METHODS ARTICLE

Determination of a Critical Size Threshold for Volumetric Muscle Loss in the Mouse Quadriceps

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Skeletal muscle has a remarkable regenerative capacity; however, after volumetric muscle loss (VML) or a loss of a large portion of the tissue, this regenerative response is diminished and results in chronic functional deficits. The critical size at which muscle will not recover has not yet been established; subsequently, the response of crucial muscle components at the critically sized threshold is unknown. In this study, we set out to determine the threshold for a critically sized muscle defect by creating full-thickness VML injuries of 2, 3, or 4 mm diameter in the mouse quadriceps. The 2, 3, and 4 mm injuries resulted in a defect of 5%, 15%, or 30% of muscle mass, respectively. At 14 and 28 days after injury, histological analyses revealed injury size-dependent differences in myofiber morphology and fibrosis; the number of small myofibers and fibers with centrally located nuclei increased with increasing injury size. The results indicated that the 3 mm injury, with 15% mass loss, was at the critical threshold point, characterized by incomplete bridging of myofibers through the defect site, persistent fibrosis and inflammation, and a temporally sustained increase in myofibers with centrally located nuclei as compared with contralateral control muscle. We further investigated the 3 mm VML for nerve and vascular regeneration. Critically sized injured muscles were accompanied by a drastic increase in denervated neuromuscular junctions (NMJs), while assessment of angiogenesis through micro-CT analysis revealed a significant increase in vascular volume primarily from small diameter vessels after the VML injury. Collectively, these data indicate that the fibrotic response and neuromotor component remain dysregulated in critically sized defects, and therefore could be potential therapeutic targets for regenerative strategies.

Keywords: skeletal muscle, volumetric muscle loss, satellite cells, neuromuscular junction

Impact Statement

The goal of this study was to determine the threshold for a critically sized, nonhealing muscle defect by characterizing key components in the balance between fibrosis and regeneration as a function of injury size in the mouse quadriceps. There is currently limited understanding of what leads to a critically sized muscle defect and which muscle regenerative components are functionally impaired. With the substantial increase in preclinical VML models as testbeds for tissue engineering therapeutics, defining the critical threshold for VML injuries will be instrumental in characterizing therapeutic efficacy and potential for subsequent translation.

Introduction

SKELETAL MUSCLE HAS a remarkable endogenous regenerative capacity; however after volumetric muscle loss (VML) this regenerative response is diminished and results in

chronic functional deficits.^{1,2} For this reason, VML remains a persistent challenge in comparison with other skeletal muscle injuries, where muscle is capable of full regeneration with virtually no fibrosis.^{3,4} VML injuries are defined as a vast loss of tissue, often due to trauma or surgery, which will not

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endogenously regenerate, resulting in chronic loss of function and permanent disability.^{5,6} VML is most commonly seen clinically as a result of extremity trauma injuries, and extremity trauma accounts for >50% of all trauma injuries among both military and civilian populations.^{7–9} The current standard of care for VML injuries is autologous free or rotational muscle flap transfer; while these techniques can be successful procedures for limb salvage, they remain largely unsuccessful in regenerating functional muscle tissue, and additionally cause donor-site complications and morbidity.^{5,10}

One major factor that sets VML injuries apart from other skeletal muscle injury models is the catastrophic loss of the basal lamina as well as all other supporting cells.^{4,5} These biophysical and biochemical components make up a specific microenvironment for muscle stem cells (MuSCs), also called satellite cells, a primary cell population required for efficient muscle regeneration.¹¹ Situated between the basal lamina and the myofiber sarcolemma, MuSCs are influenced by a unique microenvironment. The MuSC microenvironment is comprised of the myofiber,¹² basal lamina,¹³ vasculature,¹⁴ neural components (e.g., motor neuron,^{15,16} neuromuscular junction [NMJ],^{17,18} and terminal Schwann cells¹⁹), and supporting cells (e.g., fibro/adipogenic progenitors [FAPs]^{20,21}), each of which plays indispensable roles in supporting muscle and MuSCs during homeostasis and regeneration. In VML injuries there is a loss of these supporting regenerative components; however, there is currently limited understanding of what constitutes a critically sized muscle defect and which muscle regenerative components are impaired at the critical size threshold; whereas this is commonplace in other large volume defect models in other tissue types, namely bone.²² As VML models are becoming common in testing of new tissue engineering approaches, defining these components will have direct implications for both the development of new tissue-engineered therapeutics and broad testing of the efficacy of these interventions for the treatment of VML.

In this article our objective was to define the critical size in a mouse quadriceps VML defect by characterizing the response of key regenerative components. The mouse quadriceps has been used as a model site for VML^{23–25}; however the threshold for a critical size has not yet been established. We hypothesized that nonhealing VML injuries reach a critical size due to significant changes in the regenerative response of the endogenous supporting extracellular matrix (ECM), myofibers, vasculature, and neuromuscular innervation.

Materials and Methods

Animals

C57BL/6J mice were purchased from Jackson Laboratories and were used for all experiments with the exception of experiments assessing neuromotor regeneration, for which B6.Cg-Tg(Thy1-YFP)16Jrs/J (Jackson Laboratories #003709) mice were used. Adult female mice between 3 and 9 months in age were used according to the protocols approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee.

VML injury

Mice were anesthetized through inhalation of 1–3% isoflurane. Sustained-release buprenorphine (0.8 mg/mL) was administered for pain management. Hair was removed from

the incision site on the hindlimb, and disinfected using ethanol and chlorohexidine. All surgeries were performed by a single surgeon. An incision was made to expose the quadriceps muscle. Biopsy punch tools of 2, 3, or 4 mm (VWR, 21909-132, -136, -140) in diameter were used to make consistent full-thickness defects to the mid-belly region through the quadriceps, while the leg was in an extended position (Fig. 1B). Each defect consistently injured the rectus femoris, vastus intermedius, and vastus lateralis, with only 3 and 4 mm injuries injuring the vastus medialis (Fig. 1A). After injury, the skin was closed using wound clips. Animals were euthanized by CO₂ inhalation.

Tissue wet weight analysis

Immediately after euthanasia, at 7, 14, or 28 days post-injury ($n=4$ per group/time point), the animals were weighed, and the hindlimb muscles were dissected. The wet weight of the muscles was measured and was normalized to the total body weight of each animal.

Tissue histology and immunostaining

After measuring wet weight, muscles were snap frozen in liquid nitrogen cooled isopentane, and stored at -80°C . Cryosections (CryoStar NX70 Cryostat) were taken at 10 μm thickness, and stained with hematoxylin and eosin (H&E) (VWR, 95057-844, -848) or Gomori's Trichrome (Polysciences; 24205-1) according to the manufacturers' instructions. Before antibody staining, tissue sections were blocked and permeabilized using blocking buffer (5% BSA, 0.5% goat serum, 0.5% Triton-X in 1 $\times$ PBS) for 30 min. When anti-mouse secondary antibodies were used, an additional wash with Goat F(ab) antimouse IgG (Abcam; ab6668, 2 $\mu\text{g/mL}$ in blocking buffer) was performed for 1 h. Samples were washed between steps with 0.1% Triton in PBS. Primary antibodies were diluted in blocking buffer at 1:200 for dystrophin (Abcam; ab15277), von Willebrand factor (vWF) (Abcam; ab6994), and CD68 (Abcam; ab125212). Primary antibody for embryonic myosin heavy chain (eMHC) (DSHB, F1.652) and antibody for Pax7 (DSHB, PAX7) were diluted 1:10 and 1:100 in blocking buffer, respectively. All primary antibodies were incubated for 1 h. Secondary antibodies were conjugated to Alexa Fluor 488 (Thermo Fisher; Ms: A-11029, Rb: A-11034), 555 (Thermo Fisher; Ms: A-21424, Rb: A-21429), or 647 (Thermo Fisher; Ms: A-21236, Rb: A-21245). All secondary antibodies were diluted 1:250 in blocking buffer and incubated for 30 min. Alexa Fluor 647-conjugated phalloidin (Thermo Fisher; A22287) was diluted 1:250 in blocking buffer and incubated with secondary antibodies for 30 min at room temperature. Slides were mounted with Fluoroshield Mounting Medium with DAPI (Abcam; ab104139) and stored at 4°C .

Tissue preparation for neuromuscular visualization

Thy1-YFP mice received 3 mm defects and were euthanized at 14 or 28 days postinjury ($n=4$). Hindlimbs were harvested and fixed in 4% paraformaldehyde for 1 h at room temperature. Quadriceps muscles were dissected from the mouse hindlimbs and placed in blocking buffer for 1 h. Dissected muscle was then stained with α -bungarotoxin conjugated with Alexa Fluor 647 (Thermo Fisher; B35450, 1:250 in PBS) and anti-GFP conjugated with Alexa Fluor 488

(Novus Biologicals; NB600310X, 1:200 in PBS) for 30 min. The tissue within the defect site was then cut into smaller segments and whole mounted on glass slides with Fluoroshield Mounting Medium with DAPI (Abcam; ab104139) and stored at 4°C.

Tissue preparation for micro-CT angiography

Mice received 3 mm defects and were euthanized at 28 days postinjury ($n=5$). Immediately after euthanasia, mouse vasculature was perfused with saline followed by 10% neutral buffered formalin. Finally, tissue was perfused with Microfil contrast agent (Flow Tech, MV-122, 1:2 mix with diluent) until vessels were visibly perfused. Tissue was stored at 4°C until dissection.

Second harmonic generation imaging of collagen

Images were taken on a Zeiss 710 Laser Scanning Confocal Microscope using 20× objective and stitched together with Zen Black software (Zeiss) to create a complete image of the entire cross-section. Second harmonic generation (SHG) was achieved using a Pulsed IR laser at 810 nm with the confocal pinhole entirely opened. Signal from CD68 antibody conjugated with Alexa Fluor 555 secondary antibody was imaged simultaneously.

Confocal imaging and quantification of fiber cross-sectional area, eMHC⁺ fibers, and centrally located nuclei

Immunofluorescence images were taken on either a Zeiss 700 or Zeiss 710 Laser Scanning Confocal microscopes at 20× and stitched together with Zen Black software (Zeiss). The dystrophin images were analyzed using ImageJ by thresholding and using the Analyze Particle function for particles of 0.25–1.0 circularity and 150–6000 μm^2 in area to measure muscle fiber cross-sectional area. Area histograms were created in GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA). Data were grouped by injury size or contralateral control, with three replicate measurements taken from 4 (n) animals for each group. Quantification of eMHC⁺ myofibers was done on images containing all channels (eMHC, dystrophin, DAPI). The brightest fibers of entire stitched sections were counted using the ImageJ multipoint tool. Each replicate slide was counted twice, and all counts for each injury size were analyzed. Centrally located nuclei were analyzed by taking five representative regions of each section, three replicates per animal ($n=4$), and counted using the Image J multipoint tool.

Imaging and quantification of NMJs

Z-stack images were taken on a Zeiss 710 Laser Scanning Confocal using 40× objective. Z-stacks of five random fields of view were taken from whole-mounted sections from within the injured area or from a comparable region from the contralateral control. NMJs were quantified by eye by a blinded scorer.²⁶ The number of NMJs was quantified by placement in one of three categories: (1) normal, pretzel-like morphology; (2) fragmented or abnormal morphology; or (3) newly forming AChR clusters.²⁷

Micro-CT angiography imaging and analysis

Quadriceps were dissected from fixed, Microfil perfused mice and imaged on a vivaCT 40 (Scanco Medical) at 21 μm voxel size, 55 kVp, and 145 μA . Scanco software was used to analyze a volume of interest using a cylinder with a diameter of 6.429 mm and a height of 250 slices, or a total length of 5.25 mm at the middle third of the quadriceps (site of VML). A voxel density threshold of 105 was applied for segmentation, and a Gaussian filter was used ($\sigma=1.2$, support=2). The software measured vascular volume in the analyzed area, as well as vessel diameter distribution (incremental 21 μm bins).

Statistical analysis

All statistical analyses were done in GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA). Data were shown as mean $\pm$ standard error of the mean (SEM) for all figures. One-way ANOVA was used to analyze defect wet weight (Fig. 1B) and eMHC⁺ fiber quantification (Supplementary Fig. S1A), two-way ANOVA was used to analyze the wet weight of injured quadriceps over time (Fig. 1C) and centrally located nuclei (Fig. 4J), with both using Tukey's test as a *post hoc*. For each time point, the cumulative histograms for each injury size and control group were all compared with one another using multiple Kolmogorov–Smirnov tests. Paired, two-tailed *t*-test was performed to analyze vascular volume measurements (Fig. 6C). Repeated-measures two-way ANOVA with Sidak *post hoc* testing was performed to analyze vessel diameter histogram (Fig. 6D).

Results

Muscle mass following full-thickness VML model in mouse quadriceps

To determine the critical size for VML injuries in the mouse quadriceps, biopsy punches of either 2, 3, or 4 mm were used to make full-thickness defects through the mouse quadriceps (Fig. 1A, B). These injuries resulted in the removal of $4.44\% \pm 1.85\%$, $15.49\% \pm 2.04\%$, and $32.16\% \pm 5.14\%$ of the total quadriceps wet weight, respectively, as compared with the contralateral control (Fig. 1C). The mass loss of each of these injuries was significantly different from one another ($n=12$, $p<0.0001$). All injury sizes caused a significant decrease ($n=4$, 2 mm, $p<0.01$, 3 and 4 mm, $p<0.0001$) in quadriceps wet weight for the experimental groups after 7 days (Fig. 1D). After 14 or 28 days, however, only the 4 mm injury group remained significantly different ($n=4$, $p<0.0001$) from the contralateral control.

Qualitative histomorphology after VML injury of multiple sizes

Cross-sections stained with H&E were used to examine the general histomorphology of each injury size at 14 (Fig. 2A) and 28 (Fig. 2B) days postinjury, using four replicates for each injury size and time point. Contralateral control samples from both 14 and 28 days postinjury showed healthy skeletal muscle tissue: densely packed myofibers with peripherally located myonuclei. Injured samples showed vastly different morphology. Two-millimeter injuries at both time points

FIG. 1. Various sized VML injuries in mouse quadriceps. **(A)** Schematic representation of 2, 3, and 4 mm biopsy punch injuries to the mouse left quadriceps with respect to component muscle groups. **(B)** Mouse quadriceps after removal of biopsied muscle tissue. From left to right, 2, 3, and 4 mm biopsy punches. Scale bars represent 5 mm. **(C)** The wet weights of biopsied quadriceps muscle normalized to the contralateral control as plotted for each biopsy punch size. Mean percentages of contralateral control are 4.44, 15.5, and 32.2 for 2, 3, and 4 mm injuries, respectively ($n = 12$ per injury size, error bars indicate mean $\pm$ standard error of the mean (SEM), **** $p < 0.0001$ after one-way ANOVA and Tukey's test). **(D)** Wet weight of the injured quadriceps 7, 14, and 28 days postinjury normalized to total body weight. Day 0 values are calculated as an average of the defect wet weight subtracted from its respective Day 7, 14, and 28 contralateral control quadriceps wet weight and normalized to the body weight of the animal, mean is plotted $\pm$ SEM. The dotted line shows the average value of contralateral control quadriceps normalized to body weight ($n = 4$ for each group, # $p < 0.01$, † $p < 0.0001$ as compared with the control after two-way ANOVA and Tukey's test *post hoc*). RF, rectus femoris; VL, vastus lateralis; VM, vastus medialis; VI, vastus intermedius; VML, volumetric muscle loss. Color images are available online.

FIG. 2. H&E staining of each injury size at various time points. Images of quadriceps cross-sections, stained with H&E. From left to right are shown representative images of a contralateral control, 2 mm injury, 3 mm injury, and 4 mm injury, from 14-day **(A)** and 28-day **(B)** time points. Scale bars represent 200 μ m. H&E, hematoxylin and eosin. Color images are available online.

showed myofibers with centrally located nuclei surrounding areas of small, white pockets, which resemble areas of fatty infiltration. In addition, there were areas surrounding these pockets where mononuclear cells were embedded in a dense matrix. In 3 mm injuries at 14 days, there were more regenerating muscle fibers, indicated by the increased number of small, centrally nucleated myofibers compared with 2 mm injuries. There was also an increase in the area where non-muscle cell types were present in between individual myofibers. At 28 days, these differences were even more apparent in 3 mm injuries. The clearest damage was observed in 4 mm injuries after both time points. There were large areas where no myofibers could be seen; in these regions there were dense clusters of mononuclear cells with no clear tissue structure in addition to fatty infiltration. Around the area of damage, there were newly regenerating fibers with centrally located nuclei and small cross-sectional area.

Assessment of fibrosis in multiple VML injury sizes

To determine the extent of fibrosis, consecutive slides were stained with Gomori's Trichrome or imaged with SHG microscopy to visualize collagen fluorescence and stained with the pan-macrophage marker CD68 (Fig. 3). Contralateral control sections at both 14- and 28-day time points (Fig. 3A, B) showed little to no collagen signal, outside of the expected amount present in the myofiber ECM, in both Gomori's and SHG imaging. In addition, there were few CD68⁺ macrophages present in the contralateral control tissue. In contrast, 2 mm injuries (Fig. 3C, D) showed localized collagen deposition and macrophage infiltration at 14 days, while the surrounding myofibers remained largely unaffected. After 28 days, the localized fibrosis remained; however, there were few macrophages remaining. By comparison, 3 mm injuries after both 14 and 28 days (Fig. 3E, F) showed increased

FIG. 3. Assessment of fibrotic response at each injury size. Quadriceps cross-sections 14 (A, C, E, G) or 28 (B, D, F, H)-day time points postinjury. For each set of images (A–H) the *left-hand* image is a cross-section stained with CD68 antibody (shown in *red*) and imaged on a 2-photon scanning confocal microscope (at 810 nm) for second harmonic generation imaging of collagen (shown in *blue*), and the *right-hand* image is a colorimetric image of a cross-section stained with Gomori's Trichrome. The sets of images are representative sections from contralateral control (A, B), 2 mm injury (C, D), 3 mm injury (E, F), or 4 mm injury (G, H) samples. Scale bars represent 200 μ m. Color images are available online.

fibrosis, seen from both SHG collagen imaging and Trichrome staining. In addition, there was an increased infiltration and persistence of CD68⁺ macrophages out to 28 days in the 3 mm VML samples as compared with 2 mm samples or the contralateral controls. In the 4 mm VML injury animals, features of fibrosis were most prominent (Fig. 3G, H). A large region of collagen fluorescence was seen at both 14 and 28 days postinjury, with many CD68⁺ macrophages present within this tissue at both time points. With increased VML injury size, the fibrotic response increased as did the persistence of unresolved macrophages.

Myofiber regeneration in multiple VML injury sizes

To evaluate myofiber regeneration, we quantified the size distribution of the myofibers present in 2, 3, and 4 mm VML injuries. Cross-sections were immunostained for dystrophin, eMHC, and DAPI (Fig. 4A–G). Sections from the contralateral controls (Fig. 4A) showed mature myofibers with peripherally located myonuclei. The signal from the dystrophin channel was used to quantify myofiber cross-sectional area. Both cumulative (Fig. 4H, I) and relative (Supplementary Fig. S1B, C) area histograms were generated. At both 14

FIG. 4. Visualization and quantification of fiber cross-sectional area in each injury size. Representative immunofluorescence images of each time point and injury size: (A) contralateral control, (B, C, D) 14-day time point of 2, 3, and 4 mm injuries, respectively, and (E, F, G) 28-day time point of 2, 3, and 4 mm injuries, respectively. Each of the sections were stained for dystrophin (green), DAPI (blue), and eMHC (red). Scale bars represent 100 μm. Cross-sectional area was quantified using stitched images of the entire cross-section from the dystrophin channel. The measured cross-sectional areas used to create cumulative histograms 14 (H) and 28 (I)-day time points. Data were categorized by injury size. Each category (control, 2, 3, and 4 mm) had $n=4$ individual animals with three replicate slides per animal: one replicate from the beginning, one from the middle, and one from the end of the damaged area. In the case of the control, replicate slides were taken from the corresponding areas to the damaged samples. Fibers with centrally located nuclei were quantified at 14 and 28 days postinjury (J). Five representative areas from each of the same replicate slides used for cross-sectional area quantification were used to count centrally located nuclei per area. All data points shown, with bars representing mean $\pm$ SEM. Two-way ANOVA performed with Tukey's test *post hoc*, significance for $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ns, not significant. Color images are available online.

(Fig. 4H) and 28 (Fig. 4I) days post-VML, the cumulative histogram for the 4 mm injuries showed the steepest slope values at the smallest cross-sectional areas; that is, the fraction of the total number of myofibers (vertical scale) in 4 mm defects with small cross-sectional area (horizontal scale) is significantly greater in comparison with both 2- and 3 mm defects. Moreover, the steepness of the slope in the cumulative histograms decreased with decreasing injury size, indicating that as injury size decreased the number of myofibers with smaller cross-sectional areas also decreased. Control cross-sections had the lowest relative number of small myofibers, which was expected. Multiple Kolmogorov–Smirnov tests indicate that cumulative distributions of each injury size and the control are significantly different from all other groups ($p < 0.0001$). Descriptive statistics for the data displayed in these histograms can be found in Supplementary Table 1. Histogram analyses suggest that there are an increasing number of smaller diameter, newly regenerating myofibers with increasing injury size.

At both 14 and 28 days post-VML, we assessed regeneration in the defects by quantifying myofibers, which were eMHC positive and had centrally located nuclei. eMHC is a protein that is transiently expressed during the early stages of myofiber development,²⁸ and therefore myofibers that stain positive for eMHC can be classified as newly regenerated. Centrally located nuclei are another marker of newly regenerated myofibers, but the presence of centrally located nuclei persists for longer than eMHC. Our results show that at all time points and in all injury sizes there were myofibers with centrally located myonuclei, indicating that regeneration had been recently occurring (Fig. 4J). At 14 days post-VML there were significantly more centrally located nuclei in all injury groups as compared with the contralateral control. By 28 days after injury, there were persistent significant differences between the 3- and 4 mm injury groups compared with the contralateral control, but no difference between 2 mm injuries and the contralateral control ($n = 4$, significance for $p < 0.05$). Overall, there was variability in the total number of eMHC⁺ myofibers in all VML injury sizes. At 14 days postinjury, there were no significant differences in the number of eMHC⁺ fibers between any of the injury sizes (Supplementary Fig. S1A). At 28 days, there were essentially no eMHC⁺ fibers in any defect size, indicating that there are no fibers early in the regeneration process at this time point. The abundance of both eMHC staining and centrally located nuclei in 3 and 4 mm injuries indicate turnover of MuSCs postinjury. Staining for Pax7, the canonical marker of quiescent MuSCs, showed several quiescent MuSCs in the area surrounding both 3 and 4 mm injuries at both 14 and 28 days (Supplementary Fig. S2). This indicates that MuSCs are undergoing proper asymmetric division into both Pax7⁺ quiescent MuSCs and activated MuSCs, which differentiate and fuse with newly regenerating fibers.

The results from the study comparing different VML defect sizes indicated that a 3 mm injury was just past the threshold for a nonhealing critical size at the 28-day time point. This group maintained a substantial amount of unresolved fibrosis, which the regenerating muscle was unable to penetrate, evident from the Trichrome and SHG collagen imaging. 3 mm injuries at 28 days still had persistent presence of macrophages (CD68⁺ cell population), indicating

unresolved inflammation. In addition, a significant number of fibers with centrally located nuclei remained at 28 days postinjury. Each of these findings contributed to our determination that 2 mm injuries ($4.44\% \pm 1.85\%$) were subcritical defects, 4 mm injuries ($32.16\% \pm 5.14\%$) were critical defects, and that 3 mm injuries ($15.49\% \pm 2.04\%$) represent a transition point from subcritical to critically sized. This warranted further investigation into both the neuromuscular and vascular response into the injury space at this critical size.

Neuromuscular regeneration after critical VML

NMJs were quantified in Thy1-YFP mice, in which the motor neuron expresses yellow fluorescent protein (YFP),²⁹ to determine the level of denervation or reinnervation into the injury space at 14 and 28 days ($n = 4$ per time point). Muscle segments were stained with fluorophore-conjugated α -bungarotoxin (BTX) to visualize acetylcholine receptors (AChRs) on the myofibers. This channel (Fig. 5C–F) was used to quantify the number of NMJs, which fit each of the three categories: (1) normal, pretzel-like; (2) abnormal, fragmented morphology; and (3) newly formed AChR clusters. In contralateral control samples, there was an overall higher number of NMJs (Fig. 5A), 100% of which were classified as normal, innervated NMJs (Fig. 5B), as is apparent from the representative maximum intensity projection images (Fig. 5C–F). In contrast, there were far fewer NMJs of any kind in the VML-injured muscles (Fig. 5A). Of the NMJs present in VML-injured muscle at 14 days postinjury, 89% displayed fragmented morphology and therefore in group 2, and 11% were in group 3 as they were newly forming AChR clusters. At 28 days post-VML the NMJs present in injured muscle were 44.3% classified as group 2 and 55.7% classified as group 3. However, there were no normal, innervated NMJs at either time point into the injury site. Representative images of injured tissue at 14 (Fig. 5D) and 28 (Fig. 5F) days are analogous, both with some fragmented (group 2) NMJs. In addition, in these samples there was a substantial amount of autofluorescence in the YFP/GFP channel (Supplementary Fig. S3A–D), likely due to pervasive fibrotic tissue. VML-injured tissue samples also showed some newly regenerated AChR clusters (Supplementary Fig. S3E, indicated by orange arrowheads); however, there was no evidence of a regenerating nerve toward these newly formed junctions, even at 28 days.

Vascularization after critical VML

Vascularization after critical VML injury was assessed at 28 days postinjury ($n = 5$). Whole animal perfusion with Microfil contrast agent was followed by micro-CT scans on each dissected quadriceps (Fig. 6A, B). The middle third of the quadriceps (the area of injury) was chosen for analysis. Total vascular volume within the middle third showed significantly greater vascular volume in the injured quadriceps when compared with the uninjured control (Fig. 6C). In addition, there were significant differences in the diameter of perfused vessels of the injured muscle compared with its contralateral control (Fig. 6D), specifically vessels with diameters of 84, 105, and 168 μm . This increase in vascular

FIG. 5. Whole-mounted sections of neuromuscular junctions in VML compared with the contralateral control. NMJs were quantified by three classifications (**A**, **B**) as either group 1, 2, or 3 in Thy1-YFP mice 14 or 28 days post 3 mm VML injury. Group 1 NMJs were normal, pretzel-like morphology, group 2 NMJs were abnormal, fragmented morphology, and group 3 were newly forming AChR clusters. (**A**) Shows mean raw count data $\pm$ SEM for each animal and time point. (**B**) Depicts the same data as in (**A**), but displayed as a percentage of total number of NMJs in each category. Representative maximum intensity projections of z-stacks taken from the control (*left*) and injured (*right*) experimental groups are shown for 14 (**C**) and 28 (**D**)-day time points. Each image shows only the BTX channel from the image to visualize the morphology of the postsynaptic AChRs. The BTX channel was used for quantification. For images including GFP channel, see Supplementary Figure S3. All scale bars are 50 μ m. NMJ, neuromuscular junctions; BTX, α -bungarotoxin; AChR, acetylcholine receptor. Color images are available online.

volume and diameter at 28 days was confirmed with immunostaining muscle cross-sections for vWF (Fig. 6E).

Discussion

The goal of this study was to characterize the response of muscle regenerative components as a function of injury size to provide general guidelines for determining a critically sized VML threshold. We determined the threshold of a critically sized defect in the mouse quadriceps was a full-thickness biopsy punch 3 mm in diameter, as myofibers were unable to bridge the defect space in an injury of this size. These 3 mm injuries constituted a loss of $\sim 15\%$ of the muscle mass. Previously it has been suggested that a 20% muscle loss is the threshold for failure of the native regenerative process³⁰; however, the regenerative processes at a critical size is not well understood or established. As the MuSC is essential for muscle regeneration,¹¹ it is crucial that the surrounding support components, including the ECM, vasculature, motor neuron innervation, and myofibers themselves, properly modulate the microenvironment to direct successful muscle regeneration. In these studies, we show that at the critical threshold, there is a chronically increased fibrotic and inflammatory response, shown by increased collagen deposition and CD68⁺ cell populations after 28 days post-VML. In addition, there is no evidence of reinnervation of any newly regenerating myofibers, an increased number of large diameter vessels in the injury

space, and insufficient myofiber regeneration to fill the created defect.

The critically sized defects described in these experiments display the nonhealing histomorphology of a VML injury. The presence and persistence of collagen fibrosis and fatty infiltrate in the environment increased with injury size. This fibrotic and fatty infiltrate response is characteristic of skeletal muscle trauma, as has been shown consistently in previous VML studies.^{23,31,32} In addition, similar results have been seen in various ECM scaffold tissue-engineered strategies, where the scaffolds themselves become populated with fibrotic tissue comparable with an empty defect.^{33–35} While this fibrotic tissue is generally considered one of the main barriers to successful muscle regeneration, it is also important to note that complete ablation of muscle resident fibroblasts leads to altered regenerative capacity of MuSCs,³⁶ indicating that when properly regulated, fibroblasts are vital components in muscle regeneration. Therefore, determining a method for modulating this fibrotic response to be proregenerative will be essential in tissue engineering approaches for VML.

In addition to this fibrotic phenotype, the critically sized defect displayed the persistent inflammation seen after VML injury. Macrophage infiltration has been shown to be upregulated in the short term during the typical inflammatory period after VML,³⁷ and more recently it was shown that macrophages persist long-term post-VML.³⁸ In the 2 mm, noncritically sized defect, the persistent macrophage infiltration is largely resolved

FIG. 6. Micro-CT and IHC analysis of vasculature in VML compared with control quadriceps. (A) Reconstructed 3D heat map of μ CT images from Microfil perfused contralateral control and 3 mm injured quadriceps, *left to right*, respectively, from the same animal. (B) Reconstructed 3D heat map of the middle third of the same samples as in (A). Color scale is from 0.000 to 0.315 mm for vascular diameter, length scale bar is 1 mm. The middle third of each sample is what was quantitatively analyzed in (C, D). (C) Total perfused vascular volume for the middle third of each sample shown in a pairwise comparison to match injured and control from the same animal. (D) Histogram counting the number of vessels in each diameter bin. The bins represent the resolution of the measurement itself (e.g., vessels between 0 and 21 μ m are placed in the 21 μ m bin). Counts are shown as mean $\pm$ SEM from five samples. For vascular volume (C) statistical analysis, a paired, two-tailed *t*-test was performed. For comparison of injured and control values in each bin of the vascular diameter histogram (D) a two-way repeated-measures ANOVA was performed with Sidak *post hoc*, $*p < 0.05$, $\dagger p < 0.0001$. (E) Images taken of quadriceps cross-sections 28 days after a 3 mm injury (*right*) and its contralateral control (*left*). Staining was done for phalloidin (gray), nuclei (blue), and vWF (red). Color images are available online.

by 28 days. The sustained macrophage presence sets a VML injury apart from many other acute injury types and draws similarities to chronic muscle disorders, such as muscular dystrophy.^{39,40} In these disorders, chronic muscle damage results in the deposition of fibrotic tissue and fatty infiltrate between myofibers, resulting in chronic functional deficits, similar to those observed with VML injuries. In other chronic muscle conditions, the chronic inflammatory phenotype has been shown to result in a unique macrophage phenotype, which promotes the sustained proliferation of FAPs,⁴¹ which could potentially be a driving force behind the persistent and dysregulated fibrosis in VML injuries as well. For this reason, we are interested in evaluating the development of chronic inflammation over time after VML and its impact on FAPs in future studies as this could indicate the potential benefit of immune-targeted therapeutic interventions for creating a proregenerative microenvironment post-VML.

In addition, we observed a distinct temporal pattern in myofiber regeneration in the critically sized VML injuries. eMHC expression was present 14 days after injury, which is delayed in comparison with other commonly studied skeletal muscle injuries; in those, eMHC expression is not typically seen after 7 days postinjury.^{42–44} This delayed expression of eMHC could potentially indicate that post-VML there is a delayed or continued attempt at muscle regeneration, which is not seen in most other acute muscle injury models. When there is sustained muscle regeneration, or attempted muscle regeneration, over a prolonged period of time there is the potential for MuSC depletion, as occurs in aging.⁴⁵ Quantification of MuSCs within this injury model

is needed to further elucidate the mechanism of this altered regenerative timeline and will be the direction of future studies as it may be necessary to supplement the stem cell pool when designing therapeutics for these injuries.

For regenerating muscle to become functional, it is necessary that myofibers are innervated by the motor neuron. Our data indicate that there is no evidence of reinnervation of the myofibers in the defect region at 14- or 28-day post-VML in an injury that is critically sized. The fibers that are present in this area are likely a mix of myofibers that were present preinjury as well as those that are regenerating. This is indicated in the images of injured tissue at both 14 and 28 days, which shows fragmenting NMJs (class 2, Fig. 5D, F) as well as newly formed AChR clusters (class 3, Supplementary Fig. S3E). Fragmenting NMJs are likely those that were functional preinjury, but which are then denervated by the transection of the supplying motor neuron during the VML injury itself. Denervated NMJs can retain their typical morphology, and the regenerating motor neuron will reinnervate at the same location with the guidance of Schwann cells between 4 and 9 days postinjury, but will then begin to display the fragmented morphology we have shown if they are not innervated in this time frame.⁴⁶ Multiple new AChR clusters, however, will form on newly regenerating myofibers as they mature, secreting signaling factors to the motor neuron to direct innervation.⁴⁷

Our data do not show evidence of the motor neuron growing toward these junctions to reinnervate myofibers, new or old, 14 or 28 days after VML injury. This could potentially be due, in part, to the destruction of guiding

Schwann cells for motor neuron regeneration in VML, similar to the loss of the guiding basal lamina for myofibers. These findings are further supported by the persistent presence of centrally located nuclei in critically sized injuries at these time points as well (Fig. 4F), as it has been shown that myonuclei will remain centrally located until the myofiber becomes functionally mature.⁴⁸ The loss of reinnervation in the muscle regeneration process has been studied previously, both clinically⁴⁹ and preclinically,⁵⁰ indicating similar results, which also implicate the potential for postinjury physical rehabilitation to initiate reinnervation both before and after surgical placement of a therapeutic.

One of the most heavily studied thrusts in tissue engineering is the vascularization of engineered constructs and strategies for encouraging angiogenesis. There have been several studies that have shown tissue-engineered strategies, which promote successful formation of vessels after VML injury.^{51,52} Interestingly, in our critically sized VML defect with no treatment we observed increased vascular volume as compared with the contralateral control. This increase in vascularization may have potentially been driven by the large fibrotic response, as it is known that angiogenesis and fibroplasia go hand in hand in the wound healing process.⁵³ This would indicate that while vessels are key components in muscle regeneration, a decrease in the vascular volume of the injured tissue is not a requirement for a critically sized skeletal muscle injury model. When developing VML therapeutics, it may be strategic to take advantage of the native proangiogenic signals of the resulting scar tissue.

The evaluation and direct comparison of multiple defect sizes in muscle are not commonly reported. In this article we have determined and comprehensively characterized three mouse quadriceps models of VML: one below the threshold of a critically sized defect (5%), one just above that threshold (15%), and one well past the threshold (30%). These relative guidelines and techniques are believed to be applicable to many other VML models. In general, we recommend utilization of these common outcomes to define where on the spectrum of a critical size a given model is located. While there are clearly limitations in translation from small rodents to humans, we believe using this systematic characterization method in a wider variety of animal models will ease comparison across species and injury models. Based on the defining features of a critical size in VML, potential targets for future tissue-engineered interventions may be the downregulation in the fibrotic progenitor cells and controlling persistent inflammation, combined with upregulation of neural regeneration, which would be most beneficial for the recovery of functional skeletal muscle after VML and will be the direction of future studies.

Authorship

S.E.A., N.J.W., and Y.C.J. designed the study, analyzed the data, and wrote the article. S.E.A., W.M.H., M.M., V.S., and A.M. conducted experiments, analyzed the data, and reviewed the article. M.A.R., C.L.S.E., M.O., and E.A.B. provided significant contributions to methodology and data analysis, and reviewed the article. E.S. and Y.C.J. generated and maintained animals used in this study.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Table S1

Disclosure Statement

No competing financial interests exist.

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