Sustained Persistence of Transplanted Proangiogenic Cells Contributes to Neovascularization and Cardiac Function After Ischemia

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Abstract—Circulating blood–derived vasculogenic cells improve neovascularization of ischemic tissue by a broad repertoire of potential therapeutic actions. Whereas initial studies documented that the cells incorporate and differentiate to cardiovascular cells, other studies suggested that short-time paracrine mechanisms mediate the beneficial effects. The question remains to what extent a physical incorporation is contributing to the beneficial effects of cell therapy. By using the inducible suicide gene thymidine kinase to deplete transplanted cells, we determined the contribution of physical incorporation in 3 animal models. After acute myocardial infarction, depletion of cells 14 days after infusion resulted in a reduction of capillary density and a substantial deterioration of heart function. Likewise, neovascularization of Matrigel plugs and ischemic limbs was significantly suppressed when infused cells were depleted 7 days after infusion. Induction of cell death in the previously transplanted cells reduced perfusion and led to vascular leakage as evidenced by Evans blue extravasation. These results indicate that physical incorporation and persistence of cells contribute to cell-mediated improvement of neovascularization and cardiac function. Long-term paracrine activities and/or cell intrinsic mechanisms may have contributed to the maintenance of functional improvement. (Circ Res. 2008;103:000-000.)

Key Words: progenitor cells • neovascularization • cell therapy

Cell therapy is a promising option for treating ischemic diseases. Adult stem and progenitor cells from various sources have experimentally been shown to augment the functional recovery after ischemia. Clinical trials confirmed that autologous cell therapy using bone marrow–derived or circulating blood–derived progenitor cells is safe and provides beneficial effects1–3 (see also recent metaanalysis4). Endothelial progenitor cells (EPCs) were initially isolated from bone marrow or peripheral blood and were characterized by the expression of the hematopoietic stem cell marker CD34 or CD133 and endothelial markers such as vascular endothelial growth factor receptor 2 (KDR).5,6 Isolated CD34+ cells differentiate to endothelial cells in vitro and incorporate into newly formed vessels during angiogenesis in vivo.5 These pioneering studies suggested that bone marrow–derived cells can participate in vascular repair. Indeed, a single hematopoietic stem cell was shown to give rise to both blood cells and vascular endothelium.7–9 The contribution of circulating cells to the endothelium was further supported by sex-mismatched heart transplantation in humans.10,11 Other investigators used culture assays to enrich for EPC-yielding cells, which coexpress endothelial and myeloid marker (“early EPCs,” proangiogenic cells), or to show a more mature endothelial cell phenotype on further culture and expansion (“late or outgrowing EPCs”).12,13 Although the origin and the phenotype of the cultured EPCs is not entirely clear and may vary depending on the defined specific culture conditions,14 the therapeutic potential and improved neovascularization mediated by the cultured cells has been documented extensively in ischemic models.12,15,16

Progenitor cell–mediated improved neovascularization of ischemic tissue might be attributable to various therapeutic actions including a physical incorporation of the infused cells in the endothelium leading to the formation of new capillaries.16–18 Transplanted or infused hematopoietic or endothelial progenitor cells also were shown to differentiate to cardiac muscle cells.5,19,20 However, the capacity of hematopoietic or endothelial progenitor cells to acquire a cardiac phenotype is not well established.21 Bone marrow–derived cells also were detected in the perivascular region, where they were proposed...
to provide paracrine factors contributing to vessel growth. Indeed, cultured endothelial progenitor cells release proangiogenic and antiapoptotic cytokines, which may contribute to enhanced angiogenesis and endogenous repair. Based on these findings, it was speculated that short-term paracrine mechanisms account for the observed therapeutic benefits of cell therapy.

Thus, despite ample data providing evidence for functional improvements after cell therapy, the mechanisms by which cells augment the functional recovery after ischemia are not well defined, and it is not known to what extent the physical incorporation and persistence of transplanted cells contributes to the beneficial effects of cell therapy.

To determine the role of cell incorporation and persistence after cell therapy, we used the suicide gene thymidine kinase (TK), which activates the prodrug gancyclovir (GV), to induce cell death in infused cells at specific time points after ischemia and assessed the influence on neovascularization and cardiac function after induction of ischemia. Our results demonstrate that depletion of cells weeks after infusion reduces vascularization and causes a deterioration of function in 3 different animal models.

Materials and Methods
Culture of Proangiogenic Cells
Human peripheral blood mononuclear cells (MNCs) were isolated by density gradient centrifugation with Biocoll (Biochrom AG) from peripheral venous blood according to Vasa et al. Immediately following isolation, \(8 \times 10^6\) MNCs per milliliter of medium were plated on culture flask coated with human fibronectin (Sigma, Taufkirchen, Germany) and maintained in endothelial basal medium (EBM; LONZA, Verviers, Belgium) supplemented with EGM SingleQuots and 20% FBS. After 3 days in culture, nonadherent cells were removed by thorough washing with PBS, and adherent cells were incubated in fresh medium.

Cell Culture
Pooled human umbilical vein endothelial cells (HUVECs) were purchased from LONZA (Verviers, Belgium) and were cultured in EBM medium supplemented with EGM SingleQuots and 10% FBS. HEK 293 cells were maintained in DMEM containing 10% FBS and penicillin–streptomycin.

Preparation of Lentiviral Stocks
Self-inactivating lentiviral vectors containing the enhanced green fluorescent protein (GFP) gene or the viral TK gene of the herpes simplex type 2 virus and a WPRE (woodchuck posttranscriptional regulatory element) under the control of a spleen focus-forming virus (TF) promoter were generated by transient transfection in 293T cells using pCMVΔR8.91 as packaging plasmid and pMD.G for vesicular stomatitis virus–G protein (VSV-G) pseudotyping as described. Self-inactivating lentiviral vectors containing the enhanced green fluorescent protein (GFP) gene were generated by transient transfection in 293T cells using pCMVΔR8.91 as packaging plasmid and pMD.G for vesicular stomatitis virus–G protein (VSV-G) pseudotyping as described. After 8 hours, the medium was replaced by DMEM/10 mmol/L sodium butyrate for 20 hours. Lentiviral particles were collected in EBM medium supplemented with EGM SingleQuots and 20% FBS. After 8 hours, the medium was changed and EPCs were transduced with viral supernatant to the EBM supplemented with EGM SingleQuots and 20% FBS. After 8 hours, medium was changed and EPCs were transduced a second time. Transduced EPCs were used on days 5 or 6 after isolation for the following experiments.

Matrigel Plug Model
A total of 500 \(\mu\)L of Matrigel Basement Membrane Matrix (BD Biosciences) without supplements was injected subcutaneously into 6- to 8-week-old female athymic nude mice (Harlan) along the abdominal midline. After 7 or 14 days, blood vessel infiltration in Matrigel plugs was quantified by analysis of lectin and smooth muscle actin-stained sections using microscopy.

Hindlimb Ischemia Model
Eight-week-old male Balb/c nude mice (Charles River) were anesthetized with isoflurane. Right femoral artery and vein were coagulated and then cut out to induce critical ischemia. One day after operation, EPCs (1 million) were administrated via the tail vein. Laser Doppler perfusion image was taken as indicated and perfusion of each limb was calculated from mean value multiplied by the number of pixel of the region below the inguinal ligament. Data were represented as ratios of the ischemic limb to the nonischemic limb. Evans blue (30 mg/kg) was injected intravenously at day 9. Mice were euthanized 24 hours after dye injection and cardioperfused with normal saline. Tissue was harvested and frozen. Evans blue was extracted from tissue using trichloroacetic acid and ethanol. Absorbance was measured at 620 nm.

Acute Myocardial Infarction Model
Left coronary artery occlusion was performed in female nu/nu mice (8 to 9 weeks; Harlan) under mechanical ventilation and anesthesia. Acute myocardial ischemia was induced by ligating the left coronary artery. Cells (2 million) were intravenously infused 24 hours after operation.

Physiological Assessment of Left Ventricular Function
In the myocardial infarction study, transthoracic echocardiography was performed to evaluate left ventricular function by measuring ejection fraction 2, 3, and 4 weeks after myocardial ischemia.

MRI Procedure
Cardiac MRI was performed under volatile isoflurane (1.5% to 2.0%) anesthesia with a Bruker Pharmascan 7.0 T, a custom-built circularly polarized birdcage resonator and use of the Early Access Package for Self-gated Cardiac Imaging (Intragate). This measurement is based on the gradient echo method (repetition time=44.4 ms; echo time=6.0 ms; field of view=2.20×2.20 cm; slice thickness=1.0 mm; matrix=128×128; repetitions=100). The imaging plane was localized using scout images showing the 4- and 2-chamber view of the heart, followed by acquisition in short axis view, orthogonal on the septum in both scouts. Multiple contiguous short-axis slices consisting of 6 to 8 slices were acquired for complete coverage of the left ventricle. All MRI data were analyzed using Qmass digital imaging software (Medis, Leiden, The Netherlands).

Immunostainings
Sections were deparaffinized and were incubated with biotinylated Isolectin B4 (Vector B1201) followed by streptavidin Alexa Fluor 488. TUNEL assays (Roche) was performed according to the instructions of the manufacturer. Confocal microscopic analysis was performed using a Zeiss LSM 510 Meta.

Statistical Analysis
Data are expressed as means±SEM. Two treatment groups were compared by Mann–Whitney test, 3 or more treatment groups were compared by 1-way ANOVA, followed by post hoc analysis adjusted with a least significant difference correction for multiple comparisons (SPSS). Results were considered statistically significant when \(P<0.05\).

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.
vector was encoding TK. The transfection efficiency of the GFP control injected 1 day later. After 4 weeks, 1.55 coronary artery, and TK- or GFP-transduced cells were infarction was induced by permanent ligation of the left acute myocardial infarction to determine the effect of cell Next, we infused TK-expressing cells in nude mice after Suicide Gene Induction of Cell Death by Virally Transduced Adherent cells at day 4 express endothelial markers (KDR, von Willebrand factor, endothelial nitric oxide synthase), but also hematopoietic/myeloid markers such as CD31, CD45, and CD14 (see previous publications for cell characteris- Adherent cells at day 4 express endothelial markers (KDR, von Willebrand factor, endothelial nitric oxide synthase), but also hematopoietic/myeloid markers such as CD31, CD45, and CD14 (see previous publications for cell characteris-13,33). To determine the role of cell incorporation and persistence, cells were transduced using a lentiviral vector encoding TK. The transfection efficiency of the GFP control vector was >80%, and control experiments confirmed that the viral transduction did not affect cell function (viability, migration, colony forming activity) (data not shown). Addition of GV induced toxicity in the TK-transduced cells but not in the GFP control vector–expressing cells (Figure 1a). To determine potential indirect effects of GV-induced cyto- Depletion of Infused Cells Impairs Functional Recovery After Acute Myocardial Infarction Next, we infused TK-expressing cells in nude mice after acute myocardial infarction to determine the effect of cell ablation on heart function in vivo (Figure 2a). Myocardial infarction was induced by permanent ligation of the left coronary artery, and TK- or GFP-transduced cells were injected 1 day later. After 4 weeks, 1.55±0.2% of cells were positive for human specific Alu sequence in the border zone of the infarcts. Human cells were preferentially localized in or around vessels, whereas only a small number of myocytes (<0.1%) were detected (Figures I and II in the online data supplement). Two weeks after acute myocardial infarction, left ventricular ejection fraction was significantly improved in both cell-treated groups compared with PBS control mice (Figure 2b). In GFP cell-treated mice, the ejection fraction was not affected by GV treatment, which was initiated 2 weeks after cell transplantation, and the beneficial effect of cell therapy was maintained during the 4-week observation period. In contrast, left ventricular ejection fraction significantly deteriorated after injection of GV in TK cell–treated mice, indicating that ablation of the cells 2 weeks after infusing impaired heart function. To confirm these results, MRI was used as an additional means to corroborate our results. Again, left ventricular ejection fraction was significantly improved in mice treated with TK cells in the absence of GV (Figure 2c and 2e). Injection of GV after 2 weeks in TK cell–treated mice resulted in a reduced left ventricular ejection fraction at 4 weeks compared with TK cell–treated mice not receiving GV (Figure 2c). Consistent with these results, end-systolic volume was significantly reduced in mice injected with TK cells but significantly increased by additional GV treatment (Figure 2d and 2e). Because injected cells preferentially incorporated in or around capillaries, we investigated whether ablation of the cells affected capillary density. We detected a significant reduction of the increased capillary density area in TK cell–treated mice 4 weeks after myocardial infarction, when GV was injected at 2 weeks (Figure 2f; for higher magnification, see supplemental Figure IV). To obtain a more detailed view of the acute effects induced by GV treatment in TK cell–treated mice, TUNEL stainings were performed. As shown in Figure 2g and supplemental Figure III, GV treat-ment induced cell death in TK cell–treated mice 3 days after onset of GV treatment (1.7±0.28-fold compared with mice, which had received TK cells without GV treatment, P<0.05). Depletion of Infused Cells Reduces Neovascularization Having demonstrated that depletion of transplanted cells reduced capillary density, we investigated the relevance of cell incorporation in 2 models of vascularization. First, we infused TK-expressing cells in nude mice implanted with a

Figure 1. GV induces cell death in TK-expressing cells. a, GV reduces cell viability (MTT assay) dose-dependently in TK-transduced peripheral blood–derived fibronectin-adherent cells after 36 hours of incubation (N=3). b, Incubation with conditioned medium of GV-treated TK-expressing cell did not affect viability of HUVECs. MTT assay after 24 hours of incubation is shown in comparison with 1 mmol/L H2O2 treatment (N=3).

Results
Induction of Cell Death by Virally Transduced Suicide Gene
To investigate the physical contribution of proangiogenic cells, peripheral blood–derived mononuclear cells were cultivated on fibronectin-coated dishes under conditions favoring endothelial commitment as previously described.15,16 Adherent cells at day 4 express endothelial markers (KDR, von Willebrand factor, endothelial nitric oxide synthase), but also hematopoietic/myeloid markers such as CD31, CD45, and CD14 (see previous publications for cell characteris-tics13,33). To determine the role of cell incorporation and persistence, cells were transduced using a lentiviral vector encoding TK. The transfection efficiency of the GFP control vector was >80%, and control experiments confirmed that the viral transduction did not affect cell function (viability, migration, colony forming activity) (data not shown). Addition of GV induced toxicity in the TK-transduced cells but not in the GFP control vector–expressing cells (Figure 1a). To determine potential indirect effects of GV-induced cyto-toxicity, culture supernatants of the GV treated TK-expressing cells were added to HUVECs or cardiomyocytes. However, conditioned medium did not induce cell death (Figure 1b and data not shown). Moreover, TK-expressing cells were cocultured with GFP-transfected HUVECs before adding GV to induce cell death in the TK-expressing cells. The viability of GFP-transfected HUVECs was not reduced by inducing cell death in the TK-expressing cells (99.9% of control), indicating that the treatment with GV selectively kills the transduced cells.

Depletion of Infused Cells Impairs Functional Recovery After Acute Myocardial Infarction
Next, we infused TK-expressing cells in nude mice after acute myocardial infarction to determine the effect of cell ablation on heart function in vivo (Figure 2a). Myocardial infarction was induced by permanent ligation of the left coronary artery, and TK- or GFP-transduced cells were injected 1 day later. After 4 weeks, 1.55±0.2% of cells were positive for human specific Alu sequence in the border zone of the infarcts. Human cells were preferentially localized in or around vessels, whereas only a small number of myocytes (<0.1%) were detected (Figures I and II in the online data supplement). Two weeks after acute myocardial infarction, left ventricular ejection fraction was significantly improved in both cell-treated groups compared with PBS control mice (Figure 2b). In GFP cell-treated mice, the ejection fraction was not affected by GV treatment, which was initiated 2 weeks after cell transplantation, and the beneficial effect of cell therapy was maintained during the 4-week observation period. In contrast, left ventricular ejection fraction significantly deteriorated after injection of GV in TK cell–treated mice, indicating that ablation of the cells 2 weeks after infusing impaired heart function. To confirm these results, MRI was used as an additional means to corroborate our results. Again, left ventricular ejection fraction was significantly improved in mice treated with TK cells in the absence of GV (Figure 2c and 2e). Injection of GV after 2 weeks in TK cell–treated mice resulted in a reduced left ventricular ejection fraction at 4 weeks compared with TK cell–treated mice not receiving GV (Figure 2c). Consistent with these results, end-systolic volume was significantly reduced in mice injected with TK cells but significantly increased by additional GV treatment (Figure 2d and 2e). Because injected cells preferentially incorporated in or around capillaries, we investigated whether ablation of the cells affected capillary density. We detected a significant reduction of the increased capillary density area in TK cell–treated mice 4 weeks after myocardial infarction, when GV was injected at 2 weeks (Figure 2f; for higher magnification, see supplemental Figure IV). To obtain a more detailed view of the acute effects induced by GV treatment in TK cell–treated mice, TUNEL stainings were performed. As shown in Figure 2g and supplemental Figure III, GV treat-ment induced cell death in TK cell–treated mice 3 days after onset of GV treatment (1.7±0.28-fold compared with mice, which had received TK cells without GV treatment, P<0.05). Depletion of Infused Cells Reduces Neovascularization
Having demonstrated that depletion of transplanted cells reduced capillary density, we investigated the relevance of cell incorporation in 2 models of vascularization. First, we infused TK-expressing cells in nude mice implanted with a
Figure 2. Depletion of infused TK-expressing cells by GV impairs functional recovery of hearts after acute myocardial infarction. a and b, PBS-transduced (N=6), GFP-transduced (N=4), or TK-transduced cells (N=8) were injected 1 day after induction of acute myocardial infarction. GV (100 mg/kg) was injected at days 14 to 18 daily. Echocardiography was performed to assess heart function at day 14 (before GV injection), at day 21 and day 28 as indicated. c through e, Heart function and end-systolic volumes were determined by MRI in N=4 cell-treated mice per group after 28 days. *P<0.05 compared with PBS, **P<0.001 vs TK cells+GV. Representative images are shown in e, f. Vessel density was determined by automatic measurement of lectin-positive area per total area of a cross-section of the left ventricle (N=5). *P<0.05 compared with PBS and TK cells+GV. g, TUNEL staining (red) was performed in sections of mice receiving TK-transduced cells 1 day after induction of acute myocardial infarction. GV was injected at day 14 to day 17 daily, and mice were euthanized at day 17. Sections were counterstained with lectin (green) and DAPI (nuclei, blue). Representative images of the border zone are shown.
Matrigel plug to determine the effect of cell ablation on angiogenesis. Infusion of GFP- or TK-expressing cells increased the number of invading vessel-like structures in a time-dependent manner (Figure 3a and 3b). To determine whether persistence of the cells contributes to angiogenesis 7 days after infusion of the cells, we started GV infusion at day 7 and documented vessel growth at day 14 (Figure 3a and 3b). As shown in Figure 3b, GV treatment at day 7 significantly increased the number of invading vessel-like structures in a model of acute myocardial infarction. These data demonstrate that ablation of transplanted cells 1 week after infusion reduced vessel growth and perfusion recovery in 2 different models of neovascularization. Moreover, ablation of infused cells 2 weeks after administration induced a rapid decline of ejection fraction and capillary density in a model of acute myocardial infarction. These data demonstrate that ablated cells are physically incorporated into host tissue and persist weeks after administration. Thus, it appears unlikely that the beneficial effects of cell therapy using EPCs can be solely explained by short-time paracrine effects. However, the present study does not exclude that the incorporated cells may provide long term paracrine effects. However, the present study does not exclude that the incorporation of infused human EPCs in 10 to 20% of the capillaries in the ischemic hindlimb muscle.16,18 Therefore, we investigated whether ablation of the TK-transduced cells might induce capillary damage and leakage leading to edema formation and transient perfusion defects in the hindlimb ischemia model. Injections of Evans blue in TK cell–treated mice 3 days after onset of GV treatment (Figure 4c; GV treatment started at day 7) revealed multiple spots of Evans blue staining in the thigh muscle of GV-injected mice (305±78% increase quantified by dye extraction of muscle tissue compared with PBS controls, P<0.05), whereas no staining was evident in PBS-injected mice (Figure 4c), suggesting that ablation of the previously administered cells by GV injection induces vascular damage and increases permeability.

Discussion

Our data demonstrate that ablation of transplanted cells 1 week after infusion reduced vessel growth and perfusion recovery in 2 different models of neovascularization. Moreover, ablation of infused cells 2 weeks after administration induced a rapid decline of ejection fraction and capillary density in a model of acute myocardial infarction. These data demonstrate that infused cells are physically incorporated into host tissue and persist weeks after administration. Thus, it appears unlikely that the beneficial effects of cell therapy using EPCs can be solely explained by short-time paracrine effects. However, the present study does not exclude that the incorporated cells may provide long term paracrine effects. Of note, the functional consequences of cell depletion were different in the animal models used. In the angiogenesis Matrigel plug assay, vessel growth was only partially inhibited, indicating that a paracrine mechanism promoting host-

Depletion of Infused Cells Induce Vascular Leakage

Previous studies documented the incorporation of transplanted human EPCs in 10 to 20% of the capillaries in the ischemic hindlimb muscle.16,18 Therefore, we investigated whether ablation of the TK-transduced cells might induce capillary damage and leakage leading to edema formation and transient perfusion defects in the hindlimb ischemia model. Injections of Evans blue in TK cell–treated mice 3 days after onset of GV treatment (Figure 4c; GV treatment started at day 7) revealed multiple spots of Evans blue staining in the thigh muscle of GV-injected mice (305±78% increase quantified by dye extraction of muscle tissue compared with PBS controls, P<0.05), whereas no staining was evident in PBS-injected mice (Figure 4c), suggesting that ablation of the previously administered cells by GV injection induces vascular damage and increases permeability.

Figure 3. Depletion of infused TK-expressing cells by GV impairs vessel-growth in Matrigel plugs (a and b). GFP- or TK-expressing cells were injected IV in mice with subcutaneously implanted Matrigel plugs at the same day (day 0). GV (100 mg/kg) or PBS was injected daily at days 7 to 11. Matrigel plugs were explanted at days 7 (N=4), and vessels were counted in sections stained with smooth muscle actin antibodies.
derived angiogenesis within the first days after infusion significantly contributed to the vascularization of the plugs. After hindlimb ischemia, a profound increase in vascular leakage and decreased perfusion was detected after cell ablation. However, this defect was transient and mice treated with GV partially recovered at day 21, indicating that host-derived endogenous repair compensated for the loss of cells at later time points. The extent to which early paracrine mechanisms may have contributed to the rapid recovery detected later is difficult to evaluate.

In contrast to the partial inhibitory or transient effects detected in the Matrigel and hindlimb ischemia model, depletion of the cells after acute myocardial infarction induced a profound and sustained deterioration of cardiac function and capillary density was still significantly reduced in the GV treated group at 4 weeks. These findings might be explained by the crucial role of angiogenesis in the heart. Two recently published experimental models demonstrated that disruption of angiogenesis in the heart induced a decline in heart function, resulting in heart failure during pressure overload or induction of hypertrophy. In addition, the induction of cell death in cardiac myocytes derived from the injected cells might have contributed to a reduction of heart function. However, although we detected human cardiac myocytes, the number was below 0.1%, suggesting that this is unlikely to account for the deterioration of function. However, we cannot exclude that the activated prodrug GV can be transported by gap junctions to physically connected neighboring cells, although we excluded unspecific cytotoxicity of GV in vivo (supplemental Figure VI) and “kiss of death” effects imposed by TK-transduced cells on neighboring endothelial cells in culture in vitro. Because cultivated vascular progenitor cells were previously shown to connect to cardiac myocytes via gap junctions in vitro, and transplanted c-kit+ bone marrow–derived cells were shown to form interactions with host cells via gap junctions, such

Figure 4. a and b, Depletion of infused TK-expressing cells after hindlimb ischemia by GV reduces perfusion and induces vascular leakage. TK-expressing cells or PBS was injected 1 day after induction of hindlimb ischemia. GV (100 mg/kg) or PBS was injected daily at days 7 to 11, and perfusion was assessed at days 1, 7, 14, and 21 using laser Doppler (N=11 to 18 per group). P<0.05 compared with TK cells+GV and PBS. c and d, Capillary density was determined in muscle sections by lectin staining (n=5 to 7 mice per group). "P<0.05 vs PBS and TK cells+GV. e, TK-expressing cells were injected 1 day after inducing hindlimb ischemia. GV was injected at days 7 to 9 daily. At day 9, Evans blue was injected to determine vessel leakage. A representative image of thigh muscles is shown. Arrows indicate Evans blue uptake.
bystander effects might have amplified the detrimental effects caused by depletion of TK-expressing cells on cardiac function in the myocardial infarction model. Nevertheless, even if bystander effects on physically connected neighboring cells might have contributed to the observed abrogation of neovascularization recovery and deterioration of cardiac function following ablation of the administered cells, such mechanisms would require physical incorporation of the cells into the host tissue. Thus, sustained persistence of the administered cells in the host tissue contributes to the beneficial effects of cell therapy on neovascularization and recovery of cardiac function. The contribution of specific cell lineages to the maintenance of functional improvement warrants further investigations.

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Disclosures
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Supplementary Methods

Magnetic Resonance Imaging (MRI)
Dynamic contrast enhanced (DCE) MRI was performed using a 1.5 Tesla whole-body MR-scanner (Siemens Symphony, Erlangen, Germany) in combination with a custom-made radio-frequency-coil for excitation and signal reception. Contrast agent kinetics in hind limbs were recorded using a T1-weighted inversion-recovery Turbo FLASH (IRTF) sequence (TR = 13 ms, TE = 5.3 ms, TI = 300 ms, Slice Thickness = 2 mm, FOV = 60 x 22.5 mm², Matrix 128 x 48). In total, 120 dynamic scans were acquired from two sections within 14:55 min. Five seconds after starting the DCE-MRI measurement, 100 µl of the paramagnetic contrast agent Gadomer (0.1 mmol/kg body weight; Bayer-Schering Pharma, Berlin, Germany) were injected manually within 5 s into the tail vein. Data were analysed using the pharmacokinetic two compartment model of Brix (1) providing the parameters amplitude (related to relative blood volume) and exchange rate constant kep (surrogate marker of vessel permeability and perfusion). A and kep were determined pixelwise, color coded and overlaid on the morphological MR-images. In addition, region of interest (ROI) analyses of both upper thighs for each animal were performed. All postprocessing steps were conducted on the computer-based workstation Dyna Lab (Mevis Research, Germany). Dynamic MR-measurements were supplemented with morphological precontrast T2-weighted (TSE, TR = 1510 ms, TE = 59 ms, Slice Thickness = 1 mm, FOV = 50 x 38 mm², Matrix 128 x 96) and postcontrast T1-weighted (SE, TR = 600 ms, TE = 1 ms, Slice Thickness = 1 mm, FOV = 61 x 38 mm², Matrix 512 x 320) sequences.

Stainings
Paraffin embedded sections were deparaffinized and were incubated with proteinase K (15 min, 37 °C) and was hybridised with human specific fluorescein-labeled Alu probe (PR-1001-01, InnoGenex) at 60 °C (overnight). Sections were then incubated with goat anti-fluorescein followed by donkey FITC-conjugated anti-goat antibodies. Rabbit polyclonal anti-von Willbrand factor antibodies (DAKO) followed by anti-rabbit Alexa Fluor 555 antibodies were used for detecting vessel structures. Cardiomyocytes were detected by α-sarcomeric actin (Sigma) and anti-mouse IgM rhodamine (Jackson). Nuclei were counterstained with ToPro (Molecular Probes). Confocal microscopic analysis was performed using a Zeiss LSM 510 Meta.

Culture of human macrophages
CD14-positive monocytes were purified from MNCs by positive selection with anti-CD14 microbeads (Miltenyi Biotec) using a magnetic cell sorter device (Miltenyi Biotec). CD14-
positive monocytes were incubated in RPMI with 10% FCS in the presence of M-CSF (50 ng/mL) to induce macrophage differentiation.

References
Online figure I

Nuclei human cells (Alu)

vWF Merge

Nuclei human Alu 20 µm

vWF Nuclei human Alu 20 µm

Nuclei human Alu 20 µm
Online figure II
Online figure III

TK-cells

TK-cells + GV
Online figure VI

- Kidney
- Spleen
- Liver
- Lung
- Intestine
**Online figure legends**

**Online figure I:** Human Alu-positive cells (green spots) were detected in TK-cell treated mice 4 weeks after induction of myocardial infarction. Sections were additionally stained with von Willebrandt antibodies (vWF; red colour) and ToPro (blue colour) (for study design see figure 3a)

**Online figure II:** Human Alu-positive cells (green spots) were detected in TK-cell treated mice 4 weeks after induction of myocardial infarction. Sections were additionally stained with α-sarcomeric actin (red colour) and ToPro (blue colour) (for study design see figure 3a)

**Online figure III:** Lectin staining (green) was performed in mice treated with TK-cells (upper panel) or TK-cells plus GV treatment (start at 14 days; lower panel) 4 weeks after induction of myocardial infarction. Dying cells were detected by TUNEL staining (red) and nuclei were counterstained with DAPI (blue). Representative TUNEL positive cells are indicated by white arrows (for study design see figure 3a)

**Online figure IV:** Higher magnification of Lectin stainings quantified in figure 3f. Mice were treated with PBS (upper left panel), TK-cells with PBS (upper right panel), or with TK-cells plus GV (GV injections were started at day 14; lower panel) and sections were obtained 4 weeks after the induction of acute myocardial infarction (for study design see figure 3a)

**Online Figure V:** Color coded parameter maps of the exchange rate constant (kep) in the upper thighs of mice exposed to hind limb ischemia, which were treated with TK-cells plus gancyclovir (GV) at day 7 (A) or without GV (B). Images were taken at day 10 after hind limb ischemia, three days after onset of GV treatment. Corresponding T2-weighted morphological images are shown in C (TK-cells plus GV) and D (TK-cells without GV). Ischemia was induced in the right legs of the animals. On morphologic T2-weighted images the oedemas in the ischemic legs are clearly visible by enhanced signal intensities as compared with the contralateral side. When observing the parameter maps higher kep values are found in the animal that was treated with TK-cells without GV (kep= 0.38/min) as compared with the animal that was administered GV to kill the infused cells (kep= 0.77/min). Please note that kep values of the non ischemic upper thighs of both animals were comparable (kep = 0.62/min vs. kep = 0.67/min).
Online figure VI: H&E staining of tissue sections obtained from mice treated with TK-cells plus GV (injections started after 14 days after induction of myocardial infarction). Animals were sacrificed at day 18 after induction of acute myocardial infarction (for study design see figure 3a)