Characterization of embryonic stem cell transplantation immunobiology using molecular imaging

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CHAPTER 3

Timing of Bone Marrow Cell Delivery Has Minimal Effects on Cell Viability and Cardiac Recovery Following Myocardial Infarction

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Manuscript submitted

ABSTRACT

Background: Despite ongoing clinical trials, the optimal time for delivery of bone marrow mononuclear cells (BMCs) following myocardial infarction (MI) is unclear. We compared the viability and effects of transplanted BMCs on cardiac function in the acute and sub-acute inflammatory phases of MI.

Methods and Results: The time-course of acute inflammatory cell infiltration was quantified by FACS analysis of enzymatically digested hearts of FVB mice (n=12) following LAD ligation. Mac-1^{+}Gr-1^{high} neutrophil infiltration peaked at day 4. BMCs were harvested from transgenic FVB mice expressing firefly luciferase (Fluc) and green fluorescent protein (GFP). Afterwards, $2.5x10^{6}$ BMCs were injected into the left ventricle of wild-type FVB mice either immediately (Acute BMC) or 7 days (Sub-acute BMC) after MI, or after a sham procedure (n=8 per group). *In vivo* bioluminescence imaging (BLI) showed an early signal increase in the Acute BMC group at day 7, followed by a trend towards improved BMC survival in the Sub-acute BMC group that persisted until the BLI signal reached background levels after 42 days. Compared to controls (MI + saline injection), echocardiography showed a significant preservation of fractional shortening at 4 weeks (Acute BMC vs saline; $P<0.01$) and 6 weeks (both BMC groups vs saline; $P<0.05$), but no significant differences between the two BMC groups. FACS analysis of BMC injected hearts at day 7 revealed that GFP^{+} BMCs expressed hematopoietic (CD45, Mac-1, Gr-1) markers, minimal progenitor (Sca-1, c-kit), and no endothelial (CD133, Flk-1) or cardiac (Trop-T) cell markers.

Conclusion: Timing of BMC delivery has minimal effects on intramyocardial retention and preservation of cardiac function. In general, there is poor long-term engraftment and BMCs tend to adopt inflammatory cell phenotypes.

INTRODUCTION

Ischemic heart disease is the principal cause of heart failure and its prevalence continues to increase[1]. Due to the low regenerative capacity of the human heart, myocardial infarction (MI) leads to an irreversible loss of cardiomyocytes and ventricular remodeling. In recent years, treatment with autologous bone marrow-derived stem cells has been suggested to reduce myocardial damage in patients with MI[2]. Although different bone marrow cell subpopulations have been proposed to aid to cardiac repair, unfractionated autologous bone marrow mononuclear cells (BMCs) were used as donor cells in the majority of clinical trials, mainly because of the ability to safely and quickly isolate these cells. The mononuclear part of the bone marrow includes a heterogeneous mixture of cells with varying percentages of hematopoietic stem cells, endothelial progenitor cells, mesenchymal stem cells, and side population cells, as well as adult myeloid and lymphoid cells[3].

The potential mechanism(s) by which transplanted BMCs can improve cardiac function remains a subject of debate. Beyond these mechanical considerations, several basic technical issues remain to be clarified, such as the optimal cell type, route of delivery, and timing of cell transplantation. Following acute MI, a robust inflammatory response occurs that is necessary for healing and scar formation and contributes to cardiac remodeling[4]. The benefits of BMC transplantation in the acute phase after MI may thus be jeopardized by the local inflammation that renders the myocardium a hostile environment for the injected cells. On the other hand, experimental studies have demonstrated that BMC transplantation can lead to a reduction of cardiomyocyte apoptosis[5], suggesting that early timing of cell delivery might be the most efficient. Clearly, the optimal time point for cell delivery after myocardial infarction remains unknown.

To date, very few studies have addressed the timing of BMC transplantation, and those studies have relied on post-mortem analysis such as real-time PCR[6] and immunohistochemistry[7]. These methods are highly dependent on the chosen time points of animal sacrifice and provide only a limited "snapshot" representation rather than a complete picture of cell survival over time. To overcome these issues, our group has been developing and validating imaging techniques for tracking transplanted stem cells *in vivo*[8]. In this study, we investigated the viability and effects of transplanted BMCs on cardiac function in the acute and sub-acute inflammatory phases of MI using molecular imaging techniques. In addition, we analyzed the phenotype of BMCs transplanted into acute inflammatory myocardium.

MATERIALS AND METHODS

Transgenic L2G animals expressing Fluc-GFP.

The donor group consisted of male L2G85 mice (8 weeks old), which were bred on FVB background and ubiquitously expressed green fluorescent protein (GFP) and firefly luciferase (Fluc) reporter genes driven by a β-actin promoter as previously described[9]. Recipient animals consisted of syngeneic, female FVB/NJ mice (8 weeks old, Jackson Laboratories, Bar Harbor, ME, USA). Animal care was provided in accordance with the Stanford University School of Medicine guidelines and policies for the use of laboratory animals.

Preparation of bone marrow mononuclear cells (BMCs).

BMCs were harvested from the long bones of male L2G85 transgenic mice and isolated by centrifugation in a density cell separation medium (Ficoll-Hypaque; GE Healthcare, Piscataway, NJ) prior to intramyocardial injection.

BMC proliferation assay.

Proliferation was determined by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. $5x10^5$ BMCs were plated in 100 µl IMDM (10% FBS) into a 96-well plate in triplicates and were incubated under normoxic ($95\%O_2/5\%CO_2$) and hypoxic ($1\%O_2/5\%CO_2/94\%N_2$) conditions for 32 hours. 20 µl of MTT was added to each well, followed by incubation for an additional 4 hours. Absorbance was determined with a multi-well absorbance reader (Genios, Tecan Systems Inc., San Jose, CA) at 490 nm using Magellon v6.2 software.

Surgical model for acute and subacute myocardial infarction.

Female FVB mice (8 weeks old) were intubated with a 20-gauge angiocath (Ethicon Endo-Surgery, Inc. Cincinnati, OH) and placed under general anesthesia with isoflurane (2%). Myocardial infarction (MI) was created by ligation of the mid-left anterior descending (LAD) artery with 8-0 ethilon suture through a left anterolateral thoracotomy as described[10]. In the acute MI model, both the infarct and peri-infarct regions were injected with 25 µL containing $2.5x10^6$ cells or saline immediately following MI using a Hamilton syringe with a 30-gauge needle. In the subacute model, BMCs were injected after re-thoracotomy on day 7 following MI. All surgical procedures were performed in a blinded fashion by one micro-surgeon (G.H.) with many years of experience on this model.

Flow cytometric analysis of cell and/or myocardial tissue.

Freshly isolated BMCs were washed and incubated with conjugated primary antibody for 45 min at 4˚C. For tissue analysis, hearts were surgically explanted, minced and digested for 2 hours in Collagenase D (2 mg/mL; Worthington Biochemical) at room temperature in RPMI

1640 media (Sigma Chemical Co.) with 10% fetal calf serum (FCS; Life Technologies). Myocardial cell suspensions were run through a 70-m cell strainer, washed in FACS buffer (PBS 2% FCS) and incubated with conjugated primary antibody for 45 min at 4˚C. For Troponin T staining, a 30-min incubation in cell permeabilization buffer was performed prior to antibody incubation. Finally, cells were washed, incubated with 7-amino-actinomycin D (7-AAD) cell viability solution (eBiosciences), and analyzed on a FACSCalibur system (BD Biosciences). The following antibodies were used in this study: APC-conjugated CD45 (clone: 30-F11), Gr-1 (RB6-8C5) and C-kit (2B8); Phycoerythin (PE)-conjugated Mac-1 (M1/70), Flk-1 (Avas 12α1) (BD Biosciences), Sca-1 (D7) and CD133 (13A4) (eBioscience); purified goat-anti Troponin T-C (C-19) (Santa Cruz Biotechnology) followed by Alexa Fluor 647 Chicken Anti-Goat IgG (Molecular probes)

***In vivo* optical bioluminescence imaging (BLI).**

BLI was performed using the IVIS 200 (Xenogen, Alameda, CA, USA) system. Recipient mice were anesthetized with isoflurane and placed in the imaging chamber. After acquisition of a baseline image, mice were intraperitoneally injected with D-Luciferin (400 mg/kg body weight). Mice were imaged on days 2, 4, 7, and weekly until sacrifice at week 6. BLI signal was quantified in units of maximum photons per second per centimeter square per steridian (photons/s/cm^2/sr) and presented as Log$^{[photons/s/cm2/sr]}$.

Echocardiography to assess left ventricular fractional shortening (LVFS).

Echocardiography was performed using the General Electric Vivid 7 Dimension imaging system equipped with a 13-MHz linear probe (General Electric, Milwaukee). Animals were induced with isoflurane, received continuous inhaled anesthetic (1.5%–2%) for the duration of the imaging session, and were imaged in the supine position. Echocardiography was performed by an independent operator (J.A.G.) blinded to the study conditions. M-mode short axis views of the left ventricle were obtained and archived. Analysis of the M-mode images was performed using GE built-in analysis software. Left ventricular end diastolic diameter (EDD) and end-systolic diameter (ESD) were measured and used to calculate fractional shortening (FS) by the following formula: FS = (EDD – ESD)/EDD.

***Ex vivo* TaqMan PCR.**

In our protocol, the transplanted cells were derived from male mice and were transplanted into female recipients, which facilitates quantification of male cells in the explanted female hearts by tracking the *Sry* locus found on the Y chromosome. Animals were sacrificed and hearts (n=3 per group) were explanted, minced, and homogenized in 2 mL DNAzol (Invitrogen, Carlsbad, CA, USA). The DNA was isolated according to the manufacturer's protocol. The DNA was quantified on a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and 500 ng DNA was processed for TaqMan PCR using primers specific for the *Sry*

locus. RT-PCR reactions were conducted in iCylcer IQ Real-Time Detection Systems (Bio-Rad, Hercules, CA, USA). Detection levels were compared to a standard curve to assess the number of viable cells per sample. All samples were conducted in triplets.

Tissue collection, immunofluorescent, and histological analysis.

Explanted hearts were fixed in 2% paraformaldehyde for 2 hours at room temperature and cryoprotected in 30% sucrose overnight at 4°C. Tissue was frozen in optimum cutting temperature compound (OCT compound, Sakura Finetek) and sectioned at 5 µm on a cryostat. Serial sections were blocked and incubated with rat anti-CD45 (clone 30-F11) (BD Biosciences) for 1 hour at room temperature, followed by goat anti-rat Alexa 594 (Molecular Probes) for 30 min. Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) and analyzed with a Leica DMRB fluorescent microscope (Leica Microsystems, Frankfurt, Germany). Hematoxylin and Eosin (H&E) staining (Sigma) was performed according to established protocols.

Statistical analysis.

Data are presented as mean ± SEM. Comparisons between groups were done by independent sample t-tests or analysis of variance (ANOVA) with LSD post hoc tests, where appropriate. Differences were considered significant for *P*-values <0.05. Statistical analysis was performed using SPSS statistical software for Windows (SPSS)

RESULTS

Quantification of acute myocardial inflammation following myocardial infarction in mice.

Acute MI triggers an acute inflammatory phase, dominated by infiltrating neutrophils that produce reactive oxygen species and proteases that cause cardiomyocyte injury. This is followed by a proliferative phase, in which infiltrating macrophages produce cytokines and growth factors that stimulate fibroblast proliferation and neovascularization[11]. After inducing MI in our mice, conventional histology showed a robust and progressive infiltration of inflammatory cells into the infarcted area over time, followed by scar formation and subsequent remodeling of the left ventricle (Fig 1A). To determine the transition of the inflammatory into proliferative phase, we performed a quantitative analysis of intra-myocardial infiltrating cell subsets using flow cytometry of enzymatically digested hearts. MI was created by LAD ligation in FVB mice (n=12), which were sacrificed on days 2, 4, 7, and 14 following MI (n=3 per group). Progressive infiltration of $CD45^+$ infiltrating leukocytes was found to peak on day 4 and day 7 following MI (Fig 1B and D). More specifically, early infiltration of $Mac\text{-}1^+Gr\text{-}1^{high}$ neutrophils was found to peak on day 4 (Fig 1C and E), whereas $Mac\text{-}1^+Gr\text{-}1^{low}$ macrophages

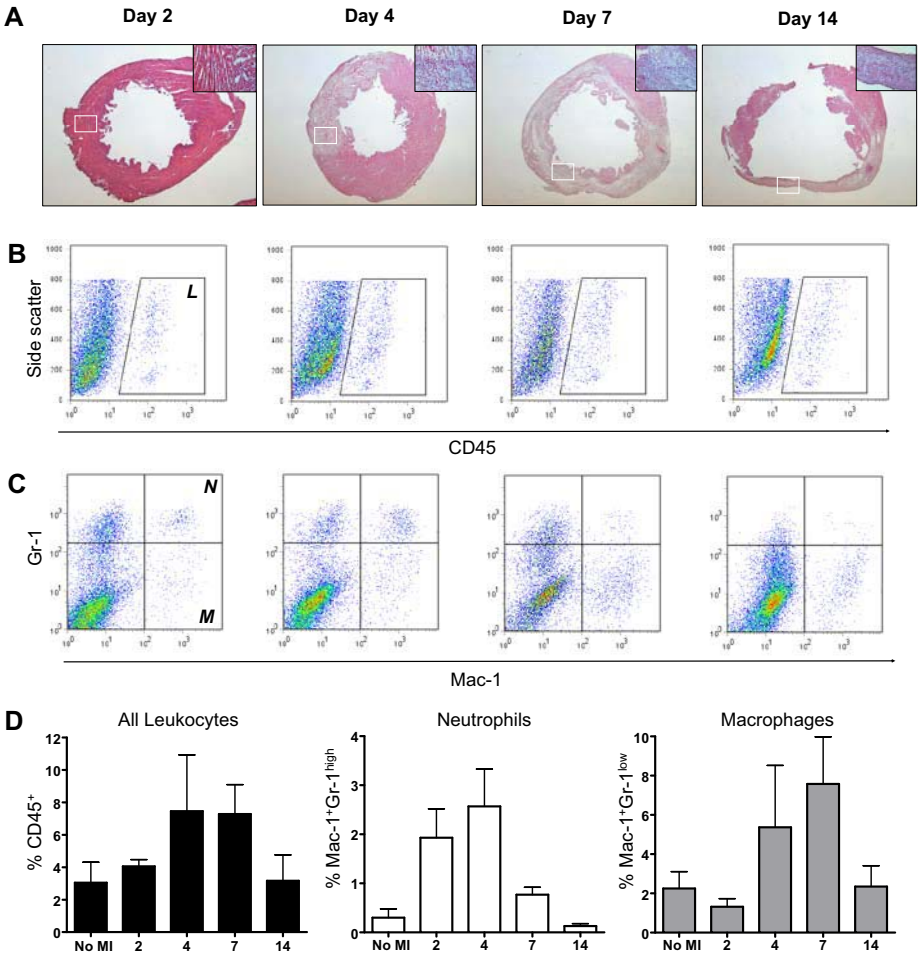

Figure 1. Quantification of myocardial inflammation following LAD ligation in FVB mice. (a) H&E staining performed on sections of the left ventricle at different time-points following LAD ligation shows increasing infiltration of mononuclear cells over time leading to ventricular remodeling. **(b)** Corresponding panels of flow cytometric analysis show that intramyocardial infiltration of $CD45^+$ leukocytes reaches a maximum at 4 to 7 days following LAD ligation. **(c)** More specifically, infiltration of $Mac\text{-}1^+Gr\text{-}1^{high}$ neutrophils (*N*) peaks on day 4, whereas $Mac\text{-}1^+Gr\text{-}1^{low}$ macrophages (*M*) peak on day 7 following LAD ligation. **(d)** Graphical representation of infiltration of inflammatory cell subsets.

infiltrated the heart most predominantly on day 7 (Fig 1C and F). These findings demonstrate that in our murine model, transition of the aforementioned phases occurs between days 4 and 7 following MI.

Characterization of $Fluc^+GFP^+$ BMC.

We have previously validated *in vivo* BLI as a reliable tool to monitor BMC engraftment into ischemic myocardium[12]. BLI measurements correlate highly with post-mortem methods of donor cell detection[12, 13]. BMC harvested from transgeneic L2G85 mice (FVB background)

exhibit a robust correlation between Fluc expression and BMC number (Fig 2A), as well as a strong expression of GFP (Fig 2B). FACS analysis confirmed the presence of stem/progenitor cells as well as adult hematopoietic cells within the BMC population (Fig 2C). The proliferation capacity of BMC was tested *in vitro*. Under hypoxic conditions, the cells showed robust proliferation when compared to BMCs kept under normoxic conditions (Fig 2D).

Effects of timing of BMC delivery following MI on cell viability.

To determine whether the survival of transplanted BMCs depends on the timing of delivery, FVB mice (n=24) were randomized into the following groups: (1) LAD ligation + immediate BMC injection (Acute BMC); (2) LAD ligation + BMC injection at 7 days post-MI (Sub-acute BMC); and (3) Sham surgery + BMC injection (BMC control). *In vivo* BLI showed an early signal

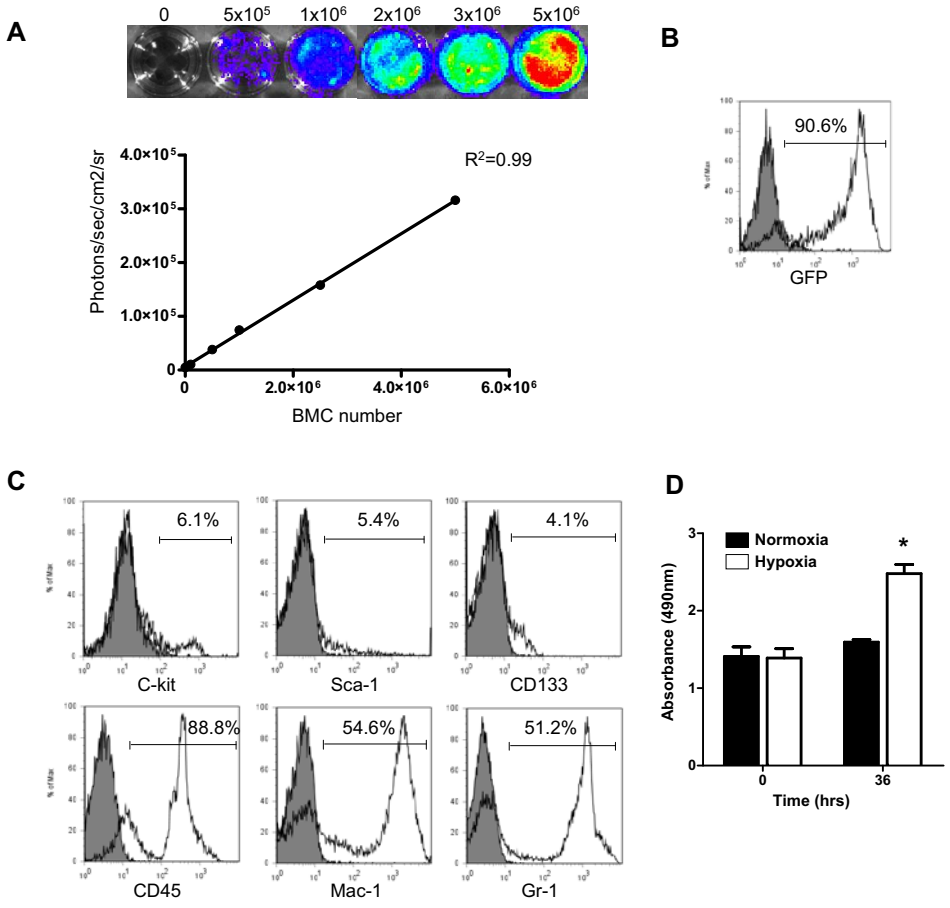

Figure 2. Characterization of firefly luciferase (Fluc) and green fluorescent protein (GFP)-positive BMCs. (a) *Ex vivo* BLI shows a robust correlation between cell number and reporter gene activity. **(b)** There is robust expression of GFP by BMCs. **(c)** Further characterization of the BMC subsets shows low numbers of stem/progenitor cells (Sca-1, c-kit, CD133) and high numbers of adult hematopoietic cells (CD45, Mac-1, Gr-1). **(d)** Viability and proliferation capacity of transgenic BMCs were confirmed *in vitro*. After 36 hours under hypoxic conditions, BMCs proliferated significantly more compared to BMCs that were maintained under normoxic conditions (*$P<0.01$).

increase in the Acute BMC group on day 7 (Acute BMC: 4.50±0.05 vs Sub-acute BMC: 4.34±0.05 $Log^{[photons/s/cm2/sr]}$; $P<0.05$), similar to validated findings in our previous study[12]. This suggests a significantly higher proliferation of donor BMCs delivered into acute MI milieu as compared to BMCs delivered in a sub-acute MI milieu. BLI further showed a trend (though not statistically

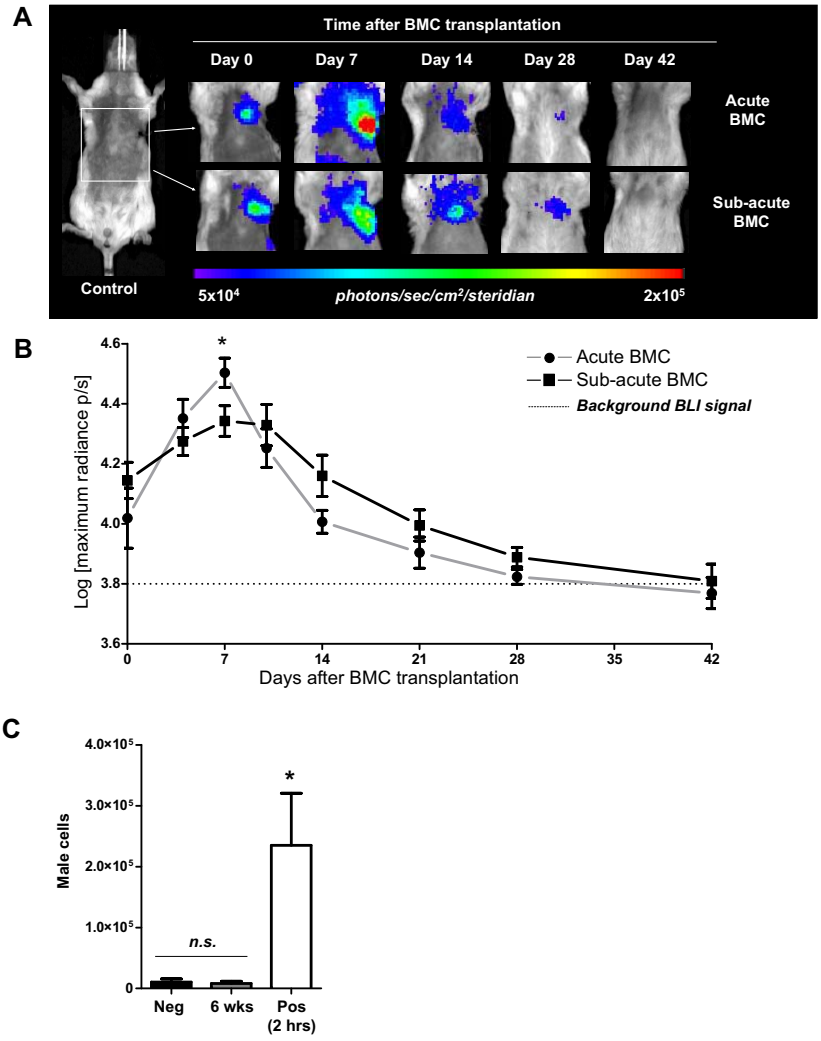

Figure 3. Longitudinal *in vivo* tracking of transplanted BMCs. (a) Representative BLI images of BMC transplanted animals either acutely (Acute BMC, upper panels) or 7 days after MI (Sub-acute BMC, lower panels) show proliferation of the cells early after transplantation. Thereafter, in both groups the BLI signal decreases gradually over time to reach background levels at day 42. Color scale bar values are in photons/s/cm^2/sr. **(b)** Graphical representation of longitudinal BLI shows a significantly increased signal intensity in the Acute BMC group at day 7 (*$P<0.05$), followed by a trend towards improved BMC survival in the Sub-acute BMC that persisted until BLI signal reached background levels at day 42 day. **(c)** *Ex vivo* quantitative TaqMan PCR found no significant differences when comparing negative control (Neg) and 6-week BMC-injected hearts. Significantly more male BMCs were found in the 2 hour positive control (Pos) hearts (n=3 per group, *n.s.* = not significant, *$P<0.001$).

significant) towards improved BMC survival in the Sub-acute BMC group that persisted until the BLI signal reached background levels in both groups at 42 days. No significant difference was in BLI signal was found between Sub-acute BMC and control BMC animals (not shown). These results suggest that, independent of timing of delivery, intramyocardial retention of BMC is limited to 6 weeks following transplant.

Ex vivo quantification of BMC survival.

To confirm BLI findings and to rule out the possibility that BMC death might have been caused by recipient immune response towards the reporter gene, we performed LAD ligation on an additional set of female FVB mice, which were randomized to receive $2.5x10^6$ non-labeled (wild-type) BMCs from male FVB donors (n=6) or saline (Negative control, n=3). BMC-injected animals were sacrificed at 2 hours (Positive control) and 6 weeks (n=3 per group). Their hearts were processed for quantitative TaqMan PCR analysis. Consistent with BLI data, there were no significant differences between 6-week BMC-injected hearts and saline-injected hearts, demonstrating that no male donor BMCs could be detected intramyocardially at the 6 week time-point. In comparison, significantly higher BMC numbers were found in 2 hour BMC hearts (Fig 3C).

Effects of timing of BMC delivery on preservation of cardiac function.

Preservation of cardiac performance was analyzed by echocardiography performed pre-operatively (base-line) and every 2 weeks following MI in the Acute and Subacute BMC animals, and compared to a control group receiving LAD ligation + saline injection (FVB, n=8). A representative M-mode tracing used for analysis is shown in Figure 4A. A significant preservation of fractional shortening was seen in BMC groups at 4 weeks (Acute BMC: 33.2±1.7% vs saline:

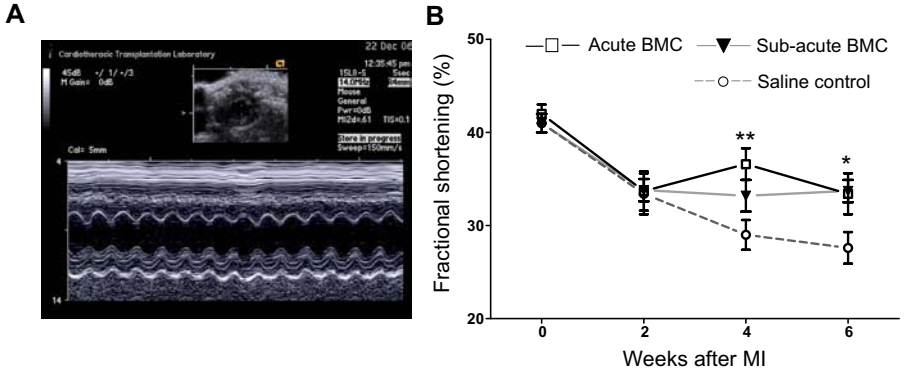

Figure 4. Echocardiographic assessment of cardiac function. **(a)** Representative M-mode echocardiogram at the level of the papillary muscle from which left ventricular diameters were measured. **(b)** Echocardiography revealed a significant preservation of left ventricular fractional shortening at 4 weeks in the Acute BMC group and 6 weeks in both BMC groups compared to saline control animals. No significant difference in cardiac performance was found between Acute and Sub-acute BMC animals. (*P<0.05, **P<0.01)

29.0±1.6%; *P*<0.01) and 6 weeks (Acute BMC: 33.7±1.2%; Sub-acute BMC: 33.4±2.2% vs saline: 27.6±1.7%; *P*<0.05 for both BMC groups vs. saline) following MI. However, no significant differences in cardiac contractility were found between both BMC groups during the 6-week study period (Fig 4B).

BMCs delivered into acute inflammatory environment adopt adult hematopoietic phenotypes.

In the first week following injection, BLI imaging showed a significantly higher proliferation rate of donor BMCs that were delivered into acute inflammatory myocardium, as compared to BMCs delivered into sub-acute inflammation (Fig 3A and B). Since BMCs represent a heterogeneous cell population, we aimed to investigate the phenotype of transplanted BMC at 7 days following injection into acute myocardial inflammation. LAD ligation + acute BMC (n=4) or saline (n=3) injection was performed in an additional set of FVB mice, which were sacrificed for heart procurement at 7 days post-transplant. Conventional H&E staining of sections of the left ventricle showed robust mononuclear cell infiltration and early signs of scar formation consistent with MI (Fig 5A). Immunofluorescent staining on a corresponding section revealed the presence of intramyocardial mononuclear cells, which appeared to be mostly of GFP$^+$ donor origin (Fig 5B). Interestingly, staining with the pan-leukocyte marker

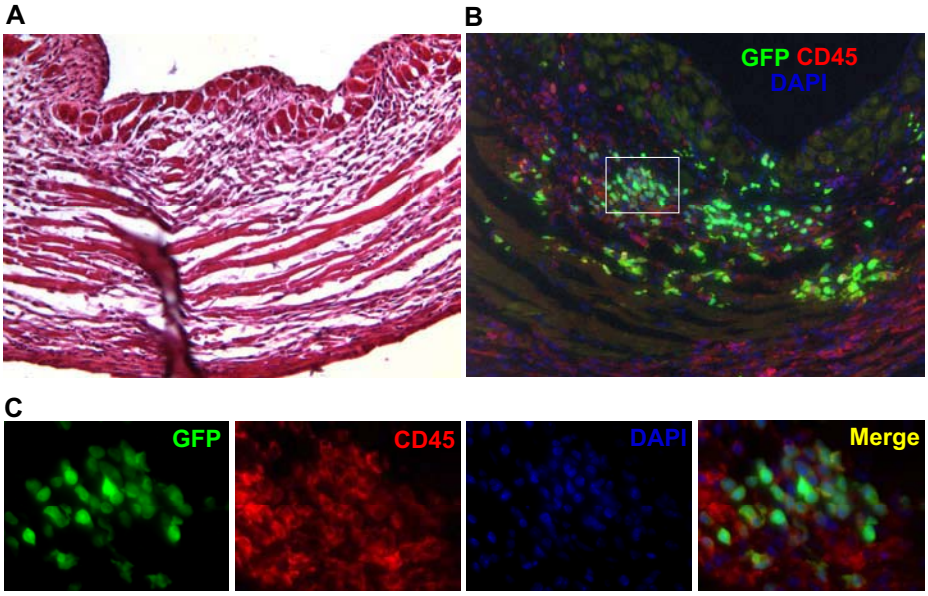

Figure 5. Immunohistochemical analysis of BMC transplanted hearts. (a) H&E staining of the left ventricular wall shows mononuclear cell infiltrates and scar formation consistent with myocardial infarction. **(b)** Immunofluorescent staining on a corresponding section reveals an abundant presence of GFP$^+$ BMCs (green) within the infarcted myocardium, which is rich in CD45$^+$ inflammatory cells (red). Counterstaining was performed with 4,6-diamidino-2-phenylindole (DAPI, blue). **(c)** High power views of the selected area (Fig 2B white square) reveal that the vast majority of donor BMCs express CD45 and retain round shapes with large nuclei, representing an inflammatory phenotype.

CD45 revealed that the vast majority of GFP+ donor cells co-expressed CD45 (Fig 5B and C). Next, we performed a systematic flow cytometric analysis on explanted hearts following enzymatic digestion (n=3 per group). Figure 6A shows representative flow cytometry panels confirming that GFP+ donor BMCs (right panel, arrow) co-expressed CD45, compared to saline-injected control hearts (left panel). Serial analysis showed that donor BMCs expressed CD45, Mac-1 and Gr-1, and minimal numbers of Sca-1 and c-kit, and remained negative for CD133, Flk-1 and/or Troponin (Trop)-T (Fig 6B). These results suggest that at 7 days following acute delivery, donor BMCs were mostly of adult hematopoietic phenotypes, and showed no signs of both endothelial (progenitor) cell (CD133 and Flk-1) and/or cardiomyocyte (Trop-T) differentiation.

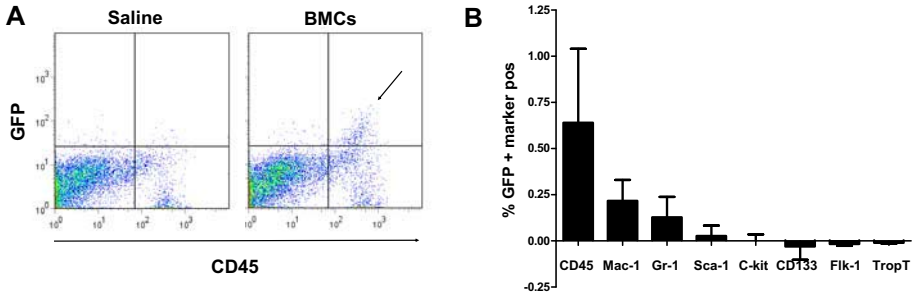

Figure 6. ***Ex vivo*** **phenotyping of donor BMCs confirms inflammatory phenotype. (a)** Representative flow cytometry panels of saline (left) and BMC (right) injected hearts at 7 days after acute BMC delivery. At this time-point GFP+ BMCs (arrow) co-express CD45, confirming their inflammatory phenotype. **(b) S**erial flow cytometric analysis reveals that GFP+ BMCs predominantly express inflammatory cell markers (CD45, Mac-1, Gr-1), rather then stem cell (Sca-1, c-kit), endothelial progenitor cell (CD133, Flk-1) or cardiomyocyte (TropT) markers. Data are presented as percentage of GFP/marker+ cells reduced by background staining.

DISCUSSION

Despite ongoing clinical trials, the optimal time-point of BMC delivery following acute MI remains a point of debate. A review of current literature reveals few studies that have systematically addressed the issue of timing of BMC transplantation[14]. This study was designed to determine the effects of timing of BMC delivery following acute MI in a standardized mouse LAD ligation model. Specifically, we have demonstrated that: (1) BMC transplantation in either acute or sub-acute myocardial infarction has a mildly positive effect on cardiac preservation, confirming earlier findings in animals models[15] and clinical trials[2]; (2) retention of BMC engraftment and preservation of cardiac function are not critically dependent on the timing of delivery; and (3) injection of BMCs into the acute inflammatory environment of myocardial infarction leads to early proliferation of donor cells that adopt adult hematopoietic phenotypes.

Earlier studies from our laboratory using *in vivo* BLI of transplanted BMCs revealed that the cells can effectively home in on and engraft into infarcted myocardium[12, 13]. Although the present study confirms the therapeutic effect of BMC transplantation in the setting of acute MI, it also clearly shows that delivery of the cells in the time-window following the hostile acute inflammatory phase–7 days after MI–does not result in extended long-term survival of donor BMCs. In addition, no significant differences were found in the preservation of cardiac function between BMCs injected groups during a 6-week period of observation.

To our knowledge, timing of BMC delivery has thus far not been investigated in experimental models. However, other cell populations, such as BM-derived mesenchymal stem cells[7] and fetal cardiomyocytes[16], showed therapeutic improvements in rat models when delivered in a the time-window of 1 to 2 weeks following MI. Most likely, the different observations made in our study are the results of the different cell populations used for transplantation. To represent the present clinical situation, we specifically used unfractioned BMCs. In addition to survival data, we show that the portion of BMCs responsible for cardiac preservation seem to be adult hematopoietic cells, rather than BM-derived endothelial, endothelial progenitor, or cardiac cells, at least following acute delivery at the time-point tested. These findings strengthen the hypothesis that preservation of cardiac performance by BMC transplantation might be attributable to modulation of the natural process of myocardial inflammation and infarct healing[17].

Investigations aimed to reveal the mechanism(s) by which stem cells might preserve cardiac function have been plenty. Early reports pointed toward the myocardial regeneration by repopulation of BM-derived endothelial cells and/or cardiomyocytes[18]; however, subsequent studies failed to support those observations[19]. Other proposed mechanisms include donor-host cell fusion and neovascularization by either vasculogenesis and/or secretion of paracrine factors leading to angiogenesis and arteriogenesis[20]. Recently, studies have focused on additional mechanisms of action of transplanted BMCs, which could be by a direct paracrine effect on the inflammatory cascade. Burchfield et al. recently reported evidence that BMCs mediate cardiac protection by release of the immunomodulatory cytokine IL-10, leading to decreased intramyocardial accumulation of T-lymphocytes, which translated into reduced LV remodeling[21]. Similarly, Ciulla et al. found transplanted BMCs to reduce serum levels of pro-inflammatory cytokines, which are known to contribute to myocardial apoptosis, necrosis, and scar formation[22]. These findings, combined with the results presented in the present study, suggest that BM progenitors could ameliorate LV remodeling following MI by continuing to differentiate along the hematopoietic lineage.

The clinical relevance of this study is significant. A recent meta-analysis of randomized clinical trials of BMC transplantation in patients suffering from acute MI found *no* significant difference in global LV function when the cells were delivered in either the <5-day or 5- to –30-day time windows[2]. The present study provides the experimental proof for these clinical findings. Specifically, we show that long-term survival and modest therapeutic efficacy

of BMCs seem to be relatively *independent* of timing of cell delivery. Clinically significant improvements of cardiac function in patients suffering from acute MI may thus be achieved by *repeated* cellular transplants, both in the acute and sub-acute phases of myocardial inflammation.

FUNDING SOURCES

This work was supported in part by NIH HL089027 and Burroughs Wellcome Foundation Career Award in Biomedical Sciences (to J.C.W), and by the ESOT-Astellas Study and Research Grant (RJS)

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