Stem cell therapy for cardiovascular disease : answering basic questions regarding cell behavior

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CHAPTER 9

Molecular Imaging of Bone Marrow Mononuclear Cell Survival and Homing in a Murine Model of Peripheral Artery Disease

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ABSTRACT

Introduction: Bone marrow mononuclear stem cell (MNC) therapy is a promising treatment for peripheral artery disease (PAD). This study aims to provide insight into cellular kinetics using molecular imaging following different transplantation methods.

Methods and Results: MNCs were isolated from F6 transgenic mice (FVB background) that express firefly luciferase (Fluc) and green fluorescence protein (GFP). Male FVB mice (n=38) underwent femoral artery ligation and were randomized into 3 groups receiving: (1) single intramuscular (i.m.) injection of $2x10^6$ MNC; (2) weekly i.m. injection of $5x10^5$ MNC; and (3) i.m. injection of PBS. To assess the biodistribution following system delivery, we also injected (1) $5x10^6$ MNCs intravenously (i.v.) and (2) PBS i.v. as control (n=10/group). Cellular kinetics, measured by *in vivo* bioluminescence imaging (BLI), revealed near-complete donor cell death 4 weeks after i.m. transplantation. Following i.v. transplantation, BLI monitored cells homed on the injured area in the limb, to liver, spleen, and bone marrow. *Ex vivo* BLI showed presence of MNCs in the scar tissue as well the adductor muscle. However, no significant effects on neovascularization were observed, as monitored by Laser-Doppler-Perfusion-Imaging and histology**.**

Conclusion: This is the first detailed study to assess the kinetics of transplanted MNCs in PAD using *in vivo* molecular imaging. MNC survival after i.m. transplantation is short-lived and MNCs do not stimulate significant improvement in perfusion in this model.

INTRODUCTION

Peripheral artery disease (PAD) currently affects over 27 million people in North America and Europe and is associated with impaired leg function and decreased quality of life, leading to significant morbidity and mortality.[1, 2] Despite a variety of treatment options, including percutaneous transluminal angioplasty, stenting, and bypass surgery, a cluster of patients do not respond to therapy, leaving no other option than amputation in one third of patients within this group.[3]

Recently, stem cell therapy has emerged from bench to bedside as a treatment for end-stage PAD, potentially offering a last option for revascularization of the ischemic limb. While results from pre-clinical experiments using bone marrow-derived mononuclear cells (MNC) appear hopeful, outcomes from clinical studies are mixed[4], raising questions about transplanted stem cell behavior and mechanisms of action involved in the benefits of stem cell transplantation. As to cell behavior, two major issues are the lack of donor cell survival after introduction into ischemic target tissue and the absence of cell homing to the injured area following systemic administration.[5] Donor cell death would hamper three mechanisms believed to be of importance for the beneficial effects seen after cell transplantation: a lasting scaffolding effect, transplanted cell-derived neovascularization, and the secretion of protective paracrine factors by the transplanted cells.

To study stem cell behavior, one must be capable of monitoring cell location, migration, proliferation, and death. Recent proof-of-principle studies have demonstrated the ability to track cell fate following cardiac injections.[5, 6] In the present study, we monitor by molecular imaging the presence of MNC after transplantation in mice with induced hind limb ischemia. These experiments are designed to answer critical questions regarding cell survival and homing patterns to the affected leg, as well as functional consequences of different transplantation strategies.

METHODS

Experimental animals. Animal study protocols were approved by the Animal Research Committees from both institutions (Stanford University and Leiden University). The donor group for imaging experiments consisted of 8-week old male F6 mice (n=10), which were bred on FVB background and ubiquitously express green fluorescent protein (GFP) and firefly luciferase (Fluc) reporter genes driven by a β-actin promoter as previously described.[7] Recipient animals (n=60) for these experiments consisted of syngeneic, male FVB mice (10-12 weeks old, Jackson Laboratories). To compare the efficacy of a single versus repeated injection with cells, animals were randomized into 3 groups: (1) single intramuscular (i.m.) injection of $2x10^6$ MNCs, (2) four

repeated injections of $5x10^5$ MNCs, and (3) i.m. injection of phosphate buffered saline (PBS) injection as control. To compare the efficacy of local versus system delivery, animals were also injected with (1) single intravenous (i.v.) injection of $2x10^6$ MNCs and (2) PBS i.v. as control.

Preparation and characterization of bone marrow mononuclear cells (MNC). The long bones were explanted, washed, and flushed with PBS using a 25-gauge needle to collect bone marrow. After passing through a 70 µm strainer, the isolate was centrifuged at 1200 rpm for 5 minutes, washed, and resuspended into PBS. To acquire the MNC fraction, the bone marrow isolate was centrifuged for 40 minutes at 1600 rpm using a 14 mL tube with 3 mL Ficoll-Paque Premium (GE Healthcare, Piscataway, NJ, USA) gradient and 4 mL cell/saline suspension, as described.[5] MNCs were prepared freshly before application.

Characterization of cells by flow cytometry. Cells were incubated in 2% FBS/PBS at 4°C for 30 min with 1 µL of APC-conjugated anti-CD31 (eBioscience), anti-CD45 (BD Biosciences), and anti-Gr-1 (BD Biosciences), or PE-conjugated anti-CD34 (eBioscience), anti-CD11b (BD Biosciences), anti-Flk-1, anti-Sca-1 (both eBioscience), and anti-NK1.1 (BD Biosciences), and processed through a FACSCalibur system (BD, San Jose, CA, USA) according to the manufacturer's protocol.

***In vivo* optical bioluminescence imaging (BLI).** BLI was performed on the IVIS 200 (Xenogen, Alameda, CA, USA) system. For *in vitro* characterization of luciferase expression, cells were suspended in different quantities in 1 mL PBS. Following administration of 10 µL (43.5 µg/mL) D-Luciferin, peak signals (photons/s/cm^2/sr) from a fixed region of interest (ROI) were evaluated and plotted versus cell number. For *in vivo* experiments, recipient mice were anesthetized with isoflurane, shaved, and placed in the imaging chamber. After acquisition of a baseline image, mice were intraperitoneally injected with D-Luciferin (400 mg/kg body weight). Mice were imaged on days 1, 3, 6, 9, 13, 20, and 27 post-injection. Peak signals (photons/s/cm^2/sr) from a fixed region of interest (ROI) were evaluated as described.[7] For *ex vivo* experiments, animals were euthanized immediately following the moment when peak signals were achieved. The organs were rapidly explanted and imaged according to the protocol described above.

Surgical model for hind limb ischemia and cell injections. Mice were placed under general anesthesia with either isoflurane (2%) or ketamine/xylazine combination. Ischemia was created by left sided electro-coagulation of the femoral artery just proximally to the superficial epigastric artery. One day postoperatively, 40 µL of cell/PBS injections were given into the adductor muscle, or 100 µL of cell/PBS solution into the tail vein using a 28-gauge syringe. Subsequently, the skin was closed using 6-0 silk sutures. Additionally, to induce a more severe model

of PAD, male C57BL/6 mice (n=20, Charles River) underwent left sided electro-coagulation of both common iliac and femoral arteries. Afterwards, animals were randomized to receive intravenous injection of $5x10^6$ MNCs or injection or PBS as control (n=10/group).

Laser Doppler Perfusion Imaging (LDPI). Neovascularization was monitored by measurements of perfusion of the hind limbs at the level of the paws and was performed in the mouse hind limb before and directly after the surgical procedure with Laser Doppler Perfusion Imaging (LDPI) (Moor Instruments) at weekly interval over 4 weeks. Eventually, perfusion was expressed as a ratio of the left (ischemic) to right (non-ischemic) paw. Before LDPI, mice were anesthetized with an intraperitoneal injection of Midazolam (5 mg/kg, Roche) and Medetomidine (0.5 mg/kg, Orion).

***Ex vivo* ELISA for apoptosis on digested muscle.** The selected muscle was explanted, digested using a stator-rotator homogenizer, and lysed. ELISA was performed directly on the supernatant to quantify histone-associated DNA fragments (mono- and oligonucleosomes), marking early apoptotic cells (Cell Death Detection ELISA, Roche Applied Science, Indianapolis, IN).

***Ex vivo* assays of reporter gene expression.** To validate *in vivo* BLI findings, the bone marrow was collected as described above and assayed for GFP expression by flow cytometry as described above.

Post-mortem immunohistochemistry. Immunohistochemistry was performed to visualize smooth muscle cell layers of collateral arteries with an antibody against smooth muscle actin. Furthermore, with an antibody against GFP, GFP^+ MNCs were traced in the ischemic skeletal muscle. Five µm-thick paraffin-embedded sections of skeletal muscle fixed with 4% formaldehyde were used. These were re-hydrated and endogenous peroxidase activity was blocked for 20 minutes in methanol containing 0.3% hydrogen peroxide. Skeletal muscle slides were stained with monoclonal anti-α smooth muscle actin (mouse anti-human, DAKO, dilution 1:800) or polyclonal anti-GFP (rabbit anti-mouse, Invitrogen, dilution 1:4000). Antigen retrieval was not necessary and sections were incubated overnight with primary antibody. Rabbit anti-mouse HRP (DAKO, dilution 1:300) or goat anti-rabbit biotin (DAKO, dilution 1:300) were used as secondary antibodies respectively. Negative controls were performed by using isotype controls instead of the primary antibody. For both stainings, the signal was detected using NovaRED substrate kit (Vector laboratories) and sections were counterstained with hematoxylin. Stainings were quantified from randomly photographed sections using image analysis (ImageJ).

Statistical analysis. Statistics were calculated using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics included mean and standard error. Comparison between groups was performed using a one-way between groups ANOVA, or one-way repeated measures ANOVA when compared over time, and significance was assumed according to the LSD procedure.

RESULTS

Cell characterization. Following isolation and Ficoll selection, the MNC population showed subpopulations of $CD31^+$, $CD34^+$, $CD45^+$, and Sca-1+, but $Flk\text{-}1^-$ cells, representing hematopoietic but not early endothelial progenitors cells. Moreover, strong expression of CD11b, Gr-1, and NK 1.1, representative of macrophages, granulocytes, and natural killer cells, indicated the largely inflammatory character of this donor cell population (**figure 1a**).

Reporter gene characterization. To be able to follow cells in an *in vivo* fashion by bioluminescence imaging (BLI), we first set out to characterize the expression of the reporter gene Fluc *in vitro*. As suggested in **figure 1b**, luciferase expression intensity increased with increasing cell numbers. When maximum expression per well was plotted versus the number of cells, a robust correlation was observed with an r^2 value equaling 0.97 (**figure 1c**). Thus, BLI signal intensity is closely representative of the number of living cells carrying the luciferase reporter gene. Moreover, the robust activity of GFP in the donor-specific Fluc-GFP double-fusion reporter gene construct was confirmed by *in vitro* fluorescence microscopy (**figure 1d**).

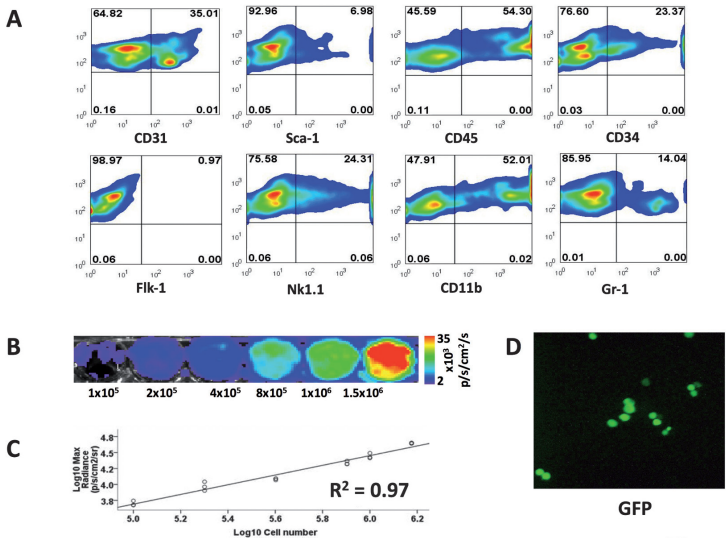

Figure 1. Bone marrow mononuclear cell (MNC) characterization. (a) Flow cytometric analysis following Ficoll-selection of MNCs indicates low numbers of stem/endothelial progenitor cells (Sca-1, flk-1) and high numbers of adult hematopoietic cells of a predominantly inflammatory phenotype (CD45, CD11-b, Gr-1, NK 1.1) (axes

present counts). (b) *In vitro* BLI signals from various numbers of Fluc$^+$ MNCs show (c) robust correlation with cell numbers (r^2=0.97). Scale bars represent BLI signal in photons/s/cm^2/sr. (d) *In vitro* fluorescence microscopy confirms the expression of GFP by the donor cells.

Monitoring kinetics of transplanted MNCs by *in vivo* bioluminescence imaging (BLI). In the current study, MNCs were injected into the left adductor muscles one day following creation of ischemia by left femoral artery occlusion. By doing so, it is possible to study the efficacy of MNCs in stimulating arteriogenesis (the process of collateral artery formation[4]), instead of studying the angiogenic effect which is less influential on restoration in blood flow.[8] To compare the efficacy of a single versus repeated injection with cells, animals were randomized into 3 groups: (1) single MNC injection, (2) repeated MNC injection, and (3) PBS injection. Following single transplantation of 2x10^6 MNCs, a short-term post-transplant increase in BLI signal from 6.6±1.5x10^4 at day 1 to 8.9±2.5x10^4 p/s/cm^2/sr at day 3 (*P*=NS), suggesting an increase in cells in the adductor muscle region during the initial days. Thereafter, however, cell death resulted in a rapid decrease in signal intensity, reaching background levels after 4 weeks (**figure 2**). In order to overcome the problem of poor long-term cell survival, a modified transplantation technique was analyzed in which a similar cumulative dose, but divided in 4 weekly doses of 5x10^5 MNCs, was transplanted. This lead to a relatively stable presence of donor cells, although there was no statistically significant difference after 4 weeks (5.1±0.8x10^3 in single vs 5.7±0.3x10^3 p/s/cm^2/sr in multiple dose group; *P*=NS).

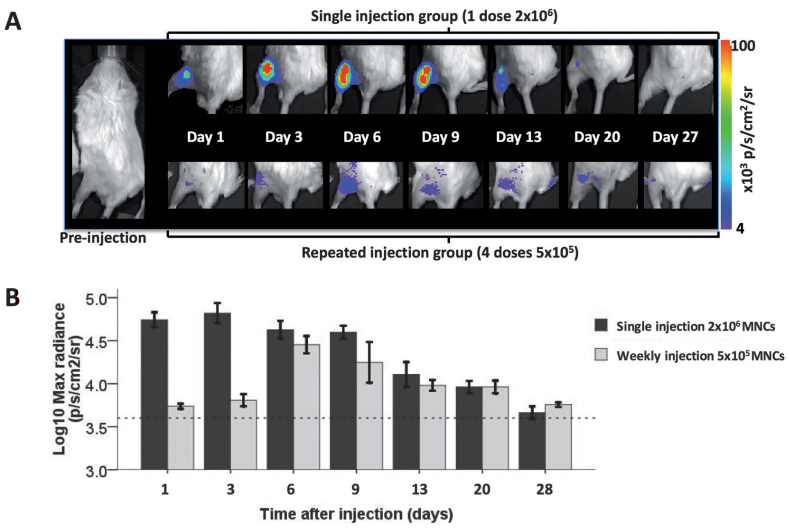

Figure 2. MNC survival following intramuscular injection into the adductor muscles of FVB mice after femoral artery ligation. (a) *In vivo* BLI pictures of mice that received either a total of 2x10^6 MNC by single injection (upper panel) or by weekly injections (lower panel) show MNC survival is short-lived as most of the signal

intensity died off at 4 weeks post-transplant. (b) Quantification of signals showed a somewhat more stable level of MNC presence following repeated injections although the difference did not reach statistical significance. Scale bars represent BLI signal in photons/s/cm^2/sr.

***Ex vivo*, postmortem localization of GFP$^+$ MNCs in the ischemic adductor muscles.** The distribution of GFP$^+$ MNCs was assessed in the post-ischemic adductor muscle of mice treated with a single injection of 2x10^6 MNCs and weekly injections of 5x10^5 MNCs. As shown in **figure 3**, skeletal muscles were harvested 28 days after the induction of ischemia. Low numbers of engrafted GFP$^+$ MNCs were observed in the adductor muscle of mice that received weekly injections of MNCs, concordant with *in vivo* BLI signals. These GFP$^+$ MNCs surrounded vessels within the muscle tissue, suggesting a potential role of these cells in inducing neovascularization. In contrast, GFP$^+$ MNCs were not observed in the adductor muscles of mice receiving a single injection of MNCs at week 4, and corresponding to BLI results.

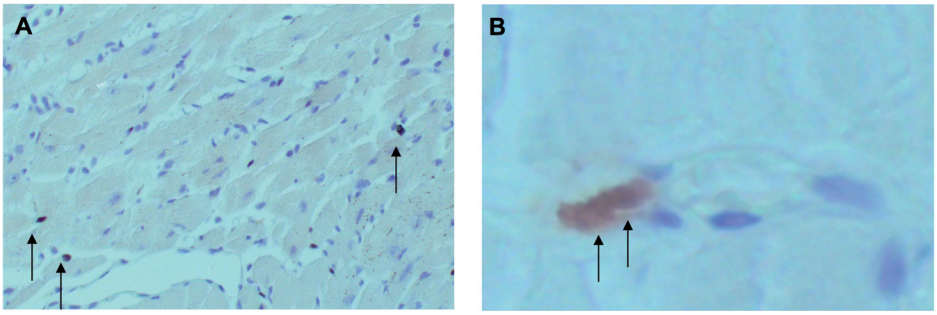

Figure 3. Immunohistochemistry of GFP$^+$ MNCs within the post-ischemic adductor muscle. Representative pictures of anti-GFP muscle staining of (a) positive control slide (magnification 20x), (b) adductor muscle of mouse receiving four weekly 5x10^5 MNC-injections, showing 2 GFP$^+$ cells near a blood vessel (magnification 80x).

Laser Doppler Perfusion Imaging (LDPI) of blood flow restoration following MNC transplantation in FVB mice. For the experiments described above, we used FVB mice to perform a syngeneic transplantation model with our transgenic FVB mice constitutively expressing Fluc$^+$/GFP$^+$ reporter genes. In these mice, femoral artery ligation resulted in a significant decrease in paw perfusion when compared to the healthy right hind limb (P<0.001 for all groups, **figure 4**). Three days following MNC transplantation, a trend was observed towards better flow recovery with increased MNC number, as the ligated/healthy paw perfusion ratios in the 2x10^6 MNC and 5x10^5 groups were 0.75±0.07 and 0.67±0.07, respectively, as compared to 0.62±0.07 in the PBS group (P=NS). However, no significant differences were observed during the prolonged follow up, with robust recovery of paw perfusion in all groups by week 4.

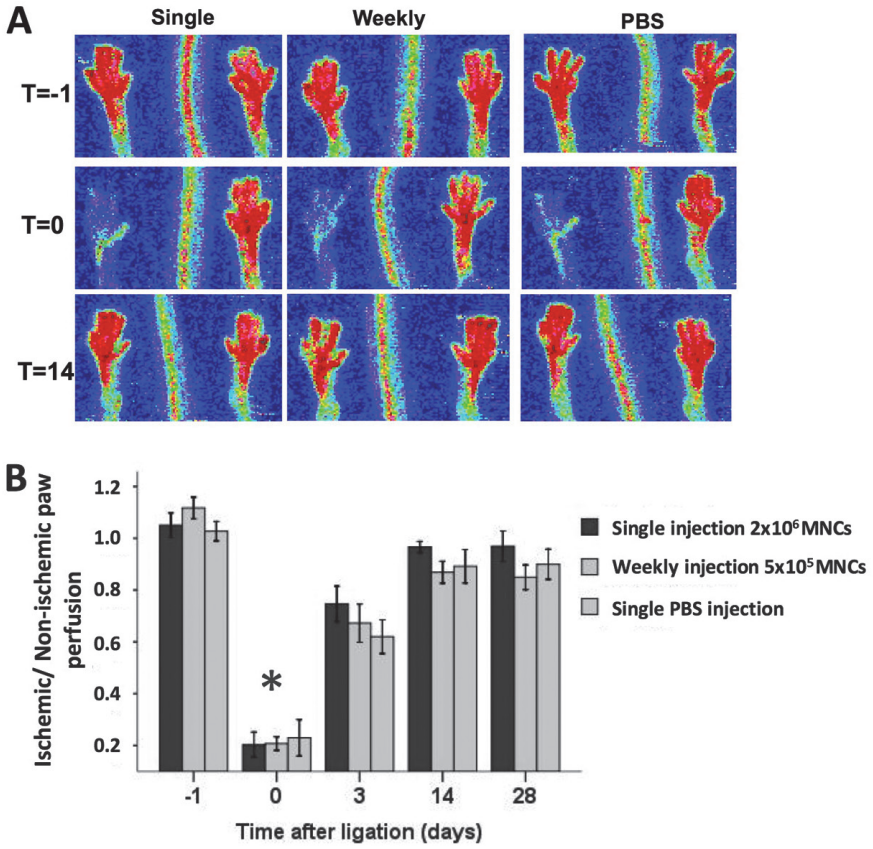

Figure 4. Laser Doppler Perfusion Imaging (LDPI) of ischemic hind limbs following intramuscular MNC therapy. (a) Graphic representation and (b) quantification of paw perfusion by LDPI show a significantly decreased perfusion in the affected left hind limbs as compared to the healthy paw. While a dose-dependent trend towards faster recovery can be observed 3 days after ligation, no significant differences were measured over a total time period of 28 days (Repeated measurements ANOVA, * indicates $P<0.05$).

Histological confirmation of short-term LDPI findings. To investigate whether *in vivo* LDPI matched the actual presence of collaterals, post-mortem histological staining for α-smooth muscle actin was performed. As shown in **figure 5,** no significant differences in collateral density and collateral size in the post-ischemic adductor muscle were found after a single MNC injection, repeated MNC injections, and saline (PBS) injection at week 4, further confirming the lack of functional improvement seen in LDPI results.

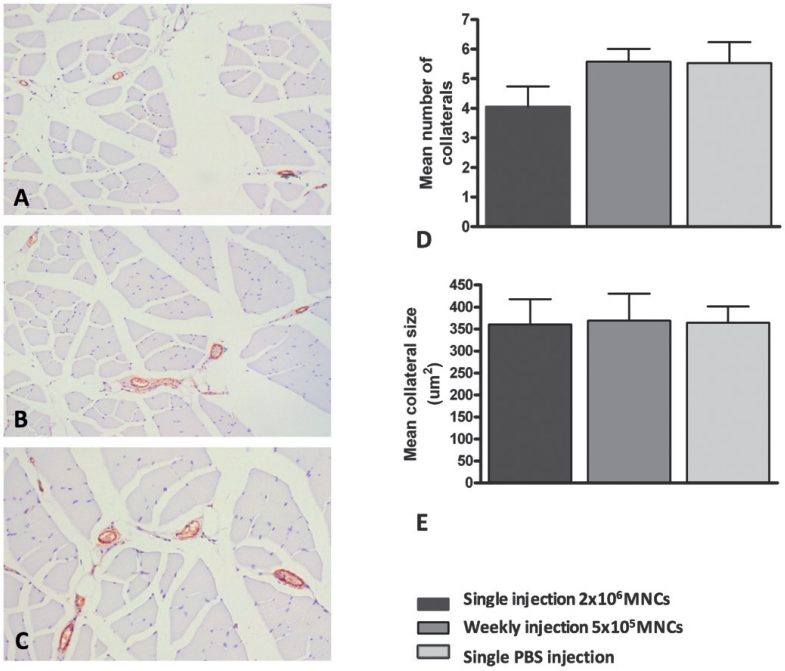

Figure 5. Immunohistochemistry analysis of arteriogenesis within the post-ischemic adductor muscle. Representative pictures of anti-α smooth muscle staining of (a) single $2x10^6$ MNC-injection, (b) weekly $5x10^5$ MNC-injection, and (c) PBS treated mice. Quantification of (d) mean number of collaterals and (e) mean collateral size showed no significant differences among different study groups four weeks after surgery(P=NS, ANOVA).

Confirmation of short-term LDPI findings. To further explore the observed short-term effect of cell therapy on paw perfusion, we performed an apoptosis specific ELISA on the affected gastrocnemius muscles. We hypothesized that increased monocytic cell numbers may have beneficial effects on ischemia-induced apoptosis in the muscular tissue. Thus, to investigate if higher perfusion ratios lead to tissue preservation, gastrocnemius muscles were assayed for DNA fragments in mono- and oligonucleosomes. As shown in **figure 6**, treatment with both single $2x10^6$ MNCs and weekly $5x10^5$ MNCs led to significantly (*P*=0.03 and P=0.02, respectively, ANOVA) decreased amount of fragmented DNA (mirroring apoptosis) as compared to the PBS group, which had an almost 3-fold higher expression than its healthy contralateral counterparts.

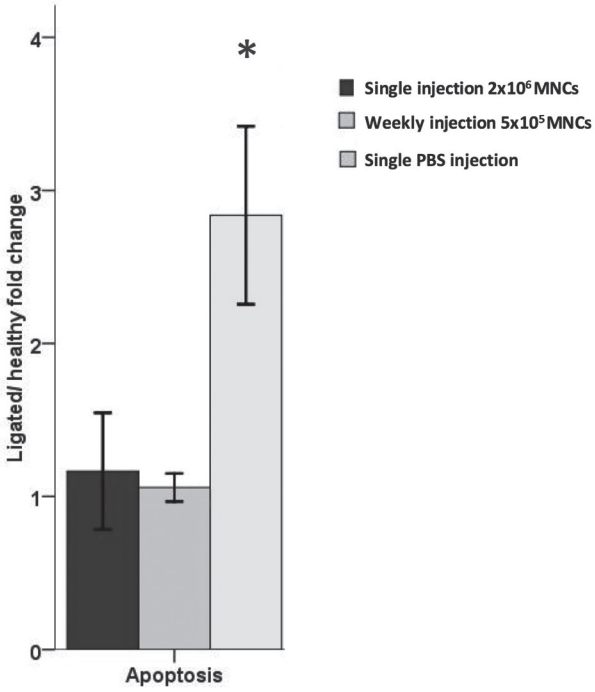

Figure 6. Quantification of short-term apoptotic rates in gastrocnemius muscles of MNC treated animals. ELISA for histone-associated DNA fragments in mono- and oligonucleosomes of digested gastrocnemius muscles revealed an almost 3-fold increase in apoptosis following left femoral artery ligation and PBS treatment as compared to the healthy contralateral muscle as well as compared to MNC treated animals (*P<0.05, ANOVA).

***In vivo* molecular imaging of MNC homing.** To date, most clinical trials have used a transplantation approach that is based on direct delivery into the affected muscle.[9] Others have chosen strategies that rely on stimulation of natural homing of progenitor cells to the affected area.[10] While the current studies with intramuscular injections of MNC suggest that low cell survival might underlie a lack of functional effect, there is no such data available for systemic injection of MNC. Therefore, FVB mice were injected i.v. with $5x10^6$ MNC 1 day following ischemia and were imaged by BLI until day 14. As shown in **figure 7**, the initial BLI signals on day 0 (1 hour after transplantation) equaled background levels, thus confirming the cells were spread out through the circulatory system, without signs of retention in the pulmonary capillaries as observed in previous studies with larger size cell types such as mesenchymal stem cells.[11] Over time, however, signal intensity to the injured area increased. In addition, signals arose from the bone marrow, spleen, and liver, which indicate homing patterns that mimic endogenous myelomonocytic pathways.[12]

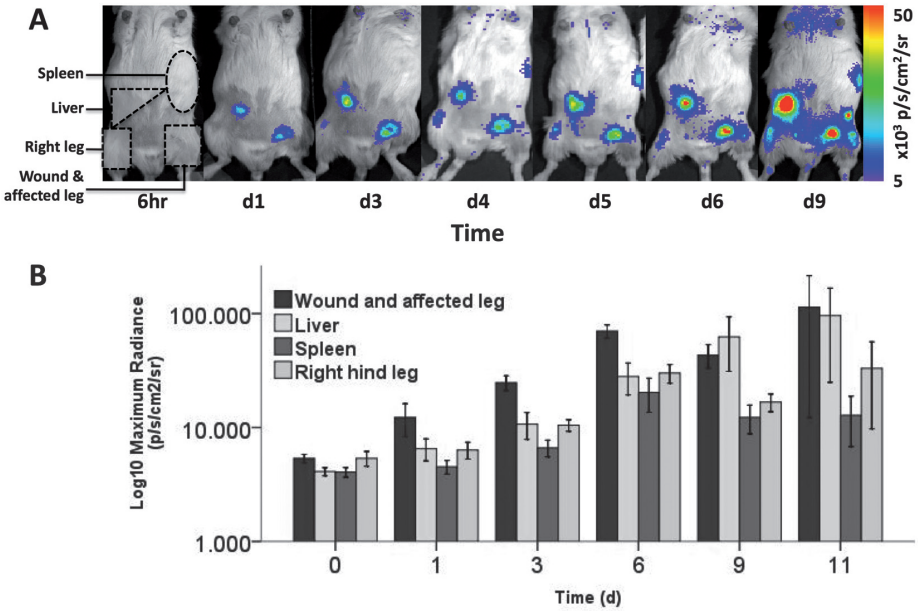

Figure 7. *In vivo* visualization of systemically injected MNC by BLI. (a,b) One day after left femoral artery ligation, 5x10^6 MNCs were injected via tail vein injection and monitored for 10 days. *In vivo* BLI pictures and signal quantification on multiple time points show that after an initial low signal period due to scattered MNCs throughout the body, cells then travelled to the injured area but also showed preference for the liver, bone marrow, and spleen. Scale bars represent BLI signal in photons/s/cm^2/sr (P=NS, ANOVA).

***Ex vivo* confirmation of *in vivo* patterns of cellular kinetics.** To validate and further specify the observed *in vivo* findings, organs were procured immediately following euthanization. As shown in **figure 8a**, BLI following dissection of the skin showed *in situ* signals from liver, spleen, and the long bones similar to *in vivo* results. However, the signals that were previously observed from the injured area *in vivo* were now largely concentrated in the subcutaneous fat pad as well as in the femoral bone. Indeed, when the different tissues were explanted, it became clear that there was only a low signal from the adductor muscle, while equally strong signals were observed from the scarred skin, the subcutaneous fat pad, and the bone marrow in the femoral bone. Thus, this *ex vivo* imaging confirmed the *in vivo* signals from liver and spleen. Moreover, the presence of GFP$^+$ donor MNCs in the bone marrow was validated with flow cytometry (**figure 8b**). Taken together, these experiments showed that BLI is a reliable method to monitor MNC trafficking in an *in vivo* fashion. Homing to the injured area was not limited to the adductor muscle, but also occurred to other areas of injury as well as more natural biological niches such as marrow, liver, and spleen.

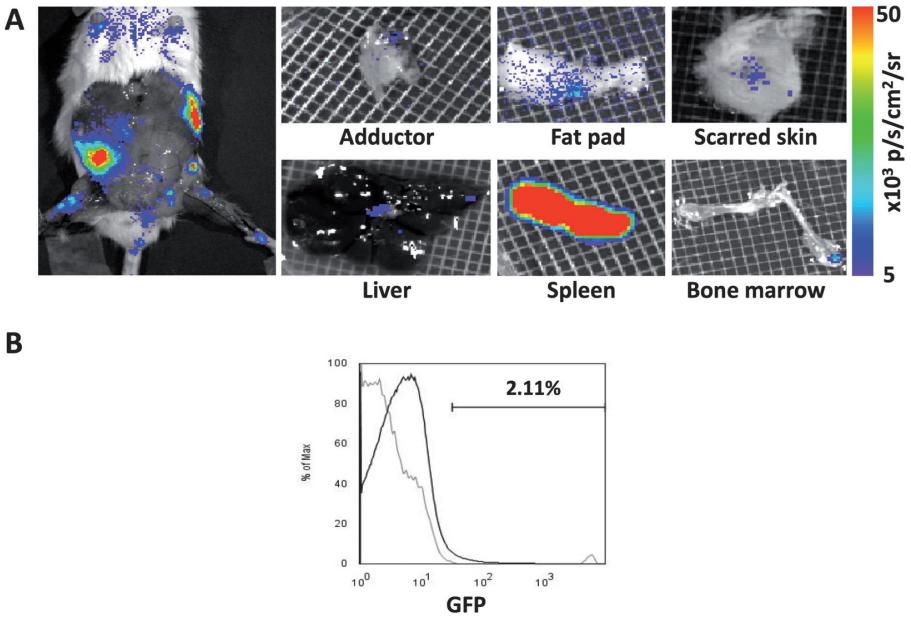

Figure 8. *Ex vivo* confirmation of *in vivo* MNC distribution patterns. (a) Graphic *in vivo* representation of MNC retention in liver, spleen, and bone marrow. Surprisingly, removal of the skin leads to a remarkable reduction of signal intensity from the scarred area. After explantation of various organs and ex vivo BLI, the signal that was previously observed from the injured area during *in vivo* experiments appeared to be a cumulative signal from MNC retention from skin, subcutaneous tissue, and muscle. Scale bars represent BLI signal in photons/s/cm^2/sr. (b) To confirm the BLI signals from the bone marrow, the marrow was flushed from the bone and processed through flow cytometry for GFP$^+$ donor cells. The flow cytometry results correlated with the BLI results, as the recipient bone marrow indeed contained Fluc$^+$/GFP$^+$ donor MNCs.

Monitoring effects of intravenously injected MNC therapy in severe PAD. The PAD model as described above has been used and validated multiple times in C57BL/6 mice.[13] The reason for using FVB mice in the previous experiments was to establish a clinically equivalent model of autologous cell transplantation as our F6 transgenic donor mice were bred on FVB background. However, we observed a robust endogenous recovery of arteriogenic response following ischemia by 4 weeks (see the PBS injection group in Figure 4B), which can be specific for FVB mice.[14] Therefore, to investigate the functional effects of intravenous injection and subsequent MNC homing to the ischemic environment, another strain of C57BL/6 mice underwent electro-coagulation of both the common iliac and femoral artery to ensure profound and more durable ischemia. One day post-operation, 5x10^6 MNCs or PBS as control were injected (n=10 per group), and paw perfusion was again measured by LDPI. As shown in **figure 9**, the ischemic/non-ischemic paw perfusion ratio decreased dramatically from an overall mean of

1.04±0.04 pre-operation to 0.04±0.01 post-operation (P<0.0001). Indeed, the current surgical model resulted in sustained loss of perfusion over 4 weeks. However, intravenous injection of MNCs was still incapable of restoring paw perfusion in a significant matter, with ratios of 0.60±0.07 in the cell group compared to 0.57±0.08 in the PBS group (P=NS) 4 weeks after operation.

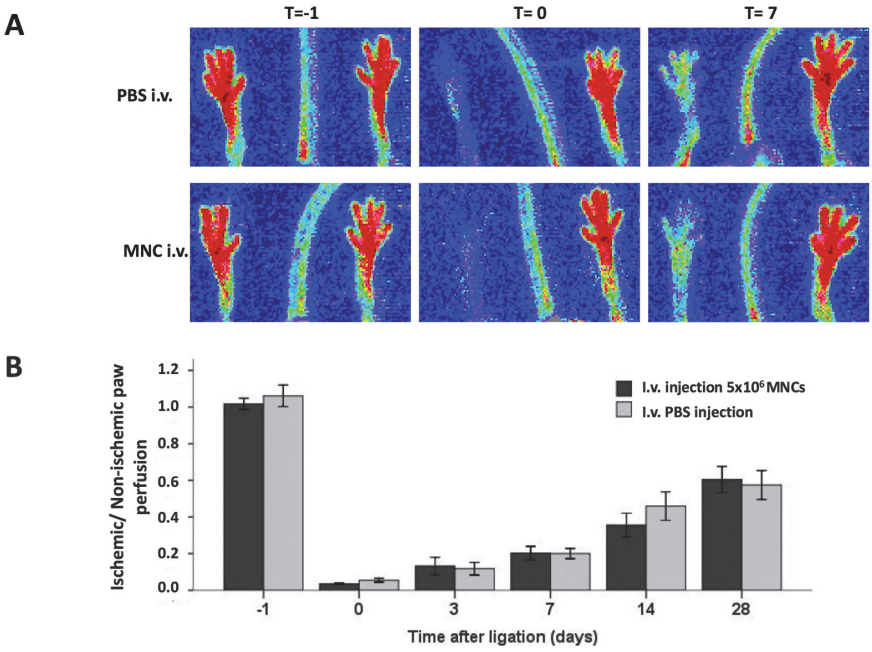

Figure 9. Functional results following systemic MNC injection after severe hind limb ischemia. (a) Following ligation of both the femoral and iliac arteries, markedly decreased paw perfusion was observed for a prolonged period. (b) Quantification of paw perfusion revealed systemic MNC injection was not capable of restoring paw perfusion significantly better than PBS treatment during. (P=NS, Repeated measurements ANOVA).

DISCUSSION

This is the first study to evaluate post-transplant MNC behavior in a murine model of PAD using *in vivo* molecular imaging techniques. The major findings can be summarized as follows: (1) BLI is a valid tool to monitor MNC survival, proliferation, and migration; (2) MNC survival following a single intramuscular injection is short-lived; (3) repeated MNC injections do not provide significantly prolonged cell survival; (4) homing of MNCs following intravenous injection is not limited to the area of injury; and (5) neither intramuscular nor intravenous injection of MNC results in an increased paw perfusion.

The clinical relevance of these findings is significant. Since the pioneering work of Tateishi-

Yuyama and colleagues[9], over 25 clinical trials have been registered on www.clinicaltrials.gov, using either intramuscular or systemic injections into the ischemic leg. Although the findings from this first study were hopeful, so far these results have not been confirmed by large randomized clinical trials. The initial thought behind the use of progenitors cells in regenerative medicine was that it could truly regenerate the damaged tissue by forming new blood vessels[15], skeletal muscle[16], or even myocardium.[17] However, since the true regenerative capacity has been questioned[18], and considering the poor survival capacity of MNC and other progenitor cells in this and other studies thus far[5], a more plausible explanation for a possible beneficial effect would be the secretion of protective cytokines as suggested before.[19] Indeed, it has recently been shown that a more profound angiogenic response can be achieved in ischemic muscle by transplanting progenitor cells overexpressing both VEGF and SDF-1.[20] Alternatively, to achieve true regeneration, one could switch to more specialized cell types rather than whole MNCs. In this respect, it has recently been shown that embryonic stem cell-derived endothelial cells can improve perfusion due to the favorable effect of engraftment and biological activity.[21] Thus, in the future, it might be a feasible approach to use a set of growth factors by gene therapy, increase survival of specialized cells (e.g., embryonic stem cell or induced pluripotent stem cell derivatives), or use a combination of these two.

Previous studies have assessed MNC function and mechanism following transplantation into the ischemic leg largely using post-mortem histological techniques.[22] However, this requires euthanizing the animal, thereby increasing inter-animal variance and preventing longitudinal studies of the same subject. Moreover, the search for scant donor cells on histological slides from all organs is extremely difficult and time consuming. As a consequence, these techniques are less suitable for studying the kinetics of cells through the body over time. In contrast, in this study we have used our molecular imaging platform based on the double-fusion reporter construct carrying $Fluc^+/GFP^+$, to yield valuable insight into longitudinal cell fate. By doing so, we were able to track the spatiotemporal kinetics of MNC homing, retention, and survival in a murine model of PAD.

Interestingly, we observed a relatively limited cell survival after intramuscular injection in the adductor muscle. After a short-term post MNC transplantation increase in BLI signal until day 3, a rapid decrease in BLI-signal intensity to background levels after four weeks was observed. The limited cell survival was confirmed by the immunohistochemical staining against GFP^+ cells. One week after the fourth transplantation of $5x10^5$ MNCs, low numbers of these cells could be found near blood vessels, suggesting a role in neovascularization, or indicating these cells prefer the adjacency of oxygenated blood. The poor survival in the adductor muscle, however, is interesting since femoral artery ligation results in less profound ischemia in the ad-

ductor muscle as compared to the gastrocnemic muscle. This suggests that even in a normoxic niche, MNCs require more biologically attractive environments to be capable of robust survival. This once again stresses the need for development of cell survival augmenting approaches such as scaffolds or transduction of cells with pro-survival factors.

Results from this study show that, following systemic injection, MNCs migrate extensively to the bone marrow, spleen, and liver. This pattern indicates MNCs travel to their natural biological niches as all of these organs play a role in intra- and extramedullary hematopoiesis. Confirming this observation, our BLI findings are concordant with previous leukocyte scans showing retention in the liver and spleen.[23] Apparently, the chemoattractant properties of these organs are stronger than the ischemic environment in the affected muscle. For future experiments, it is important to improve homing to the ischemic muscles which may increase arteriogenic response as measured by LDPI. This can be realized in two ways: 1) improving the attractiveness of the target environment with, for example, the MNC mobilizer stromal-derived factor-1 (SDF-1)[24]; or 2) manipulating the cells to become more specifically guided. In this respect, it might be a better approach to isolate a subset of the mononuclear fraction such as the $CD14^+$ expressing cells that are expected to play a more active role in the restorative process after ischemia.[25]

Taken together, this is the first study to monitor the kinetics of MNCs in PAD in an *in vivo* fashion using molecular imaging techniques. Results from this study highlight caution should be exercised when interpreting results from experimental and clinical studies. The poor survival and homing patterns warrant further research toward better retention and increased biological activity of the cells in the injured area. By doing so, cell therapy might develop as a valuable option for treating end-stage PAD.

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