Systemically Delivered Adipose Stromal Vascular Fraction Cells Disseminate to Peripheral Artery Walls and Reduce Vasomotor Tone Through a CD11b+ Cell-Dependent Mechanism

MARVIN E. MORRIS, a,b JASON E. BEARE, b ROBERT M. REED, a JACOB R. DALE, a AMANDA J. LEBLANC, a,c CHRISTINA L. KAUFMAN, d HUAIYU ZHENG, e CHIN K. NG, e STUART K. WILLIAMS, a,c JAMES B. HOYING a,c

Key Words. Cell therapy • Adipose stromal vascular fraction • Small artery • Vasomotor tone • Macrophage

ABSTRACT

Vasomotor tone, an important aspect of tissue healing, is often compromised in disease and tissue injury. Dysfunction in the smaller vasoactive arteries is most impactful, given the role of these vessels in controlling downstream tissue perfusion. The adipose stromal vascular fraction (SVF) is a mix of homoeostatic cells shown to promote tissue healing. Our objective was to test the hypothesis that autologous SVF cells therapeutically modulate peripheral artery vasoactivity in syngeneic mouse models of small artery function. Analysis of vasoactivity of saphenous arteries isolated from normal mice 1 week after intravenous injection of freshly isolated SVF cells revealed that pressure-dependent artery vasomotor tone was decreased by the SVF cell isolate, but not one depleted of CD11b+ cells. Scavenging hydrogen peroxide in the vessel wall abrogated the artery relaxation promoted by the SVF cell isolate. Consistent with a CD11b+ cell being the relevant cell type, SVF-derived F4/80-positive macrophages were present within the adventitia of the artery wall coincident with vasorelaxation. In a model of artery inflammation mimicking a common disease condition inducing vasoactive dysfunction, the SVF cells present within the adventitia of the artery wall coincident with vasorelaxation. In a rat model of myocardial infarction, adipose SVF cells, applied as an epicardial patch, improved coronary functional flow reserve, independent of increases in vessel density [7]. Whether these vascular-associated SVF cells from the patch acted directly on the vessels to influence vessel dilation or constriction was not determined.

Perfusion to a tissue bed is regulated by the vasoactive feed and resistance arteries (arteries 350 μm or less in diameter [8]) supplying the tissue. These upstream small arteries normally exist in a preconstricted state (termed “tone”) enabling dynamic increases or decreases in downstream blood flow to meet tissue demands. Increases in vasomotor tone dampens vasodilation, thereby limiting the perfusion capacity. In many diseases, particularly those associated with tissue inflammation, the ability of these small arteries to properly dilate and constrict is compromised [9–11]. In inflamed tissues, such as those with chronic ischemia or metabolic disorders (e.g., diabetes, obesity), the vessel tone is elevated, in part because of the elevated oxidant stress [12], contributing to vasoactive dysfunction [13–15]. Persistent increases in small artery tone can lead to subsequent, long-lasting, undesired...
structural changes [16], further exacerbating tissue perfusion and compromising tissue repair.

To explore the possible impact of adipose SVF cells on vascular function in normal and inflamed tissue conditions, we tested the hypothesis that freshly isolated adipose SVF cells might act directly to influence vasoactivity when used therapeutically. Thus, we examined the vasoactivity of isolated saphenous arteries from mice injected intravenously with freshly isolated, syngeneic, adipose SVF cells. Additionally, because tissue macrophages (MϕPs) reside within adipose tissue [4, 17] and regulate the vasoactivity of arteries as normal constituents of the artery wall [18], we examined the SVF-derived macrophage as a potential important cell type mediating the possible vascular responses. Finally, we determined the effect of the SVF cells on vessel diameter, as a surrogate indicator of vascular tone, in a focal model of artery inflammatory insult.

Materials and Methods

All animal studies were performed under protocols approved by the University of Louisville Institutional Animal Care and Use Committee and according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The mouse strains used in the present study included FVB/n, FVB-Tg(CAG-luc,-GFP)LG85Chco/J [19], and Tg(TIE2GFP)287Sato/J [20], either purchased from Jackson Laboratories (Bar Harbor, ME, http://jaxmice.jax.org) or obtained from in-house colonies. All reported findings were from male mice (donors and recipients) aged 10–12 weeks.

Isolation of Adipose SVF Cells

For SVF isolations, epididymal fat pads were collected, weighed, minced until paste-like, and digested with a filter-sterilized solution of lot-tested 0.1% (wt/vol) collagenase plus 0.05% (wt/vol) DNase in 5-(and-6)-choromethyl-2’,7’-dichlorodihydrofluorescein diacetate (DCF)-phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA-DCF-PBS) at 37°C for 35 minutes [4]. The digestate was centrifuged, and the SVF cell pellet was suspended and washed twice in 0.1% BSA-DCF-PBS. The washed pellet, suspended in 10 ml of 0.1% BSA-DCF-PBS, was passed through a sterile 20-µm nylon screen prewetted with 0.1% BSA-DCF-PBS to remove any incompletely digested matrix fragments and clumped cells. The flow through of single cells was collected and an aliquot counted with a NucleoCounter (LI-COR, Lincoln, NE, http://www.licor.com). The total SVF cell yield averaged 2.58 × 10⁶ ± 0.46 × 10⁶ cells per gram of fat.

Magnetic Depletion of SVF Cell Isolates

The depletion experiments used the Miltenyi MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) according to the manufacturer’s instructions. In brief, up to 1 × 10⁷ screened SVF cells were suspended in 90 µl of MACS buffer (degassed solution of PBS, pH 7.2; 0.5% BSA, and 2 mM EDTA) and incubated with 10 µl of anti-mouse CD11b antibody conjugated to iron particles (Miltenyi Biotec, catalog no. 130-049-601) at 4°C for 15 minutes. An additional 3 ml of MACS buffer was added to the cell suspension followed by centrifugation at 400g for 4 minutes. The cell pellet was resuspended with 1.0 ml of MACS buffer and loaded 0.5 ml at a time onto a Miltenyi Biotec MACS column prewetted with 0.5 ml of MACS buffer within the magnet chamber. The cell-loaded column was gravity drained and then flushed with 0.5 ml of MACS buffer at least 3 times to remove any additional cells. The effluent was collected and considered to be the CD11b⁻-depleted fraction (SVF-11bD), which represented a 38.1% ± 1.65% reduction in the total cell numbers. The column was removed from the magnet and flushed as before to collect the CD11b⁺-enriched fraction (11bE).

Flow Cytometry

Aliquots of SVF cells, CD11b⁺-depleted SVF cells (SVF-11bD), and CD11b⁺-enriched SVF cells (11bE) isolated from FVB/n tie2:GFP expressing transgenic mice were divided into polypropylene tubes for flow cytometry at a concentration of 5 × 10⁶ to 1 × 10⁶ cells in 100 µl of wash buffer (Dulbecco’s PBS containing 1% BSA and 0.025 M HEPES) per tube. Aliquots of the following antibodies (at optimized antibody dilutions) were added to label the cell surface markers: CD2-PE (catalog no. 553112; BD Biosciences, San Diego, CA, http://www.bdbiosciences.com), CD45-PerCP (catalog no. 557235; BD Biosciences), CD11b-APC (catalog no. 130-098-088; Miltenyi Biotec), Gr-1-PE (catalog no. 12-6931-83; eBioscience, San Diego, CA, http://www.ebioscience.com), FcR1-PerCP (catalog no. 46-5898-82; eBioscience), CD11b-PE (catalog no. 130-098-087; Miltenyi Biotec), CD80-APC (catalog no. 17-0801-82; eBioscience), F4/80-PerCP-Cy5.5 (catalog no. 45-4801; eBioscience), and CD301-Alexa Fluor 647 (catalog no. MCA2392A647T; AbD Serotec, Raleigh, NC, http://www.abdserotec.com). The green fluorescent protein (GFP) fluorescence (i.e., tie2 expression) was used to mark the endothelial cells. Species-matched isotypes were added to separate tubes of wild-type FVB/n SVF cell isolates. Additionally, single color tubes of FVB/n SVF cells were used as compensation controls. The cells were incubated in antibodies at 4°C for 30 minutes protected from light, lysed with PharmLyse (catalog no. 555899; BD Biosciences) for 3 minutes at 37°C, washed twice with 2 ml wash buffer, spun at 350g for 5 minutes to pellet, suspended in 400 µl wash buffer per tube, and analyzed using an LSRII flow cytometer (BD Biosciences) using FACS Diva software. Postacquisition data analyses were performed using FlowJo, version 7.6.2, software (FlowJo, Ashland, OR, http://www.flowjo.com).

Tail Vein Injection of Cells

SVF cells (1 × 10⁶ cells per mouse) and SVF-11bD cells (0.8 × 10⁶ cells per mouse), suspended in 0.2 ml of sterile saline, were injected into the tail vein using a 30-gauge needle as a single bolus.

Saphenous Artery Vasoactive Responses

Saphenous arteries were explanted, taking care to remove the extraneous connective tissue, from anesthetized (5% isoflurane/O₂ balance) recipient mice into cold, filtered physiological saline solution (PSS) (pH 7.4; containing 145 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl₂, 1.17 mM MgSO₄, 1.2 mM NaH₂PO₄, 5.0 mM glucose, 2,00 mM pyruvate, 0.02 mM EDTA, 3.0 mM 4-morpholinoepanesulfonic acid buffer, and 1% BSA). The arteries were cannulated on size-comparing resistance-matched glass pipettes in a Lucite chamber containing warm (37°C) PSS and assessed as previously described [21]. The arteries were preconstricted with phenylephrine (2 µM) to approximately 30% of the resting diameter at 50 mmHg; those that did not constrict were discarded. To assess the pressure-dependent responses, the intraluminal pressure was decreased...
to 1 mmHg and sequentially increased (waiting 3 minutes at each step), while simultaneously recording the luminal diameters throughout the procedure. The intraluminal pressure was then returned to 50 mmHg, the chamber was washed with fresh PSS and refreshed with phenylephrine, and, on tone establishment, the lumen diameter changes were recorded during drug dose-response curves for acetylcholine (doses ranged from $1 \times 10^{-9}$ to $1 \times 10^{-4}$ M, 3 minutes per dose) and, after washing, for sodium nitroprusside (doses ranged from $1 \times 10^{-10}$ to $1 \times 10^{-4}$ M, 3 minutes per dose). The chamber was then washed twice with PSS without CaCl$_2$ to measure the maximum dilation. The chamber was washed twice again without calcium and exposed to the same pressure-change regimen to assess the passive relaxation to pressure. The intraluminal diameters (measured with electronic calipers) were normalized to the maximum diameter obtained in the absence of calcium and are reported as the percentage of relaxation: (diameter/maximum diameter) $\times 100$.

**Polyethylene Glycol-Catalase Myogenic Responses and Reactive Oxygen Species Fluorescence Imaging**

After the active myogenic response assessment, the chamber was washed with PSS without albumin, and the vessels were incubated for 10 minutes in the dark with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrorhodamine 123 (5 $\mu$M) and washed with PSS without albumin to fluorescently measure the presence of H$_2$O$_2$ in the vessel walls [22]. DCF fluorescence images were captured with the same exposure times and magnifications from arteries incubated without and then with polyethylene glycol (PEG)-catalase (PEG-CAT; 500 U/ml; catalog no. C4963; Sigma-Aldrich, St. Louis, MO, http://www.sigm Aldrich.com) for 1 hour [23]. The pre- and post-PEG-CAT images were analyzed for fluorescence intensity within a 40 $\times$ 150 $\mu$m region of interest using Nikon Elements software (Nikon Instruments, Melville, NY, http://www.nikoninstruments.com). A separate group of mice were used to measure the levels of O$_2$· and nitric oxide (NO) using dihydroethidium (DHE) and 4-amino-5-methylamino-2'-difluorofluorescein diacetate (5 $\mu$M) and washed with PSS without albumin to fluorescently measure the presence of H$_2$O$_2$ in the vessel walls [22]. DCF fluorescence images were captured with the same exposure times and magnifications from arteries incubated without and then with polyethylene glycol (PEG)-catalase (PEG-CAT; 500 U/ml; catalog no. C4963; Sigma-Aldrich, St. Louis, MO, http://www.sigm Aldrich.com) for 1 hour [23]. The pre- and post-PEG-CAT images were analyzed for fluorescence intensity within a 40 $\times$ 150 $\mu$m region of interest using Nikon Elements software (Nikon Instruments, Melville, NY, http://www.nikoninstruments.com). A separate group of mice were used to measure the levels of O$_2$· and nitric oxide (NO) using dihydroethidium (DHE) and 4-amino-5-methylamino-2'-difluorofluorescein diacetate (DAF), respectively. The vessel was infused intraluminally with DHE ($1 \times 10^{-4}$ M) for 10 minutes and washed with PSS without albumin. Images of ethidium bromide and hydroethidine fluorescence were captured and subsequently analyzed using Nikon Elements software by subtracting the hydroethidine fluorescence signal from the ethidium bromide fluorescence signal to determine the relative O$_2$· levels. For NO, the vessels were infused with DAF (5 $\mu$M) for 10 minutes, protected from light, and washed with PSS without albumin. The DAF images were analyzed for fluorescence intensity as above.

**Confocal Microscopy**

Fixed saphenous arteries (2% paraformaldehyde) were washed for 2 $\times$ 5 minutes in PBS, and then permeabilized in PBS plus 0.5% Triton-X (catalog no. T8787; Sigma-Aldrich) for 15 minutes at room temperature. The vessels were blocked for 10 minutes at room temperature in DAKO Protein Block (code 0909; Dako, Glostrup, Denmark, http://www.dako.com), washed 2 times for 5 minutes in PBS and then incubated overnight with gentle shaking at 4°C in rat anti-mouse F4/80 antibody (catalog no. 14-4801; eBioscience) at 1:250 dilution in PBS plus 10% normal donkey serum plus 0.5% BSA plus 0.1% Triton-X. The next day, the arteries were washed 3 times for 15 minutes in PBS with gentle shaking at room temperature and protected from light. Following this washing, the vessels were incubated in donkey anti-rat rhodamine (catalog no. 712-026-150; Jackson ImmunoResearch, West Grove, PA, http://www.jacksonimmuno.com) at 1:200 dilution in PBS, 10% normal donkey serum, 0.5% BSA, and 0.1% Triton-X for 1 hour at room temperature, protected from light. After secondary antibody incubation, the vessels were washed for 15 minutes in PBS at room temperature and then incubated for 30 minutes at room temperature in Hoechst nuclear stain (1:2,000 dilution) and subsequently washed 15 minutes in PBS. The stained vessels were placed in Fluoromount-G (catalog no. 0100-01; Southern-Biotech, Birmingham, AL, http://www.southernbiotech.com) on a dimped slide to allow the vessels to retain their natural shape during imaging. Confocal imaging was performed using an Olympus FV1000 confocal microscope (Olympus America, Central Valley, PA, http://www.olympusamerica.com) equipped with 488 (for visualizing GFP+ SVF cells) and 543 (F4/80) laser lines and a multiphoton laser set to 800 nm to visualize Hoechst staining. Confocal stacks were obtained and subsequently washed 3 times for 15 minutes in PBS with gentle shaking and then incubated in 3% hydrogen peroxide for 4, and 6. 5-Ethynyl-2'-deoxyuridine (EdU) was detected as per the manufacturer’s instructions (Click-IT Plus EdU Alexa 647 Imaging Kit, catalog no. C10640; Life Technologies).

**Bioluminescence Imaging**

In vivo bioluminescence imaging was performed on mice using a Photon Imager (BioSpace Lab, Paris, France, http://www. biospacelab.com). Each mouse was injected intraperitoneally with 0.1 ml of warmed PBS containing a vasodilator cocktail (2.5 $\mu$g/ml single nucleotide polymorphism [SNP], 60 $\mu$g/ml papaverine, 10 U/ml heparin, and 1 mg/ml adenosine) and then with 12 ml of warmed 4% paraformaldehyde/PBS, all at 100 mmHg pressure using a pressure gauge. The harvested tissue samples were placed in 4% paraformaldehyde/PBS and kept at 4°C until processed into paraffin before sectioning and staining. For morphometry, the sections were stained with hematoxylin and eosi in using standard methods. To identify the injected cells in the saphenous arteries and select tissues, the sections were immunostained for luciferase-positive cells using an HRP kit (EnVision+
System HRP, Dako) according to the manufacturer’s instructions. The sections were treated with 3% hydrogen peroxide for 10 minutes at room temperature before incubation with an anti-firefly luciferase antibody (Abcam, Cambridge, U.K., http://www.abcam.com) at a 1:1,000 dilution in 10% goat serum (Sigma-Aldrich) for 1 hour at room temperature. All sections were then counterstained with hematoxylin and cover slipped. Tissue sections from luciferase-positive donor mice and wild-type mice that did not receive cells served as the positive and negative controls, respectively, for immunostaining.

Saphenous Artery Cuffing

All procedures were performed using a sterile technique with modifications to a previously published method [24]. The medial area of the right hindlimb of an anesthetized (isoflurane), supine mouse maintained at 37°C body temperature was depilated and wiped with Nolvasan solution (Zoetis, Florham Park, NJ, http://www.zoetis.com). A 1-cm incision on the calf side of the midline, approximately one third the distance proximal to the knee, was made and deflected to the midline to expose the femoral-saphenous vessels. The overlying fascia was blunt dissected to expose the vascular nerve sheath, which was further opened to access the vessels. The saphenous artery was carefully freed from the saphenous vein for a length of 4 mm. A 2-mm-long section of ethylene oxide-sterilized PE-50 tubing split lengthwise down the middle was placed around the freed artery section and tied closed with 5-0 silk ligature. The skin was placed back into position and closed with a single surgical clip. The sham groups were prepared as described, except that the cuff was not placed.

RESULTS

Cells From Injected SVF Disseminate Into Recipient Peripheral Tissues

Because of the practicalities of treating small arteries owing to their relatively small size, numbers, and disseminated locations, we explored intravenous delivery of the freshly isolated SVF cells as a method to distribute the cells. Our rationale was that the injected SVF cells would disperse throughout the circulation, homing to the small arteries. Whole-animal bioluminescence imaging revealed that luciferase-active SVF cells delivered to syngeneic recipient adult mice were observed throughout the body of the mouse, including in the bone marrow, 1 week and as late as 3 months after injection (Fig. 1). Furthermore, relative luminescence intensities were increased at the later time points, particularly in the visceral fat pads, suggesting SVF cell proliferation had occurred. Consistent with the imaging results, luciferase-positive cells were present within the histological sections of a variety of tissues, including around and within the walls of the small vessels, after i.v. injection (Fig. 1).
CD11b+ Cells Constitute a Relatively Large Fraction of the SVF Isolate

To define the cell constituents in our SVF preparation and possibly identify the cell types distributing to peripheral tissues, we used flow cytometry to characterize the fresh SVF isolates. Using a donor mouse expressing GFP behind the tie2 promoter [20], we identified the prevalent cell types expressing markers common to innate immune cells (CD11b), endothelial cells (tie2-GFP), tissue macrophages (F4/80), myeloid cells (Gr-1), and lymphocytes (CD2) (Fig. 2; supplemental online Figs. 1–3). The largest fractions of the SVF cells appeared to be endothelial cells (∼25%), CD11b+ cells (∼20%), and myeloid cells (∼22%). We have previously shown that mesenchymal stem cells represent a relatively small proportion of the cells in the fresh SVF isolate [4]. Although differences were present in the isolation methods, similar distributions have been described in lean adipose tissue from C57B/6 mice by others [25].

Because the CD11b+ cells represented a relatively large fraction of the fresh SVF isolate, and MΦs, a prominent CD11b+ cell type [26], are dynamic regulators of vascular function in arteries [18], we further processed the SVF cells to create a preparation depleted of CD11b+ cells as a method to investigate the role of this SVF-derived subset of cells in the hypothesized vascular activity. To do this, we used a selective magnetic separation approach (Miltenyi Biotec) targeting CD11b-expressing cells. Flow cytometry of the different fractions after magnetic depletion confirmed that ∼94% of the CD11b+ cells were removed from the SVF isolate (Fig. 2; supplemental online Figs. 1–3). Furthermore, ∼80% of the CD11b+ enriched fraction (11bD; the cells captured on the column) was highly enriched for macrophages (Fig. 2; supplemental online Figs. 1–3). The presence of tie2-GFP+ cells in this CD11b+ enriched fraction resulted from small capillary fragments that were nonspecifically trapped within the column matrix used in...
the depletion process (data not shown). Similar to Mφs, but to a lesser extent, Gr-1− cell distributions between the different fractions were also affected by the depletion process (Fig. 2). Gr-1 is a commonly used marker for myeloid cells [28]. However, a significant proportion of the Gr-1+ cells in the adipose SVF isolate were double-positive for CD11b (Fig. 2), a marker combination recently described for myeloid suppressor cells, a cell type thought to mediate lymphocyte activity [29]. Thus, although a significant majority (>80%) of the cells removed by the CD11b selection were macrophages, other cell types (likely hematopoietic in origin given the high prevalence of CD45+ cells) were also removed.

**Intravenously Injected SVF Cells Reduce Artery Vasomotor Tone in a CD11b+ Cell-Dependent Manner**

To evaluate the potential of the SVF cells and the CD11b+ cell-depleted subtraction to modulate vascular function, we directly measured the vasodilatation responses in the saphenous arteries from syngeneic recipient mice that received a single intravenous injection of these preparations. Although the saphenous artery in the mouse is in a conduit vessel position within the vascular tree, its size, medial structure and vasoactivity functionally reflect more the vasoactive, small distal arteries in humans. In an isolated, pressurized vessel preparation, saphenous arteries from untreated, SVF cell-injected, or SVF-11bΔ cell-injected mice exhibited comparable endothelium-dependent (acetylcholine) and -independent (SNP) vasodilatation responses 1 week after injection (Fig. 3). However, vessel relaxation to the sequentially increasing changes in intravascular pressure was significantly enhanced in the saphenous arteries from mice injected with the SVF cells, but not those with the SVF depleted of CD11b+ cells (Fig. 3). These differences in active tone were not observed in the absence of extravascular Ca2+ (Fig. 3), consistent with an absence of any structural remodeling of the artery wall (supplemental online Fig. 4). The influence on pressure-dependent, but not agonist-dependent, relaxation reflects changes in vessel wall tension or vasomotor tone.

**Changes in Vasomotor Tone Are Hydrogen Peroxide-Dependent**

Although potentially many pathways mediate vasomotor tone, one prevalent mechanism involves vessel wall reactive oxygen species (ROS) [30]. H2O2, one such ROS, is a potent vasodilator [31], and the reduction in super oxide anions (O2−) associated with its production preserves nitric oxide, thereby further promoting vessel relaxation [32]. Scavenging H2O2 with tissue peroxo- meent PEGylated-catalase [23] (Fig. 3C, 3D) in saphenous arteries from mice injected with the SVF cells eliminated the cell-dependent decrease in vasomotor tone, mimicking the pressure responses with CD11b-depleted SVF cell treatment. The H2O2 activity is unique to the SVF cell therapy, because baseline saphenous artery tone is mediated by NO but not H2O2 (Fig. 3E, 3F).

Also consistent with an SVF-induced H2O2 production, the saphenous arteries from mice injected with SVF cells had higher H2O2 levels, but not nitric oxide levels, in the vessel wall compared with the levels in the untreated arteries (Fig. 4). The super oxide levels were concomitantly increased (Fig. 4). The H2O2 and O2− levels in the CD11b-depleted SVF cell vessel group were not elevated (Fig. 4). Also, higher numbers of DCF-bright (H2O2-producing [33]) cells were present in the walls of SVF cell-injected arteries than in the untreated or SVF-11bΔ-injected arteries (Fig. 4). All these findings are consistent with an SVF-derived, CD11b+ cell-dependent, H2O2-dependent mechanism of small artery relaxation.

**SVF-Derived Macrophages Populate the Saphenous Artery Adventitia**

The presence of luciferase-positive SVF cells surrounding peripheral arteries after i.v. injection and the additional presence of H2O2-producing cells in the artery wall after SVF delivery suggested that the injected SVF cells were populating the saphenous artery walls. To address this directly, we examined isolated saphenous arteries via confocal microscopy for the presence and location of the SVF cells by imaging for GFP fluorescence (expressed via the GFP transgene within the SVF cells). Using rhodamine-tagged GS-1 lectin and hydrazide to simultaneously label the endothelial cells of the vaso vasorum and the elastic laminae, respectively [34], we observed GFP+ SVF cells within the artery wall near the vaso vasorum of the artery adventitia, with nearly 75% of these cells positive for the tissue macrophage marker, F4/80 (Fig. 5; supplemental online Fig. 5). GFP+ cells were also present within the adventitia of arteries from mice injected with the CD11b+ cell-depleted SVF preparation (SVF-11bΔ) (Fig. 5; supplemental online Fig. 5). However, additional analysis determined that the fraction of SVF-derived Mφs (GFP-F4/80 double-positive cells) was ~50% lower (Fig. 5B, 5C), indicating a depletion of SVF-derived Mφs in the arterial walls of the mice injected with the CD11b+ cell-depleted fraction. No difference was found in macrophage density in the walls between the two experimental groups. However, considerably more of the adventitial macrophages were derived from SVF in the SVF-treated arteries (supplemental online Fig. 5).

**Intravenously Injected SVF Cells Promote Dilation in a Vessel Insult Model**

The findings to this point indicate that SVF cells populate the adventitia of vasoactive arteries and relax vasomotor tone by modulating wall reactive oxygen species. To explore whether the SVF cells are also capable of modulating tone in a condition involving vascular insult, we used a model of focal vascular injury [35] in which a small region of the right medial saphenous artery is loosely cuffed with PE tubing to induce a materials-driven, tissue inflammation around the artery (Fig. 6). The intent with this model was to mimic the type of insult experienced by an artery present within in a chronically inflamed tissue while simultaneously minimizing any confounding systemic influences (e.g., cytokines). After inducing the insult, we intravenously delivered freshly isolated SVF cells as before and assessed the vessel morphology 1 week later. As anticipated, and consistent with a more relaxed artery wall phenotype, SVF cell delivery significantly increased the cuffed artery diameter (Fig. 6). As before, removal of the CD11b+ cells from the SVF attenuated this prodilation response (Fig. 6). Similar to the normal arteries, no significant changes were found in the other artery morphometric parameters, indicating that SVF cell-dependent wall remodeling also did not occur (supplemental online Fig. 6). Additionally, injected cells populated the adventitia of the cuffed saphenous arteries and the inflammation-induced granulation tissue surrounding the artery segment within the cuff confines (Fig. 6).
SVF cells relax vasomotor tone in normal saphenous arteries. Vessel relaxation curves of isolated, normal saphenous arteries from mice untreated or injected with SVF cells or SVF-11b\(\text{D}\) cells in response to endothelium-dependent (acetylcholine) (A) or endothelium-independent (nitroprusside) (B) dilators, progressively increased intravascular pressures in the presence (C) or absence (D) of extravascular calcium, or untreated vessels (\(n = 3\)) exposed to increasing pressures in the presence of L-NAME (to block nitric oxide production) (E) or the \(\text{H}_2\text{O}_2\) scavenger polyethylene glycol (PEG)ylated-CAT (F). \(\text{H}_2\text{O}_2\) in SVF-injected vessels was scavenged using PEGylated-CAT (C). In all cases, the measured internal vessel diameters were normalized to maximal diameters to account for intervessel variability. \(\text{p}, p < .05\), determined by \(t\) test between the untreated and SVF-injected vessels at each pressure. Abbreviations: 11b\(\text{D}\), removed CD11b\(^+\) cell-enriched fraction; ACh, acetylcholine; CAT, catalase; L-NAME, L-NG-nitroarginine methyl ester; SNP, sodium nitroprusside; SVF, stromal vascular fraction; SVF-11b\(\text{D}\), CD11b\(^-\) cell-depleted adipose SVF cells.
DISCUSSION

One week after intravenous injection, freshly isolated, adipose SVF cells had populated the walls of peripheral arteries. One such artery harboring these cells, the saphenous artery, exhibited enhanced pressure-dependent, but not endothelium-dependent and -independent, relaxation, without a change to the arterial wall structure. Concomitantly, the injected SVF cells led to increased levels of the vasoactive mediator H$_2$O$_2$ within the artery wall, the scavenging of which attenuated the relaxation induced by the SVF cells. CD11b$^+$ cells within the isolate were necessary for the change in artery tone and increase in H$_2$O$_2$. All of which indicates that a subset of CD11b$^+$ cells within the i.v. delivered adipose SVF isolate spontaneously populate the walls of existing small arteries and modulate vessel tone, likely via a mechanism involving regulation of the reactive oxygen species balance within the vessel wall.

Although we were not practically able to remove the arteries from the cuff environment for vasoactivity analysis, the increase in the diameter of the SVF cell-treated cuffed saphenous arteries

Figure 4. SVF cells alter reactive oxygen species within the walls of saphenous arteries. (A): DCF fluorescence in isolated saphenous arteries to visualize the presence of H$_2$O$_2$. Arteries were from untreated, SVF-injected, or SVF-11bΔ-injected mice. Bottom row: Images of the same vessels in the top row after treatment with the H$_2$O$_2$ scavenger polyethylene glycolylated-CAT. All images were acquired at the same camera exposure and after acquisition image processing settings. Top graph shows measurement of the intensity of DCF fluorescence for the different vessels shown at the left. Bottom graph shows measurement of the density of DCF-bright cells within the vessel wall for the different vessels shown in the top graph. (B): Normalized DHE fluorescence intensities of isolated saphenous arteries from untreated and SVF-injected mice ($n = 3$ for each) to visualize the presence of O$_2^-$. DHE (B) and DAF (C) fluorescence intensities of isolated saphenous arteries from untreated and SVF-injected mice ($n = 3$ for each) to visualize the presence of superoxide and nitric oxide, respectively. All data are shown as the mean ± SEM, $n ≥ 3$. *p value was determined by one-way ANOVA within each pre- and post-catalase groups for each of the experimental groups. Abbreviations: CAT, catalase; DAF, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DCF, 5-(and-6)-chomethyl-2′,7′-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; SVF, stromal vascular fraction; SVF-11bΔ, CD11b$^+$ cell-depleted adipose SVF cells.
fixed at a constant pressure suggests that these cells also relax the artery in the context of chronic tissue inflammation, a condition known to increase vessel tone [9, 10]. Additionally, given the anti-inflammatory potential for adipose-derived cells [6, 36–39], it is reasonable to believe that they might have modulated the inflammation in the cuffed artery setting, thereby lessening the artery insult (i.e., oxidant stress). Regardless of the mechanism, it appears that the freshly isolated SVF cells are well suited to improving vascular function in inflamed tissue environments. Other vasoactive regulatory pathways, for example endothelial-dependent and -independent processes, were unaltered, suggesting that, overall, the vascular homeostatic mechanisms are still effective. This is important when considering the patient’s condition because different vascular beds could contain small arteries that are more or less dysfunctional. If the other vasoactive regulatory pathways are intact, we would expect to find increases in perfusion only in the dysfunctional beds after the SVF cell therapy, because these other regulatory pathways would compensate for any changes brought on by the cells in normally functioning arteries.

Most of the CD11b⁺ cells that were removed from the isolate expressed F4/80, a common marker of tissue macrophages (MΦs). Also, the F4/80⁺ cell population was the most depleted population after removal of the CD11b⁺ cells, which eliminated the relaxed tone effect. Moreover, MΦs derived from the SVF population were present within the saphenous artery wall (F4/80-GFP double-positive cells in our experiments) and, importantly, were reduced in numbers in the artery adventitia in the mice injected with SVF cells depleted of CD11b⁺ cells. Macrophages normally regulate vessel form and function [18, 40], actively express the hydrogen peroxide-producing enzyme super oxide dismutase-3 [41], and are capable of moving throughout tissue compartments [42]. Although the MΦ was not the only cell type in the CD11b⁺ population in the SVF isolate (subsets of Gr-1⁺ cells were also present), our findings strongly suggest that MΦs are the relevant, vasoregulatory cell type in the SVF isolate.

**Figure 5.** SVF-derived macrophages spontaneously populate artery walls. (A): Visualization (cross-section) of GFP⁺ SVF cells (green) within regions of saphenous artery walls from untreated, SVF-injected, or SVF-11bΔ-injected mice. Macrophages were identified via F4/80 immunostaining (red) and cell nuclei by Hoechst dye (blue) (corresponding en face views are shown in supplemental online Fig. 5). In other instances, lectin GS1 (red) and hydrazide (blue) was used to identify the endothelium and elastic laminae, respectively, to locate the vaso vasorum relative to the IEL and EEL. Also shown are the density of GFP⁺ cells (B) and the percentage of the fraction of those cells also positive for F4/80 (C) in arteries from SVF-injected or SVF-11bΔ-injected mice. Data are shown as the mean ± SEM, n = 3 for all groups. p value determined by t test. Abbreviations: EEL, external elastic lamina; GFP, green fluorescent protein; IEL, internal elastic lamina; SVF, stromal vascular fraction; SVF-11bΔ, CD11b⁺ cell-depleted adipose SVF cells.
and necessary for the SVF-mediated change in vascular tone. A significant majority of the M\textsubscript{F}s in the SVF isolate were positive for CD301, a commonly used marker for the M\textsubscript{2} M\textsubscript{F}[43]. Simply based on this prevalence, it is likely that the M\textsubscript{F}s delivered to the saphenous artery wall in our experiments were these prohealing, anti-inflammatory homeostatic, CD301\textsuperscript{+} macrophages.

Because the SVF cells were injected intravenously, those observed within the saphenous artery adventitia necessarily entered the artery wall via the circulation. In arteries, shear stress at the luminal surface is sufficiently high to impede cell attachment to the intimal endothelium [44]. Conversely, the adventitia of arteries contains the vaso vasorum, a microcirculatory bed perfusing the outer and medial layers of the vessel wall [18]. Because the SVF cells were localized to the adventitia and it is unlikely that the injected cells entered the vessel wall via the intima, it is most plausible that they entered the vessel wall via the vaso vasorum. Whether the SVF cells that entered the artery adventitia were directly derived from the injected isolate (i.e., after injection, circulated to the artery wall and entered the adventitia) or they were new, hematopoietic-derived cells (e.g., monocytes) from the SVF cells that populated the recipient's bone marrow is unclear. Certainly, tissue-resident macrophages express a variety of cell adhesion and homing molecules that could mediate dissemination into the artery adventitia once
removed from the adipose environment and injected into the blood stream [42, 45]. Consistent with the increase in bioluminescence over time, SVF-derived macrophages within the artery wall incorporated EdU, administered to the recipients for the 1 week after cell delivery (supplemental online Fig. 7), indicating cell proliferation. Thus, just as with the Mφs and Mψ precursors normally residing within the artery adventitia [46], the SVF-derived Mψs likely populated the saphenous artery adventitia independent of the hematopoietic origins, propagating within this new tissue compartment. Regardless of how the SVF-derived Mψs entered the artery wall, it appears that these cells, and their vasoactive activity, can persist in the artery wall for extended periods of time.

CONCLUSION

Many are exploring the therapeutic potential of adipose-derived cells, motivated by basic and preclinical studies supporting their anti-inflammatory and proangiogenesis activities [47]. We have provided evidence for an additional therapeutic mechanism, vasorelaxation of the peripheral arteries via reduced vasomotor tone without perturbing the other vasoactive homeostatic mechanisms. This clinically favorable activity depended on CD11b⁺ cells, likely tissue-resident macrophages. Given that macrophage phenotypes are often excluded in the cultured preparations of adipose mesenchymal or stromal cells, this activity might be unique to fresh adipose cell preparations. Because the injected SVF cells were widely disseminated throughout the animal and reset the vasomotor tone in the inflamed arteries, our findings foster an autologous cell therapeutic strategy targeting disease-related, diffuse vascular dysfunction.

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ACKNOWLEDGMENTS

We thank Dr. Jill Suttles for the useful discussion concerning macrophages and Darlene Burke for assistance with the statistical analyses. This work was supported by Department of Surgery, University of Louisville funds (to M.E.M.), a grant from the Kosair Children’s Hospital Foundation (to C.L.K. and J.B.H.). This project used the Kentucky Spinal Cord Injury Research Center/Cardiovascular Innovation Institute care facilities, supported by Grant P30 GM13507 from the NIH/National Institute of General Medical Sciences. M.E.M. is currently affiliated with the Department of Vascular Surgery, Baystate Medical Center, Springfield, MA.

AUTHOR CONTRIBUTIONS

M.E.M.: conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing; J.E.B., R.M.R., and J.R.D.: collection and/or assembly of data, manuscript writing; A.J.L.: conception and design, financial support, collection and/or assembly of data, data analysis and interpretation; C.L.K.: data analysis and interpretation; H.Z. and C.K.N.: collection and/or assembly of data; S.K.W.: conception and design, data analysis and interpretation, manuscript writing; J.B.H.: conception and design, financial support, provision of study material, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.
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