Aging Impairs the Beneficial Effect of Granulocyte Colony–Stimulating Factor and Stem Cell Factor on Post–Myocardial Infarction Remodeling

Stephanie Lehrke, Ramesh Mazhari, Daniel J. Durand, Meizi Zheng, Djahida Bedja, Jeffrey M. Zimmet, Karl H. Schuleri, Andrew S. Chi, Kathleen L. Gabrielson, Joshua M. Hare

Abstract—Granulocyte colony–stimulating factor (G-CSF) and stem cell factor (SCF) are potential new therapies to ameliorate post–myocardial infarction (post-MI) remodeling, as they enhance endogenous cardiac repair mechanisms and decrease cardiomyocyte apoptosis. Because both of these pathways undergo alterations with increasing age, we hypothesized that therapeutic efficacy of G-CSF and SCF is impaired in old versus young adult rats. MI was induced in 6- and 20-month-old rats by permanent ligation of the left coronary artery. In young animals, G-CSF/SCF therapy stabilized and reversed a decline in cardiac function, attenuated left ventricular dilation, decreased infarct size, and reduced cardiomyocyte hypertrophy. Remarkably, these effects on cardiac structure and function were absent in aged rodents. This could not be attributed to ineffective mobilization of bone marrow cells or decreased quantity of c-Kit+ cells within the myocardium with aging. However, whereas the G-CSF/SCF cocktail reduced cardiac myocyte apoptosis in old as well as in young hearts, the degree of reduction was substantially less with age and the rate of cardiomyocyte apoptosis in old animals remained high despite cytokine treatment. These findings demonstrate that G-CSF/SCF lacks therapeutic efficacy in old animals by failing to offset periinfarct apoptosis and therefore raise important concerns regarding the efficacy of novel cytokine therapies in elderly individuals at greatest risk for adverse consequences of MI. (Circ Res. 2006;99:553-560.)

Key Words: aging ■ myocardial infarction ■ remodeling ■ growth substances

The administration of cytokines such as granulocyte colony–stimulating factor (G-CSF) and stem cell factor (SCF) is proposed as a potential new therapy for cardiovascular regenerative medicine. Animal studies demonstrate that G-CSF alone or in combination with SCF ameliorate post–myocardial infarction (post-MI) remodeling.1–4 Early clinical trials appeared to support these findings,5–7 and initial concerns of stimulating restenosis in the infarct-related artery8 have not been substantiated.9,10 However, a recent randomized controlled trial in patients with a mean age of 60 years of age failed to demonstrate benefit from G-CSF administration following MI, despite ample CD34+ cell mobilization.11

One of the main mechanisms by which G-CSF and SCF exert favorable effects on cardiac remodeling is enhancement of endogenous cardiac repair mechanisms that include both bone marrow stem cell mobilization, engraftment, and differentiation12–15 as well as proliferation of cardiomyocytes.16 In addition, G-CSF inhibits cardiomyocyte apoptosis17 and accelerates healing by stimulating absorption of necrotic tissue and reducing granulation and scar tissue via expression of matrix metalloproteinases.3

It is important to address whether potentially new cardiac regenerative therapies will benefit elderly patients who, despite reperfusion therapy, have increased MI-related mortality and morbidity.18,19 This higher vulnerability can only partially be attributed to comorbidities, and there is increasing evidence for impaired endogenous cardiac repair mechanisms with aging. Increased age is associated with reduced angiogenic capacity20 and diminished cell cycling of cardiac stem cells.21,22 In addition, there is increased cardiomyocyte apoptosis in the aged heart both at baseline23 and after ischemia, which could contribute to the adverse prognosis of elderly individuals.24,25

Given emerging evidence of impaired regenerative capacity of the aging heart coupled with its higher propensity for apoptotic cell death, the key question arises of whether these defects, which are the target mechanisms for the cardioprotective effect of G-CSF/SCF, will attenuate its efficacy. Here
using an established rodent model of cardiovascular aging we tested the hypothesis that G-CSF/SCF would have reduced efficacy in old compared with young adult animals because of impaired target mechanisms.

**Materials and Methods**

All procedures in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) and were approved by the Johns Hopkins University Animal Care and Use Committee.

**Rat MI Model**

MI was induced by permanent left coronary artery ligation in 6- and 20-month-old F344 rats purchased from the National Institute on Aging. Twenty-month-old Fisher rats are shown to have physiological signs of cardiovascular aging and correspond approximately to humans of 50 to 60 years of age.26,27 Rats were intubated endotracheally and ventilated with 2% isoflurane in oxygen using a rodent respirator (Model 683, Harvard Apparatus Inc). The heart was exposed via a left lateral thoracotomy, and the left anterior descending coronary artery was ligated 3 mm below its origin with a 6-0 silk suture.

Four hours post-MI, rats were randomly assigned to receive a first subcutaneous injection of recombinant human G-CSF (100 µg/kg) and rat-SCF (200 µg/kg; both Amgen, Thousand Oaks, Calif) followed by daily injections for 5 days. Placebo animals received equal volumes of isotonic saline. Rats (n=29, young; n=25, old) were followed by serial echocardiography for the development of cardiac function and geometry. The echocardiographer was fully blinded to the treatment group. The animals were allocated into treatment (n=15, young; n=12, old) and placebo (n=14, young; n=13, old) groups. A second subset of animals was euthanized 7 days after MI for the assessment of circulating c-kit+ and CD34+ cells and myocardial apoptosis and were allocated into treatment (n=5, young; n=5, old) and placebo (n=5, young; n=6, old).

Mortality within 48 hours of MI was higher in 20-month-old compared with 6-month-old Fisher rats (complete weight, g/kg; 338±11 vs 338±7, P=0.0001; absolute weight, g/kg; 12 365±16 vs 16 407±12, P=0.0001; body weight, g/kg; 338±11 vs 338±7, P=0.0001; LVESD, mm; 6.0±0.2 vs 6.0±0.2, P=0.0001; LVESD, mm; 2.9±0.3 vs 2.7±0.4, P=0.0001; AWT diast, mm; 2.1±0.1 vs 2.2±0.1, P=0.0001; PWT diast, mm; 2.2±0.1 vs 2.3±0.1, P=0.0001; FS, %; 52.4±5.6 vs 55.5±5.4, P=0.0001; 2D EF, %; 68.3±3.7 vs 69.7±3.0, P=0.0001; EDV, mL; 0.23±0.01 vs 0.23±0.02, P=0.0001; ESV, mL; 0.08±0.01 vs 0.06±0.01, P=0.0001; Young Old

HR, bpm 372±16 407±9 360±12 365±16
Bodyweight, g 338±11 338±7 410±8* 405±5*
LVESD, mm 6.0±0.2 6.0±0.2 6.3±0.3 6.0±0.3
LVESD, mm 2.9±0.3 2.7±0.4 2.8±0.5 2.6±0.5
AWT diast, mm 2.1±0.1 2.2±0.1 2.5±0.1 2.4±0.1
PWT diast, mm 2.2±0.1 2.3±0.1 2.3±0.1 2.3±0.1
FS, % 52.4±5.6 55.5±5.4 58.0±7 58.3±7
2D EF, % 68.3±3.7 69.7±3.0 76.5±4.0 72.2±3.3
EDV, mL 0.23±0.01 0.23±0.02 0.25±0.02 0.2±0.02
ESV, mL 0.08±0.01 0.06±0.01 0.06±0.01 0.1±0.01

HR indicates heart rate; LVESD, left-ventricular end-diastolic diameter; LVESD, left-ventricular end-systolic diameter; AWT diast, anterior wall thickness in diastole; PWT diast, posterior wall thickness in diastole; FS, fractional shortening; EDV, end-diastolic volume; ESV, end-systolic volume. *P<0.0001 vs young G-CSF/SCF and placebo.

**Echocardiography**

Transthoracic echocardiograms were obtained using a Sonos 5500 (Agilent, Hewlett Packard, Palo Alto, Calif) equipped with a 15-MHz linear transducer. For additional details, see the online data supplement, available at http://circres.ahajournals.org.

**Complete Blood Count/Flow Cytometric Analysis of CD34+ and c-Kit+ Cells**

Complete white blood counts and numbers of circulating CD34+ and c-kit+ cells were determined at baseline and at day 7 after MI and cytokine administration by FACS analysis (Becton Dickinson). For additional details, see the online data supplement.

**Histology**

Infarct size and myocyte width in noninfarcted remote myocardium were determined from explanted hearts. Immunohistochemistry for c-Kit (DAKO rabbit anti-human CD117 antibody; A 4502) and mast cells were performed using deparaffinized and rehydrated tissue sections. Apoptosis was quantified by TUNEL analysis. For additional details, see the online data supplement.

**Western Blot Analysis**

Western blots were performed on heart extracts as described by Razu et al. using polyclonal anti–phospho-STAT3 antibody (1:1000, Cell Signaling Technology), polyclonal anti–phospho-ERK antibodies (1:1000, Cell Signaling Technology Inc) and anti–phospho-caspase-3 (SC-7148, Santa Cruz Biotechnology). Immunoblots were detected using enhanced chemiluminescent Kits (SuperSignal; Pierce) and analyzed with a densitometer (Bio-Rad). Membranes were stripped and reprobed with polyclonal anti-STAT3 antibody (1:1000, Cell Signaling Technology), polyclonal anti-ERK antibodies (1:1000, Cell Signaling Technology Inc), or monoclonal anti-GAPDH antibodies (1:10 000, Research Diagnostics Inc).

**Results**

**Cardiac Function and Structure**

Echocardiographic parameters at baseline and at 2 days after MI were not different between age and treatment groups (Table 1). Importantly, at day 2 after MI, 2D ejection fraction (EF) showed a similar decline in all 4 groups, suggesting a comparable degree of initial myocardial injury (Table 1 and Figure 1A).

Induction of MI resulted in a substantial impairment of cardiac function, as left ventricular EF decreased significantly during the first 2 weeks in all groups (P<0.0001) without any effects of cytokine treatment or age (P=NS). Whereas ventricular function remained severely impaired during the 8-week follow-up period among young placebo animals, there was a dramatic improvement of EF from week 2 to 8 among young G-CSF/SCF–treated rats (P=0.0001; absolute increase, 22.5±3.8%), reversing the initial severe left ventricular dysfunction (Figure 1A) and representing an almost 50% recovery. Remarkably, this effect of cytokine treatment was completely absent in old animals, where EF in both treatment and placebo groups deteriorated similarly to young placebo animals and remained low until week 8 (Figure 1B).

Similarly, whereas the G-CSF/SCF cocktail ameliorated left ventricular enlargement in young animals (P=0.003), this effect was completely absent in old animals (Figure 1C and 1D).

**Effect of G-CSF/SCF on Infarct Size and Compensatory Cardiomyocyte Hypertrophy**

Induction of MI resulted in the development of substantial transmural scarring of the left ventricle. Infarct size expressed
as percentage of left ventricular perimeter was comparable between young placebo (26.1±3.1%), old G-CSF/SCF–treated (27±6%) and old placebo animals (28.1±4.9%) but was significantly reduced in young G-CSF/SCF–treated rats (14.7±3.5%; \( P=0.04 \) versus young placebo; Figure 2A).

Reactive hypertrophy of cardiomyocytes in areas remote from the scar region was evident in both age groups. Whereas the cytokine treatment completely prevented this compensatory myocyte hypertrophy in young animals (\( P<0.0001 \) versus young placebo; Figure 2B), it was without effect in the old rats.

**White Blood Count/Circulating CD34+ and c-Kit+ Cells**

Age-associated hematological changes at baseline are summarized in Table 2. These findings are in accordance with those reported previously.\(^29\) The percentage of circulating mononuclear cells expressing CD34 (0.45±0.04% young versus 0.55±0.07% old) and c-Kit (0.36±0.07% young versus 0.46±0.07% old) did not differ significantly between age groups at baseline. At 1 week post-MI, the neutrophil count was markedly elevated in G-CSF/SCF–treated groups in both young (2.17±0.25 \( \times \) 10\(^6\) /µL versus 0.87±0.05 \( \times \) 10\(^6\) /µL at baseline, \( P=0.008 \)) and old (3.16±0.69 \( \times \) 10\(^6\) /µL versus 1.19±0.28 \( \times \) 10\(^6\) /µL at baseline, \( P=0.04 \)) animals. Whereas the percentage of CD34+ cells was increased compared with baseline in all groups regardless of treatment, the percentage of circulating c-Kit+ cells remained relatively unchanged in all groups (Figure 3A and 3B). See Figure 1 in the online data supplement.

Interestingly, G-CSF/SCF treatment increased splenic weight among young (996±42 mg treated versus 828±25 mg placebo; \( P<0.01 \)) but not among old rats (1570±157 mg treated versus 1345±124 mg placebo; \( P=NS \)). This difference remained significant after correction for body weight.

**c-Kit+ Cells in the Peri-infarct Area**

Next we examined the effect of G-CSF/SCF on the number of c-Kit+ cells in the infarct region. Cells expressing membrane c-Kit were detected in the peri-infarct zone at day 7 after MI and were 2-fold more abundant in young versus old animals, although this did not reach statistical significance. Cytokine treatment increased c-Kit+ cells in young (\( \approx 14\)-fold; \( P<0.01 \) versus young placebo) and old (\( \approx 17\)-fold; \( P<0.05 \) versus old placebo; Figure 4A) rats. Although in old rats, it did not reach a level exceeding that of young rats. Thus, the lack of response to G-CSF/SCF in old rats is not attributable to the absence of c-Kit+ cell mobilization within the heart. At 8 weeks after MI, cardiac c-Kit+ cells were essentially undetectable in all groups (data not shown).

**Effect of G-CSF/SCF on Myocyte Apoptosis**

As identified by TUNEL staining, cardiomyocyte apoptosis in the infarct border zone as well as in remote areas was significantly increased with old age in both placebo and treatment groups. G-CSF/SCF treatment reduced cardiomyocyte apoptosis in the infarct border zone in both young (28±1.7% versus 36.4±2.8% young placebo; \( P<0.05 \)) and old (41.0±2.9% versus 51.3±2.5% old placebo; \( P<0.01 \); Figure 5A) animals. In areas remote from the infarct, cytokine treatment diminished cardiac myocyte apoptosis in young animals, from 37.8±3.5% to 25.7±1.4% (\( P<0.01 \)), but did not reduce apoptosis in old animals (Figure 5B). Importantly, old G-CSF/SCF–treated rats had higher apoptotic indices than young placebo animals. Thus, the response to G-CSF/SCF was profoundly impaired in old versus young hearts.

**Western Blot Analysis**

Western blot analysis of the molecular pathways involved in apoptosis revealed increased activity of STAT3, a pathway implicated in G-CSF cardiac protection, following G-CSF/
SCF treatment in young but not old infarcted rats (Figure 6A and 6B). On the other hand, activation of the proapoptotic caspase-3 pathway was greater in old versus young rats after MI ($P<0.05$ for old cytokines versus no cytokines). Importantly, G-CSF/SCF treatment did not reduce caspase-3 activation in old rats following MI (Figure 6C and 6D). Finally, the extracellular signal-regulated kinase (ERK) pathway was activated after MI in both old and young rats ($P<0.01$ in young and $P<0.02$ in old rats), and this was not affected by cytokine treatment (Figure 6E and 6F).

**Discussion**

The major new finding of this study is that the efficacy of G-CSF and SCF to attenuate post-MI remodeling is dramatically reduced with age. Whereas in young animals, the cytokine treatment markedly improved cardiac function, decreased infarct size, and ameliorated cardiomyocyte hypertrophy in residual myocytes, these effects were absent in post-MI aged rodents. Importantly, this was not attributable to reduced mobilization of stem cells in the circulation or heart. Rather, the degree of reduction in apoptosis was substantially less in old versus young hearts. In light of ongoing clinical trials, these findings have important implications for assessing novel therapies in elderly individuals at greatest risk for adverse consequences of MI.

Our findings in young animals agree with those of Orlic et al, who first demonstrated the potential of G-CSF/SCF to repair cardiac injury by mobilization of bone marrow stem cells. A number of subsequent animal studies confirmed the healing effect of G-CSF given alone or in combination with SCF and suggested additional mechanisms by which the cardioprotective effect is achieved, including antiapoptotic...
enhanced cardiomyocyte proliferation, and side population cell mobilization. Dawn et al have recently shown that either SCF or Flt-3 ligand added to G-CSF are superior to G-CSF alone in reversing post-MI remodeling and causing bone marrow mobilization.

This study offers important new insights with regard to age as a host determinant limiting responsiveness to G-CSF/SCF. MI increases in frequency as a function of age, and the elderly also experience higher mortality and morbidity. The aged heart shows marked physiological and structural alterations and varies significantly in its response to different stressors, including ischemia. Compared with young hearts, aged hearts exhibit an elevated rate of apoptotic cardiomyocytes both at baseline and after ischemia/reperfusion injury. A lower capacity for endogenous cardiac repair with aging is supported by reports of reduced ability for neoangiogenesis and cardiac stem cell senescence. G-CSF and SCF exert their effects by targeting these mechanisms that are altered in the aging heart.

The mobilization of bone marrow stem cells, their homing to the injured heart, and consequent cardiac regeneration are the main underpinnings of the beneficial effect of G-CSF/SCF on cardiac remodeling. Advanced age is a well-known limiting factor for the mobilization of bone marrow cells in donors being treated with G-CSF for leukopheresis of hematopoietic progenitor cells. We excluded poor bone marrow cell mobilization as the reason for the lack of effect seen in aged animals by demonstrating similar increases of neutrophils and circulating CD34 cells. Furthermore, the number of c-Kit cells in the peri-infarct area was similarly elevated by G-CSF/SCF treatment regardless of age. These cells have the potential to originate either from bone marrow or from the heart itself, where they represent endogenous cardiac stem cells. There is increasing support for the presence of an
endogenous population of c-Kit+/H11001 cardiac stem cells that proliferate in response to injury and that may be targets for novel approaches of activation.37

There is also evidence that cardiac stem cells, depleted after MI, are replenished by both endogenous proliferation and mobilization of bone marrow–derived stem cells. In this regard, bone marrow–derived mesenchymal stem cells have lower colony formation activity in old compared with young rats.38,39 In addition to endogenous deficits in either cardiac or bone marrow–derived cardiac precursor cells, reduced responsiveness to cellular regeneration may be another important mechanism for impaired cardiac repair in old adult animals. Support for this view is emerging, with data showing reduced responsiveness to cardiac regenerative therapy with intravenous MSC infusion in old versus young rats.40 Work is ongoing to explore these different potential mechanistic components.

In addition to ischemia induced necrosis, apoptosis contributes to cardiomyocyte loss after MI.23,41–43 Experimental studies show that apoptosis of cardiomyocytes remains elevated for months after the infarct and correlates with postinfarction left-ventricular remodeling.44 G-CSF is directly antiapoptotic in cardiomyocytes, endothelial cells, and myofibroblasts,17 activating its myocardial cell-surface receptor and leading, in turn, to JAK/Stat pathway activation and stimulation of the antiapoptotic protein bcl-2. In line with these findings, we demonstrated a reduction of apoptotic cells associated with Stat3 phosphorylation by G-CSF/SCF treatment in young animals. Whereas apoptosis of cardiomyocytes in infarct, border zone, and remote myocardium was reduced in young animals, only cardiomyocyte apoptosis in the infarct border zone was decreased in old animals. Additionally, G-CSF/SCF did not activate Stat3 or reduce phospho–caspase-3 in old hearts. Furthermore, apoptotic indices in old treated animals equaled or exceeded those of young control animals.

Whereas G-CSF/SCF increased c-Kit+/H11001 cells in the injured myocardium in old animals, they failed to reduce cardiomyocyte apoptosis to levels seen in young animals. In the context of a balance between the potential for new tissue formation through c-Kit+/H11001 cells and cardiomyocyte loss through apoptosis, the latter outweighs the former in aged animals, likely explaining the lack of cardioprotective effect in the old.

There is increasing evidence for reduced efficacy of other cytokine pathways with age, including reduced platelet-derived growth factor (PDGF)-AB–induced neoangiogenesis45 (which can be rescued by endothelial progenitor cells from young animals46) and reduced tumor necrosis factor (TNF)-α cardioprotection attributable to downregulated TNF-α receptors.46 Cytokine receptors are downregulated with aging in organs other than the heart,47 and a similar phenomenon could contribute to the present results.

Intriguingly, data from several human trials of G-CSF therapy following MI show a variance that may be related to the mean age of the study subjects. For example, 3 studies,5,9,11 each with subjects of a mean age of 60 years or more, found no statistically significant benefits in any objectively measured parameter. In these trials, the only significant clinical benefit was a reduction in subjective symptoms of myocardial ischemia in the 13 patients reported by Wang et al.5 In light of our results, it is interesting to note that the largest of these studies11 showed that older patients were capable of effectively mobilizing the CD34+/H11015 cells, suggesting that the reduced efficacy in this population can be attributed to a local defect in response to G-CSF therapy. Taken together, these reports contrast sharply with more encouraging data from 2 studies with subjects of mean ages of 507 and 586 years. The latter studies showed statistically significant

Figure 6. Activation of cardioprotective and apoptotic pathways in old and young animals and response to G-CSF/SCF treatment. G-CSF/SCF increases Stat3 phosphorylation in young (A) but not old (B) rats after MI. C, Activated caspase-3 does not increase in young rats with MI or with G-CSF/SCF. D, Activated caspase-3 increases ~3-fold with MI in old rats (P<0.05) and is unaffected by G-CSF/SCF. E and F, The ratio of p-ERK to t-ERK 42/44 is increased to a similar degree in both young (P<0.01) (E) and old (P<0.02) (F) rats treated with cytokines. Ctrl indicates control.
benefits across a breadth of objective end points, including regional wall motion, myocardial perfusion, and EF, suggesting that younger patients may benefit substantially from these therapies. In summary, we show that the beneficial effects of G-CSF/SCF on post-MI cardiac function and remodeling are dramatically diminished with age. Mobilization of circulating precursor cells and increased cardiac c-Kit+ cells were preserved in the older animals. This loss of responsiveness could be attributed, at least in part, to failure of G-CSF/SCF to adequately reduce apoptosis particularly in remote areas in rats of advanced age. At a molecular level, G-CSF failed to activate the anti-apoptotic pathways in older animals. Together, these findings have important mechanistic and therapeutic implications in an aging population at high risk for MI and its complications and need to be taken into consideration in future clinical trials.

**Acknowledgments**

The G-CSF and rat-SCF were kindly provided by Amgen (Thousand Oaks, Calif).

**Sources of Funding**

This work was supported by The Johns Hopkins University School of Medicine Institute for Cell Engineering (ICE), The Donald W. Reynolds Foundation, and NIH grants RO1 HL-65455, R21 HL-23097-36:707-710.

**Disclosures**

None.

**References**


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_Circ Res._ 2006;99:553-560; originally published online July 27, 2006;
doi: 10.1161/01.RES.0000238375.88582.d8
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/99/5/553

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**Online Detailed Methods:**

***Echocardiography.*** Transthoracic echocardiograms were obtained at baseline as well as at 48 hours and at weeks 1, 2, 4 and 8 following myocardial infarction. The studies were performed in anesthetized rats (50mg/kg ketamine plus 2mg/kg acepromazine i.p.) using a Sonos 5500 (Agilent, Hewlett Packard, Palo Alto, CA) equipped with a 15 MHz linear transducer. M-mode and 2-dimensional (2-D) echocardiographic images were obtained in parasternal long and short axis views. Anterior and posterior wall thickness as well as left ventricular end-diastolic (LVEDD) and end-systolic (LVESD) dimensions were measured and averaged from 3-5 cardiac cycles in M-mode tracings at mid-papillary muscle level. Fractional shortening (FS) was calculated as

\[ FS = \left( \frac{(LVEDD - LVESD)}{LVEDD} \right) \times 100 \]

Left ventricular end-diastolic volume (EDV), left ventricular end-systolic volume (ESV) and ejection fraction (EF) were calculated from the parasternal long-axis view using Simpson’s modified single-plane method.

***Complete blood count/flow cytometric analysis of CD34\(^+\) and c-Kit\(^+\) cells.*** Complete white blood counts and numbers of circulating CD34\(^+\) and c-Kit\(^+\) cells were determined at baseline and at day 7 after MI and cytokine administration. Leukocytes were incubated with a mouse anti-rat CD34 phycoerythrin (PE) -conjugated monoclonal antibody (sc-7324) or a rabbit anti-rat c-Kit polyclonal antibody (sc-168, both Santa Cruz Biotechnoloy Inc., Santa Cruz, CA), followed by a fluorescein isothiocyanate (FITC)- conjugated sheep anti-rabbit antibody (STAR34B, Serotec, Oxford, UK). The analysis was performed on a FACScan (Becton Dickinson) equipped with a 488 nm argon laser. Numbers of CD34 and c-Kit\(^+\) cells were expressed as percentage of the
gated lymphocyte population. Complete blood counts were obtained with an automated hematocytometer.

**Histology.** Hearts were harvested, perfused with Streck Tissue Fixative (S.T.F., Streck Laboratories Inc, Omaha, Nebraska), and cut into four 2-3 mm transverse sections. After paraffin embedment, slides were stained with H&E and Masson’s trichrome. Infarct size was calculated as percentage of left ventricular perimeter in 4 transverse slices at least 2 mm apart as previously described. Myocyte width was measured across the nuclei in non-infarcted remote myocardium.

**TUNEL staining.** To quantify apoptosis of cardiac cells, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed on paraffin embedded tissue sections according to the manufacturer’s protocol using a commercially available Kit (In Situ Cell Death Detection Kit, POD, Roche Diagnostics GmbH, Germany).

Slides were analyzed by light microscopy under 400x magnification. Apoptotic nuclei were identified by brown staining and expressed as a percentage of all nuclei per field. Nuclei were counted manually in six high power fields (HPF) from two tissue sections per animal, distinguishing between infarct borderzone and remote myocardium. See online supporting document table 1. Cardiomyocytes were identified by characteristic morphological features.

**Immunohistochemistry.** Sections were deparaffinized and rehydrated by immersion in xylenes and a graded series of ethanols. Antigen retrieval was performed by incubation with sodium citrate buffer. After blocking with 10% normal donkey serum, sections were incubated with a Dako rabbit anti-human CD117 antibody (A 4502), followed by incubation in fluorescent FITC donkey anti-rabbit antibody. Nuclei were
counterstained with DAPI. Images were obtained with both a standard fluorescence microscope and a Zeiss Axiovert 200 microscope with 510-Meta confocal laser scanning module. Double fluorescent staining for mast cell and c-Kit antibody was also performed to differentiate mast cells from bone marrow derived c-Kit positive stem cells, using purified mouse anti-rat mast cell antibody (BD cat# 551770), which is a validated antibody for detection of mast cells\(^2\). A TRITC donkey anti-mouse antibody was used as a secondary antibody for these experiments.

**Statistics.** Statistical analyses were performed using STATA 7.0™ (College Station, TX). All values are expressed as mean± S.E.M. Continuous variables were analyzed by ANOVA with post-hoc analysis (Scheffe) across multiple observations or by Student’s t-test (paired and unpaired) for comparisons between two groups. Mortality rates were compared using Fisher’s exact test. Echocardiographic parameters during the 8 week follow up period were compared within groups using ANOVA with repeated measurements and between groups using 2- way ANOVA with an interaction term. \(P\)-values <0.05 were considered significant.

**Reference List**


Online Figure 1. Example of flow cytometry for circulating CD-34⁺ and c-kit⁺ cells in a young rat at baseline and day 7 after cytokine treatment. (A) An example of flow cytometry enumeration of CD 34⁺ cells from peripheral blood at baseline, and day 7 after G-CSF/SCF treatment with an increase of the percentage of CD34⁺ cells in the gated population. (B) Flow cytometry enumeration of circulating c-kit⁺ cells at baseline and day 7 after G-CSF treatment, demonstrating an increase in the percentage of c-kit⁺ cells after G-CSF/SCF treatment.
Online Table 1. Average apoptotic indices in the border zone

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<th>apoptotic-index cardiomyocytes</th>
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G-CSF/SCF treatment reduces apoptosis of cardiomyocytes in the infarct border zone in young and old animals, *P* < 0.05 vs. respective placebo group. Cardiomyocyte apoptosis was lower in young animals, §*P* < 0.001 vs. old placebo.