin-Mediated Selective Progenitor Cell Engraftment in the Isolated-Perfused Heart

Elser –Selective Myocardial Progenitor Engraftment

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Introduction

Prior dogma asserted that the heart is a terminally differentiated organ with no capacity for generating new cardiomyocytes. However, recent evidence indicates that cardiomyocyte formation occurs throughout life, albeit at low levels [1]. Exploiting fluctuating ambient carbon-14 levels during and after an era of atmospheric nuclear bomb tests, Bergmann et. al. calculated a 1% cardiomyocyte annual turnover rate during early adulthood that eventually declines to 0.45% [2]. Analogous fate-mapping studies in mice likewise suggests low level cardiac myogenesis at baseline that is enhanced following myocardial infarction or sustained pressure overload [3]. In failing human hearts, Kubo et. al. showed that myogenic c-kit⁺ progenitor cells are enriched compared to nonfailing hearts. Most of these c-kit⁺ progenitors co-expressed the pan-leukocyte marker CD45, suggesting a bone marrow origin [4]. However, the typical scar formation following MI suggests that this native repair response is largely inadequate.

The realization that myocyte repopulation is possible has prompted a series of clinical trials aimed at augmenting the natural repair response in patients with myocardial infarction (MI). Systemic administration of chemokines such as GM-CSF induces increases in circulating bone marrow cells (BMCs) that are believed to be a source of angiogenic and cardiomyogenic progenitors. However, GM-CSF alone has not improved clinical outcomes following MI. In some studies, direct delivery of filtered, autologous bone marrow cells (BMCs) into the coronary arteries or myocardial wall resulted in statistically-significant increases in cardiac performance, but these improvements were also inadequate to improve survival. Low engraftment rates were cited as a primary limitation in some of these studies [5]. Indeed, follow-up studies determined that 93-98% of bone marrow cells delivered via coronary artery fail to engraft and cannot be detected in the heart within 1 hour [6][7].

Although these trials provide encouragement for pursuing progenitor-based therapies, full understanding of the engraftment process and the nature of the engrafted progenitor cells remains underdeveloped. In particular, little is understood about the mechanisms regulating progenitor cell engraftment immediately after myocardial injury or stress. Accordingly, we adapted an isolated-perfused mouse heart (IPMH) model to study BMC engraftment dynamics following ischemia-reperfusion injury

(IR-injury). In particular, we employed a heterogenous population of unfractionated BMCs so that ischemia-responsive engraftment would provide unbiased insights into factors affecting this process. Using this model, we identified a subset of BMCs with injury-dependent selective engraftment into the heart and also a necessary adhesion molecule that facilitates this selectivity. The mechanism for this preferential engraftment was confirmed using an established in vitro model of cell rolling dynamics. These studies provide new insights into endogenous myocardial repair processes and suggest potential improvements to future therapies for myocardial infarction.

Methods

Isolation of Mouse BMCs. Adult C57BL/6 mice (18-22 g, 10-12 weeks old) were anesthetized with 100 mg/kg ketamine and xylazine, and 1,000 units/kg of heparin was administered IP. Bone marrow was obtained by removing the femurs, and flushing them with sterile PBS over a 40 μ m filter. The cells were pelleted by centrifugation and resuspended in lysis buffer (BD Biosciences) for 1 min to lyse red blood cells. Cells were again pelleted and resuspended in 1 mL PBS to remove remaining lysis buffer. Cells were counted using a hemocytometer on an aliquotted sample, and 12 ± 3 million viable (trypan blue exclusion) BMCs were obtained to perform infusions.

Isolated Perfused Mouse Heart for Ischemia-Reperfusion Studies. Following anesthesia and heparinization, the heart was rapidly excised, the ascending aorta was cannulated and perfusion was initiated with a modified Krebs bicarbonate buffer, as previously described [8]. Perfusion was restored within 1 min of excision and maintained at 37°C and 2 mL/min. The perfusion solution was aerated with 95% O₂, 5% CO₂ in order to maintain a pH of 7.4. A side arm in the perfusion line proximal to the heart inflow cannula allowed for entrainment of labeled BMCs along with the oxygenated perfusate.

In all experiments, the perfused hearts underwent 10 min of equilibration time at a flow rate of 2 mL/min at 37°C. After equilibration, the trial cohort underwent no-flow ischemia for 15 minutes (i.e. buffer pump flow was entirely halted), whereas a control group (sham ischemia) received normal flow of buffer for 15 minutes. For all experiments, the ischemia or sham ischemia was followed by reperfusion of

plain buffer for 0, 15, 30, 45, or 60 minutes. Effects of this injury model (among others) are reviewed by Murphy and Steenbergen [9]. After this reperfusion with plain buffer, 15 min of reperfusion with freshly-harvested, PKH26GL-labeled (Sigma Aldrich, MO) BMCs occurred. At the completion of cell infusion, plain buffer was administered for 15 min to clear the circulation of non-engrafted BMCs. Most hearts were then perfused digested with collagenase (180 unit/mL for 15 min) followed by filtration for flow cytometry. A subset of hearts evaluated by histology were not perfused with collagenase.

Flow Cytometry for IPMH Ischemia-Reperfusion Studies. The heart digest was pelleted by centrifugation and resuspended in 1 mL PBS. A representative sample (0.1 mL) of the heart tissue digest and the infused BMC aliquot were stained with the viability dye DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Invitrogen, CA), an anti-CD117 (c-kit) antibody conjugated to AF-488 (Biolegend, CA) and an anti-CD45 antibody conjugated to PE-Texas Red (Abcam Inc., MA). After excluding dead cells (DAPI⁺) along with cell fragments and aggregates, gating based on PKH26GL fluorescence was employed to identify the infused BMCs. DAPI/PKH26GL⁺, size/granularity-gated cells from both the perfusate aliquot and heart digests were evaluated for c-kit and CD45 expression. Demarcation intensities for each fluorochrome were determined using the Fluorescence Minus One (FMO) technique (Supp. Fig. 1). For each immunophenotypic subtype, the percent representation in the retained cells was divided by original percent representation in the perfusate aliquot to determine a 'selectivity ratio' of engraftment: a ratio of 1 reflects non-selective engraftment while a ratio significantly greater than 1 indicates preferential engraftment.

Selectin Neutralization Studies in Isolated-Perfused Hearts. IPMHs were prepared as described above with the exception that, prior to BMC infusion, PKHGL26-labeled BMCs were incubated for 15 min with 30 µg neutralizing antibody for either P-selectin (BD Pharmingen CD62P) or L-selectin (BD Pharmingen CD62L) immediately prior to infusion. Additionally, during the final 5 min of the 30 min reperfusion, buffer flow to the heart was supplemented with 80 µg neutralizing antibody for either P-selectin or L-selectin. Hearts were then rinsed with plain buffer for 15 min and prepared for histology or flow cytometry as described above.

Histologic Analysis for IPMH Ischemia-Reperfusion Studies. Cryosectioning was used to visualize infused (PKH26GL or PKH67GL-labeled) cells retained in the heart. Following the cell infusion protocol and rinse protocol, hearts were embedded in Tissue-Tek O.C.T compound and snap frozen in liquid nitrogen. Frozen samples were stored at -20°C before sectioning. In various preparations, sections were analyzed following exposure to antibodies for c-kit (PE conjugated CD117, Abcam Inc., MA or FITC conjugated, Biolegend), CD45 (PE-Texas Red conjugated, Abcam), and/or P-selectin (FITC conjugated, BD Pharmingen, NJ). Sections were stained with DAPI-containing ProLong Gold (Invitrogen, CA) and visualized with a Nikon light microscope with DAPI, FITC, and TRITC filters.

Fluorescent Labeling & Sorting of c-kit⁺ cells from Mouse BMCs for Parallel Plate Flow Chamber Studies. Once isolated, mouse BMCs were stained with DAPI and an anti-CD117 (c-kit) antibody conjugated to PE (Abcam Inc., MA). DAPI-/c-kit⁺ and DAPI-/c-kit⁻ populations were isolated by FACS. Sorted c-kit⁻ cells were always >95% purity, and c-kit⁺ cells were >85% purity. Based on the c-kit⁺ cell recovery rate, equal concentrations of c-kit⁻ and c-kit⁺ cell suspensions of approximately 50,000 cells/mL were used in the flow chamber experiment.

Parallel Plate Flow Chamber Studies: Recombinant P- and L- selectin- IgG chimeras were purchased from R&D Systems. Modified flexiPERM wells (Sigma Aldrich, MO) were attached to 35 mm Corning non-treated culture dishes. To immobilize adhesion molecules, the surface was first incubated with recombinant protein A/G (Thermo Scientific, UK) at 4°C on a rocker overnight. The surface was then washed with a blocking solution (1% BSA in sterile PBS) 3 times and the surface was coated with 0.5 µg/mL of P-selectin and incubated at 4°C on a rocker for 5 hours. The surface was again washed 3 times with blocking solution and incubated in flowing buffer (HBSS, 2mM Ca²⁺, 2mM Mg²⁺, 10mM HEPES+0.5% BSA), until used for the flow chamber experiment. For L-selectin, which has a histidine tag on the C-terminus, the protocol was changed to include an overnight incubation with an anti-polyhistidine antibody (R&D Systems, MN), between the protein A/G and selectin coating steps. The apparatus was set up as previously described [10].

Data Analysis for Parallel Plate Flow Chamber Studies: A video of rolling and adhered cells was recorded over the same area for both c-kit⁻ and c-kit⁺ cell populations, and later processed using ImageJ

software with the MTrack2 plug-in for calculating rolling velocity and a cell counter for tracking the number of adhered cells. Rolling velocity ($\mu\text{m/s}$) was calculated by marking the change in position of the cell at every frame. Rolling flux (rolling cells/ mm^2/min) indicates the number of rolling cells, and rolling concentration (rolling flux/rolling velocity) measures rolling efficiency (slow and steady rolling indicates higher efficiency). The number of adhered cells was counted by scanning the same area for both c-kit^+ and c-kit^- cells and is expressed as a percent of the total cells that entered the flow chamber. A cell was considered firmly attached if it did not move for more than 30 seconds. Each experiment was done in triplicate ($n=3$).

Results

Histological Evidence Infused Cell Engraftment. Frozen tissue sections of IPMHs subjected to the ischemia-reperfusion protocol (15 min ischemia or sham ischemia, 30 min cell-free reperfusion, 15-min cell infusion with PKH26GL-labeled BMCs, and 15 min buffer-only wash) revealed that retained BMCs (PKH26GL^+) were distributed uniformly throughout the tissue, with a large majority in the left ventricular free wall. Cell clumping and occlusion of the coronary vasculature was rarely observed (Fig. 1). Co-staining for the myocardial marker α -actinin is shown in Supp. Fig. 2.

Identification of Engrafted Cells in Non-Ischemia Control Experiments. The goal of these experiments was to demonstrate the ability to identify infused BMCs in the IPMH and distinguish them from native heart cells. After serial gating is used to exclude dead cells and cell fragments or aggregates, flow cytometric analysis demonstrates high efficiency PKH26GL labeling of the infused BMCs, as shown in Supp. Fig. 3 (top). After identical gating, the smaller proportion of cells identified as PKH26GL^+ in the heart digest represents infused BMCs that have been engrafted while the PKH26GL^- cells represent nonengrafted heart-derived cells (Supp. Fig. 3, bottom). When identically-gated BMCs from the perfusate and heart digest are characterized based on biomarker expression (c-kit and CD45), the selectivity of retention, or lack thereof, is demonstrated. One such comparison derived from a sham ischemia experiment reveals that the four possible biomarker combinations ($\text{c-kit}^+/\text{CD45}^+$, $\text{c-kit}^+/\text{CD45}^-$, $\text{c-kit}^-/\text{CD45}^+$, $\text{c-kit}^-/\text{CD45}^-$) are similarly represented in both the sham ischemic heart and the bone marrow

perfusate aliquot, suggesting that perfusate cell retention in the heart is essentially random (nonselective) in the absence of antecedent ischemia-reperfusion injury (Supp. Fig. 3).

Injury-Dependent Engraftment Selectivity for c-kit⁺/CD45⁺ Autologous BMCs. In contrast to sham ischemia, stop-flow ischemia for 15 min followed by cell-free and then BMC infusion significantly altered the pattern of retained cells compared with the infused cells. As the representative sample in Fig. 2 shows, c-kit⁺/CD45⁺ and c-kit⁺/CD45⁻ cells comprise a far greater fraction of the retained cells in IR-injured hearts than in the sham ischemic hearts at a reperfusion duration of 30 min. Based on a series of experiments of this type, the observed proportion of ckit⁺/CD45⁺ cells increased from 3±1 percent in sham ischemia heart to 25±7 percent in the stop-flow ischemia heart after reperfusion duration of 30 min (Fig. 3, n=3). These results confirm that c-kit expression by BMCs is associated with selective retention irrespective of CD45 expression, and that a lack of c-kit expression is associated with a demonstrable reduction in engraftment compared with sham ischemia. We also examined the preferential retention of c-kit⁺ BMCs as a function of the duration of cell-free reperfusion prior to initiation of BMC administration. As shown in Supplemental Table 1, c-kit⁺ cells were found at significantly higher concentrations in ischemic hearts than in sham-ischemic hearts at reperfusion durations of 30, 45, and 60 minutes of reperfusion but not at reperfusion durations of 0 and 15 minutes. Control experiments performed without PKH26GL labeling determined that the contribution of native heart cells to the PKH26GL⁺ recorded event count was less than 11% (see Supplemental Data).

Direct comparison for selective retention between c-kit⁺/CD45⁺ and c-kit⁺/CD45⁻ populations requires the comparison of selectivity ratios (defined as the fraction in engrafted BMCs divided by the fraction in the BMC perfusate for each immunophenotypic subclass). Using this approach, the selectivity ratios for each phenotypic subtype are shown as a function of reperfusion time in Fig. 4. These data reveal that the presence of c-kit positivity alone results in average selectivity ratios that peak above 15 after 30 min of reperfusion and remain above 10 when cells are infused after 60 min of reperfusion. When the analysis focuses on both c-kit and CD45 immunophenotypes, the selectivity ratios are even higher for the subset of c-kit⁺ cells that are also CD45⁺ (Fig. 4).

In order to visualize the expression of CD45 and c-kit biomarkers on infused cells, a subset of hearts underwent 15 min of ischemia (or sham ischemia) followed by 30 min of reperfusion and then infusion with labeled BMCs (PKH67GL, a green dye, was substituted for PKH26GL, an orange dye, to further minimize spectral overlap with the other fluorophores and improve image clarity. As expected from the flow cytometric analyses, nearly all PKH67GL-labeled cells in heart sections stained positively for CD45 in ischemic and sham ischemic hearts, while approximately 25% stained positively for c-kit in the ischemic hearts and very little c-kit staining was observed in sham ischemic hearts (representative cell images shown in Supp. Fig. 4 and Supp. Fig. 5). Representative cell images co-stained with CD45 and c-kit in combination are shown in Supp. Fig. 6.

Inhibition of P-Selectin or L-selectin in Ischemic Conditions. In several hearts undergoing 15 min ischemia and 30 min reperfusion, the final 5 min of reperfusion were supplemented with soluble P-selectin or L-selectin neutralizing antibodies (80 µg). Additionally, PKH26GL-labeled BMCs were incubated with the same blocking antibody (5×10^6 BMCs with 30 µg antibody in 0.5 mL) for 15 min prior to being infused to IPMHs. As shown in Fig. 5A, P-selectin inhibition reduced the selectivity ratio for c-kit⁺/CD45⁺ BMCs in ischemic hearts to 2 ± 1 (compared to 18 ± 2 without neutralization, $p=0.002$), a result equivalent to unblocked sham ischemia trials. L-selectin inhibition in ischemic hearts produced a c-kit⁺/CD45⁺ selectivity ratio of 10 ± 4 , which was significantly greater than observed in unblocked sham ischemic trials (selectivity ratio of 2 ± 1 , $p=0.03$) and not significantly below unblocked ischemic trials, though a downward trend is observed. For BMC subtypes besides c-kit⁺/CD45⁺, no significant differences were found between any of the 4 protocols (unblocked sham ischemia, unblocked ischemia, or ischemia with either L-selectin or P-selectin blockage). To further confirm the interaction of c-kit⁺ BMCs with endothelial P-selectin, histological sections of a representative ischemic IPMH (15 min ischemia followed by 30 min reperfusion and then BMC infusion) were produced with co-staining for c-kit and endothelial P-selectin. P-selectin is observed only in ischemic, but not sham ischemic, hearts and c-kit⁺ BMCs are frequently found adjacent to regions of P-selectin (Fig. 5B, Fig. 5C, and Supp. Fig. 7).

To assess potential coexpression of c-kit and the primary P-selectin ligand (PSGL-1) on BMCs, BMCs were stained with anti-c-kit (AF700-conjugated) and anti-PSGL-1 (PE-conjugated). The odds ratio

of PSGL-1 positivity among c-kit⁺ BMCs was computed as the fraction of c-kit⁺/PSGL-1⁺ BMCs divided by the fraction of c-kit⁺/PSGL-1⁻ BMCs. The odds ratio of PSGL-1 positivity among c-kit⁻ BMCs was computed as the fraction of c-kit⁻/PSGL-1⁺ BMCs divided by the fraction of c-kit⁻/PSGL-1⁻ BMCs. The relative quotient of these ratios was 16±4, indicating that c-kit⁺ BMCs are 16±4 times more likely to be PSGL-1⁺ than are c-kit⁻ BMCs (Supp. Fig 8).

Rolling and Adhesion of c-kit⁺ versus c-kit⁻ BMCs on Selectins. To further define the interaction between c-kit⁺ cells and cell adhesion molecules, fresh BMCs were FACS sorted to obtain purified samples of c-kit⁺ and c-kit⁻ (50,000 cells/mL in 3 mL each). Using the parallel-plate flow chamber, cells were flowed over substrates coated with 0.5 µg/mL of either P-selectin or L-selectin with a shear stress of 1 dyne/cm² in the flow chamber. After a known volume of the cell suspension had run through the flow chamber, a fixed region was scanned for firmly adhered cells. A cell was considered firmly attached if it remained stationary for more than 30 seconds. The adherence of c-kit⁻ BMCs was minimally affected by the substrate (0.18±0.04% adhered on L-selectin versus 0.17±0.04% adhered on P-selectin). In contrast, c-kit⁺ BMCs adhered poorly to L-selectin and showed an approximately 9-fold increase in percentage adhered on P-selectin (0.03±0.01% adhered on L-selectin versus 0.28±0.04% adhered on P-selectin). Rolling velocities were similar for all combinations of substrate and BMC type with velocities on P-selectin (23±7 µm/s for c-kit⁻, 19±1 µm/s for c-kit⁺) and tended to be lower than velocities on L-selectin (39±14 µm/s for c-kit⁻, 64±11 µm/s for c-kit⁺). The percentage of adhered cells and rolling velocity results are summarized in Fig. 6. Rolling flux, a manual count of the number of rolling cells observed over a fixed area for a known period of time, was similar between all substrate/cell type combinations. Rolling concentration (rolling flux divided by rolling velocity) was also similar between all substrate/cell type combinations. Rolling flux and rolling concentration data are available as Supp. Fig. 9.

Discussion

These studies demonstrate, for the first time, that BMCs expressing the c-kit are preferably retained in the heart following ischemia-reperfusion injury. This selectivity varies in time following the ischemic insult with a delay in the onset of selectivity and greatest selectivity seen after 30 min of reperfusion. The

optimum engraftment time of 30 min reperfusion also corresponds with the post-ischemia time required for type I activation of the endothelium, during which P-selectin molecules surface from intracellular Weibel-Palade bodies [11]. In contrast, type II activation, which requires transcription, generally requires hours [12] and is likely not responsible for the results we report. Our results are also in excellent temporal agreement with in vivo P-selectin-dependent increases in overall leukocyte rolling following activation of cremaster endothelium conducted by Ley, et. al. [13]. P-selectin is further implicated as the mediating mechanism for this selective retention by our antibody neutralization experiments, which found that P-selectin, but not L-selectin, was required to produce selective engraftment of c-kit⁺ BMCs and by histological demonstration that engrafted c-kit⁺ cells are usually found adjacent to cells expressing P-selectin. Our analysis of the BMCs further demonstrated that c-kit⁺ BMCs are generally positive for PSGL-1, the primary P-selectin ligand in leukocytes, while only about half of c-kit⁻ BMCs express this adhesion molecule. Together, these results indicate that P-selectin/PSGL-1 mediate selective engraftment of c-kit⁺ cells to the heart early after ischemia-reperfusion stress.

Complementing the ex-vivo studies, in vitro exploration of P- and L-selectins, which have been shown to play a part in the inflammatory cell adhesion process [14], showed a strong selective interaction of c-kit⁺ BMCs with P-selectin. While c-kit⁺ cells demonstrated a statistically-significant decrease in rolling velocity along with a 9-fold increase in firm adhesion from L- to P-selectin, c-kit⁻ BMCs showed nearly identical dynamics regardless of the selectin substrate. As P-selectin is not highly expressed on resting endothelium but is known to surface when the endothelium is activated [15,16], the enhanced P-selectin interaction for c-kit⁺ BMCs supports the mechanistic hypothesis that ischemia-induced activation of the coronary endothelium results in P-selectin-mediated selective retention of c-kit⁺ BMCs.

Although the in vivo regenerative capacity of c-kit⁺ BMCs remains controversial, these cells have been shown to differentiate into cardiomyocytes in vivo [17][18]. Additional angiogenic paracrine signaling effects have been identified for c-kit⁺ BMCs in the injured heart [19]. Thus, the strong retention for these cells in response to injury (selectivity ratio of 18±2) may indicate initiation of a repair response. Together, these findings provide new insights into the dynamics of cell recruitment to the injured heart

while highlighting the utility and versatility of the IPMH model for mechanistic studies focusing on endogenous repair and translational studies aimed at developing or refining new therapeutic strategies.

The features of preferential engraftment of c-kit⁺ BMCs to the heart following ischemia-reperfusion stress in these studies provides clues to the mechanisms of this dynamic process. Most importantly, the preferential recruitment in an IPMH model obviates a requirement for systemic hemodynamic, neural or biochemical triggers for preferential engraftment. However, an auxiliary role for such extracardiac factors is not excluded by these studies. Additional factors may be causally related to the selective retention we have described. For example, because ischemia durations of 10 and 30 min. release a burst of reactive oxygen species (ROS) in isolated-perfused rabbit hearts upon reperfusion, the 15 min. ischemia duration utilized in these studies is expected to produce a significant ROS burst that could activate the coronary endothelium [20]. These factors may be causally related to cell engraftment and should be further elucidated to complete our understanding of the mechanistic pathway.

Limitations. Despite the versatility of the IPMH model for studies of cell engraftment, a few limitations deserve mention. For example, we directly infused filtered, labeled bone marrow constituents into the IPMH while the in vivo setting involves an indirect interaction in which marrow-derived cells are released into the circulation and the circulation, in turn, perfuses the myocardium. While our procedure may have allowed some marrow-derived populations greater access of to the heart than occurs in vivo, this realization does not diminish the validity of injury-triggered engraftment cues and preferential engraftment dynamics revealed by these studies. Moreover, the utility of this model is likely greatest for studying *early* engraftment dynamics before deterioration of contractile performance and edema of the IPMH model reduce its reproducibility and relevance to in vivo dynamics. Additionally, this model is critically dependent on rapid cannulation of the mouse heart since delay will induce ischemic insults that would confound the interpretation of protocol-driven ischemia-reperfusion stress. To address this concern, we routinely abort experiments if the heart cannulation and initiation of perfusion takes more than two minutes.

Conclusion. Myocardial ischemia-reperfusion stress provokes time-dependent and highly selective engraftment of c-kit⁺ cells included in the perfusate via a P-selectin-dependent mechanism. In this

context, additional applications of this model might include more extensive examinations how the type of ischemia (demand vs. supply), duration of ischemia or alternative local cytokine manipulations might alter engraftment dynamics. More detailed profiling of selectively retained cells via further immunophenotyping, sorting with gene expression studies, and/or characterization of the IPMH effluent should provide additional insights into the precise sequence of events responsible for the highly selective engraftment dynamics we observed. Refined mechanistic hypotheses raised by such studies could be testing by gain- and loss-of-function studies enabled by altering the myocardial cytokine milieu, exploiting genetically-manipulated mouse models and/or the varying the composition of the infused cells. For example, manipulations of the infused cell populations via sorting and/recombination could permit unique opportunities to examine whether interactions among infused cells affect engraftment dynamics.

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Non-standard Abbreviations: IPMH – Isolated-Perfused Mouse Heart, BMC – Bone Marrow Cell, IR – Ischemia-Reperfusion, FSC – Forward Scatter, SSC – Side Scatter

Disclosures: None

Figure Captions

Fig 1. Transverse Cryosection of Ischemia-Reperfused Mouse Heart (IPMH) Infused with PKH26GL⁺ BMCs. Histological visualization of hearts having undergone either 15 min sham ischemia (left) or ischemia (right) followed by 30 min reperfusion, 15 min PKH26GL-labeled BMC infusion, 15 min buffer-only rinse, and preparation for histology. 10 μ m slices are shown as merged images of TRITC filter (infused PKH26GL-labeled BMCs appear orange) and DAPI (blue), which represents nuclei. A) 10x of sham ischemic lateral free wall. B) 10x of ischemic lateral free wall. C) 40x of sham ischemic lateral free wall. D) 40x of ischemic lateral free wall.

Fig 2. Biomarker Expression of Retained BMCs Following Sham-Ischemic and Ischemic Protocol. Bone marrow perfusate aliquots and heart digests were serially gated as described in Figure 2 (FSC: Forward Scatter, SSC: Side Scatter). A) Aliquot from BMCs infused into a sham ischemic heart (results of corresponding heart digest displayed in graph C below). B) Aliquot from BMCs infused into an ischemic heart (results of corresponding heart digest displayed in graph D below). C-kit⁺ BMCs made up a greater percentage of retained BMCs in ischemia-reperfusion injured hearts than in sham-ischemic hearts despite similar c-kit⁺ representation in infused BMCs.

Fig 3. Cell Subtype Distribution in Bone Marrow, Sham-Ischemic Hearts, and Ischemic Hearts. For each cell subtype, the percent composition of viable, infused cells (DAPI⁺, FSC&SSC-gated, PKH26GL⁺) isolated from bone marrow, sham-ischemic hearts, and ischemic hearts are displayed as histograms. A histogram set for each possible reperfusion duration (0, 15, 30, 45, 60 min) was computed; the histogram set for reperfusion time of 30 min (n=3) is shown as an example. The c-kit⁺/CD45⁺ subpopulation is more strongly represented in heart than in bone marrow are considered to be preferentially recruited.

Fig 4. Engraftment Ratios for Various BMC Subtypes at Reperfusion Durations of 0, 15, 30, 45, and 60 min. The ordinate plots the percent composition of the retained BMCs found in the heart (DAPI⁺, FSC&SSC-gated, PKH26GL⁺) divided by the percent composition of that BMC subtype in the perfusate (i.e. the BMC subtype's Selectivity Ratio). Thus comparisons between selective engraftment of different

BMC subtypes can be drawn even when the absolute percent representation of these subtypes in the bone marrow are highly disparate. (n=3 for each data point)

Fig 5. Neutralizing Antibodies and Co-localization Implicate P-selectin in c-kit Selective Cell Engraftment. A) Ischemia induces strong selective retention of c-kit⁺/CD45⁺ BMCs compared to sham ischemic trials. Concomitant incubation of infused BMCs with and infusion of neutralizing antibodies for P-selectin eliminates ischemia-induced selective engraftment. Identical treatment with L-selectin neutralizing antibodies in ischemic hearts fails to significantly eliminate selectivity (although a trend towards reduction is observed). (n=3 for each data point). (* p<0.05, ** p<0.01). B&C) Histological visualization of heart having undergone 15 min sham ischemia (B) or ischemia (C) followed by 30 min reperfusion, 15 min PKH67GL-labeled BMC infusion, 15 min buffer-only rinse, and preparation for histology with no neutralizing antibodies. P-selectin (green) is not observed in sham ischemic hearts but is co-localized with c-kit (orange) cells in ischemic hearts.

Fig 6. Rolling and Adhesion Dynamics of c-kit⁺ and c-kit⁻ cells on P-Selectin and L-Selectin. A) The number of cells found to be adhered to a substrate coated with either L-selectin or P-selectin is graphed for a perfusate sample containing either c-kit⁻ or c-kit⁺ BMCs. While the adherence percentage for c-kit⁻ cells does not vary with substrate, c-kit⁺ BMC demonstrate significantly stronger adherence to P-selectin than to L-selectin. B) Rolling velocities are graphed for c-kit⁻ and c-kit⁺ BMCs over L-selectin or P-selectin. Rolling velocities for both cell types trend lower for L-selectin than P-selectin and c-kit⁺ BMCs show a stronger sensitivity to choice of selectin (* p < 0.05 between marked groups, one-way ANOVA, student's t-test). (n=3 for each data point)

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