

## Original Article

# Severe muscle trauma triggers heightened and prolonged local musculoskeletal inflammation and impairs adjacent tibia fracture healing

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## Abstract

**Objectives:** Complicated fracture healing is often associated with the severity of surrounding muscle tissue trauma. Since inflammation is a primary determinant of musculoskeletal health and regeneration, it is plausible that delayed healing and non-unions are partly caused by compounding local inflammation in response to concomitant muscle trauma. **Methods and results:** To investigate this possibility, a Lewis rat open fracture model [tibia osteotomy with adjacent tibialis anterior (TA) muscle volumetric muscle loss (VML) injury] was interrogated. We observed that VML injury impaired tibia healing, as indicated by diminished mechanical strength and decreased mineralized bone within the fracture callus, as well as continued presence of cartilage instead of woven bone 28 days post-injury. The VML injured muscle presented innate and adaptive immune responses that were atypical of canonical muscle injury healing. Additionally, the VML injury resulted in a perturbation of the inflammatory phase of fracture healing, as indicated by elevations of CD3<sup>+</sup> lymphocytes and CD68<sup>+</sup> macrophages in the fracture callus at 3 and 14d post-injury, respectively. **Conclusions:** These data indicate that heightened and sustained innate and adaptive immune responses to traumatized muscle are associated with impaired fracture healing and may be targeted for the prevention of delayed and non-union following musculoskeletal trauma.

**Keywords:** Skeletal Muscle Injury, Volumetric Muscle Loss, Trauma, Musculoskeletal, Inflammation, Innate, Adaptive, Immune Response, Composite Injury

## Introduction

Musculoskeletal injuries are highly prevalent in civilian and military trauma and can impart permanent disability<sup>1,2</sup>. A volumetric loss of muscle tissue adjacent to fractured bone

is frequently encountered (e.g., type IIIa/b open fracture). Volumetric muscle loss (VML) injuries are irrecoverable in humans and animal models- owing to the complete loss of crucial elements for skeletal muscle regeneration. Since there are no therapies clinically available that can regenerate the lost musculature, VML is often untreated and considered part of the natural sequelae of trauma. As a result, VML injury manifests a permanent loss of contractile tissue, extensive fibrosis, and a chronic loss of function<sup>3-7</sup>, which may directly lead to disability in open fracture patients<sup>8</sup> that may progressively worsen with time<sup>9</sup>.

While VML injuries result in chronic reduction of limb functional capacity, acutely after trauma these severe muscle injuries may also impair healing of adjacent fractured bone. This is exemplified by the clinical grading of open fractures whereby severe fracture with predictably worse healing outcomes are predicated on the extent of surrounding soft tissue comorbidity<sup>10</sup>. In some of these cases (e.g., type IIIb), free or

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rotational muscle flaps are transferred to the site of VML, signifying the importance of restoring muscle-bone interactions to fracture healing. In support of clinical observations, animal models have unequivocally demonstrated that the loss of adjacent musculature impairs fracture healing. For instance, a series of studies performed in a rat self-healing tibia fracture model demonstrated that tibialis anterior (TA) muscle ablation delayed recovery of tibia mechanical properties<sup>11-13</sup>. Additionally, in a rat femur critical size defect model, adjacent quadriceps VML (~10% loss of muscle) impaired rhBMP-2 mediated fracture healing<sup>14</sup>. Lastly, in a mouse simple fracture model TA muscle dissection and further isolation of the fracture site from the posterior musculature impaired fracture healing<sup>15</sup>. All told, it is clear that the traumatic or surgical loss of surrounding musculature has a deleterious effect on fracture healing.

Mechanisms bridging VML with compromised bone healing intuitively involve compromised regional blood flow, but also may be affected by localized VML-associated effects on fracture inflammation. Dysregulated inflammation is associated with diminished bone strength and impaired fracture healing. For example, disease conditions that present chronic systemic inflammation, such as diabetes mellitus, inflammatory bowel syndrome, and rheumatoid arthritis are associated with increased susceptibility to fracture<sup>16-19</sup>. Moreover, elevated systemic inflammation following polytrauma (e.g., fracture concomitant with blunt chest trauma) has been associated with delayed fracture healing<sup>20,21</sup>. Notably, there is also evidence that local muscle inflammation may also mediate fracture healing. In a mouse model of Duchenne muscular dystrophy, dystrophin deficiency results in chronic muscle inflammation, diminished bone strength, and impaired fracture healing<sup>22-24</sup>. To date very little is understood of the immune response to traumatic VML injury and its potential role in local musculoskeletal healing. Therefore, the purpose of this study was to characterize the immune response to VML injury and to assess whether concomitant VML altered macrophage or T-lymphocyte cellular infiltration within an adjacent diaphyseal fracture in a rat muscle-bone composite injury model that presents VML-dependent delayed fracture healing.

## Materials and methods

### *Surgical protocol*

All animal procedures were approved by the Institutional Animal Care and Use Committee and were conducted in compliance with the Animal Welfare Act and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals. Inbred male Lewis rats (350-400 g) were housed in a specific-pathogen-free animal facility at the US Army Institute of Surgical Research. Rats underwent an osteotomy (OST) and, to create an open fracture in selected groups, a volumetric muscle loss injury (OST+VML) as described elsewhere<sup>5</sup>. Briefly, rats were anesthetized and a longitudinal incision was made along the lateral aspect of the leg and

the skin and fascia covering the tibialis anterior (TA) muscle were bluntly separated from the underlying musculature. The tissue covering the middle third of the tibia was separated bluntly from the bone. An osteotomy was performed approximately 5 mm proximal to the tibia-fibula junction and stabilized with a 1.25 mm Kirschner pin inserted in the medullary cavity from the tibial plateau. To surgically create a volumetric muscle loss, the TA muscle and underlying extensor digitorum longus (EDL) muscle were separated using blunt dissection and a punch biopsy (6 mm) was executed through the middle third of the TA muscle and the tissue was removed, as we have previously reported<sup>3,5</sup>. Controls consisted of either naïve housing controls or sham surgery animals that underwent all surgical procedures except osteotomy or VML (n=3-6 per group).

### *Micro-computed tomography*

Microcomputed tomography ( $\mu$ CT) scans (Viva-CT40, Scanco Medical) were performed on a set of rat tibias at 28d post-injury. Osteotomy regions were scanned at medium resolution with a 21  $\mu$ m voxel size at a voltage of 55 kVp and a current of 145  $\mu$ A. A volume of interest of 400 slices was analyzed (~8.4 mm) with a centralized osteotomy site. Bone and callus were segmented by applying a threshold corresponding to 455 mg hydroxyapatite/cm<sup>3</sup> and a low-pass Gaussian filter (sigma=1.2, support=1.0) was applied to suppress noise. Contours were first drawn around the entire specimen to obtain a total volume and then drawn around the cortical bone to obtain bone (original) volume. Callus volume was calculated by subtracting bone (original) volume from total volume, expressed as mm<sup>3</sup>.

### *Three-point bending mechanical testing*

Three-point bending was performed on a set of rat tibias at 28d post-injury. For these samples, after harvesting, bones were stripped of muscles and adjoined fibulas, and placed in phosphate buffered saline (PBS). To eliminate any equipment variability, all samples were frozen at -80°C until testing could be performed at one time. Prior to mechanical testing, samples were thawed in a water bath at 37°C for approximately 1 hour. Briefly, tibias were placed in a materials bending system (Lloyd LRX) along the sagittal plane, with the medial (flat) portion of the tibia resting on the lower points, separated by 20 mm. Samples were deflected by lowering the apparatus at the midpoint of the lateral side of the bone. Samples were loaded at a constant speed of 2 mm/min until failure. Tibias from contralateral legs (without injury) were tested as controls. Load-deflection curves were used to obtain maximum load (N) and stiffness (N/mm).

### *TA muscle histology*

Comparative histopathology and immunofluorescence staining methods were conducted with tissue sections from the middle third of the TA muscle that comprised the VML defect as well as bone tissue. TA muscle tissue isolation and

**Table 1.** Nucleotide sequence for primers used for quantitative RT-PCR.

	Forward sequence	Reverse sequence	Amplicon length, bp
<b>ARG1</b>	5'-GTGAAGAACCCACGGTCTGT-3'	5'-GTGAGCATCCACCCAAATG-3'	180
<b>CCR7</b>	5'-GCTCTCTGGTCATTTTCCA-3'	5'-AAGCACACCGACTCATACAGG-3'	107
<b>CD163</b>	5'-TCATTTTGAAGAAGCCCAAG-3'	5'-CTCCGTGTTTCACTTCCACA-3'	101
<b>iNOS</b>	5'-ACTGTGTGCCTGGAGGTTCT-3'	5'-GACACAAGGCTCCAACCT-3'	193
<b>MRC1</b>	5'-CAAAACAAAGGGACGTTTCG-3'	5'-CCTGCCACTCCAGTTTTCAT-3'	116
<b>18S</b>	5'-GGCCGAAGCGTTTACTT-3'	5'-ACCTCTAGCGGCGCAATAC-3'	173

freezing was performed as described previously<sup>3,5</sup>. Tissue sections (10 µm thickness) were stained with hematoxylin and eosin (H&E) by standard procedure or probed for laminin, CD68, and nuclei (DAPI) using previously reported methodology<sup>3,5</sup>. Composite images of the complete TA muscle were acquired using Axio Scan.Z1 microscope (Zeiss) and ZEN imaging software (Zeiss). Sections were also examined by brightfield and fluorescence microscopy (Olympus IX71) and images of muscle tissue were acquired and analyzed using cellSens Standard software (Ver 1.4.1, Olympus).

#### Real-time PCR

RNA was extracted from the muscle defect as previously described<sup>3</sup>. Briefly, the defect was excised and flash frozen in liquid nitrogen. Approximately 50 mg of muscle tissue was removed and homogenized. RNA was isolated using TRIzol and purified using an RNeasy Mini Kit (Qiagen) per manufacturer's protocol. RNA was converted into complementary DNA (cDNA) using RT2 First Strand Kit (Qiagen). A vendor-designed RT<sup>2</sup> Profiler PCR array (PARN052Z, SABiosciences) was employed to examine rat innate and adaptive immune responses. Custom designed primers (Sigma-Aldrich) with the sequences presented in Table 1 were used for select genes. A C1000 Touch Thermo Cycler equipped with a CFX96 Real-Time System (BioRad) was used to determine gene expression. Data analysis was carried out either with the RT<sup>2</sup> Profiler PCR Array Data Analysis version 3.5 or expression levels for each mRNA transcript were determined by the 2<sup>-ΔΔCT</sup> method. Gene expression in OST+VML muscle tissue was relative to expression in contralateral control muscle tissue and normalized to reference gene, *Rplp1* or *Rn18s* (n=3-6 per group).

#### Isolation of cellular infiltrates from muscle defect

Cells were isolated from complete TA muscle defect by enzymatic digestion. The defect was surgically isolated and the mass was determined. In 1.5 ml tubes, tissue was incubated with 1% collagenase type II and 2.4 U/mL of dispase dissolved in phosphate buffered saline (PBS) plus 2.5 mM calcium chloride for 90 minutes at 37°C. Following enzymatic digestion, cells were further released in a tissue culture plate containing RPMI 1640 media plus L-glutamine and 5% heat-

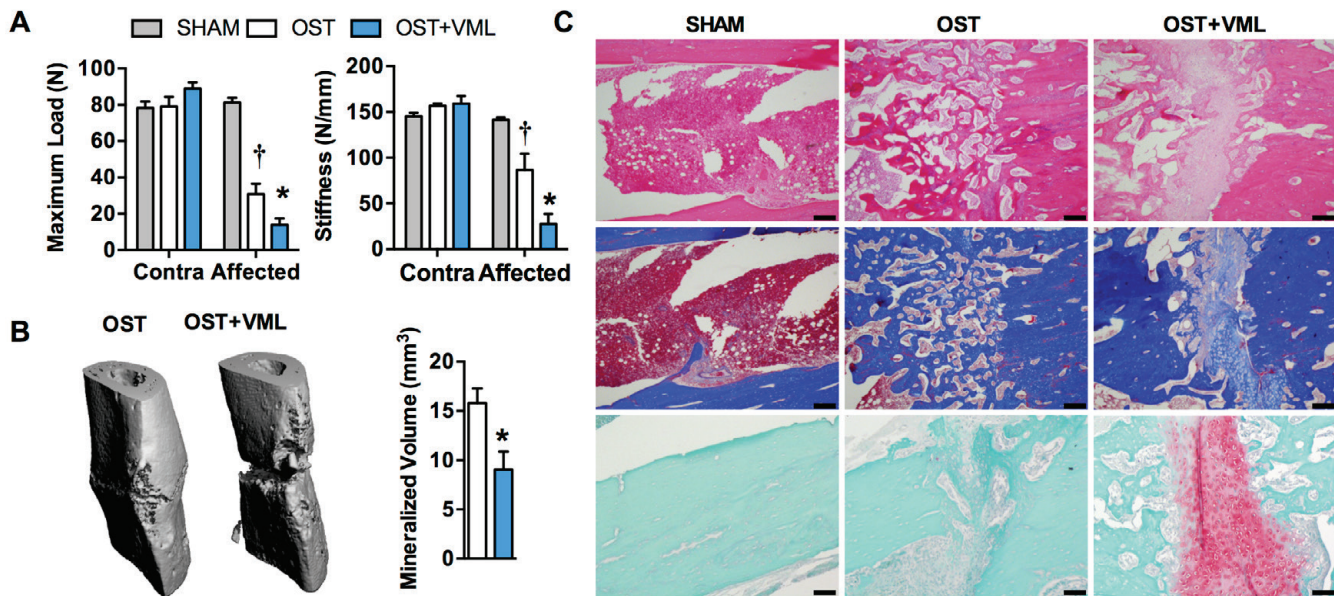
**Table 2.** Antibodies used for flow cytometry.

Antigen	Antibody Clone	Vendor
<b>CD3</b>	IF4	BD Pharmingen
<b>CD4</b>	OX-35	BD Pharmingen
<b>CD8α</b>	OX-8	BD Pharmingen
<b>CD11b</b>	WT.5	BD Pharmingen
<b>CD45</b>	OX-1	BD Pharmingen
<b>CD68</b>	ED1	AbD Serotec
<b>CD86</b>	24F	BD Pharmingen
<b>CD163</b>	ED2	AbD Serotec

inactivated fetal bovine serum (FBS) by gentle mechanical disruption using the back of a sterile 3-ml syringe plunger and filtered through a cell strainer (70 µm pore size). Erythrocytes were lysed with ammonium-chloride-potassium (ACK) lysing buffer and cells were pelleted by centrifugation at 1500 RPM for 10 min, 4°C. Cells were subsequently filtered through a cell strainer (40 µm pore size), washed, and resuspended in wash buffer that consisted of PBS containing 0.5% FBS and 0.1% sodium azide. Viable cells were visualized by trypan blue exclusion and quantified by light microscopy using a hemocytometer.

#### Quantification of cellular infiltrates in muscle defect

Approximately 2-4 x 10<sup>5</sup> cells were incubated with anti-CD32 antibody to block Fc receptors. Cells were washed and then labeled for 30 min with one of two cocktails of fluorochrome-conjugated monoclonal antibodies described in Table 2 to identify myeloid cells or T lymphocytes. The myeloid cell cocktail included anti-CD45, anti-CD11b, anti-CD86, and anti-CD163. The T lymphocyte cell cocktail consisted of anti-CD3, anti-CD4, and anti-CD8α. Labeled cells were washed twice and fixed with 1% paraformaldehyde. Labeled cells (100,000 or 200,000 cells per myeloid or lymphoid samples, respectively) were enumerated by fluorescence-activated cells sorting (FACS) using a MACSQuant flow cytometer (Miltenyi Biotec). Data were analyzed using MACSQuantify software (Miltenyi Biotec). Gating strategies to identify cellu-



**Figure 1. Traumatized muscle impairs bone healing after open fracture.** Three-point bending mechanical testing and  $\mu$ CT imaging of tibiae were performed at 28 days post-injury. A) Contralateral tibia maximum load and stiffness were similar among groups and no significant difference was observed between contralateral and affected tibia of the SHAM group. Significant differences were detected among affected limb groups. † affected OST vs. contralateral OST & affected SHAM; \* affected OST+VML vs. contralateral OST+VML & affected SHAM and OST groups ( $p$ -values  $<0.05$ ). B) Representative  $\mu$ CT images of OST and OST+VML tibiae are presented from which callus volume was calculated; \*  $p < 0.05$ . Values are means  $\pm$  SEM;  $n = 4 - 6$  per group. C) Histology of tibia of affected limb. Top, middle, and bottom rows: H&E, Masson's Trichrome, and Safranin O. Scale bars = 200  $\mu$ m.

lar populations included the following: CD45<sup>+</sup>CD11b<sup>+</sup> myeloid cells, CD45<sup>+</sup>CD11b<sup>+</sup>CD86<sup>+</sup> M1-like cells, CD45<sup>+</sup>CD11b<sup>+</sup>CD163<sup>+</sup> M2-like cells, CD3<sup>+</sup>CD4<sup>+</sup>, and CD3<sup>+</sup>CD8 $\alpha$ <sup>+</sup> T lymphocytes. CD45<sup>+</sup>CD11b<sup>+</sup> cells were confirmed as  $>99\%$  CD68<sup>+</sup> in a separate control experiment. To determine the cell numbers of each myeloid cell population, the numbers of hematopoietic cells were first determined by multiplying the percentage of CD45<sup>+</sup> cells by the total number of viable cells recovered from the respective defect. The cell numbers of myeloid cells were then determined by multiplying the percentage of CD11b<sup>+</sup> cells by the total number of calculated hematopoietic cells. The cell numbers of M1- or M2-like cell populations were then determined by multiplying their percentage by the total number of calculated myeloid cells. The cell numbers of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocyte populations were determined by first multiplying the percentage of CD3<sup>+</sup> cells by the total number of viable cells followed by multiplying this number by the percentage of CD4<sup>+</sup> or CD8 $\alpha$ <sup>+</sup> cells. All cell numbers were normalized per gram of excised muscle tissue.

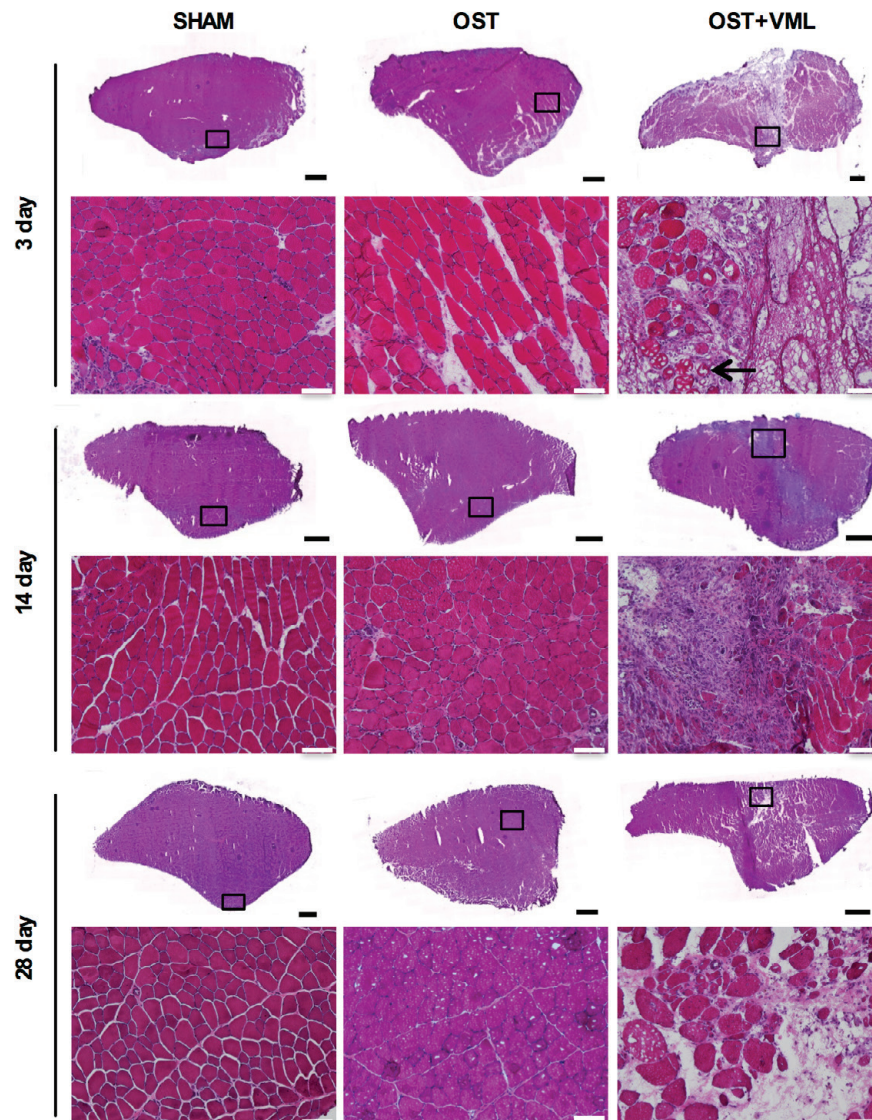
#### Tibia histology

Histology was conducted on a set of rat tibiae at 28d post-injury. Bones were harvested from rats and fixed in 10% neutral buffered formalin for a minimum of 7 days. Bones were then rinsed in water and decalcified for 7d in

a formic acid bone decalcifier (Immunocal, Decal Chemical Corp, Tallman NY). Samples were processed in paraffin and embedded in a longitudinal orientation. Bone was cut in 8  $\mu$ m sections, deparafinized, and was stained with hematoxylin and eosin (H&E), trichrome, and safranin O by standard procedure. Sections were also stained with CD68 or CD3 primary antibodies with biotinylated IgG secondary antibody. Detection using 3,3'-diaminobenzidine (DAB) was performed to visualize the stain. For analysis, 4 images (10x magnification) were taken in the osteotomy and callus region (not including the marrow cavity) of each sample. Three samples were analyzed for each group at 3 and 14d. Images were converted to 8bit black and white images (ImageJ, NIH) and a threshold was set to include positively stained cells (evident with brown chromogen staining). Thresholds were applied to all images within a batch and ImageJ software was used to count positive cells.

#### Statistics

Dependent variables were analyzed using ANOVA or independent samples t-tests. Post-hoc means comparisons testing was performed when a significant ANOVA was observed. Alpha was set at 0.05. Statistical testing was performed with Prism 6 for Mac (Graphpad, La Jolla, CA, USA).



**Figure 2.** VML injury leads to increased cellular infiltration to TA muscle. The TA muscle was isolated at 3, 14, and 28 days post-trauma from SHAM, OST, and OST+VML groups. Tissue sections were stained with H&E. Representative images of TA cross-sections and magnified regions (inset black boxes) displaying overall muscle morphology and cellular recruitment were collected. Black arrows denote degenerating muscle fibers.  $n=4$  per group. Black scale bars=1.0 mm and white scale bars=100  $\mu\text{m}$ .

## Results

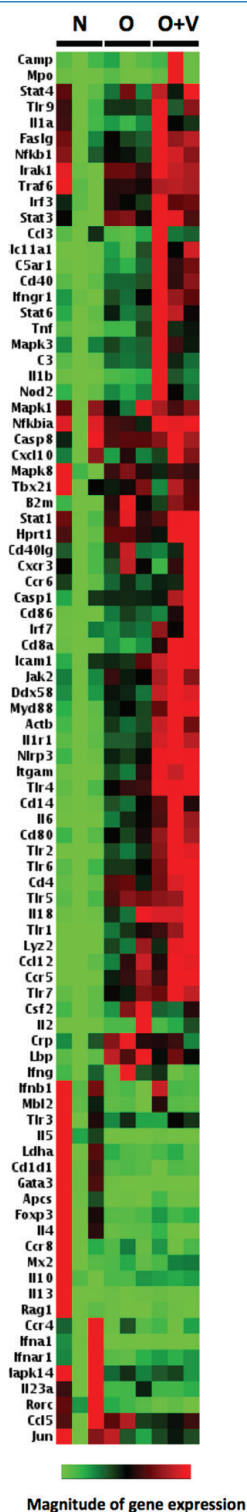
### *Severe muscle trauma impairs adjacent bone healing*

A rat model of bone-muscle composite injury was interrogated, in which the tibia and the overlying tibialis anterior (TA) muscle underwent osteotomy (OST) and VML injury, respectively<sup>5</sup>. Tibia mechanical strength and stiffness (Figure 1a) and bone volume within the fracture callus (Figure 1B) were reduced 28d post-injury in the presence of adjacent TA muscle VML injury. These findings are corroborated histologically by the heightened presence of cartilage instead of

woven bone within the fracture callus 28d post-injury (Figure 1c). Overall, these findings indicate concomitant VML injury impairs adjacent tibia healing.

### *Severe muscle trauma invokes cellular recruitment*

As an initial assessment of immune cell infiltration following VML injury, H&E sections of TA muscles were analyzed (Figure 2). TA muscles from OST groups presented modest cellular infiltration and muscle fiber damage that was resolved by 28d post-injury following stereotypical muscle fiber repair<sup>25,26</sup>. Consistent with previous observations of iso-



**Figure 3.** Increased expression of genes involved in innate and adaptive immune responses following complex musculoskeletal injury. Clustergram analysis displaying gene expression values in TA muscle tissue from naïve, OST, and OST+VML groups 3 d post-trauma as determined by PCR array (PARN-O52Z, SABiosciences). n=3 per group. Magnitude of gene expression is depicted by color. Green represents reduced expression, black represents no change, and red represents increased expression according to the average value of the gene in all samples.

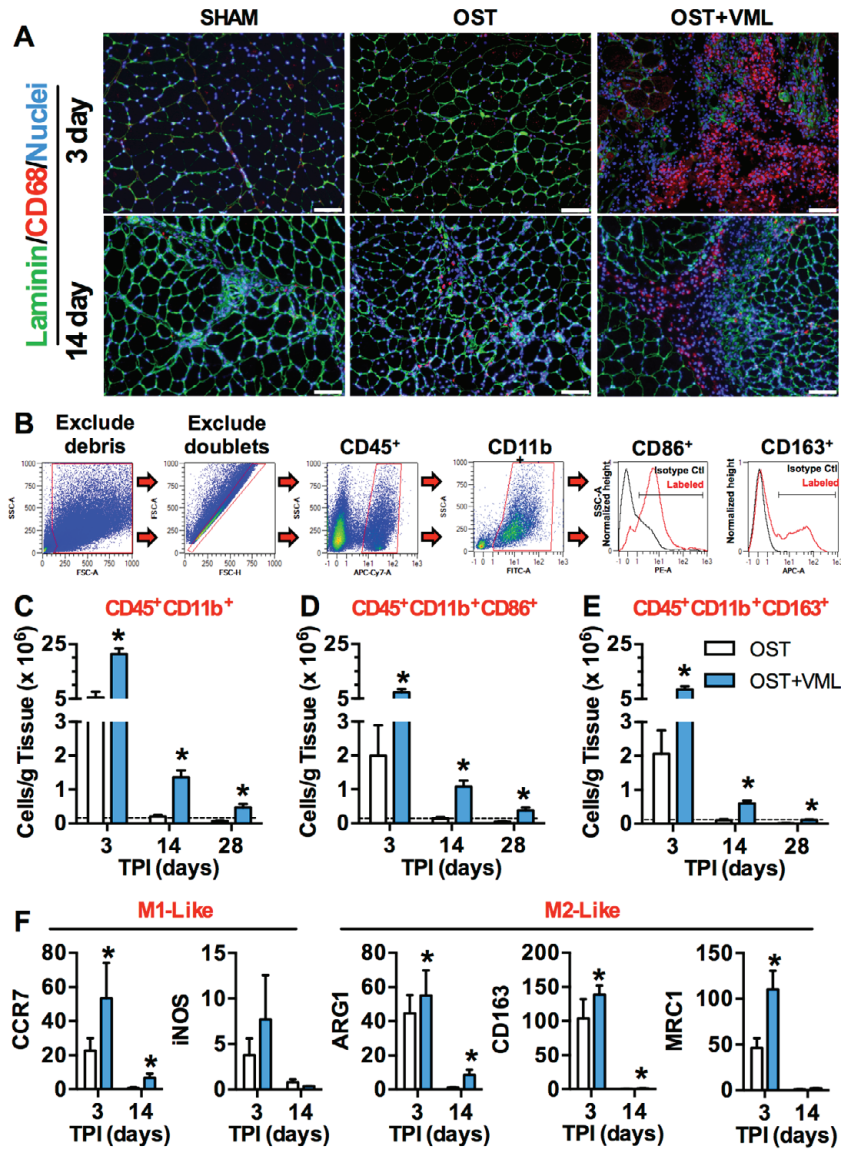
lated VML injury<sup>3,27</sup>, OST+VML presented extensive cellular infiltration to the muscle defect 14d post-injury, which was partly reduced 28d post-injury.

*Increased expression of genes involved in innate and adaptive immune responses in skeletal muscle following composite injury*

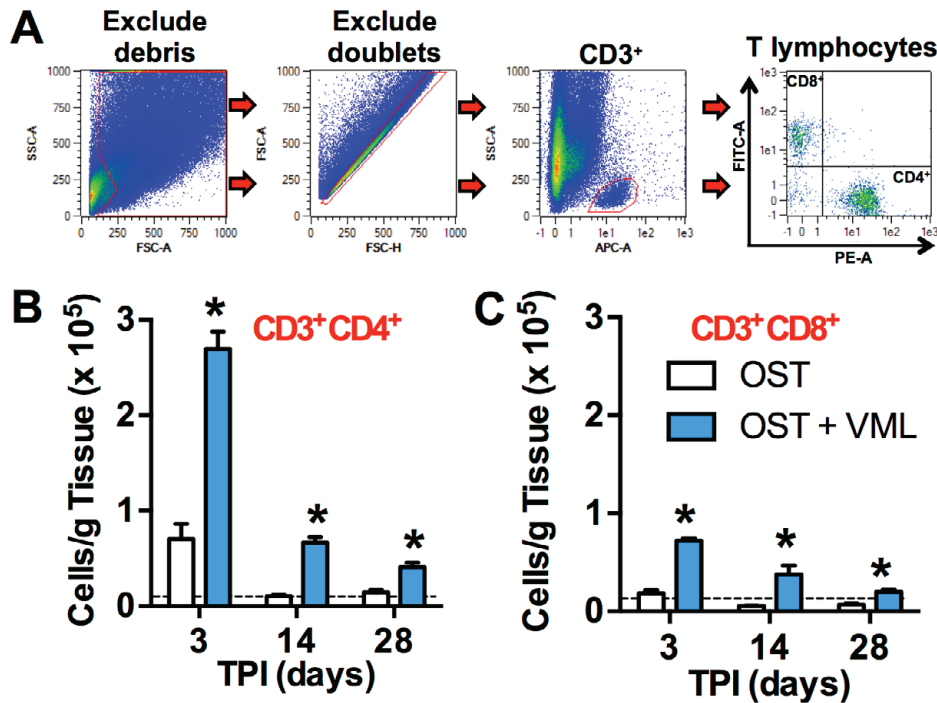
The immune response in VML injured muscle was further compared to the modest, resolvable TA muscle fiber damage in OST groups 3d post-injury by PCR array. Clustergram analysis revealed that VML injury broadly upregulates expression of genes involved in innate and adaptive immune responses (Figure 3). More specifically, expression of genes involved in the acute phase response, inflammasome, pattern recognition receptors (PRRs), downstream components of intracellular signaling pathways, effector functions including cytokine/chemokine production, and other innate genes trended towards or were significantly up-regulated following OST+VML injury (Table 3). Moreover, gene expression of T-cell markers and adhesion molecules involved in T-cell-antigen presenting cell (APC) interaction trended towards or were significantly greater after OST+VML than OST-only.

*Heightened innate immune response consisting of a mixed M1/M2 macrophage phenotype is triggered in muscle tissue adjacent to a fracture*

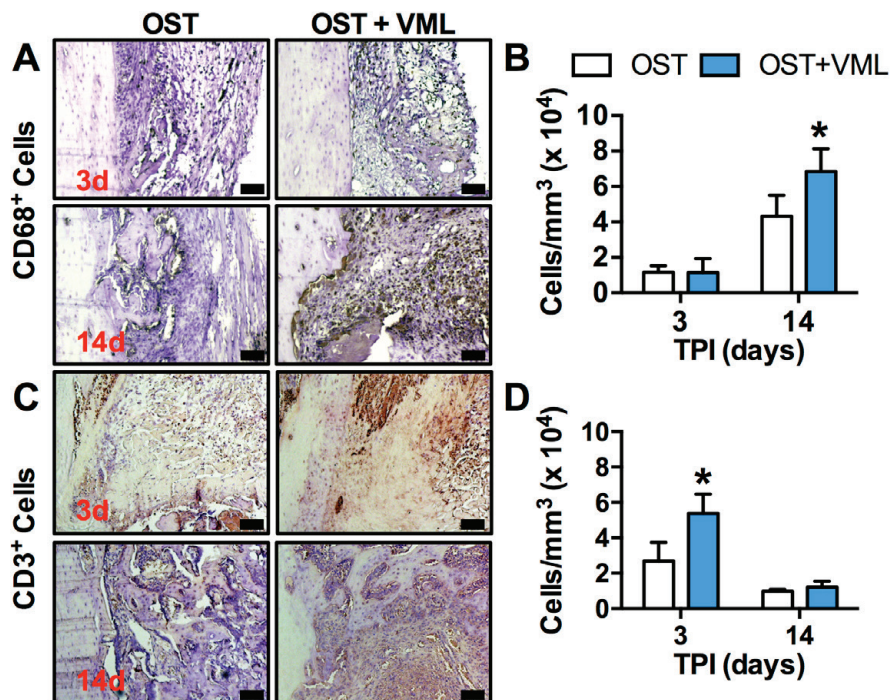
Initial immunofluorescence staining of TA muscle sections revealed increased recruitment of CD68<sup>+</sup> macrophages at 3 and 14 days post-trauma following OST+VML (Figure 4A). To accurately quantitate cellular recruitment over a longer period of time, flow cytometric analysis of cells isolated from the middle third of the TA muscle adjacent to the tibia osteotomy was conducted through 28d post-trauma. Cells were initially gated for CD45<sup>+</sup> hematopoietic cells followed by CD11b<sup>+</sup> myeloid cells that were determined to be CD68<sup>+</sup> at 3d (data not shown) and either CD86<sup>+</sup> M1-like or CD163<sup>+</sup> M2-like macrophages (Fig 4B). Overall, the majority of the cells recruited following open fracture were CD45<sup>+</sup>CD11b<sup>+</sup> myeloid cells (92.30 ±0.41%). At 3, 14, and 28d post-injury, OST+VML injured muscles had 3.86, 6.77, and 6.50 times greater CD45<sup>+</sup>CD11b<sup>+</sup> cells, respectively, than OST muscles (Figure 4C). The cell numbers of M1-like (CD11b<sup>+</sup>CD86<sup>+</sup>) to M2-like (CD11b<sup>+</sup>CD163<sup>+</sup>) macrophages in OST+VML versus OST groups were elevated through 28d (Figures 1D&E). Ratios (mean±SEM) of M1/M2 cell numbers were similar at 3d (0.86±0.08 vs. 0.85±0.12; p=0.97), but were higher at 14d (1.78±0.25 vs. 1.33±0.02; p=0.01) and 28d (3.25±0.53 vs. 2.10±0.18; p=0.09) post-injury, highlighting a prolonged M1-like phenotype after open fracture (i.e., OST+VML). Gene expression analysis of M1 (CCR7 and iNOS) and M2 (ARG1, CD163, and MRC1) markers revealed a mixed M1/M2 response at 3 and 14 d post-trauma (Figure 4F). Collectively, these data show elevated and mixed recruitment of M1- and M2-like macrophages to TA muscle through 28d in animals that underwent OST+VML when compared to simple fracture.



**Figure 4.** Heightened and prolonged recruitment of innate immune cells displaying mixed M/M2 phenotype within VML injured muscle. The TA muscle was isolated from OST, OST+VML, and specified control groups at indicated times post-injury (TPI) for A) immunofluorescence, B-E) flow cytometric, and F) transcriptional analyses. A) For immunofluorescence analysis tissue sections were stained for laminin, CD68, and nuclei (DAPI); n=4 per group. Scale bars=50 μm. B) For flow cytometric analysis, the middle third of the TA muscle that contains the defect and neighboring area was collected, digested, and cellular content isolated. Muscle-derived cells were labeled with antibodies to identify myeloid cells and analyzed by flow cytometry. To identify pro- and anti-inflammatory myeloid cells, populations were initially gated (red polygon) to exclude cell debris and doublets. Selected single cell populations were gated based on CD45 expression, followed by CD11b to identify myeloid cells. Pro- and anti-inflammatory myeloid cells were subsequently gated (black bar) based on their expression of CD86 or CD163, respectively. Gates were established by fluorescence-minus-one plus isotype controls. Relative cell numbers of C) CD45<sup>+</sup>CD11b<sup>+</sup> myeloid cells, D) CD45<sup>+</sup>CD11b<sup>+</sup>CD86<sup>+</sup> pro-inflammatory myeloid cells, and E) CD45<sup>+</sup>CD11b<sup>+</sup>CD163<sup>+</sup> anti-inflammatory myeloid cells were determined. Dashed-lines represent average cell numbers detected within contralateral control tissue. Data are normalized per gram of tissue and presented as mean ± SEM. n=4 per group. \*P<0.05 versus OST group. (F) Gene expression of M1 (*CCR7* and *iNOS*) and (F) M2-like (*ARG1*, *CD163*, and *MRC1*) macrophage markers were determined in TA muscle isolated from OST and OST+VML groups at 3 and 14 days post-trauma by qPCR. Tissue isolated from contralateral TA muscle was used to normalize data. Data are presented as mean ± SEM. \*P<0.05 versus OST group.



**Figure 5.** VML injury triggers increased recruitment of T helper and cytotoxic lymphocytes to muscle defect region. The TA muscle was isolated from OST, OST+VML, and specified control groups at indicated times points for flow cytometric analysis. A) To identify T lymphocytes (red polygon), samples were gated to exclude debris and doublets (as previously described), followed by gating for CD3 expression. CD3<sup>+</sup> cells were gated based on CD4 and CD8 $\alpha$  expression to identify T helper and cytotoxic lymphocytes, respectively. Cells were analyzed by flow cytometry for B) CD3<sup>+</sup>CD4<sup>+</sup> and C) CD3<sup>+</sup>CD8<sup>+</sup> expression. Dashed-lines represent average cell numbers detected within contralateral control tissue. Data are normalized per gram of tissue and presented as mean  $\pm$  SEM. n=4 per group. \*P<0.05 versus OST group.



**Figure 6.** VML injury directly influences immune responses in the adjacent fracture callus. Affected tibiae from OST and OST+VML groups were harvested 3 and 14d post-injury for immunohistological analysis of CD68<sup>+</sup> macrophage and CD3<sup>+</sup> T-lymphocyte infiltration in the fracture callus. A & C) Representative images from the callus region for each group are presented. Scale bars=100  $\mu$ m. (B & D) The number of CD68<sup>+</sup> and CD3<sup>+</sup> cells present in the callus was quantified from immunohistological sections. \*p<0.05 vs. OST & OST+VML within the respective time point. Values are means  $\pm$  SEM; n=3 per group.



**Table 3.** Transcriptional fold changes of innate and adaptive genes following OST and OST+VML injuries.

INNATE IMMUNE RESPONSE			
Pattern Recognition Receptors (PRRs) and Co-receptors			
Gene	OST	OST+VML	p-value
<i>CD14</i>	53.27±7.99	101.41±18.76	0.08
<i>DDX58 (RIG-1)</i>	<b>3.65±0.57</b>	<b>8.16±0.97</b>	<b>0.001</b>
<i>NOD2</i>	11.14±1.25	29.9±10.14	0.14
<i>TLR1</i>	<b>59.27±10.39</b>	<b>114.15±15.9</b>	<b>0.04</b>
<i>TLR2</i>	<b>92.39±11.39</b>	<b>227.31±18.77</b>	<b>0.004</b>
<i>TLR3</i>	0.86±0.12	0.99±0.15	0.56
<i>TLR4</i>	<b>7.61±0.38</b>	<b>12.78±0.06</b>	<b>0.0002</b>
<i>TLR5</i>	9.72±0.47	11.73±1.04	0.15
<i>TLR6</i>	<b>4.9±0.3</b>	<b>9.59±0.94</b>	<b>0.01</b>
<i>TLR7</i>	12.02±1.66	17.66±1.79	0.08
<i>TLR9</i>	2.88±0.08	4.81±1.27	0.2
Signaling Molecules and Transcription Factors			
Gene	OST	OST+VML	p-value
<i>IRAK1</i>	<b>4.19±0.23</b>	<b>5.69±0.34</b>	<b>0.02</b>
<i>IRF-3</i>	3.21±0.31	5.22±0.7	0.06
<i>IRF-7</i>	<b>2.8±0.22</b>	<b>6.7±1.12</b>	<b>0.03</b>
<i>Jak2</i>	<b>4.39±0.34</b>	<b>7.06±0.61</b>	<b>0.02</b>
<i>Jun</i>	2.14±0.91	1.87±0.14	0.78
<i>Mapk1</i>	1.71±0.55	2.09±0.14	0.54
<i>Mapk3</i>	3.12±0.2	6.2±1.38	0.09
<i>Mapk8</i>	3.43±0.26	3.03±0.3	0.37
<i>Mapk14</i>	1.21±0.16	1.16±0.17	0.85
<i>MYD88</i>	<b>12.97±0.99</b>	<b>25.71±2.9</b>	<b>0.01</b>
<i>NFKB1</i>	<b>3.22±0.29</b>	<b>6.13±0.5</b>	<b>0.01</b>
<i>NFKBIA</i>	<b>1.28±0.11</b>	<b>2.02±0.03</b>	<b>0.003</b>
<i>Stat1</i>	4.19±0.64	5.06±0.63	0.39
<i>Stat4</i>	2.41±0.76	4.26±0.82	0.17
<i>Stat3</i>	19.58±1.87	25.49±3.3	0.19
<i>Stat6</i>	<b>15.08±2.3</b>	<b>33.42±5.87</b>	<b>0.04</b>
<i>TRAF6</i>	<b>2.14±0.04</b>	<b>3.19±0.04</b>	<b>0.0001</b>
Cytokine/chemokine and Related Receptors			
Gene	OST	OST+VML	p-value
<i>CCL12</i>	33.26±10.22	47.74±7.9	0.32
<i>CCR5</i>	36.94±5.72	51.64±7.63	0.2
<i>CCL3</i>	2.4±0.85	11.85±5.04	0.14
<i>CCL5 (RANTES)</i>	1.39±0.21	1.18±0.09	0.42
<i>CSF2</i>	7.28±2.36	4.64±0.86	0.35
<i>CXCL10</i>	2.27±0.69	4.54±1.13	0.16
<i>Ifngr1</i>	<b>24.94±3.74</b>	<b>51.63±6.2</b>	<b>0.02</b>
<i>IL1A</i>	0.36±0.05	1.03±0.28	0.08
<i>IL1R1</i>	<b>9.18±0.66</b>	<b>22.45±1.08</b>	<b>0.0005</b>
<i>IL6</i>	<b>142.3±24.7</b>	<b>278.99±36.59</b>	<b>0.04</b>
<i>CXCR3</i>	2.33±0.57	2.28±0.97	0.97
<i>IFNAR1</i>	<b>0.68±0.21</b>	<b>1.32±0.09</b>	<b>0.05</b>
<i>TNF</i>	7.06±2.88	20.43±5.84	0.11

Acute-phase Response			
Gene	OST	OST+VML	p-value
<i>C3</i>	17.65±0.68	32.65±11.89	0.28
<i>C5AR1</i>	<b>21.38±4.41</b>	<b>68.65±10.9</b>	<b>0.02</b>
<i>CAMP</i>	4.62±2.69	19.21±15.5	0.41
<i>CRP</i>	2.55±0.41	1.58±0.36	0.15
<i>LBP</i>	21.97±2.69	14.79±1.91	0.09
Inflammasome			
Gene	OST	OST+VML	p-value
<i>NLRP3</i>	<b>15.11±0.87</b>	<b>33.02±0.3</b>	<b>0.00004</b>
<i>IL1B</i>	9.27±1.22	35.3±15.19	0.16
<i>IL18</i>	43.56±15.87	72.93±2.29	0.14
<i>Casp1</i>	2.42±0.01	3.97±0.74	0.1
<i>Casp8</i>	3.09±0.04	4±0.35	0.06
Unclassified			
Gene	OST	OST+VML	p-value
<i>MX2</i>	1.36±0.48	2.7±0.66	0.18
<i>ITGAM (CD11b)</i>	<b>89.12±18.99</b>	<b>205.71±8.14</b>	<b>0.005</b>
<i>Lyz2</i>	69.25±21.81	113.48±25.62	0.26
<i>SLC11A1</i>	<b>34.37±13.99</b>	<b>129.43±24.34</b>	<b>0.03</b>
<i>MPO</i>	3.72±3.32	53.39±48.03	0.36
<i>Faslg</i>	<b>0.95±0.12</b>	<b>2.02±0.15</b>	<b>0.01</b>
ADAPTIVE IMMUNE RESPONSE			
T lymphocytes - Activation and Adhesion Molecules			
Gene	OST	OST+VML	p-value
<i>CD1D1</i>	0.39±0.02	0.32±0.07	0.44
<i>CD4</i>	19.16±2.27	27.8±2.67	0.07
<i>CD40</i>	<b>7.22±0.29</b>	<b>16.55±2.51</b>	<b>0.02</b>
<i>CD40LG</i>	3.18±0.97	3.37±1.24	0.91
<i>CD8A</i>	<b>28.43±4.33</b>	<b>116.96±19.67</b>	<b>0.01</b>
<i>CD80</i>	<b>11.33±1.16</b>	<b>20.6±1.67</b>	<b>0.01</b>
<i>CD86</i>	28.24±2.73	50.37±18.06	0.29
<i>ICAM1</i>	<b>5.39±0.84</b>	<b>10.04±0.37</b>	<b>0.01</b>
<i>IL2</i>	119.51±88.48	63.96±23.72	0.58
<i>IL23A</i>	0.9±0.37	0.6±0.05	0.47
T lymphocyte Phenotype - Th1/Th2/Th17/Treg			
Gene	OST	OST+VML	p-value
<i>CCR6</i>	5.82±0.66	10.16±2.94	0.22
<i>Foxp3</i>	0.43±0.04	0.73±0.1	0.06
<i>GATA3</i>	0.06±0.01	0.04±0.02	0.4
<i>IFN-g</i>	6.19±2.86	2.79±1.45	0.35
<i>IL4</i>	0.31±0.03	0.24±0.11	0.61
<i>IL10</i>	0.49±0.14	0.67±0.03	0.27
<i>IL13</i>	0.27±0.08	0.29±0.11	0.86
<i>RORC</i>	<b>0.28±0.05</b>	<b>0.1±0.02</b>	<b>0.04</b>
<i>Tbx21 (Tbet)</i>	0.82±0.11	0.83±0.24	0.97

**BOLD** p<0.05, Student's t-test of OST vs. OST+VML; Values are means ± SEM.

### *Complex musculoskeletal injury triggers increased recruitment of T helper and cytotoxic lymphocytes in adjacent soft tissue*

The role of the adaptive immune response in sterile wound healing is an active area of research<sup>28-30</sup>. While animals that lack functional B and T lymphocytes display improved fracture healing<sup>31</sup>, indicating an inhibitory response in bone regeneration, in skeletal muscle the adaptive immune response appears to be beneficial to regeneration in healthy mice but detrimental in dystrophic mice<sup>32</sup>. Flow cytometric analysis was carried out on cells isolated from the TA muscle, which were gated for CD3<sup>+</sup> T lymphocytes followed by CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocyte markers (Figure 5A). At 3, 14, and 28d following trauma, CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes were 3.83, 6.35, and 2.85-times higher, respectively, in OST+VML than OST muscles. At the same time points, CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes were 3.95, 6.56, and 2.91-times higher, respectively, in the VML defect region of the OST+VML group compared to the OST group (Figures 5B&C). The OST+VML group displayed CD3<sup>+</sup>CD4<sup>+</sup>/CD3<sup>+</sup>CD8<sup>+</sup> ratios (mean  $\pm$ SEM) of  $3.79 \pm 0.28$ ,  $2.02 \pm 0.42$ , and  $2.11 \pm 0.28$  at 3, 14, and 28d post-trauma, respectively. These ratios were similar compared to the OST group and indicate that initially CD4<sup>+</sup> T cells comprise a larger portion of the T lymphocyte population, but at later time points there is a progressive increase in the proportion of CD8<sup>+</sup> T cells recruited to the site of injury. Altogether open fracture promoted a heightened and prolonged innate and adaptive inflammatory response through the initial 28d post-injury.

### *Macrophage and T-lymphocyte recruitment to fracture callus is exacerbated by muscle trauma*

The immune response to traumatized bone is crucial to regeneration, but can be deleterious if unbalanced or temporally disrupted<sup>33</sup>. Within the callus region, CD68<sup>+</sup> macrophages were similar among all groups 3d post-injury, but were significantly elevated 14d post-injury in non-repaired OST+VML tibia (Figures 4A&C). Meanwhile, CD3<sup>+</sup> cells were significantly elevated 3d post-injury after OST+VML, but were similar among all groups 14d post-injury (Figures 4B&D). These findings suggest that heightened inflammation within VML injured muscle directly influences immune cell infiltration within the adjacent fracture callus.

## Discussion

The immune response to isolated injuries of muscle or bone is known to play a fundamental role in their regeneration<sup>19,34</sup>. In the context of complex fractures, investigations of the cellular components and dynamics of the ensuing inflammatory response have almost completely focused on the fracture site, with very little attention given to the surrounding traumatized musculature. Yet, the incidence of delayed and non-union rates is greatest in fracture cases that pre-

sent complex injuries that often involve significant trauma or loss of surrounding muscle tissue<sup>10,21</sup>. Moreover, sterile “open fracture” studies in animal models unequivocally demonstrate that targeted irrecoverable muscle injury (i.e., volumetric muscle loss) or whole muscle ablation delays fracture healing via mechanisms unrelated to revascularization<sup>11-15,35</sup>. The current study corroborates with these earlier findings, demonstrating that TA muscle VML injury impaired fractured tibia healing in the otherwise healthy male Lewis rat. We observed heightened and prolonged myeloid and lymphocyte infiltration with the VML injured tissue and the adjacent tibia fracture callus, making it plausible that the VML injury triggered a dysregulated immune response that impaired fracture healing. These findings are in line with other conditions in which comorbid heightened inflammation is associated with impaired fracture healing<sup>16-20,24</sup>. The current findings therefore broadly identify the immune response to traumatized muscle, particularly VML injury, as a potential therapeutic target for advancement of open fracture orthopaedic care. These initial findings are significant because current clinical practice lacks targeted therapeutics for the systemic or local impact of traumatized skeletal muscle inflammation on musculoskeletal healing.

Volumetric muscle loss presents distinct injury characteristics that challenge mammalian skeletal muscle regeneration. Prolonged outcomes of VML include extensive fibrosis and chronic losses of muscle fibers, strength, and joint range of motion<sup>3,5,6</sup>. In contrast, other forms of muscle trauma in rodent models, such as eccentric-, crush-, freeze-, and toxin-induced injuries typically endogenously regenerate and fully recover muscle strength<sup>26,36-38</sup>. Regeneration among these recoverable injuries is partly modulated by the amplitude and dynamics of a predominantly innate immune response<sup>34,39-42</sup>. As an example, the osteotomy-only group presented muscle injury (likely caused by surgery) that regenerated in tandem with a typical canonical immune response, which has been characterized for recoverable eccentric and freeze injury<sup>34,43</sup>. Comparatively, VML injury ( $\approx 20\%$  loss of TA muscle) incited an amplified, prolonged, and progressively pro-inflammatory innate and adaptive immune response. It is possible that the inflammatory response following VML injury was triggered by a heightened release of endogenous danger-associated molecular patterns (DAMPs) from damaged muscle fibers<sup>44</sup>, which are recognized by TLRs and the inflammasome and initiate cell signaling pathways that drive inflammatory responses that include the production of proinflammatory cytokines. In support, TA muscle transcript levels for several PRRs (TLR2 and TLR4), the inflammasome (NLRP3) and pro-inflammatory cytokines (IL-1, IL-6 and TNF- $\alpha$ ) were elevated in VML injured compared to canonically healing muscle in osteotomy-only rats. Given that other recoverable muscle injuries, such as crush and ischemia-reperfusion<sup>13,45</sup>, have not impaired fracture healing, it would appear either the sheer magnitude or composition of the immune response to VML injury is a primary determinant of musculoskeletal healing.

The cellular immune response to VML overwhelmingly consisted of macrophages, which has been highly characterized in

wound healing and shown to play phenotype-specific roles<sup>46</sup>. Classically activated, proinflammatory M1 macrophages directly facilitate wound repair by participating in the initial inflammatory response as debris/cell clearing phagocytes, produce reactive oxygen species (ROS) and proinflammatory cytokines (IL-1, IL-6, and TNF- $\alpha$ ), and support a proinflammatory T helper cell phenotype (Th1). The alternative phenotype (M2) contributes to wound resolution by production of anti-inflammatory molecules (IL-4, IL-10, IL-13, IL-1R antagonist, decoy IL-1 receptor type II) and growth factors (VEGF, TGF- $\beta$ , and IGF-1) and supports an anti-inflammatory T helper 2 (Th2) phenotype<sup>46,47</sup>. Temporal macrophage polarization is critical for successful wound healing, wherein prolonged M1 or early M2 responses result in poor regenerative outcomes. In addition, *in vivo* phenotypes are more diverse than general M1/M2 phenotypes and likely influenced by the type and/or level of injury. For example, following skeletal muscle laceration mice presented early transcriptional upregulation and macrophage surface marker expression were indicative of a mixed M1/M2 phenotype, suggesting severe muscle injury does not trigger canonical macrophage polarization<sup>48</sup>. Our data are in agreement in that complex musculoskeletal trauma involving VML injury does not trigger canonical polarization, as we also observed a mixed M1/M2 phenotype through day 28 based on CD86 (M1) and CD163 (M2) markers. We speculate that the inability to properly polarize local macrophages to the required phenotypes at the required times or extending presence of M1 macrophages impairs complex musculoskeletal healing partly by extending the inflammatory phase and delaying the repair and remodeling phases of bone healing. As support, Grundnes and Reikeraas showed that fracture healing was impaired following local macrophage activation with glucan, a proinflammatory molecule<sup>49</sup>.

While the macrophage response to muscle injuries is well-studied, the T cell response is poorly characterized. We observed that VML injury induced an amplified and prolonged recruitment of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes within the traumatized musculature, as well as elevated gene expression of co-stimulatory molecules required for lymphocyte activation and infiltration. It is unknown if this response is non-specific (inflammatory) or antigen-specific. In sterile recoverable muscle injuries, antigen specific CD8<sup>+</sup> T cell responses are promptly regulated<sup>50</sup>, although it is possible that a severe or persistent injury may incite an antigen-based CD8<sup>+</sup> T cell response<sup>34</sup>. In other muscle-based pathologies including polymyositis and Duchenne muscular dystrophy, there is marked, prolonged T lymphocyte infiltration and activity, raising the potential that VML injury possesses characteristics akin to a myopathic condition<sup>34,51</sup>. Notably, VML injury also resulted in elevated T lymphocytes in the adjacent fracture callus, which has previously been associated with impaired fracture healing<sup>31</sup>. For example, CD8<sup>+</sup> T cell recruitment and terminal differentiation in fracture hematomas are associated with impaired healing<sup>28,52</sup>, while depletion improved bone repair<sup>53</sup>. Impaired musculoskeletal healing may be due to modulation of various activation and effector functions of CD8<sup>+</sup> T cells including polarization (cytokine production), cell lysis via per-

forin and granzyme B, and cellular interactions. We therefore hypothesize that VML injury prompts a CD8<sup>+</sup> T cell response that impairs musculoskeletal regeneration that may be effectively targeted by immunomodulatory therapies.

Similar to CD8<sup>+</sup> T cells, we show CD4<sup>+</sup> T cells were recruited at increased numbers through 28 d following OST+VML. Although depletion studies in normal fracture healing suggest a beneficial effect<sup>53</sup>, literature also suggests that during pathologies (arthritis, infection, cancer) CD4<sup>+</sup> T lymphocytes are detrimental in bone repair processes<sup>54</sup>. Whether CD4<sup>+</sup> T cells are beneficial or detrimental to fracture repair likely depends on the magnitude and duration of T cell recruitment, T helper cell polarization (Th1/Th2/Th17) and production of pro- or anti-inflammatory cytokines. For example, T helper polarization impacts osteoblasts and osteoclasts and influence cellular activation and function, all which can indirectly uncouple the bone repair process<sup>54</sup>. Lymphocytes can also directly impair fracture healing by the release of soluble factors including RANKL, which activate osteoclasts as well as direct cell-to-cell (CD40-CD40L) contact of stromal cells, which impact RANKL and OPG levels<sup>54</sup>. Impaired healing could also be tied in to the recruitment, or lack of T regulatory cells (Tregs). Tregs make up a small percentage of CD4<sup>+</sup> T lymphocytes and modulate the immune response by anti-inflammatory mechanisms (IL-10, TGF- $\beta$ , direct cell-to-cell interactions). Recently, Tregs have been shown to infiltrate injured muscle and secrete amphiregulin, which was required for successful muscle repair<sup>55</sup>. Additionally, Tregs modulate regeneration following recoverable toxin injury via a putative interaction with resident satellite cells<sup>29</sup>. We found transcriptional expression of Foxp3, the master regulator of Treg development and function, was not significantly upregulated in injured muscle following complex musculoskeletal injury. These differences suggest Treg induction/recruitment and amphiregulin production may be low following complex musculoskeletal injury and partly explain impaired healing. It is essential that future studies are carried out to evaluate the impact of modulating T lymphocyte recruitment, activation, polarization, and secretion of factors (e.g., amphiregulin) in both traumatized muscle and bone to favor tissue repair.

The current data provide the most extensive characterization of the immune response to VML injury to date. As predicted by the irrecoverable nature of VML, the ensuing immune response deviates from that described for canonical muscle regeneration. Notably, VML presents a prolonged mixed M1/M2 response and heightened lymphocyte infiltration that resembles pathological muscle tissue. The data indicate that VML results in heightened macrophage and lymphocyte infiltration within the fracture callus of adjacently fractured bone and impairs fracture healing. It is important to note that this rat model of open fracture did not include blunt trauma, and it is likely that the magnitude and temporal characteristics of the dysregulated immune response to VML are further exacerbated in the clinic<sup>56</sup>. We posit that VML presents various targets that may be targeted by immunomodulatory therapies for the improvement of bone and muscle regeneration following complex musculoskeletal injuries.

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