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ORIGINAL RESEARCH

Satellite Cell Expression of RAGE (Receptor for Advanced Glycation end Products) Is Important for Collateral Vessel Formation

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BACKGROUND: The growth and remodeling of vascular networks is an important component of the prognosis for patients with peripheral artery disease. One protein that has been previously implicated to play a role in this process is RAGE (receptor for advanced glycation end products). This study sought to determine the cellular source of RAGE in the ischemic hind limb and the role of RAGE signaling in this cell type.

METHODS AND RESULTS: Using a hind limb ischemia model of vascular growth, this study found skeletal muscle satellite cells to be a novel major cellular source of RAGE in ischemic tissue by both staining and cellular sorting. Although wild-type satellite cells increased tumor necrosis factor-α and monocyte chemoattractant protein-1 production in response to ischemia in vivo and a RAGE ligand in vitro, satellite cells from RAGE knockout mice lacked the increase in cytokine production both in vivo in response to ischemia and in vitro after stimuli with the RAGE ligand high-mobility group box 1. Furthermore, encapsulated wild-type satellite cells improved perfusion after hind limb ischemia surgery by both perfusion staining and vessel quantification, but RAGE knockout satellite cells provided no improvement over empty capsules.

CONCLUSIONS: Thus, RAGE expression and signaling in satellite cells is crucial for their response to stimuli and angiogenic and arteriogenic functions.

Key Words: collateral vessels ■ RAGE ■ satellite cells

Peripheral artery disease is most commonly the result of atherosclerosis in the arteries of the limbs (primarily the legs), impairing blood flow to the extremities, or in the carotid arteries, impairing flow to the head and brain. Limb peripheral artery disease often first manifests as pain with physical exertion, but chronic oxygen deficiency attributable to inadequate blood supply can lead to tissue damage that results in ulcers, infections, and amputations. However, the ischemic insult does promote the growth of new blood vessels and the remodeling of existing ones to help restore blood flow and prevent further damage. Indeed, the development of collateral vessels and vascular networks is correlated with better patient outcomes.[1]

The regulation of this vascular response is likely multifactorial, with many common cardiovascular risk factors, such as smoking, hypertension, hypercholesterolemia, and diabetes, playing a negative role in vessel growth and development.[2,3] One protein that has been thought to play a role in the poor outcomes observed in patients with diabetes is RAGE (receptor for advanced glycation end products). RAGE is a member of the immunoglobulin superfamily of receptors and has several ligands, including s100B, high-mobility group box 1 (HMGB1), and advanced glycation end products, that are often studied in the context of diabetes.[4,5] Several studies have investigated the importance of RAGE in vascular injury responses in diabetic animals. RAGE

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CLINICAL PERSPECTIVE

What Is New?

- RAGE (receptor for advanced glycation end products) is highly expressed in skeletal muscle satellite cells in ischemic tissue.
- RAGE signaling is critical for proper cytokine signaling in satellite cells.

What Are the Clinical Implications?

- RAGE expression and signaling is imperative for satellite cells to increase vascular response.
- The cellular source of RAGE matters, with RAGE signaling in satellite cells having a beneficial rather than detrimental effect on vascular growth.
- Satellite cells have the potential to be a therapeutic cell source in vascular diseases.

Nonstandard Abbreviations and Acronyms

HLI	hind limb ischemia
HMGB1	high-mobility group box 1
IL	ischemic limb
LDPI	laser Doppler perfusion imaging
MCP-1	monocyte chemoattractant protein-1
NIL	nonischemic limb
RAGE	receptor of advanced glycation end products
SC	satellite cell
WT	wild type

activation and signaling has been shown to result in endothelial dysfunction in coronary arterioles in diabetic mice.[6] RAGE signaling has also been shown to be associated with neointimal hyperplasia, and treatment with sRAGE (the soluble form of the receptor) decreases the medial thickening in both diabetic and nondiabetic rats.[7] In an ischemia-reperfusion model of myocardial infarctions, both RAGE knockout mice and RAGE inhibition by treatment with sRAGE showed decreased ischemic damage and improved myocardial function compared with wild-type (WT) or untreated animals.[8] Specifically related to angiogenesis and vessel growth, the blockage of advanced glycation end product formation with aminoguanidine improved reperfusion in a femoral artery ligation model.[9] Diabetic RAGE knockout mice or mice that overexpress sRAGE have increased angiogenesis in Matrigel plugs compared with WT mice with diabetes.[6] More recent studies have also shown that RAGE knockout mice have improved outcomes following hind limb ischemia (HLI) procedures.[10,11] One interesting finding in these recent studies was RAGE seemed to play a role in even the nondiabetic mice, likely attributable to signaling from increases in the RAGE ligand HMGB1.[10] Expression of RAGE in cells, such as endothelial cells, smooth muscle cells, and macrophages, is currently thought to be the major player in RAGE signaling; however, we have identified a novel cell type with significant RAGE expression: satellite cells. The current study investigates unique role of RAGE signaling in skeletal muscle satellite cells and the paradoxical, proangiogenic effects.

Satellite cells are skeletal muscle stem cells that play an important role in the repair of muscle tissue. Healthy muscle contains a quiescent pool of satellite cells that are capable of repairing by the muscle by proliferating and differentiating into myotubes in response to injury as well as self-renewing to maintain their population. Yet, the importance of RAGE signaling by these cells in relation to vascular growth and repair has not been studied. The objective of this study was to investigate the role of RAGE signaling and vessel formation in a nondiabetic setting. We identified skeletal muscle satellite cells as a novel cellular site of RAGE expression in an HLI model of vessel formation and studied the function of RAGE expression in skeletal muscle satellite cells.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Surgical Model

All animal studies were conducted under the approval of the Emory University Institutional Animal Care and Use Committee. Mice were either bred in house (RAGE knockout) or obtained from Jackson Laboratories (C57Bl/6) or Charles River (129Sv2). All animals were randomized to treatment groups. The prevalence of peripheral artery disease is higher in males; thus, for this study, male mice were used.[12] Male mice between 8 and 10 weeks of age were anesthetized with 1% to 2% isoflurane via inhalation in a chamber and then maintained through a nose cone during the procedure. The animals received buprenorphine (Buprenex; 0.1 mg/kg, subcutaneous) preoperatively for analgesia, and aseptic techniques were used. The HLI procedure was performed unilaterally, and the other limb was used as the contralateral control. An incision was made over the left thigh of the mouse, exposing the superficial femoral artery and vein. Ligations were made with 6-0 silk suture proximal to the deep femoral artery branch point and just proximal to the branching of the tibial arteries. Following ligation, the length of the artery and vein between the 2 points was excised. The skin was closed with monofilament nylon suture.

Satellite Cell (SC) Isolation and Culture

Satellite cells for both the establishment of primary cell cultures and for protein and gene analysis were isolated from the adductor and gastrocnemius muscle (the 2 major muscles affected by the HLI model). The muscles were excised and digested using both mechanical and enzymatic methods.[13] Muscles were first minced before digestion in 0.1% Pronase (Calbiochem). The digested cells were dissociated and passed through a 100-μm filter. Satellite cells were purified from this cell suspension using magnetic bead separation (SC separation kit from Miltenyi Biotec). For protein and gene expression assays of freshly isolated cells, the cells were pelleted and stored in a −80 ℃ freezer until use in the assays described below. This process also resulted in viable cells, which were capable of continued growth and proliferation in culture. The primary cell cultures were maintained in Hams/F-10 media (Hyclone) with 20% fetal bovine serum (Sigma), penicillin/streptomycin (Hyclone), and HEPES (Hyclone). These cells were then used for cell delivery and in vitro experiments.

Histology, Immunohistochemistry, and in Situ Hybridization

Mice were euthanized at day 14 and prepared for histology by perfusion with saline, followed by 10% buffered formalin for fixation. For immunohistochemistry of paired box protein 7 (Pax7) and RAGE, the fixed muscles were processed, paraffin embedded, and cut in 5-μm sections for staining. Antigen retrieval using citrate buffer (pH=6) with pressure cooker was used before incubation with antibodies. Sections were stained for satellite cells with a PAX7 antibody (Developmental Studies Hybridoma Bank Pax7 concentrate), and RAGE was stained using an anti-RAGE antibody (Gentex GTX23611) before incubation together overnight. Secondary antibodies, Qdot 655 goat anti-mouse IgG conjugate for Pax7 and Qdot 585 goat anti-rabbit IgG conjugate for RAGE (Invitrogen), were incubated for 2 hours at room temperature. Slides were washed and coverslipped with Vectashield mounting media with 4′,6-diamidino-2-phenylindole (Vector Labs). Pictures were acquired using a Zeiss LSM 800 Airyscan laser scanning confocal microscope with a 63× 1.4 numerical aperture Plan-Apochromat objective. A region of interest was drawn around cells expressing Pax7, and its colocalization with RAGE was determined by calculating the Pearson correlation coefficient using the Costes threshold method.[14] The average Pearson correlation coefficient±SEM was calculated from ≈40 cells per mice from 4 different animals (n=4).

For histology and immunohistochemistry of blood vessels, the limbs were demineralized in a formic acid–based solution (Cal-Ex II; Fisher Scientific) for 48 hours before paraffin processing, embedding, and sectioning (5 μm). Capillaries were visualized by staining with lectin (Biotinylated Griffonia simplifolia Lectin 1; Vector Lab), followed by a Streptavidin Qdot 655 (Invitrogen). Images were acquired using a Zeiss Axioskop 2 with Axiocam Camera with a 20× Plan-Neo 0.5 numerical aperture objective with 6 random fields per animal. Vessels were counted using ImageJ (National Institutes of Health). Arterioles and arteries were stained for smooth muscle α-actin with a mouse monoclonal antibody (Sigma) using the avidin–biotin–alkaline phosphatase method (Vectastain ABC-AP; Vector Laboratories) and a hematoxylin counterstain. Images of the entire limb cross-section were obtained using a Hamamatsu Nano-Zoomer SQ Whole Slide Scanner. Vessels were counted using the affiliated NDP.veiw2 software (Hamatsu).

Perfused blood vessels were quantified using in vivo lectin staining. Fluorescein isothiocyanate–conjugated lectin (Griffonia Simplicifolia Lectin I, Fluorescein; Vector Labs) was injected IV (1 mg/mL in 100 μL) via the retro-orbital sinus, and all were allowed to circulate for 10 minutes. Mice were then euthanized and perfused with saline and 10% formalin. The hind limb muscles were fixed for 24 hours in 10% formalin, followed by 24 hours in a 30% sucrose solution. The tissue was then embedded in optimal cutting temperature media, frozen, and cut into cryosections in 50-μm thick sections. The sections were mounted with Vectashield with 4′,6-diamidino-2-phenylindole (Vector Labs). Z-stacks throughout the thickness were acquired on a Zeiss LSM 800 Airyscan laser-scanning confocal microscope with a 10× 0.45 numerical aperture Plan-Apochromat objective. A total of 5 to 7 random fields per animal were acquired. A maximum intensity projection was made for each z-stack and used for analysis. Blood vessels were analyzed in batch mode using ImageJ (Fiji). All pictures were preprocessed using a Median filter and Subtract Background functions. Subsequently, images were binarized, applying a threshold followed by the Analyze Particles function to eliminate small artifacts (<20 pixels). The blood vessel area was measured as the percentage of pixels different than zero in the binarized image. Vessel length and number of branches were automatically calculated with a script after skeletonizing the binary image, as described before.[15,16]

Polymerase Chain Reaction

Gene expression was quantified using quantitative reverse transcription–polymerase chain reaction analysis. RNA was isolated using RNeasy (Qiagen) from homogenized adductor and gastrocnemius muscle tissue, SC pellets isolated from hind limbs, or cultured satellite cells. cDNA was purified with QiaQuick

(Qiagen), and expression was quantified on an Applied Biosystems StepOnePlue Real-Time PCR system. All primers were Quantitect Primer Assays purchased from Qiagen.

Cytokine Array

A UPlex assay (Meso Scale Diagnostics) was used to quantify the protein levels of several cytokines. The array contained granulocyte-macrophage colony-stimulating factor, interleukin-1β, interleukin-6, tumor necrosis factor (TNF)-α, vascular endothelial growth factor, and monocyte chemoattractant protein-1 (MCP-1). The protein lysates for this study were obtained by isolating and sorting satellite cells from either the ischemic limb (IL) or nonischemic limb (NIL) at post-operative surgical day 7. The cells were lysed in radioimmunoprecipitation assay buffer containing protease inhibitors, and equal amounts of total protein lysate were added to each well of the assay plate. The assay was performed using the standard protocol from Meso Scale Diagnostics.

HMGB-1 Stimulation in Vitro

Cultured WT and RAGE knockout satellite cells (passage 5-passage 8) were plated in collagen-coated, 6-well plates with technical replicates for each group. Cells were stimulated with 250 nmol/L HMGB-1 (Sigma) for 24 hours before RNA isolation. RNA was isolated, as described above, using an RNeasy kit (Qiagen); and cDNA was purified with a QiaQuick kit (Qiagen). Qiagen Quantitect primers were used to quantify expression using an Applied Biosystems StepOnePlue Real-Time PCR system.

Alginate Encapsulation and Cell Delivery

To test the differential effects of satellite cells with and without RAGE on collateral growth and recovery, we delivered encapsulated satellite cells at the time of HLI surgery. We selected this approach as we have previously shown that alginate encapsulation enhances cell survival.[17,18] Cultured satellite cells were suspended in 1% ultrapure low-viscosity alginate (Novamatrix) and collected in a gelling solution of 50 mmol/L $BaCl_2$. An electrostatic encapsulator (Nico) with a 0.17-mm nozzle, 10-mL/h flow rate, and 7-kV voltage generated microcapsules ≈200 to 250 μm in diameter. Capsules were washed in 0.9% saline solution and stored in saline on ice during the HLI surgery until implantation. To deliver the cells, a small secondary incision medial to the proximal end of the ligation incision was made to create a pocket to deliver 1 million encapsulated cells per animal. Both WT and RAGE knockout primary SC lines, generated as described above, were used in addition to empty capsules as controls. For this study, the cells were delivered to 129Sv2 mice (Charles River) as this strain has less robust collateral vessel growth, thus allowing a better opportunity to observe a therapeutic effect.[19]

Assessment of Cell Viability

The viability of the cells at the time of encapsulation was verified using a LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) and imaged using a Zeiss LSM 800 laser-scanning confocal microscope. Luciferase imaging was used to monitor the viability of cells in vivo. For these experiments, GFP (green fluorescent protein)/luciferase dual-expressing satellite cells were derived from transgenic mice from Jackson Labs. Mice received 20 mg/mL of luciferin (GoldBio) via an intraperitoneal injection 45 minutes before imaging. Mice were then anesthetized with isoflurane and placed in the In-Vivo Xtreme II whole animal imager (Bruker). Both luminescence and X-ray images were acquired at each time point. Mean luminescence intensity was quantified as a measure of cell viability over time.

Laser Doppler Perfusion Imaging

Perfusion of the hind limbs was monitored noninvasively using a laser Doppler perfusion imaging (LDPI) system (Moor Instruments). Mice were anesthetized with isoflurane and placed on a heating pad for 4 minutes. Hair on both hind limbs was removed with depilatory cream and washed with water. Perfusion was assessed as the ratio of the mean perfusion value from equivalent regions of interest in the IL to NIL.

Statistical Analysis

Data were collected and quantified blinded to experimental groups before statistical analysis. Sample sizes were calculated a priori using G*Power software with effect sizes determined using previous data from our laboratory as well as pilot studies. For all studies, we used an ANOVA:fixed-effect, omnibus, one-way analysis with input parameters of α=0.05 and power=0.8. Post hoc data analysis was performed using GraphPad Prism 9. Data were checked for a normal distribution using the Shapiro-Wilk and Kolmogorov-Smirnov tests before statistical analysis. The data were analyzed using *t*-tests for pairwise comparisons or ANOVA for multiple comparisons with an appropriate post hoc test (Tukey multiple-comparison test) to determine significance between individual groups when data passed normality tests. Data for which we could not assume normality were analyzed using a Kruskal-Wallis test with Dunn multiple-comparison test. Note, the in vitro study had multiple end points (LDPI and histology) without specific correction for type I error. $P<0.05$ was considered

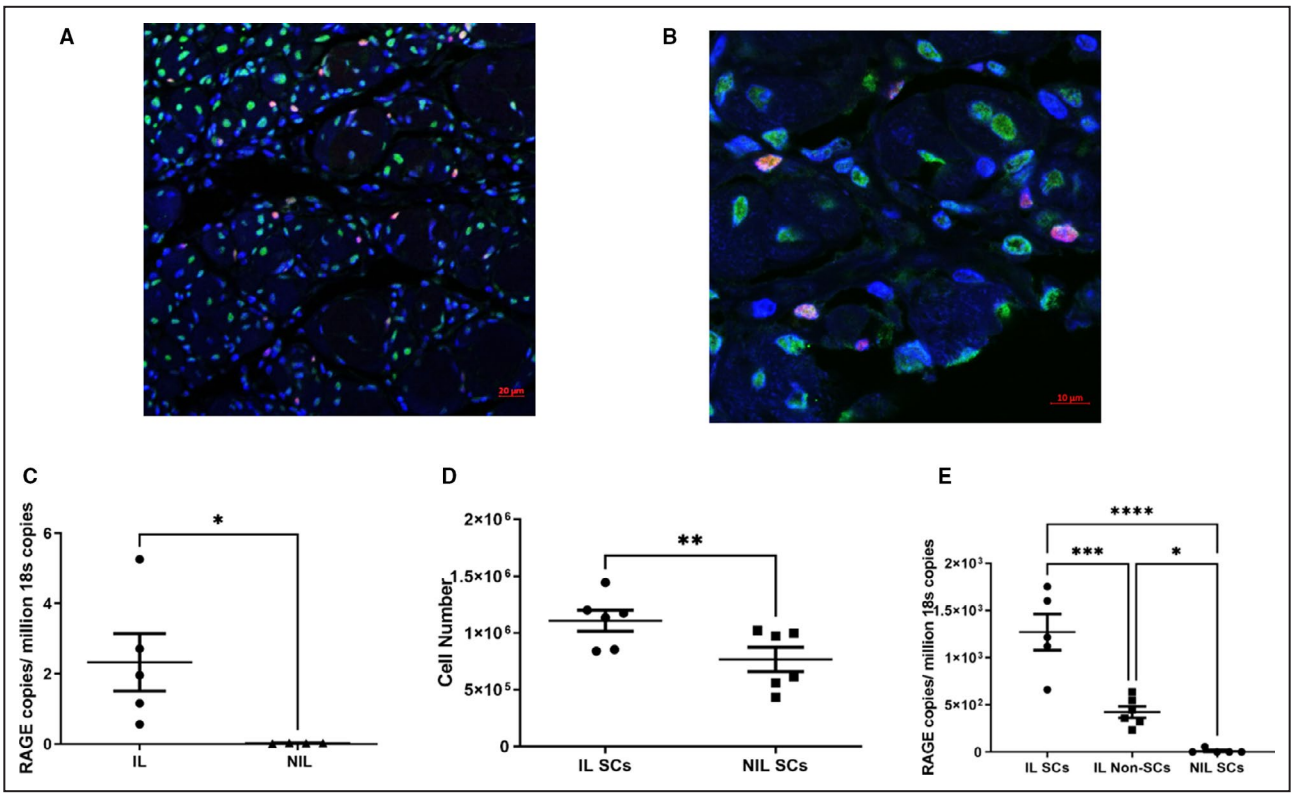

Figure 1. RAGE (receptor for advanced glycation end products) is increased in satellite cells (SCs) in the hind limb.
A and **B**, Hind limb muscle was harvested 7 days after hind limb ischemia (HLI), and immunohistochemistry was used to visualize RAGE within the muscle tissue. The representative images show expression of RAGE (green) in Pax7 (red) SCs (**A**, 20×; **B**, 63×). **C**, RAGE expression in the hind limb gastrocnemius muscles was determined using quantitative reverse transcription–polymerase chain reaction in wild-type mice. The ischemic limb (IL) had increased RAGE expression compared with the nonischemic limb (NIL) (n=6; **P*<0.05). **D** and **E**, SCs from the limbs following HLI were purified using magnetic bead separation. **D**, The number of SCs was increased in the IL compared with the NIL (n=6; ***P*<0.01). **E**, SCs in the IL had increased RAGE expression compared with SCs in the nonischemic and the non-SC fraction in the ischemic limb (n=6; **P*<0.05, ****P*<0.001, *****P*<0.0001). **C** through **D**, circles, triangles, and squares are individual data points for that group. The center line is the mean and the error bars are SEM.

significant, and data are reported as mean±SEM and adjusted *P* values.

RESULTS

HLI surgery was performed on mice, and the adductor and gastrocnemius muscles were isolated 7 days after surgery for RNA isolation to quantify RAGE expression. Muscles from the IL had significantly increased RAGE expression compared with the muscles isolated from the nonischemic contralateral limb (n=5; *P*=0.0417) (Figure 1C). However, the relatively low expression levels raised the possibility that the source of RAGE was a rare cell type. Immunohistochemistry of the ischemic tissue was used to visualize the RAGE expression in the ischemic tissue. The staining showed a pattern similar to that of satellite cells and costaining for RAGE and Pax7 (a SC marker) confirmed expression of RAGE in satellite cells (Figure 1A and 1B). Colocalization analysis between Pax7 and RAGE is shown in Figure S1, and the Pearson correlation coefficient was 0.75±0.02 (mean±SEM; n=4), indicating a strong colocalization efficiency. To further confirm this observation, satellite cells were isolated from hind limbs following the HLI procedure using magnetic bead sorting and analyzed for RAGE expression. Figure 1D shows that the number of satellite cells was increased in the IL (n=6; *P*=0.0033). This increase in satellite cells was consistent in both WT and RAGE knockout mice (Figure S2), n=6: *P*=0.0065 for WT NIL versus WT IL, *P*=0.0009 for WT NIL versus RAGE knockout IL, *P*=0.0011 for RAGE knockout NIL versus RAGE knockout IL, *P*=0.0077 for WT IL versus RAGE knockout NIL, and no significant difference (*P*=0.9998) for WT NIL versus RAGE knockout NIL and no significant difference (*P*=0.828) for WT IL versus RAGE knockout IL. Satellite cells isolated from the IL had increased RAGE expression compared with the non–SC populations from the IL, and satellite cells from the NIL had almost no RAGE expression (Figure 1E; n=6; *P*=0.0003 for IL SCs versus IL non-SCs, *P*<0.0001 for IL SCs versus NIL SCs, and *P*=0.0481 for IL non-SCs versus NIL SCs).

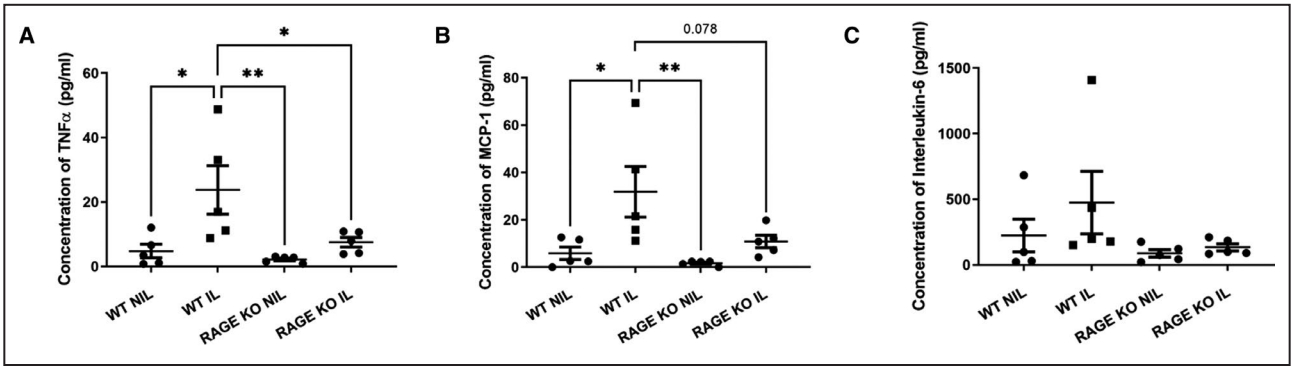

Figure 2. Wild-type (WT) satellite cells from the ischemic limb (IL) have increased cytokine expression.
Satellite cells were isolated using magnetic bead sorting from hind limb muscles at 7 days after hind limb ischemia. Protein cytokine levels were quantified using a Uplex immunoassay (Meso Scale Diagnostics). **A**, WT satellite cells from the IL had significantly greater tumor necrosis factor (TNF)-α protein production than satellite cells from WT nonischemic limb (NIL) and RAGE (receptor for advanced glycation end products) knockout (KO) NIL and IL (n=5; **P*<0.05, ***P*<0.01). **B**, WT satellite cells from the IL also had increased MCP-1 (monocyte chemoattractant protein-1) compared with WT and RAGE KO NILs. WT IL satellite cells trended toward an increase over RAGE KO IL (*P*=0.078) (n=5; **P*<0.05, ***P*<0.01). **C**, Interleukin-6 followed similar trend but was nonsignificant.

We have previously shown that RAGE negatively impacts collateral vessel formation.[10] Therefore, we hypothesized that the satellite cells may have a counteracting effect influencing vascular regeneration through the production and secretion of several cytokines and growth factors. We used a multiplex immunoassay cytokine array to quantify cytokine expression in satellite cells from the IL and NIL of WT mice. Both TNF-α and MCP-1 protein levels were significantly increased in the satellite cells from the IL of WT mice compared with the NIL (n=5; TNF-α: P=0.019 for WT NIL versus WT IL; MCP-1: P=0.0236 WT NIL versus WT IL), whereas interleukin-6 appeared to trend toward an increase but was nonsignificantly different (n=5; P=0.5754 for WT NIL versus WT IL) (Figure 2). TNF-α expression in satellite cells from the ILs of RAGE knockout mice was significantly less than that of WT for TNF-α (n=5; P=0.0487), whereas MCP-1 (n=5; P=0.078) and interleukin-6 appeared to trend toward differences, although not statistically significant (n=5; P=0.3228). Vascular endothelial growth factor, however, was not significantly different between any of the groups (data not shown). The levels of granulocyte-macrophage colony-stimulating factor and interleukin-1β were

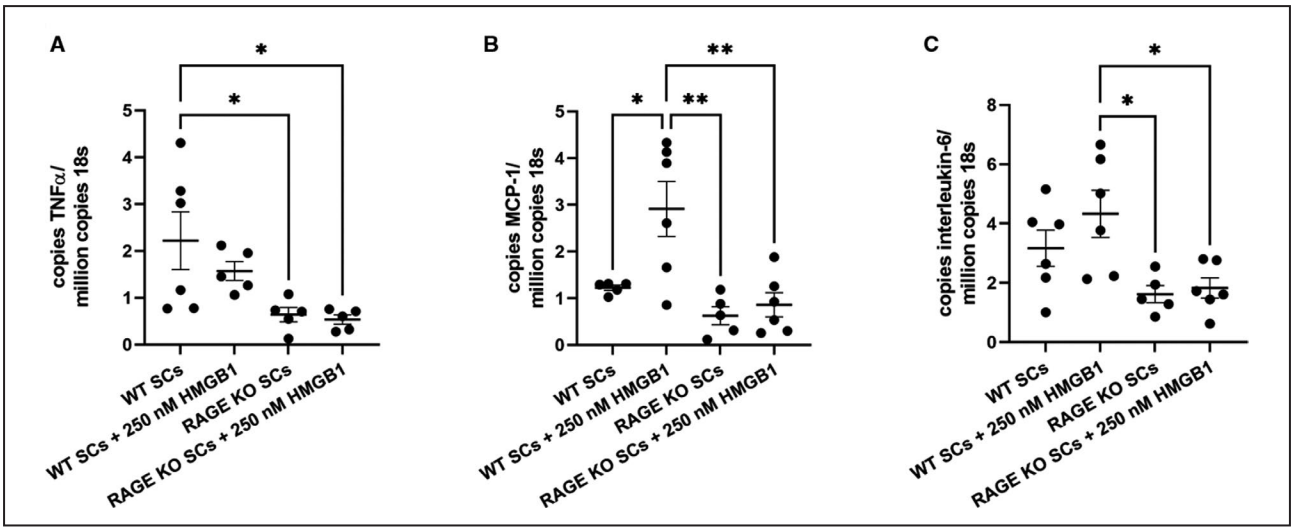

Figure 3. Wild-type (WT) satellite cells (SCs) have increased cytokine response to high-mobility group box 1 (HMGB1) stimulation.
Primary cultures of WT or RAGE (receptor for advanced glycation end products) knockout (KO) SCs were stimulated with 250 nmol/L HMGB1, and cytokine expression was quantified by polymerase chain reaction. **A**, WT SCs had increased tumor necrosis factor (TNF)-α expression compared with RAGE KO SCs. **B**, HMGB1-stimulated WT SCs had increased MCP-1 (monocyte chemoattractant protein-1) expression compared with all other groups. **C**, WT SCs stimulated with HMGB1 also had significantly more interleukin-6 expression compared with control and stimulated RAGE KO cells (n=6; **P*<0.05, ***P*<0.01).

below the detection range of our assay (1 and 15 pg/mL, respectively).

We further examined the expression of these same factors in cultured WT and RAGE knockout satellite cells in response to stimulation with the RAGE ligand, HMGB-1 (Figure 3). Similar to the array data, WT satellite cells had increased expression of TNF-α, MCP-1, and interleukin-6 compared with RAGE knockout satellite cells. Specifically, TNF-α was increased in WT satellite cells compared with both control and HMGB1-stimulated RAGE knockout satellite cells (n=6; *P*=0.0369 for WT SCs versus RAGE knockout SCs, and *P*=0.0248 for WT SCs versus RAGE knockout SCs+250 nmol/L HMGB1). WT satellite cells stimulated with HMGB1 had increased MCP-1 expression compared with all 3 other groups (WT, RAGE knockout, and RAGE knockout+HMGB1) (n=6; *P*=0.0205 for WT SCs+250 nmol/L HMGB1 versus WT SCs, *P*=0.0017 for WT SCs+250 nmol/L HMGB1 versus RAGE knockout SCs, and *P*=0.0030 for WT SCs+250 nmol/L HMGB1 versus RAGE knockout SCs+250 nmol/L HMGB1). Interleukin-6 expression was also increased in stimulated WT satellite cells compared with both the untreated and control RAGE knockout satellite cells (n=6; *P*=0.0187 for WT SCs+250 nmol/L HMGB1 versus RAGE knockout SCs and *P*=0.0234 for WT SCs+250 nmol/L HMGB1 versus RAGE knockout SCs+250 nmol/L HMGB1).

Next, we studied the effects of WT versus RAGE knockout satellite cells on collateral vessel formation in response to ischemia. Alginate encapsulated satellite cells were delivered near the proximal ligation point at the time of HLI surgery.[17,18] The viability of the delivered cells was monitored by quantifying the luminescence emitted from luciferase-expressing satellite cells. Figure 4 shows good viability for 10 days (≈50% of mean luminescence detected at day 0), with mean luminescence diminished by day 14 in most animals. The effects of vascular recovery were monitored via LDPI for perfusion and immunohistochemically staining for vessels. Figure 5 shows that animals that received WT satellite cells had improved perfusion, as measured by LDPI, compared with animals that received empty capsules (n=11; *P*=0.0422). The mice that received RAGE knockout satellite cells did not show differences compared with the empty capsule–treated group (n=11; *P*=0.9621). Figure S3 is an LDPI time course that indicates that the group that received WT satellite cells has improved perfusion at day 7 that persists out to day 14 (n=11; *P*=0.0465 for empty

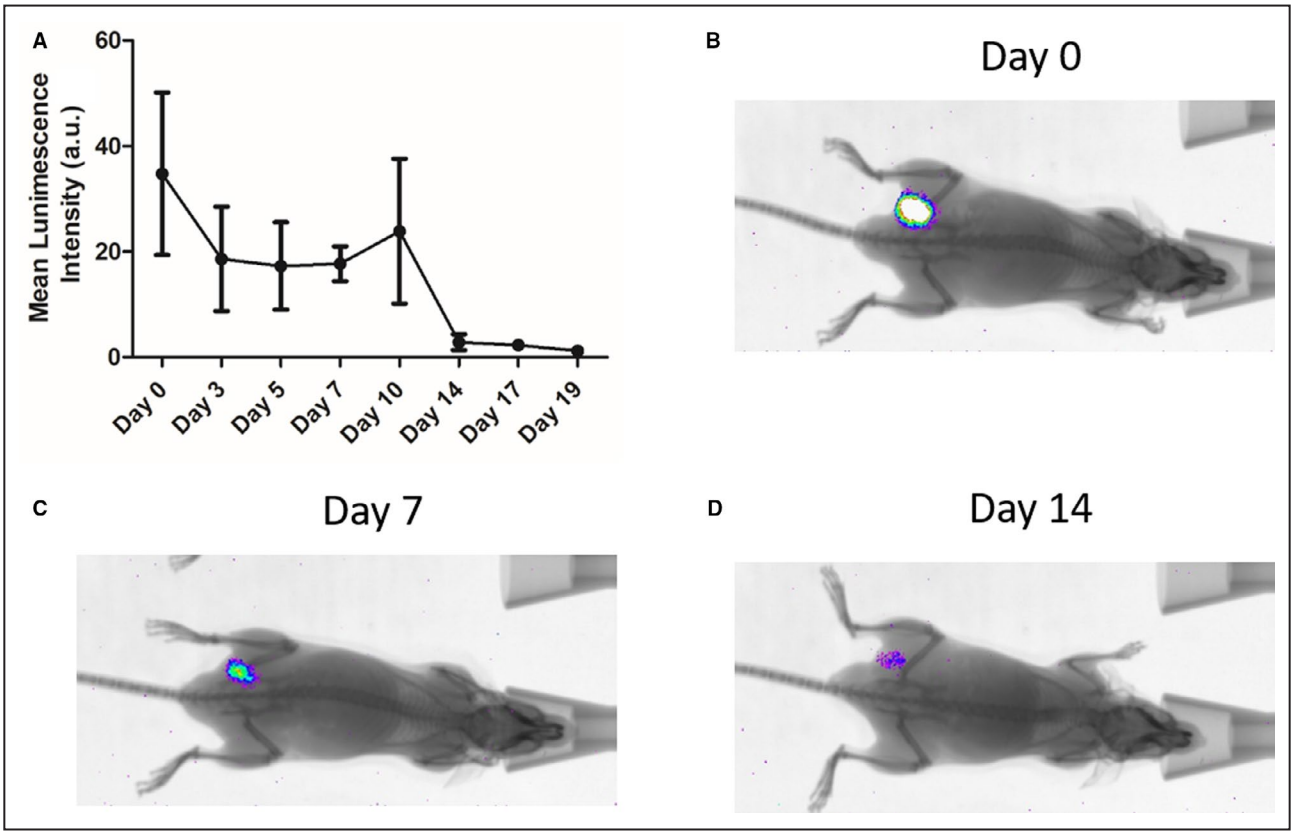

Figure 4. Implanted satellite cells are viable for >10 days.
Luciferase-expressing satellite cells were tracked in vivo using a whole animal in vivo imager (Bruker). **A**, Quantification of the luminescence showed good viability up to 10 days, with some cells detected up to 19 days after implantation. **B** through **D**, Representative images at days 0, 7, and 14. A.u. indicates arbitrary unit.

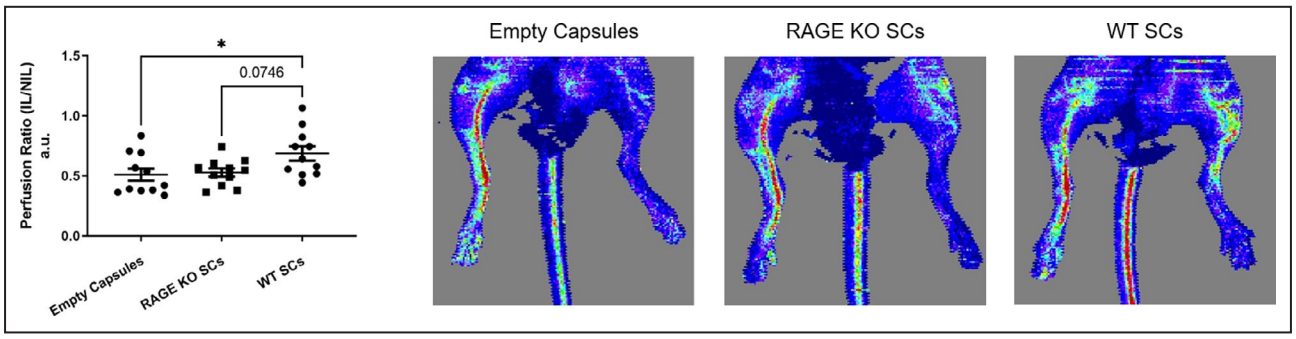

Figure 5. Delivery of wild-type (WT) satellite cells (SCs) improves perfusion in a hind limb ischemia model.
Mice received either WT or RAGE (receptor for advanced glycation end products) knockout (KO) SCs in alginate capsules or empty capsules at the time of hind limb ischemia surgery. Perfusion recovery was measured via laser Doppler perfusion imaging over time. Mice that received WT SCs had improved perfusion over mice that received empty capsules or RAGE KO SCs at both 7 and 14 days after surgery (n=11; **P*<0.05 for empty capsules vs WT SCs, day 14 data shown). IL indicates ischemic limb; and NIL, nonischemic limb.

capsules versus WT SCs at day 7, *P*=0.0086 for empty capsules versus WT SCs at day 14, and *P*=0.0206 for WT SCs versus RAGE knockout SCs at day 14). In addition to LDPI, recovery was assessed by quantifying the lectin-positive capillaries and small vessels as well as smooth muscle actin–containing vessels, including arteries and arterioles, via histological staining. Similar to the LDPI results, the group that received the WT satellite cells had increased lectin-positive vessels compared with the empty capsule control group (n=6; *P*=0.0029 using Kruskal-Wallis test and Dunn multiple-comparison test), with the group with RAGE knockout satellite cells not different from either of the other 2 groups (Figure 6A through 6D). Similarly, smooth muscle actin–stained vessels were increased in the mice that received WT satellite cells compared with empty capsules (n=4–5; *P*=0.0093 using Kruskal-Wallis test and Dunn multiple-comparison test; Figure 6E through 6H). Figure S4 is representative images of the smooth muscle α actin staining, which illustrates that

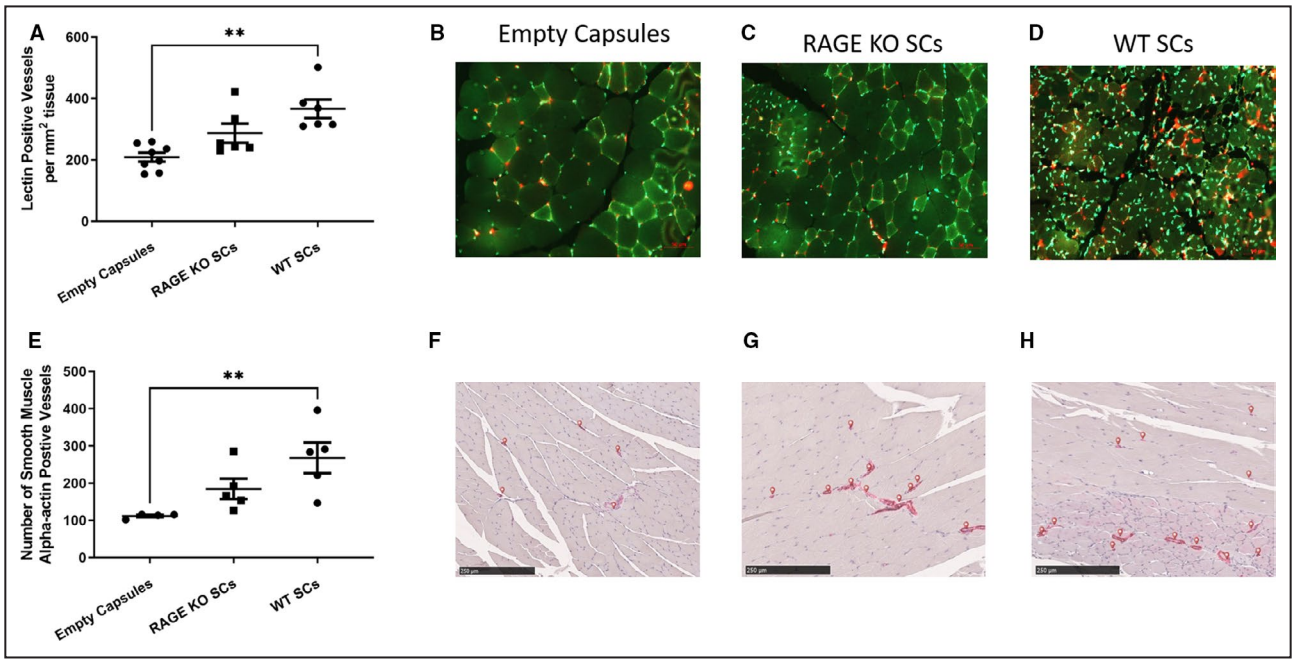

Figure 6. Delivery of wild-type (WT) satellite cells (SCs) increases revascularization.
Mice received either WT or RAGE (receptor for advanced glycation end products) knockout (KO) SCs in alginate capsules or empty capsules at the time of hind limb ischemia surgery, and vascular growth was assessed via immunohistochemistry at day 14 after surgery. **A** through **D**, Capillaries and small vessels were quantified using lectin staining. WT SCs resulted in increased small vessels over empty capsules (n=11; ***P*<0.01; red=lectin). **E** through **H**, Larger vessels, including arterioles and arteries, were quantified by staining for smooth muscle α-actin. As with capillaries, animals that received WT SCs had more vessels than animals that received empty capsules (n=5; ***P*<0.01; red markers indicate vessels).

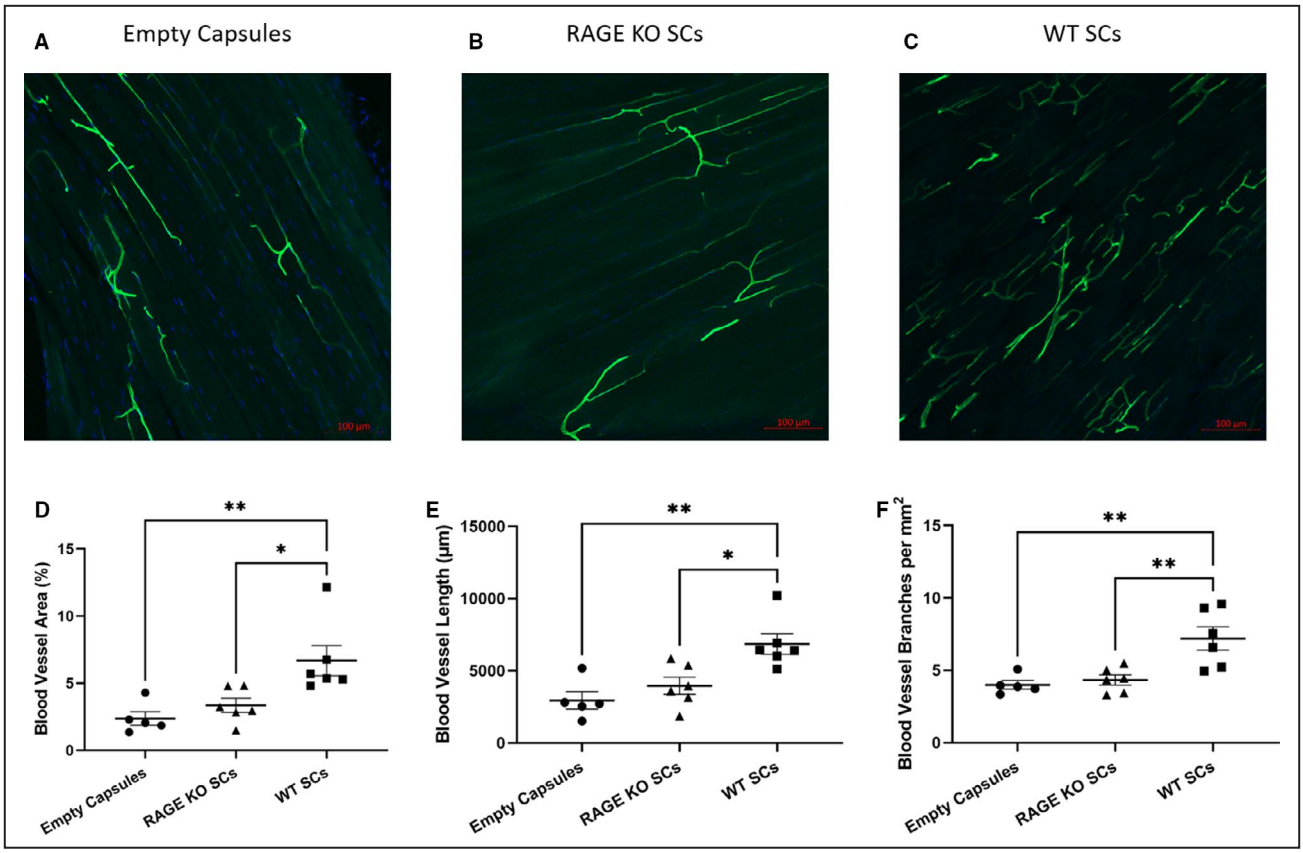

Figure 7. Delivery of wild-type (WT) satellite cells (SCs) increases perfused vascular growth.
Lectin perfusion was used to assess vascular density at day 14 in mice that received either WT or RAGE (receptor for advanced glycation end products) knockout (KO) SCs in alginate capsules or empty capsules at the time of hind limb ischemia surgery. **A** through **C**, Representative maximum intensity projection images of each group are shown. **D** through **F**, Quantification of vessel parameters, including vessel area, length, and branches, found that animals that received encapsulated WT cells had significantly increased measures of all 3 parameters compared with the empty capsule and RAGE KO cell treated group (n=5–6; **P*<0.05, ***P*<0.01).

the sections analyzed were muscles adjacent to the implanted capsules. Finally, to look at the functional perfused vasculature, fluorescein isothiocyanate–lectin was injected intravenously and allowed to circulate before euthanasia. Quantification of thick sections of the muscle tissue found increases in blood vessel area, vessel length, and vessel branches in mice that received WT satellite cells compared with both empty capsules and RAGE knockout cell delivery (n=5–6; blood vessel area: *P*=0.0062 for empty capsules versus WT SCs and *P*=0.0241 for WT SCs versus RAGE knockout SCs; vessel length: *P*=0.0025 for empty capsules versus WT SCs and *P*=0.0151 for WT SCs versus RAGE knockout SCs; vessel branches: *P*=0.0038 for empty capsules versus WT SCs and *P*=0.0062 for WT SCs versus RAGE knockout SCs; Figure 7).

DISCUSSION

We have demonstrated the presence of a previously unappreciated role for RAGE-mediated SC function in collateral vessel formation. In contrast to our previous studies showing that global RAGE deficiency led to improved collateral vessel function,[10] the current work shows that RAGE stimulation of satellite cells exerts a positive and paradoxical effect on collateral vessel formation. We show that satellite cells release cytokines and growth factors previously shown to enhance collateral vessel formation and that increased RAGE expression in the satellite cells extracted from ischemic tissue is associated with increased secretion of some of these factors. In addition to increased expression of cytokines by satellite cells in ischemic tissue, there is also an increase in the number of satellite cells in these tissues, thus augmenting the potential impact of satellite cells on collateral vessel formation. Finally, our cell delivery studies suggest that satellite cells may prove to be a viable therapeutic strategy for ischemic tissues, and this response is dependent on the presence of RAGE in the satellite cells.

Our finding that satellite cells are a major source of RAGE in the IL is a novel finding as most work on the role of RAGE in vascular biology and ischemia

has focused on RAGE expression by macrophages, endothelial cells, and smooth muscle cells.[11,20–23] Yet, these findings do not exclude a variable role for RAGE signaling in vascular and inflammatory cells. However, our work is in agreement with work in muscle biology, which shows that although RAGE is expressed in skeletal muscle during development and in cultured myoblasts, healthy mature skeletal muscle has little basal RAGE expression.[21,24] We also show in Figure 1 that satellite cells from the control limb had almost no RAGE expression, whereas the cells in IL had robust expression and were increased in number. Thus, we sought to explore the role of SC RAGE and its subsequent downstream signaling in our model of ischemic-induced vascular growth.

We determined, in addition to increasing in number, satellite cells from the ischemic hind limb muscles had increased TNF-α and MCP-1, suggesting that ischemia-stimulated satellite cells have a proinflammatory phenotype (Figure 2). We compared the expression profiles of satellite cells from WT and RAGE knockout mice and interestingly found that satellite cells from RAGE knockout mice had decreased cytokine expression (Figure 2). We further explored these differences by stimulating cultured satellite cells with the RAGE ligand HMGB1. Similar to the freshly isolated cells, WT satellite cells produced more expression of TNF-α than stimulated and control RAGE knockout satellite cells, and stimulated wild-type satellite cells expressed more MCP-1 and interleukin-6 than RAGE knockout and stimulated RAGE knockout satellite cells (Figure 3). Taken together, the results in Figures 2 and 3 show that WT satellite cells are responsive to stimuli with increased cytokine expression, whereas RAGE knockout satellite cells are less responsive and express lower levels of cytokines. Thus, RAGE knockout satellite cells appear to have a less inflammatory phenotype when compared with WT cells. This finding is contrary to the typical dogma that RAGE signaling leads to negative effects but is consistent with the concept that collateral vessel formation is driven by inflammatory responses.[22,25–31] We speculate that stimulation of RAGE on satellite cells is an endogenous repair mechanism. Specifically, the ligand HMGB1 has been shown to be increased in the IL and may be responsible for RAGE signaling even in nondiabetic settings, such as the HLI model in this study.[10] HMGB1 is a transcription factor that is released from dead and damaged cells; thus, the potential connection between released HMGB1 in the setting of ischemic tissue and stimulation of satellite cells makes teleological sense as a repair mechanism.

We hypothesized that the cytokine production by the satellite cells might play a role in regulating angiogenesis and arteriogenesis because it has been shown that increases in SC number and capillary number are correlated in acute exercise-induced muscle growth and muscle repair following injury.[32,33] Although previous studies have shown that RAGE knockout animals recover better in the HLI model,[10,11] the results in Figures 2 and 3 show that satellite cells from the RAGE knockout mice have decreased production of several key cytokines and growth factors. Thus, we hypothesized that RAGE knockout satellite cells are less responsive to the ischemic insult, not capable of producing the same degree of cytokines and growth factors, and therefore less potent stimuli for vascular growth. To test this hypothesis, we delivered satellite cells to the IL at the time of surgery. The cells were encapsulated in alginate, which has been previously shown to improve the retention and survival of mesenchymal stem cells in both an HLI and myocardial infarction model.[17,18] Figure 4 shows that the satellite cells were viable for up to 10 to 14 days, which also correlates with the peak differences observed in LDPI analysis of perfusion and assessment of vessel number. This suggests the viability was sufficient to result in a positive effect on collateral vessel formation and function. In agreement with our hypothesis, the WT satellite cells showed significant improvement over the empty capsules in several assessments of vascular growth, whereas the animals with RAGE knockout satellite cells had no significant improvement over empty capsules (Figures 5 through 7). We do note these measures quantify vessel numbers and do not take into effect the health of endothelial cells as RAGE signaling has been associated with endothelial dysfunction and dilation.[34–36] However, the LDPI data in Figure 5, in addition to the histological studies in Figures 6 and 7, demonstrate that these vessels are functional and able to increase blood flow.

Although this finding of the WT satellite cells having better outcomes than RAGE knockout cells may seem contrary to previous studies that have implicated RAGE as having negative effects (and thus positive effects when RAGE is knocked out), these studies focused on other cellular sources of RAGE.[6,8–11,37–39] This suggests that perhaps the negative effects of RAGE signaling are not attributable to satellite cells but other cells expressing RAGE. More important, our findings are consistent with multiple previous studies showing that RAGE signaling upregulates cytokine expression and secretion.[5,31,40] In Figures 2 and 3, we show that that RAGE knockout satellite cells produce fewer cytokines in both an in vivo and an in vitro system. Specifically, others have shown that RAGE activates several mitogen-activated protein kinase signaling cascades, including p38, leading to nuclear factor-κB translocation, and signaling upregulation of multiple cellular responses, including RAGE itself, interleukin-6, and TNF-α.[27,40–44] We also showed that stimulation of WT cells with a RAGE ligand leads to increases in cytokine expression, including interleukin-6 and TNF-α, whereas the

lack of RAGE produces a blunted response to the same stimuli (Figures 2 and 3).

Although increased inflammation is typically viewed as a negative factor, it is known that some degree of inflammation and reactive oxygen species are required for proper collateral vessel formation.[45–51] Indeed, although studies have shown endothelial dysfunction, specifically impaired NO-dependent vasodilation, associated with RAGE signaling, this change is often attributed to increases in reaction oxygen species, leading to NO synthesis uncoupling.[34–36,52,53] However, other studies have shown that reactive oxygen species are crucial to proper vessel growth.[46,50,54,55] Therefore, we purpose that satellite cells produce a physiological inflammatory response to ischemia and increased vascular growth and that RAGE is a critical mediator of this process. This suggests that in certain cell types, RAGE signaling might play a physiological rather than pathophysiological role in producing inflammatory signals. HMGB1-mediated RAGE signaling in rat myoblasts has been shown to increase p38 phosphorylation, which is required for myogenic differentiation and myogenesis.[21] In the C2C12 myoblast cell line, RAGE was shown to be required for differentiation through the p38–mitogen-activated protein kinase pathway to upregulate myogenesis and downregulate Pax7.[20] The mitogen-activated protein kinase pathway is upstream of myogenin, the transcription factor that marks terminal differentiation of the SC into a myoblast. Thus, satellite cells that lack RAGE fail to properly activate, proliferate, and differentiate into myoblasts, but instead have decreased differentiation and an increased number return to the quiescent state.[20,21] This is consistent with dual function of RAGE-mediated inflammation in ischemic muscle injury, leading to both muscle and vascular regeneration. In our study, the satellite cells were encapsulated in nondegradable alginate, so although molecules are still able to diffuse to and from the cells, their lack of ability to differentiate and fuse to repair the muscle is not a factor in our results as only paracrine mechanisms are involved. These findings suggest that in addition to RAGE being required for proper renewal and differentiation of the SC population, it is also required for production of cytokines and factors that drive vascular growth.

Our study demonstrates that in addition to their well-known role in muscle repair, satellite cells play an important role in regulating vascular growth. We found that ischemia both increases SC number and changes their function to a more inflammatory phenotype. Interestingly, RAGE expression and signaling, which is also increased in response to ischemia, is an absolutely required mediator of this phenotypic change. Specifically, RAGE knockout satellite cells were not effective at increasing vascular regrowth. These findings corroborate with the literature that shows that the lack of RAGE changes the phenotype of the satellite cells and results in a more quiescent state. We believe that this phenotype change also results in decreased inflammatory signaling that impairs the vasculogenic potential of the satellite cells. Taken together, it demonstrates that RAGE signaling is important for proper SC function in terms of both muscle repair and coordinating the vascular response. Thus, although satellite cells appear to be a major source of RAGE in the ischemic hind limb, RAGE-mediated inflammatory responses appear to be required for their proper function. Furthermore, these studies raise the interesting possibility that administration of autologous satellite cells is a potential therapeutic strategy for the treatment of peripheral artery disease.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Material

Figures S1–S4

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Supplemental Material

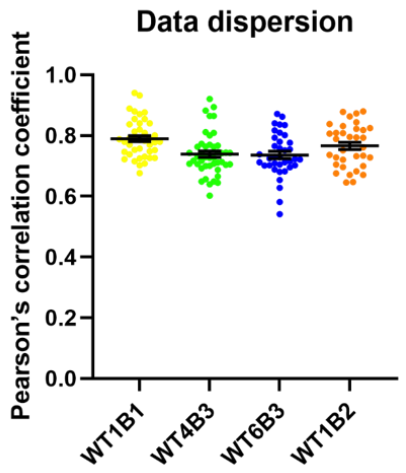

Figure S1: Pax7 and RAGE have a strong colocalization efficiency. The Pearson's correlation coefficient was 0.75 ± 0.02 (mean ± s.e.m., n=4) for RAGE and Pax7 in the ischemic limb.

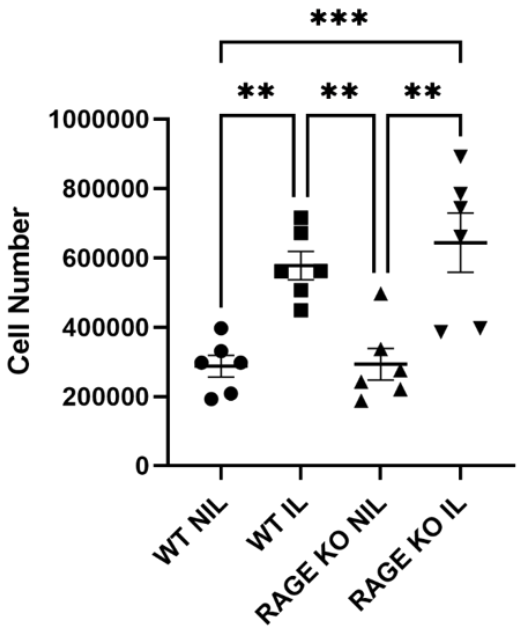

Figure S2: Satellite cells increase with ischemia. Satellite cells were purified at day 7 post HLI from both the nonischemic (NIL) and ischemic limb (IL) in both wild type and RAGE KO

mice. Cells increased with ischemic with no differences between genotypes. (n=6, ** indicates p<0.01 and *** indicates p<0.001)

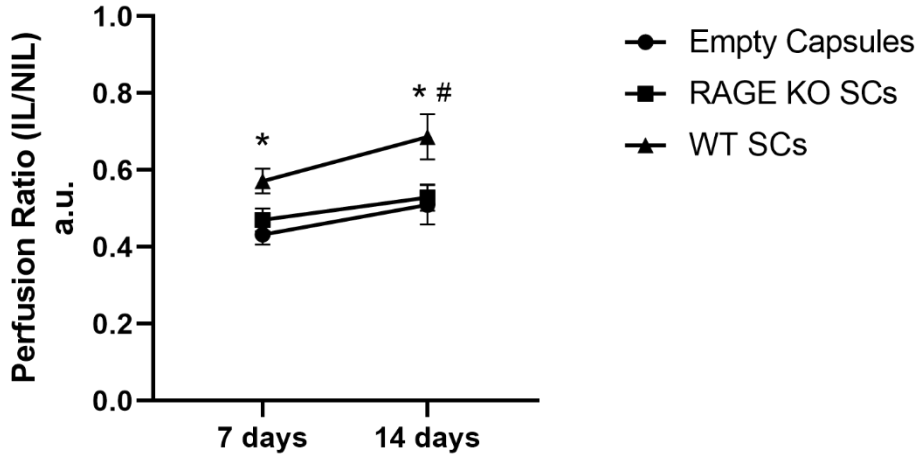

Figure S3: Wild type satellite cells improve perfusion over time. Mice that received wildtype cells had increased perfusion at both days 7 and 14. (n=11, * indicates p<0.5 for Empty Capsules vs WT SCs, # indicates p<0.05 for RAGE KO SCs vs WT SCs)

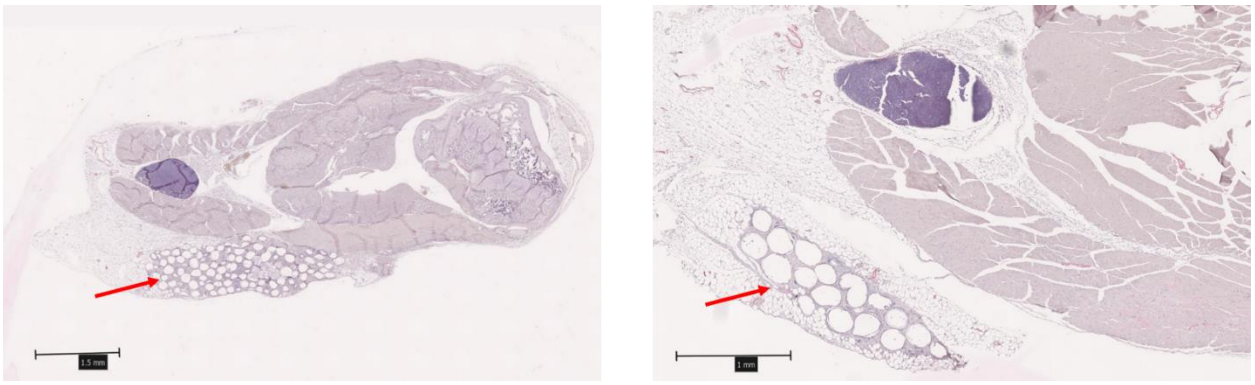

Figure S4: Histological analysis was performed in the same plane as the capsules. Representative images of smooth muscle alpha actin staining show that histological analysis of vessels was done in the muscle sections adjacent to subcutaneously implanted alginate capsules. Red arrows indicate capsules.