ABERRANT CELLULAR RESPONSE AND MUSCLE REGENERATION FOLLOWING VOLUMETRIC MUSCLE LOSS

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To my parents, Eric and Maria, for a lifetime of limitless support.

To Leo, for believing in me and walking with me every step of the way.
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**LIST OF SYMBOLS AND ABBREVIATIONS**

<table>
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<tbody>
<tr>
<td>AChR</td>
<td>Acetylcholine receptor</td>
</tr>
<tr>
<td>AM</td>
<td>Anti-inflammatory Monocyte</td>
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<td>AMPK</td>
<td>5' adenosine monophosphate-activated protein kinase</td>
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<tr>
<td>ANGPT</td>
<td>Angiopoietin</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ASC</td>
<td>Adipose-derived Stem Cell</td>
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<tr>
<td>αSMA</td>
<td>Alpha Smooth Muscle Actin</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>BDNF</td>
<td>Brain Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>β1-HI</td>
<td>β1-integrin high expressing FAPs</td>
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<td>β1-LO</td>
<td>β1-integrin low expressing FAPs</td>
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<tr>
<td>BTX</td>
<td>Bungarotoxin</td>
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<tr>
<td>Cav</td>
<td>Caveolin</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CRIT</td>
<td>Critical</td>
</tr>
<tr>
<td>CTRL</td>
<td>Control</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EDAC</td>
<td>1-ethyl-3-[3- dimethylaminopropyl] carbodiimide hydrochloride</td>
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<tr>
<td>Abbreviation</td>
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</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>eMHC</td>
<td>Embryonic Myosin Heavy Chain</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FAP</td>
<td>Fibro-adipogenic Progenitor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FSC-A</td>
<td>Forward Scatter Area</td>
</tr>
<tr>
<td>GCS-F</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GS</td>
<td>Goat Serum</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; Eosin</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
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<tr>
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<td>Horse Serum</td>
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<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IGF</td>
<td>Insulin-like Growth Factor</td>
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<td>IGTB1</td>
<td>Integrin beta-1</td>
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<td>Immunohistochemical</td>
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<td>Interferon-induced protein</td>
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<tr>
<td>IM</td>
<td>Inflamatory Monocyte</td>
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<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>KC</td>
<td>Keratinocyte Chemoattractant</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency Associated Protein</td>
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LIF Leukemia Inhibitory Factor
LOX Lysyl Oxidase
LV Latent Variable
Mφ Macrophage
M1 Inflammatory Macrophage
M2 Anti-inflammatory Macrophage
MAPK Mitogen Activated Protein Kinase
MCP Monocyte Chemoattractant Protein
MHC Myosin Heavy Chain
MIG Monokine-induced by interferon gamma
MMP Matrix metalloproteinase
mRNA Messenger Ribonucleic Acid
MuSC Muscle Stem Cell
NHS N-hydroxysulfosuccinimide
NMJ Neuromuscular Junction
PAI Plasminogen Activator Inhibitor
Pax7 Paired box transcription factor 7
PBS Phosphate Buffered Saline
PC Principal Component
PCA Principal Component Analysis
PCL Polycaprolactone
PCR Polymerase Chain Reaction
PDGF Platelet-Derived Growth Factor
PDGFR Platelet-Derived Growth Factor Receptor
PDMS Polydimethylsiloxane
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PLSDA</td>
<td>Partial Least Squares Discriminant Analysis</td>
</tr>
<tr>
<td>PM</td>
<td>Primary Myoblast</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SDF</td>
<td>Stromal cell-derived factor</td>
</tr>
<tr>
<td>SHG</td>
<td>Second Harmonic Generation</td>
</tr>
<tr>
<td>SPADE</td>
<td>Spanning-tree Progression Analysis of Density-normalized Events</td>
</tr>
<tr>
<td>SSC-A</td>
<td>Side Scatter Area</td>
</tr>
<tr>
<td>SUB</td>
<td>Subcritical</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming Growth Factor β1</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteases</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>µCT</td>
<td>Micro-Computed Tomography</td>
</tr>
<tr>
<td>UI</td>
<td>Uninjured</td>
</tr>
<tr>
<td>UMAP</td>
<td>Uniform Manifold Approximation and Projection</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VML</td>
<td>Volumetric Muscle Loss</td>
</tr>
<tr>
<td>VOI</td>
<td>Volume of Interest</td>
</tr>
<tr>
<td>vWF</td>
<td>vonWillebrand Factor</td>
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YFP  Yellow Fluorescent Protein
SUMMARY

Skeletal muscle is a plastic tissue with robust regenerative capacity following minor injury. However, following the traumatic loss of a large portion of tissue, known as volumetric muscle loss (VML), the muscle is unable to recover and will have chronic functional deficits. Regeneration of skeletal muscle requires on the proliferation and differentiation of a population of muscle stem cells (MuSCs), which are present throughout the tissue in a quiescent state through adulthood. Minor muscle injury initiates a coordinated response of key cellular components in the MuSC niche. Immune cells begin the process of removing damaged or necrotic muscle and fibro-adipogenic progenitors (FAPs) lay down a regenerative matrix, each secreting factors which prime the MuSCs differentiation and fusion. Subsequently, damaged blood vessels and motor neurons will regenerate and remodel returning the muscle to a state nearly indistinguishable to that of uninjured muscle. However, VML results in the concurrent loss of extracellular matrix (ECM), MuSCs, FAPs, blood vessels, and motor neurons, eliciting a cellular response which results in chronic fibrosis and inflammation. The dysregulated response which leads to critical VML has not previously been well characterized, and the overall objective of this thesis was to characterize the cellular response which leads to the accumulation of non-functional fibrotic tissue following critically sized VML injury model.

First, we characterized the tissue response to three different sizes of VML injuries to determine the threshold of a critical sized VML in the mouse quadriceps. We defined the critical threshold as the point where regenerating myofibers were unable to bridge the created muscle defect, replaced instead by a persistent fibrosis and macrophage infiltration.
We further characterized this critical threshold and saw substantial revascularization through the fibrotic tissue, but persistent denervation both within and distal to the localized injury. To understand the immune environment which led to these later stage outcomes, we used flow cytometry to quantify the dynamics of myeloid and lymphoid cell subtypes at earlier time points in critical compared to subcritical injuries. Critical injuries had distinct immune infiltration dynamics from subcritical injuries, as they exhibited significantly higher concentrations of lymphoid cell infiltration as well as a sustained significant elevation of anti-inflammatory monocytes and macrophages.

We then sought to understand the response of FAPs and MuSCs, two populations key to proper muscle regeneration. FAPs, which are considered to be the main progenitor population in muscle fibrosis, were of particular interest to us in further understanding the pathology of VML. We determined that critical VML induced a FAPs population which was present at significantly elevated concentrations and were most abundant in the VML injury space. FAPs isolated from VML injured tissue had different secretomes, responsiveness to pro-fibrotic stimuli, and differentiation capacity in vitro compared to FAPs from uninjured tissue. Cell surface markers were tracked over time in subcritical and critical VML, revealing a subset of FAPs which highly expressed β1-integrin that are persistently elevated in critical but not subcritical VML. Increased β1-integrin expression in FAPs was associated with increased gene expression of several pro-fibrotic genes and increased sensitivity to differentiate down a fibrogenic lineage in vitro. In addition to TGF-β1, a known pro-fibrotic signaling molecule, our data indicate TIMP1 may play a role in the fibrotic differentiation of FAPs. Together, these signaling molecules were also inhibitory of myogenesis in vitro.
These data indicated that critical VML results in a cellular response and local environment with sustained immune cell presence and pro-fibrotic FAPs propagating a hostile environment for muscle regeneration. We then assessed whether a structurally aligned, porous, myoblast seeded collagen scaffold would improve muscle function after critical VML. Aligned collagen scaffolds were able to improve maximal isometric torque over time and with the addition of exogenous cells. Long term engraftment of transplanted cells into the surrounding tissue was evident, but within the scaffold blood vessels and immune cells were more abundant than exogenous cells four weeks after transplantation.

Overall, this thesis has contributed a critical VML injury model with well-defined cellular and tissue pathology for use in preclinical studies. We have identified a subpopulation of pro-fibrotic FAPs specific to critical VML which can aid in future development of targeted therapeutics. Finally, we have tested the feasibility of use of a versatile biomaterial scaffold for cell transplantation in critical VML.
CHAPTER 1. INTRODUCTION

1.1 Motivation

Skeletal muscle has remarkable regenerative capacity due to the coordinated response of both circulating and local cells following minor acute injury. In commonly studied experimental skeletal muscle injury models, including cardiotoxin, barium chloride, or freeze injury, the myofiber extracellular matrix, or basal lamina, remains intact and myofibers will functionally regenerate with little to no persistent fibrotic tissue accumulation. However, following volumetric muscle loss (VML) injuries, where a large portion of skeletal muscle is lost through traumatic injury or surgical resection, the tissue cannot properly regenerate and results in permanent functional deficits. These functional deficits are largely attributed to the inability of myofibers to regenerate across the injured region, which is instead filled by non-contractile fibrotic tissue. A multipotent muscle resident mesenchymal cell population, fibro-adipogenic progenitors (FAPs), are the main cell source implicated in the generation of this fibrotic scar.

The current standard of care for VML injuries is autologous free or rotational muscle flap transfer; while these procedures are successful for limb salvage, they are unsuccessful in regenerating functional muscle. Additionally, there are several complications associated with skeletal muscle autograft including donor-site morbidity and graft failure. As clinical options for VML remain limited, there has been interest in the field of tissue engineering to develop both acellular and cell-laden scaffolding materials to encourage functional regeneration following VML. Some of these techniques have shown promise in preclinical models but have yet to be adapted for clinical applications. Additionally, scaffolds
engineered to mimic the native skeletal muscle tissue architecture have shown potential in models of skeletal muscle injury and disease and may be beneficial in the context of VML.

The overall objective of this thesis is to characterize the cellular response which leads to the accumulation of non-functional fibrotic tissue following critically sized VML injury model. We hypothesize that the endogenous response to critical VML promotes the proliferation of fibrogenic progenitors and limits myogenesis; and treatment with a structurally aligned myoblast-seeded scaffold will improve functional muscle recovery.

1.2 Specific Aims

**Specific Aim 1: Define and characterize the threshold of critical VML injury in the mouse quadriceps.** The primary objective of this aim was to determine the threshold for a critically sized VML injury and evaluate the response of key regenerative cell populations. We hypothesized that a critically sized VML defect would result in significant fibrosis and the dysregulated response of immune cells, fibro-adipogenic progenitors (FAPs), MuSCs, vasculature, and motor neurons. Full-thickness quadriceps VML injuries of three sizes (5%, 15%, and 30% muscle mass) were compared. Muscle cross-sections were used to assess regenerating myofibers and fibrosis. Micro-CT angiography was used to quantify vascularization. Transgenic mice with fluorescently tagged motor neurons combined with α-bungarotoxin staining of acetylcholine receptors (AChR) were used to visualize and quantify neuromuscular junction (NMJ) re-innervation. Flow cytometry at early time points (day 1, 3, and 7) was used to evaluate the temporal response and heterogeneity of myeloid cells, T cells, FAPs, and MuSCs in critical and sub-critical injuries.
Specific Aim 2: Determine the role of aberrant fibro-adipogenic progenitors in impaired myogenesis following critical volumetric muscle loss. The primary objective of this aim was to identify the cell subpopulation causing persistent fibrosis following critical VML and evaluate the impact of these cells on myogenesis. We hypothesized that a sub-population of aberrantly upregulated FAPs were the cause of fibrosis and detrimental to myofiber regeneration in our critical VML model. Flow cytometry was used to evaluate the quantity and surface marker expression of FAPs at day 7 after critically sized VML. Cytokine multiplex assays were used to evaluate changes in FAPs cytokine secretion following VML. MuSCs were co-cultured in transwell with FAPs from injured and uninjured tissue to determine the impact of FAPs secreted factors on MuSC differentiation. FAPs were sorted using fluorescence activated cell sorting (FACS) on the level of β1-integrin expression. A mouse fibrosis PCR array was used to compare pro-fibrotic gene expression of β1-Hi with β1-Lo FAPs and secretion of TGF-β1 and TIMP1 were measured. In vitro culture was used to measure proliferation and differentiation differences among FAP subpopulations. MuSCs were treated with TIMP1, TGF-β1, and FAPs subpopulation conditioned media and differentiation was measured. In vitro treatment of FAPs with tyrosine kinase inhibitor, nilotinib, was used to reduce fibrotic differentiation, but did not reduce the FAPs population in vivo.

Specific Aim 3: Evaluate the therapeutic efficacy of syngeneic myoblast transplantation seeded on a structurally aligned collagen scaffold for the treatment of critical volumetric muscle loss. The primary objective of this aim was to determine the impact of alignment on efficacy of primary isolated myoblast transplantation in various scaffolding materials to improve functional muscle regeneration following critical VML. We hypothesized that
pre-seeded aligned collagen scaffolds would improve muscle function by encouraging myofiber fusion across the VML defect. MuSCs in polyethylene-glycol-4-maleamide (PEG-4MAL) hydrogels were transplanted into VML injured tissue as an initial screen for therapeutic potential. Collagen scaffolding with varying degrees of alignment were evaluated in vitro for their ability to support myogenic proliferation and differentiation. Anisotropic collagen scaffolds were implanted in vivo with and without fluorescently labeled myoblasts. Maximal isometric torque about the knee was used to quantify functional recovery and muscle mass was measured. Engraftment of myoblasts into the host muscle was quantified via cross-sectional and whole mount histology. Migration of other cell types, including myeloid cells, FAPs, and blood vessels, were also measured by immunohistochemical staining of cross-sections.

1.3 Significance

The establishment and thorough characterization of the critical threshold for VML in the mouse quadriceps was accomplished in Aim 1. Utilizing this model in Aim 2, we were able to identify a novel FAP subpopulation which leads to persistent fibrosis and limits myogenesis following critical VML. Aims 1 and 2 illustrated a pathological cellular response to critical VML. In the absence of treatment, we quantified persistent immune cell and pro-fibrotic FAPs populations which limit the fusion of MuSCs in the VML defect. Further, aberrant FAPs down-regulate the degradation of the dense, fibrotic scar they produce. In Aim 3, we developed a myoblast-seeded, structurally aligned collagen scaffold which enhanced functional muscle recovery. Overall, this thesis has elucidated the impact
of injury size on the cellular response to VML in a mouse model. The identification of novel FAPs subpopulation which highly expresses β1-integrin as a driver of fibrosis is a significant contribution to understanding the role of FAPs and fibrosis in VML. These findings can be utilized in the development of both cellular and acellular clinical therapeutics for VML, increasing muscle functionality and quality of life.
CHAPTER 2. BACKGROUND AND LITERATURE REVIEW

2.1 Skeletal Muscle

2.1.1 Structure and Function

Skeletal muscle makes up 40% of the total body weight of humans and contains 50-75% of all body proteins [1]. Some of the key functions of skeletal muscle are to generate force, maintain posture, and produce movement. However, the skeletal muscle also plays an important role in whole-body protein metabolism and acts as a reservoir of amino acids for other organs, indicating that maintaining muscle health is important for overall organism health [2]. Muscle architecture is characterized by a highly organized structure of closely associated and directionally aligned myofibers. The number and size of these post-mitotic and multinucleated myofibers are directly responsible for the size of healthy muscle at the tissue level. The myofiber membrane is referred to as the sarcolemma and its cytoplasm referred to as the sarcoplasm [1].

Skeletal muscle produces force through the myofiber’s contractile apparatus, composed of myosin and actin filaments which are organized into sarcomeres. Briefly, skeletal muscle contraction is stimulated by innervating motor neurons, which transmit acetylcholine to the myofiber at the neuromuscular junction (NMJ). Upon stimulation, there is an intracellular depolarization and release of calcium ions into the sarcoplasm. The presence of calcium causes an exposure of the actin filament active site, allowing myosin to bind. Through the binding and hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP), myosin pulls on the actin filaments resulting in the contraction of the
sarcomere [3]. Each of these individual units contracting in unison causes the overall contraction of the muscle and production of force.

Myofibers can be classified into four major fiber types in humans (I, IIA, IIB, IIC) [4] and four in rodents (IIA, IIB, IIX) [5]. These fiber types are separated broadly into fast (type II) or slow (type I) fibers which are distinguished by the myosin subtype, myoglobin content, speed of shortening, degree of fatiguability, and their predominant method of energy consumption. While type II fast fibers tend to utilize glycolysis for energy production, type I slow fibers bias towards oxidative phosphorylation. As such, slow fibers have increase myoglobin production, slower speed of shortening, but are more fatigue resistant [1]. The relative composition of fast and slow fibers is dependent on the muscle being studied [5] as well as the innervating motor neurons. Following denervation, re-innervation of myofibers can cause a change in fiber type [6].

Directing the maintenance each myofiber’s complex protein structures are hundreds of post-mitotic myonuclei. It has been traditionally thought that each myonuclei maintains a specific region of the myofiber, called a myonuclear domain [7], [8]. The myonuclear domain size is not consistent across all myofibers, as more oxidative type I fibers have the smallest myonuclear domain and glycolytic, type II fibers have larger myonuclear domains [8], [9]. Similarly, it has been shown that type II fibers have the ability to hypertrophy without adding myonuclei, affectively increasing their myonuclear domain with myofiber growth [9]. Additionally, while myonuclei are distributed throughout the myofiber they have been shown to be a heterogeneous population. Myonuclei which localized beneath the NMJ or near myotendinous junctions are functionally different than the myonuclei outside of these regions [10], [11]. Recent studies have identified a subset
of myonuclei responsible for sarcomere assembly [11]. These diverse myonuclei are positioned near the sarcolemma of each myofiber, pushed to this peripheral location by the contraction of the sarcomere in the center of the cell [12].

Immediately surrounding the myofiber sarcolemma is a basement membrane called the basal lamina. The basal lamina associates closely with the endomysium, the extracellular matrix (ECM) surrounding each myofiber. Bundles of myofibers are surrounded by the perimysium, these bundles are in turn surrounded by the epimysium at the whole muscle level [13]. The major structural protein in the skeletal muscle ECM is collagen, with types I and III being most abundant. However, the basal lamina is mainly composed of collagen type IV and laminin [14]. The structural integrity of the muscle ECM is crucial for force transduction through the tissue to the myotendinous junction to produce movement [15]. Additionally, the muscle ECM houses a population of adult stem cells called muscle stem cells (MuSCs) which are required for the maintenance and growth of skeletal muscle, replenishing myonuclei over the lifespan.

Situated between the basal lamina and the myofiber sarcolemma, MuSCs are influenced by a unique microenvironment, termed the MuSC niche. This niche is comprised by the myofiber [16], basal lamina [17], vasculature [18], neural components (e.g. motor neuron [19], [20], neuromuscular junction (NMJ) [21], [22], and terminal Schwann cells [23]), and supporting cells (e.g. fibro-adipogenic progenitors [FAPs] [24], [25]) which each play indispensable roles in supporting muscle and MuSCs during homeostasis and regeneration.

2.1.2 Muscle Stem Cells and the Muscle Stem Cell Niche During Homeostasis
MuSCs, also called satellite cells, are an adult stem cell population capable of self-renewal and differentiation into myoblasts through both asymmetric and symmetric division [26]. While the majority of MuSCs are in a quiescent state during homeostasis, it has been shown that MuSC activate and fuse into existing multi-nucleated myofibers or form de novo myofibers in order to maintain skeletal muscle mass and growth during adulthood [27], [28]. Quiescent MuSCs can be identified by their anatomical location underneath the basal lamina and their canonical marker, the paired box transcription factor 7 (Pax7) [29]. Quiescent MuSCs are cells which have reversibly exited the cell cycle, have a relatively small cell volume, and low levels of protein synthesis [30], [31]. To actively maintain quiescence, MuSCs directly interact with myofibers through N- and M-cadherins and Notch signaling [32], [33] and with the basal lamina by binding laminin [34]. A loss of these signals from their niche will result in the activation of MuSCs, marked by increased expression of myogenic transcription factors Myf5 and MyoD, and the cell will enter the cell cycle [33]. It is important to note that while MuSCs share many common traits, they are also a heterogeneous cell population. For example, MuSCs can express varying levels of Pax7, with Pax7-Hi MuSCs being less likely to enter the cell cycle in comparison to Pax7-Lo expressing MuSCs [35]. Upon entering the cell cycle, MuSCs can divide symmetrically, into 2 activated daughter cells which will continue to proliferate, or asymmetrically, into 1 activated daughter cell which will proliferate and 1 daughter cell that will return to quiescence [26].

2.1.2.1 Fibro-adipogenic progenitors

There are various mesenchymal stromal cells which interact with MuSCs in their niche during homeostasis. One such population resident to skeletal muscle are fibro-
adipogenic progenitors (FAPs) [24], [25]. As their name would imply, FAPs can differentiate into fibroblasts or adipocytes, but exist during homeostasis as non-committed progenitor cells in the interstitial space between myofibers [36]. FAPs can be identified by their surface marker expression as the CD45−/CD31−/Sca1+/PDGFRα+ population and, as such, can be isolated through fluorescence activated cell sorting (FACS) [24], [25], [37]. While FAPs can contribute to the accumulation of pathological tissue in muscle, they have also been identified as regulators of skeletal muscle during homeostasis and regeneration. FAPs are known to secrete various pro-myogenic factors including WISP1 and follistatin [38], [39] and the ablation of FAPs from the muscle has been shown to result in skeletal muscle atrophy [36]. These studies have implicated that FAPs play a role in maintaining the MuSC pool in healthy muscle [40].

2.1.2.2 Vasculature

Skeletal muscle is a highly vascularized tissue, where every 3-4 adjacent myofibers are associated with 5-10 capillaries [41]. MuSCs and blood vessels are known to be located in close proximity to one another, with 88% of MuSCs located within 20 μm of a capillary [18]. Similarly, the number of vessels around a muscle fiber is highly correlated with the number of MuSCs surrounding the same fiber [18]. In vitro, there is evidence of interactions between MuSCs and endothelial cells (ECs) as factors such as insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF)-BB, and vascular endothelial growth factor (VEGF) secreted from ECs have been shown to promote MuSC growth and, conversely, differentiating myoblasts have shown to be pro-angiogenic [18], [42], [43]. Additionally, EC-derived Notch and Ang1/Tie-2 signaling have been implicated in maintaining MuSC
quiescence [44], [45]. Pericytes, another vascular-associated cell population, also play a role in MuSC regulation and quiescence through insulin-like growth factor 1 (IGF-1) and angiopoietin-1 (ANGPT-1) secretion [46]. Finally, it has been shown that increasing the vascularity of muscle ameliorates muscle’s disease phenotype in a mouse model of muscular dystrophy [47], [48]. Therefore, in addition to the vasculature’s role in bring nutrients and oxygen to the muscle, it also plays a critical role in the maintenance of the MuSC population.

2.1.2.3 Motor neurons

Voluntary muscle movement requires the innervation of skeletal muscle fibers by peripheral motor neurons at the NMJ. At the NMJ, there are densely packed acetylcholine receptors (AChRs) which closely associate in a distinct pretzel-like pattern on the myofiber membrane when functionally mature [49], [50]. The motor neuron is closely associated with a specific subset of MuSCs which aid in NMJ maintenance and regeneration [22]. This proximity allows for cross-talk between the motor neuron and MuSCs via nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) which encourage MuSC differentiation and quiescence, respectively [51],[52]. The cross-talk between neuron and MuSC is evident in the case of long-term denervation, which leads to a decrease in MuSC number [20]. In turn, MuSC depletion, either via transgenic modification or due to age, leads to impaired NMJ repair and lack of myofiber reinnervation following nerve injury [21], [22].

2.2 Muscle Regeneration
In many acute muscle injuries, muscle is capable of full regeneration with virtually no fibrosis [53], [54]. This is due, in part, to the basal lamina, a crucial factor in the proper regeneration of muscle, often remaining intact [55]. Within the basal lamina, MuSCs differentiate into myoblasts which can fuse with existing, damaged myofibers or with each other to form new myofibers [56]. As myofibers are regenerating, there is active remodeling of the ECM and vascular networks [57], [58]. In the final stage, regenerated myofibers are innervated by the regenerating motor neuron and recover function of the contractile apparatus [49]. This regenerative process is visually summarized in Figure 2.1 A.

2.2.1 Activation and Differentiation of Muscle Stem Cells

It is well-documented that MuSCs are indispensable for skeletal muscle regeneration, as the ablation of this cell population will result in a total loss of muscle regeneration after injury [59]. Just three hours following cardiotoxin injury, MuSCs will reduce expression of Pax7 and begin expressing Myf5 and MyoD [60], indicating their activated state. Many cytokines are important in the activation of MuSCs, including FGF [61], [62], HGF [63], IGF-1 [64], and tumor necrosis factor-α (TNFα) [65], which can be released from damaged myofibers or circulating immune cells which localize to the area of injury [63], [65], [66]. Damage of the myofiber and the release of intracellular contents including nitric oxide (NO) [67] and, recently, Tenascin-C [68] have also been shown to play a role in the activation of MuSCs. It is not only the MuSCs immediately surrounding the injury site which will contribute to regeneration, but it has been shown that MuSCs
along the injured myofibers will activate and migrate towards the site of injury [69]. Once activated, the expansion myogenic progenies will occur with spatiotemporal concurrence to the clearance of necrotic myofibers by phagocytic immune cells [65] (discussed further in the following section).

Following the expression of MyoD and Myf5, proliferating myoblasts will begin to downregulate these early myogenic transcriptional factors and begin upregulating the expression of Mrf4 and Myogenin indicating the terminal differentiation of MuSCs into post-mitotic myoblasts [56], [70], [71]. Following injuries where the basal lamina remains intact, myoblasts will migrate along the direction of the matrix where the necrosed myofiber used to be, dividing and fusing to create a new myofiber in this empty space [72]. These newly regenerating myofibers can be identified by their transient expression of embryonic myosin heavy chain (eMHC) as well as by the central location of the myonuclei [73], [12]. Muscle regeneration is considered complete when the myofibers rescue functional performance and will be evident by the movement of the myonuclei to the periphery of the myofiber [12], [74].

While MuSCs are required for the regeneration of myofibers [59], the success of this regenerative process requires cooperation and communication of all niche components. VML injury results in the catastrophic loss of the entire niche and therefore each component must be considered in the design of a therapeutic.

2.2.2 Immune Cell Infiltration

Following acute muscle injury, there is degeneration and necrosis of damaged myofibers [75], triggering the invasion of neutrophils, peaking between 12 and 24 hours
before steeply dropping off [76]. As neutrophils secrete TNF-α and interferon-γ (IFN-γ), classically activated inflammatory monocytes (IMs) and phagocytic (M1) macrophages will quickly begin to populate the injury area, sourced either from their resident location in muscle or from circulating blood [77], [78], and peak in number between 1 and 2 days post-injury [79]. M1 macrophages aid in the removal of tissue debris and also contribute to the secretion of pro-inflammatory cytokines including TNF-α, IFN-γ, interleukin (IL)-1β, IL-22, and IL-23 [65], [80]. Both TNF-α and IFN-γ also play a role in the regulation of skeletal muscle regeneration by silencing Pax7 but also preventing the expression of MyoD, maintaining MuSCs in an activated, proliferative state [65]. Once in tissue, IMs and M1s can differentiate into anti-inflammatory or alternatively activated, monocytes (AMs) or macrophages (M2s) [81]. By 4-7 days post-injury, there is a peak in the concentration of AMs and M2 macrophages present in muscle [82]. The transition to an IL-10 and transforming growth factor (TGF)-β rich environment corresponds with both a transition to M2 phenotype macrophages as well as the differentiation and growth stages of myogenesis [83], however IL-10 and TGF-β have been reported to be either ineffective or inhibitory, respectively, with regard to myoblast differentiation [83], [84]. Conversely, IGF-1 and 5’-adenosine monophosphate-activated protein kinase (AMPK)-α1 signaling by macrophages promote both the M1 to M2 transition as well as increase myoblast differentiation [85]–[87]. As myofibers regenerate, myeloid cells will traffic out of the tissue, resolving by about two weeks post-injury [65], [88]. This regulated response of myeloid cells is crucial for the proper regeneration of skeletal muscle, as the depletion or altered polarization of macrophages has been shown to increase adipose and fibrotic tissue deposition while reducing regenerated myofiber cross-sectional area [89], [90].
In addition to myeloid derived cells, lymphoid-derived T-cells also respond to muscle injury. Following cardiotoxin injury, peak infiltration of CD4+ and CD8+ T cells occurs at 3 days post-injury, returning to baseline levels gradually by day 14 [91]. This study also reported that depletion of CD8+ T cells in mice results in impaired muscle regeneration, implicating a role for CD8+ T cells in recruiting Gr1\textsuperscript{high} inflammatory macrophages through monocyte chemoattractant protein 1 (MCP-1), which in turn promote MuSC proliferation [91]. Regulatory T cells (T\textsubscript{reg} cells, CD4+/Foxp3+) infiltrate the muscle after acute injury with similar kinetics, peaking at day 4 post-injury, to that of M2 macrophages [92]. Depletion of T\textsubscript{reg} cells impairs muscle repair and prolongs inflammation [92], which could be attributed to need of T\textsubscript{reg} cells for the transition from M1 to M2 macrophage phenotype, the ability of T\textsubscript{reg} cells to limit IFN-γ and macrophage accrual, or the need for T\textsubscript{reg} derived IL-10 [92]–[94]. Other CD4+ T cells have also been implicated in promoting myogenesis; specifically the secreted cytokines from T helper (Th) type 2 and Th17 cells increased myoblast and endothelial cell proliferation in vitro and in vivo [95]. Taken together, the regulation of myeloid and lymphoid immune cell infiltration and clearance works in concert with the stages of myogenesis for prompt and proper muscle regeneration after acute injury.

2.2.3 Activation of Fibro-adipogenic Progenitors

During typical muscle regeneration, FAPs play a positive role in the regenerative process. Following injury FAPs rapidly proliferate, peaking between 3 and 4 days post injury, and create a transitional niche by depositing collagen and other ECM proteins before returning to pre-injury levels by 14 days post-injury [24], [25]. FAPs are also the source of several cytokines in this transitional niche, promoting the switch in the
regenerative cascade to myogenic differentiation. Some of these cytokines include IGF-1, IL-6, Wnt1, Wnt3A, and Wnt5A [24]. In co-culture in vitro, FAPs have been shown to decrease MuSC quiescence related genes, but increase differentiation related genes, including MyoD and Myogenin [24]. FAPs ablation before injury resulted in impaired myofiber regeneration shown by significantly smaller myofiber cross-sectional area [36] indicating that FAPs are required for proper muscle regeneration. FAPs are also the main source of IL-33 in the muscle, which aids in the recruitment and maintenance of T_{reg} cells, promoting muscle repair [96]. It is through chronic damage and aging that FAPs persist and result in pathological fibrotic and adipocyte infiltration [24], [37], [97].

An important factor in the appropriate regulation of FAPs after injury is a normal inflammatory cycle. Pro-inflammatory macrophage secretion of TNF-α is required for efficient FAP clearance by apoptosis [98]. Following this pro-inflammatory phase, pro-regenerative macrophages lead to a switch from a TNF-rich environment to a TGF-β1 rich environment, leading to the survival and differentiation of FAPs down a pro-fibrotic lineage [98]. If inflammation is not resolved, however, the persistent presence of both TNF-α and TGF-β1 leads to persistent FAPs survival [98] and is characteristic of chronic inflammation and improper macrophage polarization [99].

2.2.4 Vascular Regeneration and Remodeling

Skeletal muscle injury often causes secondary injury to the highly connected network of blood vessels in the tissue. Similarly, vascular injury often results in the injury of muscle myofibers. As skeletal muscle is a highly vascularized tissue, regeneration and subsequent remodeling of blood vessels following injury is crucial to the proper healing of
skeletal muscle. Immediately following injury, many blood vessels undergo initial damage and degeneration which will begin to recover within days after a chemical injury (notexin, cardiotoxin, barium chloride) or within the first week following freeze injury [53]. This initial angiogenic response will continue to increase capillary density to levels higher than in uninjured controls. This increase in capillary density and anastomoses will remain elevated for months following injury as the vasculature continues to remodel and mature [53]. Critical hindlimb ischemia, caused by transection of the supplying femoral artery, will induce widespread skeletal muscle damage within 3 days following injury. Muscle regeneration will follow, coinciding with vascular regeneration and reperfusion of the limb, returning to control levels at 56 days post-ischemia [100]. Additionally, following cardiotoxin injury angiogenesis and myogenesis have been shown to be linked in part via cross-talk of secreted factors including apelin, oncostatin M, and periostin [101]. Local delivery of VEGF has also been shown to improve muscle regeneration following ischemia and contusion injury [102]. These studies indicate a synergistic regenerative relationship between myofibers and vasculature.

2.2.5 Neuromuscular Re-innervation

Like the relationship with the vasculature, muscle is impacted by motor neuron injuries and, in turn, the motor neurons can be impacted by muscle injury. Barium chloride injection induces injury within the first 24 hours to motor neurons, post-synaptic terminals, AChRs, and surrounding non-myelinating terminal Schwann cells [103]. Peripheral nerves are surrounded by terminal Schwann cells, essential for NMJ formation and maintenance [23], which help guide regenerating motor neurons back to mature NMJs [104]. Interestingly, in the case of critical limb ischemia, the resulting muscle injury will also lead
to motor neuron denervation. The regeneration of motor neurons coincides with both the regeneration of the skeletal muscle and vascular reperfusion [100]. Conversely, when the motor neuron is injured and denervates the myofiber MuSCs will respond by activating and increasing myogenesis [19], [105]. Interestingly, this effect will actually prime muscle for enhanced regeneration in the case of mild nerve injury [105]. While these studies show the importance for nerve-muscle regenerative cooperation, the initial stages of muscle regeneration do not depend on re-innervation to the extent that the final maturation and functionality of regenerated myofibers do. Motor neuron reinnervation in the later stages of muscle regeneration, on the order of weeks post-injury, impacts the expression of mature myosins in the regenerating myofibers [106].
Figure 2.1. Muscle regenerative response in minor and VML injury. Minor injury regenerative cascade outlined in (A) compared to the hypothesized cellular response to VML which we aim to characterize in this thesis. Timing of key cell populations are highlighted including M1 and M2 macrophages, T cells, fibro-adipogenic progenitors (FAPs), muscle stem cells (MuSCs),

2.3 Skeletal Muscle Trauma

Extremity trauma injuries account for more than 50% of all trauma injuries among both military and civilian populations [107]–[109]. Following extremity injury, the
treatment focus is typically on bone healing and preventing or resolving infection [110]. Even under circumstances where bone healing and infection management are successful, many patients still experience persistent functional deficits due to a large volume of soft tissue loss, called volumetric muscle loss (VML) [110]. For veterans with VML injuries, the resulting lifetime disability costs nearly $350,000 per person [111].

2.3.1 Volumetric Muscle Loss

Within the field of musculoskeletal injuries, volumetric muscle loss (VML) remains a persistent challenge. VML injuries are defined as an abrupt, traumatic or surgical loss of tissue which will not endogenously regenerate, resulting in chronic loss of function and permanent disability [54], [110], [111]. Clinically, these injuries are difficult to define as the locations and types of wounds vary patient to patient [110]. Similarly, while there have been many previously established pre-clinical models for VML in a variety of animals and muscle groups [54], [112]–[116], the threshold for a critical size in VML injury models has not yet been established. Previously it has been suggested that a 20% muscle loss is the threshold for failure of the native regenerative process [117], however the regenerative processes at a critical size were previously not well understood.

2.3.2 Current Standard of Care

The current gold standard of care for VML injuries is autologous free or rotational muscle flap transfer; while these are successful procedures for limb salvage, these techniques remain largely unsuccessful for regenerating functional muscle tissue and additionally can result in donor-site morbidity and graft failure [110], [118]. The failure of muscle regeneration in these large muscle grafts is in part due to the lack of quick, effective
revascularization of the grafted tissue, ultimately resulting in fibrosis [119]. Following surgical treatment, the use of advanced bracing techniques [110] and physical rehabilitation regimens [120], [121] are used for patients to regain some functional use of their limb. Due to these persistent functional deficits after limb reconstruction, VML injury to the lower extremities leads to high rates of requested late amputation [110]. The limited options for care following VML provide motivation for a tissue engineered regenerative strategy to improve functional outcomes after VML injuries.

2.3.3 Cellular Response to VML

Traumatic injuries, including VML, display the dysregulated dynamics of chronic inflammation [122]–[124]. This includes the sustained presence of CD4+ helper T cells and CD8+ killer T cells as well as improper macrophage polarization [98], [122], [123], resulting in the chronic upregulation of TNF-α [122] and TGF-β1 after VML injuries [116]. This creates an environment rich with both pro- and anti-inflammatory factors, which has been shown in other muscle injuries and diseases lead to the proliferation of FAPs and fibrosis [98], [125].

Pathological fibrosis and the development of a stiff, fibrotic scar is a hallmark of VML injury. Surgical debridement of fibrotic tissue, which is sometimes used clinically to improve range of motion, resulted in no improvement in muscle force production, results in a recurrent fibrotic scar of similar magnitude to the removed tissue, and had similar gene regulation profiles to untreated animals in a porcine mode of VML [126]. This indicates that pharmacologically modulating fibrosis in chronic inflammatory muscle injury environments, such as VML, may be a better method for limiting fibrosis and encouraging
muscle regeneration. However, it should be noted that the ablation of fibroblasts or FAPs impairs muscle regeneration [36], [127] and the downregulation of fibroblasts by blocking TGF-β1 after VML has does not enhance functional recovery [128]. It is likely that the temporal regulation, but not ablation, of fibrotic cell populations would need to be combined with other pro-regenerative muscle therapeutics to result in enhanced muscle recovery. Further discussion of FAPs and pathological fibrosis can be found in section 2.4.

Due to the nature of VML injuries, the myofiber associated vasculature is removed with the skeletal muscle defect. This limited vascular supply is a major concern when treating VML injuries clinically, and attempting to mitigate this is evident in the careful choice of donor tissue based on vessel locations when comparing to the defect site [129]. Similar efforts to encourage vascularization have been seen pre-clinically, with the intension of encouraging muscle regeneration through the crosstalk between angiogenesis and myogenesis discussed previously. However, it is important to not there is also a link between angiogenesis and chronic inflammation [130], which could explain significant vascularization seen even without treatment [131].

In VML injuries, it is expected that there is a concurrent loss of neural components in the defect region, indicated by sustained loss of muscle function after injury [132]. It has been estimated clinically that at least 20-30% of extremity trauma injuries involve an injury to the peripheral nerve [133]. Pre-clinically, neuromotor damage and recovery following VML has not been particularly well characterized. Recently, it has been reported that a VML injury to the rat TA causes ~69% axotomy, or denervation by serving an axon, of the motor neurons innervating that muscle [132]. This would indicate that axotomy is likely a compounding factor in the sustained functional deficiencies seen following VML. As noted
previously, sustained denervation also has a negative effect on the MuSC number over time, which could further inhibit muscle regeneration. Teasing out the interactions between MuSCs, newly regenerating myofibers, and motor neuron innervation will likely be important in promoting functional regeneration after VML.

It is likely that the impaired regenerative response of each of these niche components to VML negatively impacts the ability of MuSCs to proliferate, differentiate, and fuse to form de novo myotubes which can bridge the defect left by the VML injury itself. The background literature contributed to our working hypothesis of the cellular response to VML, visualized in Figure 2.1 B, prior to characterization in this thesis.

2.4 Chronic Inflammation and Pathological Fibrosis in Skeletal Muscle

2.4.1 Chronic Inflammation

Chronic, or non-resolving, inflammation has been linked at the systemic level to diseases including atherosclerosis and cancer [134], [135] as well as at the tissue level to non-healing wounds, tissue fibrosis, and organ failure [136], [137]. The term chronic inflammation can refer to the aberrant presence of immune cells, their associated paracrine signaling molecules, or both. In skeletal muscle, chronic inflammation has been most thoroughly characterized in the context of idiopathic inflammatory myopathies, dystrophies, or aging, in each of these cases leading to fibrotic accumulation and poor skeletal muscle regeneration [98], [138]–[140].

The temporal imbalance in pro-inflammatory versus pro-resolving cytokines has been characterized as a driver of impaired tissue regeneration and fibrosis in many tissue
Persistent immune cells, including macrophages, are often the source of these cytokines. Alternatively-activated M2 macrophages are considered to be pro-resolving in normal wound-healing conditions, but secrete a number of pro-fibrotic cytokines including TGF-β1 and PDGF. Conversely, while classically activated M1 macrophages secrete traditionally considered anti-fibrotic cytokines, including IL-1β and TNF-α, they have both been shown to accentuate pro-fibrotic differentiation in the presence of TGF-β1. Interestingly, the presence of a mixed M1 and M2 polarized macrophage population, along with a mixture of their associated cytokines is a hallmark of chronic inflammation in skeletal muscle.

2.4.2 Fibrotic signaling and fibro-adipogenic progenitors (FAPs)

It is well known that chronic muscle injury, disease, and aging result in the infiltration and accumulation of fibrous and adipogenic tissues which, in turn, compromise muscle function. As previously discussed, skeletal muscle resident, PDGFRα+ FAPs are the main source for fibrotic and adipogenic lesions in skeletal muscle. TGF-β is considered to be the main driver of fibrotic differentiation of FAPs.

TGF-β is a growth factor which commonly acts as a pro-fibrotic signaling molecule in many tissues. In skeletal muscle, TGF-β signaling has been linked to a number of myopathies that result in fibrotic accumulation including amyotrophic lateral sclerosis (ALS) and several forms of muscular dystrophy. Canonical TGF-β signaling bind the receptor tyrosine kinase TGF-β type I receptor, which activates the Smad pathway, with Smad2/3 activation playing a particularly important role in fibrosis.
There are also several non-canonical TGF-β signaling pathways, including mitogen activated protein kinase (MAPK) pathways ERK, p38, and JNK which have also been linked to muscle diseases [150], [152], [155]. In FAPs TGF-β signaling can lead to the transcription of fibrosis related genes, including those to produce ECM proteins, promoting mature fibroblast differentiation [156] and limit adipocyte differentiation [157]. It is important to note that the link between TGF-β and muscle disease is two-fold: TGF-β directly drives fibrotic differentiation of muscle-resident FAPs as well as inhibits myoblast differentiation and fusion.

PDGFs are another family of growth factors capable of pro-fibrotic signaling. These signal through binding one of two receptor tyrosine kinases, PDGFRα or β, and have a variety of downstream pathways which can affect cell proliferation, survival, and differentiation [158]. Expression of and signaling through PDGFRs is typically tightly controlled, but aberrant over-activation of PDGFRα has been linked to tissue hyperplasia and chronic fibrosis in many organs, including skeletal muscle [159]. FAPs express high levels of PDGFRα, and PDGF ligands have been shown to affect their proliferation and differentiation [160]. Recently, PDGFRα has been found to be a target gene of TGF-β. As TGF-β signaling increases, PDGFRα expression of FAPs decreases, as they differentiate down a fibrogenic lineage. However, there is also evidence of crosstalk between the TGF-β and PDGFRα signalling pathways, with a PDGFRα inhibitor decreasing the pro-fibrotic response of FAPs to TGF-β stimulation [161].

In addition to the increase in the production of ECM proteins during fibrogenesis, changes in the activity of proteins which degrade and remodel the ECM also contribute to fibrosis. Matrix metalloproteinases (MMPs) are a large family of enzymes which degrade
collagen and other ECM components. Tissue inhibitor of metalloprotease 1 (TIMP1), as the name implies, has mainly been studied for its inhibitory effect on MMPs, which by limiting ECM degradation contributes to the accumulation of collagen and other proteins during fibrosis [162]. However, more recently TIMP1 has been shown to bind cell surface receptors directly, signaling through CD63 and β1-integrin complex [163]. Expression of TIMP1 has been linked to tissue fibrosis in the lung, liver, and myocardium [164]–[166]. In the myocardium, intracellular crosstalk of TIMP1 and TGF-β signaling was found to increase nuclear translocation of Smad2/3 and β-catenin, leading to the increased production of collagen [166]. The impact of TIMP1 signaling on FAPs has not yet been elucidated.

2.4.3 Anti-fibrotic therapeutics

Following muscle injury, the use of a variety of anti-fibrotic agents have been studied to reduce pathological fibrosis with varying results. In the mouse model of DMD, the use of a TGF-β1 neutralizing antibody was found to reduce fibrotic protein deposition without impairing myogenesis, but increased the amount of CD4+ T lymphocytes [167]. In the same murine dystrophy model, halofuginone hydrobromide was used to inhibit the TGF-β-dependent activation of Smad3 which reduced collagen expression and myofiber damage during exercise [154]. These examples show promise for anti-fibrotic agents in muscular dystrophy, where the consistent degeneration and regeneration of muscle leads to the accumulation of collagen in the intra-fibrillar space.

Some anti-fibrotic strategies in the skeletal muscle have specifically focused on the impact on FAPs. In murine muscular dystrophy, a tyrosine kinase inhibitor, nilotinib, was
used to block TGF-β signaling [97]. This study demonstrated an additional for TGF-β in skeletal muscle fibrosis, providing evidence for TGF-β as a survival cue for FAPs not only as a promoter of collagen deposition. Another group studied the expression of and signaling through PDGFRα in FAPs. They identified a transcriptional variant which resulted in a PDGFRα isoform with a truncated kinase domain; the overexpression of this variant was ultimately found to reduce muscle fibrosis [168]. Together, these data reveal novel, FAPs-specific therapeutic targets for skeletal muscle fibrosis.

In the context of VML, similar anti-fibrotic strategies have been used. Losartan, primarily an angiotensin II receptor blocker, has the downstream effect of blocking TGF-β1 activation and signalling [169]. When utilized following VML injury in a rat model, Losartan was able to reduce fibrotic gene expression and collagen deposition, but did not improve muscle regeneration and reduced functional muscle force generation [128]. The use of a tyrosine kinase inhibitor, nintedanib, which targets the FGF, PDGF, and VEGF receptors, and has been shown to reduce fibrosis in pulmonary fibrosis was used in a porcine VML model. Nintedanib was found to reduce the stiffness of the fibrotic scar, reduced the magnitude of expression of fibrosis-related genes, but also presented a greater strength deficit than untreated VML injured muscles [170]. These results demonstrate a phenomenon of force transmission through fibrotic tissue seen following VML and highlight the need for concurrent muscle regeneration with fibrotic tissue reduction in these traumatic injuries.

2.5 Skeletal Muscle Tissue Engineering
Due to the lack of clinical therapeutic options for VML, there have been significant efforts to design tissue engineered skeletal muscle constructs that can be used for implantation into a VML defect. Tissue engineering is a relatively new concept, defined in 1993 as an intersection of engineering and life sciences working toward “the development of biological substitutes that restore, maintain, or improve tissue function” [171]. The first tissue engineered therapeutics utilized scaffolding materials which could support growth of biological tissues. Today, tissue engineered strategies utilize scaffolds with finely tuned characteristics including: physical properties, such as degradation, stiffness, and anisotropy; bioactive properties, such as the release of growth factors or pharmacological compounds; and functional tissue properties, such as seeded stem cells, differentiated cell networks, or pre-vascularized or pre-innervated constructs. Many of these characteristics have been modulated, either separately or in combination, in the effort to create engineered skeletal muscle.

2.5.1 **Scaffolding materials**

One common tissue engineered strategy for musculoskeletal regeneration is the use of scaffolding materials which can be derived from natural or synthetic sources. Utilization of decellularized skeletal muscle ECM scaffolds from a variety of tissue sources has been done both pre-clinically [112], [121], [172] and clinically [120], [121], [173]. While some of these approaches can result in an increase in mass and functional force generation, however this is not accompanied by myofiber regeneration into the scaffold material, and increased force is likely due to force transfer over an increased mass of fibrotic scar tissue within the implanted ECM [173]–[175].
A wider variety of scaffolding materials have been used exclusively preclinically, \textit{in vitro} and \textit{in vivo}. Many ECM derivatives have also been widely used for muscle regeneration including collagen [176], fibrin [177], and laminin [178]. These tissue native materials provide the benefit of being recognizable by the host tissue and enzymatically degradable [179]. Synthetic polymers, which provide higher degree of reproducibility and more precisely controllable degradability properties, are also often used in skeletal muscle regeneration. Some of these include poly(lactic-co-glycolic acid) (PLGA) [114], [180], polycaprolactone (PCL) [181], [182], polyethylene glycol (PEG) [183]. Interestingly, many of these synthetic polymer examples incorporate natural ECM proteins or cell binding motifs to enhance their interaction with host muscle or seeded cells. Outside of these commonly studied materials, the first study using an inorganic material, bioactive glass, was recently published for use in a rat model of VML [184], demonstrating the vast range of materials that may be of use in these complex injuries.

\textbf{2.5.2 Bioactive Factors}

To improve upon passive scaffolding materials, some approaches incorporate bioactive factors with the goal of interacting with the endogenous cell response to enhance myogenesis. The addition of bioactive growth factors or drugs to natural or synthetic scaffolding materials can allow for effective local delivery by passive diffusion or in bulk as the scaffold is actively degraded [185]. Growth factors, such as VEGF, IGF-1, HGF, SDF-1, and basic FGF [102], [186]–[188], and other signaling molecules, such as Wnt-7a [189], have shown improvement in myogenic regeneration in several injury models.
Immunomodulating compounds have also been incorporated into biomaterials for use following muscle injury. Sphingosine-1-phosphate (S1P) analog FTY720 in PLGA has been used to bias macrophages towards the M2, pro-regenerative phenotype, which was beneficial for myogenesis [114], [190]. Additionally, the use of immunosuppressant FK506 (Tacrolimus) in sponge-like scaffolds made of gelatin, collagen, and laminin-111 was shown to improve myogenesis and functional muscle recovery following VML, potentially due to altered T cell dynamics [191].

2.5.3 Seeded Cell Types and Sources

Seeding cells into biomaterial scaffolds can alleviate some of the need to recruit endogenous cells from the host muscle for skeletal muscle regeneration. However, the use of transplanted, exogeneous cells comes with its own distinct challenges, including seeded cell survival, integration of seeded cells into the host muscle, cell sourcing, and immunogenicity.

2.5.3.1 Myogenic progenitors

One of the most used cell types in skeletal muscle regeneration are myogenic precursors, or myoblasts. The most attractive source for myogenic precursors in preclinical studies is primary isolated, quiescent MuSCs. These can be isolated through FACS [192], [193]. In culture, the isolated MuSCs will begin to spontaneously differentiate and eventually fuse into multinucleated myotubes [194], however freshly isolated, Pax7+ MuSCs have the greatest therapeutic potential [195]. Due to the small number of cells per tissue mass and difficulty to expand MuSCs outside the body after isolation leads to issues generating the cell concentration needed to see functional improvement after
transplantation. Recently, the use of co-transplantation of MuSCs in a PEG hydrogel has been shown to improve MuSC engraftment following injury and in disease [183]. MuSCs used with and without muscle resident support cells in a decellularized ECM biomaterial in a murine TA model of VML showed the ability to engraft and improve functional muscle recovery [196].

Other myogenic progenitor sources have also been used preclinically in the treatment of VML injuries. These include the murine myoblast cell line C2C12s [197], [198] and primary myoblasts (PMs) [177]. C2C12s are an immortalized cell line, distinct from MuSCs as they will not fuse with the native host tissue in immune competent mice. PMs are similar to MuSCs as they are isolated from primary muscle, but this is done in vitro by sequential plating, resulting in an activated myoblast, not quiescent MuSC, population [199]. Also, the cell yield by pre-plating is much higher than by FACS. Importantly, it has been shown that syngeneic transplantation of PMs does not elicit an immune response and the transplanted cells can integrate to the host muscle of immune competent mice both by differentiating to fuse into host myofibers as well as by self-renewing to replenish the pool of muscle progenitor cells [200].

The use of primary muscle as a cell source is translationally challenging as a strategy for VML as it would require a large biopsy or graft of the patients’ muscle. Induced pluripotent stem cells (iPSCs) provide a potential solution to this problem. iPSCs are stem cells which can be generated by reprogramming mature human cells and then subsequently induced to differentiate into any cell type [201], including muscle progenitors [202], [203]. iPSC derived muscle cells have been used to generate 3D skeletal muscle constructs in vitro to model healthy and diseased muscle [204]. Human iPSC derived muscle progenitors
were transplanted in fibrin hydrogels into immunodeficient mice following a VML injury to the TA. These constructs resulted in donor-derived innervated myofibers and increase functional force generation [205]. In this way, it may be possible in the future to create constructs like these for use as grafts in VML without having to take a large muscle graft from the patient. For this reason, iPSCs have come to the forefront as a cell source for skeletal muscle tissue engineered strategies looking to translate to the clinic.

2.5.3.2  Muscle Niche Supporting Cells

While the use of myogenic progenitor cells is the most common approach in skeletal muscle tissue engineering, myofibers require additional support cell populations (vessels, motor neurons) to be functional. Taking this into consideration, several studies have used a combination of muscle support cells as therapeutic strategies.

One such approach is the use of autologous minced muscle in the defect. While this approach incorporates all cell components of the removed muscle, it results in little regenerative benefit or functional improvement [116], [124], [206]. Taking a slightly different approach than using whole minced muscle, one study utilized FACS to isolate additional muscle resident cell populations for in vivo transplantation. A decellularized scaffold was seeded with MuSCs alone or MuSCs with hematopoietic cells, endothelial cells, FAPs, and a population of fibroblast-like cells. Comparing these groups, MuSCs seeded with the additional muscle resident cells had enhanced engraftment potential and improved functional muscle recovery [196].

Vascularization of the VML defect is a common outcome measure of success for therapeutics. The use of adipose-derived stem cells (ASCs) [197], [207], and microvessel
fragments [131], each were able to increase vascularization within the defect space. However, with an increase in vascularization, there was not always an increase in myofiber formation within the injury.

2.5.4 Biomechanical Properties and Alignment Cues

The overall structure of the scaffolds discussed thus far vary widely from study to study, with some formed via lyophilization into dry, porous materials [176], others are solubilized polymers or proteins which are then cross-linked to form hydrogels [177]. These different structures will also result in vastly different mechanical properties, altering the response of cells they interact with. Native skeletal muscle is a relatively soft (~12 kPa) and anisotropic ECM alignment in the direction of the myofibers. It has been shown that MuSC self-renewal is responsive to the stiffness [208] and three-dimensional structure [195] of the matrix to which they are adhered. The stiffening of the native ECM over time following injury, which is known to occur following VML [170], will persistently activate MuSCs, which can lead to MuSC pool depletion [209]. The overall structure of the matrix is important for native muscle regeneration as MuSCs typically proliferate and differentiate within the empty, elongated scaffold of basement membrane following myofiber necrosis [72]. Scaffolds which recreate these aligned, tubular structures may provide an environment more conducive to myofiber regeneration than amorphous hydrogels.

With these biophysical properties in mind, there have been biomaterials designed for specifically for skeletal muscle. Hydrogels often can closely replicate the elastic modulus of whole muscle, with natural [210] and synthetic [183] hydrogels tuned specifically to replicate this parameter at or around 12 kPa. Lyophilized scaffolds have
been reported to have compressive moduli in the range of ~10 to 800 kPa [176], [211]. Anisotropy can be achieved through porous alignment by controlled lyophilization or through fibrillar alignment by controlled extrusion of material. Collagen scaffolds with porous alignment have been successfully used for the culture of tenocytes [211]. Electrospun fibrin fibers with an elastic modulus of 17 kPa were used to create aligned bundles for use in a VML defect [212]. Similarly, extruded collagen fibers [213], [214] and a decellularized muscle-PCL blend [182] were also used for in vivo myogenesis support.

For skeletal muscle constructs grown outside the body, active tensile forces can be placed on the biomaterial to align cells in culture. Recently, through the addition of passive stress in a cultured material, engineered muscle constructs were generated by principles of anisotropic morphogenesis [215]. Further, there has been progress in creating active hydrogel materials which can create alignment through contractile biomaterials [216]–[218]. The active nature of these materials results in the alignment of cells in the direction of contraction, similar to how native muscle contracts.

2.5.5 Rehabilitation and Functional Outcome Metrics

The material, bioactive factors, cell incorporation, and biophysical properties of a tissue engineered construct are all chosen with the intent of functional muscle recovery. Clinically, the use of rehabilitative exercise regimens for skeletal muscle regeneration are prescribed to patients following lower extremity VML and has been shown to improve functional use of the limb [219], [220]. Additionally, exercise therapy has also been used in the clinic to reduce chronic inflammation [147], [221], [222], which, as discussed previously (see Section 2.3.3), is a hallmark of tissue following VML.
Preclinically, rehabilitation in rodent VML models can include voluntary running wheels, range of motion therapy, electrical stimulation, or high intensity interval training [196], [214], [223]–[225]. Following exercise treatment, function was assessed by force production by the injured muscle and muscle force generation was improved in some studies where running was used [196], [224]. It should be noted that each of these studies used the tibialis anterior as the location for VML, a muscle group which is actively used during running in rodents. These data indicate that the exercise and the muscle of interest should be chosen in a complementary way to see beneficial results. Additionally, in experiments which incorporated cell-seeded scaffolds in combination with exercise training, vascularization, innervation, and myogenesis within the scaffolds were also improved concurrently with force generation [214], [225], but in the case where no scaffold was used, there were not differences in myofiber cross-sectional area in the VML defect [224].

Measuring functional muscle recovery preclinically is often done by stimulating the innervating motor neuron and measuring the force production of the injured muscle. Stimulation protocols can be varied to measure different force production parameters including maximal isometric tetanic force, twitch force, or fatigue [206], [226], [227]. The force production of the injured muscle is typically compared to an uninjured contralateral limb to determine whether force generation has been restored. Another functional output measure which can be used to evaluate recovery following VML is the animals’ gait pattern including joint kinetics, stride length, duty cycle, and paw print area [206], [228], [229]. These analyses are useful as they can be done longitudinally throughout the healing process, unlike force generation measurements which tend to be terminal procedures. The
restoration of muscle force generation or gait patterns following treatment of VML are indicative that there has been functional muscle regeneration. However, myofiber regeneration as seen through histological tissue analyses and improved force generation do not always correlate due to force transmission across fibrotic tissue [128]. This highlights the importance of following up any functional assessments with analyses of tissue morphology.
CHAPTER 3.  DEFINITION OF A CRITICALLY SIZED VOLUMETRIC MUSCLE LOSS DEFECT IN THE MOUSE QUADRICEPS

3.1 Introduction

Skeletal muscle has a remarkable endogenous regenerative capacity, however after VML this regenerative response is diminished and results in chronic functional deficits [55], [72]. For this reason, VML remains a persistent challenge in comparison to other skeletal muscle injuries, where muscle is capable of full regeneration with virtually no fibrosis [53], [54]. VML injuries are defined as a vast loss of tissue, often due to trauma or surgery, which will not endogenously regenerate, resulting in chronic loss of function and permanent disability [110], [111]. VML is most commonly seen clinically as the result of extremity trauma injuries, and extremity trauma accounts for more than 50% of all trauma injuries among both military and civilian populations [107]–[109]. 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 [110], [118].

One major factor which 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 [54].

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These biophysical and biochemical components make up the specific microenvironment for MuSCs, also called satellite cells, a primary cell population required for efficient muscle regeneration [59]. Situated between the basal lamina and the myofiber sarcolemma, MuSCs are influenced by a unique microenvironment. The MuSC microenvironment is comprised by the myofiber [16], basal lamina [17], vasculature [18], neural components (e.g. motor neuron [19], [20], NMJ [21], [22], and terminal Schwann cells [23]), and supporting cells (e.g. fibro/adipogenic progenitors [24], [25]) which each play 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 [230]. 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 chapter 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 [112], [113], [231], however the threshold for a critical size has not yet been established. We hypothesized non-healing VML injuries reach a critical size due to significant changes in the regenerative response of the endogenous supporting ECM, myofibers, vasculature, and neuromuscular innervation.
3.2 Methods

3.2.1 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 3-9 months in age were used according to the protocols approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee.

3.2.2 Volumetric Muscle Loss Injury

Mice were anesthetized via 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. 3.1 B). Each defect consistently injured the rectus femoris, vastus intermedius and vastus lateralis, with only 3 and 4 mm injuries injuring the vastus medialis (Fig. 3.1 A). After injury, the skin was closed using wound clips. Animals were euthanized by CO₂ inhalation.

3.2.3 Tissue Wet Weight Analysis
Immediately after euthanasia, at 7, 14, or 28 day 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.

### 3.2.4 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 (VWR, 95057-844, -848) or Gomori’s Trichrome (Polysciences, 24205-1) according to the manufacturers’ instructions. Prior to antibody staining, tissue sections were blocked and permeabilized using blocking buffer (5% BSA, 0.5% goat serum, 0.5% Triton-X in 1X PBS) for 30 minutes. When anti-mouse secondary antibodies were used, an additional wash with Goat F(ab) Anti-Mouse IgG (Abcam, ab6668, 2 µg/ml in blocking buffer) was performed for 1 hour. 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 hour. 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 minutes. Alexa Fluor 647-conjugated phalloidin (Thermo Fisher, A22287) was diluted 1:250 in blocking buffer and incubated with
secondary antibodies for 30 minutes at room temperature. Slides were mounted with Fluoroshield Mounting Medium with DAPI (Abcam, ab104139) and stored at 4°C.

3.2.5 Tissue preparation for neuromuscular visualization

Thy1-YFP mice received 3 mm defects and were euthanized at 14 or 28 days post-injury (n=4). Hindlimbs were harvested and fixed in 4% paraformaldehyde for 1 hour at room temperature. Quadriceps muscles were dissected from the mouse hind limbs and placed in blocking buffer for 1 hour. 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 minutes. 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.

3.2.6 Tissue preparation for microCT angiography

Mice received 3 mm defects and were euthanized 28 days post-injury (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.

3.2.7 Second Harmonic Generation Imaging of collagen

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

3.2.8 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 20X 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 \(\mu\text{m}^2\) in area to measure muscle fiber cross sectional area. Area histograms were created in GraphPad Prism 7. Data were grouped by injury size or contralateral control, with 3 replicate measurements taken from an \(n=4\) 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 5 representative regions of each section, 3 replicates per animal (\(n=4\)) and counted using the Image J multipoint tool.

3.2.9 Imaging and quantification of neuromuscular junctions

Z-stack images were taken on a Zeiss 710 Laser Scanning Confocal using 40X objective. Z-stacks of 5 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 [232]. The number of NMJs were quantified by placement in one of three categories: (1) normal, pretzel-like morphology, (2) fragmented, or abnormal morphology, or (3) newly forming AChR clusters [233].

3.2.10 MicroCT angiography imaging and analysis

Quadriceps were dissected from fixed, Microfil perfused mice and imaged in 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 (VOI) 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 21µm bins).

3.2.11 Statistical Analysis

All statistical analyses were done in GraphPad Prism 7. Data shown as mean +/- standard error of the mean (S.E.M.) for all figures. One-way ANOVA was used to analyze defect wet weight (Fig. 3.1 B) and eMHC$^+$ fiber quantification (Fig. 3.4 H), two-way ANOVA was used to analyze the wet weight of injured quadriceps over time (Fig. 3.1 C) and centrally located nuclei (Fig. 3.4 K), 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 to one another using multiple Kolmogorov-Smirnov tests. Paired, two-tailed $t$-test was done to analyze vascular volume measurements (Fig. 3.7 C). Repeated measure
two-way ANOVA with Sidak post-hoc testing was done to analyze vessel diameter histogram (Fig. 3.7 D).

3.3 Results

3.3.1 Muscle mass following full-thickness VML model in mouse quadriceps

Figure 3.1. Various sized VML injuries in mouse quadriceps. (A) Schematic representation of 2, 3, and 4 mm biopsy punch injuries (RF: rectus femoris, VL: vastus lateralis, VM: vastus medialis, and VI: vastus intermedius). (B) Mouse quadriceps after removal of biopsied muscle tissue. From left to right, 2 mm, 3 mm, 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 percentage of contralateral control is 4.44%, 15.5%, and 32.2% for 2, 3, and 4 mm injuries, respectively. (n=12 per injury size, error bars indicate mean +/- 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 post injury 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 +/- SEM. The dotted line is the average value of contralateral control quadriceps normalized to body weight. (n= 4 for each group, # = p<0.01, † = p<0.0001 as compared to the control after two-way ANOVA and Tukey’s test post-hoc).
To determine a 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. 3.1 A,B). These injuries resulted in the removal of 4.44 ± 1.85%, 15.49 ± 2.04%, and 32.16 ± 5.14% of the total quadriceps wet weight, respectively, as compared to the contralateral control (Fig. 3.1 C). The mass loss of each of these injuries were 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. 3.1 D). After 14 or 28 days, however, only the 4 mm injury group remained significantly different (n=4, p<0.0001) from the contralateral control.

3.3.2 Qualitative histomorphology following VML of multiple injury sizes

Cross-sections stained with hematoxylin and eosin (H&E) were used to examine the general histomorphology of each injury size at 14 (Fig. 3.2 A) and 28 (Fig. 3.2 B) days post-injury, using 4 replicates for each injury size and time point. Contralateral control samples from both 14 and 28 days post-injury showed healthy skeletal muscle tissue: densely packed myofibers with peripherally located myonuclei. Injured samples showed vastly different morphology. 2 mm injuries at both time points showed myofibers with centrally located nuclei surrounding areas of small, white pockets which resemble areas of fatty infiltration. Additionally, 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 to 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 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.

![Figure 3.2](image)

**Figure 3.2.** 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 representative images of a contralateral control, 2mm injury, 3mm injury, and 4mm injury, from 14 day (A) and 28 day (B) time points. Scale bars represent 200 µm.

### 3.3.3 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 Second Harmonic Generation (SHG) microscopy to visualize collagen fluorescence and stained with the pan-macrophage marker CD68 (Fig. 3.3). Contralateral control sections at both 14 and 28 day time points (Fig. 3.3 A,B) showed little to no collagen signal, outside of the expected amount present in the myofiber ECM, in both Gomori’s and SHG imaging. Additionally, there were few CD68+ macrophages present in the contralateral control tissue. In contrast, 2 mm injuries (Fig. 3.3 C,D) showed localized
collagen deposition and macrophage infiltration at 14 days, while the surrounding myofibers remained largely unaffected.

Figure 3.3. Assessment of fibrotic response in each injury size. Quadriceps cross-sections 14 (A, C, E, G) or 28 (B, D, F, H) day time points post-injury. For each set of images (A-H) the left-hand is an image of 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 colorimetric image of a cross-section stained with Gormori’s Trichrome. The sets of images are representative sections from contralateral control (A,B), 2mm injury (C,D), 3mm injury (E,F), or 4mm injury (G,H) samples. Scale bars represent 200 μm.

After 28 days, the localized fibrosis remained, however there were few macrophages remaining. By comparison, 3mm injuries after both 14 and 28 days (Fig. 3.3 E,F) showed increased fibrosis, seen from both SHG collagen imaging as well as Trichrome staining.
Additionally, there was an increased infiltration and persistence of CD68+ macrophages out to 28 days in the 3 mm VML samples as compared to 2 mm samples or the contralateral controls. In the 4 mm VML injury animals, features of fibrosis were most prominent (Fig. 3.3 G,H). A large region of collagen fluorescence was seen at both 14 and 28 days post-injury, 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.

3.3.4 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. 3.4 A-G). Sections from the contralateral controls (Fig. 3.4 G) showed mature myofibers with peripherally located myonuclei. The signal from the dystrophin channel was used to quantify myofiber cross-sectional area. Both cumulative (Fig. 3.4 I,J) cross-sectional area histograms were generated. At both 14 (Fig. 3.4 H) and 28 (Fig. 3.4 I) 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 to both 2 mm 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 Table 3.1. 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 which is transiently expressed during the early stages of myofiber development [73], and therefore myofibers which stain positively 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. 3.4 K). At 14 days post-VML there were significantly more centrally located nuclei in all injury groups as compared to the contralateral control. By 28 days after injury, there were persistent significant differences between the 3 and 4 mm injury groups compared to 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 post-injury, there were no significant differences in the number of eMHC$^+$ fibers between any of the injury sizes (Fig. 3.4 H). 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.
Figure 3.4. Visualization and quantification of fiber cross-sectional area in each injury size.

(A-F) Representative immunofluorescence images of each time point and injury size, (G) contralateral control. Sections stained for dystrophin (green), DAPI (blue), and eMHC (red). Scale bars represent 100µm. (H) eMHC+ fibers were counted and quantified for 4 biological sample. 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 (I) and 28 (J) day time points. Data were separated by injury size. Each category (control, 2, 3, & 4mm) had n=4 individual animals with 3 replicate slides per animal. (K) Fibers with centrally located nuclei were quantified at 14 and 28 days post-injury. 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 +/- 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.

Table 3.1. Myofiber cross-sectional area descriptive statistics by injury size

<table>
<thead>
<tr>
<th>14 days post-VML</th>
<th>Control</th>
<th>2 mm</th>
<th>3 mm</th>
<th>4 mm</th>
</tr>
</thead>
</table>

50
The abundance of both eMHC staining and centrally located nuclei in 3 and 4 mm injuries indicate turnover of MuSCs post-injury. 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 (Fig. 3.5). This indicates that MuSCs are undergoing proper asymmetric division into both Pax7+ quiescent MuSCs as well as 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 non-healing 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. Additionally, a significant number of fibers with centrally located nuclei remained at 28 days post-injury. Each of these findings contributed to our determination that 2 mm injuries (4.44 ± 1.85%) were subcritical defects,

<table>
<thead>
<tr>
<th></th>
<th>28 days post-VML</th>
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<tbody>
<tr>
<td>Total Number of Values</td>
<td>32926</td>
</tr>
<tr>
<td>Median (µm²)</td>
<td>1801.70</td>
</tr>
<tr>
<td>Mean (µm²)</td>
<td>1982.01</td>
</tr>
<tr>
<td>Std. Deviation (µm²)</td>
<td>1231.77</td>
</tr>
<tr>
<td>Std. Error of Mean (µm²)</td>
<td>6.79</td>
</tr>
</tbody>
</table>
4 mm injuries (32.16 ± 5.14%) were critical defects, and that 3 mm injuries (15.49 ± 2.04%) represent a transition point from sub-critical to critically sized. This warranted further investigation into both the neuromuscular and vascular response into the injury space at this critical size.

Figure 3.5. Pax7 staining in 3 and 4 mm injuries 14 and 28 days post-VML
Representative images of IHC staining for DAPI (blue), phalloidin (grey), and Pax7 (red) are shown for 3 and 4 mm injuries at 14 (A) and 28 (B) days post-VML injury. In the injured samples, images were taken at the periphery of the fibrotic region. Scale bars represent 50 µm.

3.3.5 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 re-innervation 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. 3.6 D,E, top images)
was used to quantify the number of NMJs which fit each of the 3 categories: (1) normal, pretzel-like, (2) abnormal, fragmented morphology, and (3) newly formed AChR clusters (shown in Fig. 3.6 A). In contralateral control samples, there was an overall higher number of NMJs (Fig. 3.6 B), 100% of which were classified as normal, innervated NMJs (Fig. 3.6 C) as is apparent from the representative maximum intensity projection images (Fig. 3.6 D,E). In contrast, there were far fewer NMJs of any kind in the VML injured muscles (Fig. 3.6 B). Of the NMJs present in VML injured muscle at 14 days post injury, 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. 3.6 D) and 28 (Fig. 3.6 F) days are analogous, both with some fragmented (group 2) NMJs. Additionally, in these samples there was substantial amount of autofluorescence in the YFP/GFP channel (Fig. 3.6 D,E, bottom images), likely due to persistent fibrotic tissue.
Figure 3.6. Whole mount imaging of neuromuscular junctions in VML and contralateral control quadriceps.

NMJs were quantified by three classifications defined in (A) as either group 1, 2, or 3 in Thy1-YFP mice 14 or 28 days post 3 mm VML injury. (B) Shows mean raw count data +/- SEM for each animal and time point. (C) Depicts the same data in (B), but displayed as a percentage of total number of NMJs in each category. Representative maximum intensity projections of z-stacks taken of the control and injured experimental groups are shown for 14 (C,D) and 28 (E,F) day time points. Top images show only the BTX channel from the image to visualize the morphology of the post-synaptic AChRs. The BTX channel was used for quantification. Images including all channels shown directly below BTX images. All scale bars are 50 µm.

3.3.6 Vascularization after critical VML

Vascularization following critical VML injury was assessed at 28 days post injury (n=5). Whole animal perfusion with Microfil contrast agent was followed by MicroCT scans on each dissected quadriceps (Fig. 3.7 A,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 to the uninjured control (Fig. 3.7 C). Additionally, there were significant differences in the diameter of perfused vessels of the injured muscle compared to its contralateral control (Fig. 3.7 D), specifically vessels with diameters of 84, 105, and 168 µm. This increase in vascular volume and diameter at 28 days was confirmed with immunostaining muscle cross-sections for von Willebrand factor (vWF) (Fig. 3.7 E).

Figure 3.7. MicroCT and IHC analysis of vasculature in VML compared to control quadriceps.

(A) Reconstructed 3D heat map of µCT images from microfil perfused contralateral control and 3mm injured quadriceps, left to right, 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) and (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 µm are placed in the 21 µm bin). Counts are shown as mean +/- SEM from 5 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, † 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).
3.4 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. For our full-thickness quadriceps defect model in mice, we determined the threshold of a critically-sized defect was a 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 approximately 15% of the muscle mass. Previously it has been suggested that a 20% muscle loss is the threshold for failure of the native regenerative process [117], however the regenerative processes at a critical size is not well understood or established. As the MuSC is essential for muscle regeneration [59], 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 postVML. Additionally, there is no evidence of re-innervation 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 non-healing 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 [112], [177], [206]. Additionally, similar results have been seen in
various ECM scaffold tissue engineered strategies, where the scaffolds themselves become populated with fibrotic tissue comparable to an empty defect [172]–[174]. 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 satellite cells [127], indicating that when properly regulated, fibroblasts are a vital component in muscle regeneration. Therefore, determining a method for modulating this fibrotic response to be pro-regenerative will be essential in tissue engineering approaches for VML.

In addition to this fibrotic phenotype, the critically sized defect also 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 [114], and more recently it was shown that macrophages persist long-term post-VML [124]. In the 2 mm, non-critically sized defect, the persistent macrophage infiltration is largely resolved 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 [234], [235]. 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 [98], 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 pro-regenerative microenvironment post-VML.

Additionally, we observed a distinct temporal pattern in myofiber regeneration pattern in the critically sized VML injuries. eMHC expression was present 14 days after injury, which is delayed in comparison to other commonly studied skeletal muscle injuries; in those, eMHC expression is not typically seen after 7 days post-injury [236]–[238]. 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 injury 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 [239]. 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 which is critically sized. The fibers that are present in this area are likely a mix of myofibers that were present pre-injury as well as those which are regenerating. This is indicated in the images of injured tissue at both 14 and 28 days which shows fragmenting NMJs (class 2, Fig. 3.6 D,E) as well as newly formed AChR clusters (class 3). Fragmenting NMJs are likely those which were functional pre-injury, 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 post-injury, but will then begin to display the fragmented morphology we have shown if they are not innervated in this time frame [104]. Multiple new AChR clusters, however, will form on newly regenerating myofibers as they mature, secreting signaling factors to the motor neuron to direct innervation [49].

Our data do not show evidence of the motor neuron growing towards 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. 3.4 K), as it has been shown that myonuclei will remain centrally located until the myofiber becomes functionally mature [12]. The loss of re-innervation in the muscle regeneration process has been studied previously, both clinically [240] and pre-clinically [196], indicating similar results and which also implicate the potential for post-injury physical rehabilitation to initiate re-innervation 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 which have shown tissue engineered strategies which promote successful formation of vessels after VML injury [131], [241]. Interestingly, in our critically sized VML defect with no treatment we saw increased vascular volume as compared to 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 [57]. This would indicate that while vessels are a key component 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 pro-angiogenic signals of the resulting scar tissue.

3.5 Conclusions

The evaluation and direct comparison of multiple defect sizes in muscle is not commonly reported. In this chapter we have determined and comprehensively characterized three mouse quadriceps models of VML: one below the threshold of a critical 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 down-regulation in the fibrotic progenitor cells and controlling persistent inflammation, combined with an up-regulation of neural regeneration, that would be most beneficial for the recovery of functional skeletal muscle after VML and will be the direction of future studies.
CHAPTER 4. ALTERED DYNAMICS AND HETEROGENEITY OF IMMUNE CELL SUBTYPES FOLLOWING CRITICAL VOLUMETRIC MUSCLE LOSS

4.1 Introduction

Following acute muscle injury, skeletal muscle’s robust regenerative response relies on the prompt and proper coordination of immune cells. The cellular dynamics of myeloid and lymphoid cell trafficking into and out of the muscle coincide with each stage of the muscle regenerative process [65]. Following muscle injury, phagocytic, inflammatory monocytes and macrophages (IMs and M1s, respectively) infiltrate the tissue peaking in number between 1 and 2 days post-injury [79]. These cells aid in the removal of necrotic muscle fibers and other debris while secreting pro-inflammatory cytokines [65], [80]. These pro-inflammatory cytokines, including TNFα and IFN-γ, play a role in the regulation of skeletal muscle regeneration by promoting the activation and proliferation of a required pool of skeletal muscle progenitor cells called MuSCs [65].

Once in tissue, IMs and M1s can differentiate into anti-inflammatory or alternatively activated, monocytes (AMs) or macrophages (M2s), which peak in concentration between 4-7 days following muscle injury [81], [82]. The transition to an anti-inflammatory, interleukin (IL)-10 and transforming growth factor (TGF)-β rich environment corresponds with both a transition to M2 phenotype macrophages as well as the differentiation and growth stages of myogenesis [83]. As the skeletal muscle regenerates, myeloid cells traffic out of the tissue by two week post-injury [88]. This
regulated response of myeloid cells is crucial for the proper regeneration of skeletal muscle, as the depletion or altered polarization of macrophages has been shown to increase adipose and fibrotic tissue deposition while reducing regenerated myofiber cross-sectional area [89], [90].

In addition to myeloid derived cells, lymphoid-derived T-cells also respond to muscle injury. Peak infiltration of CD4+ and CD8+ T cells occurs at 3 days post-injury, returning to baseline levels gradually by day 14 [91]. Regulatory T cells (T\textsubscript{reg} cells) infiltrate the muscle after acute injury with similar kinetics, peaking at day 4 post-injury, to that of M2 macrophages [92]. The T cell response has been implicated in the maintenance of myeloid cell infiltration. The depletion of CD8+ T cells have been shown to reduce skeletal muscle regeneration through a reduction in the recruitment of inflammatory macrophages [91]. Similarly, depletion of T\textsubscript{reg} cells impairs muscle repair and prolongs inflammation [92], which could be attributed to need of T\textsubscript{reg} cells for the transition from M1 to M2 macrophage phenotype, the ability of T\textsubscript{reg} cells to limit IFN-\gamma and macrophage accrual, or the need for T\textsubscript{reg} derived IL-10 [92]–[94]. The regulation of myeloid and lymphoid immune cell infiltration and clearance works in concert with the stages of myogenesis for prompt and proper muscle regeneration after acute injury.

By contrast, following VML, muscle does not properly regenerate but is instead replaced by non-contractile fibrotic tissue resulting in chronic loss of function and permanent disability [54], [110], [111]. In addition to persistent fibrosis, chronic inflammation is a hallmark of VML [173], however the initial immune cell response following a critical muscle defect have not been elucidated. Previously, we worked to characterize multiple muscle biopsy punch injuries in the mouse quadriceps as a model of
VML. We found that a 2mm biopsy punch caused damage to the tissue but the muscle was able to regenerate without significant fibrotic scarring, while a 3mm injury caused persistent fibrotic scarring and inflammation through four weeks following injury [242].

The objective of this study was to characterize the early immune cell dynamics at early time points which lead to the pathological phenotype following critical VML injury. We hypothesized that critical VML would lead to persistent elevation of several immune cell populations, particularly those which are associated with promoting fibrosis.

4.2 Methods

Figure 4.1. Graphical experimental workflow for immune cell quantification and characterization following sub-critical and critical VML. Animals were given either sub-critical (2mm diameter biopsy) or critical (3mm diameter biopsy) VML injuries unilaterally to the left quadriceps. At 1, 3, or 7 days, tissue was excised, digested, and flow cytometric analyses were performed. Multi-dimensional surface marker analyses were conducted including Uniform Manifold Projection (UMAP) and Spanning-tree Progression Analysis of Density-normalized Events (SPADE).

4.2.1 Animals

C57BL/6J mice were purchased from Jackson Laboratories and were used for all experiments. Adult male mice, referred to as “young”, were on average 6.1±0.5 (mean ± standard deviation) months in age at the time of euthanasia. All animals were used according to the protocols approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee (IACUC).
4.2.2 *Volumetric muscle loss injury*

Surgical procedure performed as previously reported [242]. Briefly, the left hindlimb was prepped and sterilized. A single incision was made above the quadriceps and either a 2mm or 3mm biopsy punch (VWR, 21909-132 [2mm] or 21909-136 [3mm]) was used to make a full-thickness muscle defect. Skin was closed and muscle was left to recover without intervention for 1, 3, or 7 days before euthanasia by CO₂ inhalation.

4.2.3 *Tissue harvest and flow cytometry*

Samples were prepared for flow cytometry analysis on a FACS AriaIII flow cytometer (BD Biosciences) as previously reported [243]. Briefly, entire injured, left quadriceps were harvested and digested with 5,500U/ml collagenase II and 2.5U/ml Dispase II for 1.5 hours in a shaking 37°C water bath. The digested muscles were filtered through a cell strainer to obtain a single cell suspension. Single-cell suspensions were stained for live cells using Zombie NIR (Biolegend) dyes in cell-culture grade PBS per manufacturer instructions. Cells were stained with cell phenotyping antibodies in a 1:1 volume ratio of 3% FBS and Brilliant Stain Buffer (BD Biosciences) according to standard procedures. The following antibodies were used for T cell phenotyping: BV605-conjugated CD4 (Biolegend), BV785-conjugated CD8 (BioLegend), BV421-conjugated CD3ε (Biolegend), PerCP-Cy5.5-conjugated CD25 (BioLegend), APC-conjugated CD127 (BioLegend), and PE-conjugated FoxP3. The following antibodies we used for myeloid cell phenotyping: BV421-conjugated anti-CD11b (BioLegend), BV510-conjugated Ly6C (BioLegend), BV711-conjugated anti-CD64 (BioLegend), PE-conjugated anti-MerTK (BioLegend), PE-Cy7 conjugated anti-CD206 (BioLegend), FITC-conjugated anti-
Ly6A/E (BioLegend), APC-conjugated Lineage antibody cocktail (BD Pharmigen), APC-conjugated anti-CD31 (BioLegend), PE-Cy5 conjugated anti-CD29 (BioLegend), and PerCP-Cy5.5-conjugated anti-CXCR4 (BioLegend). Cells were then fixed in 4% PFA for 10 minutes. 30μL of CountBright Absolute Counting Beads (C36950, Invitrogen) were added per sample for absolute quantification of cell populations.

4.2.4 Biplot gating strategies

For each fluorescent stain, a tube with cells from other hindlimb muscles were digested and stained (as reported above) with all fluorophores except one for fluorescence minus one (FMO) controls. These controls were recorded and used to inform biplot gating. All biplot gating was done in FlowJo v10 software (Becton, Dickinson and Company, Franklin Lakes, NJ). Gated data were exported as cell counts and normalized to bead counts to get concentration (cells/milligram) and as .fcs files for SPADE and UMAP analyses.

Table 4.1. Immune Cell Profiling Flow Markers

<table>
<thead>
<tr>
<th>Panel 1 Cells</th>
<th>Panel 1 Surface Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>CD11b+, CD64+, Mertk+</td>
</tr>
<tr>
<td>M1</td>
<td>CD206-, Ly6C+</td>
</tr>
<tr>
<td>M2</td>
<td>CD206+, Ly6C-</td>
</tr>
<tr>
<td>Monocytes</td>
<td>CD11b+ CD64+ Mertk-</td>
</tr>
<tr>
<td>IM</td>
<td>Ly6C high</td>
</tr>
<tr>
<td>AM</td>
<td>Ly6C low</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel 2 Cells</th>
<th>Panel 2 Surface Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>CD3+</td>
</tr>
<tr>
<td>Helper</td>
<td>CD4+</td>
</tr>
<tr>
<td>Killer</td>
<td>CD8+</td>
</tr>
<tr>
<td>Regulatory</td>
<td>CD4+, CD25+</td>
</tr>
</tbody>
</table>

4.2.5 Uniform Manifold Approximation and Projection (UMAP)
UMAPs generated as previously reported [243]. Briefly, UMAP was used to embed high-dimensional flow cytometry data into a space of two dimensions, cells are visualized in a scatter plot where similarity is demonstrated via proximity to other points. Prior to UMAP dimensional reduction, each flow cytometry sample was pre-gated to select cellular subsets of interest (i.e. CD11b+ myeloid cells and CD3+ T cells) and then imported into Python 3.7 using fcsparser (https://github.com/eyurtsev/fcsparser) and Pandas 2.5. Each channel except for FSC and SSC was normalized by applying arcsinh/150. A composite UMAP projection that utilized data points from all desired samples was generated using Matplotlib (https://github.com/lmcinnes/umap). Each cell was then phenotyped by overlaying the pre-gated cell subset onto the UMAP projection.

4.2.6 Spanning-tree Progression Analysis of Density-normalized Events (SPADE)

SPADE trees generated as previously reported [243]. Briefly, SPADE was performed through MATLAB and the source code is available at http://pengqiu.gatech.edu/software/SPADE/. MATLAB-based SPADE automatically generates the tree by performing density-dependent down-sampling, agglomerative clustering, linking clusters with a minimum spanning-tree algorithm and up-sampling based on user input. The SPADE tree was generated by exporting uncompensated pre-gated live, single cells or select pre-gated cellular subsets (i.e. CD3+ T cells). The following SPADE parameters were used: Apply compensation matrix in FCS header, Arcsinh transformation with cofactor 150, neighborhood size 5, local density approximation factor 1.5, max allowable cells in pooled downsampled data 50000, target density 20000 cells remaining, and number of desired clusters 50-100, depending on cell population size.
4.2.7 Statistical Analyses

All statistical analyses were done in GraphPad Prism 8. To correct for instances of unequal variance and non-normality of cell frequency data, 2-way ANOVA with Tukey test for multiple comparisons was performed on log-10 or square root transformed data, \( p<0.05 \) considered significant. Data points displayed with outlined bars representing the mean, error bars are ± Standard Error of the Mean (SEM).

4.3 Results

4.3.1 Myeloid cells remain elevated one week following critical VML

Following subcritical (2mm) and critical (3mm) VML, myeloid cell populations were quantified at 1, 3, and 7 days post-injury via biplot gating of flow cytometry data. Monocyte (CD11b+/CD64+/Mertk-/SSC-lo) cell concentration was not different between injury sizes at 1 or 3 days post-injury, but significantly higher in critical VML compared to subcritical injury at 7 days post-injury (Fig. 4.2 A, \( p<0.01 \), n=4 biological replicates per group). When broken down into Ly6C<sup>hi</sup> (IM, Fig. 4.2 B) and Ly6C<sup>lo</sup> (AM, Fig 4.2 C) subtypes, differences were again only seen at 7 days post-injury, with critical injuries having significantly higher concentrations of IMs and AMs when compared to subcritical injury (\( p<0.01 \), n=4). The overall rise and fall of total monocytes and AMs is similar between subcritical and critical injuries, but the trajectory of IMs is different, peaking at day 3 in critical injuries but at day 1 in subcritical injuries. Compared to uninjured (UI)
muscle, all groups post-injury had significantly higher concentrations of monocytes at all time points \( (p<0.0001, \text{n}=4) \).

Macrophage \( \text{CD11b}+/\text{CD64}+/\text{Mertk}^+ \) concentrations were also quantified from flow cytometry data. Similar to monocytes, differences in macrophage concentrations were only significant at 7 days post-injury, with critical injuries having a higher concentration of macrophages than subcritical injuries \( (\text{Fig. 4.2 D, } p<0.05, \text{n}=4) \). Macrophages were then grouped into M1 \( (\text{Fig. 4.2 E, CD206}^-/\text{Ly6C}^{\text{hi}}) \) and M2 \( (\text{Fig. 4.2 F, CD206}^+/\text{Ly6C}^{\text{lo}}) \) subtypes. M1 dynamics are also visually different between subcritical and critical injuries, with M1s peaking at 1 days post-injury in subcritical injuries, but at fairly consistent levels from 1 to 7 days post-injury in critical injured tissue. By contrast, M2 macrophages showed expected cell dynamics in subcritical injuries, peaking at 3 days post-injury, but altered dynamics in critical injuries, maintaining high concentrations through 7 days post-injury. At 7 days post-injury, the concentration of M2 macrophages in critically injured tissue were significantly higher than those following subcritical injury \( (\text{Fig. 4.2 F, } p<0.05, \text{n}=4) \). All macrophage subtypes were at significantly higher concentrations in injured muscle when compared to macrophages in UI tissue \( (p<0.0001, \text{n}=4) \).
4.3.2 **UMAP dimensionality reduction reveals similar myeloid subpopulation trajectory**

*by surface marker expression*
Figure 4.3. UMAP visualization of myeloid cell dynamics by marker expression. CD11b+ live cell UMAPs. (A) Marker expression level overlaid onto each analyzed event, dark blue indicating relatively low expression, yellow indicating relatively high expression. (B) cells from uninjured (UI) animals overlaid in light green, dark grey arrow indicating observed movement of events following injury shown (C), where events overlaid in blue are from subcritical injuries and red represents events from critical injuries at 1, 3, or 7 days post-injury (left to right). D1: day 1, D3: day 3, D7: day 7, SUB: subcritical, CRIT: critical.

All CD11b+ live cell events from UI and injured animals were used to generate the UMAP space in Figure 4.3. Surface markers used to cluster events were overlayed on the UMAP (Fig. 4.3 A), where events are represented as dots colored according to the gradient shown (dark blue to yellow representing low to high expression, respectively). The marker expression overlay UMAPs can be compared to the UMAPs with the overlaid locations of cells from each group (UI: Fig 4.3B, VML: Fig. 4.3 C). The dark grey dashed arrow shown in Fig 3.4B indicates the observed general movement of cells over time through the UMAP space. Each colored dot over the light grey UMAP represents a single analyzed CD11b+ live cell event, with blue dots representing cells from subcritical injuries and red dots representing cells from critically sized injuries.
Using UMAP visualization, myeloid cells have similar cell phenotypes over time. This is indicated by the movement of events within the UMAP (grey dashed arrow), as events from subcritical and critical injuries follow similar trajectories through UMAP space. Myeloid cells qualitatively appear to show movement towards the top right portion of the UMAP space with time, indicating an increase in overall population expression of Mertk, CD206, Sca-1, CXCR4, and CD29 (Fig. 4.3 A). In both subcritical and critical injuries, a cell subset appears to move back to the location of immune cells present in UI tissue (Fig. 4.3 B, C). While overall trajectories in subcritical and critical VML are similar, the density of events are visually different. Critical injury overlays show a higher density of events within the UMAP at day 3 and day 7 (Fig. 4.3 C), indicating cell concentration differences within cells subtypes. To quantify concentration differences by subtype, SPADE trees were generated.

4.3.3 SPADE identifies unique monocyte subpopulations in critical VML
Figure 4.4. SPADE identification of heterogeneous monocyte subtypes.
All monocyte events from all biological replicates and timepoints were used to generate a SPADE tree with 50 nodes in (A), where each node is color-coded to its subtype determined by marker expression as represented in the heatmap (B). Each column in the heatmap represents a node of the SPADE tree and average relative expression at each node is shown in rows labeled at right. Each color-coded subtype which had significant differences between injury sizes are shown (C-E) with (C) as subtypes of Ly6C$^{hi}$ monocytes, (D) Ly6C$^{int}$ monocytes, and (E) Ly6C$^{lo}$ monocytes. SUB: subcritical, CRIT: critical, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

A SPADE tree generated from all gated monocytes displayed in Figure 4.4A shows the 50 nodes where monocytes were clustered. From left to right the nodes move from Ly6C$^{hi}$ to Ly6C$^{lo}$. Each node is clustered based on average marker expression, shown in the heatmap (Fig 4.4 B). Each column in the heatmap correlates to one node on the SPADE tree, with the color of the node in Figure 4.4A corresponding to the color underneath the
columns in the heatmap. The groups of nodes break down different subtypes of the larger subpopulations typically used in gating flow cytometry data which were quantified in Figure 4.2B and 4.2C. Subtypes with significant differences in concentration between injury size are shown in Figure 4.4 C-E.

Ly6C$^{\text{hi}}$ monocytes were broken into 3 subtypes based on their expression of other markers in the panel. Hot-pink Ly6C$^{\text{hi}}$/Sca-1$^{\text{hi}}$ monocytes and light pink Ly6C$^{\text{hi}}$/Sca-1$^{\text{hi}}$/CD29$^{\text{hi}}$ were both present at significantly increased concentrations in quadriceps which had received critical injuries at 3 days post-injury (Fig. 4.4 C, $p<0.05$). Similarly, Ly6C$^{\text{int}}$ monocytes – which would typically be left out of traditional gating methods in between “high” and “low” expression gates – were broken into four groups (Fig. 4.4 B). Light purple and dark purple Ly6C$^{\text{int}}$ monocytes were significantly increased in concentration at 3 days post-injury, which represent monocytes highly expressing all other measured markers (CD206, Sca-1, CXCR4, and CD29, Fig 4.4 D, $p<0.05$). Finally, Ly6C$^{\text{lo}}$ monocytes were broken into 4 subtypes (Fig. 4.4 B). Dark green Ly6C$^{\text{lo}}$ monocytes had high expression of CD206, CXCR4, and CD29 and were significantly elevated in critical injuries only at 3 days post-injury (Fig. 4.4 E, $p<0.001$). Light green Ly6C$^{\text{lo}}$ monocytes had CD206$^{\text{int}}$ expressing but showed low expression of all other markers and were present at significantly increased concentrations at 3 days post-injury (Fig. 4.4 E, $p<0.001$). The only Ly6C$^{\text{lo}}$ subtype which was significantly increased at 7 days post-injury were dark blue Ly6C$^{\text{lo}}$ monocytes which were low for all other markers (Fig. 4.4 E, $p<0.05$).

4.3.4 SPADE identifies unique macrophage subpopulations in critical VML
All macrophage events analyzed were used to generate a SPADE tree with 50 nodes (Fig. 4.5 A). Surface marker expression at each node shown in the heatmap (Fig. 4.5 B) color-codes each subtype of macrophage. The macrophage (Mφ) SPADE tree clustered nodes into 4 separate branches, with initial M1-like Mφ (Ly6C<sup>hi</sup>/CD206<sup>lo</sup>) at the top left, moving down towards an unpolarized Mφ trajectory in the bottom right, as indicated by the dotted grey arrows (Fig. 4.5 A). In the middle of the tree, the Mφ trajectory splits into an M2-like, or CD206<sup>hi</sup>/Ly6C<sup>lo</sup>, population, or into a CD206<sup>hi</sup>/Ly6C<sup>hi</sup> subtype which we refer to as “hybrid Mφ” subtype.

Based on expression of other surface markers, these typical macrophage populations were broken down further into other subtypes. Subtypes with significant differences between injury size are shown in Figure 4.5C-E. M1-like Mφs at the very end of the branch, colored yellow, which were Sca-1<sup>hi</sup>/CXCR4<sup>lo</sup>/CD29<sup>hi</sup>, showed varied dynamics between injury size. While the yellow M1-like Mφs following a subcritical injury were elevated and trend lower with time, this same population started lower and peaks at 3 days post-injury, where they are present at significantly increased concentrations following critical injuries (Fig. 4.5 C, p<0.05). Unpolarized Mφs did not show any significant differences between injury sizes. M2-like Mφs were a relatively homogeneous population by marker expression (orange colored) each node being generally Sca-1<sup>lo</sup>/CXCR4<sup>hi</sup>/CD29<sup>lo</sup> (Fig. 4.5 B). Notably, this marker expression is opposite of the yellow M1-like Mφ subtype. M2-like Mφs were significantly elevated at 7 days post-injury (Fig 4.5 E, p<0.01), as was shown with biplot gating quantification previously (Fig. 4.2 F). Hybrid Mφs were present in two locations on the SPADE tree, colored pink on the right and red on the left (Fig. 4.5A). Both hybrid Mφ subtypes had were present at significantly
increased concentrations at 7 days post-injury in critically injured quadriceps (Fig 4.5 E, \( p<0.05 \))

Figure 4.5. Macrophage SPADE tree identifies unique Mϕ trajectories and subtypes. All macrophage events from all biological replicates and timepoints were used to generate a SPADE tree with 50 nodes in (A), where each node is color-coded to its subtype determined by marker expression as represented in the heatmap (B). Nodes with similar surface marker expression were grouped as cell subtypes and those subtypes with significant differences between injury sizes are displayed in (C-E). (C) Initial M1-like Mϕ yellow subtype, (D) M2-like Mϕ, and (E) hybrid (CD206\(^{hi}\)/Ly6C\(^{hi}\)) Mϕ. SUB: subcritical, CRIT: critical, * \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \).

4.3.5 Lymphoid cells are transiently present at elevated concentrations following critical VML
Figure 4.6. T cell concentrations following subcritical and critical VML.

Biplot quantification of (A) all CD3+ T cells, (B) CD4+ helper T Cells, (C) CD8+ cytotoxic T cells, (D) CD4+ CD25+ Regulatory T cells. Cell count normalized to bead count and analyzed tissue mass to yield cells per mg. Statistics performed on log transformed data as 2-way ANOVA to determine differences between injury sizes and one-way ANOVA between UI and all groups of each injury size. UI: uninjured, SUB: subcritical, CRIT: critical, * p<0.05, ** p<0.01, # indicates significantly different from all other groups.

General T cell concentrations (cells/mg) over time were quantified using biplot gating and showed a distinct peak at 3 days post-injury in critical injuries (Fig. 4.6). Total T cell concentration quantified as all CD3+ cells per milligram of muscle was significantly higher at 3 and 7 days post-injury in critically injured quadriceps compared to subcritically injured quadriceps (Fig. 4.6 A, p<0.05). From this general T cell population, three subpopulations were quantified using biplot gating based on expression of CD4, CD8 and CD25. CD4+ helper T cells and CD4+/CD25+ regulatory T cells (T_{reg} cells) were present...
at significantly increased concentrations at 3 days post-injury in critical injuries (Fig. 4.6 B,D, \(p<0.05\)). CD8+ cytotoxic T cells were not significantly different between injury size at any time point (Fig. 4.6 C). When compared to T cell concentrations in UI quadriceps, all T cell subtypes at all days following injuries of either size were significantly increased (Fig. 4.6 A-D).

4.3.6 **UMAP visualization of T cell dynamics**

Each T cell measured from all biological replicates were used to generate a UMAP space, clustering similar cells based on surface marker expression (Fig. 4.7). Relative expression level of each measured marker was overlaid on the UMAP to visualize the subpopulations present at each location in the two-dimensional space (Fig. 4.7 A). T cells present in UI quadriceps were overlaid in green on the light grey UMAP (Fig. 4.7 B). The dotted grey arrow drawn over this UMAP (Fig. 4.7 B) indicates the observed temporal dynamics of T cells through the UMAP over time, as seen in Figure 4.7 C. T cell events from subcritical injured quadriceps are overlaid in blue, by time point (Fig. 4.7 C, top, left to right 1 to 7 days post-injury), whereas T cells from critical injured quadriceps are overlaid in red, by time point (Fig. 4.7 C, bottom, left to right 1 to 7 days post-injury). The density and location of overlaid dots give a visual representation of T cell subtype dynamics following subcritical and critical injury.

Qualitatively, T cells from subcritical and critical VML overlay in similar locations in UMAPs space with time, indicating that they have overall similar population surface marker dynamics (Fig. 4.7 C). Comparing the location of overlaid events (Fig. 4.7 C) to
the surface marker expression levels (Fig. 4.7 A), it appears that T cells increase their expression level of CD127 and CD4 over time, with CD25 remaining relatively constant moving from left to right along the dotted grey arrow (Fig. 4.7 B). While overlaid events are in similar locations in UMAP space between subcritical and critical VML, the overall density of events is higher in critical VML (Fig. 4.7 B). This increased density can be seen throughout the UMAP at day 3, there are two specific clusters on the bottom left in critical injuries at day 7. To quantify concentration differences in specific subpopulations, SPADE trees were generated for all T cell events.

Figure 4.7. UMAP visualization of lymphoid cell dynamics by marker expression. All lymphoid cell events were used to create a two-dimensional UMAP, clustering cells by marker expression similarities. (A) Marker expression level overlaid onto the UMAP, from dark blue to yellow representing low to high expression, respectively. (B) T cells in UI quadriceps overlaid onto the UMAP and a dotted grey arrow indicating observed T cell movement in (C). (C) T cells present after subcritical (top, blue) and critical (bottom, red) injuries from 1, 3, and 7 days post-injury (left to right). SUB: subcritical, CRIT: critical, D1: day 1, D3: day 3, D7: day 7.

4.3.7 SPADE identifies unique T cell subpopulations in critical VML

A SPADE tree with all CD3+ T cell events clustered at 100 nodes (Fig. 4.8 A) was used to characterize T cells subtypes, as described previously for myeloid cells. Clustering
T cells according to surface marker expression resulted in the events localizing by time point. This indicates that surface marker expression generally changes with time. The SPADE tree nodes were colored accordingly, red nodes are the initial T cell response, green are transition T cells, and blue are final T cell fates (Fig. 4.8 A). To verify the time-associated clustering, the percentage of total T cell events located in each colored region was quantified and plotted (Fig. 4.8 B). On average 79% of CD3+ T cells from 1 days post-injury, 54% of CD3+ T cells from 3 days post-injury, and 18% of CD3+ T cells from 7 days post-injury were clustered at red initial T cell response nodes from both subcritical and critical injuries (Fig. 4.8 B, left). The percentage of total T cells from 1 days post-injury in this initial response population was significantly higher than the percentage of T cells from 3 and 7 days post-injury (Fig. 4.8 B, left, $p<0.0001$). Additionally, the percentage of T cells from 3 days post-injury at red nodes was significantly higher than the percentage of T cells at 7 days post-injury (Fig. 4.8 B, left, $p<0.0001$). The same quantification was done for the green transition nodes and the blue final T cell fates nodes. The average percentage of CD3+ T cells at transition nodes from both subcritical and critical injuries was on average 14% from 1 days post-injury, 25% from 3 days post-injury, and 29% from 7 days post-injury. The percentage of T cells at transition fate nodes at 3 and 7 days post-injury were significantly higher than those from 1 days post-injury (Fig. 4.8 B, middle, $p<0.0001$). The average percentage of T cells at final fate nodes from subcritical and critical injuries was 7% at 1 days post-injury, 19% at 3 days post-injury, and 53% at 7 days post-injury, with the percentage at 7 days post-injury being statistically significantly higher than the percentage at 1 and 3 days post-injury (Fig. 4.8 B, right, $p<0.0001$). There were
no differences in the percentage of T cells between injury sizes within any time point or SPADE node grouping.

The marker expression at each SPADE node was z-scored and displayed in the heatmap in Figure 4.8C. The columns are organized from left to right in the same progression as the tree in Figure 4.8A, with the colors denoted at the bottom. A few subpopulations of interest were denoted with separate colors below the heatmap and quantified. The pink colored subtype of T cells had generally low expression of all measured surface markers as well as low forward scatter area (FSC-A) and side scatter area (SSC-A), which indicate small relative cell size and low intracellular granularity, respectively. Due to these characteristics, this subtype was determined to be unpolarized T cells. The cells present at these nodes were quantified as concentration (cells/mg) over time. There were significantly more unpolarized T cells present at 3 days post-injury in critically sized injuries when compared to subcritical injuries (Fig. 4.8 D, $p<0.01$). Marked by an orange bar on the heatmap, we recognized a subtype of CD8+ cytotoxic T cells which were low for all other markers, FSC-A, and SSC-A. These cells were present at significantly higher concentrations following critical injury at 3 and 7 days post-injury (Fig. 4.8 D, $p<0.05$). It is notable that SPADE was able to capture this population of CD8+ T cells as traditional gating did not show any statistically significant differences in CD8+ T cells between injury sizes (Fig. 4.6C).
Figure 4.8. T cell SPADE tree shows cell subtypes by marker expression over time. All T cell events clustered into 100 nodes of a SPADE tree by marker expression (A). Nodes colored according to the relative percentage of T cells present from each time point. Grey arrow indicates general movement over time following injury. (B) The percentage of total T cells from each time point within each colored region of the SPADE tree, as indicated by the colored bar and title of each graph. (C) Heatmap of z-scored expression at each node in the SPADE tree, grouped by the same colors as in A and B. Each column is one node and each row is one surface marker, FSC-A, or SSC-A, as labeled on the right. Other colored bars mark the grouped nodes for each subtype quantified in D. (D) Subtype cell counts quantified as cell concentration (cells/mg). Statistics performed on log transformed data as 2-way ANOVA to determine differences between injury sizes. SUB: subcritical, CRIT: critical, * p<0.05, ** p<0.01, **** p<0.0001.

T cells in the transition and final fate had larger cell size and higher intracellular granularity as measured by FSC-A and SSC-A (Fig. 4.8 C). A subtype of T cells within the transition population was a group of cells positive for all surface markers. This yellow all-positive T cell population was present at significantly higher concentrations at 3 and 7 days post-injury in critical injuries (Fig. 4.8 D, p<0.05). Within the nodes representing final T cell fates, there were two notable subtypes. The teal colored CD4+ helper T cell subtype also highly expressed CD127. These CD127+ helper T cells were present at significantly increased concentrations in critical injuries at 3 and 7 days post-injury (Fig 4.8 D, p<0.05).
Lastly, a group of CD127+ T\textsubscript{reg} cells (CD4+ CD25+) were also present at higher concentrations at 3 and 7 days post-injury in critical injuries (Fig. 4.8 D, p<0.05).

4.4 Discussion

Immune cell regulation following minor muscle injury is a crucial component of the regenerative process. Muscle injuries typically elicit an immune response which progresses from inflammatory to pro-regenerative as polarized immune cells influence the activation and differentiation stages of MuSCs [89], [90]. In this chapter, our data revealed key differences in the concentration and dynamics of immune cell subtypes following critical and subcritical VML. Subcritical VML elicited an immune response similar to what is expected in acute muscle injury. However, critical VML resulted in abnormal immune cell dynamics including the sustained elevation of anti-inflammatory myeloid cells and transiently elevated lymphoid cell concentrations. These results were summarized in Figure 4.9, where the average concentration data for each subtype and injury size were plotted and connected with continuous hinge functions. These plots demonstrate the altered immune response after critical VML.
Figure 4.9. Summary of immune cell response to subcritical and critical VML injuries in the first week after injury.

Cell population dynamics in subcritical and critical VML injuries represented as lines fit as a continuous hinge functions (GraphPad Prism 8) through the quantified concentration data (shown in Fig. 4.2 and 4.6) for monocytes (A), macrophages (B), and T cells (C). Axes values were kept consistent between cell populations.
Previous literature has characterized that the pro-inflammatory M1 macrophages (Mϕ) response following minor muscle injury peaks around 1-2 days post injury [79]. While our data showed M1s peak at day 1 in subcritical VML, critical VML injuries show a consistent concentration of M1 Mϕs over time through day 7 (Fig. 4.2 E). The persistence of M1 Mϕs may lead to the sustained presence of pro-inflammatory cytokines, including TNF-α and IFN-γ, which have been previously shown to maintain MuSCs in an activated, proliferative but non-differentiated state [65].

With time, the Mϕ transition from M1 to M2 polarized states is coordinated with the differentiation stage of myogenesis [83], peaking around 4 days post muscle injury [82]. Comparing these temporal dynamics to our data, we saw that M2s did indeed peak at day 3 in subcritical VML but remain significantly elevated following critical VML through day 7.

In minor injury, the elevation of AMs and M2s has been linked with improved muscle healing [83], [114]. However, our data indicate that there is a sustained presence of M1 and M2 Mϕs in critical VML. It would follow that these cells would lead to an environment rich in inflammatory and anti-inflammatory cytokines, the presence of which are known to lead to chronic inflammation and tissue fibrosis [99], [141]. Further, we characterized a population of hybrid CD206 Ly6C co-expressing Mϕs. Previously, Mϕs simultaneously expressing pro- and anti-inflammatory cytokines have characterized in chronic inflammation [98]. In future studies, we plan to investigate the role of cytokine signaling in fibrosis in VML to further our understanding of the role of persistent Mϕs in critical VML. Of particular interest would be the cytokine TGF-β1, a cytokine known to be both secreted from M2 Mϕs and promote the fibrotic differentiation of FAPs [98].
While CD206 and Ly6C expression can be used to capture bulk changes in myeloid cell phenotype, it is well known that myeloid cell polarization is far more heterogeneous than classical biplot gating can indicate [244]. The use of multidimensional reduction techniques UMAP and SPADE allowed for the in-depth investigation into surface marker expression of non-traditional myeloid markers. While there is limited understanding of the specific roles of these immune cell subsets, these data implicate important shifts in immune cell populations.

In Mφs, previous studies have linked CD29 (β1-integrin) expression with Mφ phagosome maturation in the clearance of microbes [245] which are important for their ability to clear debris following injury. Further studies would be needed to determine whether β1-integrin highly expressing myeloid cells are more phagocytic than their β1-integrin low counterparts in the context of VML. However, this seems a reasonable hypothesis as CD29 was expressed highly in both M1 and hybrid Mφs where Ly6C was highly expressed. M1 Mφs are known to be phagocytic, and phagocytosis of muscle cell debris has been implicated in promoting their switch to anti-inflammatory phenotype [81]. The expression of CD29 may provide insight into the functionality of our hybrid classification as well, implicating phagocytic capability.

A subpopulation of M1 Mφs which expressed Sca-1 were recently characterized in a model of sepsis. These Mφs secreted lower levels of inflammatory cytokines and were detrimental to animal survival. Depletion of Sca-1 Mφs in this model was shown to decrease mortality and organ damage from sepsis [246]. In our results, we do see Sca-1 expression concurrently with Ly6C, our M1 marker indicating there may be similarities between M1 subtypes in VML and the previously studied sepsis model. Future studies
would be required to explore the functionality of Sca-1+ Mϕs following VML to determine if they are detrimental to healing in models other than sepsis.

Finally, CXCR4hi Mϕs have been studied in the context of aging and injury in several organs. They have been shown to be linked to fibrosis, in part through their expression and secretion of tissue inhibitor of metalloprotease 1 (TIMP1) [247]. This is particularly relevant in the context of our injury model and results. Our data showed CXCR4 expression elevated concurrently with CD206 in M2s as well as in Ly6Cint and Ly6Clo monocytes. It is notable that CXCR4 was elevated in M2s and AMs, as these are known to express pro-fibrotic cytokines, including TGF-β1. Further, CXCR4 is highly expressed in the hybrid Mϕs, indicating a potential role for CD206+ Ly6C+ Mϕs in fibrosis. If VML results in M2s, AMs, and hybrid Mϕs which also secrete TIMP1 this could exacerbate fibrosis by downregulating ECM turnover.

In muscle healing, T cells are known to play an important role aiding Mϕ trafficking and polarization [91], [94]. Additionally, as phagocytic Mϕs populate the injury site, they are responsible for presenting antigens on their cell surface and secreting cytokines which will lead to T cell activation [248]. With these cell interactions in mind, since we see disrupted myeloid response in our data following critical VML, it would follow that there may be altered T cell dynamics as well. Overall T cell dynamics looked relatively similar in critical and subcritical injuries, peaking around 3 days post-injury. This is the time course which is generally expected for T cells following muscle injury [91]. Despite seemingly appropriate dynamics, our data do reveal significantly increased T cell concentrations following critical injuries overall and in several subtypes.
T cell characterization through SPADE allowed us to identify unique lymphoid subtypes and dynamics in critically injured muscle. Notably, while SPADE clusters cells based on surface marker expression, SPADE tree branching can also be used to indicate cell differentiation through pseudotime [243]. Indeed, we saw that SPADE clustered T cells such that cells from day 1 post-injury were clustered towards the left and cells from day 7 were towards the right, indicating that there were overall surface marker changes with time (Fig. 4.8 B). This is a similar pattern of clustering that was seen in the T cell UMAP (Fig. 4.7).

As the SPADE T cell heatmap moves from left to right with time as the SPADE tree clustered, we qualitatively observed a trend of increasing FSC-A over time. An increase FSC-A indicates increase in T cell size and increased cell size, in turn, indicates antigenic stimulation and T cell activation [249]. On the left side of the heatmap, we characterized a subpopulation of unpolarized T cells, which were low expressing for all measured surface markers, except CD3, and had low FSC-A. As these cells are present in the initial T cell response, these small, unpolarized T cells made up a large relative proportion of the T cells at 1 and 3 days post-injury in VML. Conversely, with time the proportion of T cells in the muscle which are activated (FSC-A high) increases. These dynamics from unpolarized to activated T cells with time can be interpreted in the context of our characterization of phagocytic Mϕ subpopulations. Our data may indicate an immune environment in critical VML where the persistence of phagocytic hybrid Mϕs leads to the aberrant and continued activation of T cells. Notably, the three subtypes characterized in this area of the heat map were all significantly elevated in critical injuries at day 3 and 7 post-injury (Fig. 4.8).
One such activated T cell population highly expressed all measured markers. These CD4+/CD8+ T cells have been previously reported in cancer and autoimmune diseases but are not well characterized in the literature. Double positive T cells have been previously shown to be both immunosuppressive and highly cytotoxic depending on the biological context [250]. Whether an increased concentration of CD4+CD8+ T cells is detrimental to muscle regeneration in critical VML would require additional investigation.

The two other quantified T cell populations present in the FSC-A high expressing “Final T Cell Fates” section of the heatmap also highly expressed CD127. One was a population of CD4+ helper T cells and the other a population of CD4+ CD25+ T regulatory cells. Previously, CD127, the IL-7 receptor, has been shown to be a expressed on activated T regulatory cells with their survival promoted by the presence of IL-7 [251]. The presence of CD127 on CD4+ T cells in our injury model may be indicative of the level of IL-7 present following injury. IL-7 is known to be secreted by skeletal muscle myotubes, and has been found to reduce myoblast differentiation and fusion [252]. Therefore, a possible future direction would be to measure whether there is increased IL-7 after critical VML leading to an upregulation of CD127+ T cells and downregulation of myogenesis.

The current work is limited in that our characterization of immune cell dynamics and subtype are based solely on data provided by flow cytometry, namely cell size and surface marker expression. Additionally, cell dynamics were only measured at 3 discrete time points. As immune cells concentrations are changing continuously after an injury, it could be beneficial to measure cell concentrations at additional timepoints. Particularly, in the context of critical VML, it would be interesting to measure these cell populations at later timepoints, even out to 28 days, to directly measure which cell populations are present.
during the chronic inflammatory stage. Despite these limitations, our methodology provides important groundwork in a workflow where cell subtypes, identified through biplot gating and dimensionality reduction techniques, could then be isolated using FACS. Isolated cell subtypes could be used for functional immune cell assays in vitro or for messenger ribonucleic acid (mRNA) isolation and gene expression analyses. Additionally, our results show clear differences in the overall concentration of many immune cell subtypes in subcritical versus critical VML, which provides a basis for future work in understanding the injury threshold at which endogenous skeletal muscle healing is no longer possible.

4.5 Conclusions

The results of this study indicate that the imbalance in immune cell concentration following critical VML contributes to non-healing outcomes and chronic inflammation. Relative to subcritical injuries, immune cell concentrations are elevated and populations are present for longer periods following injury (Fig. 4.9). Among the increased concentration of immune cells per tissue mass, there are also unique cell subtypes following critical injury. Modulating the subtype and concentration of immune cells following VML may be crucial to creating an environment conducive to healing and muscle regeneration.
CHAPTER 5. ABERRANT FIBRO-ADIPOGENIC PROGENITORS LEAD TO FIBROSIS AND IMPAIR MYOGENESIS FOLLOWING CRITICAL VOLUMETRIC MUSCLE LOSS

5.1 Introduction

The interaction between MuSCs and FAPs is critical to the regenerative process following skeletal muscle injury [24]. FAPs, a muscle-resident population of mesenchymal cells can differentiate into fibroblasts or adipocytes, but exist during homeostasis as non-committed progenitor cells in the space between myofibers [36]. While FAPs can contribute to the accumulation of pathological tissue in muscle, they have also been identified as regulators of skeletal muscle during homeostasis and regeneration. FAPs are known to secrete various pro-myogenic factors, including WISP1 and follistatin [38], [39], and the ablation of FAPs from the muscle has been shown to result in skeletal muscle atrophy [36]. Following muscle injury, FAPs typically proliferate, peaking around 4 days post injury, and create a transitional niche for MuSCs before returning to pre-injury levels by 14 days post-injury [24], [25]. However, during chronic damage or aging, FAPs have been shown to persist and contribute to pathological fibrotic and adipocyte infiltration [24], [37], [97].

Previous studies have shown that FAPs survival is closely related to the inflammatory state of the tissue. Pro-inflammatory macrophage secretion of TNF-α is required for efficient FAP clearance by apoptosis however, the persistent presence of both
TNF-α and TGF-β1 leads to persistent FAPs survival [98]. Environments containing both pro- and anti-inflammatory cytokines are characteristic of chronic inflammation and dysregulated macrophage polarization [99], similar to the immune environment in VML as characterized in Chapter 4. Further, FAPs have been identified as a heterogeneous cell population. In a mouse model of muscular dystrophy, a Vcam1-expressing, pro-fibrotic subtype of FAPs was previously identified to accumulate following a disruption in the immune response [253].

Outside of chronic muscle disease, the accumulation of fibrotic tissue, persistent inflammation [122], [124], [242], and chronic upregulation of TNF-α [122] and TGF-β1 are all hallmarks of VML injuries [116]. In Chapter 3, we defined a critically sized VML defect in the mouse quadriceps, in which we characterized this critical threshold by persistent inflammation, fibrosis, and a lack of re-innervation [242]. The persistent inflammation and eventual fibrosis implicate a role for FAPS in VML injury, however, this has not been well characterized. Recently, it was reported that FAPs following VML increase in number and that those FAPs have increased gene expression related to proliferation and fibrosis but no significant differences in pro-myogenic cytokines IGF-1 or follistatin [254]. While FAPs contribute to fibrosis after minor acute injury, the ablation of FAPs or fibroblasts impairs muscle regeneration [36], [127]. Further, the downregulation of fibroblasts by blocking TGF-β1 after VML did not enhance functional recovery [128]. This previous literature outlines dueling roles for FAPs as either a pro-myogenic or pro-fibrotic population. As FAPs have previously been found to be a heterogeneous population in muscle disease, it is likely that the promotion of myogenesis over fibrosis requires the balancing of FAPs subpopulations.
The objective of this study was to first characterize the temporal systemic and local response of MuSCs, FAPs, and related cytokines following critical VML. We then sought to investigate cell heterogeneity of FAPs following VML to identify a subpopulation which may contribute to fibrosis and impair myogenesis. We hypothesized that an aberrantly upregulated FAPs subpopulation would be biased towards fibrosis following critical VML.

5.2 Methods

5.2.1 Animals

B6.129S4-Pdgfra<sup>tm11(EGFP)Sor</sup>/J (PDGFRα<sup>EGFP</sup>), in which cells containing the surface marker PDGFRα have green fluorescent protein (GFP) expressing nuclei, were purchased from Jackson Laboratories and used for histological quantification and cell sorting and in vitro culture experiments. Male and female adult mice 4-7 months old were used. Pax7<sup>Cre;RsR<sup>tdTomato</sup></sup> (Pax7TdT) mice were bred by crossing Pax7<sup>tm1(cre)Mrc</sup>/J and B6.Cg-Gt(Rosa)26Sor<sup>tm9(CAG-tdTomato)Hze</sup>/J mice from Jackson Laboratories. The resulting Pax7TdT mice expressed TdTomato red fluorescent protein in Pax7+ cells following tamoxifen treatment. C57BL/6-Tg(CAG-EGFP)1Osb/J (b-actin GFP) mice expressing green fluorescent protein (GFP) under control of the b-actin promoter were purchased and bred for utilization in MuSC-FAPs transwell culture experiments. C57BL/6J mice were purchased from Jackson Laboratories and were used for cell sorting, in vitro culture experiments, and subcritical versus critical injury characterization. Adult male mice, referred to as “young”, were on average 6.1±0.5 (mean ± standard deviation) months in
age at the time of euthanasia. All animals were used according to the protocols approved by the Georgia Institute of Technology’s IACUC.

5.2.2 Volumetric muscle loss injury

Surgical procedure performed as previously reported [242] and used in Chapters 3 and 4. Briefly, mice were anesthetized with 2% isoflurane, the left hindlimb was prepped and sterilized. A single incision was made above the quadriceps and a 3mm biopsy punch (VWR, 21909-136) was used to make a full-thickness muscle defect. Skin was closed and muscle was left to recover without intervention for 1, 3, 7, or 14 days before euthanasia by CO₂ inhalation.

5.2.3 Quadriceps tissue histology and immunostaining

Tissue processing and histology done as previously reported [242]. Briefly, muscle was dissected, weighed, and snap frozen in liquid nitrogen cooled isopentane. 10 µm cryosections were taken. Samples were blocked and permeabilized before staining with Alexa Fluor 647 phalloidin (ThermoFisher, A22287, 1:250) and Alex Fluor 488 conjugated anti-GFP (ThermoFisher, A21311, 1:250) for 1-hour incubation at room temperature. Slides were mounted with Fluoroshield Mounting Medium with DAPI (Abcam, ab104139) and stored at 4°C.

5.2.4 Whole mount imaging

Hindlimbs were fixed with muscles attached in 4% PFA for 45 minutes, washed with PBS, and kept in PBS at 4°C until dissection. Quadriceps were dissected and incubated in 20% w/v sucrose overnight at 4°C. Quadriceps were washed in PBS, dried,
and pinned to a polydimethylsiloxane (PDMS) filled petri dish. Two razor blades were placed on the lateral and medial sides of the muscle and a scalpel was used to create longitudinal sections. Sections were stained in microcentrifuge tubes on a rotating rack.

5.2.5 Imaging and quantification of percent GFP+ nuclei

Images were taken at 20x magnification using a Zeiss fluorescent microscope. Images were imported into ImageJ/FIJI, converted to 8-bit, thresholded using “Moments” built in method, and then analyze particles. This was done for GFP and DAPI channels. The area occupied by particles was summed for “GFP occupied area” metric and number of GFP+ particles was then divided by number of DAPI+ particles to give “Percent GFP+ nuclei” metric.

5.2.6 Enzyme-linked immunosorbent assays (ELISAs) and Luminex multiplex cytokine assays

Media was collected at either 6 or 10 days in culture, centrifuged at 10,000xg for 5 minutes. Supernatant was collected and frozen at -80°C. MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex Assay (32-plex mouse cytokine assay, Millipore Sigma, MCYTOMAG-70K) was used with for cytokine/chemokine multiplex measurements according to kit instructions. TGF-β1 (ThermoFisher, BMS608-4) and TIMP1 (ThermoFisher, EMTIMP1) ELISAs were used according to manufacturer instructions.

5.2.7 Flow cytometry and fluorescence activated cell sorting (FACS)
Samples were prepared for flow cytometry analysis on a FACS AriaIII flow cytometer (BD Biosciences) as previously reported [243]. Entire injured, left quadriceps were harvested and digested with 5,500U/ml collagenase II and 2.5U/ml Dispase II for 1.5 hours on a shaker at 37°C. Muscle digest solution was filtered through a cell strainer. Single-cell suspensions for flow cytometry were stained for live cells using Zombie NIR (Biolegend) dyes in cell-culture grade PBS per manufacturer instructions before fixation with 4% paraformaldehyde (PFA). If cells were being sorted, antibody staining immediately followed filtration. Cells were stained with cell phenotyping antibodies. The following antibodies we used: FITC-conjugated anti-Ly6A/E (BioLegend), APC-conjugated Lineage antibody cocktail (BD Pharmigen), APC-conjugated anti-CD31 (BioLegend), PE-Cy5 conjugated anti-CD29 (BioLegend), and PerCP-Cy5.5-conjugated anti-CXCR4 (BioLegend). 30µL of CountBright Absolute Counting Beads (C36950, Invitrogen) were added per sample for absolute quantification of cell populations. For FACS, propidium iodide (PI) was added immediately before sorting for viability. Cells were sorted into whole cell growth media supplemented with b-FGF at 2.5 ng/ml. If FAPs sorting on β1-integrin expression, “β1-integrin-Hi” was considered to be the 30% highest expressing FAPs and “β1-integrin-Lo” were considered the lowest 30% expressing FAPs.

5.2.8 Fibrosis PCR Array

FAPs were isolated by FACS as described above from uninjured (UI) animals as controls, experimental groups were β1-integrin-Hi and β1-integrin-Lo expressing VML-derived FAPs. Immediately following sorting, cells were centrifuged at 300xg for 5 minutes at 4°C, supernatant was removed. Cells were lysed with 1% 2-Mercapto-ethanol
in RNeasy Plus lysis buffer (Qiagen, 1030963) and freeze-thawed 3x in liquid nitrogen. Final thawed solution was centrifuged at 18,000xg for 10 minutes at 4°C. Supernatant was collected and stored at -80°C until RNA purification. RNA purification was completed using RNeasy Mini Kit (Qiagen, 74104) according to kit instructions. RNA was diluted in RNase-Free Water (Qiagen, 1017979) normalized to cell number collected via FACS. RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, 4368814) according to manufacturer instructions with SUPERase-In RNasae Inhibitor (ThermoFisher, AM2694). Mouse Fibrosis RT²Profiler PCR Array (Qiagen, PAMM-120ZC, genes in Table A1 below) with PowerUp SYBR Green Master Mix (ThermoFisher, A25777). Cycle run per manufacturer instructions on Applied Biosystems StepOnePlus qPCR system. Results were analyzed with Qiagen supplied data analytics tools available at: https://geneglobe.qiagen.com/us/analyze/. B2m, Gapdh, Gusb, and Hsp90ab1 were used as reference genes. Data presented as Normalized Fold Regulation over average UI control expression levels.

5.2.9  Cell culture

24-well plates (Ibidi, 82401) were coated in collagen and laminin at 9 μg/ml and 10 μg/ml, respectively, in PBS for at least 1 hour at 37°C before aspirating and allowed to dry. Freshly sorted FAPs were seeded at a density of 10,000 cells/well, MuSCs were seeded at 5,000 cells/well. FAPs growth medium was DMEM (ThermoFisher, 11965-118), with 20% FBS (Atlanta Biologicals, S11150H), 1% penicillin-streptomycin (PS, ThermoFisher, 15140-122), and 1% GlutaMAX (ThermoFisher, 35050061). Each day in growth media, cells were supplemented with 2.5 ng/ml b-FGF (Sigma, F0291-25UG). MuSC growth medium was Ham’s F10 Nutrient Mix (ThermoFisher, 11550043) with 20% Horse Serum
(Atlanta Biologicals, S12150H), 1% GlutaMAX, 1% PS. MuSCs were supplemented with 2.5 ng/ml b-FGF for days 0-3 in culture. FAPs differentiation medium was DMEM, 5% HS (Atlanta Biologicals, S12150H), 1% PS, 1% GlutaMAX. 50% FAPs media by volume was changed every other day. When noted, TGF-β1 (R&D Systems, 7666-MB-005), TIMP1 (Biolegend, 593702), Ultra-LEAF anti-CD29 (Biolegend, 102235), or Ultra-LEAF isotope control (Biolegend, 400940) were added to FAPs differentiation media or directly to MuSC media. MuSCs were allowed to self-differentiate by serum consumption, media was not changed throughout the culture timeline. For transwell experiments, MuSCs were cultured as described with FAPs added in transwell after 3 days in culture. Transwells had 0.4 μm pores (VWR, 29442-129). FAPs were seeded at 10,000 cell/transwell density.

5.2.10 Partial least squares discriminant analysis and principal component analysis

Partial least squares discriminant (PLSD) computational analysis were done in MATLAB (Mathworks, Natick, MA) using the PLS or function written by Cleiton Nunes (available on the MathWorks File Exchange) according to previously reported protocols [255]. Briefly, the data were z-scored and then input into the PLS script. D-PLSRs were run for the 32-plex mouse cytokine Luminex assay and the qPCR Fibrosis Array. Orthogonal rotations were applied to maximally separate groups on latent variables in two-dimensions (LV1 and LV2) created by the PLS algorithm. LV loading plots show the mean and standard deviation of each cytokine or gene’s weighting in each latent variable. Standard deviations for loading plots were calculated by iteratively excluding and replacing samples 1000 times and calculating the D-PLSR model each time.
Principal component analysis (PCA) was done using the PCA function in MATLAB. Orthogonal rotations were applied to maximally separate groups on principal components in two-dimensions (PC1 and PC2).

5.2.11 Uniform Manifold Approximation and Projection (UMAP):

UMAPs were generated as previously reported [243]. Briefly, UMAP was used here to embed high-dimensional data into a space of two dimensions, and cells are visualized in a scatter plot, where similarity is demonstrated via proximity to other points. Prior to UMAP dimensional reduction, each flow cytometry sample was pre-gated to select for FAPs and then imported into Python 3.7 using fcsparser (https://github.com/eyurtsev/fcsparser) and Pandas 2.5. Each channel except for FSC and SSC was normalized by applying arcsinh/150. A composite UMAP projection that utilized data points from all desired samples was generated using Matplotlib (https://github.com/lmcinnes/umap). Pre-gated cell subsets were overlaid by group onto the UMAP projection to identify the clustered events.

5.2.12 Spanning-tree Progression Analysis of Density-normalized Events (SPADE)

SPADE trees generated as previously described [243]. Briefly, SPADE was performed through MATLAB and the source code is available at http://pengqiu.gatech.edu/software/SPADE/. MATLAB-based SPADE automatically generates the tree by performing density-dependent down-sampling, agglomerative clustering, linking clusters with a minimum spanning-tree algorithm and up-sampling based on user input. The SPADE tree was generated by exporting uncompensated pre-gated FAPs. The following SPADE parameters were used: Apply compensation matrix in
5.2.13 Statistical Analyses

All statistical analyses were done in GraphPad Prism 8. For continuous data with normal distributions and equal variance, students t-tests, one-way ANOVA, or two-way ANOVA were used. Where applicable, Tukey test for multiple comparisons was performed, and $p<0.05$ was considered significant. To correct for instances of unequal variance and non-normality of cell frequency data, 2-way ANOVA with Tukey test for multiple comparisons was performed on log-10 or square root transformed data, $p<0.05$ considered significant. Data points are displayed with outlined bars representing the mean, error bars are $\pm$ Standard Error of the Mean (SEM).

5.3 Results

5.3.1 Systemic cytokine changes elevated at early time points following VML

Unilateral VML injuries have mainly been studied at a local tissue level, however previous studies have shown that other unilateral muscle injuries can impact contralateral MuSCs [256]. To determine whether critical VML induces a marked systemic immune response, we measured the cytokine content in blood serum following injury using a Luminex multiplex assay utilizing highly specific fluorescent beads. Twelve cytokines
were present at detectable levels (Fig. 5.1 A). PLSD analysis was used to separate each injury size and time point along two latent variables (Fig. 5.1 B).

Figure 5.1. Blood serum cytokines fluctuate similarly over time following subcritical and critical VML.

Luminex cytokines multiplex assay was used to measure the cytokine concentration in serum of mice following critical and subcritical VML. Measured fluorescence was normalized to the level in uninjured control serum, values reported as fold change. All cytokine levels were plotted by their z score expression value in a heat map (A) with red color indicating higher expression and blue indicating lower expression. (B) PLSDA was used to reduce dimensionality and plot each sample based on their score on latent variables (LV) 1 and 2. Scores of each sample of LV1 and 2 are plotted (C, D) and the loading of each cytokine in each LV are shown (D, F). Fluorescence fold change are compared between groups for significant differences and plotted for IL-6 (G), G-CSF (H), IP-10 (I), KC (J), and MIG (K). Two-way ANOVA performed in GraphPad Prism 8 with Sidak post-hoc test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.
Individual sample scores on LV1 and LV2 were grouped by injury size and timepoint and compared (Fig. 5.1 C, E). There were no significant differences in scores between groups on LV1 (Fig. 5.1C). On LV2, there were significant differences between each time point in both subcritical and critical injuries, with scores on LV2 increasing with time (Fig. 5.1 E, \( p<0.05 \)). At day 1 post-injury, subcritical injuries had significantly lower LV2 scores than critical injuries (Fig. 5.1 E, \( p<0.05 \)). Loading plots were generated to show the relative weighting of each cytokine used to separate samples along LV1 and LV2 (Fig. 5.1 D, F). IL-1α, and MIG contributed to positive LV2 scores (Fig. 5.1 F). Conversely, IL-6, GCS-F, KC, IP-10, TNFα, IL-5, IL-13, and IL-17 contributed to negative scores of LV2 (Fig. 5.1 F).

We evaluated individual cytokines over time and between groups by normalizing the measured relative fluorescence in each group to the average of the uninjured control. IL-6, granulocyte colony stimulating factor (GCS-F), and keratinocyte chemoattractant (KC) had similar cytokine expression patterns over time in subcritical and critical injuries, with significant differences only within injury sizes between time points (Fig. 5.1 G, H, J). Interferon gamma-induced protein (IP-10), interestingly, was expressed at significantly higher levels in subcritical injuries at 1 days post-injury than at later time points in the same injury size and critical injuries at the same time point (Fig. 5.13 I, \( p<0.05 \)). Monokine induced by gamma interferon (MIG, also called CXCL9) was present at consistent levels following subcritical injury, but at levels which increased over time following critical injury reaching a concentration at 7 days post-injury which was significantly greater than 1 days post-injury (Fig. 5.1 K, \( p<0.05 \)).
5.3.2 *MuSCs and FAPs are significantly increased in critical VML injury*

Flow cytometry analyses were conducted on mice following a subcritical (2mm biopsy) or critical (3mm biopsy) injury to the quadriceps at 1, 3, or 7 days post-injury (n=4 per group, Fig. 5.2 A). Biplot gating of analyzed events was done to determine the number of MuSCs and FAPs present per milligram of muscle tissue, or cell concentration within the injured muscles. The concentration of MuSCs increased over time both subcritical and critical injuries, but at 7 days post-injury, MuSC concentration was significantly elevated in critically injured quadriceps (Fig. 5.2 B, *p*<0.01). FAPs in subcritical and critical injuries were present at relatively low levels at 1 days post-injury (Fig. 5.2 C). By 3 days post-injury, the number of FAPs in critically sized injuries increased to a level significantly greater than in subcritical injuries (*p*<0.001). Four days later, subcritically injured muscle had no significant difference in FAPs concentration to muscle at 1 day post-injury, but critical injuries had a persistent significant FAPs elevation (*p*<0.001).

![Figure 5.2](image.png)

**Figure 5.2.** Flow cytometry analysis shows significantly elevated MuSCs and FAPs at Day 7 following critical VML.

Experimental was conducted as described (A). Biplot gating was used to quantify MuSCs (B) and FAPs over time following subcritical (SUB) or critical (CRIT) injury. Cells and serum were from day 1, day 3, or day 7 (D1, D3, D7 respectively) following injury. Cells were normalized to the weight of the muscle using counting beads to normalize. Two-way ANOVAs were performed on log transformed raw data to correct for inequality in variance using Sidak post-hoc test for multiple comparisons. **=*p*<0.01, ***=*p*<0.001.
5.3.3 Activated MusC fuse adjacent to VML defect site

The dimensionality reduction technique SPADE was used to cluster MuSC flow cytometry data at all time points to determine changes in the MuSC population after injury. The SPADE tree showed a delineation between FSC-A high and FSC-A low MuSCs, which indicates a difference in cell size (Fig. 5.3 A). The events at the FSC-A high nodes were then broken down by their respective experimental groups and compared. The percentage of total MuSCs which were FSC-A high (cells which were larger in size), increased over time from 1 days post-injury to 3 days post-injury similarly in subcritical and critical injuries. However, from 3 to 7 days post-injury, the percentage of FSC-A high MuSCs remained constant in subcritical injuries but continued to increase in critically injured muscle. At 7 days post-injury, the percentage of FSC-A high MuSCs in from critical injuries was significantly greater than those in subcritical injuries (Fig. 5.3 B, p<0.001). The percentage of FSC-A high MuSCs in critical injuries was significantly elevated above the percentage in uninjured controls at all time points, whereas the same was only true in subcritical injuries at 3 and 7 days post-injury (Fig. 5.3 B, p<0.05). When MuSCs leave quiescence and become activated their cell size increases (Fig 5.3 C) [256], suggesting that critical VML specifically induces an increased concentration of predominantly activated MuSCs compared to subcritical VML.

To further investigate the fusion capacity of MuSCs following subcritical and critical VML, we utilized a mouse model with tamoxifen inducible TdTomato in Pax7 positive MuSCs. This mouse model allowed for the tracking of MuSCs. 14 days following injury, tissue was used for whole mount, longitudinal imaging (Fig. 5.3 D). CTRL muscle at both low and high magnification (Fig. 5.3 E, F) show TdTomato+ MuSCs along
myofibers, and some dimly fluorescent TdTomato+ myofibers, indicating MuSC fusion. Following subcritical injury, bright TdTomato+ myofibers can be clearly seen, even at lower magnification (Fig. 5.2 G, H). The brightest fibers were located in the middle of the quad, nearest to where the injured would have been induced (Fig. 5.3 H, middle) indicating the highest levels of MuSC fusion proximal to the injury. Conversely, further away from the injury, the fibers are less brightly red and more quiescent MuSCs can be seen along myofibers (Fig. 5.3 H, distal and proximal).
Figure 5.3. Subcritical and critical VML injuries induce MuSC activation and fusion into nearby myofibers.

A SPADE tree with 25 nodes was created to cluster MuSCs after critical and subcritical injuries over time (A), and was used to segment MuSCs into FSC-A high and low groups. The percentage of MuSCs located at FSC-A high nodes was quantified by group (B). FSC-A measures cell size, which in the case of MuSCs correlates with activation out of a quiescent state (C). Pax7TdT mice were used for whole mount longitudinal imaging (D) to track MuSC presence and fusion. Confocal images were taken of each group at different locations along the quadriceps. Sections from uninjured control (CTRL, E,F), subcritical injury (SUB, G,H), critical injury (CRIT, I,J). Two-way ANOVAs was performed using Sidak post-hoc test for multiple comparisons, ***=p<0.001, # indicates difference between uninjured (UI) control, p<0.05.
Critically injured muscle also shows bright TdTomato+ myofibers at low magnification, but noticeably these myofibers do not bridge across the defect area (Fig. 5.3 I). At higher magnification, there are bright TdTomato+ myofibers at all regions along the length of the muscle (Fig. 5.3 J distal, middle, proximal). Quiescent MuSCs can be seen in high abundance along the myofibers of distal and middle regions of the tissue, but fewer at the proximal end. Taken together, these data show that MuSCs are proliferating, activating, and fusing following both critical and subcritical VML injury, indicating that MuSCs are responding to the injury and attempting to regenerate muscle following VML.

5.3.4 FAPs make up the majority of cells within the defect 7 days following critical VML

While critical defects have a significantly elevated concentration of MuSCs (Fig. 5.2) the majority were adjacent to the defect site (Fig. 5.3). To further assess resident muscle cells in the injured tissue and in the defect region, we next focused on FAPs. To evaluate the localization of FAPs within critically injured muscle, we utilized a reporter mouse which expresses GFP within the nuclei of cells expressing PDGFRα (referred to as PDGFRα-GFP mice). Therefore, within the skeletal muscle of PDGFRα-GFP mice, tissue resident PDGFRα+ FAPs can be visualized through their GFP+ nuclei. Unilateral critical injuries were performed on PDGFRα-GFP mice and the location of FAPs at 7 days post-injury throughout the tissue was imaged (Fig. 5.4 A).
Figure 5.4 FAPs are present at an elevated percentage within the site of critical VML and throughout the injured quadriceps.

Experimental timeline (A) of PDGFRα-GFP unilateral critical VML with tissue analysis at 7 days post-injury. Quantification of cross section images as percent GFP occupied area (B) and percent GFP+ nuclei (C). Representative cross-section images (D) of contralateral control (CTRL) and critical VML (VML) injured muscle. Representative longitudinal images (E) of contralateral (left) and critical VML injured (right) tissue. Quantification of GFP+ FAPs in longitudinal sections as percent GFP+ nuclei (F) and average pixel value versus position (distal to proximal, 0-13 mm). Fluorescence signal colored so nuclei are blue, f-actin is grey, and FAPs PDGFRα-GFP+ nuclei are in green. Statistics in GraphPad Prism 8, students t-test, *=p<0.05, **=p<0.01, ***=p<0.001.

At 7 days post-injury, the location and number of GFP+ FAPs nuclei were quantified through tissue cross sections (Fig. 5.4 D) and longitudinal whole mount sections (Fig. 5.4 E). The percentage GFP+ occupied image area was quantified in contralateral control (CTRL) sections and VML injured sections. In VML injured tissue, GFP+ signal
occupied about 6% of the total image area, a significantly higher percentage than in CTRL tissue (Fig. 5.4 B, \( p < 0.001 \)). Additionally, the percentage of nuclei which were GFP+ was measured and compared between VML and CTRL sections. In the VML injury area, approximately 60% of nuclei were GFP+, indicating they were FAPs or FAPs-derived cells. This was significantly higher than in CTRL tissue, where approximately 20% of nuclei were GFP+ (Fig. 5.4 C, \( p < 0.05 \)).

Longitudinal whole mount sections allowed for visualization of FAPs throughout the length of the quadriceps muscle in critically injured and CTRL tissue (Fig. 5.4 E, CTRL: left, VML: right). The percentage of GFP+ nuclei was quantified in these sections. Approximately 15% of nuclei along the length of critically injured muscle were GFP+, significantly higher than the 5% of GFP+ nuclei in CTRL tissue (Fig. 5.4, \( p < 0.05 \)). The GFP fluorescence intensity was also measured as average pixel value and plotted along the length of the muscle from distal (0mm) to proximal (13mm) (Fig 5.4 G). The increased fluorescence GFP intensity illustrates the higher concentration of FAPs distally and within the injury compared to proximally. Overall, that this intensity measure is higher than the UI quad at all locations reinforces the qualitative observation that there is stronger GFP signal throughout the VML injured tissue. FAPs are pervasive throughout the muscle and within the defect site following critical VML.

5.3.5  *MuSC fusion is inhibited by VML derived FAPs*

Given the elevated MuSC presence within injured muscle (Fig. 5.2), MuSC fusion surrounding the defect (Fig. 5.3), and an increased concentration of FAPs in areas of non-
bridging following critical VML, we wanted to characterize the crosstalk of FAPs and MuSCs in vitro in the context of VML. MuSCs isolated from b-actin-GFP mice were cultured and allowed to self-differentiate either alone, cultured with FAPs in transwell from uninjured (UI) muscle, or FAPs in transwell from critical VML muscle (VML) (Fig. 5.5 A). The number of multinucleated myotubes and total nuclei were quantified after eight days in culture (Fig. 5.5 B,C). MuSCs showed a significant increase in myotube and nuclei number when UI FAPs were cultured in transwell (Fig. 5.5 B,C, $p<0.05$), a phenomenon which has been previously reported. However, VML derived FAPs did not result in a significant increase in myotube or nuclei number when cultured in transwell. There data indicated that after VML injury FAPs may have an altered cytokine secretory profile compared to FAPs from uninjured muscle.

To measure differences in FAPs secreted factors, we collected culture media from the transwells of each group before fixation at day 8 in culture and measured the cytokine concentrations in each group (Fig. 5.5 D). IL-6 and Eotaxin, were significantly elevated with either UI or VML FAPs in transwell compared to untreated MuSCs. Monocyte chemoattractant protein (MCP-1) and VEGF were significantly increased with VML FAPs in transwell compared to UI FAPs. Additionally, MCP-1 and G-CSF were significantly increased with VML FAPs in transwell from the untreated controls. KC secreted in the UI FAPs transwell condition was significantly elevated above untreated and VML. These differences in cell crosstalk between MuSCs and UI versus VML FAPs may be important in the progression of fibrosis rather than myogenesis in the injury environment following critical VML.
Figure 5.5. Critical VML derived FAPs do not increase myogenesis and have altered cytokine expression when cultured in transwell with MuSCs. Representative IHC images of GFP-MuSCs following culture with FAPs in transwell (A) stained for nuclei (blue), myosin heavy chain (MHC, red), and anti-GFP for amplification of b-actin-GFP signal (green). Multinucleated myotubes (B) and total nuclei (C) were counted and compared. Cytokines present in media at day 8 were measured and detected cytokines are shown (D). One-way ANOVA, Tukey’s test post-hoc, *=p<0.05, ***=p<0.001.

5.3.6 Fibrotic response of FAPs following VML to TGF-β1

After characterizing the interaction of UI and VML derived FAPs when in culture with MuSCs, we wanted to investigate the secretory and differentiation behaviors of FAPs cultured alone. These functional differences were evaluated on FAPs sorted via FACS from
UI mice or from CRIT VML PDGFRα-GFP mice and cultured in vitro (Fig. 5.6 A). Conditioned media was collected immediately before differentiation was induced at 6 days in culture, and cytokine concentrations were measured. Cell culture media from wells with VML FAPs had significantly elevated concentrations of KC, VEGF, MCP-1, and TGF-β1 compared to media from wells with UI FAPs (Fig. 5.6 B, p<0.05). Fibrotic differentiation was induced by a reduction in serum content in the media with or without the addition of TGF-β1. IHC staining following differentiation for myofibroblast marker αSMA or adipocyte marker perilipin (Fig. 5.6 C) showed that VML FAPs had a higher mean fluorescence expression of αSMA in the presence of TGF-β1 when compared to UI FAPs (Fig. 5.6 D). However, without TGF-β1 stimulus, VML FAPs had increased perilipin expression, which was significantly reduced by TGF-β1 stimulus (Fig. 5.6 E). The fraction of GFP+ nuclei was also quantified. GFP+ nuclei is indicative that FAPs still express PDGFRα. UI FAPs stimulated with TGF-β1 showed a significant decrease in the fraction of nuclei expressing GFP, but there was no such reduction in VML derived FAPs.

Following differentiation, conditioned media was collected for Luminex cytokine analysis. VML derived FAPs stimulated with TGF-β1 expressed significantly higher levels of IL-6, MCP-1, IP-10, VEGF, and LIF when compared to VML derived FAPs not stimulated with TGF-β1 (Fig. 5.6 G, p<0.05). By contrast, UI derived FAPs did not significantly increase their expression of any of these cytokines with TGF-β1 stimulus. In the case of IP-10, VEGF, and LIF TGF-β1 stimulated VML derived FAPs had significantly increased expression over stimulated UI FAPs as well. Eoxtaxin was the only cytokine which significantly decreased with TGF-β1 stimulus and did so regardless of whether they were sourced from VML or UI tissue (Fig. 5.6 G).
Figure 5.6. VML-derived FAPs have altered secretome and increased responsiveness to TGF-β1.

Experimental design (A), briefly FAPs were isolated from uninjured (UI) or critical VML injured (VML) tissue, seeded, and cultured for 10 days. Cytokine concentrations in conditioned media were measured at day 6 in culture (B) and cytokines with significant differences are shown. Representative IHC staining (C) for nuclei (blue), αSMA (red), FAPs nuclei (green), and perilipin (grey). Mean fluorescence intensity per area measured was quantified for αSMA (D) and perilipin (E) signal. The fraction of nuclei expressing GFP (F) was quantified. Cytokines in conditioned media at day 10, immediately before fixation, were measured (G) and cytokines with significant differences are shown. Statistics performed in GraphPad Prism 8, students t-test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001. Asterisks above individual groups indicates a difference between that group’s unstimulated control, stars above comparison bars indicate differences between UI and VML in either stimulated or unstimulated condition.

5.3.7 FAPs from critical VML express higher levels of β1-integrin than FAPs from subcritical VML

As FAPs following VML had an increased responsiveness to the pro-fibrotic stimuli TGF-β1 and FAPs are known to be a heterogeneous cell population, we hypothesized that a subset of FAPs might be contributing to the sustained fibrosis following
VML. To evaluate this, we looked at our flow cytometry data to determine if there was fluctuation in surface marker expression that was unique to FAPs following critical VML. We utilized two dimensionality reduction techniques, UMAP and SPADE, to look at these surface marker trends. First, with UMAP, we created a two-dimensional space which clustered all FAPs events from UI, SUB, and CRIT muscle tissue at 1, 3, and 7 days post-injury. Events from each group were then overlaid on the UMAP to visualize each groups’ clustering and movement over time. FAPs from UI muscle clustered in the bottom left corner of the UMAP (Fig. 5.7 A), but over time after injury overlaid FAPs are located more predominantly towards the upper right quadrant of the UMAP space (Fig. 5.7 B). At 1 and 3 days post-injury, FAPs from SUB and CRIT injuries are located in similar locations on the UMAP. However, at 7 days post-injury, SUB FAPs shift back to the left of the UMAP, but CRIT FAPs remain on the right. CRIT FAPs are also present at qualitatively higher densities on the UMAP than SUB FAPs, illustrating the previously quantified difference in concentration (Fig. 5.2). To visualize the phenotypic differences in FAPs clustering within the UMAP, we overlaid the surface markers used to create the UMAP. The surface marker which showed a distribution most closely matching the movement of FAPs with time was β1-integrin. When overlaid, β1-integrin expression increases from high to low in the same pattern as CRIT FAPs, from bottom left to top right (Fig. 5.7 C).

To further investigate this β1-integrin highly expressing (β1-HI) FAPs population, a SPADE tree was generated to quantify the concentration and relative percentage of β1-HI FAPs in SUB versus CRIT muscle (Fig. 5.7 D). Nodes shown in color were considered β1-HI and used for quantification. The number of FAPs per tissue mass was quantified, showing a significantly greater concentration of FAPs in CRIT injured tissue at 3 and 7
days post-injury were β1-HI compared to SUB (Fig. 5.7 E, p<0.001). Notably, the percentage of total FAPs which were β1-HI was quantified the proportion of β1-HI FAPs in CRIT injuries remained elevated at a level which was significantly higher than in SUB injured tissue (Fig. 5.7 F, p<0.001). As persistent fibrosis is a hallmark of critical VML, this population of FAPs which was specifically increased following CRIT injury warranted further investigation as a potential pro-fibrotic subpopulation.

Figure 5.7. Dimensionality reduction techniques UMAP and SPADE identify a subpopulation of FAPs in critically injured muscle which highly express β1-integrin. A UMAP was generated for all FAPs analyzed via flow cytometry and overlaid with events from each group, uninjured muscle (A, UI, black), subcritical injured (SUB, blue) and critical injured (CRIT, red) (B). Grey dotted line drawn on after to illustrate the β1-integrin high region, shown by the surface marker expression overall (C). A SPADE tree was generated (D) from the same flow cytometry data used for the UMAP. Colored nodes were considered β1-integrin highly expressing and quantified as concentration (cells/mg, E) and percentage total FAPs (F). Statistics performed in GraphPad Prism 8, two-way ANOVA with Sidak test post-hoc, ***=p<0.001, ****=p<0.0001.
5.3.8  \( \beta_1 \)-integrin expression is associated with pro-fibrotic gene expression in VML FAPs

Figure 5.8 Partial least squares discriminant analysis of FAPs qPCR mouse fibrosis array separates UI, \( \beta_1 \)-HI, and \( \beta_1 \)-LO FAPs on two latent variables. Gene expression measured via qPCR for FAPs isolated from uninjured (UI) or critically injured tissue segmented into \( \beta_1 \)-integrin high expressing (\( \beta_1 \)-HI) and \( \beta_1 \)-integrin low expressing (\( \beta_1 \)-LO) was analyzed using PLSDA and plotted according to their scores on two latent variables (LV) 1 and 2 (A). LV1 scores plotted and compared (B) and some select high and low gene loadings on LV1 are shown (C). The same was done for LV2 (D, E). One-way ANOVA with Tukey’s test for post-hoc, \(*=p<0.05, **=p<0.01.\)

FAPs at 7 days post-injury from CRIT tissue were sorted based on their \( \beta_1 \)-integrin expression. Isolated \( \beta_1 \)-HI and \( \beta_1 \)-LO cells were then used to determine differences in gene expression and in vitro differentiation. A mouse fibrosis qPCR array was used to quantify differences in gene expression in UI, \( \beta_1 \)-HI, or \( \beta_1 \)-LO FAPs. 84 genes were evaluated, and dimensionality reduction techniques were used to determine differences between groups and which genes contributed to those differences. PLSDA, a supervised clustering technique, was used first to look for differences between the 3 measured groups. UI, \( \beta_1 \)-
HI, and β1-LO separated along two LVs, with injured versus uninjured separating along LV1 and β1-HI versus β1-LO separating along LV2 (Fig. 5.8 A). β1-HI and β1-LO FAPs had significantly higher LV1 scores than UI FAPs (Fig. 5.8 B, p<0.01). On LV2, β1-HI FAPs had significantly higher scores than both UI and β1-LO FAPs (Fig. 5.8 D, p<0.05). The relative contributions of each gene to each latent variable can give indication of the meaning of each of these LV scores (Fig. 5.8 C, E). Of note, genes for collagen 3 (Col3a1), tissue inhibitor of metalloproteases (TIMP1), thrombospondin 1 (Thbs1), and β1-integrin (IGTB1) contributed to higher scores on LV1 and LV2; suggesting these genes may be upregulated with injury and with β1-integrin expression. On LV1 specifically, the Tnf gene was associated with low scores, indicating that UI FAPs express more TNF than VML derived FAPs. On LV2 specifically, the genes for αSMA (Acta2) and TGF-β1 (Tgfb1) were both associated with higher scores and therefore with β1-HI FAPs. Conversely, Mmp3 was one gene associated with β1-LO FAPs specifically.

A principal component analysis (PCA) clustering algorithm was also used to analyze the qPCR array data. PCA is an unsupervised algorithm in which groups are not given in advance to maximize clustering effects. This can give more powerful indications of differences between groups. With PCA, UI, β1-HI, and β1-LO separated along a single principal component (PC, the PCA equivalent of a latent variable, Fig. 5.9 A). Scores for each sample on PC1 and PC2 were compared (Fig. 5.9 B, C) and scores for PC1 were significantly different between all groups (Fig. 5.9 B, p<0.05). Scores on PC1 increased for each group increased from UI to β1-LO to β1-HI. There were no differences between groups on PC2. The loading plot for PC1 revealed similar genes of interest for β1-HI FAPs to those from PLSDA including Timp1, Acta2, and Tgfb1.
Figure 5.9. Principal component analysis of FAPs fibrosis qPCR array separates UI, β1-LO, and β1-HI FAPs on one principal component. Gene expression measured by qPCR from isolated FAPs from uninjured (UI) or injured tissue, with injured FAPs segmented into β1-LO and β1-HI expressing groups was used for PCA and each sample was plotted according to its principal component (A). Scores on PC1 (B) and PC2 (C) were plotted and compared. Differences were seen along PC1, so the loading plot for relative gene contribution to PC1 for select genes is shown (D). One-way ANOVA with Tukey’s test for post-hoc, *=p<0.05, **=p<0.01.

As clustering methods were able to separate UI, β1-HI, and β1-LO FAPs, we then directly compared the expression levels of specific genes. Values are reported as normalized fold regulation relative to UI FAPs. Genes with notable differences based on β1-integrin expression are shown in a heatmap (Fig. 5.10 A) and those genes with significant differences were plotted individually (Fig. 5.10 B, p<0.05). Tgfb1, the gene for the pro-fibrotic growth factor TGF-β1, was significantly elevated in β1-HI versus β1-LO
FAPs ($p<0.001$). Thbs1 encodes thrombospondin 1 which can activate latent TGF-β1 and is also upregulated in β1-HI compared to β1-LO FAPs ($p<0.05$). Timp1, Col1a2, Serpine1, Thbs2, and Lox were all significantly upregulated in β1-HI compared to β1-LO FAPs ($p<0.05$). These genes are for proteins which collectively contribute to ECM deposition and cross-linking. Acta2 and Cav1 were also significantly elevated in β1-HI FAPs compared to β1-LO FAPs ($p<0.01$) and are related to cell structure. Acta2, specifically, encodes αSMA, a marker of myofibroblast differentiation (used in Fig. 5.6 IHC). The only gene which was significantly downregulated in β1-HI FAPs was a gene for ECM degradation, Mmp3. These data indicate β1-HI FAPs may be more biased to differentiate down a fibrotic lineage and promote a pro-fibrotic environment.

Figure 5.10. β1-HI FAPs have significantly higher expression of pro-fibrotic genes. Heatmap showing relative gene expression from qPCR analysis quantified as normalized fold regulation (A). Significantly different genes are plotted (B) and labelled as “Protein Name (Gene Name)”. Paired student’s t-test performed for biologically matched samples, *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$.

5.3.9 β1-HI FAPs are more responsive than β1-LO FAPs to pro-fibrotic TGF-β1
To further investigate the β1-HI subpopulation of FAPs following VML, we cultured β1-HI and β1-LO from PDGFRα-GFP mice *in vitro* under the same growth and differentiation protocols as in Section 5.3.6. The goal of these experiments was to determine whether VML derived FAPs would have altered cytokine secretion or differentiation potential dependent on β1-integrin expression. GFP+ nuclei were tracked over time to quantify proliferation (Fig. 5.11 A). There were no differences in proliferation between β1-HI and β1-LO FAPs. The concentration of TGF-β1 in the culture media was quantified after 6 days in growth media; there were no significant differences in TGF-β1 levels in the media between β1-HI and β1-LO FAPs (Fig. 5.11 B). However, when stimulated with TGF-β1, β1-HI FAPs expressed more αSMA than the unstimulated control, indicating increased myofibroblast differentiation (Fig. 5.11 D, *p*<0.01). Conversely, β1-LO FAPs did not show a significant increase in αSMA when treated with TGF-β1 (Fig. 5.11 D, *p*>0.05). β1-HI and β1-LO FAPs showed a similar reduction in the fraction of GFP+ nuclei when treated with TGF-β1 compared to the unstimulated controls (Fig. 5.11 E, *p*<0.05).
β1-HI FAPs show increased myofibroblast differentiation when stimulated with TGF-β1 than β1-LO FAPs. The GFP+ nuclei per well in each group were imaged and quantified over time (A) as a surrogate measurement for proliferation over time. The concentration of TGF-β1 present in media at day 6 in culture was measured (B) immediately before the differentiation protocol was started. Representative images after differentiation with or without TGF-β1 stimulus (C) IHC staining performed for αSMA (red) nuclei (blue), FAPs nuclei (green), and perilipin (grey). αSMA fluorescence was quantified per area of images taken (D) and the fraction of GFP+ nuclei per total nuclei was calculated (E). Two-way ANOVA, Sidak test post-hoc, *=p<0.05, **=p<0.001. Asterisks indicate differences within groups, stimulated vs. unstimulated control.

**5.3.10 TIMP1 may play a role in pro-fibrotic signaling following critical VML**

From our qPCR and in vitro differentiation analyses, β1-HI FAPs appeared to be a pro-fibrotic subset of FAPs derived from CRIT VML. While TGF-β1 has been shown here and in previous studies to be a clear pro-fibrotic stimulus in FAPs culture [37], the stark increase in β1-HI TIMP1 gene expression (Fig. 5.10) drove us to investigate whether TIMP1 played a role in FAPs differentiation. While TIMP1 is typically studied for its role in inhibiting the degradation of ECM proteins through the enzymatic action of MMPs, it has recently been studied for its ability to function as a signaling molecule [257]. In order
to impact intracellular cell signaling, TIMP1 binds a surface receptor complex of β1-integrin and CD63 [163].

Figure 5.12. TIMP1 is secreted by β1-HI FAPs and increases pro-fibrotic differentiation of FAPs when added with TGF-β1. Mean fluorescence intensity of CD63 staining as measured by flow cytometry of UI and VML FAPs (A) or of β1-HI and β1-LO FAPs (B). TIMP1 concentration in conditioned media from UI and VML (C) or β1-HI and β1-LO FAPs (D). Representative IHC images of UI FAPs cultured with either TGF-β1, TIMP1, neither, or both (E) with staining for nuclei (blue), FAPs nuclei (green), αSMA (red), perilipin (grey). Mean αSMA fluorescence (F) and the fraction of GFP+ nuclei (G) were quantified. One-way ANOVA was used with Tukey’s test (F) or Dunnett’s test (G) post-hoc, *=p<0.05, **=p<0.01, ****=p<0.0001.

Surface expression of CD63 was measured via flow cytometry on the surface of FAPs which were from either UI or critical VML injured tissue. CD63 expression was significantly upregulated in VML versus UI FAPs (Fig. 5.12 A, p<0.01). Additionally, FAPs from UI and VML tissue gated on β1-integrin expression showed a significant increase in CD63 fluorescence in β1-HI FAPs (Fig. 5.12 B, p<0.001). To investigate whether TIMP1 gene expression resulted in increased TIMP1 secretion, the concentration of TIMP1 was measured in the cell culture media of UI or VML derived FAPs at day 6 in culture. VML FAPs were found to secrete significantly increased levels of TIMP1 in culture (Fig. 5.12 C, p<0.01). VML-derived FAPs which were sorted based on β1-integrin
expression also had significantly different TIMP1 secretion in vitro, with β1-HI FAPs secreting more TIMP1 than their β1-LO counterparts (Fig. 5.12 D, p<0.001).

To compare the pro-fibrotic signaling capabilities of TIMP1 to TGF-β1, UI FAPs were cultured for 6 days before beginning differentiation in media containing TGF-β1, TIMP1, or both. IHC staining was done to measure FAPs differentiation (Fig. 5.12 E) and αSMA expression increased significantly when FAPs were stimulated with TIMP1 and TGF-β1 over TGF-β1 alone (Fig. 5.12 F, p<0.01). TIMP1 alone did not significantly increase αSMA expression (Fig. 5.12 F). TGF-β1 with or without TIMP1 reduced the fraction of PDGFRα-GFP+ nuclei similarly when compared to unstimulated FAP controls (Fig. 5.12 G).

5.3.11 Anti-Integrin-β1 blocks fibrotic differentiation induced by TGF-β1

While it has been characterized that β1-integrin is required for TIMP1 signaling, β1-integrin has also been shown to play a role in kidney and liver fibrosis in the context of TGF-β1 stimulation [258], [259]. We therefore wanted to investigate whether inhibiting binding to β1-integrin using a blocking antibody (anti-ITGB1) would decrease the fibrotic differentiation of FAPs stimulated with TGF-β1. Following FAPs differentiation with TGF-β1 with or without anti-ITGB1 supplemented in the media, IHC staining was done, and the mean αSMA fluorescence was quantified (Fig. 5.13). These results showed FAPs cultured with TGF-β1 had a significant increase in αSMA fluorescence, consistent with previous experiments (Fig. 5.13 D, p<0.05). However, when treated with both TGF-β1 and anti-ITGB1, FAPs did not have a significant increase in fibrotic differentiation compared
to the untreated control (Fig. 5.13 D, p>0.05). These preliminary results, consistent with findings in tissue fibrosis of other organs, provides a potential target for reducing fibrotic differentiation of FAPs following critical VML.

Figure 5.13. Anti-integrin-β1 blocks pro-fibrotic differentiation of FAPs. FAPs were isolated, cultured, and differentiated before fixation and IHC staining. Representative images are shown for FAPs with no additives (A), TGF-β1 (B), or TGF-β1 plus an antibody for Integrin-β1 (Anti-ITGB1, C). Staining was for nuclei (blue), αSMA (red), and ITGB1 (white). Mean αSMA fluorescence was quantified (D). One-way ANOVA, Tukey’s test post-hoc, *=p<0.05.

5.3.12 TGF-β1 and TIMP1 impact the growth and differentiation of MuSCs in vitro
Figure 5.14. MuSCs cultured with TGF-β1 do not fuse to form multinucleated myotubes. Representative images of MuSCs cultured with TGF-β1, TIMP1, neither, or both following fixation and IHC staining (A) stained for nuclei (blue), myosin heavy chain (MHC, red), and f-actin (grey). The number of multinucleated myotubes (B) and nuclei (C) were quantified. One-way ANOVA, Dunnett’s test (B) and Tukey’s test post-hoc (C), *=p<0.05, ****=p<0.0001.

Following our characterization of TIMP1 and TGF-β1 as pro-fibrotic signaling molecules secreted by β1-HI FAPs, we wanted to investigate whether these cytokines may play a role in the inability of VML derived FAPs in transwell to increase the number of multinucleated myotubes (Fig. 5.5). MuSCs were cultured with TGF-β1, TIMP1, neither, or both (Fig. 5.14 A), as was done with FAPs (Fig. 5.12). MuSCs culture with TIMP1 alone did not cause a significant change in the number of multinucleated myotubes compared to untreated MuSCs (Fig. 5.14 B, p>0.05). However, MuSCs cultured with TGF-β1 were unable to fuse to form any multinucleated myotubes, a significant reduction compared to the untreated control (Fig. 5.14 B, p<0.0001). When comparing the number of nuclei, neither TGF-β1 nor TIMP1 alone caused a significant change in nuclei from the untreated
control. However, when TGF-β1 and TIMP1 were added concurrently, there was a significant reduction in the number of nuclei compared to the TGF-β1 alone treated MuSCs (Fig. 5.14 C, *p*<0.05). Taken together, these data may indicate that TGF-β1 and TIMP1 could be working to slow MuSC proliferation and differentiation following VML.

### 5.4 Discussion

VML results in substantial fibrosis rather than functional muscle regeneration. While FAPs have been identified as a source of fibrosis in muscle myopathies, they have not been specifically studied in the context of VML. In these experiments we have laid the groundwork for understanding the interplay between FAPs and MuSCs following a critical VML injury. While there is a high overall concentration of MuSCs and evidence of fusion events following critical injury, we show that the injury environment has an abundance of pro-fibrotic FAPs that secrete signaling molecules which are simultaneously pro-fibrotic and anti-myogenic. These data implicate a role β1-integrin and TIMP1 in the chronic fibrotic process and therefore may be novel targets following VML reduce pro-fibrotic FAPs and enhance muscle regeneration.

While critical VML is defined by the lack of muscle regeneration in the defect, the impact of VML on endogenous MuSCs has not been well characterized. We measured an overall increase in the concentration of MuSCs in critically injured VML at 7 days post-injury (Fig. 5.2), which we then were able to segment into activated (FSC-A high) and inactivated (FSC-A low) MuSCs (Fig. 5.3) [260]. Finding a significantly higher proportion of activated MuSCs following critical VML is consistent with our previous data which
showed that critical VML injury resulted in prolonged muscle regeneration (see Chapter 3). This sustained activation of MuSCs could be due to a number of factors, including a sustained inflammatory environment, which is known to activate and drive proliferation of MuSCs [261]. Additionally, there was substantial endogenous MuSC fusion adjacent to both critical and subcritical injuries (Fig. 5.3). While MuSC activation and fusion are required for proper muscle regeneration, in cases of chronic inflammation or aging, continuous activation can lead to MuSC depletion and impaired regeneration over time [262].

FAPs are a resident skeletal muscle progenitor population which both of support MuSC function but also drive fibrosis and adipogenesis. With an understanding of the activation of MuSCs and location of fusion events following critical VML, we then sought to determine the relative abundance (Fig. 5.2) and location (Fig. 5.4) of FAPs in these injuries. FAPs concentration increased in muscle from critical VML injuries and FAPs nuclei made up the majority of nuclei within the VML defect site. While one study has previously reported the localization of FAPs nuclei to the VML defect [254], this is the first report comparing FAPs concentration in subcritical and critical VML. Our data indicate that FAPs may play an important role in the switch from recoverable (subcritical) to irrecoverable (critical) VML injury. Additionally, finding an abundance of FAPs where there is a lack of muscle regeneration and prolonged immune cell presence led us to ask questions about the role of FAPs’ paracrine signaling following critical VML. More broadly, measurements of systemic cytokine presence also may contribute to the chronic inflammation and fibrosis characteristic of critical VML.
Both circulating and locally secreted cytokines can play important roles in muscle regeneration and, conversely, chronic fibrosis and inflammation. While several cytokines were measured throughout our experiments, a subset of those were significantly elevated. First, we found that IL-6 was elevated systemically in blood serum following critical and subcritical injuries at early timepoints and is reduced over time (Fig. 5.1). Additionally, IL-6 was found at elevated levels during FAPs differentiation and when FAPs were co-cultured with MuSCs in transwell (Fig. 5.5, Fig. 5.6). Increased IL-6 signaling through STAT3 has previously been characterized to be associated with muscle atrophy and FAPs accumulation following denervation, in skeletal muscle following spinal cord injury, and in muscle with amyotrophic lateral sclerosis (ALS) [263]. Additionally, IL-6 was found to be a driver of chronic inflammation and fibrosis in the peritoneum [264]. If, as we found in vitro, FAPs undergoing fibrotic differentiation following VML secrete additional IL-6 in vivo, this may be yet another molecule biasing the environment towards fibrosis. However, the impact on myogenesis is less clear from our data, as increased levels of IL-6 were found when both UI and VML FAPs were cultured in transwell with MuSCs. It is known that muscle secretes IL-6 following exercise, and has been associated with muscle growth and hypertrophy [265]. These dichotomous roles suggest that the function of IL-6 may be context dependent.

G-CSF is another cytokine which was found at high levels at 1 days post-injury systemically, regardless of injury size (Fig. 5.1). Following tissue injury, G-CSF recruits granulocytes and promotes the maturation to neutrophils [266]. The presence of G-CSF in serum would indicate recruitment of neutrophils, which is an expected and crucial part of the muscle regenerative process. When FAPs were cultured in transwell with MuSCs there
was an elevation in G-CSF as well, but this was only significantly different in VML derived FAPs (Fig. 5.5). Previously, the G-CSF receptor has been detected on myoblasts in regenerating muscle and the addition of exogenous G-CSF was reported to increase myoblast proliferation in vivo in regenerating muscle [267]. The detected expression of G-CSF only in transwell culture experiments may illustrate that there is crosstalk between MuSCs and FAPs promoting G-CSF secretion for muscle regeneration. This may also contribute to the sustained MuSC activation and proliferation seen in critical VML.

KC, also called C-X-C motif ligand (CXCL)1, was detected in blood serum and followed similar trends in critical and subcritical injuries, with the highest concentration at 1 days post-injury (Fig. 5.5). In vitro, KC was significantly increased when UI FAPs were cultured in transwell with MuSCs, but when cultured alone, VML derived FAPs secreted more KC than UI FAPs. This may indicate crosstalk with MuSCs impacts FAPs secretion of KC, in an injury dependent manner. In the context of tissue fibrosis KC has been shown to promote the chemotaxis of neutrophils, and, when KC-dependent recruitment was blocked, pulmonary fibrosis was reduced [268]. KC reduction was correlated with pulmonary fibrosis reduction following treatment with a TGF-β1 inhibitor [269]. However, similar to IL-6, KC has also been shown to be secreted from muscle following exercise and have metabolic benefits [270].

We found that IP-10, also called CXCL10, was only increased in serum following subcritical injury at 1 days post-injury. However, IP-10 was maintained at a constant level in the serum of critically injured mice (Fig. 5.1). In contrast, we also found that IP-10 was upregulated in VML derived FAPs when stimulated with TGF-β1 (Fig. 5.6). IP-10 is involved in T helper (Th1), monocyte, and natural killer cell recruitment and regulation
following skeletal muscle injury and in inflammatory myopathies [271], [272]. The dynamic presence of IP-10 specifically in subcritical injury may indicate a dysregulated recruitment of inflammatory cells following critical VML. IP-10 has been reported to increase MuSC differentiation [272] and, conversely, amplify Th1 infiltration and chronic muscle inflammation [271]. While IP-10 secretion from skeletal muscle cells can stimulated by inflammatory cytokines TNFα and interferon-gamma (IFNγ) [271], we see IP-10 upregulated in VML FAPs after stimulus with an anti-inflammatory cytokine, TGF-β1. It is possible that IP-10 is secreted as compensatory mechanism for FAPs to attempt to down-regulate fibrosis. A similar result was seen in renal fibrosis where a blockage of IP-10 resulted in increased fibrosis and TGF-β1 expression [273]. However, chronic inflammatory conditions, such as VML, are characterized by an upregulation in both traditionally inflammatory and anti-inflammatory cytokines. Therefore, the precise role of IP-10 in the context of critical VML will require further investigation.

The cytokine MIG, or CXCL9, significantly increased over time in critical, but not subcritical, VML injuries (Fig. 5.1). MIG has the same receptor as IP-10 (CXCR3) and is similarly associated with macrophages and T cells in inflammatory myopathies [274]. Serum increases in MIG have been clinically associated with juvenile idiopathic arthritis, a disease which is linked to macrophage activation syndrome (MAS), and uncontrolled and dysfunctional immune response [275]. While MAS is a severe, systemic condition unlike the local immune dysregulation characterized in critical VML (see Chapter 4), the increase of MIG over time in blood serum implicates potential systemic inflammatory effects of critical VML that have not yet been elucidated and may exacerbate chronic inflammation and fibrosis within the muscle itself.
Both systemic and local cytokines may impact the overall healing outcomes in critical VML. While these data do not directly measure the cell source of systemic cytokines, the relative concentration of immune cells in the muscle at day 1 post-injury (Chapter 4) is higher than FAPs (Fig. 5.2). However, it is possible that elevated serum cytokines at 1 day post-injury could be acting to mobilize FAPs within the muscle to proliferate and migrate to the injury, which could explain the increased number of FAPs throughout the muscle as seen in our longitudinal sections (Fig. 5.4). Subsequently, increased cytokine secretion from FAPs within the injury site may then play a role in perpetuating local chronic inflammation, through factors such as IL-6, G-CSF, IP-10, MIG, and MCP-1.

To directly measure the cytokine secretion of FAPs we utilized conditioned media from in vitro experiments. VEGF, a cytokine which is a crucial promoter of angiogenesis across tissue types, showed consistent significant elevation in vitro in VML derived FAPs. When cultured in transwell with MuSCs (Fig. 5.5) and when cultured alone before differentiation, VEGF was present at similar levels (Fig. 5.5 and 5.6), however following stimulation with TGF-β1 VEGF levels increased in VML derived FAPs 20-fold over unstimulated FAPs, significantly higher than the level from culture of UI FAPs (Fig. 5.6). VEGF has been most extensively characterized as a potent promoter of angiogenesis, however it has also been studied for its dysregulated role in tissue fibrosis [276]. VEGF has been shown to stimulate fibrotic differentiation by increasing αSMA and Col1a1 in liver cells in vitro [277]. In future studies we would like to determine whether the increase in VEGF in culture media of VML derived FAPs is also driving pro-fibrotic differentiation of these cells. While VEGF-A was measured in this study, previous studies have found an
effect of VEGF-C on fibrogenesis through activation of the TGF-β1 pathway and increased secretion of TIMP1 in cardiac myofibroblasts [278]. VEGF-A and VEGF-C share a common receptor, VEGFR-2 [279], therefore FAPs expression of VEGF-C and VEGFR-2 specifically would be relevant in this context. VEGF has also been studied in the context of MuSCs and has been shown to play a role in the maintenance of MuSC quiescence [44]. If the secretion of VEGF is increased by VML FAPs in vivo as it is in vitro, this may play an additional role in reducing myofiber fusion in the VML defect space.

Similar to VEGF, MCP-1 also was significantly elevated in VML derived FAPs in vitro. MCP-1 is a chemokine known to be produced by many cell types, including fibroblasts and immune cells, and aids in the regulation and recruitment of monocytes and macrophages [280]. In the context of chronic inflammation and fibrosis, MCP-1 and TGF-β1 have been shown to stimulate expression of one another via positive crosstalk, resulting in excessive inflammation and ECM deposition [281]. In muscular dystrophy, a reduction in the presence of chronic M1 polarized macrophages resulted in reduced MCP-1 in the muscle and increased muscle repair [138]. Our results indicate FAPs may be an additional source of MCP-1 in VML injured muscle, which may feed forward the recruitment of immune cells and TGF-β1 within the muscle and ultimately impair muscle regeneration.

A member of the IL-6 family of cytokines [282], LIF was found to be upregulated only in VML derived FAPs stimulated with TGF-β1. In muscular dystrophy, leukocytes with increased transgenic expression of LIF was found to reduce M2 phenotype macrophages, TGF-β signaling, FAPs, and overall fibrosis [283]. While this may seem counterintuitive, it is possible that differentiation to myofibroblasts stimulates the release of LIF as a compensatory mechanism which under normal healing circumstances could
properly limit tissue fibrosis. Since we still see substantial myofibroblast differentiation in VML derived FAPs even with this increase in LIF, the concentration of LIF may not be high enough to have anti-fibrotic effects in this setting. Additionally, since LIF signals through the same receptor as IL-6 and activates downstream STAT3 [284], it is likely there is crosstalk between LIF and IL-6 in critical VML and potentially overlap in the effect of IL-6 STAT3 signaling in FAPs accumulation. STAT3 has also been reported to have downstream crosstalk with TGF-β1 activated Smad3 [285]. Understanding the downstream effect of LIF from FAPs in VML will require additional studies.

Eotaxin is a chemokine which drives the recruitment of eosinophils. Eotaxin was detected at elevated levels in both UI and VML FAPs when cultured in transwell with MuSCs as well as following differentiation. Notably, with the addition of TGF-β1 during differentiation suppressed the expression of eotaxin. In muscular dystrophy, cytotoxic eosinophils and eotaxin are present at elevated levels in the muscle, but ablation of eosinophils did not improve muscle quality indicating a non-driving role of eosinophils in muscle damage in this disease [286]. Whether shifting the muscle cytokine profile away from the recruitment of eosinophils contributes to fibrosis in vivo warrants further studies.

TGF-β1 and TIMP1 were measurable both in conditioned media and via qPCR for mRNA expression in FAPs from UI or VML injured tissue. The increased secretion of both TGF-β1 and TIMP1 in VML versus UI FAPs suggested that VML derived FAPs are polarized towards myofibroblast differentiation. TGF-β1 is considered to be the main driver of myofibroblast differentiation of FAPs [150]. TIMP1 has been primarily characterized for its activity as an inhibitor of MMPs, which can limit cell migration and exacerbate fibrosis by preventing the degradation and remodeling ECM proteins [287].
Recently, TIMP1 has also been found to work synergistically with TGF-β1 in the development of myocardial fibrosis and increased collagen deposition [166]. In order to function as a signaling molecule, TIMP1 binds to a CD63 and β1-integrin surface receptor complex [163]. Notably, we found that β1-integrin highly expressing FAPs were present at significantly higher concentrations specifically in critical compared to subcritical injuries. Additionally, we saw increased CD63 surface expression in VML derived FAPs, which correlated with increased β1-integrin expression. Fibrosis gene expression levels were analyzed using PLSDA, which showed Tgfb1 and Timp1 gene expression were both associated with higher scores on LV1. This is notable as UI vs. VML derived FAPs separated along LV1, with VML derived FAPs having significantly higher LV1 scores than UI FAPs.

Fibrosis gene expression analyses were also performed to assess changes in gene expression within two subpopulations of VML derived FAPs, either β1-integrin high or expressing (β1-HI or β1-LO, respectively). PLSDA showed separation of FAPs based on β1-integrin expression along LV2 (Fig. 5.8). Notably, Tgfb1 and Timp1 were associated with high scores on LV2, indicating further enrichment of these genes in the β1-integrin high subpopulation. In addition to clustering by the supervised PLSDA algorithm, we utilized an unsupervised PCA algorithm. PCA showed separation of each group of FAPs along one principal component, PC1 (Fig 5.9). FAPs scored from low to high on PC1 from UI, to β1-LO, to β1-HI. Again, higher scores on PC1 were associated with higher levels of Tgfb1 and Timp1 gene expression. When directly compared on the normalized fold expression of Timp1 and Tgfb1, β1-HI FAPs had significantly higher expression of both
genes (Fig. 5.10). This common observation of upregulated TIMP1 in addition to TGF-β1 in a specific subset of β1-HI FAPs is one which has not been previously characterized.

Several other genes were strongly associated with β1-HI and β1-LO FAPs in the loadings plots of PLSDA and PCA. Some of these showed significant differences in normalized fold regulation when β1-HI and β1-LO FAPs were compared. These comparisons were done in a pairwise manner as β1-HI and β1-LO FAPs were isolated from the same 4 biological samples. Two additional genes which were upregulated in β1-HI FAPs were Col1a2, a gene for collagen type I, and Acta2a, the gene for αSMA. Collagen type I has previously been reported to be expressed by FAPs [125] and is known to be increased in the intrafibrillar space in chronic muscle fibrosis [288]. It has been shown previously that as FAPs differentiate to myofibroblasts, expression of αSMA also increases [24], as was demonstrated in the current work. In contrast to the increased expression of TIMP1 and Col1a2 in β1-HI FAPs, Mmp3 was upregulated in β1-LO FAPs. MMP3 has been found to degrade a variety of ECM proteins including collagen, laminin, and fibronectin [289]. There were a variety of additional fibrosis related genes which showed differential expression based on β1-integrin segmentation which broadly support the hypothesis of β1-HI FAPs as a pro-fibrotic FAPs subtype.

Thbs1 & 2, genes which encode the proteins thrombospondin (TSP) 1 and 2, respectively, were both significantly elevated in β1-HI FAPs. TSP1 is an ECM protein which is known to be an activator of latent TGF-β1 [290], [291]. In addition, TSP1 and 2 have been shown to suppress the ECM degradation activity of MMPs [292], [293]. Taken together, an increase in TSP1 and 2 may intensify fibrosis following VML by increasing the availability of active TGF-β1 and inhibiting the degradation of ECM proteins.
Serpine1, the gene for plasminogen activator inhibitor (PAI)-1, was expressed at significantly higher levels in β1-HI FAPs. PAI-1 is a potent inhibitor of plasminogen activators; plasminogen, in turn, is the inactive form of the active plasmin. Plasmin works to degrade fibrin or can activate proteases downstream which will degrade other ECM proteins [294]. By inhibiting the activation of plasminogen, pathological elevation of PAI-1 can lead to the accumulation of fibrin and other ECM proteins in tissue fibrosis [295]. Both pro- and anti-inflammatory signaling through TGF-β1 [296], [297] or TNFα [298], respectively, can lead to an upregulation of Serpine1 transcription. Plasminogen activator has been shown to be required for MuSC fusion, and blockage of this through PAI-1 has been found to hinder muscle regeneration [299].

Another gene significantly upregulated in β1-HI FAPs was Lox, which encodes for lysyl oxidase (LOX) protein. LOX proteins react with ECM proteins, including elastin and collagen, to induce the formation of lysine-derived crosslinks and increases the stiffness of the ECM [300]. Clinically, LOX expression was found to be associated with idiopathic pulmonary fibrosis [301]. With the increase in Col1a2 and Lox in β1-HI FAPs, this subpopulation may help to elucidate the mechanism which leads to tissue stiffening which has been reported following VML [170].

Cav1 showed significant upregulation in β1-HI FAPs but is downregulated in β1-LO FAPs. Caveolin-1 (Cav-1), the protein encoded by the Cav1 gene, is an integral cell membrane protein found in caveolae. Caveolae play important roles in cell signaling, endocytosis, and cell-matrix interactions [302]. Cav-1 has been shown to play multiple roles in the context of fibrosis. Numerous studies have found Cav-1 to be inversely correlated to TGF-β1 [303], [304] and treatment with overexpression of Cav-1 has been
shown to ameliorate fibrosis in the lung [305]. However, Cav-1 also plays an important role in focal adhesions and has been found to co-localize with β-integrins [306]. Through this mechanism, an increase in Cav-1 in β1-HI FAPs may be aiding the increased levels of β1-integrin to form successful focal adhesions with the surrounding ECM.

β1-integrin has been shown to be a receptor for multiple ligands, not only TIMP1 but also TGF-β1. As mentioned previously, TGF-β1 is found in vivo in a latent form, bound to latency associated proteins (LAP). These LAPs have RGD-peptide sequences and have been shown to bind to various integrins, including β1-integrin with αV and α8, which could serve to localize TGF-β1 near the surface of β1-HI FAPs [307], [308]. This local reservoir of latent TGF-β1 could be activated through TSP-1 [290], also secreted by β1-HI FAPs, or through contraction of the ECM [309]. Integrins have also been shown to behave synergistically TGF-β1 signal transduction [310]. Targeting β1-integrin with blocking antibodies reduced the capacity of TGF-β1 activation in vitro and in vivo [258], [259]. Our results indicate that blocking β1-integrin reduced TGF-β1 induced myofibroblast differentiation of FAPs in vitro, however the mechanism of this phenomenon was not determined. Whether there was an effect blocking TIMP1 binding, directly interfering with TGF-β1 signaling, or impairment of TGF-β1 activation are all potential explanations.

As TGF-β1 and TIMP1 were characterized for their in vitro effect of enhancing FAPs fibrotic differentiation, we assessed whether these specific cytokines had an impact on MuSC proliferation and fusion. TGF-β1 has previously been shown to inhibit myogenic differentiation [311]. We were able to replicate these results as we saw no MuSC fusion when treated with TGF-β1. TIMP1 has not been directly studied for its impact on MuSC proliferation, but a small molecule MMP inhibitor, SB-3CT, has been shown to reduce
MuSC motility [312]. In other cell types, TIMP1 has varied effects on cell proliferation. In fibroblasts, TIMP1 was shown to promote cell growth [313], but TIMP1-mediated cell cycle arrest reduced breast epithelial cell growth [314]. We tested TIMP1 in combination with TGF-β1 on MuSCs and saw the same inhibition of fusion consistent with TGF-β1 treatment alone. However, the addition of TIMP1 resulted in a significant decrease in the number of MuSC nuclei. This indicates a potential negative effect on MuSC proliferation when TIMP1 and TGF-β1 are present simultaneously.

These results lay the groundwork for understanding pro-fibrotic FAPs and their effect on muscle regeneration following critical VML. However, there are several limitations of the current work. Our studies focused on FAPs at day 7 following critical VML, at which point we characterized abnormal sustained FAPs concentration and elevated β1-HI subtype. However, FAPs are clearly a dynamic population, which if measured at a different time point would likely exhibit differences in gene expression and cytokine secretion. Further, there are numerous cytokine and gene differences which were not fully explored in this work. To understand the role each of these factors has in VML related fibrosis and myogenesis they would need to be studied individually. Additionally, the characterization of altered gene expression would need to be verified at the protein level to determine if there is translation of the measure mRNA in FAPs following VML. Lastly, each of these genes and cytokines are components in intracellular signaling cascades, the downstream effects of which were not evaluated in these studies that we hope to further elucidate in the future.

5.5 Conclusions
Our results provide insight at the cellular level as to the response of two progenitor populations, MuSCs and FAPs, following a critical VML injury in the mouse quadriceps. We have provided an understanding of the temporal regulation of each of these populations as well as their relative locations within an injured muscle. We have shown that non-healing outcomes results despite a significant increase in activated MuSCs following critical injury and elucidated possible cell crosstalk which may, in part, drive poor outcomes and substantial fibrosis. Our characterization of a FAPs subpopulation which highly expresses β1-integrin and CD63 on their cell surface and remains significantly elevated following critical VML. These VML derived FAPs were shown to have a distinct secretome and differentiation behavior in vitro, and β1-HI FAPs specifically were seen to have a distinct gene expression profile. This work implicates an aberrant, pro-fibrotic FAPs subpopulation as a driver of chronic fibrosis and inflammation while impairing myogenesis within VML injured tissue (Fig. 5.15).
Figure 5.15. β1-HI FAPs summary and working hypothesis. As discussed in the text, β1-HI VML derived FAPs have distinct secretome and gene expression profile which may prolong inflammation by increasing immune cell recruitment, promote fibrotic differentiation of FAPs, increase ECM deposition, and impair myogenesis following critical VML. TIMP: tissue inhibitor of metalloproteases, PAI: plasminogen activator inhibitor 1, TSP: thrombospondin, MMP: matrix metalloprotease, Cav1: caveolin 1, LOX: lysyl oxidase, TGF: transforming growth factor, IL: interleukin, VEGF: vascular endothelial growth factor, G-CSF: granulocyte colony stimulating factor, KC: keratinocyte chemoattractant, MIG: monokine induced by interferon gamma, MCP: monocyte colony stimulating factor.
CHAPTER 6. CELL-LADEN ALIGNED COLLAGEN SCAFFOLDS LEAD TO FUNCTIONAL IMPROVEMENT IN CRITICAL VOLUMETRIC MUSCLE LOSS

6.1 Introduction

VML presents a distinct clinical challenge with limited treatment options. While skeletal muscle has robust endogenous regenerative capacity, following the frank loss of tissue which constitutes VML patients experience chronic functional deficits and disability [111]. The standard of care for VML injuries is autologous free or rotation muscle flap transfer and while these procedures can aid in wound closure and prevent the need for amputation there are a number of associated complications at both the donor and graft sites [110], [118]. Due to the lack of options for treatment, there have been numerous attempts to design tissue engineered therapeutics for use in VML injuries.

While there have been various materials designed for use in VML, the three main design criteria of relevance to the current chapter are scaffold type, scaffold structure, and the presence or absence of cells. There are two main categories of materials for tissue engineered skeletal muscle: synthetic or natural. While synthetic materials, typically polymers, have the advantage of being more tunable and scalable, natural derived materials, typically ECM derived proteins have the benefit of being both recognizable by the host and enzymatically degradable without alteration [179]. Collagen sponges have been previously used as a scaffolding material in muscle [176], [191] and is a protein which natively gives structure to the skeletal muscle ECM [14].
The multinucleated myofibers of the skeletal muscle are arranged in a tightly aligned fashion to produce force along the axis of the muscle. Typically during muscle regeneration, MuSCs proliferate, migrate, and fuse within the aligned ECM that remains following myofiber necrosis [72]. Porous collagen scaffolds have been developed which have aligned microstructure which have been shown to give contact guidance cues to tenocytes [315] may have promise as a scaffolding material for skeletal muscle as well [316]. Previous preclinical studies utilizing aligned matrices have had success in improving functional muscle regeneration and vascularization in vivo following VML [214].

Strategies with or without the incorporation of exogenous cells have both been studied in the context of VML. As MuSCs are required for the regeneration of myofibers [59], various muscle progenitor populations have been used in tissue engineered scaffolds. Primary isolated quiescent MuSCs have the greatest engraftment potential in vivo [195], but low cells yields make transplantation a challenge. Primary isolated myoblasts (PMs), distinct from MuSCs as they are isolated by sequential plating in vitro, are not quiescent but still retain the ability to engraft into host muscle of syngeneic animals without eliciting immune response [199], [200]. With the advantage of higher cell yields while maintaining engraftment potential, PMs were chosen as the cell source for this study.

The overall objective of this chapter was to test whether a pre-seeded aligned collagen scaffold would encourage myofiber regeneration across a VML defect and thereby improve muscle function. We hypothesized that cell seeded aligned collagen would improve force generation of VML injured muscle over blank scaffolds.

6.2 Methods
6.2.1 **Animals**

B6.Cg-Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J} mice constitutively expressed TdTomato fluorescent protein were used as transplantation recipients in PEG-4MAL studies. C57BL/6-Tg(CAG-EGFP)1Osb/J (b-actin GFP) mice expressed GFP under control of the b-actin promoter were used as MuSC donors in PEG-4MAL studies. Pax7^{Cre};RsR1^{IslTdTomato} (Pax7TdT) mice were bred by crossing Pax7^{tm1(cre)Mrc/J} and B6.Cg-Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J} mice from Jackson Laboratories. The resulting Pax7TdT mice expressed TdTomato red fluorescent protein in Pax7+ cells following tamoxifen treatment. The resulting Pax7+ MuSC derived primary myoblasts were isolated from these mice were used in transplantation experiments. C57BL/6J mice were purchased from Jackson Laboratories and were used for VML injured empty defect, aligned collagen blank, or collagen + cells recipients. All animals were used according to the protocols approved by the Georgia Institute of Technology’s or Atlanta Veteran’s Affairs Medical Center’s IACUC depending on location of the study.

6.2.2 **PEG-4MAL hydrogel MuSC transplantation**

PEG-4MAL hydrogels were fabricated and utilized for cell transplantation as previously described [183]. Briefly, four-arm PEG-4MAL macromer (molecular weight, 22,000 or 10,000; Laysan Bio) was dissolved in 1× phosphate-buffered saline (PBS) containing 10mM Hepes (pH 7.4). Cell adhesive peptide (GRGDSPC, >95% purity; GenScript) were dissolved in 1× PBS containing 10 mM Hepes and added to PEG-4MAL solution to produce functionalized PEG-4MAL precursors. Freshly isolated MuSCs were then added to the solution containing functionalized PEG-4MAL precursors. To synthesize cell-
encapsulated hydrogels, the solution containing functionalized PEG-4MAL precursors and cells was mixed with protease-degradable cross-linking peptide (GCRDVPMSMRGGDRCG; GenScript) immediately before transplantation into the VML defect. MuSCs were isolated using FACS as previously described in Section 5.2.7. PEG-4MAL degradation study was conducted by incorporating AlexaFluor 750 conjugated RGD peptide into the gel solution.

6.2.3 Primary myoblast (PM) isolation

Primary myoblasts (PMs) were isolated through a modified pre-plating method previously described [317]. Briefly, hindlimb muscles were minced and digested in 0.2% collagenase type II (Worthington, LS004176) in high glucose DMEM (ThermoFisher, 11965-118) at 37°C for 1 hour. Digestion was stopped by adding 2x volume of PBS once digested muscle could easily be passed through a 23G syringe needle. Cells were pelleted and resuspended in cell culture media. Culture media was high glucose DMEM, 20% FBS (Fisher, MT35011CV), 1% GlutaMAX (ThermoFisher, 35050061), 1% penicillin-streptomycin (ThermoFisher, 15140-122). Cells were plated sequentially to isolate PMs, first on a rat tail collagen (0.1 mg/ml) coated plate for 16 hours and then the supernatant of the first plate is seeded on a new Matrigel (VWR, 47743-715, at 1:50 dilution) coated plate for 48 hours. Cells are then detached with trypsin-EDTA, plated on a new collagen dish, shaken every 5-10 minutes 6-10 times, before plating the supernatant on a new Matrigel dish containing the purified PMs.

6.2.4 Aligned collagen scaffold preparation and hydration
Aligned collagen-glycosaminoglycan (GAG) scaffolds were created as previously described [315]. Briefly, a suspension of 1.5 g of type 1 collagen from bovine achilles tendon (Sigma C9879) was homogenized in 250 ml of 0.05 M acetic acid for 1 hour. A solution of 0.133 g of chondroitin sulfate sodium salt from shark cartilage (Sigma C4384) in 50 ml of 0.05 M acetic acid was added drop wise to collagen solution. The collagen-GAG slurry was lyophilized after overnight hydration at 4˚C in 6mm wide wells in a polytetrafluoroethylene (PTFE)-copper mold. Unidirectional heat transfer was promoted by the thermal conductivity difference between the PTFE and copper of the mold. This heat transfer difference resulted in porous collagen scaffolds with aligned microstructure [211].

Prior to cell seeding, scaffolds were cut to 3mm in height then treated with a crosslinking and hydration protocol as previously described [315], [318]. Scaffolds were then immersed in 100% ethanol overnight, washed with PBS, and further crosslinked using carbodiimide chemistry in a solution of 1-ethyl-3-[3- dimethylaminopropyl] carbodiimide hydrochloride (EDAC) and N-hydroxysulfsuccinimide (NHS) at a molar ratio of 5:2:1 EDAC:NHS:COOH. Scaffolds were then placed in PM cell culture media for 48 hours prior to cell seeding.

6.2.5 Collagen scaffold seeding

Cells (either C2C12 myoblasts [Fig. 6.1] or PMs [all in vivo studies]) were seeded on aligned collagen or collagen sponge. Pelleted cells were split into 20 μl droplets in aliquots with the total cell seeding density for one scaffold. Six-well plates were coated with sterile 2% w/v agarose to prevent cell adherence to the well bottom. Hydrated collagen
scaffolds were tapped on a sterile absorbent wipe to eliminate excess culture media prior to placement on the agarose. 10 μl of the cell solution was seeded on one side of the scaffold and allowed to adhere at 37˚C for 30 minutes. After the initial seeding, the scaffold was flipped over and the remaining 10 μl of cell solution was seeded and allowed to incubate and adhere for 1-2 hours before filling the well with 6 ml of cell culture media. For *in vitro* studies, cells were cultured for several weeks until myotubes formed. For *in vivo* studies, cells were left to culture on scaffolds for 1 week prior to transplantation.

6.2.6 *Volumetric muscle loss injury and scaffold implantation*

Surgical procedure performed as previously reported [242]. Briefly, the left hindlimb was prepped and sterilized. A single incision was made above the quadriceps and a 3mm biopsy punch (VWR, 21909-136) was used to make a full-thickness muscle defect. Scaffolds were kept in culture media at 37˚C until approximately 5 minutes before implantation. At that time, scaffolds were transferred to 1 ml aliquots of sterile saline to rinse cell culture media. Scaffolds were then tapped on sterile gauze to eliminate excess saline, and placed into the defect in alignment with muscle fibers of the quadriceps. Skin was closed and muscle was left to recover for 14 or 28 days before torque testing and euthanasia by CO₂ inhalation.

6.2.7 *Isometric torque testing and analysis*

Quadriceps function was assessed isometric torque about the knee, with a set-up and protocol adapted from previous studies in rat quadriceps VML [206]. Briefly, mice were anesthetized with 2% isoflurane. Hindlimbs were shaved and a 1 cm incision was made in the skin on each side to expose the quadriceps and medial vasculature and motor
neurons. The femoral nerve was isolated from the surrounding connective tissue. Mice were fixed in place in a supine position on the testing box and the ankle of one limb at a time was secured to a force transducer (Harvard Apparatus No. 60-2996) underneath the animal such that the knee was secured at a 90° angle. The femoral nerve was stimulated with a nerve cuff attached to a Grass S11 Stimulator set to 0.2 ms pulse duration, 5.7 ms pulse interval, and 500 ms train duration. The stimulus was directed through a Grass Stimulus Isolation Unit (SIU) before connecting to the nerve cuff. Force data was recorded using a data acquisition board (USB-1608G) and DASYLab software. Voltage was varied until maximal torque was measured, then 3 trials were recorded.

Data analysis was done using MATLAB (Mathworks, Natick, MA). The findpeaks function was used to pick out the 3 peaks from the testing file. Voltage values were converted to force and then multiplied by an average mouse tibia length (2 cm) to get torque about the knee.

6.2.8 Quadriceps tissue histology and immunostaining

Tissue processing and histology done as previously reported [242]. Briefly, muscle was dissected, weighed, and snap frozen in liquid nitrogen cooled isopentane. 10 µm cryosections were taken. Samples were blocked and permeabilized before staining. Primary antibodies used included anti-COL1A1 (Santa Cruz, sc-293182, 1:100), PDGFRa (Cell Signaling Technology, 3174, 1:200), and vWF (abcam, ab6994, 1:200), diluted in blocking buffer for 1 hour at room temperature. Secondary antibodies used included Alexa Fluor anti-Mouse 647 (ThermoFisher, A21236, 1:250), Alexa Fluor anti-Rabbit 647 (ThermoFisher, A21245, 1:250), or Alexa Fluor anti-Mouse 488 (ThermoFisher, A11029,
Conjugated antibodies used included Alexa Fluor 647 phalloidin (ThermoFisher, A22287, 1:250), Alex Fluor 488 phalloidin (ThermoFisher, A12379, 1:250), and Alexa Fluor 647 Bungarotoxin (ThermoFisher, B35450, 1:200) for 30 minute incubation at room temperature. Slides were mounted with Fluoroshield Mounting Medium with DAPI (Abcam, ab104139) and stored at 4°C.

6.2.9 Whole mount imaging

Hindlimbs were fixed with muscles attached in 4% PFA for 45 minutes, washed with PBS, and kept in PBS at 4°C until dissection. Quadriceps were dissected and incubated in 20% w/v sucrose overnight at 4°C. Quadriceps were washed in PBS, dried, and pinned to a polydimethylsiloxane (PDMS) filled petri dish. Two razor blades were placed on the lateral and medial sides of the muscle and a scalpel was used to create longitudinal sections. Sections were stained in microcentrifuge tubes on a rotating rack.

6.2.10 Imaging and Quantification

Images of scaffolds and tissue cross sections were taken on a Zeiss 710 confocal microscope. Directionality of myotubes seeded on scaffolds was quantified using the Directionality plugin on Fiji-ImageJ (written by Jean-Yves Tinevez, available on GitHub). The number of TdTomato myofibers within scaffolds were quantified in Fiji-ImageJ by converting exported TdTomato channel images to 8-bit, cropping the image so only the area where the scaffold was located would be analyzed, thresholding using the MaxEntropy method, and then Analyze Particles function. TdTomato+ fibers outside the scaffold were counted by eye with the Multi-point tool.
6.2.11 Statistical Analyses

All statistical analyses were done in GraphPad Prism 8. For continuous data with normal distributions and equal variance, students t-tests, one-way ANOVA, or two-way ANOVA were used. Where applicable, Tukey test for multiple comparisons was performed, $p<0.05$ considered significant. Data point displayed with outlined bars representing the mean, error bars are ± Standard Error of the Mean (SEM).

6.3 Results

6.3.1 PEG-4MAL hydrogel promotes MuSC engraftment but not bridging across defect

PEG-4MAL hydrogels with GFP+ MuSCs were transplanted into critical VML defects of TdTomato mice. GFP+ MuSCs in saline were used controls. Whole mount longitudinal sections (Fig. 6.1 A) from 28 days following injury and transplantation showed a qualitatively higher number of GFP+ myofibers when MuSCs were transplanted in PEG-4MAL compared to in saline. However, from H&E cross sections it was observed that the PEG-4MAL hydrogel did not allow for myofiber bridging across the defect (Fig. 6.1 B). This was confirmed by stitched longitudinal images of the PEG-4MAL hydrogel condition (Fig. 6.1 C), where there is substantial engraftment into the tissue surrounding the defect, indicated by green and yellow myofibers, but no evidence of myofibers bridging through the defect. The number of GFP+ myofibers was quantified in each condition (Fig. 6.1 D), but limited sample size in this initial study resulted in no significant differences.
To investigate the degradation of the PEG-4MAL gel over time, Alexa Fluor 750 conjugated RGD was incorporated into the PEG-4MAL gel before transplantation. Free conjugated-RGD in saline was used as a control. 28 days following injury and transplantation, whole quadriceps were imaged (Fig. 6.1 E) and there was significantly increased fluorescence in the PEG-transplanted muscle (Fig. 6.1 F, p<0.05). This indicated that the PEG-4MAL hydrogels were not being fully degraded over a 28-day period in vivo.

Figure 6.1 PEG-4MAL hydrogels do not promote myofiber bridging in VML. GFP+ MuSCs were transplanted with or without PEG-4MAL hydrogels. Longitudinal (A) and H&E cross sections were compared (B). Representative stitched longitudinal image (C) of a quadriceps 28 days after GFP MuSC containing PEG-4MAL transplantation. GFP+ myofibers were quantified from longitudinal images (D). Fluorescently labeled PEG-4MAL gels were used to measure degradation, representative images were taken (E) and signal was quantified (F). Statistics in GraphPad Prism 8, unpaired t-test, *p<0.05.

6.3.2 Aligned collagen scaffolds promote aligned, fused myotubes in vitro

The use of a porous, natural biomaterial was next investigated. C2C12 myoblasts were cultured on aligned collagen scaffolds. Following C2C12 culture and differentiation on unaligned or aligned collagen scaffolds, myotubes were evaluated for their directional
alignment. Staining for nuclei, f-actin, and MHC revealed many mature, MHC positive, multinucleated myotubes on aligned collagen compared to unaligned collagen (Fig. 6.2 A, B). Additionally, the directionality of myotubes on aligned collagen was quantified. The angle of actin filaments of the myotubes was measured, binned, plotted in histograms, and fit with a gaussian distribution (Fig. 6.2 C, D). The center of the gaussian fits were compared and unaligned collagen scaffolds had a significantly higher variance in their distribution than aligned collagen. Myotubes on aligned collagen were all centered near the angle of scaffold alignment at 0˚ (Fig. 6.2 E, p<0.01). The goodness of fit of the gaussian curves were also significantly different between aligned and unaligned scaffolds. Aligned scaffolds had significantly increased goodness of fit, with a value of 1.0 being a perfect gaussian distribution about the axis of alignment (Fig. 6.2 F, p<0.05).

Figure 6.2. Aligned collagen scaffolds significantly increase myotube alignment *in vitro*. Representative maximum intensity projections of confocal images of myotubes cultured on collagen scaffolds (A), nuclei in blue, f-actin in green, myosin heavy chain (MHC) in red. Angle of directionality of f-actin images binned in histograms (C, D), black bars are
the histogram values, red line is gaussian fit. Center of gaussian fits of multiple scaffolds (E) and the goodness of fit (F) were quantified and compared. F test used to determine differences in variance (G), #=$p<0.01$, Welch’s t test to correct for unequal variance used to compare goodness of fit (F), *=p<0.05.

In preparation for \textit{in vivo} studies, it was necessary to determine the survival and differentiation ability of PMs on aligned collagen. PMs were needed for cell seeded \textit{in vivo} studies as they are capable of engraftment into the host muscle with minimal immune response. Similar to C2C12s, PMs cultured on aligned collagen were able to proliferate and form multinucleated, aligned myotubes on the aligned collagen scaffolds (Fig. 6.3 A). PMs were isolated from Pax7-TdT mice for \textit{in vivo} transplantation to allow for cell tracking. Prior to scaffold implantation, they were imaged to verify that there was a high density of undifferentiated TdTomato+ myoblasts on the scaffolds (Fig. 6.3 B).

**Figure 6.3.** Primary myoblasts proliferate and differentiate on aligned collagen \textit{in vitro}. Primary myoblasts (PMs) proliferate and differentiate to MHC+ myotubes on aligned collagen (A), stained for nuclei (blue), MHC (red), and f-actin (green). PMs isolated from Pax7-TdTomato mice proliferate on aligned collagen (B), imaged just prior to implantation.

6.3.3 \textit{Muscle function improves with time and cell seeding}
At 14 or 28 days following injury and scaffold transplantation, the maximal isometric torque about the knee was used to assess functional muscle recovery of the muscle. Maximal isometric torques of contralateral control quadriceps were significantly higher than any injured muscles at both timepoints (Fig. 6.4 A & E, \( p<0.0001 \)). Similarly, the masses of all injured muscles were significantly lower than the mass of contralateral controls (Fig. 6.4 B & F, \( p<0.05 \)). However, muscle mass did trend upwards with the addition of collagen scaffolds at both timepoints. When normalized to mass, contralateral controls had significantly higher torque per mass values at day 14 than all other groups (Fig. 6.4 C, \( p<0.05 \)). However, at day 28, the reduced mass of untreated controls resulted in a torque per mass value which was not different from the uninjured control group. By contrast, the increased mass of the collagen treated groups resulted in significantly lower torque per mass values, indicating that the increasing mass did not necessarily mean increasing functional muscle tissue (Fig. 6.4 G, \( p<0.01 \)). When the torques of injured quadriceps were normalized to their respective contralateral controls, there were no significant differences between treatment groups at day 14 or 28 post-VML (Fig. 6.4 D, H, \( p>0.05 \)).

In looking at these data, it appeared that while injured group attained functional recovery to the level of uninjured controls there may have been an effect of cell seeding compared to acellular scaffolds. Therefore, quadriceps treated with acellular scaffolds were compared to cell seeded scaffolds over time. While there were no significant differences between groups in muscle weight (Fig. 6.4 J), the maximal isometric torque of muscle treated with cell seeded collagen was significantly higher at day 28 than that of acellular collagen at day 14, regardless of normalization (Fig. 6.4 I, K, L, \( p<0.05 \)). These findings
gave preliminary indication that the PMs seeded into the aligned collagen scaffolds may have functionally integrated into the host muscle over time.

Figure 6.4. Cell seeded collagen scaffolds improve functional recovery over acellular scaffolds with time.

Quadriiceps torque and mass data from 14 (A-D) and 28 (E-H) days post-injury. Collagen with and without cells were compared separately over time (I-L). Data displayed as raw maximum torque (A, E, I), muscle mass (B, F, J), torque per mass (C, G, K), and injured quadriceps torque normalized to the contralateral torque (D, H, L). One way ANOVA with Dunnett’s test post hoc in A-C, E-G, Tukey’s test post hoc for D and H. Two way ANOVA with Sidak’s test post hoc in I-L. Significance level represented as *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

6.3.4 Tissue morphology is similar between cell seeded and blank collagen scaffolds
H&E staining allowed for visualization of the overall tissue morphology and cell infiltration into aligned collagen scaffolds (Figure 6.5, A-H). Stitched images showed that collagen scaffolds were effective in holding the structure of the quadriceps, integrating well within the defect space at both time points (Fig. 6.5 A-D). Qualitatively, there are cell nuclei present throughout acellular and cell seeded scaffolds. At 14 days post-injury, the cell density within the scaffolds was similar regardless of whether they were pre-seeded with cells (Fig. 6.5 A, C, E, G). However, by 28 days post-injury, there were qualitatively more gaps in the acellular scaffolds compared to the cell seeded scaffolds which were dense with nuclei and ECM (Fig. 6.5 B, D, F, H). Regardless, the number of nuclei present in acellular scaffolds indicates that the scaffolds are capable of supporting endogenous cell infiltration from the surrounding host tissue (Fig. 6.5 A, B, E, F).

![Figure 6.5. Collagen scaffolds qualitatively integrate well into injured quadriceps and support endogenous cell infiltration. Stitched images of the entire cross sections qualitatively show collagen scaffold integration and overall muscle structure (A-D). Cell nuclei within the scaffolds can be more easily seen in cropped higher magnification images (E-F). Sections were taken from muscle at day 14 (A, C, E, G) and 28 (B, D, F, H) days following injury, as well as from acellular and cell seeded scaffolds.](image-url)
Transplanted myoblasts show short term survival within the scaffold and long term engraftment into surrounding myofibers

Figure 6.6. TdTomato+ myofibers present within the scaffold at 14 days but engrafted into surrounding tissue after 28 days. Images were taken in the middle (A,B) and at the edge (D,E) of the scaffolds. Stained for nuclei (blue) and f-actin (grey), collagen (green), and TdTomato visualized from transgenic cells (red). The number of TdTomato+ myofibers within the scaffolds (C) as well as engrafted into the surrounding tissue (F) were counted and compared. Unpaired t tests performed on square-root transforms of data to correct for unmet assumptions (unequal variance, non-normal distribution), *=p<0.05.

Tissue cross sections of cell seeded scaffolds were measured for the presence of TdTomato+ myofibers. TdTomato+ myofibers were quantified by location, either within the collagen scaffold (Fig. 6.6 A,B) or engrafted into the surrounding muscle (Fig. 6.6 C-F).
D, E). At day 14 post-implantation, TdTomato+ myofibers were most abundant within the collagen scaffold and at significantly higher numbers than at day 28 post-implantation (Fig. 6.6 C, p<0.05). However, 28 days following implantation, we observed more TdTomato+ fiber outside the scaffolds than within the scaffold. These TdTomato+ myofibers indicated PM engraftment into the surrounding muscle (Fig. 6.6 E). The number of TdTomato+ myofibers outside the scaffolds was significantly greater at 28 days post injury compared to 14 days prior (Fig. 6.6 F, p<0.05).

Figure 6.7. TdTomato myofibers appear aligned and elongated in the direction of the host muscle. Longitudinal muscle sections of fixed tissue were images in whole mount and shown as stitched images (A, C) from distal (left) to proximal (right). Cropped, zoomed images were show the TdTomato+ myofibers and their integration into the surrounding tissue (B, D) with the TdTomato channel shown alone (far left, B, D). Stained for nuclei (blue) and f-actin (grey), TdTomato visualized from transgenic cells (red).

Muscle was also sectioned longitudinally to visualize the location and length of TdTomato+ myofibers within and adjacent to the scaffold (Fig. 6.7). 14 days following implantation, TdTomato+ myofibers can be seen within the scaffold as well as at the
junction between the host tissue and the scaffold (Fig. 6.7 A, B). Notably, the TdTomato+ fibers appear to be aligned in the direction of the scaffold and the host tissue. Muscle at 28 days following injury also has TdTomato+ myofibers (Fig. 6.7 C, D), but qualitatively there appears to be fewer fibers overall than at 14 days. However, as was seen in cross sections, the TdTomato+ myofibers at day 28 appear to be more mature based on their length, width, and location in the tissue. Taken together with the cross-section images and quantification, our results indicate that by 28 days the seeded PMs have engrafted into host tissue but it would not appear that they survived within the scaffold. This, however, is despite seeing many cell nuclei within the scaffolds at the same timepoint.

Figure 6.8. Acetylcholine receptors present on cells throughout the collagen scaffolds.

Staining for AChR visualization in cross sections at day 14 (A) and 28 (B). Stained for nuclei (blue), f-actin (grey), and AChRs (green). TdTomato protein visualized in red.

To investigate whether the transplanted myoblasts were functional, the AChRs were stained with α-BTX. Within the scaffolds, there were a number of punctate AChR clusters at both 14 and 28 days post injury transplantation (Fig. 6.8 A,B). These AChRs were not, however, co-localized with TdTomato+ transplanted myoblasts. Additionally, the positive staining within the scaffolds does not resemble what would be expected of
mature NMJs. Positive staining of mature NMJs can be seen in the adjacent muscle tissue in the image from 28 days (Fig. 6.8 C). The localization of AChRs within the scaffolds could indicate immature NMJ development within the scaffolds, similar to those seen on regenerating myofibers in critical VML as characterized in Chapter 3. However, this staining did not indicate functional NMJs within the collagen scaffolds.

6.3.6 Blood vessels, macrophages, and FAPs populate the cell seeded scaffolds

To determine what other cell types were present within the scaffolds, additional IHC staining was done for vessels, macrophages, and FAPs. Blood vessels, visualized with vWF, can be seen throughout the scaffolds at both 14 and 28 days (Fig. 6.9). Qualitatively, the vessels at day 28 look more aligned as the cross section shows smaller, circular vessel lumens in the scaffold (Fig. 6.9, F). Conversely, at 14 days, many vessels have been cut transversely (Fig. 6.9 C), indicating that they were not aligned in the direction of the muscle. This may be indicated vascular remodeling within the scaffold between 14 and 28 days.
Figure 6.9. Aligned collagen scaffolds support vascularization through 28 days following injury. Cross sections from 14 (A-C) or 28 (D-E) post transplantation are shown as entire stitched sections (A, D) or representative higher magnification images to visualize blood vessels within the scaffolds (B, C, E, F). Fluorescence imaging of nuclei (DAPI, blue), f-actin (grey), von Willebrand factor (vWF, green), and TdTomato protein from transgenic cells (red).

Macrophages and FAPs were visualized at 14 and 28 days following injury using the pan-macrophage marker CD68 (Fig. 6.10) and PDGFRα (Fig. 6.11), respectively. While macrophages can be seen within the scaffolds at both time points, qualitatively, there are more macrophages within the scaffolds at 14 days (Fig. 6.10, A-C) than at 28 days (Fig. 6.10, D-F). Notably, the macrophages are distributed throughout the scaffolds at each time point, not clustered around the outside. This, taken with the previous histological characterizations, suggest that the cell seeded collagen scaffolds are not eliciting a foreign body response from the host immune system.
FAPs can be seen at both 14 and 28 days within the scaffolds (Fig. 6.11). Qualitatively, there are more FAPs with time, as there is more ubiquitous PDGFRα signal at 28 days (Fig. 6.11 B) compared to day 14 (Fig. 6.11 A). This may indicate that FAPs continue to proliferate or migrate into the scaffold with time.

Figure 6.10. Macrophages are present throughout the collagen scaffolds at 14 and 28 days. Cross sections from 14 (A-C) or 28 (D-E) post transplantation are shown as entire stitched sections (A, D) or representative higher magnification images to visualize blood vessels within the scaffolds (B, C, E, F). Fluorescence imaging of nuclei (DAPI, blue), f-actin (grey), macrophages (Mϕ, CD68, green), and TdTomato protein from transgenic cells (red).
Figure 6.11. FAPs migrate into and populate collagen scaffolds over 28 days. FAPs can be seen within cell seeded collagen scaffolds at 14 (A) and 28 (B) days in cross sections. Stained for nuclei (blue), f-actin (grey), and FAPs (green). TdTomato protein is colored red.

6.4 Discussion

VML is a complex injury with limited clinical treatment options. Due to the inability of muscle to recover following VML, tissue engineered strategies are an attractive option for encouraging functional muscle regeneration. In this chapter, we tested pre-seeded aligned collagen scaffolds which were able to modestly improve muscle function with time following critical VML when compared to acellular scaffolds. While it appears most transplanted myoblasts were unable to survive within the scaffold 28 days following injury, there was improved engraftment of transplanted cells into the surrounding tissue with time, indicating functional incorporation. PM engraftment into the host muscle is likely one explanation for the improved force generation of cell containing scaffolds compared to acellular scaffolds. Previous studies have shown increased engraftment of transplanted MuSCs is associate with improve functional muscle recovery [319].
It is well-characterized that the alignment of myofibers is crucial to the overall function of the tissue and its ability to generate directional force [320]. The structure of the muscle ECM is crucial for force transduction through the tissue to the myotendinous junction to produce movement [15]. Therefore, it is notable that our transplanted PMs seeded in muscle ECM mimicking aligned, porous collagen scaffolds can be seen in longitudinal whole mount sections of the injured muscle and appear to be aligned with the host muscle. This provides further qualitative support that the transplanted PMs may contribute to the increased force production of acellular scaffolds.

In order for myofibers to be functional, they need to be innervated by a motor neuron at the NMJ. AChRs are present in high density at the post-synaptic membrane of NMJs and therefore can be used in muscle as an indicator of innervation or re-innervation in skeletal muscle [232]. To further interrogate transplanted myotube functionality, AChRs were localized. However, as the structure of the AChR staining in our sections does not match the structure of mature NMJs, it is possible these AChRs are not on the surface of regenerating myofibers. Immune cells, including macrophages and T cells, are both known to express AChRs and acetylcholine. AChRs have been implicated in the cholinergic anti-inflammatory pathway in macrophages and in T cell activation and polarization [321], [322]. Whether the AChRs in the scaffold are on the surface of immune cells would require additional staining. Investigating the interaction between acetylcholine, the immune response, and re-innervation in VML would be worthwhile in future studies.

Vascularization of muscle grafts is one of the most important outcomes for successfully limb salvage clinically [119]. Our results indicate successful vascularization of the scaffolds; however, we did not see continued transplanted myotube survival within
the scaffolds through 28 days. It is possible that the degree of vascularization was insufficient to support TdTomato myofiber survival in the scaffold for 4 weeks. Encouraging further vascularization of the scaffolds could be achieved through co-transplantation of vascular support cells or vascular fragments in addition to PMs \([131]\) or through inclusion of cytokines, such as VEGF, into the scaffold to provide a pro-angiogenic gradient \([323]\). However, our previous characterization of the pro-fibrotic environment following VML indicates there may already be elevated local levels of VEGF (Chapter 5), complicating the overall impact of this approach.

In the transplantation of any scaffolding material and exogenous cell source, the immune response is always a concern. As previous studies have shown the lack of a foreign body response to collagen sponges \([176]\) and PMs \([199]\), \([200]\) \textit{in vivo} in mice, we did not expect a detrimental immune reaction to our treatment. Indeed, while there are persistent macrophages in the scaffolds through 28 days, we postulate that this a result of the VML injury environment (Chapters 3 & 4) rather than a response to the scaffold itself. This would be a reasonable hypothesis as the visualized macrophages are spread throughout the tissue and the scaffold and do not localize at the scaffold edges or specifically near transplanted TdTomato+ PMs. Further characterization of the subtypes of the immune cells present in these scaffolds would give us additional information of how these scaffolds impact the native response to VML characterized in Chapter 4. Additionally, as has been discussed previously (Chapters 4 and 5) macrophage polarization and cytokine secretion both play important roles in both myogenesis and fibrosis \([65]\), \([98]\).

In future studies, we plan to improve the specificity and bioactivity of the aligned collagen scaffolds to enhance functional outcomes. Previous utilization of the collagen
scaffolds used in this chapter have shown their tunability for specific applications on several parameters that were not studied here. These parameters include pore density, degree of pore anisotropy, overall scaffold stiffness, and electrical conductivity [211], [315], [316], [318]. Additionally, the protein content can be altered to provide additional binding domains specific to muscle. For example, collagen was chosen as a candidate material for skeletal muscle as the major structural protein in the skeletal muscle ECM is collagen, with types I and III being most abundant. However, the basal lamina, to which MuSCs bind, is mainly composed of collagen type IV and laminin [14]. Therefore, it may be valuable to include laminin which has previously been shown to improve muscle regeneration when incorporated into scaffolding materials [176], [178].

Further, while the scaffold promoted endogenous cell migration, the scaffolds were still present and not enzymatically degraded by 28 days post transplantation. Altering the various cross-linking steps in fabrication may be important for promoting scaffold degradation, including modifying the molar ratio of EDC:NHS [318]. Additionally, in the context of an aberrant pro-fibrotic FAPs population (Chapter 5), it should be determined whether the scaffolds promote the proliferation or migration of this FAPs subtype. As pro-fibrotic FAPs secrete elevated levels of TIMP1, they may be impeding degradation of the collagen scaffold. In previous studies, inhibiting TIMP1 has been shown to increase cell migration and vascularization of three-dimensional scaffolds [287]. In future applications in VML, inhibiting TIMP1 could be an approach to increase both scaffold and endogenous matrix turnover. This, in turn, could make room for additional myoblasts to penetrate and bridge through the scaffolds, and potentially increase transplanted cell survival by increasing angiogenesis.
6.5 Conclusion

Aligned, porous collagen scaffolds seeded with exogenous primary myoblasts present an attractive and promising option for skeletal muscle tissue engineering following critical VML. Cell seeded scaffolds showed modest improvement of muscle torque, aided in maintaining overall muscle structure, supported the engraftment of transplanted myoblasts, and promoted infiltration of blood vessels, immune cells, and FAPs into the scaffolds. These studies provide a basis for the \textit{in vivo} use of tunable aligned collagen scaffolds for skeletal muscle regeneration.
CHAPTER 7. CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Overall Conclusions

Overall, this thesis has contributed a thoroughly characterized VML injury model in the mouse quadriceps for use as a platform for testing biomaterial-based tissue-engineered constructs for VML. Through the definition of a critically sized VML injury, we established a system for comparing subcritical and critical muscle trauma to elucidate the key cellular and tissue components which are significantly altered following critical VML. This provided a basis for which we were able to identify a novel pro-fibrotic subpopulation of FAPs specific to critical VML injured muscle as a potential target for anti-fibrotic and pro-regenerative therapeutics. Finally, we used this critical VML model to assess the use of a cell-seeded, structurally aligned biomaterial for the improvement of functional muscle regeneration. These findings can be leveraged in the future for the creation of informed therapeutics for critical VML.

7.1.1 Specific Aim 1: Define and characterize a critically sized VML injury model in the mouse quadriceps.

In Chapter 3, we directly compared three different sizes of biopsy punch defects to determine the threshold of a critical VML injury in the mouse quadriceps. Utilizing histological analyses at 14 and 28 days following injury, we determined that while 2mm injuries were able to heal with little to no fibrotic scarring, 3 and 4mm injuries left a persistent fibrotic region where myofibers were unable to bridge. Myofiber regeneration was measured adjacent to the injury and eMHC expression was prolonged in critical
injuries. 3mm injuries, determined to be the threshold of critical VML, were further characterized. 3mm VML resulted in increased vascularization but no appreciable neuromuscular regeneration at 4 weeks post injury.

In Chapter 4, we sought to distinguish the difference in immune response dynamics and heterogeneity between subcritical 2mm and critical 3mm injuries. Using flow cytometry, we found that critical injuries elicit an increased and sustained concentration of myeloid cells, particularly type M2 macrophages. Additionally, critical injuries resulted in a significantly higher concentration of lymphoid cells, most notably at 3 days post injury but sustained through day 7. These cells were segmented by their surface marker expression using dimensionality reduction techniques, UMAP and SPADE. One key result was the quantification of a subpopulations of monocytes and macrophages which highly expressed CXCR4 following critical VML. This is significant because CXCR4 expressing myeloid cells have previously been characterized to contribute to tissue fibrosis.

Taken together, the results in chapters 3 and 4 defined the critical threshold of VML and characterized the pathology of critical VML at the tissue and cellular levels from day 1 to day 28 following injury.

7.1.2 Specific Aim 2: Determine the role of aberrant fibro-adipogenic progenitors in impaired myogenesis following critical volumetric muscle loss.

FAPs and MuSCs are key progenitor populations in muscle regeneration, but FAPs are also the main source of fibrosis in skeletal muscle. In Chapter 5, we demonstrated that FAPs make up a majority of the mononuclear cells found within a critical VML defect and determined that a subtype of FAPs which highly express the surface marker β1-integrin are
specifically upregulated in critical VML. Our analyses of β1-HI FAPs identified a novel population of FAPs which are biased towards fibrotic differentiation. Gene expression and cytokine secretion analyses revealed that β1-HI FAPs may play a crucial role in the progression of fibrosis and chronic inflammation in VML while inhibiting myogenesis. Additionally, TIMP1 was identified as a pro-fibrotic signaling molecule which provides a new potential target for in vivo anti-fibrotic therapeutics.

7.1.3 Specific Aim 3: Evaluate the therapeutic efficacy of syngeneic myoblast transplantation seeded on structurally aligned biomaterials for the treatment of critical volumetric muscle loss.

Utilizing aligned, porous collagen scaffolds in Chapter 6 with or without pre-seeded primary myoblasts, we were able to show modest functional improvement of cell-seeded aligned collagen over time compared to acellular scaffolds. While the scaffolds did not degrade over 28 days, they were able to improve the overall muscle structure as visualized through H&E histology when compared to the histology in previous chapters of untreated critical VML defects. Utilizing fluorescently labeled cells for transplantation, we were able to show that the scaffolds promoted aligned myoblast engraftment into the host muscle. IHC staining indicated that there was endogenous cell migration of blood vessels, macrophages, and FAPs into the collagen scaffolds.

7.2 Future Directions

7.2.1 Functional characterization of immune subpopulations in critical VML
The sustained immune response in critical VML was characterized in Chapter 4 of this thesis by quantification of cell concentrations over time. While several myeloid and lymphoid cell subtypes were quantified through dimensionality reduction techniques, the functional differences of those subtypes were not elucidated. In future studies, it would be beneficial to study these subpopulations in more detail. Additional studies could include measuring subpopulation location within the tissue following injury relative to other cell types utilizing a relatively new technique called IHC multiplexing. IHC multiplexing has been most widely used in the context of cancer immunology and is quite powerful for its ability to image 40-100 biomarkers at one time on a single sample [324]. Using these techniques would provide spatial information about the subtypes we have characterized using flow cytometry.

Another intriguing future direction would be to utilize intracellular cytokine analyses in combination with some of the key flow cytometry markers for our identified subpopulations. Some preliminary work has been done with collaborators in this effort. Using intracellular cytokine staining for TNFα and TGF-β, some immune cell subtypes were characterized for their relative expression of these cytokines (Fig. 7.1). In these studies, neutrophils were characterized, a population which we did not measure in Chapter 4. These results showed a TNFα+ population of neutrophils is upregulated at day 7 following critical VML (Fig. 7.1 C, \( p=0.0509 \)). Similarly, M2 macrophages, which we found to be significantly elevated in critical injuries at day 7, can be further segmented into TNFα/TGF-β double negative or TNFα/TGF-β double positive populations (Fig. 7.1 D). These results provide additional insights into our characterization of a chronic inflammatory environment following critical VML where the balance of pro- and anti-
inflammatory cells phenotypes and associated cytokines are not maintained and impairs muscle regeneration [89].

Figure 7.1. Intracellular cytokine flow cytometry analyses provide additional immune cell subtype insights. Neutrophil concentration at 3 and 7 days post-injury (A), percent TNFα positive neutrophils at 3 (B) and 7 (C) days post-injury. M2 macrophage concentration, segmented by intracellular presence of TNFα and TGF-β. T test performed, significance at $p<0.05$. Figure courtesy of Lauren Hymel.

Taking this a step further, using mass cytometry would provide a more in depth look at immune cell subpopulations following critical VML. Mass cytometry allows for the use of up to 40 intracellular and cell surface markers simultaneously and has been previously used for myogenic lineage mapping as well as monocyte derived macrophage characterization [325], [326]. These studies would be possible as we have access to a Fluidigm Helios mass cytometer. Additionally, we have shown that our cell isolation protocol that we follow for flow cytometry is suitable for use with the Helios machine as we were able to measure a simple DNA stain with low levels of heavy metal contamination.
Figure 7.2. Single cell isolation protocol suitable for mass cytometry. Rain plot indicates measurements in each heavy metal “channel” for single cells isolated from hindlimb muscle using flow cytometry cell isolation protocol. Cells stained only for DNA as proof of concept and to determine if there was heavy metal contamination that would require additional protocol optimization. Samples run and figure provided by Laxmi Krishnan.

7.2.2 Targeted FAPs subpopulation modulation in critical VML

The identification of a pro-fibrotic FAPs subpopulation in Chapter 4 would support future studies in the modulation of this subpopulation. Our results indicate that cytokine signalling of TGF-β1 and TIMP1 play a role in the fibrotic differentiation of FAPs and are secreted at increased levels from FAPs following VML as well as specifically in β1-integrin-HI pro-fibrotic FAPs. These findings provide several potential avenues for the reduction of pro-fibrotic FAPs in critical VML.

A method to pharmacologically inhibit FAPs has been previously investigated in the context of chronic muscle disease (Duchenne muscular dystrophy) and minor acute injury (notexin injection) through the use of tyrosine kinase inhibitor, nilotinib [98], [125].
Nilotinib has shown promise as an antifibrotic agent in skeletal muscle as well as other tissues, including the lung and liver, by inhibiting TGF-β1 signaling [98], [125], [327], [328]. Specifically, nilotinib was shown to inhibit fibrosis by increasing the apoptosis and clearance of persistent FAPs as well as by decreasing collagen deposition [98], [125]. Preliminary results in the use of nilotinib in our critical VML model have been inconsistent.

Our preliminary data in Figure 7.3 show the results from two initial experiments. In each experiment, nilotinib or solvent were delivered to mice following VML injury through Alzet osmotic pumps. The first study, beginning nilotinib treatment at day 3 post-VML (Fig. 7.3 A) resulted in promising results in which nilotinib treated mice did not have a significant increase in β1-integrin-HI FAPs when compared to the UI control (Fig. 7.3 B). This was not true of solvent treated mice, which had a significant increase in FAPs at day 7 over UI solvent treated controls (Fig. 7.3 B, p<0.001). With these promising results, a fully powered follow up experiment was done to determine whether treatment with nilotinib starting the day of injury would further reduce FAPs concentration (Fig. 7.3 C). However, the flow cytometry data from these experiments did not show any differences between untreated, solvent, or nilotinib treated VML (Fig. 7.3 D, p>0.05). These results were confirmed at day 14 with histology which showed similar tissue morphology and mononuclear cell infiltrate into the VML defect regardless of treatment (Fig. 7.3 E, F). These results are inconclusive and may be the result of improper dosing of nilotinib treatment or ineffectiveness of systemic delivery in this injury model. Future studies would be required to determine whether nilotinib could have therapeutic potential in critical VML.
Nilotinib treatment results in inconsistent FAPs modulation following critical VML.

Initial pilot experiment was conducted to determine the effect of nilotinib versus solvent in critical injured and uninjured tissue as described (A). Flow cytometry results indicated a non-significant increase over uninjured muscle in FAPs concentration when treated with nilotinib (B). A larger study was conducted (C) with nilotinib delivered immediately following injury. Flow cytometry revealed no significant differences between treatment groups (D) and histology showed no detectable qualitative differences in tissue morphology at day 14 following injury.

Pro-fibrotic FAPs were characterized as highly expressing β1-integrin, but β1-integrin is an extremely broadly expressed surface receptor throughout many cell types. Therefore, in future studies the specific alpha subunit which colocalizes with β1 on the surface of pro-fibrotic FAPs should be evaluated. Our data indicate the possibility that gene expression αV subunit contributes more than any other alpha subunit measured. Despite non-significant differences in gene expression in a pairwise comparison of β1-HI and β1-LO FAPs (Fig. 7.4 A, \(p>0.05\)), αV expression was as strongly associated with high scores on PC1 and LV2 as β1-integrin (referencing PLSDA and PCA from Chapter Fig. 5.8 and 5.9, adapted results in Fig. 7.4 B, C). These results provide a starting point for future studies.
to investigate the level and role of $\alpha V$ integrin subunit on the surface of FAPs following injury.

Figure 7.4 Integrin $\alpha V$ is associated with $\beta 1$-HI FAPs indicated by higher LV2 and PC1 loadings. Normalized fold regulation of the Igtav gene comparing $\beta 1$-HI and $\beta 1$-LO FAPs from VML injured muscle (A). Data in B & C adapted from Chapter 5, referencing LV2 (Fig. 5.8) and PC1 (Fig. 5.9). LV2 (B) and PC1 (C) loadings of all measured integrin subunit genes in fibrosis PCR array. Relative meaning of loadings indicated in grey arrows to the left of y axes. Paired t test conducted in A, significance level $p<0.05$ not met.

In addition to our results, there are previous studies which have shown the $\alpha V\beta 1$ complex is highly upregulated on activated fibroblasts, binds the TGF-$\beta 1$ latency associated peptide, and mediates TGF-$\beta 1$ activation. This study also developed a blocking antibody which specifically binds the $\alpha V\beta 1$ integrin complex, reducing pulmonary and liver fibrosis in vivo [259]. This may be a promising approach in critical VML and would have the benefit of targeting a specific integrin complex and would therefore have fewer off target affects than targeting $\beta 1$ alone.

Lastly, our analysis of pro-fibrotic FAPs indicated the role of TIMP1 as a potential driver of fibrosis in critical VML. Previously, inhibition of TIMP1 using a commercially available blocking antibody has been shown to increase cell migration and vascularization into subcutaneous three-dimensional scaffolds [287]. In the liver, small interfering RNA
(siRNA) was used to block TIMP1 and its promoter, c-Jun, to downregulate the expression of TIMP1 following induction of hepatic fibrosis [165]. Additionally, TIMP1 knockout transgenic mice resulted in reduced myocardial fibrosis through a reduction in phosphorylated SMAD2/3, a downstream signaling molecule of pro-fibrotic TGF-β1 [166]. In future studies we could utilize these TIMP1 knockdown or inhibition strategies in critical VML and assess the impact on fibrosis and muscle regeneration.

7.2.3 Utilizing aligned collagen scaffolds for combinatorial therapeutic approaches

Our work in Chapter 6 provided the groundwork for the use of cell-seeded aligned collagen in VML injuries. These aligned collagen scaffolds have been used in several applications and are able to be highly customized. In the future, tuning these scaffolds to improve myogenesis and myofiber bridging may lead to further enhanced functional recovery after VML.

Previously, a method to covalently attach spatially patterned biomolecules to the aligned collagen scaffolds used in Chapter 6 was developed [329]. This photochemical patterning method can be broadly applied to a variety of biomolecules as it only requires the presence of a C-H bond. This method could be used in VML for sequestering other ECM proteins for myoblast binding or for immobilizing growth factors onto the matrix [329], [330]. One limitation of this photo-patterning method is the limited depth penetration of the pattern into the scaffold. Another method for incorporating growth factors or other biomolecules into the collagen scaffolds is during the EDAC-NHS crosslinking step, which covalently binds biomolecules throughout the bulk of the scaffold [331]. Utilizing either of these methods could useful for our applications for the addition
of myogenic factors which may increase MuSC migration into the scaffold, such as previous work in our lab has shown with Wnt7a containing hydrogels [189]. Additionally, anti-fibrotic molecules, potentially TIMP1 or αVβ1 blocking antibodies (discussed in 7.2.2) could be delivered this way.

Specifically for use in skeletal muscle, these aligned collagen scaffolds were recently tuned for C2C12 culture in vitro [316]. These scaffolds were customized by including the electrically responsive polymer polypyrrole (PPy) into the collagen solution prior to lyophilization which did not negatively impact myoblast growth and differentiation. This study indicates another potential future direction for this work through the incorporation of electrically conductive materials. Similarly, in future work we could include mechanically active materials capable of providing contractile forces to further stimulate functional muscle regeneration [216], [218]. The versatility of this platform is a distinct advantage of the current work as it will be conducive to numerous modifications in future work.
APPENDIX A. PROTOCOLS

A.1 Isometric Torque Protocol

Materials
- Force Transducer
  o Harvard Apparatus No. 60-2996
- Stimulus Generator
  o Grass S11 Stimulator
- Stimulus Isolation Unit
  o Grass SIU5
- Nerve Cuffs:
  o Silicone tubing
    ▪ A-M Systems Cat# 806700 (.025” X.027” X.011”, 20FT)
  o Wire
    ▪ A-M Systems Cat# 793500 (Stainless Steel Wire – Thicker)
    ▪ Pyrofuse MEDWIRE.281-9/#49 PTFE COATED (Platinum-Iridium Wire – Thinner than the A-M systems)
  o Silicone clone
    ▪ Factor II, Inc. Product Code FX-302
- Animal Immobilization Apparatus
  o See figure below for torque about knee
    ▪ Box dimensions: 4” high x 12” long x 6” wide
    ▪ Box made of acrylic from McMaster Cat# 8560K191 Clear Cast Acrylic Sheet 12"x12"x7/64"
      ● Laser cut and glued together
    ▪ Bar to hold transducer below from McMaster Cat# 8560K274 Clear Cast Acrylic Sheet 6"x6"x0.5"
      o Should be customized for each application
- Laptop with DASYLab software & USB port
- Data Acquisition Board (DAQ)
  o USB-1608G Series (I need to verify which one it is)
- 2 cables with alligator clip attachment on one end
  o Connects SIU to nerve cuff
- 1 cable with BNC -> split cable to connect to DAQ
  o Connects force transducer to DAQ
- Suture to attach point of measurement on animal to force transducer
  o Rats: want 4-0 or thicker (so suture does not snap under tension)
  o Mice: can use as low as 6-0 without snapping

Methods
1. Connect all electronic components
   a. Force transducer needs to be plugged into outlet, placed into fixation device
   b. Computer should be plugged into wall outlet
   c. Connect DAQ to force transducer (BNC)
      i. Use electrical tape to connect ground wire from DAQ to metal table/object
   d. Connect DAQ to computer (USB)
   e. Connect SIU to Stimulus Generator
      i. Use electrical tape to connect ground wire from SIU to metal table/object
   f. *If you want to measure and record stimulus, connect from SIU to DAQ as well (this hasn’t worked in the past, unclear why – sampling frequency?)*

2. Once DAQ is connected to computer, open DASYLab program
   a. Open file -> “Force Input Only”

3. Set stimulation pulse duration, frequency, and train duration for the specific application and set Stimulus Generator accordingly
   a. Femoral nerve stimulation (for quadriceps torque production)
      i. Rats:
         1. Pulse duration: 0.5 ms
         2. Pulse interval: 5.7 ms (Frequency: 175 Hz)
         3. Train duration: 500 ms
      ii. Mice:
         1. Pulse duration: 0.2 ms
         2. Pulse interval: 5.7 ms (Frequency: 175 Hz)
            a. Works, should be validated with Force-Frequency curve
         3. Train duration: 500 ms
   b. Supraspinatus direct muscle stimulation
      i. Rats:
         1. Pulse duration: 1 ms
         2. Pulse interval: 10 ms
         3. Train duration: 300 ms
   c. Note: When setting up a new protocol, voltage with max force production should be done first (see step 10), then a frequency-force relationship should be established to determine a stimulus frequency within the plateau.
      i. Useful references:
         2. G. S. Lynch, Measuring isometric force of isolated mouse skeletal muscles in situ, (2009), SOP ID# DMD_M.2.2.005
Alternatively, you can find previously reported stimulation parameters for your muscle of interest.

4. Put animal under isoflurane
5. Dissect to expose nerve or muscle of interest
6. Attach nerve cuff around nerve (if applicable)
7. Move animal to secure in place on immobilization box
8. Turn on heat lamp to maintain animal body temperature
9. Attach nerve cuff to SIU
10. Attach animal to force transducer
   a. For torque about the knee, attach suture to animal ankle using a zip tie so suture can be re-used
   b. For direct muscle measurement, will likely need to knot suture to secure to the muscle and would likely not be reusable
   c. FOR ALL:
      i. Make sure the suture connection to force transducer is taut (under slight tension, no slack in line between point of measurement and transducer)
      ii. Make sure the suture is in line with the axis of measurement
11. Zero the force transducer
12. Determine voltage required for maximal twitch force
   a. Turn down the isoflurane for about a minute immediately prior to testing to increase animal responsiveness to stimuli
   b. Start by stimulating at 5V (5-15V should be sufficient for direct nerve stimulation)
      i. If using S11 Stimulator and SIU5 – Voltage out does not match printed settings use this table:

<table>
<thead>
<tr>
<th>Desired Output Voltage</th>
<th>S11 Voltage Setting</th>
<th>S11 Multiplier Setting</th>
<th>SIU5 Multiplier Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>5V</td>
<td>6V</td>
<td>10x (SIU)</td>
<td>0.1x</td>
</tr>
<tr>
<td>7V</td>
<td>8V</td>
<td>10x (SIU)</td>
<td>0.1x</td>
</tr>
<tr>
<td>10V</td>
<td>1.8V</td>
<td>10x (SIU)</td>
<td>1x</td>
</tr>
<tr>
<td>12V</td>
<td>13V</td>
<td>10x (SIU)</td>
<td>0.1x</td>
</tr>
<tr>
<td>15V</td>
<td>2.4V</td>
<td>10x (SIU)</td>
<td>1x</td>
</tr>
</tbody>
</table>

   ii. Hit play on DASYLab to record Force Data
   iii. Hit the “Pulse - Single” Trigger
      1. If a signal is being transmitted, a light will go on
   iv. Hit 3x waiting 30 seconds between pulses
   v. Stop DASYLab recording
   c. Increase at 2V intervals
   d. Do not exceed 15V
e. Reference DASYLab graphs for maximal force
f. You will need to re-do this measurement for every muscle you test!
13. Take 3 tetanic contraction measurements
   a. Hit play on DASYLab to record force data
   b. Switch the “Train – Single” Trigger to send just one train stimulus for recording
   c. Record the time shown on the computer when each measurement is taken (files are saved with a timestamp, but the computer is not synced to the current time)
   d. Wait 45 seconds between each repeat stimulus
      i. There is also a “Train Interval” Setting which would appear to control the spacing between Trains. When set to 45000 ms, there was not a reliable additional impulse at 45 seconds. If we want to use this setting, need to validate output.
   e. Stop DASYLab recording
14. Repeat steps 5-13 on contralateral side
15. Following data collection, the animal should be euthanized according to IACUC or ACORP protocols without regaining consciousness as no pain medication is administered and this is a terminal procedure.
REFERENCES


[34] K. Ishii et al., “Recapitulation of Extracellular LAMININ Environment Maintains


[47] M. Verma *et al.*, “Flt-1 haploinsufficiency ameliorates muscular dystrophy


R. N. Cooper, S. Tajbakhsh, V. Mouly, G. Cossu, M. Buckingham, and G. S. Butler-Browne, “In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse


[73] S. Schiaffino, A. C. Rossi, V. Smerdu, L. A. Leinwand, and C. Reggiani,


[131] M.-T. Li et al., “Skeletal Myoblast-Seeded Vascularized Tissue Scaffolds in the
Treatment of a Large Volumetric Muscle Defect in the Rat Biceps Femoris Muscle,”


of Skeletal Muscle: Mechanisms and Comparisons with Bone Marrow Adiposity,”


155. A. Muchir, P. Pavlidis, G. Bonne, Y. K. Hayashi, and H. J. Worman, “Activation of MAPK in hearts of EMD null mice: Similarities between mouse models of X-linked


B. T. Corona and S. M. Greising, “Challenges to acellular biological scaffold mediated skeletal muscle tissue regeneration,” *Biomaterials*, vol. 104, pp. 238–246,


[201] K. Takahashi et al., “Induction of Pluripotent Stem Cells from Adult Human


[258] Y. Chang et al., “Pharmacologic Blockade of a v b 1 Integrin Ameliorates Renal


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damage but is not necessary for muscle regeneration,” Physiol. Rep., vol. 6, no. 8,

Nephron Exp. Nephrol., vol. 107, no. 1, pp. e12–e21, 2007, doi:
10.1159/000106505.


significance of serum CXCL9 levels as a biomarker for systemic juvenile idiopathic


[277] J. Luo et al., “Vascular endothelial growth factor promotes the activation of hepatic


[297] A. L. Eun et al., “Reactive oxygen species mediate high glucose-induced plasminogen activator inhibitor-1 up-regulation in mesangial cells and in diabetic


