

# Gangrene, revascularization, and limb function improved with E-selectin/adeno-associated virus gene therapy

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## ABSTRACT

**Objective:** Novel therapeutic angiogenic concepts for critical limb ischemia are still needed for limb salvage. E-selectin, a cell-adhesion molecule, is vital for recruitment of the stem/progenitor cells necessary for neovascularization in ischemic tissues. We hypothesized that priming ischemic limb tissue with E-selectin/adeno-associated virus (AAV) gene therapy, in a murine hindlimb ischemia and gangrene model, would increase therapeutic angiogenesis and improve gangrene.

**Methods:** FVB/NJ mice were given intramuscular hindlimb injections of either E-selectin/AAV or LacZ/AAV and then underwent induction of gangrene via femoral artery ligation and concomitant systemic injections of the nitric oxide synthesis inhibitor L-NAME (L-N<sup>G</sup>-Nitro arginine methyl ester; 40 mg/kg). Gangrene was evaluated via the Faber hindlimb appearance score. The rate of ischemic limb reperfusion and ischemic tissue angiogenesis were evaluated using laser Doppler perfusion imaging and DiI perfusion with confocal laser scanning microscopy of the ischemic footpads, respectively. The treadmill exhaustion test was performed on postoperative day (POD) 8 to determine hindlimb functionality.

**Results:** The E-selectin/AAV-treated mice ( $n = 10$ ) had decreased Faber ischemia scores compared with those of the LacZ/AAV-treated mice ( $n = 7$ ) at both PODs 7 and 14 ( $P < .05$  and  $P < .01$ , respectively), improved laser Doppler perfusion imaging reperfusion indexes by POD 14 ( $P < .01$ ), and greater gangrene footpad capillary density ( $P < .001$ ). E-selectin/AAV-treated mice also had improved exercise tolerance ( $P < .05$ ) and lower relative muscular atrophy ( $P < .01$ ).

**Conclusion:** We surmised that E-selectin/AAV gene therapy would significantly promote hindlimb angiogenesis, reperfusion, and limb functionality in mice with hindlimb ischemia and gangrene. Our findings highlight the reported novel gene therapy approach to critical limb ischemia as a potential therapeutic option for future clinical studies. (JVS—Vascular Science 2021;2:20-32.)

**Clinical Relevance:** In the United States, >150,000 limb amputations are performed annually for critical limb ischemia (CLI) despite the use of medical and surgical therapy. Thus, novel therapeutic angiogenic concepts for CLI are still needed for limb salvage. We used a novel gene therapy approach in a mouse model of gangrene, the most severe form of CLI, to demonstrate the efficacy of our gene therapy with E-selectin. Preclinical studies such as ours are a vital step in the development of new therapies for CLI patients with threatened limbs and no further medical or surgical options.

**Keywords:** Critical limb ischemia; E-selectin; Gangrene; Therapeutic angiogenesis

Peripheral artery disease (PAD) affects  $\leq 10$  million people in the United States. It is often incurable and can progress to the end-stage condition, critical limb ischemia (CLI), which develops in  $\sim 20\%$  of all PAD patients.<sup>1</sup> Patients with CLI have an overall mortality approaching 50% at 5 years and 70% at 10 years.<sup>2,3</sup>

Patients with diabetes mellitus have an increased risk of PAD<sup>4-6</sup> and CLI, with the development of lower extremity ischemic ulcers or gangrene (tissue death due to lack of blood supply), which leads to greater rates of lower extremity amputation.<sup>7-9</sup> Our laboratory first described a deficit in stromal cell-derived factor-1-alpha

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(SDF-1 $\alpha$ ; an important angiogenic factor) in the diabetic phenotype, which accounts for the more severe impairment in vascularization seen in patients with diabetes mellitus.<sup>10</sup> Current therapies, such as combined medical and surgical approaches, are often ineffectual, and >150,000 lower limb amputations occur annually in the United States owing to the failure currently available therapeutic options.<sup>11</sup> Thus, novel therapeutic angiogenic concepts are needed to attempt limb salvage and preservation of limb function in this vulnerable patient population.

Therapeutic angiogenesis, which induces postnatal neovascularization and the promotion of collateral vessel formation, is a promising modality that can lead to improved clinical outcomes in patients with CLI. This concept has been shown feasible via gene- and cell-based therapies, which include recombinant proteins, genes, and stem cells.<sup>1,12-17</sup> However, adhesion molecules have not been tested as a therapeutic proangiogenic and proregenerative gene therapy modality. Thus, we sought to test alternative gene targets that do not encode soluble factors yet are critical elements for therapeutic angiogenesis and tissue healing and regeneration. E-selectin, an inducible cell-adhesion molecule, is involved in the regulation of angiogenesis and inflammatory responses. It was originally known to mediate adhesive interactions of the activated vascular endothelium with circulating leukocytes/monocytes in response to inflammatory processes such as rheumatoid arthritis and atherosclerosis<sup>18,19</sup> and plays a role in the homing of hematopoietic stem cells<sup>20,21</sup> and endothelial progenitor cells (EPCs).<sup>22,23</sup> We have recently demonstrated the critical role of cell-bound E-selectin expression in ischemic tissue, regulated by SDF-1 $\alpha$ , for the recruitment of EPCs and EPC-dependent angiogenesis<sup>24</sup> and have demonstrated improved wound healing after gene therapy with an adeno-associated virus (AAV) vector, which encodes E-selectin.<sup>25</sup> Therefore, we sought to test the effect of priming hindlimb tissue with an E-selectin/AAV vector to induce overexpression of cell surface E-selectin and modify the ischemic limb tissue microenvironment in a mouse model of gangrene, the most severe form of CLI. We hypothesized that E-selectin-primed tissue would ultimately promote angiogenesis and regeneration in ischemic, gangrenous tissue and could improve clinically relevant signs and symptoms such as muscular atrophy and functionality.

## METHODS

**AAV production.** Full-length murine genes of *E-selectin* and *LacZ* were inserted into multiple cloning sites in the pZac vector and confirmed by gene sequencing. E-selectin/pZac and LacZ/pZac plasmids were then sent to University of North Carolina Gene Therapy Vector Core, where AAV serotype 2/9 (AAV<sup>2/9</sup>) was packaged. The preparations were performed per standard protocol

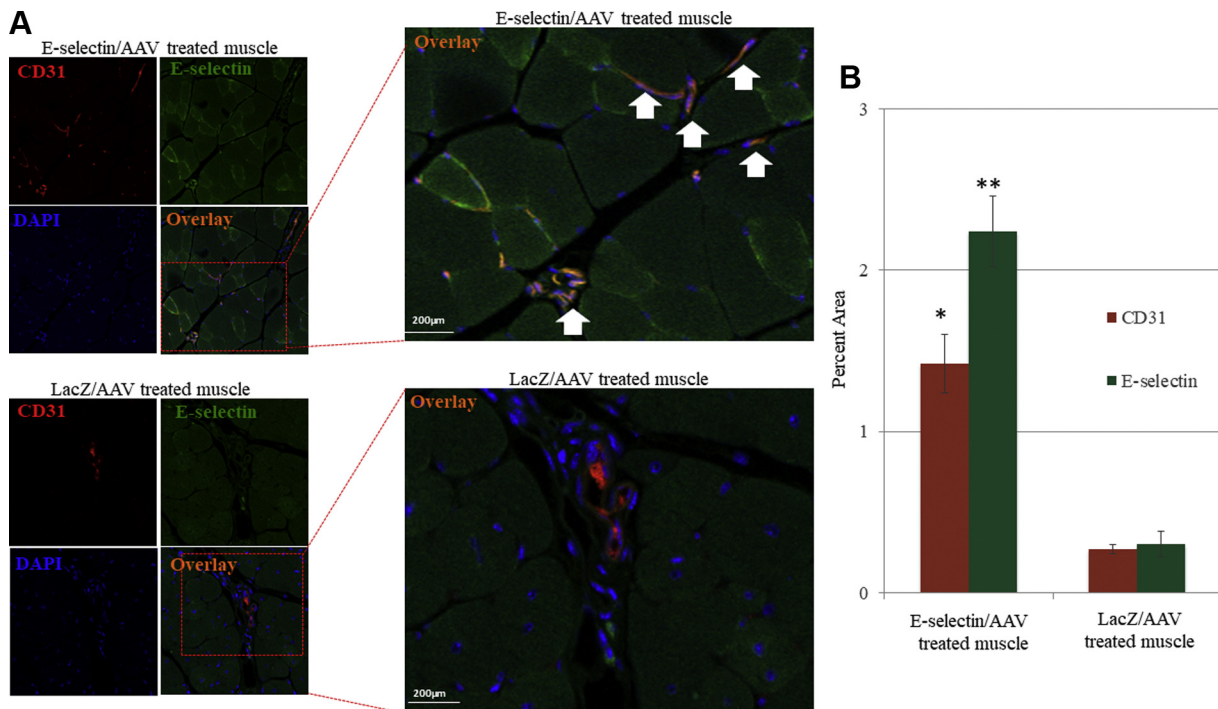
## ARTICLE HIGHLIGHTS

- **Type of Research:** A mouse model of tissue gangrene
- **Key Findings:** Gene therapy using an E-selectin/adeno-associated virus vector improves gangrene, tissue perfusion, and limb functionality.
- **Take Home Message:** The results of our preclinical study have demonstrated the novel usage of an adeno-associated virus vector coupled with E-selectin as an effective and biosafe therapeutic angiogenic and regenerative concept.

using the three-plasmid transfection into HEK293 cells.<sup>26</sup> Quality assurance and control testing included quantitative polymerase chain reaction quantification of AAV genomes, determination of the infectivity titer, and tests for replication competent AAV.<sup>26</sup>

**Gene therapy priming.** FVB/NJ male mice (catalog no. 001800; Jackson Laboratory, Bar Harbor, Me) were given in vivo intramuscular injections of  $5 \times 10^{12}$  viral genome of either E-selectin/AAV<sup>2/9</sup> or LacZ/AAV<sup>2/9</sup> suspended in phosphate-buffered saline (PBS) at five sites into the left medial thigh and calf tissue (20  $\mu$ L per injection site using a 31-gauge needle). The local injection was performed preoperatively (4 and 2 days before surgery) and again immediately after femoral artery and vein ligation (FAL). Gene therapy was administered before surgery to compensate for the lag time from vector injection to E-selectin gene expression in vivo.<sup>27</sup>

**Creation of a mouse CLI gangrene model.** Creation of the hindlimb ischemia and foot gangrene mouse model was performed as previously described.<sup>28</sup> The model uses a combination of FAL and intraperitoneal injections of L-NAME (L-N<sup>G</sup>-Nitro arginine methyl ester), which causes systemic vascular constriction and, overall, results in a reliable murine model of hindlimb gangrene without causing autoamputation of the hindlimb. This model allows for investigators to test treatment strategies to determine whether tissue salvage is feasible with gangrene. In brief, 8- to 10-week-old male FVB/NJ mice (Jackson Laboratory) were cared for and treated in accordance with the Policy on Humane Care and Use of Laboratory Animals and received institutional animal care and use committee approval at the University of Miami Miller School of Medicine (approval no. 16-188). Male mice were used to decreased the potential confounding effects of ovarian steroid hormones on angiogenesis.<sup>29</sup> Before surgery, L-NAME (40 mg/kg), a nitric oxide synthesis inhibitor, was given by intraperitoneal injection 2 hours before surgery and, thereafter, on postoperative days (PODs) 1 to 3 after FAL. For FAL, the mice were anesthetized with ketamine 100 mg/kg



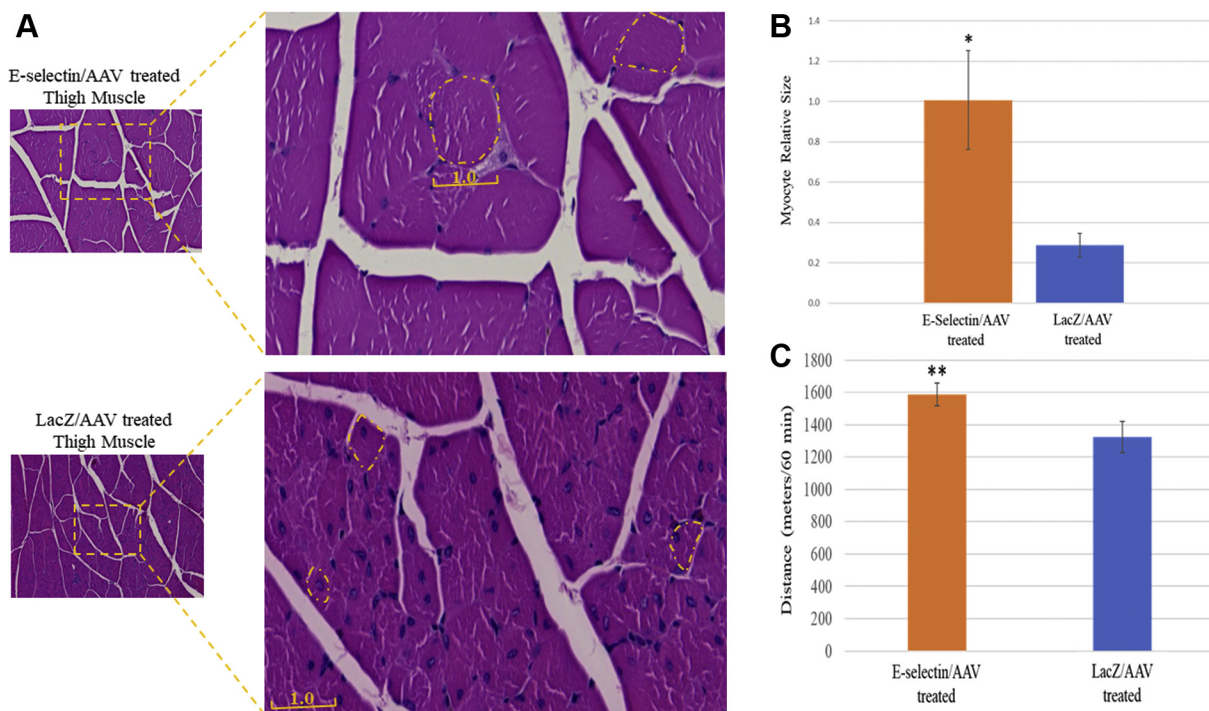
**Fig 1.** Expression of transgene E-selectin in ischemic tissue. **A**, Representative image of immunofluorescence of CD31 (red) and E-selectin (green). Overlay images demonstrate higher expression of E-selectin in endothelial cells in ischemic tissue of E-selectin/adeno-associated virus (AAV)-treated muscle vs LacZ/AAV-treated muscle. **B**, Quantitatively, a two-tailed Student's *t* test was used to compare the mean values of each treatment group. E-selectin/AAV-treated ischemic tissue had a significantly greater amount of E-selectin (\*\**P* < .01) and CD31 (\**P* = .01) expression. LacZ/AAV treatment, *n* = 7; E-selectin/AAV treatment, *n* = 10.

and xylazine 10 mg/kg intraperitoneally. The hair was sheared from the hindlimbs, and an incision was made to expose the femoral sheath. The femoral artery and vein were dissected and separated from the femoral nerve distally at the saphenopopliteal bifurcation and proximally below the inguinal ligament. The artery and vein were both ligated at these two locations using 7-0 silk sutures, and the intervening segment between sutures was severed with a single incision (Supplementary Fig 1, A). Finally, the skin incision was closed using 7-0 silk suture in a continuous fashion. A single dose of sustained-release buprenorphine (1.0 mg/kg; ZooPharm, Wedgewood Pharmacy, Swedesboro, NJ) was subcutaneously administered for postoperative analgesia.

**Evaluating hindlimb ischemia.** All FVB mice were evaluated daily for 2 weeks postoperatively for signs of distal hindlimb ischemia. The Faber hindlimb ischemia score was used (0, normal; 1-5, cyanosis or loss of nails, with the score reflecting the number of nails affected; 6-10, partial or complete atrophy of digits, with the score reflecting the number of affected digits; and 11-12, partial atrophy or gangrene of forefoot).<sup>30,31</sup> The ischemia scores were compared between the E-selectin/AAV- and LacZ/AAV-treated mice in a blinded manner.

**Laser Doppler imaging.** Laser Doppler imaging (LDI) was performed using the LD12-HR System (Moor Instruments, Wilmington, Del). LDI was performed in a blinded manner on the ligated and nonligated legs preoperatively, immediately after FAL, and on PODs 7 and 14. As previously reported, the mice were lightly anesthetized<sup>28,30,31</sup> and placed on a heating pad to maintain their core body temperature at 37°C. Images of the plantar foot were of particular interest because this area most effectively demonstrates reperfusion on LDI. The hindlimb reperfusion index was defined as a ratio of the ligated to nonligated leg for each individual mouse.

**Live animal Dil perfusion and confocal laser scanning microscopy.** The lipophilic carbocyanine dye Dil (1,1'-di-octadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) was used for vascular perfusion and staining, as previously described.<sup>28,32,33</sup> In brief, intracardiac perfusion of filtered PBS, followed by 10 mL of Dil, and, finally, 10% neutral formalin for tissue fixation was performed on POD 14. The area of interest was the footpad, given our interest in distal hindlimb reperfusion. The skinned murine footpad was compressed between two glass microscopic slides using binder clips to allow for adequate imaging using the Zeiss LSM 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany). The

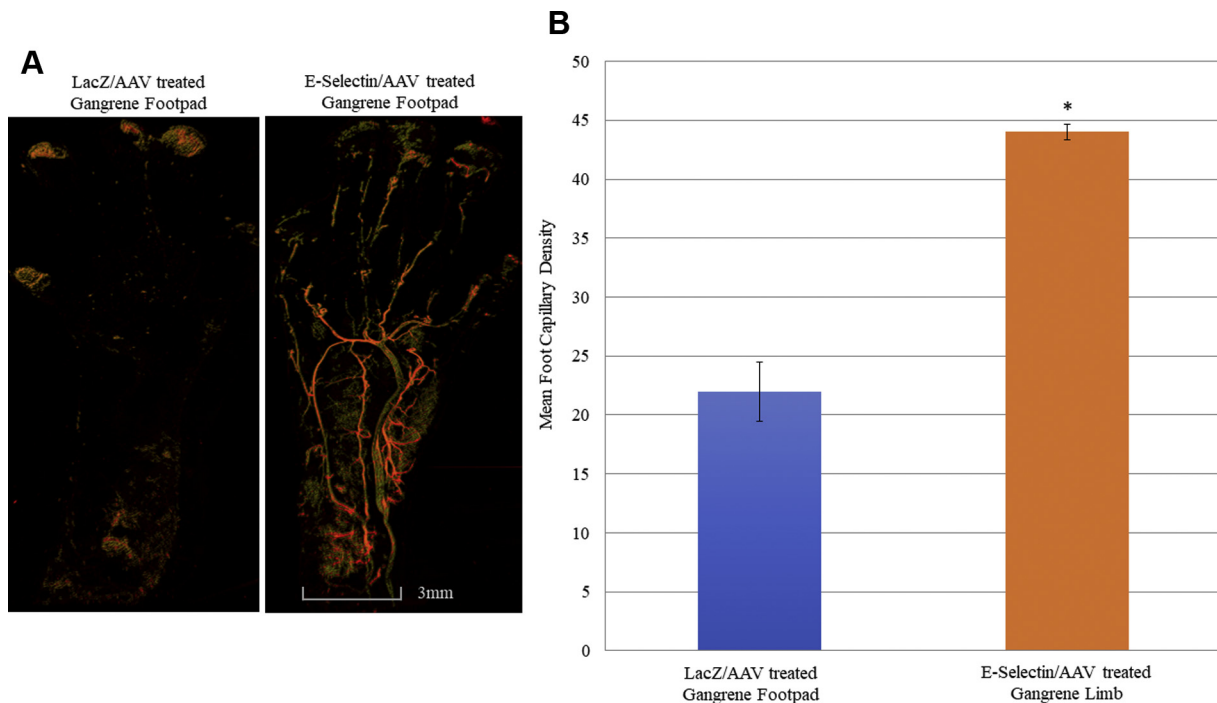


**Fig 2.** Increased muscular stamina and decreased atrophy in mice treated with E-selectin/ adeno-associated virus (AAV) gene therapy. **A**, Representative images of transverse thigh muscle section with hematoxylin-eosin staining. **B**, Cross-sectional size of individual myocytes. E-selectin/AAV-treated myocytes size set to 1.0. LacZ/AAV-treated myocytes were 30% less than the size of E-selectin/AAV-treated myocytes; the size was significantly larger with E-selectin/AAV treatment (\*\* $P < .01$ ). **C**, Treadmill exhaustion function test in both E-selectin/AAV-treated mice and LacZ/AAV-treated mice on postoperative day (POD) 8. The distance traveled in meters within 60 minutes was measured and compared between groups. The E-selectin/AAV-treated mice had run a significantly greater distance in 60 minutes compared with the LacZ/AAV-treated mice (\* $P < .05$ ). Statistical analysis was performed via the two-tailed Student  $t$  test. E-selectin/AAV-treated mice,  $n = 10$ ; LacZ/AAV-treated mice,  $n = 7$ .

Z-series obtained from confocal imaging were reconstructed into three-dimensional images and analyzed using ImageJ, an image processing program developed at the National Institutes of Health (Bethesda, Md), and the Laboratory for Optical and Computational Instrumentation (University of Wisconsin, Madison, Wis). The intricate network of vessels regenerated in the foot was captured, quantified, and represented by the mean capillary density (volume of total Dil-stained vessels) for each mouse.

**Histologic examination.** Immunofluorescent staining and hematoxylin-eosin staining were performed in accordance with a previously reported protocol.<sup>24,34</sup> Tissue section slides were deparaffinized, hydrated, washed, and blocked with protein-blocking serum (Dako, Carpinteria, Calif) for 1 hour at 25°C. To verify E-selectin transgene expression, the slides were incubated with anti-E-selectin/CD62E (Alexa Fluor 488; Novus Biologicals, Centennial, Colo) 5  $\mu\text{g}/\text{mL}$  and endothelial cell marker CD31/PECAM-1 (Alexa Fluor 647; Novus Biologicals) 15  $\mu\text{g}/\text{mL}$  in protein blocking serum for 12 hours

at 4°C. The slides were then washed with 0.1% Tris-buffered saline plus Tween 20 before DAPI (4',6-diamidino-2-phenylindole) staining (Vector Laboratories, Inc, Burlingame, Calif) for nuclei visualization. A confocal laser scanning microscope with  $\times 20$  to  $\times 40$  magnification was used to image immunofluorescence. To detect EPCs (defined in the present study as CD34<sup>+</sup> endothelial cells), we co-stained hindlimb tissue sections with CD34 (Alexa Fluor 488; Santa Cruz Biotechnology, Dallas, Tex) and endothelial cell marker CD31/PECAM-1 (Alexa Fluor 647; Novus Biologicals). To evaluate the potentially proinflammatory nature of E-selectin, we stained control and treatment hindlimb tissue sections with Mac-2/galectin 3 (Alexa Fluor 594; BioLegend, San Diego, Calif), an inflammatory marker for monocytes/macrophages. For quantification of E-selectin, CD31, CD34, and Mac-2, at least five random  $\times 10$  or  $\times 20$  magnification fields for each section were blindly scored and/or measured using ImageJ (National Institutes of Health) or Adobe Photoshop CC (Adobe, San Jose, Calif). To determine the amount of muscular atrophy in the ligated limbs of the mice after FAL, hematoxylin-eosin staining was



**Fig 3.** Evaluation of vessel density within the ischemic, gangrenous footpads of the murine ligated hindlimb with E-selectin/adeno-associated virus (AAV) vs control (LacZ/AAV) gene therapy. Treatment with E-selectin/AAV gene therapy resulted in increased footpad vessel density. **A**, Representative images showing DiI perfusion capillary density was improved in treatment vs control groups on postoperative day (POD) 14. **B**, Quantification of capillary density in ischemic, gangrene footpads of both E-selectin/AAV treatment (n = 5) and LacZ/AAV treatment (n = 5); E-selectin/AAV–treated ischemic footpad capillary density was 44 capillaries per myofibril cross-sectional area, and LacZ/AAV–treated footpad capillary density was 22 capillaries per myofibril cross-sectional area (\* $P < .001$ , two-tailed Student's  $t$  test).

performed on paraffin-embedded transverse muscle tissue sections of both treatment groups, as previously described.<sup>35</sup> Images were then acquired at  $\times 10$  magnification, and the relative size of the E-selectin/AAV–treated myocytes were set and compared with the myocyte size of the LacZ/AAV–treated group.

**Treadmill exhaustion test.** Exercise treadmill testing was performed using a rodent treadmill, as previously described<sup>36</sup> (model, Exer 3/6; Columbus Instruments, Columbus, Ohio), 8 days after FAL to determine the degree of hindlimb functionality and recovery after gene therapy. In the present study, our protocol started with a  $10^\circ$  slope at a speed of 10 m/min for 5 minutes. The mice were placed on a horizontal treadmill, and the speed was increased by 5 m/min every 5 minutes until a maximum speed of 30 m/min had been reached. The mice were allowed to run for a total of 60 minutes, at which point the test was concluded. The total distance the mice had run during the 60 minutes was recorded and compared between the treatment and control groups. If the mouse remained on the stimulation plate for 10 seconds without any attempt to reengage the treadmill,<sup>36</sup> the time to exhaustion (expressed in minutes) and total distance traversed was recorded, as this was defined as exhaustion. Thus, use of the treadmill test

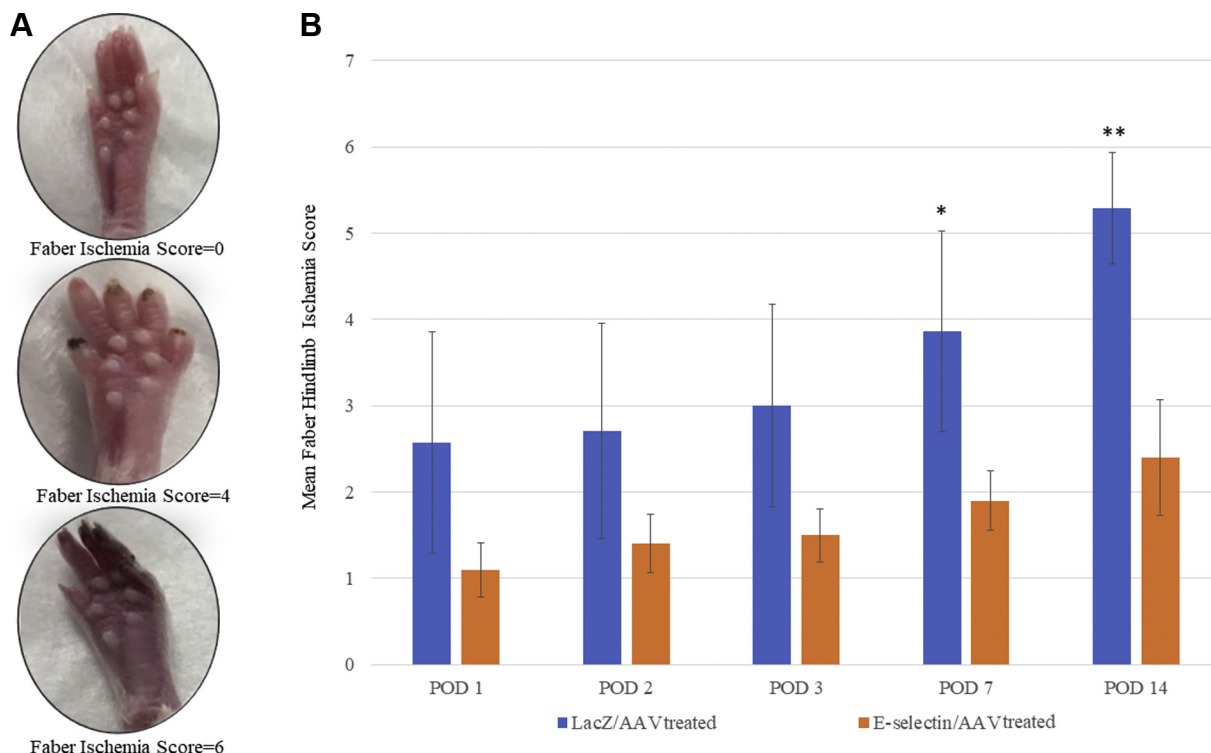
allowed us to investigate the running capability of the mice in both treatment and control groups postoperatively.

**Evaluating potential prothrombotic effects of E-selectin gene therapy delivered intramuscularly to ischemic limb.** To evaluate the potentially prothrombotic effects of E-selectin limb intramuscular gene therapy in vivo, the D-dimer levels were measured in serum. Blood sampling of the E-selectin/AAV– and LacZ/AAV–treated mice was performed on POD 5 to determine the D-dimer levels in serum.

**Statistical analysis.** The data were parametric and are presented as the mean  $\pm$  standard deviation. Two-tailed Student's  $t$  tests were performed to compare the mean values of two groups. Probability values of  $P < .05$  were interpreted to denote statistical significance.

## RESULTS

**Effective method of gene delivery after in vivo injection of E-selectin/AAV and LacZ/AAV viral particles.** We observed that the E-selectin/AAV–treated mice manifested a greater confluence of the E-selectin (green) and endothelial cell CD31 (red) co-staining, shown by orange, compared with that of the LacZ/AAV–treated mice (Fig 1, A). Quantitatively, the percent area of E-



**Fig 4.** Postoperative Faber ischemia scores in murine gangrene model improved with E-selectin/adeno-associated virus (AAV) treatment vs LacZ/AAV treatment. **A**, Representative images of Faber score 0, 4, and 6 (top to bottom). **B**, E-selectin/AAV treated mice demonstrated a significantly lower ischemia score in their ischemic limbs on postoperative day (POD) 7 (\* $P < .05$ ) and POD 14 (\*\* $P < .01$ ) compared with the ischemic limbs of LacZ/AAV-treated mice using a two-tailed Student's *t* test. LacZ/AAV,  $n = 7$ ; E-selectin/AAV,  $n = 10$ .

selectin and CD31 co-staining was significantly greater statistically in the E-selectin/AAV-treated muscle vs the LacZ/AAV-treated muscle (Fig 1, B). These results confirmed that AAV<sup>2/9</sup>-mediated gene delivery effectively increased E-selectin expression of the microvasculature within the ischemic limb tissues of the mice.

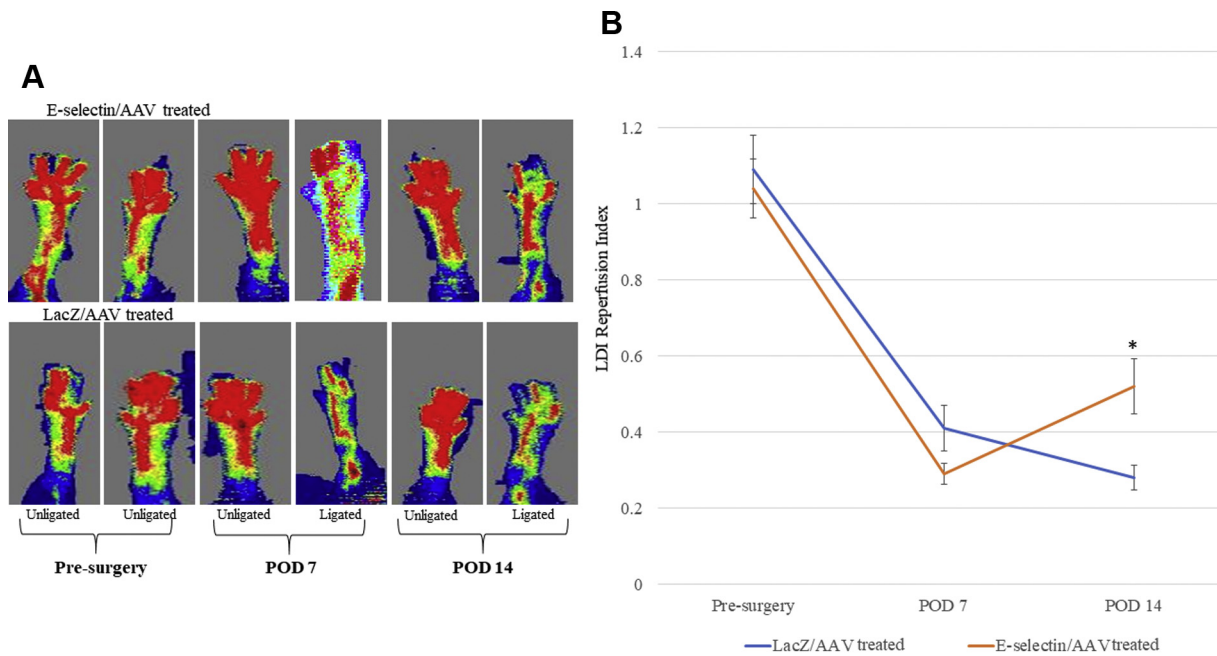
**E-selectin/AAV gene therapy increased limb function performance, enhanced exercise tolerance (running distance), and decreased limb skeletal muscle atrophy.**

We observed less muscle atrophy in the ischemic limbs treated with E-selectin/AAV gene therapy compared with that in those treated by LacZ/AAV. The myocytes in the E-selectin/AAV-treated mice appeared healthier with a larger relative size ( $1 \pm 0.24$ ) compared with those in the control mice ( $0.3 \pm 0.6$ ; Fig 2, A and B). The E-selectin/AAV-treated mice were found to traverse a statistically significantly greater distance in 60 minutes compared with the LacZ/AAV-treated mice (Fig 2, C). Specifically, the mice in the treatment group were able to run a mean of 1590 m within 60 minutes and the mice in the control group were able to run a mean of 1325 m within 60 minutes ( $P < .05$ ). Overall, our data have demonstrated that gene therapy with E-selectin/AAV improves limb function recovery and mitigates skeletal

muscle atrophy in this murine model of induced CLI and gangrene.

**E-selectin/AAV gene therapy increased vessel density in the murine gangrene footpad.**

Based on confocal microscopy images of DiI-stained blood vessels, the unligated limbs of both E-selectin/AAV- and LacZ/AAV-treated mice showed comparable vessel densities in the normal footpad postoperatively (Supplementary Fig 2). However, a statistically significant difference was found in the number of capillaries in the gangrene footpads of the ligated hindlimbs of the E-selectin/AAV-treated mice compared with the LacZ/AAV-treated mice (Fig 3, A and B). The LacZ/AAV-treated mice showed a scarcity in capillary density postoperatively in the ischemic and gangrenous footpad of the ligated hindlimb (Fig 3, A). Quantitatively, the capillary density within the gangrene footpad of the E-selectin/AAV- and LacZ/AAV-treated mice was 44 and 22 capillaries per myofibril cross-sectional area, respectively (Fig 3, B). Taken together, these data have demonstrated that gene therapy with E-selectin/AAV induces increased vessel density in the footpad in mice with surgically induced hindlimb ischemia and distal gangrene of the digits.



**Fig 5.** Laser Doppler imaging (LDI) demonstrated improved ischemic limb reperfusion in E-selectin/adeno-associated virus (AAV)-treated mice vs LacZ/AAV-treated mice by postoperative day (POD) 14. **A**, Representative images of LDI at various days between treatment and control groups. **B**, Quantitative reperfusion index at various days for treatment and control groups (reperfusion index = ligated/unligated hindlimb). A two-tailed Student's *t* test was used to compare the mean values of each treatment group. By POD 14, the E-selectin/AAV-treated mice demonstrated significantly greater foot reperfusion vs LacZ/AAV-treated mice (\* $P < .01$ ). LacZ/AAV,  $n = 7$ ; E-selectin/AAV,  $n = 10$ .

### E-selectin/AAV gene therapy improved the hindlimb ischemia score in the murine model of distal gangrene.

The E-selectin/AAV-treated mice demonstrated fewer visual signs of ischemia/gangrene to the distal hindlimb and digits compared with the LacZ/AAV-treated mice (Fig 4, A and B) and demonstrated lower Faber hindlimb ischemia scores on each postoperative day compared with the LacZ/AAV-treated mice. The difference in CLI severity between the treatment and control groups reached statistical significance by POD 7 ( $P < .05$ ) and was maintained through POD 14 ( $P < .01$ ; Fig 4, B). On POD 14, the E-selectin/AAV mice had a mean Faber ischemia score of  $\sim 2$ , equating to two ischemic or gangrene nails. In contrast, the LacZ/AAV mice had a mean Faber ischemia score of  $\sim 5$ , equivalent to five ischemic or gangrene nails (Fig 4, B). These data revealed that E-selectin/AAV gene therapy, not only promoted therapeutic angiogenesis, but also reduced the signs of CLI, such as the extent of gangrene and tissue loss related to severe limb ischemia.

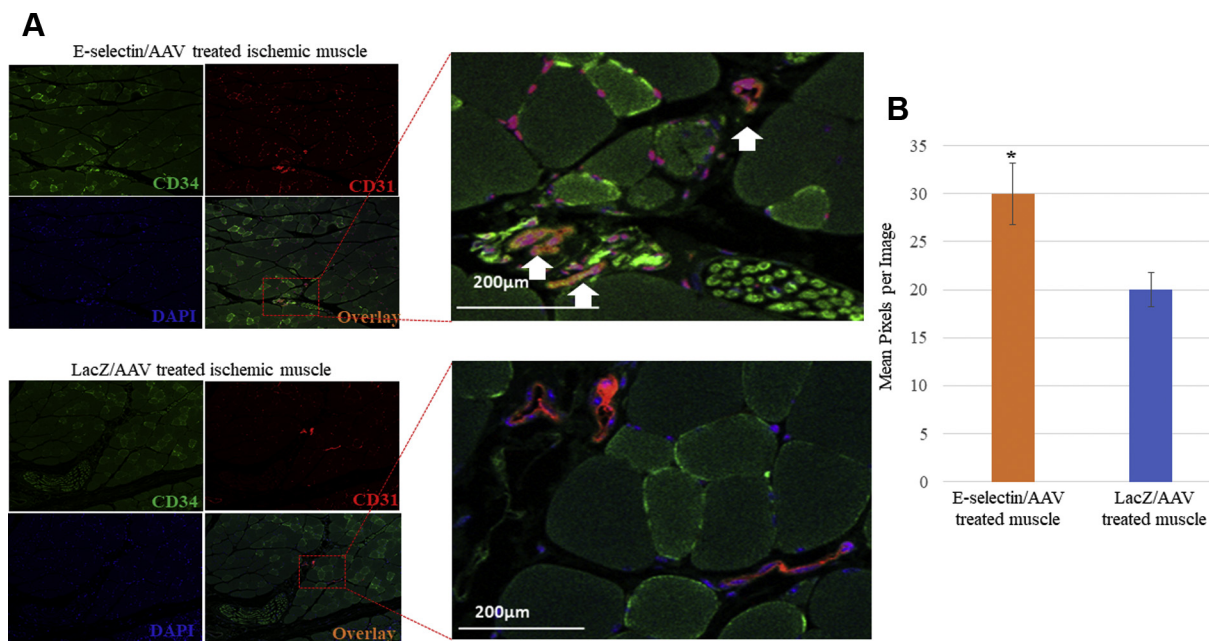
**E-selectin/AAV gene therapy increased hindlimb skin reperfusion.** The LDI measurements performed preoperatively and immediately postoperatively were comparable between the two groups, with both groups of mice manifesting normal perfusion to the foot before surgery and severely diminished perfusion to the foot

immediately after surgery (Supplementary Fig 1, B). However, by POD 14, the mice treated with E-selectin/AAV demonstrated statistically significantly increased distal hindlimb reperfusion indexes on LDI compared with their LacZ/AAV counterparts (Fig 5, A and B;  $P < .01$ ). Overall, these results revealed an increased level of reperfusion in the ischemic hindlimbs treated with E-selectin/AAV, as evidenced by the increased perfusion seen on LDI and that the increased reperfusion occurred at the skeletal muscle, skin, and subcutaneous levels (Figs 1, 3, and 5).

### E-selectin/AAV gene therapy increased recruitment of EPCs.

We found greater amounts of CD34<sup>+</sup>/CD31<sup>+</sup> cells, which are surrogate markers of EPCs, in mice treated with E-selectin/AAV compared with those treated with LacZ/AAV (Fig 6, A and B). This suggests that the increased availability of EPCs in the ischemic hindlimb of mice primed with E-selectin/AAV might play a key mechanistic role in postnatal limb neovascularization and skeletal muscle function preservation, which was imputed in previous studies.<sup>37-39</sup>

**E-selectin/AAV gene therapy did not appear to induce inflammation, thrombosis, or systemic toxicity in the ischemic mouse model.** These two experimental groups of mice (E-selectin/AAV and LacZ/AAV) showed



**Fig 6.** Gene therapy with E-selectin/adeno-associated virus (AAV) increased recruitment of endothelial progenitor cells (EPCs) in ischemic tissue. **A**, Representative images of immunofluorescent staining of CD34 (green) and CD31 (red) of ischemic muscle tissues of E-selectin/AAV-treated mice and LacZ/AAV-treated mice. E-selectin/AAV-treated ischemic tissue demonstrates increased expression of CD34 and CD31. **B**, Quantitative levels of CD34/CD31 per image in mice undergoing E-selectin/AAV treatment vs LacZ/AAV treatment. Student's *t* test revealed a significant increase in CD34/CD31 levels in E-selectin/AAV-treated ischemic tissue vs LacZ/AAV-treated ischemic tissue ( $P < .05$ ). E-selectin/AAV sections,  $n = 15$ ; LacZ/AAV sections,  $n = 15$ .

comparable expression of the Mac-2<sup>+</sup> cell marker (Fig 7, A and B), indicating that E-selectin/AAV gene therapy does not appear to induce a stronger proinflammatory response. Our analysis revealed no statistically significant differences in this marker of thrombosis *in vivo* between the E-selectin/AAV- and LacZ/AAV-treated mice (Fig 7, C), suggesting that mice receiving E-selectin/AAV gene therapy are not more likely to develop clots than mice treated with the LacZ control vector. We did not find any abnormalities in liver and kidney function test results among the mice treated with E-selectin/AAV, LacZ/AAV, or PBS (control, no virus), with no statistically significant differences among the three groups (Supplementary Table). These data together have demonstrated that E-selectin/AAV gene therapy does not appear to induce inflammation or thrombosis or cause major organ toxicity in the ischemic/gangrene mouse model and suggest that this novel gene therapy is generally nontoxic to these key metabolic organs in the short term.

## DISCUSSION

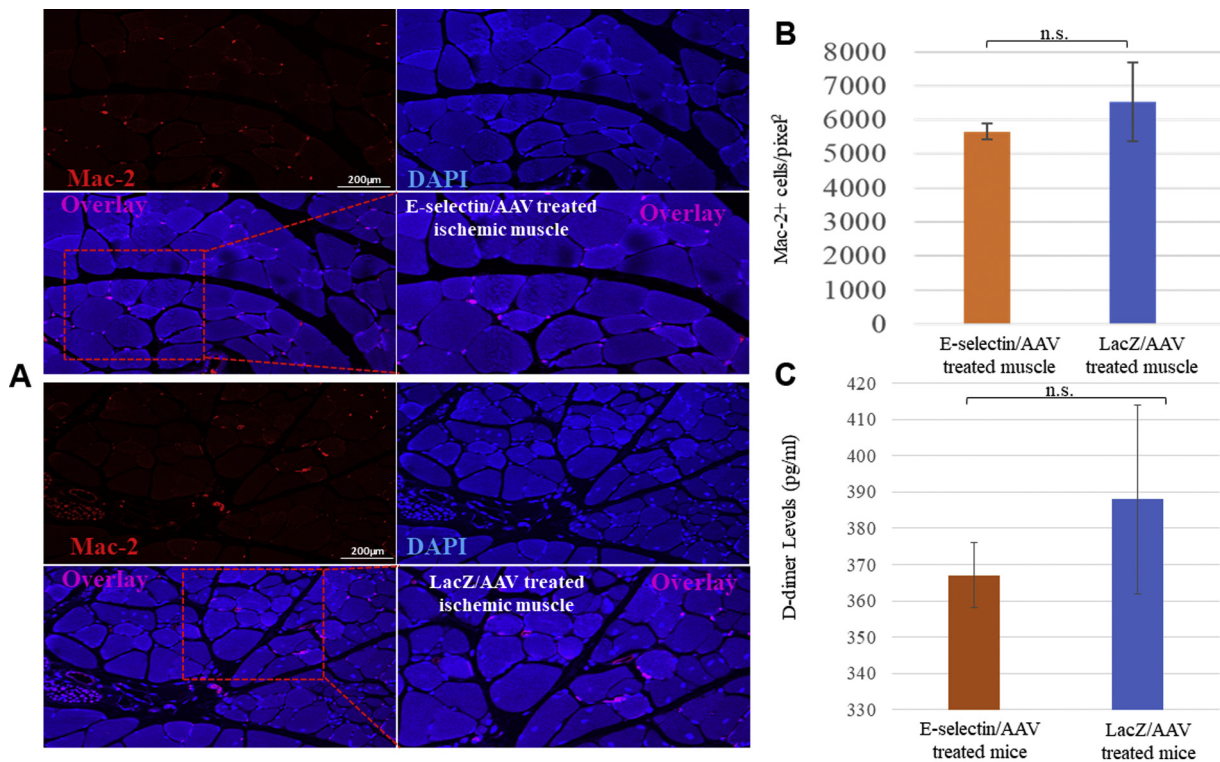
In the present study, we have provided a novel approach for postnatal neovascularization of severely ischemic limb tissues by the usage of gene therapy with E-selectin to prime the ischemic tissue microenvironment via a safe and efficient AAV<sup>2/9</sup> vector to augment the body's own neovascularization

mechanisms through the enhancement of recruitment of CD34<sup>+</sup> progenitor cells.

Under hypoxic conditions, the endothelium in ischemic tissues expresses certain adhesion molecules, including E-selectin owing to stimulation of several hypoxia-inducible factor-1 $\alpha$ -induced cytokines and chemokines.<sup>23</sup> We previously reported that SDF-1 $\alpha$  can upregulate E-selectin expression on endothelial cells in tumor and wound vasculature<sup>22</sup> and that E-selectin is involved in EPC-dependent neovascularization via recruitment of circulating leukocytes and hematopoietic stem cells through interaction with counterpart ligands.<sup>23,24</sup> In the present study, we have demonstrated, for the first time, to the best of our knowledge, that effective overexpression of the cell-bound E-selectin transgene into ischemic gangrenous hindlimb tissues, not only improves angiogenesis, limb perfusion, and recruitment of CD34<sup>+</sup> progenitor cells, but also improves mouse exercise tolerance and ameliorates the atrophy effects of severe ischemia in limb skeletal muscle. Ostensibly, this gene therapy approach primes the microenvironment of the ischemic tissue via overexpression of the cell adhesion molecule E-selectin, which then allows for increased capture of stem/progenitor cells to maximize the neovascularization and healing process.

The results of the present study have demonstrated that gene therapy with E-selectin/AAV confers hindlimb





**Fig 7.** Gene therapy via E-selectin/adeno-associated virus (AAV) did not seem to significantly induce inflammation or thrombosis. **A**, Representative images of immunofluorescence of Mac-2<sup>+</sup> cells in E-selectin/AAV–treated ischemic muscle and LacZ/AAV–treated ischemic muscle. **B**, Quantification of Mac-2<sup>+</sup> cells per square pixel in ischemic muscle in E-selectin/AAV–treated mice vs LacZ/AAV–treated mice. Student's *t* test revealed no significant differences in expression of Mac-2 in ischemic muscle tissues between E-selectin/AAV treatment and LacZ/AAV treatment ( $P = .602$ ). **C**, Serum D-dimer levels for E-selectin/AAV–treated mice and LacZ/AAV–treated mice. No significant difference was found in serum D-dimer levels at postoperative day (POD) 5 between treatment and control groups ( $P = .6$ ). E-selectin/AAV sections,  $n = 15$ ; LacZ/AAV sections,  $n = 15$ . DAPI, 4',6-Diamidino-2-phenylindole; *n.s.*, not significant.

tissue salvage, regeneration of hindlimb blood vessels, and reperfusion of ischemic hindlimbs, with an improved running distance and decreased skeletal muscle atrophy. The tests performed in the CLI mouse model were designed to correlate clinically with how human patients with CLI are monitored. Most patients with CLI are monitored via physiologic testing such as the ankle brachial index (ABI)<sup>40</sup> and pulse volume recording waveform analysis<sup>41</sup> owing to their relative cost-effective, low-risk nature and typical congruence with clinical features. However, these relatively simple diagnostic tests are not feasible in the mouse; therefore, we used LDI as a correlate test because it allows for determination of blood flow to the limb, much like the ABI and pulse volume recording waveform analysis. Morphologic examinations of the human vasculature such as computed tomography angiography, magnetic resonance angiography, or procedural angiography are used in patients with CLI to determine the vascular anatomy and location of arterial blockage, often before an attempt at revascularization. These examinations are also impractical in the mouse model; therefore, we used confocal microscopy after Dil

perfusion as a proxy to determine whether the foot had had increased blood vessel density. Overall, the acceleration of tissue regeneration after CLI in the treatment mice can be appreciated by many different modalities that underscore the beneficial biologic and functional effects of E-selectin/AAV gene therapy. These promising preclinical results suggest potential translatable effects in humans. The ABI could serve as a compass to identify patients in need of treatment who are neither surgical nor endovascular candidates. Although further testing is necessary, we believe that no-option patients with a certain ABI could potentially qualify for preemptive treatment with gene therapy in initial clinical studies to deter disease progression and, potentially, reverse tissue ischemia, thus preventing amputation.

In vivo, E-selectin–mediated adhesion and signaling can contribute to adverse pathophysiologic outcomes such as atherosclerosis, arterial and deep vein thrombosis, ischemia–reperfusion injury, and, conceivably, other cardiovascular diseases.<sup>42,43</sup> Because of the potential proinflammatory and thrombogenic nature of either increased soluble E-selectin or systemic generalized

cell-bound E-selectin expression, determining the safety profile of this locoregional limb intramuscular gene therapy approach is important. Monocyte/macrophage staining demonstrated comparable levels of Mac-2<sup>+</sup> cells between the hindlimbs in the E-selectin/AAV– and LacZ/AAV–treated mice. It is important to recognize, however, that macrophages of differing phenotypes exist, including an activated, proinflammatory macrophage (M1) and a proregulatory macrophage (M2). We did not differentiate between these two macrophage phenotypes, which is a potential area of further study to determine whether E-selectin/AAV treatment results in a change in ischemic tissue macrophage differentiation.<sup>44</sup> Furthermore, based on our histologic analysis of the tissue samples, no evidence of microthrombi was noted within the blood vessels, and the serum D-dimer levels were normal and similar between the groups. These results have shown that this novel adhesion molecule-based gene therapy approach is effective and appears to be generally safe in preclinical testing.

## CONCLUSION

The present study has demonstrated the novel usage of an AAV vector coupled with E-selectin as an effective and biosafe therapeutic angiogenic and regenerative concept. The intramuscular injection of E-selectin/AAV in mice with CLI significantly promoted hindlimb angiogenesis and reperfusion, attenuating and reversing overall ischemia to the hindlimb, increasing exercise tolerance, and reducing distal gangrene and muscle atrophy. Our study has highlighted this novel gene therapy approach to CLI as a potential therapeutic option to be tested in future clinical studies.

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## AUTHOR CONTRIBUTIONS

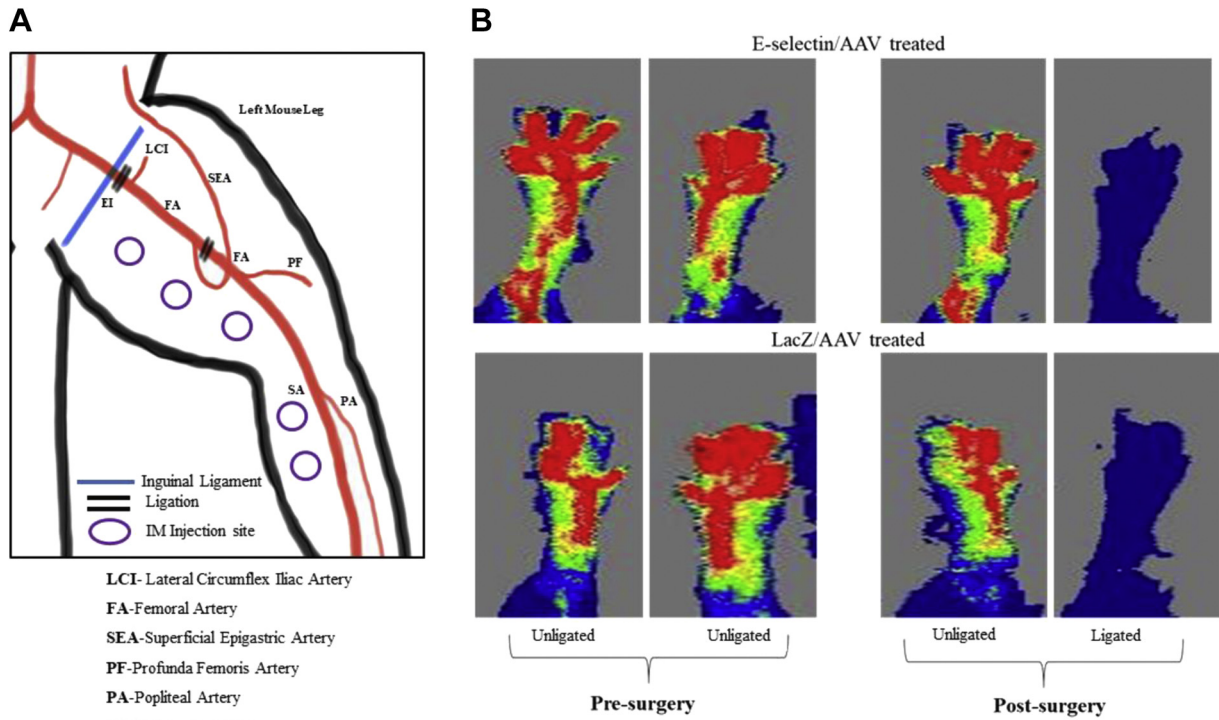
Conception and design: RV, JP, ZL, OV  
Analysis and interpretation: HQ, PP, RLS, YL, HS, ZL, OV  
Data collection: HQ, PP, RLS, MR, YL, HS  
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HQ and PP contributed equally to this article and share co-first authorship.

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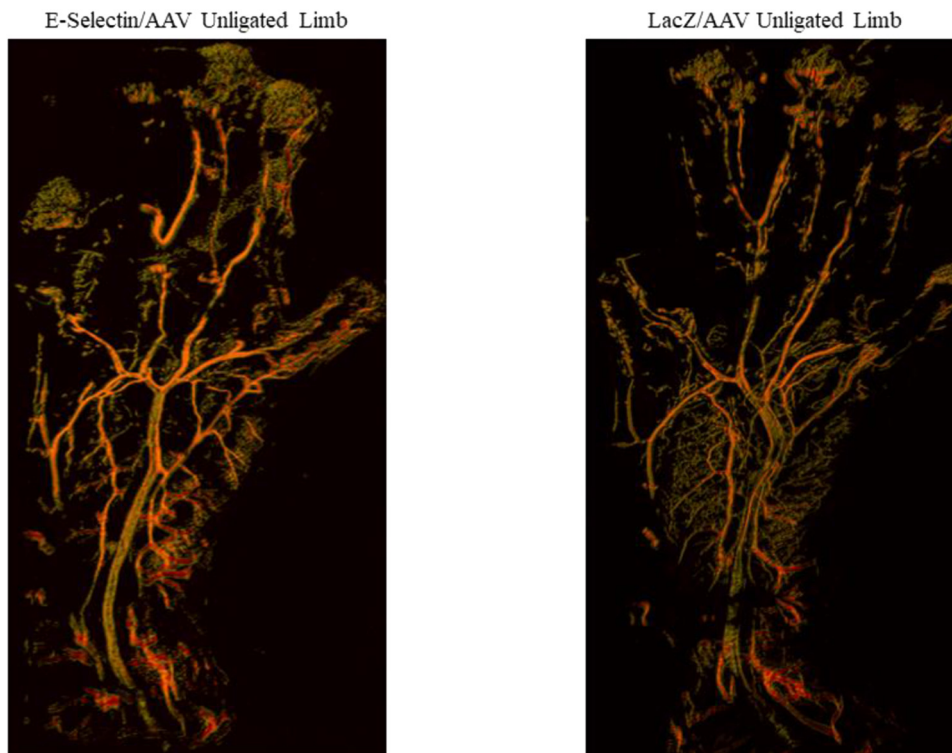
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**Supplementary Fig 1. A**, Peripheral vascular anatomy in mice. Femoral artery ligation (FAL) was performed via proximal and distal ligation (*double lines*) located below the inguinal ligament and immediately above the saphenopopliteal bifurcation. The injection sites were in 5 areas in the left (ligated) medial thigh and calf muscles. **B**, Preoperative laser Doppler images showing normal perfusion distribution in bilateral limbs of the mice and postoperative laser Doppler images showing successful cessation of limb perfusion in the FAL limb (E-selectin/ adeno-associated virus [AAV], n = 10; LacZ/AAV, n = 7).



**Supplementary Fig 2.** Representative images via confocal laser scanning microscopy of the normal, unligated footpads of mice treated with E-selectin/adeno-associated virus (AAV) and LacZ/AAV. Analysis using two-tailed Student's *t* test showed no statistically significant difference in the capillary density between the two groups (E-selectin/AAV treatment, *n* = 5; LacZ/AAV treatment, *n* = 5).

**Supplementary Table.** Liver and kidney function blood test results<sup>a</sup>

| Biochemistry test | E-selectin/AAV | LacZ/AAV | PBS (no virus) | <i>P</i> value | Normal range |
|-------------------|----------------|----------|----------------|----------------|--------------|
| AST, U/L          | 123            | 118      | 117            | .91            | 50-270       |
| ALT, U/L          | 32             | 40       | 67             | .1             | 29-77        |
| ALP, U/L          | 29             | 33       | 31             | .84            | 51-285       |
| Bilirubin, mg/dL  |                |          |                |                |              |
| Total             | 0.5            | 0.5      | 0.8            | .21            | 0.1-0.9      |
| Direct            | 0.5            | 0.5      | 0.8            | .46            | 0.1-0.9      |
| Albumin, g/dL     | 1.6            | 1.9      | 1.6            | .17            | 2.5-2.8      |
| BUN, mg/dL        | 23             | 24       | 21             | .24            | 18-29        |
| Creatinine, mg/dL | 0.1            | 0.2      | 0.1            | .27            | 0.1-0.4      |

AAV, Adeno-associated virus; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; PBS, phosphate-buffered saline.

<sup>a</sup>Intracardiac blood samples were taken before animal sacrifice; no differences were found among mice treated with E-selectin/AAV (*n* = 4), LacZ/AAV (*n* = 7), or PBS (no virus; *n* = 5) via analysis of variance statistical analysis.