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

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

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) after left anterior descending artery ligation. Mac-1+ Gr-1+ neutrophil infiltration peaked at day 4. BMCs were harvested from transgenic FVB mice expressing firefly luciferase (Fluc) and green fluorescent protein (GFP). Afterward, 2.5 × 10⁶ BMCs were injected into the left ventricle of wild-type FVB mice either immediately (acute BMC) or 7 days (subacute BMC) after myocardial infarction, or after a sham procedure (n = 8 per group). In vivo bioluminescence imaging showed an early signal increase in both BMC groups at day 7, followed by a nonsignificant trend (P = 0.203) toward improved BMC survival in the subacute BMC group that persisted until the bioluminescence imaging signal reached background levels after 42 days. Compared with controls (myocardial infarction + saline injection), echocardiography showed a significant preservation of fractional shortening at 4 weeks (acute BMC versus saline; P < 0.01) and 6 weeks (both BMC groups versus saline; P < 0.05) but no significant differences between the 2 BMC groups. FACS analysis of BMC-injected hearts at day 7 revealed that GFP⁺ BMCs expressed hematopoietic (CD45, Mac-1, Gr-1), 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. (Circ Cardiovasc Imaging. 2010;3:77-85.)

Key Words: bone marrow mononuclear cells ■ myocardial infarction ■ delivery timing ■ bioluminescent imaging

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The potential mechanisms 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. After acute MI, a robust inflammatory response occurs that is necessary for healing and scar formation and contributes to cardiac remodeling. 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 environ-
ment for the injected cells. On the other hand, experimental studies have demonstrated that BMC transplantation can lead to a reduction of cardiomyocyte apoptosis, suggesting that early timing of cell delivery might be the most efficient. Clearly, the optimal time point for cell delivery after MI 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 polymerase chain reaction (PCR) and immunohistochemistry. 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. In this study, we investigated the viability and effects of transplanted BMCs on cardiac function in the acute and subacute 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. Recipient animals consisted of syngeneic, female FVB/NJ mice (8 weeks old, Jackson Laboratories, Bar Harbor, Maine). Animal care was provided in accordance with the Stanford University School of Medicine guidelines and policies for the use of laboratory animals.

Preparation of BMCs

BMCs were harvested from the long bones of male L2G85 transgenic mice (n = 12) and isolated by centrifugation in a density cell separation medium (Ficoll-Hypaque; GE Healthcare, Piscataway, NJ) before intramyocardial injection.

BMC Proliferation Assay

Proliferation was determined by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; 5 × 10^5 BMCs were plated in 100 μL IMDM (10% FBS) into a 96-well plate in triplicates and were incubated under normoxic (95% O2/5% CO2) and hypoxic (1% O2/5% CO2) 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 multiwell absorbance system with a standard curve to assess the number of viable cells per sample. All samples were conducted in triplets.

Flow Cytometric Analysis of Cell and Myocardial Tissue

Freshly isolated BMCs were washed and incubated with conjugated primary antibody for 45 minutes 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-μm cell strainer, washed in FACS buffer (PBS 2% FCS), and incubated with conjugated primary antibody for 45 minutes at 4°C. For Troponin T staining, a 30-minute incubation in cell permeabilization buffer was performed before 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 number of events was set at 20,000. Next, live cells were gated from dead cells and debris based on negative 7-AAD cell viability solution staining, as well as on side and forward scatter characteristics. The following antibodies were used in this study: APC-conjugated CD45 (clone: 30-F11), Gr-1 (RB6-8C5), and c-kit (2B8); Phycoerythrin (PE)-conjugated Mac-1 (M1/70), Fli-1 (Avas 12a1) (BD Biosciences), Sca-1 (D7), and CD133 (13A4) (eBiosciences); 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

Bioluminescence imaging (BLI) was performed using the IVIS 200 (Xenogen, Alameda, Calif) 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 wt). Mice were imaged on days 2, 4, 7, and weekly until killing at week 6. BLI signal was quantified in units of maximum photons per second per centimeter square per steridian (photons/s/cm^2/ster) and presented as Log [photons/s/cm^2/ster].

Echocardiography to Assess Left Ventricular Fractional Shortening

Echocardiography was performed using the General Electric Vivid 7 Dimension imaging system equipped with a 13-MHz linear probe (General Electric, Milwaukee, Wis). Animals were induced with isoflurane, received continuous inhaled anesthetic (1.5% to 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 by the following formula: Fractional shortening = (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 facilitate quantification of male cells in the explanted female hearts by tracking the Sry locus found on the Y chromosome. Animals were euthanized and hearts (n = 3 per group) were explanted, minced, and homogenized in 2 mL DNAzol (Invitrogen, Carlsbad, Calif). The DNA was isolated according to the manufacturer’s protocol. The DNA was quantified on a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del) and 500 ng DNA was processed for TaqMan PCR using primers specific for the Sry locus. Reverse transcription–PCR reactions were conducted in iCycler IQ Real-Time Detection Systems (Bio-Rad, Hercules, Calif). Detection levels were compared with a standard curve to assess the number of viable cells per sample. All samples were conducted in triplets.
Tissue Collection, Immunofluorescence Staining, 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 minutes. 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 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. BLI results were analyzed with a 3×8 repeated-measures ANOVA with group and days as fixed factors, with days as the repeated factor. A Greenhouse-Geisser correction was used for nonsphericity. Differences were considered significant for probability values <0.05. Statistical analysis was performed using SPSS statistical software for Windows (SPSS, Inc. Chicago, IL).

Results
Quantification of Acute Myocardial Inflammation After MI 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 (Figure 1A). To determine the transition of the inflammatory into proliferative phase, we performed a quantitative analysis of intramyocardial infiltrating cell subsets using flow cytometry of enzymatically digested hearts. MI was created by LAD ligation in FVB mice (n=12), which were euthanized on days 2, 4, 7, and 14 after MI (n=3 per group). Progressive infiltration of CD45+ infiltrating leukocytes was found to peak on day 4 and day 7 after MI (Figure 1B and 1D). More specifically, early infiltration of Mac-1+ neutrophils was found to peak on day 4 (Figure 1C and 1D), whereas Mac-1−Gr-1low macrophages infiltrated the heart most predominantly on day 7 (Figure 1C and 1D). These findings demonstrate that in our murine model, transition of the aforementioned phases occurs between days 4 and 7 after MI.

Characterization of Fluc+cGFP+ BMCs
We have previously validated in vivo BLI as a reliable tool to monitor BMC engraftment into ischemic myocardium.12,13 BLI measurements correlate highly with postmortem methods of donor cell detection.12,13 BMC harvested from transgenic L2G85 mice (FVB background) exhibit a linear relationship between Fluc expression and BMC number (Figure 2A), as well as a strong expression of GFP (Figure 2B). FACS analysis confirmed the presence of stem/progenitor cells as well as adult hematopoietic cells within the BMC population (Figure 2C). The proliferation capacity of BMC was tested in vitro. Under hypoxic conditions, the cells showed robust proliferation when compared with BMCs kept under normoxic conditions (Figure 2D).

Effects of Timing of BMC Delivery After 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 after MI (subacute BMC); and (3) sham surgery+BMC injection (BMC control). In vivo BLI showed an early signal increase in both BMC groups on day 7 (acute BMC: 4.50±0.05 versus subacute BMC: 4.34±0.05 Log10(photons/s/cm2/sr)), similar to validated findings in our previous study.12 Although there was a highly significant effect of day (P<.0001), there was no significant effect of group (P=0.717) nor group-by-day interaction (P=0.357). These results suggest that independent of timing of delivery, intramyocardial retention of BMCs is limited to ≈6 weeks after transplantation. In addition, we injected lower cell number (1×106, n=4) into the peri-infarct region immediately after MI. BLI data showed 2.44±0.7×10^3 (day 2), 5.68±1.34×10^3 (day 4), 5.70±0.6×10^3 (day 7), 4.63±0.9×10^3 (day 10), 6.45±2.01×10^3 (day 14), 4.23±0.4×10^3 (day 21), 2.45±0.2×10^3 (day 28), 1.73±0.3×10^3 (day 35), and 1.58±0.2×10^3 photons/s/cm²/sr (day 42). Overall, the cell survival followed a trend similar to that observed with transplantation of higher cell number (2.5×10^6). Specifically, an early signal increase followed by a progressive decline of BLI signal to background levels by ≈day 42. Thus, the poor donor cell survival also does not appear to be dependent on the number of cells that were injected.

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 toward the reporter gene, we performed LAD ligation on an additional set of female FVB mice, which were randomized to receive 2.5×10^6 nonlabeled (wild-type) BMCs from male FVB donors (n=6) or saline (negative control, n=3). BMC-injected animals were euthanized at 24 hours (positive control) and 6 weeks (n=3 per group). Their hearts were processed for quantitative TaqMan PCR analysis. Consistent with BLI data, only background levels of BMCs were detected 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, drastically higher BMC numbers were found in 24-hour relative to 6-week BMC-injected hearts (Figure 3C).

Effects of Timing of BMC Delivery on Preservation of Cardiac Function
Preservation of cardiac performance was analyzed by echocardiography performed preoperatively (baseline) and every 2 weeks after MI in the acute and subacute BMC animals, and compared with 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% versus saline: 29.0 ± 1.6%; P < 0.01) and 6 weeks (acute BMC: 33.7 ± 1.2%; subacute BMC: 33.4 ± 2.2% versus saline: 27.6 ± 1.7%; P < 0.05 for both BMC groups versus saline) after MI. However, no significant differences in cardiac contractility were found between both BMC groups during the 6-week study period (Figure 4B). Similarly, no significant difference in ESS and/or EDD were found between BMC groups at the time points measured (data not shown).

BMCs Delivered Into Acute Inflammatory Environment Adopt Adult Hematopoietic Phenotypes

In the first week after injection, BLI imaging showed a significantly higher proliferation rate of donor BMCs that were delivered into acute inflammatory myocardium, as

Figure 1. Quantification of myocardial inflammation following LAD ligation in FVB mice. A, Hematoxylin and eosin staining performed on sections of the left ventricle at different time points after LAD ligation shows increasing infiltration of mononuclear cells over time leading to ventricular remodeling (magnification ×16). Higher magnification images (×200) taken from the area within the white boxes are shown in the upper right corner of each image. B, Corresponding panels of flow cytometric analysis show that intramyocardial infiltration of CD45⁺ leukocytes reaches a maximum at 4 to 7 days after LAD ligation. C, More specifically, infiltration of Mac-1⁻Gr-1(high) neutrophils (N) peaks on day 4, whereas Mac-1⁻Gr-1(low) macrophages (M) peak on day 7 after LAD ligation. D, Graphical representation of infiltration of inflammatory cell subsets. Error bars indicate standard error of the mean.
compared with BMCs delivered into subacute inflammation (Figure 3A and 3B). Because BMCs represent a heterogeneous cell population, we aimed to investigate the phenotype of transplanted BMC at 7 days after 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 euthanized for heart procurement at 7 days after transplant. Conventional hematoxylin and eosin staining of sections of the left ventricle showed robust mononuclear cell infiltration and early signs of scar formation consistent with MI (Figure 5A). Immunofluorescent staining on a corresponding section revealed the presence of intramyocardial mononuclear cells, which appeared to be mostly of GFP+ donor origin (Figure 5B).

The majority of cells which were GFP+ also coexpressed the pan-leukocyte marker CD45 (Figure 5B and 5C, controls included in supplemental Figure 1). Next, we performed a systematic flow cytometric analysis on explanted hearts after enzymatic digestion (n=3 per group). Figure 6A shows representative flow cytometry panels confirming that the
majority of GFP\(^+\) donor BMCs (upper two quadrants) coexpressed CD45 (upper right quadrant, arrow). Serial analysis showed that GFP\(^+\) 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 (Figure 6B). These results suggest that at 7 days after acute delivery, some of the donor BMCs adopted an adult hematopoietic phenotype but showed no significant differentiation into endothelial (progenitor) cell (CD133 and Flk-1) and/or cardiomyocyte (Trop-T) lineages.

**Discussion**

Despite ongoing clinical trials, the optimal time point of BMC delivery after 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.\(^1\) This study was designed to determine the effects of timing of BMC delivery after acute MI in a standardized mouse LAD ligation model. Specifically, we have demonstrated that: (1) BMC transplantation in either acute or subacute myocardial infarction has a mildly positive effect on cardiac preservation, confirming earlier findings in animal models\(^1\) 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 inflammatory environment of myocardial infarction leads to early proliferation of donor cells, some of which 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.\textsuperscript{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 after 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 BMC-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 bone marrow–derived mesenchymal stem cells\textsuperscript{7} and fetal cardiomyocytes,\textsuperscript{16} showed therapeutic improvements in rat models when delivered in a time window of 1 to 2 weeks after 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 unfractionated BMCs. In addition to the survival data, we show that some of the BMCs that engraft intramyocardially appear to be of adult hematopoietic cell phenotype rather than bone marrow–derived endothelial, endothelial progenitor, or cardiac cells, at least after 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.\textsuperscript{17} These experiments, however, only provide insight into the phenotype of the transplanted GFP\textsuperscript{+} donor BMCs, not at the endogenous BMC recruitment process, paracrine action of these cells, or their potential capability to activate resident progenitor cells that may aid in the cardiac repair process.

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 bone marrow–derived endothelial cells and/or cardiomyocytes\textsuperscript{18}; however, subsequent studies failed to support those observations.\textsuperscript{19} Other proposed mechanisms

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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 with saline control animals. No significant difference in cardiac performance was found between acute and subacute BMC animals. Error bars indicate standard error of the mean. *$P<0.05$, **$P<0.01$.

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Figure 5. Immunohistochemical analysis of BMC transplanted hearts. A, Hematoxylin and eosin 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\textsuperscript{+} BMCs (green) within the infarcted myocardium, which is rich in CD45\textsuperscript{+} inflammatory cells (red). The majority of the GFP\textsuperscript{+} cells coexpressed CD45, confirming an inflammatory phenotype. Counterstaining was performed with 4,6-diamidino-2-phenylindole (DAPI, blue). C, High-power views of the selected area (Figure 2B, white square) reveal that the majority of donor BMCs express CD45 and retain round shapes with large nuclei, representing an inflammatory phenotype.
include donor-host cell fusion and neovascularization by either vasculogenesis and/or secretion of paracrine factors leading to angiogenesis and arteriogenesis. 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.21 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 left ventricular remodeling. Similarly, Ciulla et al.122 found transplanted BMCs to reduce serum levels of proinflammatory cytokines, which are known to contribute to myocardial apoptosis, necrosis, and scar formation. These findings, combined with the results presented in the present study, suggest that BM progenitors could ameliorate left ventricular remodeling after 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 with acute MI found no significant difference in global left ventricular function when the cells were delivered in either less than 5 days or the 5- to 30-day window period.2 The present study provides similar conclusions to the majority of clinical studies analyzed by Abdel-Latif et al.2 Specifically, long-term survival and modest therapeutic efficacy of BMCs appear to be relatively independent of timing of cell delivery. Clinically significant improvements of cardiac function in patients with acute MI may be achieved by repeated cellular transplants, both in the acute and subacute phases of myocardial inflammation. Further studies will be needed to test this hypothesis.

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Disclosures
None.

References
In the majority of clinical trials investigating cardiac stem cell–based therapy, autologous bone marrow mononuclear cells (BMCs) have been the most common chosen cell type. However, a clinical controversy exists regarding the optimal time point to transplant BMCs after myocardial infarction. In the acute phase after myocardial infarction, a robust inflammatory response occurs that may jeopardize the viability of transplanted cells compared with the subacute phase. Yet, the majority of cardiomyocyte apoptosis actually occurs in the earlier stages after myocardial infarction. Experimental studies have demonstrated that BMC transplantation can lead to a reduction of cardiomyocyte apoptosis, which points to the importance of determining the exact timing of optimal cell delivery. In this study, we address this critical issue by using molecular imaging technology to investigate the viability of transplanted BMCs in both acute and subacute myocardial infarction settings and to determine their subsequent effects on cardiac recovery.
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Supplemental Figure 1. Immunofluorescent controls for CD45 staining. (a) Representative image of negative control staining for CD45 on a mouse heart slide; only very few CD45\(^+\) cells reside within the healthy myocardium. (b) Positive control for CD45 staining on a mouse spleen slide; abundant presence of CD45\(^+\) cells is seen.